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Air pollution and the onset of balance problems: The Canadian longitudinal study on aging☆

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ABSTRACT

Purpose: To understand the relationship between ambient air pollution and the onset of balance problems.
Design: Population-based prospective cohort study.
Methods: Baseline and 3-year follow-up data were used from the Canadian Longitudinal Study on Aging. The Comprehensive Cohort included adults aged 45–85 years old recruited from 11 sites across 7 provinces. Data on air pollution came from the Canadian Urban Environmental Health Research Consortium. Annual mean levels of ozone, fine particulate matter ($PM_{2.5}$), and sulfur dioxide for each participant's postal code were estimated from satellite data. Balance was measured at both time points using the one-leg balance test with those who could not stand on one leg for at least 60 s defined as failing the balance test. Our outcome was the new development of failing the balance test at follow-up in those who passed the balance test at baseline. Logistic regression was used.
Results: Of the 12,158 people who could stand for 60 s on one leg at baseline, 18% were unable to do so 3 years later. In single pollutant models, living in an area with higher ozone levels was associated with the 3-year onset of balance problems (odds ratio (OR) = 1.13 per interquartile range of ozone, 95% CI 1.02, 1.24) after adjustment for demographic, lifestyle, and health variables. In a multipollutant model, the association with ozone increased slightly (OR = 1.16, 95% CI 1.04, 1.30). There were no associations with $PM_{2.5}$ or sulfur dioxide.
Conclusion: Our findings provide longitudinal evidence that higher ozone levels are associated with the odds of developing balance problems over a 3-year period. Further work should attempt to confirm our findings and explore the potential mechanism of action.

1. Introduction

There is extensive evidence that air pollution has a major impact on human health including the cardiovascular system, the respiratory system, the central nervous system, and the risk of cancer (Thurston et al., 2017). Air pollution levels are associated with inflammation, oxidative stress, metabolic disorders, and epigenetic changes, all of which contribute to pathological aging (Fougere et al., 2015). The Global Burden of Disease study ranks air pollution fourth in risk factors for global disease and mortality behind hypertension, smoking, and dietary factors (Collaborators, 2020). Given the growing recognition of the harmful role of air pollution on human health, The World Health Organization established lower recommended limits for air pollutants in 2021 (WHO, 2022).

Despite all that is known about the risks of air pollution, research on the impact of air pollution on balance is minimal. The ability to maintain balance is required for successful aging as studies have found that balance problems are associated with an increased risk of falls, nursing home admission, and mortality (Mihailovic et al., 2020; Vellas et al., 1997; Sourdet et al., 2012). 22% of older adults are affected by poor balance (Stevens et al., 2008). Multiple systems are involved in the regulation of balance including the visual system, the vestibular system, and the proprioceptive system. Risk factors for poor balance include older age, diabetes, arthritis, poor eyesight, and grip strength (Stevens et al., 2008). Four studies suggest that there may be an association between air pollution and balance. First, in a controlled crossover

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experiment, acute diesel exposure showed a trend towards reduced postural stability in 28 healthy adults in Vancouver, Canada (Curran et al., 2018). Second, in a study of 1,762 older adults in the Netherlands, researchers found that greater exposure to fine particulate matter was associated with worse performance on the Short Physical Performance Battery (SPPB), which includes a balance assessment (de Zwart et al., 2018). Third, a study of 2,912 older adults in Korea found that higher levels of particulate matter and ozone were associated with lower performance on the SPPB (Shin and Choi, 2021). Finally, greater exposure to fine particulate matter was associated with the development of worse performance on a series of increasingly difficult balance stances in a population-based study of 10,823 adults in China (Wang et al., 2021).

There is very limited evidence on the relationship between ambient air pollution and balance. More large, longitudinal studies from across the world that examine multiple pollutants at a time are needed. The Canadian Longitudinal Study on Aging (CLSA), a study of 30,097 older adults, can be used to provide valuable data on this topic.

2. Methods

2.1. Study design

The CLSA is a population-based prospective cohort study (Raina et al., 2019). This analysis focuses on the adults in the Comprehensive Cohort of the CLSA because they visited a data collection site and performed a balance test (Fig. 1). There were 30,097 people in the Comprehensive Cohort at baseline with 27,765 people providing follow-up data 3 years later (92%). Of these 27,765 people, 26,475 people attempted the balance test at baseline with 12,929 standing for 60 s (48%). Of the 12,929 who could stand for 60 s at baseline, 12,158 attempted the test at follow-up, which is our analysis sample. Participants were recruited in part through provincial health registries. This was done by sending out letters introducing the study to randomly chosen, age-eligible persons. Consent forms to be returned were enclosed. Participants were also recruited through random digit dialing by taking a random sample of landline telephone numbers for a given geographic area. Once a call was answered, eligibility was verified and written consent was obtained. Stratified sampling was used to have adequate representation of certain demographic groups. Strata were defined by age group, sex, and distance from the data collection site. Baseline data were collected between 2012 and 2015 while the follow-up data were collected between 2015 and 2018. A follow-up rate of 92% was achieved.

2.2. Study population

The inclusion criteria for the CLSA are as follows: between the ages of 45 and 85 years old, community-dwelling, and living near one of the 11 data collection sites in 7 provinces (Victoria, Vancouver, Surrey, Calgary, Winnipeg, Hamilton, Ottawa, Montreal, Sherbrooke, Halifax, and St. John's). Exclusion criteria included: being in an institution, living on a First Nations reserve or settlement, being a full-time member of the Canadian Armed Forces, not speaking French or English, not being a Canadian citizen or permanent resident, or having cognitive impairment at baseline. The study was approved by institutional review boards of all sites. Written informed consent was obtained for all participants.

2.3. Data collection

Data were collected from either an interviewer-administered questionnaire given at home or from procedures performed at a data collection site. CLSA personnel received detailed training sessions to standardize the data collection process.

2.3.1. Balance assessment

The one-leg balance test was used to assess standing balance. This test has acceptable reliability (Giorgetti et al., 1998; Beauchamp et al., 2021) and is predictive of injurious falls (Vellas et al., 1997) and incident disability (Michikawa et al., 2009). Participants who could not stand unassisted or who used a cane or walker regularly were excluded from this test. Participants removed their shoes, stood 1 m away from the wall, and lifted their right leg to the calf while placing their hands on their waist. The timer was started when the right foot left the ground and was stopped when it touched the ground, when the participant lost balance and/or touched the wall, or once 60 s had passed. This procedure was then repeated for the left leg. The better time of the right and left legs was used. For our analyses, a person was considered to have failed the balance test if they could not stand for 60 s.

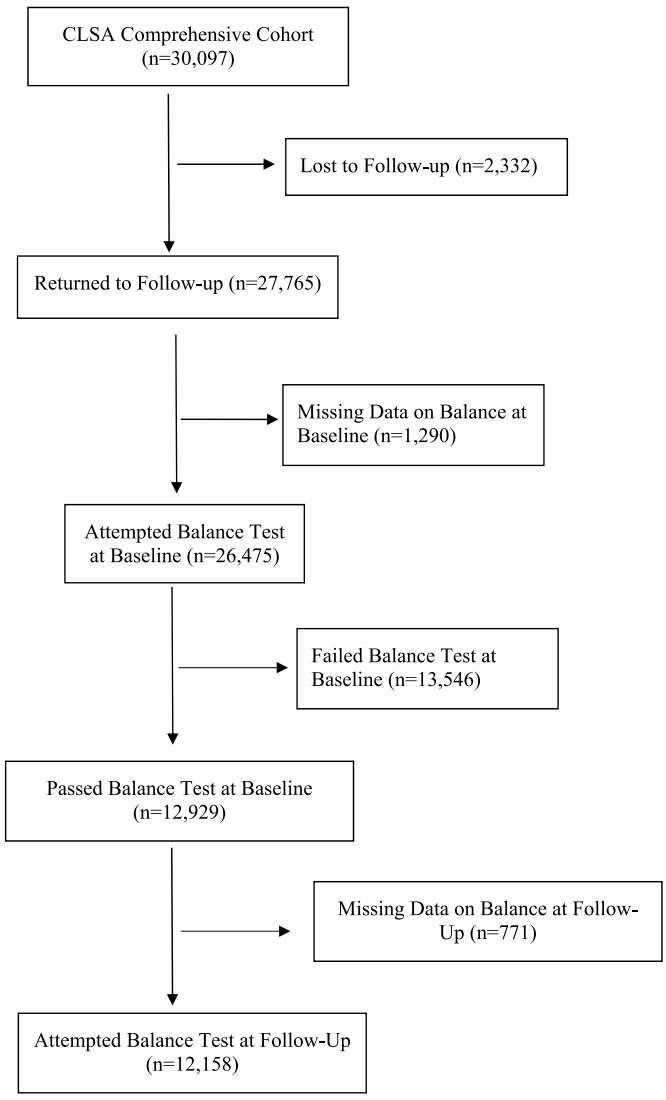

Fig. 1. Flow chart of participants included in the analysis.

2.3.2. Air pollution

Data on air pollution came from the Canadian Urban Environmental Health Research Consortium (CANUE), which was merged into CLSA data by postal code, which was collected for each participant by CLSA researchers (Brook et al., 2018). Ground level $PM_{2.5}$ concentration levels were estimated by satellite by combining aerosol optical depth retrievals using the GEOS-Chem chemical transport model from the following NASA instruments: Moderate Resolution Imaging Spectroradiometer, Multi-angle Imaging SpectroRadiometer, and Sea-viewing Wide Field-of-view Sensor. These measurements were then calibrated to regional ground-based observations using geographically weighted

regression. These 0.01° × 0.01° gridded surface datasets were used to assign values of annual mean concentration of $PM_{2.5}$ in μg/m^3 to the postal code of each CLSA participant (van Donkelaar et al., 2015; Boys et al., 2014; CanMap Postal Code Suite v2015, 2015).

Hourly ground-level ozone concentrations were estimated with the Global Environmental Multi-Scale Modelling Air Quality and Chemistry model by staff at Environment and Climate Change Canada. Estimates incorporate ground-level observation data. These datasets were used to assign values of annual mean concentration of ozone in parts per billion to the postal code of each CLSA participant (Robichaud, 2014; Robichaud et al., 2016; Data from, 2017a; Data from, 2017b).

Ground-level sulfur dioxide concentrations were estimated from the Ozone Monitoring Instrument satellite data using sulfur dioxide profiles from the Global Environmental Multi-scale – Modelling Air quality and Chemistry model over North America. These annual gridded datasets were aggregated to 3-year running averages and used by CANUE staff to assign values of annual mean concentration of sulfur dioxide in parts per billion to the postal code of each CLSA participant (CanMap Postal Code Suite v2015, 2015; McLinden et al., 2014; Kharol et al., 2017; Data from, 2017c).

2.3.3. Demographic, health, and lifestyle data

Sociodemographic data including age, sex, race/ethnicity, and household income were collected by self-report. Categories for race/ethnicity were collapsed into 2 categories (White/non-White) to avoid small sample sizes. Body mass index (BMI) was calculated by taking measured weight in kilograms and dividing by measured height (in meters (Fougere et al., 2015)). BMI was divided into 4 categories: underweight (<20 kg/m^2), normal weight (20–24 kg/m^2), overweight (25–29 kg/m^2, and obese (≥30 kg/m^2) in order to examine non-linearity. An illuminated Early Treatment of Diabetic Retinopathy Study (ETDRS) visual acuity letter chart at a 2-m distance was used to assess binocular visual acuity. Participants wore their normal correction for distance, if any. Visual acuity was scored as the number of letters read correctly. Visual impairment was defined as visual acuity worse than 20/40, as has been done previously (Aljied et al., 2018). Limitations in activities of daily living (ADL) were determined by the number of activities that participants could only do with help or were unable to do at all. Some examples of activities included eating, walking, getting dressed or out of bed. A participant who needed help with or was unable to do 1 or more activities was defined as having ADL limitations. Participants were classified as having diabetes or stroke if they reported ever having a physician diagnosis of those conditions. A current smoker was defined as a person who reported smoking at least 100 cigarettes in life and currently smokes daily or occasionally. A former smoker was someone who reported smoking at least 100 cigarettes in life but had not smoked in the last 30 days.

2.3.4. Statistical analysis

To examine the new onset of balance problems at follow-up, the analysis was restricted to those who were able to stand for 60 s at baseline. Baseline characteristics of those who were unable to stand for 60 s at follow-up (defined as a failure) were compared to those who were able to stand for 60 s (defined as passing). Wilcoxon rank sum tests were used to compare air pollution levels between those who passed and those who failed the balance test at follow-up. Lowess plots were used to examine the shape of the relationship between each air pollutant and the outcome. If nonlinearity was detected, spline terms were used and the fit of the model was compared to a model without spline terms using AIC and BIC statistics. The models that fit the data the best were retained. Multiple logistic regression models were used to determine whether the air pollution variables were independently related to failing the balance test. First, single pollutant models were run. The collinearity of the air pollutants was checked by calculating Spearman's correlation coefficients and variance inflation factors. Then, a multipollutant model was run. Models were adjusted for variables thought to be related to both air pollution and balance based on prior literature (Grant et al., 2021; Kahiel et al., 2021) including: demographic variables (age, sex, race, household income, province) and lifestyle variables (body mass index, smoking). We were unsure if health variables like diabetes, stroke, limitations in ADL, and visual impairment would be mediators (in the causal pathway) or confounders so we adjusted for them in a separate model being careful to keep the sample size constant between models (Baron and Kenny, 1986). We also examined models stratified by diabetes and visual impairment. Interaction terms were created and were tested in the regression models. The complex survey design was accounted for in all analyses using sample weights and the strata variable within the suite of SVY commands of Stata SE Version 16 (College Station, Texas).

3. Results

We compared the characteristics of those 12,158 people who attempted the balance test to those 17,939 who did not (Supplemental Table 1). Those who did not attempt the balance test at baseline or at follow-up were older, more likely to be women, to have lower incomes, to smoke, to have higher BMI, and to have more health conditions like diabetes, stroke, visual impairment, and to have ADL limitations. They were also more likely to live in areas with higher mean levels of $PM_{2.5}$ and ozone.

The overall analysis cohort was middle-aged, on average, and was fairly healthy with 20% being obese, 10% having or being suspected of having diabetes, 0.5% having had a previous stroke, 3% having ADL limitations, and 3% having visual impairment. The characteristics of those who passed and failed the balance test at follow-up are shown in Table 1. Of the 12,158 people who could stand for 60 s on one leg at baseline, 18% were unable to do the same 3 years later at follow-up. Demographic, lifestyle, and health characteristics of those more likely to fail the balance test at follow-up included older age, lower household income, current smoking, obesity, diabetes, stroke, ADL limitations, and visual impairment.

The median levels of air pollution in the postal codes of the participants who passed and failed the balance test are presented in Table 2. Those who failed the balance test at follow-up lived in areas with higher levels of ozone ($P < 0.01$) and $PM_{2.5}$ ($P < 0.01$) compared those who passed the balance test. There was no difference in exposure to sulfur dioxide levels between those who failed and those who passed the balance test ($P = 0.61$).

In single pollutant models, participants living in areas with higher levels of ozone were more likely to fail the balance test at follow-up (odds ratio (OR) = 1.13 per interquartile range (IQR), 95% CI 1.02, 1.24) (Table 3). Other variables that were associated with failing the balance test included older age, female sex, non-White ethnicity, lower household incomes, current smoking, higher BMI, diabetes, visual impairment, ADL limitations, and visual impairment. When the health variables were added to the model, the odds ratio for ozone was unchanged. In contrast, in single pollutant models, $PM_{2.5}$ and sulfur dioxide levels were not associated with failing the balance test (Supplemental Tables 2–3).

There was some heterogeneity in the association between ozone and balance although we did not detect statistically significant interaction. When stratifying by diabetes, ozone was more strongly associated with balance in those with diabetes (OR = 1.60, 95% CI 1.20, 2.13) than in those without diabetes (OR = 1.08, 95% CI 0.97, 1.19) although the difference in the associations was not statistically significant (interaction term $P = 0.212$). Similarly, when stratifying by visual impairment, ozone was more strongly associated with balance in those with visual impairment (OR = 1.37, 95% CI 0.81, 2.31) than in those without visual impairment (OR = 1.11, 95% CI 1.01, 1.23). However, again, the difference in the associations was not statistically significant (interaction term $P = 0.082$).

The 3 pollutants were not highly correlated with each other (all r <

Table 1
Descriptive characteristics of participants by ability to pass the balance test.

	Passed Balance Test at Follow-Up n = 9,528 Mean (SD) or %	Failed Balance Test at Follow-Up n = 2,630 Mean (SD) or %
Age, Years (n = 12,158)	54.2 (6.2)	59.4 (9.2)
Sex		
Male (n = 6,304)	53%	52%
Female (n = 5,854)	47%	48%
Race		
White (n = 11,476)	93.9%	93.7%
Non-White (n = 672)	6.1%	6.4%
Household income		
>$150,000 (n = 5,910)	58%	42%
$50,000 -$100,000 (n = 3,897)	28%	35%
$20,000 - $50,000 (n = 1,556)	9%	16%
<$20,000 (n = 268)	1%	2%
Refused/Don't Know (n = 527)	4%	5%
Smoking		
Never (n = 6,410)	56%	48%
Former (n = 4,896)	37%	43%
Current (n = 822)	7%	9%
Body Mass Index		
<20 kg/m^2 (n = 421)	3%	3%
20–24 kg/m^2 (n = 4,333)	37%	26%
25–29 kg/m^2 (n = 5,050)	42%	42%
≥30 kg/m^2 (n = 2,348)	18%	29%
Diabetes		
No (n = 10,833)	91.5%	84.8%
Type 1 (n = 36)	0.2%	0.7%
Types 2 (n = 494)	2.9%	6.8%
Neither/Suspect (n = 728)	5.4%	7.7%
ADL Limitations		
No (n = 11,763)	98%	95%
Yes (n = 366)	2%	5%
Stroke		
No (n = 12,072)	99.6%	99.0%
Yes (n = 68)	0.4%	1.0%
Visual Impairment		
No (n = 11,661)	97.4%	95.0%
Yes (n = 453)	2.7%	5.0%

SD = standard deviation; ADL = activities of daily living.
The following variables had missing data: race/ethnicity (n = 10), smoking (n = 30), BMI (n = 6), diabetes (n = 67), ADL limitations (n = 29), stroke (n = 18), visual impairment (n = 44).

Table 2
Median levels of air pollution in postal codes of participant's residence by ability to pass the balance test.

	Passed Balance Test at Follow-Up n = 9,528 Median [IQR]	Failed Balance Test at Follow-Up n = 2,630 Median [IQR]	P-value
Ozone, ppb (n = 12,143)	25.6 [5.8]	26.1 [6.2]	<0.01
$PM_{2.5}$, μg/m^3 (n = 11,727)	6.4 [2.1]	6.5 [2.9]	<0.01
Sulfur dioxide, ppb (n = 10,952)	0.26 [0.22]	0.24 [0.22]	0.61

$PM_{2.5}$ = fine particulate matter; ppb = parts per billion; IQR = interquartile range.

Table 3
Multiple logistic regression results of relationship between ozone and failure on the balance test in single pollutant models.

	Model 1[b] Failed Balance Test at Follow-Up n = 11,948 OR[a] (95% CI)	Model 2[c] Failed Balance Test at Follow-Up n = 11,948 OR[a] (95% CI)
Ozone, Per IQR	1.13 (1.02, 1.24)	1.13 (1.02, 1.24)
Age, Per Year	1.11 (1.10, 1.12)	1.11 (1.10, 1.12)
Sex		
Male	1.00	1.00
Female	1.22 (1.10, 1.36)	1.19 (1.07, 1.34)
Race		
White	1.00	1.00
Non-White	1.43 (1.15, 1.79)	1.39 (1.11, 1.74)
Household income		
>$150,000	1.00	1.00
$50,000 -$100,000	1.25 (1.10, 1.42)	1.25 (1.09, 1.41)
$20,000 - $50,000	1.43 (1.22, 1.69)	1.39 (1.18, 1.64)
<$20,000	1.42 (1.02, 1.97)	1.34 (0.96, 1.88)
Refused/Don't Know	1.31 (1.01, 1.69)	1.30 (1.00, 1.68)
Smoking		
Never	1.00	1.00
Former	1.06 (0.95, 1.19)	1.05 (0.94, 1.18)
Current	2.00 (1.63, 2.45)	1.96 (1.60, 2.41)
Body Mass Index		
<20 kg/m^2	1.12 (0.82, 1.54)	1.10 (0.80, 1.51)
20–24 kg/m^2	1.00	1.00
25–29 kg/m^2	1.53 (1.34, 1.74)	1.49 (1.31, 1.70)
≥30 kg/m^2	3.15 (2.71, 3.66)	2.94 (2.53, 3.43)
Diabetes		
No		1.00
Type 1		3.72 (1.70, 8.13)
Types 2		1.59 (1.26, 2.00)
Neither/Suspect		1.22 (1.0.99, 1.51)
ADL Limitations		
No		1.00
Yes		1.97 (1.50, 2.59)
Stroke		
No		1.00
Yes		1.95 (0.93, 4.09)
Visual Impairment		
No		1.00
Yes		1.35 (1.03, 1.77)

OR = odds ratio; CI = confidence interval; ADL = activities of daily living; IQR = interquartile range.
[a] Also adjusted for province.
[b] Model 1 included 11,948 people (2,573 of whom failed the balance test) and included ozone, age, sex, race, income, smoking, body mass index, and province.
[c] Model 2 included 11,948 people (2,573 of whom failed the balance test) and included ozone, age, sex, race, income, smoking, body mass index, diabetes, ADL limitations, stroke, visual impairment, and province.

Table 4
Multiple logistic regression results of relationship between air pollutants and failure on the balance test in a multiple pollutant model.

	Failed Balance Test at Follow-Up n = 10,423 Adjusted OR[a]	95% CI
Ozone, Per IQR	1.16	1.04, 1.30
$PM_{2.5}$, Per IQR	1.01	0.91, 1.13
Sulfur dioxide, Per IQR	1.01	0.93, 1.10

OR = odds ratio; CI = confidence interval; ADL = activities of daily living; IQR = interquartile range.
[a] Adjusted for age, sex, race, income, smoking, BMI, diabetes, ADL limitations, stroke, visual impairment, and province.

0.3) and variance inflation factors were all under 4 indicating variance inflation was not a concern by combining them in a model together. In a multi-pollutant model (Table 4), the association between higher levels of ozone and failing the balance test slightly increased (OR = 1.16, 95% CI 1.04, 1.30). $PM_{2.5}$ and sulfur dioxide levels were still not associated with failing the balance test ($P > 0.05$).

4. Discussion

We found that higher levels of ozone were associated with the development of balance problems while $PM_{2.5}$ and sulfur dioxide were not. Ozone is one of the main components of smog, "a noxious mixture of gases and particles that often appears as haze in the air" (Go, 2022). Ozone forms at the ground level when pollutants such as oxides and volatile organic compounds emitted by cars, power plants, refineries, and other sources chemically react in sunlight.

Previous studies have found that higher ozone levels are associated with a higher risk of mortality (Bell et al., 2004; Vicedo-Cabrera et al., 2020), emergency department visits (Zheng et al., 2021), hospital admissions (Zheng et al., 2021), frailty (Shin and Choi, 2021), diabetes (Jerrett et al., 2017), platelet activation (Day et al., 2017), worse lung function (Wang et al., 2019), and blood pressure increases (Day et al., 2017). To our knowledge, there is no research that indicates a direct effect of ozone on balance. Rather, we speculate that ozone is related to balance by its effect on conditions known to affect balance. For example, higher ozone levels may be related to balance by their impact on diabetes-related complications (Jerrett et al., 2017). Diabetes was strongly related to the new onset of failing the balance test in our data. Adjusting for diabetes did not attenuate the odds ratio for ozone as one would expect if diabetes mediated this association., However, the diabetes data were based on self-report and we did not have data on the severity of diabetes so we may not have removed the whole effect of diabetes from the odds ratio for ozone. Diabetes-related complications like peripheral neuropathy could have an impact on balance by affecting the proprioceptive system. In addition, there is evidence that air pollution may impact the visual and vestibular systems (Grant et al., 2021; Chua et al., 2019; Mun et al., 2021), which help to regulate balance. However, adjusting for visual impairment did not attenuate the odds ratio for ozone and we did not have data on vestibular function. If the association between ozone and balance is causal, further work is needed to understand the biological mechanism.

To our knowledge, no prior studies have examined the relationship between ozone and standing balance by itself. Our finding is somewhat inconsistent with Shin et al. who reported that higher levels of ozone were not associated with worse scores on the SPPB, which includes a balance assessment, time to complete a 4-m walk, and time to rise from a chair. In that study, those in the highest exposure quartile for ozone did not have a statistically significantly higher odds of having a worse score on the SPPB (OR = 1.75, 95% CI 0.90, 3.39) although the odds ratio was elevated. Instead, those in the highest exposure quartile for fine particulate matter had a higher odds of having a worse score on the SPPB (OR = 1.87, 95% CI 1.15, 3.05).

Strengths of this work are the use of a very large, population-based sample with longitudinal data collection and the measurement of multiple pollutants. However, some limitations must be acknowledged. First, air pollution is measured at the level of the postal code of the participant's residence. There may be variability in air pollution levels within a postal code depending on its size. Also, if people spend very little time at their residence, the air pollution measured at their postal code may not accurately reflect their actual air pollution exposure resulting in measurement error. Further research should collect information on how much time people spend at their residence or in their postal code. Second, the risk of residual confounding should be acknowledged. CLSA participants who were older, racial minorities, and had lower household incomes lived in areas exposed to slightly higher levels of air pollution according to previous work (Grant et al., 2021). Although we adjusted for age, race, and household income, there may be other differences in areas with higher levels of air pollution that we did not adjust for that may be confounding our results. Missing data were a limitation for $PM_{2.5}$ and SO_2. Finally, some people were excluded from the CLSA including those in institutions and those with obvious cognitive impairment at baseline. Our results may not generalize to these populations.

In conclusion, our results indicate that people living in areas with higher levels of ozone are more likely to develop balance problems. Our results should be confirmed and an exploration of the mechanism of action underlying this finding should be performed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114114.

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Alteration of oral microbiome composition in children living with pesticide-exposed farm workers

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ABSTRACT

Our prior work shows that azinphos-methyl pesticide exposure is associated with altered oral microbiomes in exposed farmworkers. Here we extend this analysis to show the same association pattern is also evident in their children. Oral buccal swab samples were analyzed at two time points, the apple thinning season in spring-summer 2005 for 78 children and 101 adults and the non-spray season in winter 2006 for 62 children and 82 adults. The pesticide exposure for the children were defined by the farmworker occupation of the cohabitating household adult and the blood azinphos-methyl detection of the cohabitating adult. Oral buccal swab 16S rRNA sequencing determined taxonomic microbiota proportional composition from concurrent samples from both adults and children. Analysis of the identified bacteria showed significant proportional changes for 12 of 23 common oral microbiome genera in association with azinphos-methyl detection and farmworker occupation. The most common significantly altered genera had reductions in the abundance of *Streptococcus*, suggesting an antimicrobial effect of the pesticide. Principal component analysis of the microbiome identified two primary clusters, with association of principal component 1 to azinphos-methyl blood detection and farmworker occupational status of the household. The children's buccal microbiota composition clustered with their household adult in ~95% of the households. Household adult farmworker occupation and household pesticide exposure is associated with significant alterations in their children's oral microbiome composition. This suggests that parental occupational exposure and pesticide take-home exposure pathways elicit alteration of their children's microbiomes.

1. Introduction

Apple orchard farmworker occupation and azinphos-methyl (AZM) pesticide exposure have been shown to be associated with alterations of the oral buccal microbiome (Stanaway et al., 2017). AZM, and other organophosphates, have an acute mechanism of toxicity by covalently bonding to the serine-200 residue in the active site of cholinesterase (Fukuto, 1990). It is likely AZM can interact with other similar serine hydrolase protein moieties in many organisms, including bacteria. This drives our scientific inquiry to assess the compositional changes of the microbiome which may occur in association with AZM exposure. We have previously shown a robust association between blood detection of AZM and alterations of the buccal microbiome composition, particularly reductions in *Streptococcus* with the adults in this cohort (Stanaway et al., 2017). This report extends this investigation to include the children cohabitating with the adults. While AZM has been banned in the United States, the continued use of structurally related organophosphates (OPs) makes this report of Public Health importance for the well-being of agricultural workers and their children.

These farmworkers have children who are likely to be exposed to pesticides as workers come home after work, bringing pesticide contamination with them (Coronado et al., 2006). This gives the potential for household pesticide exposure to similarly alter the microbiomes of these children cohabitating with pesticide exposed adults.

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Table 1
Cohort demographics and exposure groups.

Adults	Spring-Summer 2005 Thinning Season		Winter 2005–2006	
	N = 101		N = 82	
	Farmworker	Non-farmworker	Farmworker	Non-farmworker
	N = 54	N = 47	N = 42	N = 40
Male	9 (17%)	8 (17%)	7 (17%)	7 (18%)
Female	45 (83%)	39 (83%)	35 (83%)	33 (82%)
Adult Age				
under 25	4 (7%)	8 (17%)	3 (7%)	3 (8%)
25 to 29	11 (20%)	9 (19%)	8 (19%)	10 (25%)
30 to 34	23 (43%)	13 (28%)	18 (43%)	12 (30%)
35 to 44	14 (26%)	16 (34%)	11 (26%)	13 (33%)
45 to 54	2 (4%)	1 (2%)	2 (5%)	1 (3%)
55 or over	0	0	0	1 (3%)
AZM detected in blood[a]				
Yes	29 (54%)	0	0	1 (3%)
No	25 (46%)	47 (100%)	42 (100%)	39 (97%)
Reported Adult Race and Ethnicity				
White	1 (2%)	0	1 (2%)	0
Hispanic	53 (98%)	47 (100%)	41 (98%)	40 (100%)
Children				
	N = 78		N = 62	
	N = 42	N = 36	N = 27	N = 35
Male	19 (45%)	23 (64%)	9 (33%)	21 (60%)
Female	23 (55%)	13 (36%)	18 (67%)	14 (40%)
Child Age				
2	9 (21%)	7 (19%)	2 (5%)	2 (6%)
3	9 (21%)	9 (25%)	7 (26%)	5 (14%)
4	17 (40%)	7 (19%)	8 (27%)	13 (37%)
5	2 (5%)	5 (14%)	5 (19%)	3 (9%)
6	3 (7%)	7 (19%)	3 (11%)	8 (23%)
7	2 (5%)	0	2 (5%)	4 (11%)
AZM detected in parent blood				
Yes	23 (55%)	0	0	1 (3%)
No	19 (45%)	36 (100%)	27 (100%)	34 (97%)

[a] LOD for AZM <0.04 ng/g.

This longitudinal cohort consists of pome fruit (apples and pears) farmworker and non-farmworker households from the Yakima Valley, Washington. The households of this cohort are predominantly Hispanic with each represented by an adult farmworker or non-farmworker and one cohabitating child between the ages of 2–6 years old at the time of data collection. This cohort aimed to study the potential for childhood exposures by agricultural pesticide residuals via their cohabitating paired adults' occupational exposures taken home on clothing and residual dust. Previous studies of this cohort identified and characterized pesticide exposures of pome fruit orchard workers directly by measured blood pesticide concentration in adults, and in their children by urine pesticide metabolites, and by household dust pesticide detection (Coronado et al. 2006, 2009, 2010, 2011, 2012; Holme et al., 2016; Nonnenmann et al., 2012; Smith et al. 2015b, 2017; Thompson et al., 2014; Tamaro et al., 2018; Bennett et al., 2019). AZM was a widely used organophosphate pesticide in the Washington State pome fruit orchard industry. The Environmental Protection Agency has removed AZM from registration with legal applications ceasing in the United States as of September 30, 2013.

2. Material and methods

2.1. Agricultural setting and comparison groups

Participants were recruited from 199 Yakima Valley agricultural community households as reported in detail by Thompson et al. (2014). Written and informed consent was obtained from each adult and consent of the guardian for the child. The Fred Hutchinson Cancer Research Center Institutional Review Board approved the study collection (File IR 5946). During April to July 2005 (spring-summer) pome farmworkers thinned excess fruit and branches of pome fruit trees treated with OPs giving the potential for exposure to pesticide residues (Holme et al., 2016; Nonnenmann et al., 2012; Smith et al., 2017; Stanaway et al., 2017; Thompson et al., 2014; Tamaro et al., 2018; Bennett et al., 2019). Whole blood from adult participants and buccal swabs from paired adult and child participants in the same household were collected. Samples were collected in two distinct agricultural seasons: the thinning season in the spring-summer of 2005 and the non-spray season of winter 2006. Sample attrition allowed for the analytic cohort to be 78 children and 101 adults in the thinning season (spring-summer 2005) and 62 children and 82 adults in the non-spray season (winter 2006). Household exposure groups were defined by pome fruit orchard farmworker occupation and measurement of AZM in the adults' blood as two measures of exposure. In the thinning season we analyzed 54 adult farmworkers and 47 non-farmworkers. In the winter non-spray season 42 farmworkers and 40 non-farmworkers were analyzed. Twenty-nine of these adults has AZM detected in their blood. Children were categorized to the farmworker status of the enrolled cohabitating household adult, and the adults' AZM blood detection exposure groups. In the spring-summer thinning season, we analyzed 42 children of farmworkers and 36 children of non-farmworkers. In the winter non-spray season 27 children of farmworkers and 35 children of non-farmworkers were analyzed. Twenty-three of the children lived with adults with AZM detected in their blood. The exposed and unexposed groups were compared to the microbiota composition while stratified by adult and child status, and when adult and child sample groups are combined.

2.2. Adult blood AZM

Blood samples were collected from adult participants. The blood AZM concentrations were determined by mass spectrometry as described by Barr et al., (2002). Briefly, 4-g aliquots of blood plasma with spike in of stable isotope labeled internal standards (100 μL of 100 pg/uL) were denatured in ammonium sulfate, centrifuge pelleted (3400 rpm, 5 min) and the supernatant solid-phase extracted on OASIS SPE columns with methylene chloride elution. The resulting eluates were passed through anhydrous sodium sulfate cartridges. Analysis with isotope dilution gas chromatography–high-resolution mass spectrometry was performed on a Hewlett-Packard 6890+ gas chromatograph connected to a MAT 900 trap mass spectrometer (ThermoFinnigan). A calibration curve (0.25, 0.5, 2, 5, 10, 20, 50, 100, 200 and 400 pg/uL) was used to quantify the AZM in blood. Individual participants were categorized to detect or non-detect for statistical analysis. The limit of detection is 0.04 ng/g plasma.

2.3. Microbiome 16S rRNA buccal swab sequencing

Oral buccal samples were collected using the Catch-all™ Sample Collection Swab (Epicentre, Madison, WI) and stored at −80 °C. Samples were thawed then diluted with 9 mL of phosphate buffered saline (PBS), centrifuged, aspirated and DNA extracted using the Maxwell® 16 Buccal Swab LEV DNA Purification Kit (Promega, Fitchburg, Wisconsin) kit. The Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) determined DNA concentrations. PCRs were loaded with 10 ng of DNA. Eurofins (Eurofins Scientific, Luxembourg) primers were diluted to 18 μM. Primers and the EmeraldAmp GT PCR Master Mix (Clontech Laboratories, Inc., Mountain View, CA) were added to each PCR at final primer concentrations of 0.9 μM. Primer sequences targeted the 16S rRNA gene variable region 5 (V5, ATTAGATACCCNGGTAG) and variable region 6 (V6, CGACAGCCATGCANCACCT) of the 16S ribosomal subunit DNA gene as in Cai et al. (2013). Primers were designed to be bidirectional with four oligonucleotides including the reverse complements, the Ion Torrent™ sequencing adaptors and 96 unique DNA barcodes. PCRs were performed with a MJ Research PTC 200 Peltier Thermal Cycler (MJ

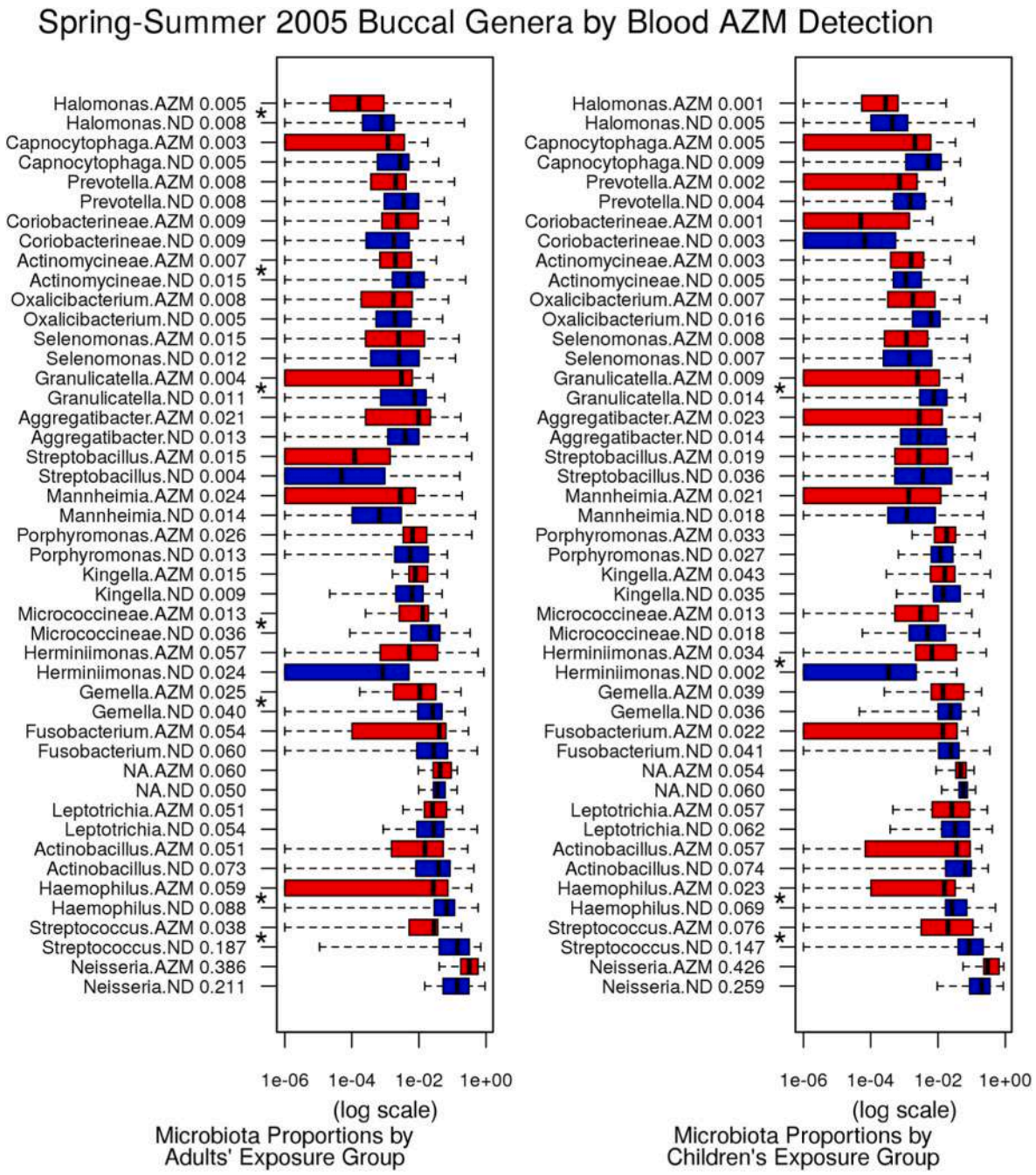

Fig. 1. Spring-summer Adult and Child Buccal Bacteria by Azinphos-methyl (AZM) Detection
Spring-summer buccal swab sample sequencing of 23 common (>0.5% sample mean) microbiota dichotomized by spring-summer blood sample Azinphos-methyl blood mass spectrometry detection. Panel boxplots with zero genera proportion samples are set to 1e-6. Black bars, boxes and whisker bars respectively show the median, the central quartiles and the extreme values. The panel label texts report the graphed group labels and mean proportions. Genera are ordered from most abundant at the bottom to least abundant at the top. Genera labeled with a * are significant (Wilcoxon's FDR<0.1) between AZM exposed (adults, n = 29; children n = 23) and unexposed (ND, adults, n = 72; children, n = 55) groups.

Research, St. Bruno, Quebec, Canada) using the temperature cycle protocol presented in (Stanaway et al., 2017). The Agencourt AMPure XP PCR purification system (Beckman Coulter, Brea, CA) purified the PCR amplicons. Sample PCR DNA amplicons were bioanalyzed for fragment length and quantified using the Agilent High Sensitivity DNA Kit (Agilent Technologies Inc., Santa Clara, CA) and diluted in Low TE buffer to yield ~26pM for sequencing. PCR amplicons were pooled for sequencing with the Ion Torrent™. Quality control discarded reads without primer sequences detected and those with >3 expected errors based on the PHRED score (Edgar 2013; Ewing and Green 1998; Ewing et al., 1998). We generated a de-novo 16S rRNA Operational Taxonomic Unit (OTU) reference at 97% sequence identity with USEARCH/UPARSE following the software manual (Edgar 2013). Assignment of OTU reference sequences to bacterial taxa was performed with the RDP Bayesian Classifier v2.6 java program default settings (Wang et al., 2007). Alignment of 16S rRNA read sequences to the OTU references was performed with USEARCH to generate taxonomic read counts per sample.

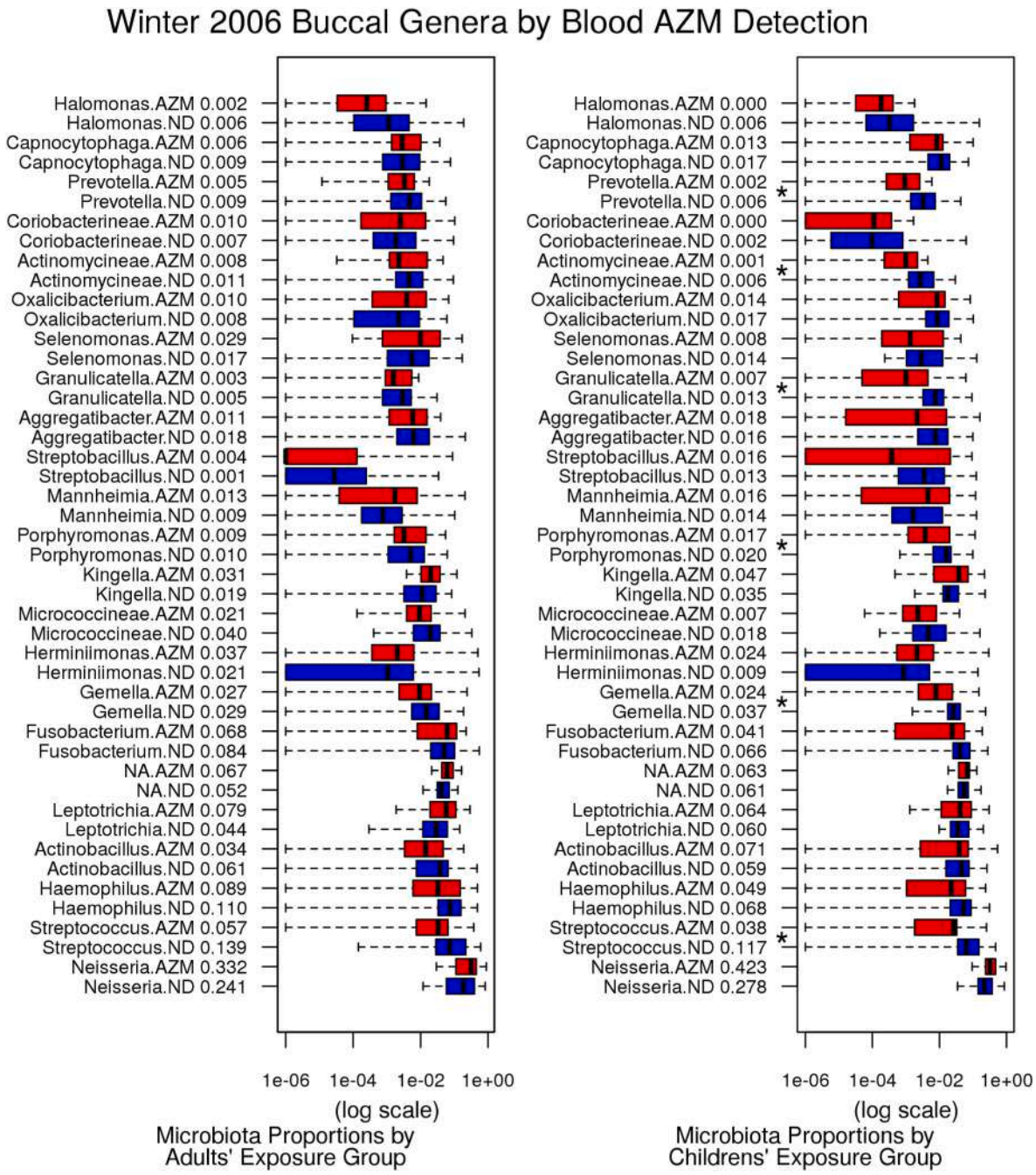

Fig. 2. Winter Adult and Child Buccal Microbiota by Azinphos-methyl (AZM) Detection
Winter buccal swab sample sequencing common (>0.5% sample mean) microbiota dichotomized by the previous summer's Azinphos-methyl blood mass spectrometry detection. Panel boxplots with zero genera proportion samples are set to 1e-6. Black bars, boxes and whisker bars respectively show the median, the central quartiles and the extreme values. The panel label texts report the graphed group labels and mean proportions. Genera are ordered from most abundant at the bottom to least abundant at the top. Genera labeled with a * are significant (Wilcoxon's FDR<0.1) between AZM exposed (adults, n = 26; children, n = 19) and unexposed (ND, adults, n = 56; children, n = 43) groups.

2.4. Statistical methods

Hypothesis testing was conducted by comparing dichotomized exposure groups determined using two variables, adult AZM detection (≥LOD) versus not detected groups and household adult pome fruit orchard farmworker occupation versus non-farmworker groups. The children were assigned to the same dichotomized exposure group as their cohabitating adult. Binary dichotomized classification of exposed and unexposed states has been proposed as an appropriate model to test temporally sparse exposure sampling data over life course (Smith et al., 2015a).

The negative binomial identity, Pr[missed taxon] = e^{-pn}, with p proportion of taxon reads and n sample read depth of sequencing, quantifies the probability of not observing a taxonomy member if it truly exists in a sample (Hodges and Le Cam, 1960; Turnbaugh et al., 2009). Microbiome samples were considered for analysis if the depth of sequencing was >2500 reads to ensure the probability of detection of common genera at this sequencing depth. The molecular taxonomy of de-novo OTU clustering was defined by the Hamming distances between DNA read sequences clustered with homology at 97% sequence identity.

Table 2
Wilcoxon's Rank Sum Tests of Spring-Summer Thinning Season 2005) Buccal Microbiota by Adult Azinphos-methyl Blood Detection
AZM exposed (adults, n = 29; children n = 23) and unexposed (ND, adults, n = 72; children, n = 55).

Genera	Adults and Children Combined					Adults					Children				
	Loc. Dif.	lower	upper	p-value	FDR	Loc. Dif.	lower	upper	p-value	FDR	Loc. Dif.	lower	upper	p-value	FDR
Streptococcus	−1.70	−2.39	−1.05	0.000002	[a]0.00004	−1.98	−2.74	−1.08	0.00005	[a]0.001	−1.37	−2.59	−0.40	0.009	[a]0.095
Herminiimonas	1.22	0.71	1.85	0.000054	[a]0.0006	1.01	−0.04	1.62	0.081	0.18	1.85	0.97	2.95	0.00003	[a]0.001
Haemophilus	−1.13	−1.75	−0.49	0.000251	[a]0.002	−1.05	−1.92	−0.24	0.008	[a]0.05	−1.08	−2.15	−0.21	0.01	[a]0.095
Granulicatella	−0.89	−1.50	−0.29	0.001	[a]0.007	−0.89	−1.64	0.00	0.026	[a]0.09	−0.89	−1.83	−0.16	0.02	[a]0.096
Micrococcineae	−0.88	−1.46	−0.31	0.004	[a]0.02	−1.06	−1.74	−0.43	0.002	[a]0.02	−0.53	−1.59	0.57	0.34	0.6
Gemella	−0.82	−1.44	−0.25	0.005	[a]0.02	−0.99	−1.96	−0.25	0.009	[a]0.05	−0.53	−1.51	0.26	0.17	0.4
Capnocytophaga	−0.57	−1.04	−0.04	0.02	[a]0.05	−0.57	−1.16	0.00	0.040	0.11	−0.61	−1.35	0.12	0.13	0.4
Actinobacillus	−0.80	−1.55	−0.08	0.03	[a]0.08	−0.98	−2.09	0.13	0.09	0.18	−0.62	−1.55	0.24	0.16	0.4
Halomonas	−0.54	−1.10	0.00	0.03	[a]0.08	−0.80	−1.49	−0.07	0.01	[a]0.06	−0.12	−1.02	0.43	0.6	0.8
Mannheimia	0.49	0.00	1.16	0.05	0.12	0.79	0.00	1.55	0.04	0.11	0.13	−0.49	1.16	0.5	0.8
Neisseria	0.35	−0.02	0.72	0.06	0.12	0.45	−0.08	1.07	0.09	0.18	0.25	−0.25	0.73	0.3	0.6
Actinomycineae	−0.54	−1.13	0.02	0.06	0.12	−1.00	−1.72	−0.20	0.01	[a]0.06	0.12	−0.56	0.71	0.7	0.8
Fusobacterium	−0.49	−1.16	0.00	0.08	0.14	−0.27	−1.16	0.30	0.37	0.6	−0.70	−1.68	0.10	0.10	0.3
Oxalicibacterium	−0.42	−1.13	0.05	0.09	0.15	−0.12	−0.90	0.64	0.62	0.7	−0.92	−2.06	0.03	0.06	0.3
Prevotella	−0.46	−0.98	0.08	0.10	0.16	−0.68	−1.47	0.13	0.11	0.2	−0.03	−0.87	0.65	0.8	0.8
otu_NA	−0.26	−0.64	0.10	0.16	0.2	−0.05	−0.68	0.43	0.8	0.9	−0.45	−1.00	0.07	0.08	0.3
Leptotrichia	−0.22	−0.70	0.27	0.4	0.5	0.00	−0.59	0.59	1.0	1.0	−0.51	−1.39	0.30	0.17	0.4
Selenomonas	−0.18	−0.80	0.38	0.4	0.6	−0.22	−1.11	0.81	0.6	0.7	−0.09	−1.06	0.60	0.6	0.8
Porphyromonas	−0.16	−0.64	0.37	0.5	0.6	−0.19	−0.90	0.62	0.5	0.7	−0.16	−0.87	0.57	0.7	0.8
Aggregatibacter	0.07	−0.40	0.75	0.6	0.7	0.12	−0.60	1.13	0.6	0.7	0.07	−0.72	1.05	0.7	0.8
Streptobacillus	0.00	−0.57	0.47	0.8	0.8	0.00	−0.25	0.56	1.0	1.0	−0.34	−1.56	0.78	0.6	0.8
Coriobacterineae	0.00	−0.41	0.69	0.8	0.8	0.13	−0.62	0.94	0.6	0.7	0.00	−0.47	0.76	0.9	0.9
Kingella	0.02	−0.46	0.51	0.9	0.9	0.35	−0.24	0.98	0.19	0.3	−0.44	−1.17	0.33	0.3	0.5

[a] Significant at an FDR<0.1 between Azinphos-methyl exposed and unexposed groups, lower and upper are the 95% confidence intervals of the location difference (Loc. Dif.).

OTU clustering provides a physio-chemical DNA homology-taxonomy independent of the assumed traditional Linnaean nomenclature. We overlay on the molecular sequence OTU ontology the Linnaean taxonomy by assigning the OTU groups to genera using the RDP Bayesian Classifier (Wang et al., 2007). Exposure group differences in microbiome composition were tested in genera detected at >0.5% mean proportions of the adult and children's samples by Wilcoxon's Rank-Sum Tests. A false discovery rate (FDR<0.1) was used in significance testing (Hochberg and Benjamini 1990). Microbiome 16S DNA count data simplex geometry posits a compositional taxa's perturbation (i.e., reductions due to exposure) which has auto-correlated compensatory proportional increases in other taxa. The remaining count of taxa reads are occupied by other remaining compositional members (Fernandes et al., 2014). To adjust for proportional bias, we applied the 'centered-log-ratio' transformation (Aitchison, 1982; Fernandes et al., 2014; Lovell et al., 2015; Mclaren et al., 2019) to the taxonomic proportions.

Principal Component Analyses (PCA) used the molecular taxonomy for common OTUs >0.1% sample mean proportion to capture perturbations in the moderately rare OTUs. PCA of the centered-log-ratio transformed OTU proportions and cluster fitting using a model-based algorithm in the R mclust package (Fraley and Raftery 2002) defined microbiome groups for hypothesis testing with Fisher's Exact test. The adult spring-summer 2005 buccal sample collection was fitted to establish a decision value from Principal Component 1 (PC1) discriminating two microbiome cluster phenotypes. To avoid overfitting the data, the slope loadings from the adults' spring-summer PCA fit was used to project the winter data for all other samples (adults and children) including the children's samples for summer. AZM detection in adult blood and pome farmworker occupation groups for both the adults and children were compared to PC1 scores by Welch's *t*-test and between clusters by Fisher's Exact Test to test the hypothesis that agricultural pome work and pesticide exposure is associated with the microbiome composition of individuals in these groups.

3. Results

3.1. Blood AZM ascertainment among microbiome sequenced samples

We report the blood AZM detection counts among adult samples where we also have quality controlled oral microbiome data. The children's AZM exposure was defined by their cohabitating adults' blood AZM values. See Table 1 for a summary of ascertained samples in each season. All microbiome sequenced adults (n = 101) from the spring-summer collection had mass-spectrometry blood data. Seventy-two (72) of these adults (farmworkers and non-farmworkers) with microbiome data in the spring-summer, had no detected AZM in the blood. Twenty-nine (29) of the adult farmworkers had detection of blood AZM and microbiome data in the spring-summer. One (1) adult sample has a non-zero AZM result (0.021 ng/g) below the limit of detection (0.04 ng/g) and was treated as a non-detect. Of the 101 sampled spring-summer adults with AZM and microbiome data, 78 also had a sampled child with microbiome data. The adult AZM detected values allowed for the classification of 23 AZM exposed children in the spring-summer portion of the cohort by using the parent's AZM detection as a proxy. In the winter blood collection, 82 adults also had microbiome sequence data. One of these was an adult non-farmworker with detected AZM and this individual was a non-detect in the summer blood collection. Thirteen (13) adults with microbiome data from the winter collection had no winter blood AZM data but were sampled in the spring-summer. These adults and their children were removed from the winter analysis leaving 82 adults and 62 children for hypothesis testing of the winter portion of the analysis. Twenty-six (26) adults from the winter microbiome data had AZM detected in either spring-summer or winter blood collections. This adult AZM data allowed for the AZM exposure group classification of 19 children in the winter microbiome data based on the adult values from either season.

Table 3
Wilcoxon's Rank Sum Tests of Winter (2006) Buccal Microbiota by Adult Azinphos-methyl Blood Detection
AZM exposed (adults, n = 26; children, n = 19) and unexposed (ND, adults, n = 56; children, n = 43).

Genera	Adults and Children Combined					Adults					Children				
	Loc. Dif.	lower	upper	p-value	FDR	Loc. Dif.	lower	upper	p-value	FDR	Loc. Dif.	lower	upper	p-value	FDR
Streptococcus	−1.33	−2.06	−0.66	0.0001	[a]0.002	−1.19	−2.11	−0.30	0.01	0.14	−1.68	−3.05	−0.63	0.002	[a]0.03
Gemella	−0.99	−1.59	−0.33	0.003	[a]0.02	−0.65	−1.58	0.33	0.17	0.4	−1.37	−2.28	−0.45	0.004	[a]0.03
Actinomycineae	−1.01	−1.65	−0.33	0.003	[a]0.02	−0.70	−1.49	0.10	0.09	0.3	−1.43	−2.24	−0.30	0.006	[a]0.03
Halomonas	−0.82	−1.43	−0.23	0.004	[a]0.02	−1.04	−1.83	−0.19	0.01	0.14	−0.41	−1.24	0.10	0.17	0.3
Granulicatella	−0.72	−1.22	−0.14	0.008	[a]0.04	−0.23	−0.86	0.38	0.48	0.79	−1.36	−2.37	−0.44	0.004	[a]0.03
Prevotella	−0.86	−1.49	−0.20	0.01	[a]0.04	−0.49	−1.25	0.32	0.21	0.4	−1.48	−2.49	−0.51	0.006	[a]0.03
Haemophilus	−0.88	−1.68	−0.15	0.01	[a]0.04	−0.88	−1.97	0.07	0.1	0.3	−0.89	−2.20	0.08	0.07	0.20
Micrococcineae	−0.82	−1.50	−0.16	0.02	[a]0.04	−0.78	−1.59	−0.09	0.03	0.2	−0.93	−1.89	0.19	0.10	0.24
Neisseria	0.45	0.01	0.89	0.04	0.103	0.46	−0.17	1.11	0.13	0.4	0.45	−0.26	1.06	0.2	0.3
Porphyromonas	−0.58	−1.14	−0.01	0.04	0.103	−0.18	−0.97	0.60	0.7	0.8	−1.11	−1.88	−0.19	0.01	[a]0.04
Actinobacillus	−0.59	−1.32	0.05	0.09	0.19	−0.67	−1.70	0.24	0.1	0.4	−0.47	−1.70	0.48	0.41	0.50
Fusobacterium	−0.52	−1.26	0.07	0.10	0.2	−0.17	−1.26	0.60	0.59	0.8	−1.00	−2.08	−0.03	0.03	0.11
Mannheimia	0.48	−0.06	1.13	0.13	0.2	0.55	−0.25	1.31	0.28	0.5	0.47	−0.45	1.62	0.3	0.5
Kingella	0.35	−0.16	0.88	0.16	0.3	0.60	−0.07	1.30	0.1	0.3	0.14	−0.89	1.02	0.77	0.77
Capnocytophaga	−0.35	−1.01	0.19	0.19	0.3	−0.07	−0.93	0.63	0.8	0.8	−0.79	−1.58	0.06	0.1	0.2
Aggregatibacter	−0.35	−1.05	0.25	0.3	0.4	−0.01	−1.06	0.73	0.8	0.8	−0.83	−1.94	0.26	0.16	0.3
Selenomonas	−0.41	−1.19	0.38	0.3	0.4	−0.23	−1.27	1.02	0.73	0.8	−0.73	−1.89	0.45	0.2	0.4
Herminiimonas	0.17	−0.29	0.75	0.4	0.6	0.14	−0.60	0.87	0.6	0.8	0.17	−0.63	1.28	0.6	0.6
Leptotrichia	0.12	−0.35	0.57	0.7	0.8	0.42	−0.16	1.06	0.2	0.4	−0.36	−1.10	0.41	0.3	0.5
Streptobacillus	0.00	−0.54	0.24	0.8	0.8	0.00	0.00	0.47	0.7	0.8	−0.43	−1.68	0.63	0.4	0.5
Oxalicibacterium	−0.06	−0.82	0.61	0.8	0.8	0.13	−0.83	1.37	0.7	0.8	−0.37	−1.42	0.54	0.5	0.5
Coriobacterineae	−0.01	−0.74	0.61	0.8	0.8	0.12	−0.88	1.03	0.8	0.8	−0.03	−0.89	0.43	0.5	0.5
otu_NA	0.03	−0.29	0.37	0.9	0.9	0.18	−0.26	0.62	0.4	0.8	−0.24	−0.61	0.28	0.4	0.5

[a] Significant at an FDR<0.1 between Azinphos-methyl exposed and unexposed groups, lower and upper are the 95% confidence intervals of the location difference (Loc. Dif.).

3.2. Microbiome census of 16S rRNA

We identified 2520 OTUs with >7.41 nucleotide differences between OTUs which classify to 286 genera. Twenty-three (23) of these genera were common at >0.5% mean proportion among child and adult samples. The median buccal sample read depth is 34,212 reads. The depth of sequencing parameters, minimum (>2500) and median depth of sequencing, gives us a >0.999 probability of detecting the common genera present in >0.5% of reads.

3.3. Microbiome genera associated with AZM exposure groups

The household adult blood detection of AZM allowed for categorization of the children's exposure by proxy. The Wilcoxon's Rank Sum tested the adult and child microbiome samples both independently and combined to assess which microbiota are perturbed between the AZM exposed and unexposed groups. Twelve common (>0.5% mean proportion) genera are significantly (FDR <0.1) associated in the spring-summer 2005 and/or winter 2006 with AZM exposure among the children and adult tests. These genera included *Streptococcus, Herminiimonas, Haemophilus, Granulicatella, Micrococcineae, Gemella, Capnocytophaga, Actinobacillus, Halomonas, Actinomycineae, Prevotella,* and *Porphyromonas*. Genera proportional abundances and association statistics are summarized in Fig. 1 (spring-summer) and Fig. 2 (winter) and Table 2 (spring-summer) and Table 3 (winter) for the AZM exposure groups. The spring-summer adult data show *Streptococcus, Haemophilus, Granulicatella, Micrococcineae, Gemella, Halomonas,* and *Actinomycineae* (Table 2) are significantly reduced in those with AZM detected in blood. The spring-summer microbiomes of the children cohabitating with adults with AZM exposure also showed a reduction of *Streptococcus, Haemophilus, Granulicatella,* but an increase in *Herminiimonas*. When the adults' and children's data is combined *Streptococcus, Haemophilus, Granulicatella, Micrococcineae, Gemella, Capnocytophaga, Halomonas,* and *Actinobacillus* are reduced in abundance while *Herminiimonas* is increased (FDR <0.1). No significant differences were identified as FDR = 0.1 in the winter 2006 microbiome composition between adults with and without AZM detection. However, children maintained robust differences in the composition of their microbiome in winter between the AZM detect and non-detect exposure groups with six significantly perturbed genera (*Streptococcus, Gemella, Actinomycineae, Granulicatella, Prevotella,* and *Porphyromonas*). When adults and children's samples are combined, *Streptococcus, Gemella, Actinomycineae, Halomonas, Granulicatella, Prevotella, Haemophilus,* and *Micrococcineae* are significantly reduced in abundance by AZM exposure (FDR<0.1, See Fig. 2 and Table 3 for winter 2006 proportional abundances and summary statistics).

3.4. Microbiome genera associated with household occupation and Co-habitation

Significant perturbations in 11 common (>0.5% mean proportion) genera detected in children and adults are associated with household orchard farmworker agricultural occupation. The microbiota includes *Streptococcus, Neisseria, Haemophilus, Gemella, Micrococcineae, Granulicatella, Herminiimonas, Actinobacillus, Leptotrichia, Kingella*, and *Halomonas*. Proportional abundances and statistics are summarized in Fig. 3 and Fig. 4 and Table 4 (spring-summer) and Table 5 (winter) for the occupational exposure groups. The adult farmworkers and the children cohabitating with farmworkers both showed significant reductions in *Streptococcus* and increases in *Neisseria* compared to non-farmworker households with the spring-summer data. The children cohabitating with farmworkers also showed significant spring-summer reductions in *Haemophilus, Gemella* and *Granulicatella* and increases in *Herminiimonas*. When adult and child spring-summer 2005 samples are combined in analysis *Neisseria* is increased in abundance, while, *Streptococcus, Haemophilus, Gemella,* and *Micrococcineae* are all significantly reduced (FDR<0.1) (see Table 4).

Adult farmworkers winter samples had significant reductions in the genera *Halomonas, Streptococcus, Actinobacillus*, and increases in *Leptotrichia, Kingella,* and *Neisseria*. The microbiomes of children in the winter had no significant genera associated with co-habitation with farmworkers detected at FDR = 0.1, although *Halomonas* and *Streptococcus* had low p-values (p~0.03) and decreased proportional abundance similar to the adults. When the adults' and children's winter microbiome samples are combined for analysis, farmworker associated genera include decreased proportions of *Streptococcus* and *Halomonas* (see

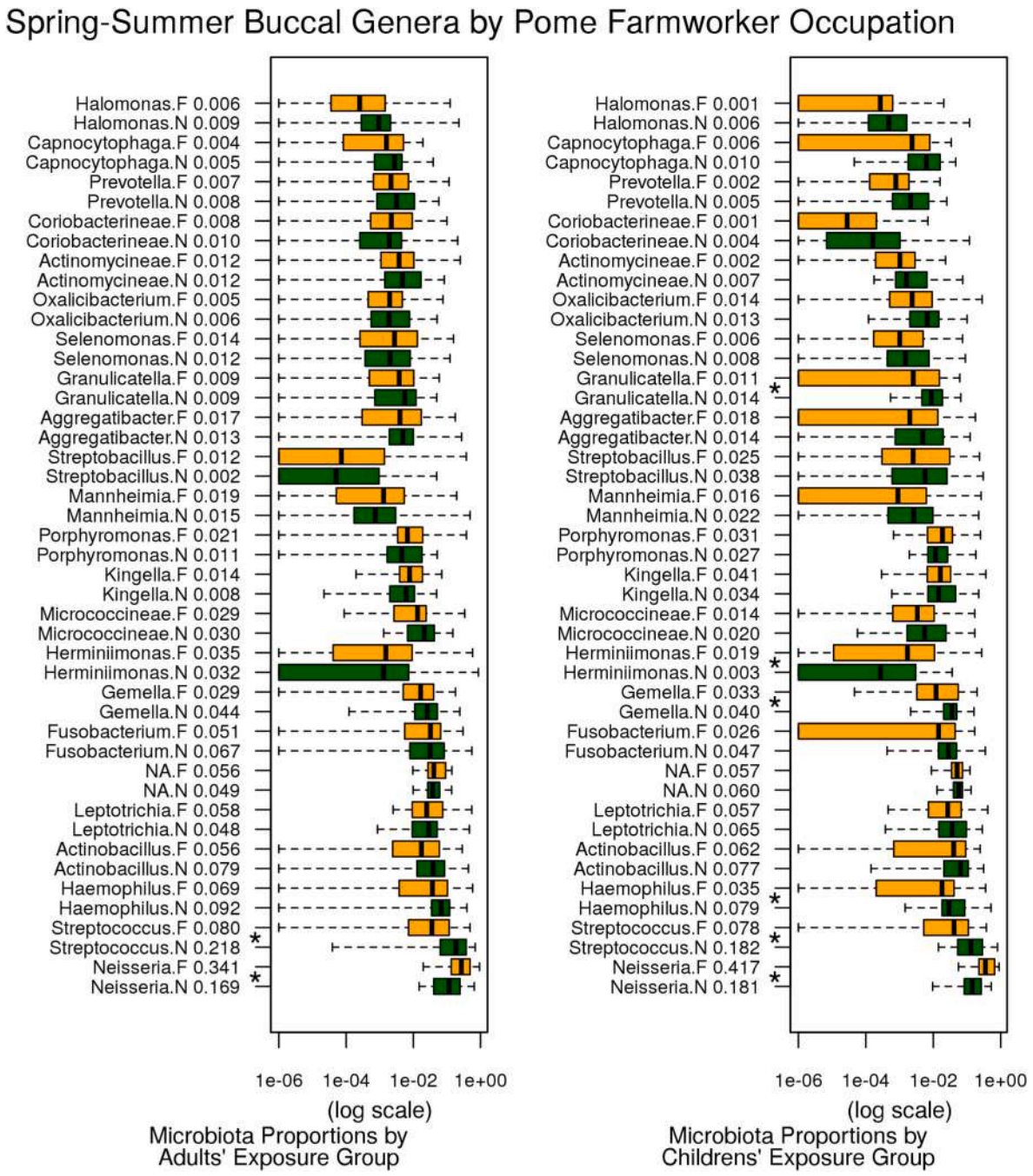

Fig. 3. Spring-summer Adult and Child Buccal Microbiota for Farmworker (F) and Non-Farmworker (N) Category Household
Spring-summer buccal swab sample sequencing common (>0.5% sample mean) microbiota dichotomized by farmworker versus non-farmworker households. Left panel boxplots with zero genera proportion samples are set to 1e-6. Black bars, boxes and whisker bars respectively show the median, the central quartiles and the extreme values. The panel label texts report the graphed group labels and mean proportions. Genera are ordered from most abundant at the bottom to least abundant at the top. Genera labeled with a * are significant (Wilcoxon's FDR<0.1) between exposed (F, adults, n = 54; children, n = 42) and unexposed (N, adults, n = 47; children, n = 36) groups.

Table 5).

3.5. Principal component analysis of microbiomes

PCA was used to identify microbiome clusters and test association with farmworker and AZM exposure groups. The spring-summer common OTU (>0.1% mean proportion) adult PCA is presented in Fig. 5 and shows PC1 with 12.6% and PC2 with 5.8% of variance explained in microbiome composition. To not overfit the data, the spring-summer adults' PC loadings were used to project the adults' winter and children's OTU proportions in both seasons PCA plots (See Fig. 6 for Winter PCA). Inspection revealed two distinct primary clusters in each season. The left cluster generally has a more diverse score in PC2 suggesting those individuals have more dysbiotic variation in microbial community structure than the tighter grouping of the right cluster. The R mclust() model based clustering algorithm (Fraley and Raftery 2002) generated a PC1 decision value of 0.189 from the spring-summer data to discriminate these left and right clusters. Using this PC1 directionality and PC1 decision value, we classified the individuals to modeled "Exposed" and "Unexposed" cluster groups (See Fig. 7). The

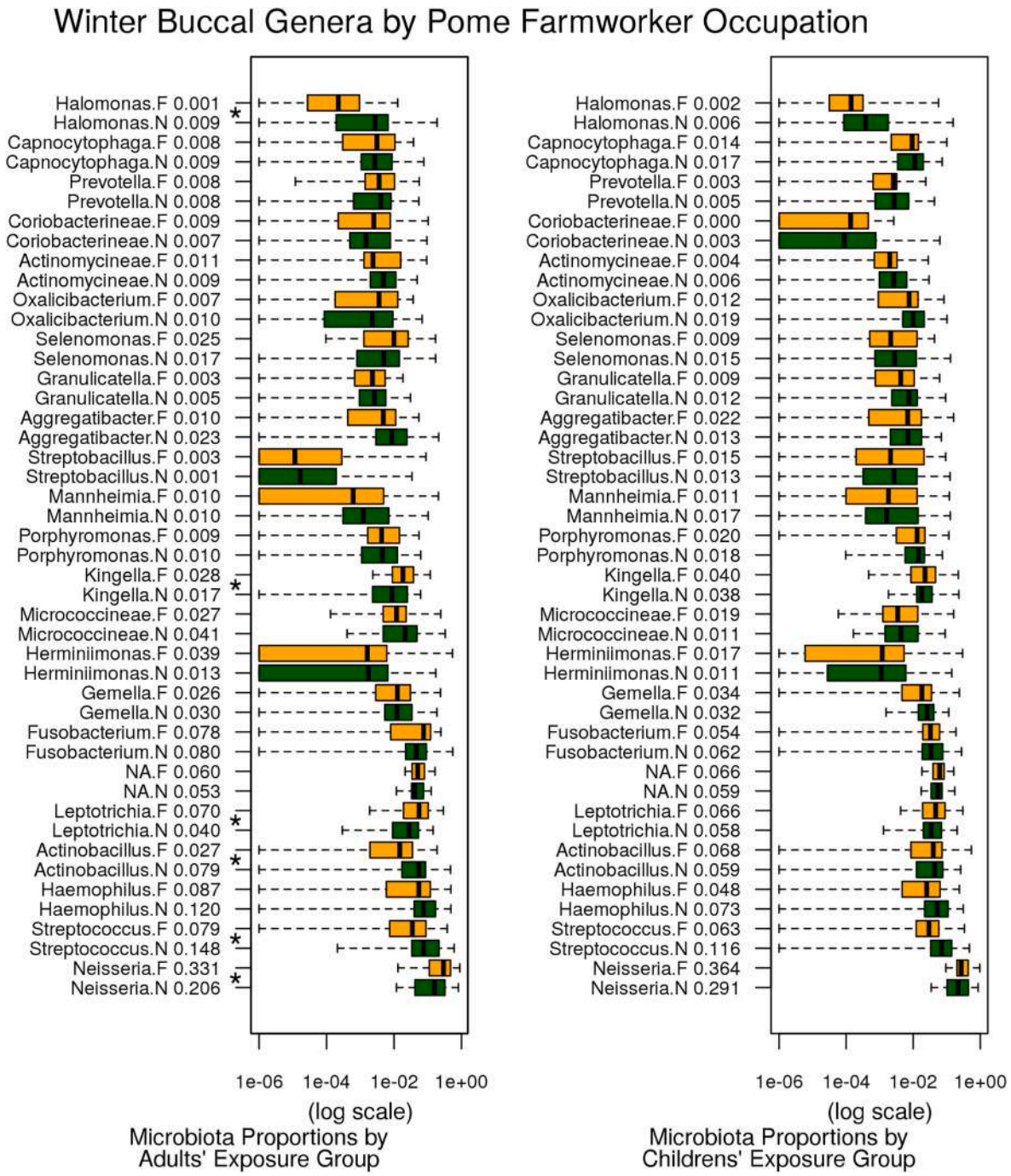

Fig. 4. Winter Adult and Child Buccal Microbiota for Farmworker (F) and Non-Farmworker (N) Category Households
Winter buccal swab sample sequencing common (>0.5% sample mean) microbiota dichotomized by farmworker versus non-farmworker households. Left panel boxplots with zero genera proportion samples are set to 1e-6. Black bars, boxes and whisker bars respectively show the median, the central quartiles and the extreme values. The panel label texts report the graphed group labels and mean proportions. Genera are ordered from most abundant at the bottom to least abundant at the top. Genera labeled with a * are significant (Wilcoxon's FDR<0.1) between exposed (F, adults, n = 42; children, n = 27) and unexposed (N, adults, n = 40; children, n = 35) groups.

spring-summer left cluster group is more likely to have children of adults with detection of blood AZM (Children's Fisher's exact test p~3×10^{-3}, OR = 5.6, See Table 6) and is enriched for children in farmworker households (Children's Fisher's exact test p~0.022, OR = 3.1, See Table 7). In the spring-summer 2005 microbiome PCAs, Welch's t-tests of detected AZM exposure groups showed significant (p~0.009) differences in the PC1 score of the children of adults with AZM detected in blood. This spring-summer microbiome PC1 score difference is suggestive (Welch's *t*-test, p~0.1) with the children based on the farmworker occupational classification exposure groups (see Fig. 5). Fig. 6 depicts the winter 2006 microbiome PCA. Similarly, exposed samples are more abundant to the left of PC1. The association with blood AZM detection maintains significance (Welch's *t*-test, p~0.02) with the winter microbiome PC1, but occupation becomes non-significant (Welch's *t*-test, p > 0.32) in the winter children's PC1 score. Cluster based PCA analysis with Fisher's Exact test shows significant exposure associated differences in the children's winter microbiomes with AZM exposure but is similarly underpowered for the farmworker occupational classification group.

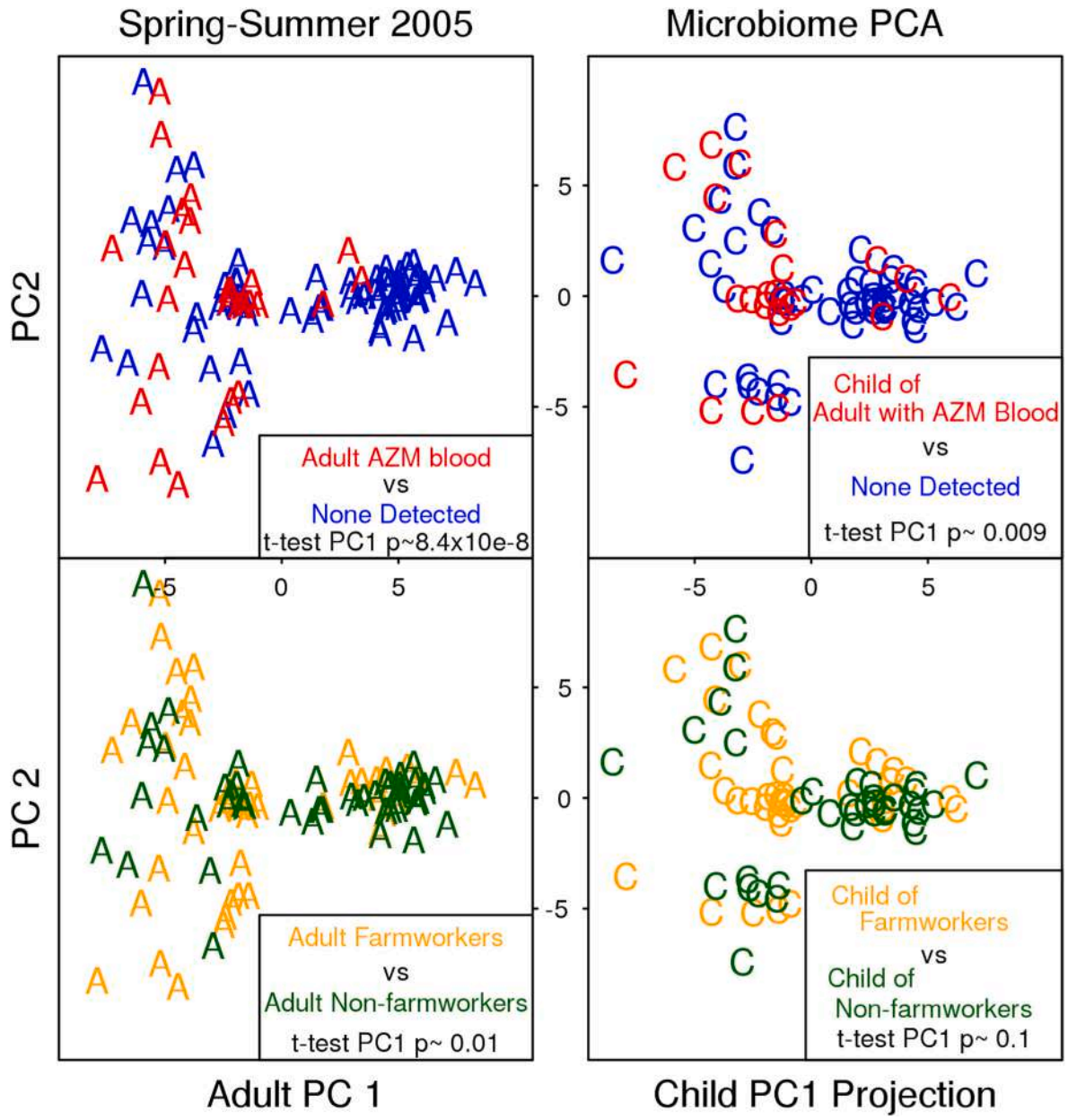

Fig. 5. Spring-summer Adult and Child Microbiome PCA
The common spring-summer OTU (>0.1% mean, n = 153) adult PCA is presented in the left two panels explain 12.6% (PC1) and 5.8% (PC2) of the variance. The spring-summer adult PC loadings were used to project the children's OTU PCs in the right panels.
Welch's *t*-test was used with PC1 for hypothesis testing groups.

The winter exposed left hand cluster is enriched with the children of households whose previous spring-summer adult blood found AZM detection (Children's Fisher's p~0.006, OR = 6.0, See Table 8) and farmworker occupation (Children's Fisher's p~0.12, OR = 2.5; adults-children combined Fisher's p~0.018, OR = 2.3, See Table 9). We detect the occupational exposure difference in the microbiome when the adults and children are combined in the winter samples.

The microbiome cluster phenotype appears to be very stable between seasons. Very few individuals change clusters from summer to winter with 111 of 116 (96%) participants with data in both seasons remaining in the same cluster.

3.6. Top OTU loadings in the principal components analysis

In Fig. 7 (top left panel) we show the top 10 OTUs driving the PCA are mainly dominated in the right-hand direction of PC1, with nearly all (9 of the 10 OTUs) increased to the right unexposed cluster. This increase of nearly all the top driver PC loadings towards the unexposed cluster suggests these individuals have more of these OTUs' representative organisms, as seen in the Wilcoxon's tests. These OTUs classify to the genera *Streptococcus, Haemophilus, Granulicatella, Fusobacterium,* and *Actinobacillus*. The OTU25 *Streptococcus* taxon is the top ranked driver loading. To illustrate the effects of over four orders of magnitude reduction in the abundance of the genera *Streptococcus* we shaded the PCA with the proportion of sample read alignments matching a *Streptococcus* OTU in the bottom two panels of Fig. 7. Of particular interest, individuals in the left exposed cluster direction of PC1 contain very low proportions (~<0.01) of this normally very proportionally common (>0.1) genus.

Table 4
Wilcoxon's Rank Sum Tests of Spring/Summer 2005 Buccal Microbiota by Household Pome Farmworker Occupation (Farmworker, adults, n = 54; children, n = 42 and Non-farmworker, adults, n = 47; children, n = 36).

Genera	Adults and Children Combined					Adults					Children				
	Loc. Dif.	lower	upper	p-value	FDR	Loc. Dif.	lower	Upper	p-value	FDR	Loc. Dif.	lower	Upper	p-value	FDR
Streptococcus	−1.48	−2.11	−0.89	0.000001	[a]0.00003	−1.48	−2.33	−0.61	0.001	[a]0.01	−1.46	−2.54	−0.63	0.001	[a]0.02
Neisseria	0.70	0.36	1.05	0.00004	[a]0.0005	0.67	0.20	1.24	0.01	[a]0.06	0.73	0.29	1.22	0.002	[a]0.02
Haemophilus	−0.80	−1.36	−0.26	0.003	[a]0.02	−0.58	−1.31	0.00	0.06	0.3	−0.96	−1.88	−0.15	0.02	[a]0.08
Gemella	−0.73	−1.27	−0.22	0.004	[a]0.03	−0.58	−1.30	0.11	0.09	0.4	−0.95	−1.82	−0.18	0.01	[a]0.08
Micrococcineae	−0.68	−1.21	−0.11	0.02	[a]0.08	−0.59	−1.18	0.05	0.07	0.3	−0.66	−1.57	0.44	0.21	0.35
Actinobacillus	−0.67	−1.38	−0.05	0.03	0.13	−0.78	−1.84	0.21	0.13	0.4	−0.57	−1.43	0.18	0.14	0.29
Prevotella	−0.52	−1.01	0.00	0.04	0.14	−0.32	−1.04	0.35	0.4	0.8	−0.73	−1.39	0.03	0.08	0.21
Actinomycineae	−0.52	−1.05	0.00	0.05	0.15	−0.47	−1.19	0.22	0.2	0.5	−0.42	−1.10	0.23	0.19	0.34
Granulicatella	−0.41	−0.96	0.02	0.09	0.2	−0.01	−0.79	0.59	0.8	0.9	−0.86	−1.71	−0.19	0.02	[a]0.08
Oxalicibacterium	−0.38	−0.90	0.04	0.09	0.2	−0.12	−0.73	0.65	0.7	0.9	−0.80	−1.72	0.02	0.06	0.17
Fusobacterium	−0.42	−1.00	0.05	0.10	0.2	−0.16	−0.88	0.41	0.6	0.9	−0.76	−1.67	0.00	0.05	0.15
Capnocytophaga	−0.32	−0.75	0.07	0.16	0.3	−0.16	−0.67	0.34	0.5	0.9	−0.60	−1.34	0.22	0.16	0.32
Halomonas	−0.27	−0.79	0.05	0.16	0.3	−0.61	−1.20	0.00	0.0	0.3	0.08	−0.62	0.59	0.71	0.75
Herminiimonas	0.19	0.00	0.76	0.2	0.3	0.00	−0.63	0.57	0.7	0.9	0.69	0.00	1.46	0.02	[a]0.08
Leptotrichia	−0.18	−0.61	0.28	0.4	0.6	0.07	−0.45	0.67	0.8	0.9	−0.54	−1.25	0.18	0.10	0.24
Streptobacillus	0.00	−0.66	0.28	0.5	0.7	0.00	−0.38	0.08	0.6	0.9	−0.49	−1.53	0.63	0.55	0.72
Kingella	0.13	−0.31	0.56	0.5	0.7	0.34	−0.21	0.91	0.2	0.5	−0.17	−0.87	0.50	0.63	0.72
otu_NA	−0.08	−0.36	0.21	0.6	0.8	0.04	−0.41	0.40	0.9	1.0	−0.19	−0.64	0.27	0.43	0.65
Mannheimia	0.06	−0.35	0.64	0.6	0.8	0.38	−0.23	1.15	0.3	0.6	−0.19	−1.11	0.57	0.60	0.72
Selenomonas	−0.07	−0.66	0.45	0.7	0.8	0.00	−0.82	0.88	1.0	1.0	−0.11	−0.98	0.55	0.60	0.72
Coriobacterineae	0.00	−0.50	0.39	0.7	0.8	0.07	−0.57	0.80	0.8	0.9	0.00	−0.70	0.29	0.56	0.72
Porphyromonas	0.08	−0.35	0.57	0.7	0.8	0.26	−0.33	0.99	0.4	0.8	−0.15	−0.77	0.52	0.70	0.75
Aggregatibacter	0.00	−0.61	0.40	0.8	0.8	0.00	−0.76	0.65	0.9	1.0	0.02	−1.09	0.71	0.90	0.90

[a] Significant at an FDR<0.1 between farmworker and non-farmworker occupation groups, lower and upper are the 95% confidence intervals of the location difference (Loc. Dif.).

Table 5
Wilcoxon's Rank Sum Tests of Winter (2006) Buccal Microbiota by Household Pome Farmworker Status (Farmworker, adults, n = 42; children, n = 27 and Non-farmworker, adults, n = 40; children, n = 35).

Genera	Adults and Children Combined					Adults					Children				
	Loc. Dif.	lower	upper	p-value	FDR	Loc. Dif.	lower	Upper	p-value	FDR	Loc. Dif.	lower	Upper	p-value	FDR
Halomonas	−1.03	−1.59	−0.50	0.00004	[a]0.0009	−1.49	−2.29	−0.79	0.0001	[a]0.002	−0.52	−1.43	0.00	0.039	0.4
Streptococcus	−0.94	−1.55	−0.34	0.002	[a]0.027	−0.98	−1.84	−0.10	0.024	[a]0.09	−0.92	−1.93	−0.06	0.03	0.4
Neisseria	0.52	0.10	0.94	0.01	0.11	0.74	0.13	1.30	0.016	[a]0.07	0.27	−0.34	0.86	0.4	0.8
Actinobacillus	−0.70	−1.32	−0.06	0.03	0.16	−1.11	−2.04	−0.21	0.013	[a]0.07	−0.16	−1.07	0.61	0.7	0.9
Haemophilus	−0.67	−1.39	−0.01	0.04	0.2	−0.60	−1.62	0.20	0.2	0.6	−0.77	−1.84	0.09	0.08	0.6
Leptotrichia	0.41	0.01	0.81	0.05	0.2	0.67	0.13	1.18	0.014	[a]0.07	0.07	−0.63	0.74	0.9	0.9
Kingella	0.45	−0.02	0.90	0.06	0.2	0.86	0.25	1.49	0.006	[a]0.07	−0.02	−0.78	0.69	0.95	1.0
Gemella	−0.35	−0.95	0.15	0.18	0.5	−0.21	−1.07	0.64	0.63	0.83	−0.46	−1.24	0.17	0.2	0.8
Granulicatella	−0.23	−0.74	0.28	0.3	0.7	0.00	−0.65	0.63	0.9	0.9	−0.48	−1.43	0.34	0.2	0.8
Actinomycineae	−0.30	−0.89	0.27	0.3	0.7	−0.23	−0.94	0.48	0.6	0.8	−0.49	−1.53	0.35	0.3	0.8
Oxalicibacterium	−0.27	−0.93	0.31	0.4	0.8	0.01	−0.84	1.05	0.9	0.9	−0.54	−1.46	0.25	0.19	0.8
otu_NA	0.12	−0.20	0.40	0.5	0.9	0.21	−0.19	0.63	0.3	0.7	−0.11	−0.53	0.35	0.7	0.9
Micrococcineae	−0.17	−0.77	0.46	0.6	0.9	−0.37	−1.01	0.28	0.3	0.7	−0.23	−1.24	0.78	0.6	0.9
Aggregatibacter	−0.18	−0.89	0.34	0.5	0.9	−0.28	−1.19	0.46	0.5	0.8	−0.19	−1.10	0.71	0.7	0.9
Selenomonas	0.22	−0.48	0.97	0.5	0.9	0.50	−0.44	1.47	0.3	0.7	−0.32	−1.25	0.90	0.6	0.9
Coriobacterineae	0.12	−0.43	0.74	0.6	0.9	0.35	−0.50	1.19	0.4	0.7	0.00	−0.94	0.43	0.6	0.9
Porphyromonas	−0.10	−0.65	0.41	0.7	0.9	0.13	−0.64	0.88	0.7	0.8	−0.35	−1.06	0.37	0.4	0.8
Herminiimonas	0.00	−0.43	0.33	0.8	1.0	0.00	−0.70	0.55	0.8	0.9	0.00	−0.80	0.61	0.8	0.9
Streptobacillus	0.00	−0.45	0.33	0.8	1.0	0.00	0.00	0.55	0.5	0.8	−0.07	−1.28	0.95	0.7	0.9
Capnocytophaga	−0.02	−0.62	0.48	0.8	1.0	0.32	−0.50	1.08	0.4	0.7	−0.43	−1.26	0.34	0.2	0.8
Fusobacterium	0.00	−0.61	0.63	1.0	1.0	0.16	−0.77	1.04	0.7	0.8	−0.19	−1.06	0.61	0.53	0.9
Mannheimia	0.00	−0.54	0.58	1.0	1.0	0.00	−0.61	0.87	0.9	0.9	−0.06	−1.00	0.82	0.8	0.9
Prevotella	0.00	−0.63	0.55	1.0	1.0	0.21	−0.62	0.98	0.6	0.8	−0.39	−1.34	0.51	0.4	0.8

[a] Significant at an FDR<0.1 between farmworker and non-farmworker occupation groups, lower and upper are the 95% confidence intervals of the location difference (Loc. Dif.).

3.7. Adult-child PCA model based Co-clustering

Inspection of adult-child pairs in the PCA derived exposed and unexposed clusters showed a pattern where children co-cluster with their household adult. In the spring-summer collection 70 adult-child cohabitating household pairs were classifier to the two clusters. Sixty-seven (67) of these adult-child pairs were in the same microbiome cluster. Thirty-seven (37) of 38 cohabitating adult-child pairs clustered with each other in the modeled AZM exposed cluster (See Table 10). One child of an adult (1 of 38) in the AZM exposed spring-summer cluster had a child PC1 score in the unexposed PC1 cluster group. In the summer unexposed PC1 cluster, 30 of 32 adult-child cohabitating pairs with microbiome data were in the same cluster. Two children (2 of 31) with a cohabitating adult in the unexposed summer PC1 cluster, clustered with

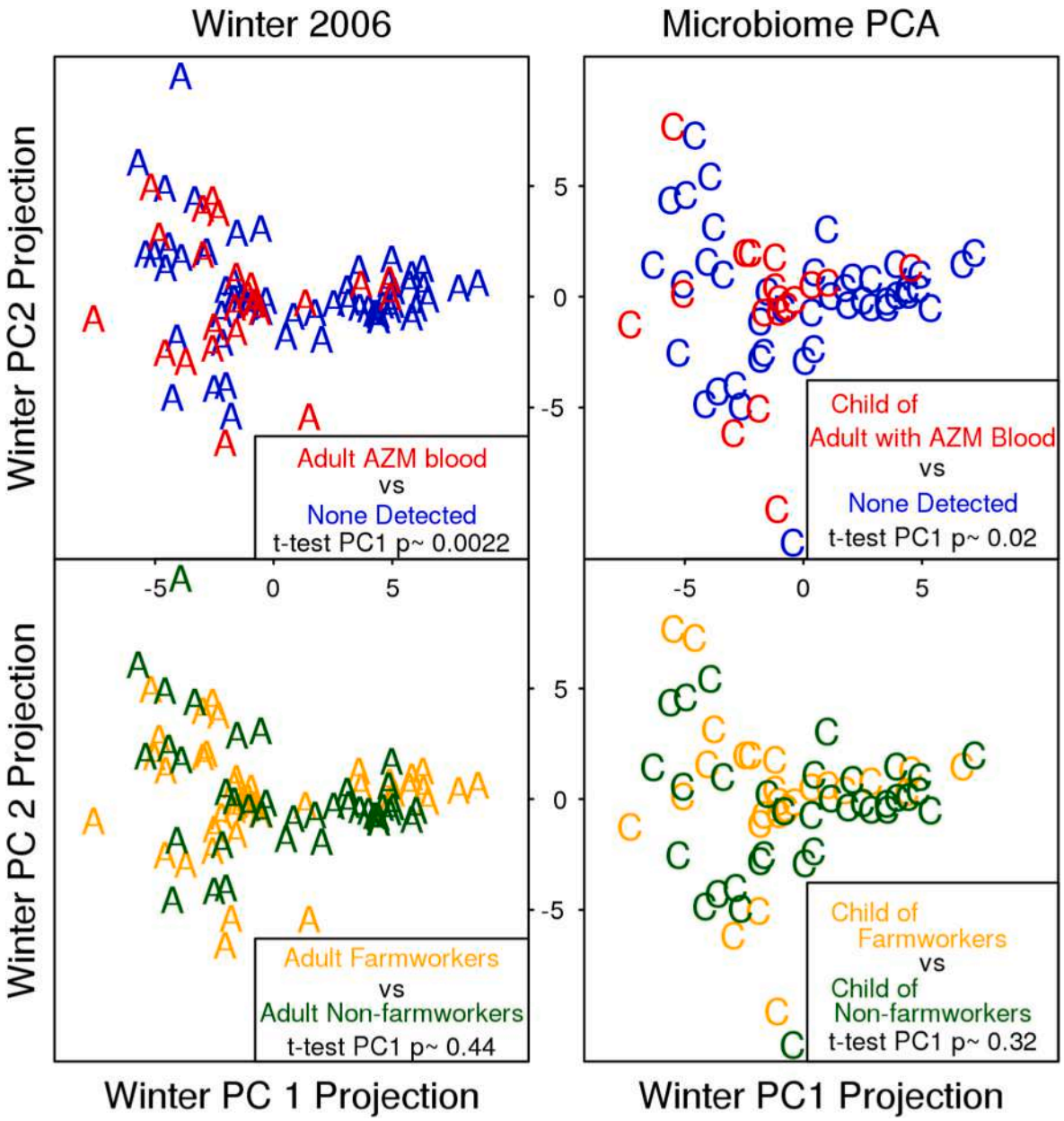

Fig. 6. Winter Adult and Child Microbiome PCA
The winter OTU adult and child PCA projections. The spring-summer adult PC loadings were used to project the winter OTU PCs. Welch's *t*-test was used with PC1 for hypothesis testing groups.

those individuals in the AZM exposed summer cluster group. In the winter, 53 adult-child household pairs were sequenced where both child and the adult had microbiome data. Of these, 50 of 53 adults and their cohabitating child were in the same winter PC1 clusters. In the exposed microbiome winter PC1 cluster, 31 of 33 children clustered with their cohabitating adult. Nineteen of 20 children clustered with their adult in the winter PC1 unexposed cluster. We assessed the significances (p-values $\sim< 3.3\times10^{-11}$; ORs>~217) by the Fisher's Exact Test of the non-random microbiome type co-clustering of children with respective household adults as shown in Table 10 for both spring-summer and winter collections. Forty-one (41) adult-child household pairs with microbiome data spanned both seasons' collections. Of these adult-child cohabitating pairs, 38 of 41 co-clustered in their respective clusters with the same respective household member for both seasons. Two (2) adult-child household pairs moved from the exposed to unexposed cluster.

3.8. Diversity analysis of PC clusters

The diversity estimation measure of Chao (Chao and Shen 2003; McMurdie and Holmes 2013) was compared between PC1 exposure groups. The adult exposed PC clusters had significant reductions in diversity in both seasons (Welch's *t*-test, $p < 0.02$, summer and winter). The children's PC1 winter exposed cluster showed significant (p~0.01) reduced diversity, whereas in the spring-summer is suggestive (p~0.09, Fig. 8).

4. Discussion

The microbiome varies in association with many common phenotypes, diseases, and environmental stimuli (Carmody and Turnbaugh 2014; Choi et al., 2013; Clarke et al., 2013; Davari et al., 2013; Desbonnet et al., 2014; Lu et al., 2014; Mason et al., 2015; Mayer et al.,

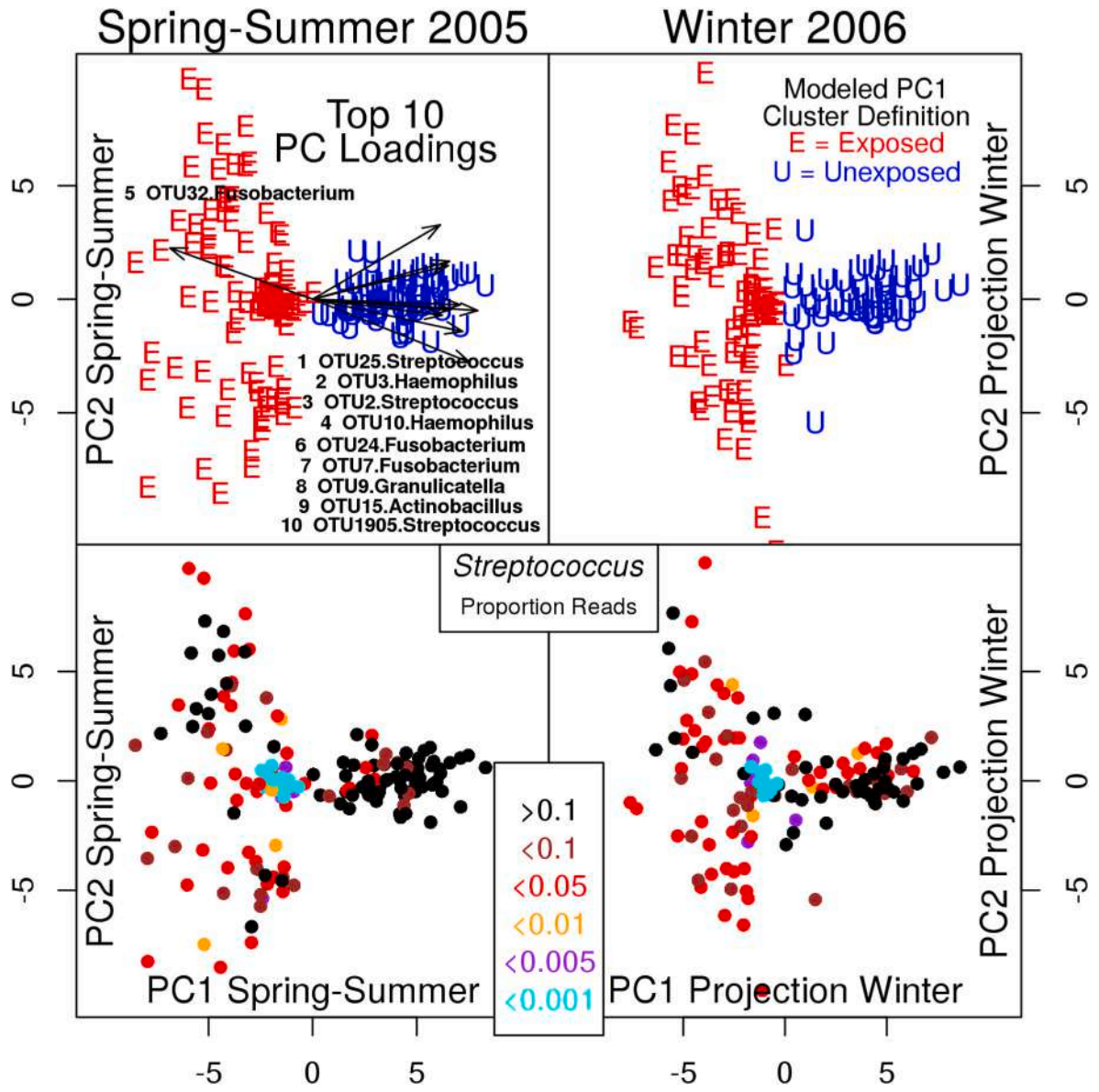

Fig. 7. PCA Loadings, Cluster Membership and Streptococcus Abundance
The top left panel shows the top 10 PCA loading driving the PC clustering. Both the top two panels show the model based clustering of inferred exposed (E) and unexposed (U) subjects in spring-summer 2005 (top left panel) and winter 2006 (top right panel) PCA projections. The bottom two panels show the proportion of reads mapping to Streptococcus OTUs in spring-summer 2006 (bottom left panel) and winter 2006 (bottom right panel) in the given PCA score distribution.

Table 6
Azinphos-methyl Fisher's exact tests by spring-summer microbiome PC 1 clusters.

Fisher's Exact Tests	[a]Combined PC Cluster		[b]Adults PC Cluster		[c]Children PC Cluster	
Groups	Exposed	Unexposed	Exposed	Unexposed	Exposed	Unexposed
AZM	45	7	26	3	19	4
ND	55	72	30	42	25	30

PC: Principal Component.
CI: Confidence Interval.
AZM: Azinphos-Methyl detected.
ND: None Detected.
[a] p-value ~5.77 × 10-8; Odds Ratio: 8.3, 3.3–24 (95% CI).
[b] p-value ~ 6.75 × 10-6; Odds Ratio: 11.8, 3.2–66 (95% CI).
[c] p-value ~ 0.003; Odds Ratio: 5.6, 1.6–26 (95% CI).

2015; Rosenbaum et al., 2015; Ussar et al., 2015; Zheng et al., 2015). Interactions of the microbiome with both endogenous and exogenous chemical compounds have been recognized for many years. Antibiotics (Mayer et al., 2015), probiotics (Dassi et al., 2014) and diet (De Filippo et al., 2010; Wang et al., 2015) are all well understood mechanisms by which we can change our microbiomes.

Table 7
Farmworker household Fisher's exact tests by spring-summer microbiome PC 1 clusters.

Fisher's Exact Tests	[a]Combined PC Cluster		[b]Adults PC Cluster		[c]Children PC Cluster	
Groups	Exposed	Unexposed	Exposed	Unexposed	Exposed	Unexposed
Farmworkers	67	29	38	16	29	13
Non	35	50	18	29	15	21

PC: Principal Component.
CI: Confidence Interval.
[a] p-value ~ 0.000078; Odds Ratio: 3.47, 1.8–6.8 (95% CI).
[b] p-value ~ 0.001; Odds Ratio: 3.8, 1.5–9.5 (95% CI).
[c] p-value ~ 0.022; Odds Ratio: 3.1, 1.1–8.8 (95% CI).

Table 8
Azinphos-methyl Fisher's exact tests by winter microbiome PC 1 clusters.

Fisher's Exact Tests	[a]Combined PC Cluster		[b]Adults PC Cluster		[c]Children PC Cluster	
Groups	Exposed	Unexposed	Exposed	Unexposed	Exposed	Unexposed
AZM	37	8	21	5	16	3
ND	46	53	26	30	20	23

PC: Principal Component.
CI: Confidence Interval.
AZM: Azinphos-Methyl detected.
ND: None Detected.
[a] p-value ~ 0.00005; Odds Ratio: 5.3, 2.1–14 (95% CI).
[b] p-value ~ 0.004; Odds Ratio: 4.8, 1.5–18 (95% CI).
[c] p-value ~ 0.006; Odds Ratio: 6.0, 1.4–36 (95% CI).

Table 9
Farmworker household Fisher's exact tests by winter microbiome PC 1 clusters.

Fisher's Exact Tests	[a]Combined PC Cluster		[b]Adults PC Cluster		[c]Children PC Cluster	
Groups	Exposed	Unexposed	Exposed	Unexposed	Exposed	Unexposed
Farmworkers	47	22	28	14	19	8
Non	36	39	19	21	17	18

PC: Principal Component.
CI: Confidence Interval.
[a] p-value ~ 0.02; Odds Ratio: 2.3, 1.1–4.8 (95% CI).
[b] p-value ~ 0.12; Odds Ratio: 2.2, 0.82–5.9 (95% CI).
[c] p-value ~ 0.12; Odds Ratio: 2.5, 0.78–8.4 (95% CI).

Table 10
Co-clustering of household matched adult-child pairs by PC cluster groups.

Fisher's Exact Tests	[a]Spring/Summer PC Clusters		[b]Winter PC Clusters	
Groups	Adult-Exposed	Adult-Unexposed	Adult-Exposed	Adult-Unexposed
Child-Exposed	37 match	2 non	31 match	1 non
Child-Unexposed	1 non	30 match	2 non	19 match

PC: Principal Component.
CI: Confidence Interval.
[a] p-value <2.6 × 10-16; Odds Ratio: 407, 40–16384 (95% CI).
[b] p-value <3.3 × 10-11; Odds Ratio: 217, 21–11202 (95% CI).

Prenatal human exposure to the structurally similar organophosphate pesticide chlorpyrifos has been associated with a loss of working memory (Horton et al., 2012; Lovasi et al., 2011; Rauh et al. 2006, 2011, 2012). Controlled animal organophosphate pesticide dosing experiments have replicated similar neurological phenotypes related to these findings (Yan et al., 2012). In this report, we add to these toxicologic properties of OPs by examining pesticide exposure associated alterations of children's oral buccal microbiome composition. We observed many of the same genera of bacterial abundance to be altered in the children's microbiomes in association with the adult exposures, whether we examined by specific AZM pesticide exposure or by the occupational farmworker status of the household. This assumes a take home occupational household exposure pathway scenario. Previous studies of this same cohort have examined urinary organophosphate pesticide metabolite and blood pesticide profiles that confirm co-exposure profiles for the adults and cohabitating children (Coronado et al. 2006, 2010; Thompson et al., 2014).

There is a high percentage (~95%) of microbiome PCA co-clustering of children with their cohabitating household adult. This points at the buccal microbiome as a possible non-invasive biomarker of organophosphate pesticide exposure that could be used to indirectly assess children's exposures. Both the adults and children also show significant reductions in Chao diversity between microbiome PCA clusters which are associated with pesticide exposure. The clustering associated with AZM and farmworker exposure groups has a lower abundance of the very common oral bacterium, *Streptococcus*.

The two most common bacterial genera (*Streptococcus* and *Neisseria*) in our agricultural cohort microbiome study are significantly altered in abundance in association with pesticide exposure. *Streptococcus* is a member of the order *Lactobacillales* as is the also significant genera *Granulicatella*, suggesting this exposure may affect *Lactobacillales* in general. There are many commensal organisms beneficial to host life in the order *Lactobacillales*. As an example, strains of *Lactobacillus*

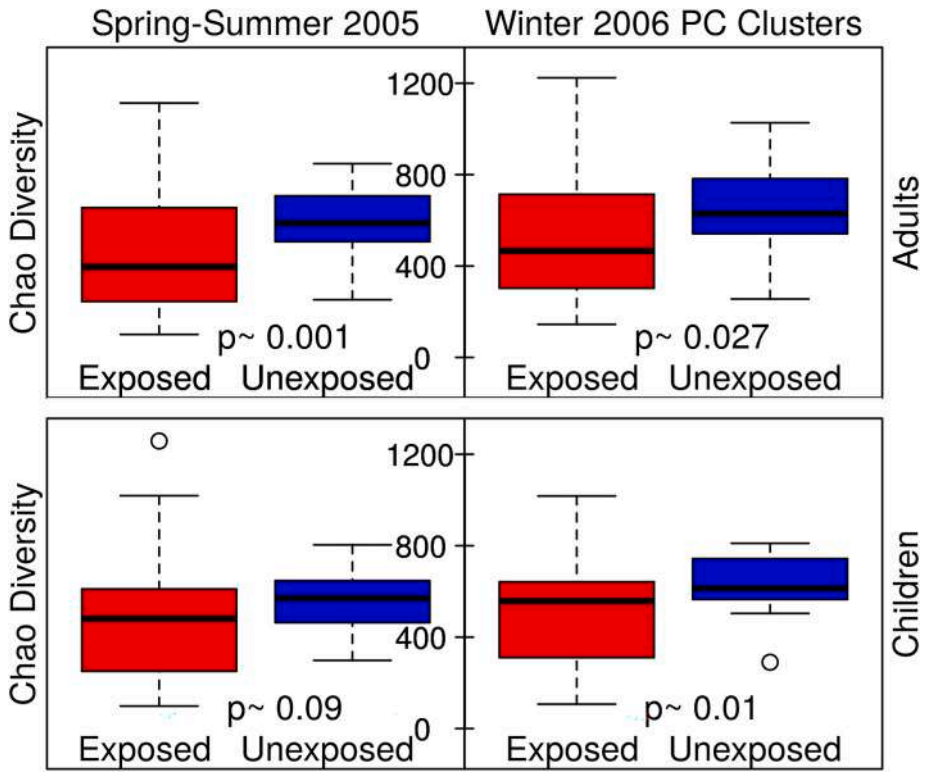

Fig. 8. Adult and Child Alpha Diversity by PCA Cluster.
Summer and winter PC clusters by the Chao diversity of children and adults. Clusters are as shown in Fig. 7. P-values are by Welch's *t*-test.

plantarum help to maintain normal growth in chronically underfed infant mice (Schwarzer et al., 2016). The current investigation does not have the ability to link these genera changes with beneficial or pathological functional changes, but such studies will be very important going forward. Based on these seasonal studies when agriculturally based pesticide exposures are reduced in the winter, our participants (including children) retain an altered microbiome profile. It has been found that microbiota extinctions in the rat gut microbiome induce altered persistent states unless recolonization occurs via inoculation of the live organisms (Sonnenburg et al., 2016). The impact of these changes to the microbiome profiles remains a matter of active research and should be investigated. Interpreting the potential health impacts for the oral microbiome are complex. *Neisseria* is a large genus with many non-pathogenic members and therefore an increase may not be associated with or causal of adverse health outcomes (Liu et al., 2015).

These results in children join a growing body of recent evidence identifying organophosphate pesticide induced perturbations in the microbiome. The commensal microbiota of experimental animals is similarly altered when exposed to organophosphate pesticides (Crisol-Martínez et al., 2016; Fang et al., 2018; Gao et al., 2017; Gao et al., 2018; Joly et al., 2013; Joly Condette et al., 2015; Joly Condette et al., 2014). One of these studies replicated their observation of changes in the rat gut microbiome in an in-vitro bioreactor (Joly et al., 2013) suggesting a host-independent antibiotic mechanism of action for organophosphates. Four of these orthogonal reports utilized rodent in-vivo gut microbiome model systems (Gao et al., 2017; Joly et al., 2013; Joly Condette et al., 2015; Joly Condette et al., 2014) and one studied the Japanese quail (*Coturnix japonica*) (Crisol-Martínez et al., 2016). These studies identified members of the order *Lactobacillales* as being reduced under organophosphate pesticide exposure conditions. *Streptococcus* and *Granulicatella* are common oral buccal members of *Lactobacillales*. These taxa were consistently detected at reduced abundances in this agricultural population of farmworkers' children.

There appears to be a microbiome phenome associated with environmental exposures in many contexts (Carmody and Turnbaugh 2014; Choi et al., 2013; Dassi et al., 2014; De Filippo et al., 2010; Korpela et al., 2016; Lu et al., 2014; Mason et al., 2015; Mayer et al., 2015; Wang et al., 2015). Microbiome associated states induced by continued antibacterial challenges (Yee and Gilbert 2016) have the potential to induce disease-related dysfunction phenotypes (Gilbert et al., 2016). There are known microbiome connections to periodontal disease, obesity, cardiovascular disease, and diabetes (Branchereau et al., 2016; Duparc et al., 2017; Emoto et al., 2016; Emoto et al., 2017; Feng et al., 2016; Ferguson et al., 2016; Gerardi et al., 2016; Kobyliak et al., 2016; Mazidi et al., 2016; Wilson Tang and Hazen, 2016; Wang et al., 2016; Xin et al., 2015; Zhang et al., 2016; Zhu et al., 2016). Hispanics carry a disproportionate disease burden of diabetes and cardiovascular disease (Below and Parra 2016; Below et al., 2016; Castañeda et al., 2016; Kaiser et al., 2016; Meyer, 2016; Pflederer et al., 2016; Qato et al., 2016; Rana et al., 2016; Silverman et al., 2016). These phenotypes are connected to the metabolic dysfunction endophenotype which has been shown to be inducible by the microbiome (Komaroff 2017). Pesticide exposure and alterations of the microbiome could have multivariate causal pathways of influence on pleotropic common and rare diseases. The children with altered microbiomes as shown in this analysis could have the potential to develop microbiome associated diseases early in life. Our results point to the need to examine additional health outcomes within our cohort to begin to determine the health significance of our studies, especially with a focus on the development of these young children (~6 years old) in 2005–2006, as they exit adolescence to become young adults. While AZM has been banned in the United States, other organophosphate pesticides with the same mechanism of action are still in common use and our results open a significant area of research. It has been suggested that organophosphate pesticide use should be greatly reduced due to childhood effects (Hertz-Picciotto, I. et al., 2018).

5. Conclusions

The results presented here show an association between agricultural AZM pesticide exposure and alteration of the oral buccal microbiome of children and their parents. The children's microbiome alterations are concordant with the alterations observed in cohabitating adult farmworkers when compared to non-farmworker households. Given our expanding understanding of the microbiome's role in disease, these results suggest the importance of further study of organophosphate pesticide exposure on microbiomes in exposed adult and child populations.

Conflicts of interest

The authors declare they have no actual or potential competing financial interests.

Declaration of competing interest

None.

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Assessing the risk of acute gastrointestinal illness attributable to three enteric pathogens from contaminated private water wells in Ontario

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A B S T R A C T

The province of Ontario compromises the largest groundwater reliant population in Canada serving approximately 1.6 million individuals. Unlike municipal water systems, private well water is not required to meet water quality regulatory standards and thus source maintenance, treatment and testing remains the responsibility of the well owner. Infections associated with private drinking water systems are rarely documented given their typically sporadic nature, thus the human health effects (e.g., acute gastrointestinal illness (AGI)) on consumers remains relatively unknown, representing a significant gap in water safety management. The current study sought to quantify the risk of waterborne AGI attributed to *Giardia*, shiga-toxin producing *E. coli* (STEC) and norovirus from private drinking water sources in Ontario using Monte Carlo simulation-based quantitative microbial risk assessment (QMRA). Findings suggest that consumption of contaminated private well water in Ontario is responsible for approximately 4823 AGI cases annually, with 3464 (71.8%) and 1359 (28.1%) AGI cases predicted to occur in consolidated and unconsolidated aquifers, respectively. By pathogen, waterborne AGI was attributed to norovirus (62%; 2991/4823), *Giardia* (24.6%; 1186/4823) and STEC (13.4%; 646/4823). The developed QMRA framework was used to assess the potential health impacts of partial and total well water treatment system failure. In the unlikely event of total treatment failure, total mean annual illnesses are predicted to almost double (4217 to 7064 cases per year), highlighting the importance of effective water treatment and comprehensive testing programs in reducing infectious health risks attributable to private well water in Ontario. Study findings indicate significant underreporting of waterborne AGI rates at the provincial level likely biasing public health interventions and programs that are effective in monitoring and minimizing the health risk associated with private well water.

1. Introduction

Approximately 1.6 million Ontarians rely on a private well water supplies, with most residing in rural regions (Statistics Canada, 2019). Individuals using private wells as their primary drinking water source are at an elevated risk of acute gastrointestinal illness (AGI) (Uhlmann et al., 2009; Bradley et al., 2021). Private water wells are subject to contamination by enteric pathogens consequent to factors including improper land application/disposal of manure, septic system leakage, and contaminated run-off (overland flow) resulting from extreme weather events (e.g., flooding, high-intensity rainfall, snowmelt) (Murphy et al., 2017). Further, private water wells are not required to meet the regulatory standards of the Ontario Safe Drinking Water Act (2002) or the Ontario Clean Water Act (2006), and thus source maintenance, testing and other measures to ensure potability remain the responsibility of the well owner (Kreutzwiser et al., 2011). Navigating guidelines and recommendations can prove challenging for property owners, as can the cost of efficacious treatment systems, making access to clean water a critical health equity issue in Ontario. Quantifying the true extent of AGI attributable to consumption of contaminated private well water also remains a challenge as cases of laboratory-confirmed AGI reported in Ontario's integrated Public Health Information System (iPHIS) do not distinguish infections based on their likely source of transmission (e.g., drinking water, food, animal contact or person-to-person transmission) (Vrbova et al., 2012). Moreover, the estimated number of AGI cases associated with waterborne pathogens is severely under-reported and likely significantly under-represents the true burden of infection; private groundwater contamination events are often prolonged (i.e., ongoing), sporadic, and/or limited to a single household (i.e., groundwater source) and are therefore difficult to identify (Haagsma et al., 2013). Compounding this, individuals experiencing gastrointestinal infection frequently do not seek medical care as disease mild and self-limiting, resulting in a lack of laboratory confirmation and source identification (Hrudey and Hrudey, 2007; Haas et al., 2014; Graydon et al., 2020).

The susceptibility of private drinking water sources to microbial contamination and associated outbreaks has been reported throughout

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the literature (Hynds et al., 2014a; Murphy et al., 2017). A recent global review examining contamination of domestic groundwater systems by shiga-toxin producing *E. coli* (STEC) found that private (i.e., unregulated, serving a single household) groundwater samples (15/800; 1.9%) and supplies (15/631; 2.4%) were characterised by higher detection rates compared to equivalent municipal samples (0/92) and supplies (0/32) (Chique et al., 2021). Likewise, three recent Canadian studies have reported that the risk of sporadic infections caused by five enteric pathogens (*Campylobacter, Salmonella, STEC, Giardia* and *Cryptosporidium*) differ by drinking water source type, with highest rates encountered among private (ground)water consumers (Uhlmann et al., 2009; Galanis et al., 2014; Murphy et al., 2016). Further, Borchardt et al. (2021) sampled 131 wells for fecal contamination in Wisconsin, United States; approximately 40% (32/79) of *E. coli* positive wells had markers concurrently present for pathogens including enteric viruses and protozoa. Illness associated with private drinking water systems is rarely documented given the sporadic nature of infections, thus the human health effects (e.g., acute gastrointestinal illness, hospitalizations) remain relatively unknown, representing a recognized gap in water safety and quality management strategies.

Quantitative microbial risk assessment (QMRA) represents a valuable tool for estimating the burden of disease associated with waterborne illness and thus supports water safety planning and management (Petterson and Ashbolt, 2016; Owens et al., 2020). QMRA aims to employ best available information, using an iterative (simulation-based), probabilistic approach to account for variability and uncertainty (via sensitivity analysis) to understand the potential human health effects from microbial exposures and ultimately provide guidance for evidence-based policy development (Ramírez-Castillo et al., 2015). Several previous studies have utilized QMRA to estimate the human health risk posed by private drinking water sources (Hunter et al., 2011; Hynds et al., 2014a; Balderrama-Carmona et al., 2015; Murphy et al., 2016; Balderrama-Cormona et al., 2015). However, due to data scarcity these studies have resulted in very high levels of uncertainty and variability with the potential to either over or underestimate human health risk. For example, Murphy et al. (2016) utilized QMRA to estimate the human health risks associated with private wells and small water systems across Canada, concluding that private groundwater consumers had a higher risk of contracting enteric infections than those supplied by publicly managed systems. However, QMRAs are inherently generalised, and rarely account for site-specific conditions or temporal variations in real time (e.g., contaminant ingress mechanisms), thus may misrepresent the inherently complex and fluid nature of groundwater contamination (Smeets et al., 2010; Bichai and Smeets, 2013). Accordingly, the need for larger, increasingly adapted databases that account for contamination pathways (e.g., microbiological, physical, hydrogeological, temporal) are essential to minimize the limitations of previous QMRAs (Latchmore et al., 2020; White et al., 2021; Borchardt et al., 2021).

The objective of the current study was to quantify the risk of waterborne AGI attributed to *Giardia*, shiga-toxin producing *E. coli* and norovirus from private drinking water sources in Ontario as these represent the most frequently reported protozoan, bacterial and viral water-borne pathogens in Canada, and contribute to significant morbidity and mortality, both regionally and internationally (Hynds et al., 2014b; Wallender et al., 2014; Murphy et al., 2016; Health Canada 2019; Owens et al., 2020; Sorensen et al., 2021). The presented QMRA was hydrogeologically delineated and employed a large spatiotemporal groundwater quality dataset (>700,000 samples) (Latchmore et al., 2020), permitting spatio-temporally specific exposure distributions for several model inputs, including private well water consumption, annual contamination duration and contamination event overlap (i.e., re-occurrence) (Lavallee et al., 2021a; Latchmore et al., 2022). Study findings will identify the human health risk attributable to private drinking water systems in rural communities in Ontario. Finally, presented models will serve as an effective template for public health agencies and provincial governments to safeguard private drinking water systems and diminish health risks.

2. Methods

2.1. Model framework

Previous research has found that *E. coli* detection rates in Ontario private well water differ significantly based on hydrogeological setting (i.e., consolidated vs. unconsolidated aquifers) (Latchmore et al., 2020; White et al., 2021). Latchmore et al. (2020) report significantly higher detection rates associated with consolidated aquifers, suggesting that human exposure to waterborne pathogens is likely mediated by hydrogeological setting. Further, given the importance of accounting for sensitive sub-populations due to differing dose-response relationships, and the need for age-adjusted incidence rates for public health planning, QMRA models have been separated based on age (</>10 years of age). Accordingly, the overarching risk estimation framework comprised 12 models (three pathogens x two hydrogeological settings [i.e., consolidated, and unconsolidated aquifers] x two age categories [i.e., adults and children <10]) for estimating the exposure and subsequent human health risks associated with private well water consumption in Ontario.

In the current study, several structural and algorithmic amendments have been made to the traditional four-tiered QMRA approach as model components/inputs and algorithms are bespoke depending on the specific objectives of the risk assessment. The major mathematical tasks of a QMRA are exposure assessment, hazard characterization and risk characterization (Haas et al., 2014). For the purpose of the current study, where appropriate, all terminology and descriptions have been adopted from Haas et al. (2014). The 3-step province-scale stochastic QMRA model was developed as follows (Fig. 1):

Step 1. Exposure Assessment

$$ED = C_{ecoli} \text{ x } 1/R \text{ x } PI \text{ x } V_{DW} \quad (1)$$

where ED = daily exposure per person (cfu/day), C_{ecoli} = *E. coli* loading (cfu/mL), R = detection sensitivity (%), PI = pathogen contribution (%), Vdw = Daily water consumption (mL/day).

Step 2. Hazard Characterization

$$PDinf_{STEC} \text{or } PDinf_{norovirus_{adults}} = [1 - (1 + ED / \beta)]\alpha \quad (2a)$$

$$PDinf_{giardia} = 1 - \exp(-ED * r) \quad (2b)$$

$$PDinf_{norovirus_{children}} = P * (1 - \exp(-ED)) \quad (2c)$$

where: PDinf = daily probability of infection (%), ED = daily exposure per person (CFU/day), β = Beta Poisson parameter value, α = Beta Poisson parameter value, *r* = Exponential parameter value, *P* = Fractional Poisson parameter

$$PA\ inf = \left[1 - (1 - PDinf)^{DUR}\right] \text{x } Fc \quad (2d)$$

where PA*inf* = annual probability of infection (%), DUR = contamination duration (days/annum), Fc = contamination frequency (%)

Step 3. Risk Characterization

$$\text{Health burden per annum} = PAinf \text{ x } Pop\ (1 - NCon) \text{ x } AR \quad (3)$$

where PA*inf* = probability of infection per annum (%), Pop = affected population/population at risk (number with well water as primary drinking source), NCon = rate of non-consumption (%), AR = attack rate (%) Statistical distributions were developed and tested using R (version 3.2.1) and RStudio (version 1.1.447) with the add-on packages ggplot2 (version 3.2.1), fitdistrplus (1.0–14) and mc2d (version 0.1–21). Anderson-Darling and Kolmogorov-Smirnov statistics were used to examine goodness-of-fit between fitted parametric distributions and

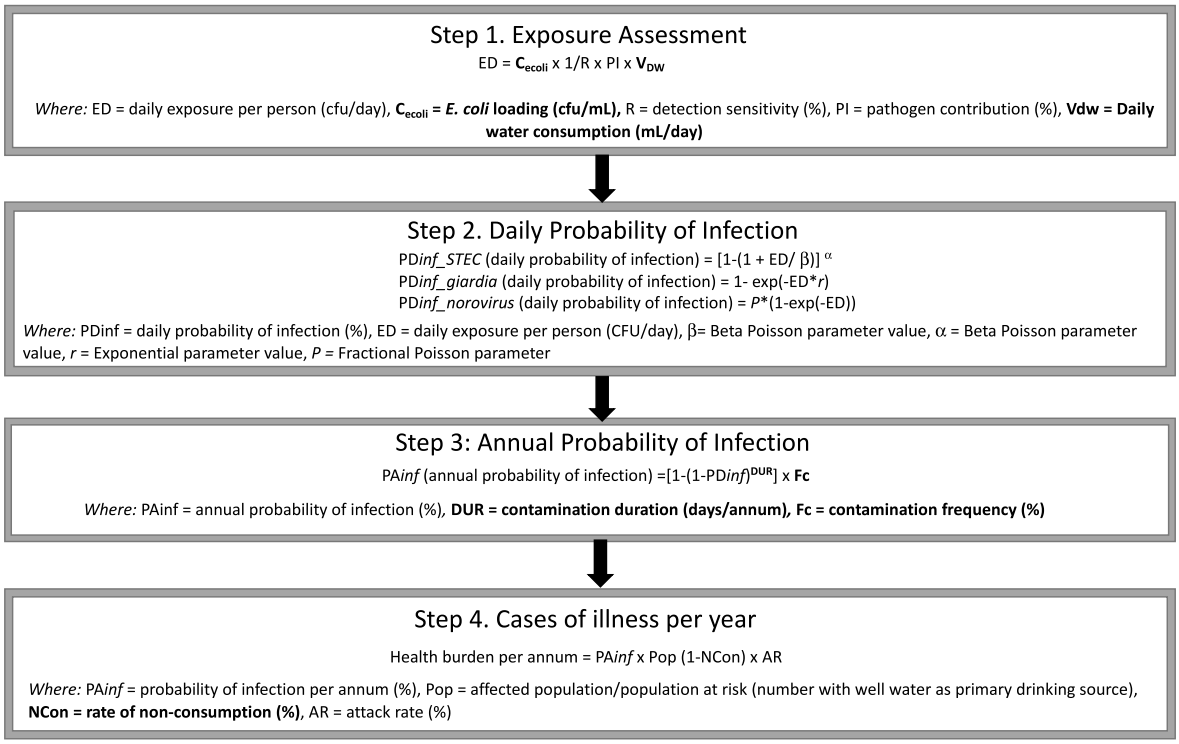

Fig. 1. Schematic diagram of QMRA model used to estimate the disease burden of STEC, Giardia and norovirus from consumption of contaminated private well water in Ontario (adapted from Haas et al., 2014; Murphy et al., 2016).

empirical data for all statistical distributions (Delignetter-Muller and Dutang, 2015). All simulations were run in R using 2-dimensional Monte Carlo (2DMC) simulations with the package mc2d (Pouillet and Delignetter-Muller, 2010). This approach was chosen based on previous risk estimates from private groundwater wells (Burch et al., 2021) as it permits individual and concurrent measurement of the effects of parameter variability and uncertainty on risk estimates and distributions. More specifically, variability in this case refers to irregularity in natural conditions and subsequent exposures (e.g., water consumption patterns, *E. coli* loading), while uncertainty refers to discrepancies associated with simulated model parameters (e.g., pooling of pathogen contribution literature) (Pouillet and Delignetter-Muller, 2010). Each 2DMC simulation was based on 10,000 iterations. A summary of model inputs is provided in Table 1. Sensitivity analysis was carried out using Spearman's rank order correlation coefficient to determine the significant input variables contributing to uncertainty in the risk calculations (Cummins et al., 2010; Hunter et al., 2011). Scenario analysis is a well-recognized tool in environmental change research and is the process of modeling potential future outcomes by considering the impact of alternative events on model inputs (Schweizer and Kurniawan, 2016). The developed QMRA simulations assessed the potential health impact of a private well water treatment system completely failing or partially failing (e.g., range of 0%–100% treatment efficacy). It is important to note that the majority of private wells in Ontario do not have microbiological treatment systems installed. Scenario analysis was undertaken using the multivariable nodes as a third dimension in the mc2d package (Delignetter-Muller and Dutang, 2015).

2.2. Model inputs

2.2.1. Private well data sources

The current study combined the Well Water Information System (WWIS) and the Well Water Testing Dataset (WWTD) from 2010 to 2017, inclusive. The WWIS is the dataset of well records maintained by the Ontario Ministry of the Environment, Conservation and Parks (MECP). Well records include details pertaining to well construction, location, pump test results, source-specific geological profile, and general information regarding well water quality (MECP, 2019). The WWTD comprises all results of bacteriological testing performed at one of the eleven provincial laboratories. A full description of dataset development has previously been described in Latchmore et al. (2020); briefly, the WWTD-WWIS dataset was created by merging the WWTD with the WWIS via an interactive, nearest-neighbour, fuzzy logic-based (inexact string matching) custom algorithm. Private well water samples were specifically matched to sources using minimum haversine distance, with a "one well-one distance" approach used to ensure accuracy. A total of 156,033 wells (458,910 samples) were geographically associated with consolidated aquifers and 83,211 wells (243,951 samples) were associated with unconsolidated aquifers. According to the Ministry of the Environment, Conservation and Parks (2014), approximately 70% and 30% of private well users have wells constructed in consolidated and unconsolidated aquifers, respectively. All submitted well water samples were processed and analyzed for Total Coliforms (TC) and *Escherichia coli* via direct membrane filtration and culture, based on MECP Method #E3407 (membrane filtration method using DC Agar for the simultaneous detection and enumeration of TC and *E. coli* in Drinking Water (Ministry of the Environment, Conservation and Parks, 2010). Description and analyses of the WWTD-WWIS have been presented in Latchmore et al. (2020), and White et al. (2021) (Figs. S8–S9). Further, only *E. coli* results (excluding TC results) have been included in the current study as it represents a specific indicator of recent fecal ingress and, if present, suggests a potential threat to public health and is used in waterborne QMRA. The recommended acceptable limit of *E. coli* is 'none' (<1) detectable in a 100 mL sample (Health Canada, 2019).

2.2.2. Population at risk

The total at-risk population was estimated from Census data (2019; Table 1) and comprised individuals whose primary drinking water source is an inadequately treated or untreated private well. Based on Census data, approximately 34% of individuals who treat their well

Table 1
Summary of QMRA input variables, descriptions, and distributions.

Input	Variable	Distribution	Source
E. coli loading	Cecoli	Consolidated – Negative Binomial (r = 0.753, p = 6.81) Unconsolidated – Negative Binomial (r = 0.739, p = 7.29)	Current Study
Contamination Frequency (day/year)	Fc	Consolidated – Beta (α = 0.0106, β = 0.322) Unconsolidated – Beta (α = 0.00739, β = 0.323)	Current Study (supplementary material)
Contamination Duration	^{DUR}	Consolidated – Log-normal (μ = 0.0772, σ = 0.0546) Unconsolidated – Weibull (μ = 1.4544, σ = 28.0393)	Current Study
Pathogen Contribution *STEC* *Giardia* *Norovirus*	PI	Uniform (min = 0.014, max = 0.019)	Murphy et al. (2016) Allen et al. (2017) Borchardt et al. (2021) Pang et al. (2021)
Detection Sensitivity	R	Uniform (min = 0.93, max = 1)	Hynds et al., (2014a) Unpublished Data
Consumption Volume (mL/day)	VDW	Adult: Exponential (rate = 0.00088; 1132 mL/day; SD = 649 mL/day) Children (<10 years) Exponential (rate = 0.00111; 905 mL/day; SD = 519 mL/day)	Lavallee et al. (2021a) Lavallee et al. (2021a) Pintar et al. (2009)
Dose Response: *STEC* *Giardia* *Norovirus*	β:α	Beta-Poisson (α = 398.9 β = 3.96 × 10^4) Exponential (r = 0.0199) Beta-Poisson (α = 0.04 β = 0.05) Fractional Poisson (P = 0.72)	Teunis et al. (2008a) Teunis and Havelaar (2002) Regli et al. (1991) Teunis et al. (2008b) Messner et al. (2014)
Attack Rate: *STEC* *Giardia* *Norovirus*	AR	See Table 4	Pooled-analyses (n = 50)
Affected Population	Pop	0.11 (11% of the Ontario population on private well water) 1,031, 800 at-risk (after considering treatment)	Statistics Health Canada (2019)
Rate of non-consumption	NCon	0.185	Lavallee et al. (2021a)

water, use a treatment system effective against microbial contamination (e.g., reverse osmosis, membrane filtration, shock chlorination) (Statistics Canada, 2019); the total at-risk population was estimated to be approximately 1,031,800 (Table 2), presuming treatment systems are properly maintained and managed.

Vulnerable sub-populations, namely, immunocompromised individuals, the elderly, women during pregnancy, and children, are characterized by an increased risk in terms of severity (including hospitalisations, development of sequelae, and prolonged effects) and mortality when exposed to enteric pathogens (Gerba et al., 1996). Vulnerable sub-populations in the current QMRA comprised infants (<1 year), children (1–10 years) and elderly (>65 years), mirroring a previous study by Hynds et al. (2014b). Due to insufficient data regarding the distribution of immunocompromised individuals and pregnant women, these sub-populations have not been accounted for in the current study. According to Census data, and assuming these

Table 2
Total and vulnerable population served by private water wells in Ontario (as of 2019; adapted from Statistics Canada, 2019).

Age category	Total	Proportion of provincial population served by private well water (%)
Infants (<1)	13,775	1.1
Children (1–10)	147,421	8.5
Adults (11–64)	1,119,200	72
Elderly (>65)	293, 379	19.2
Total Vulnerable Population	454,575	28.8
Total population	1,573,775	
Total at-risk population (after water treatment)	1,031,800	

sub-populations are randomly distributed across the province, this population compromises approximately 28.8% (454,575 individuals) of the total population (Statistics Canada, 2019): Infants – 1.1%, Children – 8.5%, Elderly – 19.2% (Table 2).

2.2.3. Contamination frequency

Determination of contamination frequency in private well water in Ontario was undertaken using the WWTD-WWIS dataset from 2010 to 2017, inclusive, via analyses of the proportion of wells contaminated at least once per year (Table 3). Private wells had *E. coli* present on 4.0% and 2.9% of sampling occasions in consolidated and unconsolidated aquifers, respectively (Table 3). Correspondingly, contamination frequency was estimated using detection rates reported by Latchmore et al. (2020); an estimated 3 and 4 contamination events per 100 wells per year is expected to occur in consolidated and unconsolidated aquifers, respectively, thus the majority of contaminated private wells are characterized by one contamination event per year. Sampling results from both aquifer types were fit to determine the most appropriate distribution. Goodness of fit tests indicate the beta distribution provided the best fit for both consolidated (α = 0.0106, β = 0.322) and unconsolidated aquifers (α = 0.00739, β = 0.323) (Figs. S1 and S2).

2.2.4. Escherichia coli concentration

E. coli concentration (CFU/100 mL) in private well water was fit and distributed using the WWTD-WWIS dataset. To estimate *E. coli* loading, two probabilistic distributions (i.e., consolidated, and unconsolidated aquifers) were developed using data from all contaminated private wells (≥1 CFU/100 mL; Table 3). The negative binomial distribution provided

Table 3
E. coli occurrence in private well water in Ontario stratified by aquifer type and year.

Year	Consolidated (Annual detection rate)	Average *E. coli* concertation for positive samples (CFU/100 mL)	Unconsolidated (Annual detection rate)	Average *E. coli* concentration for positive samples (CFU/100 mL)
2010	3004/63,954 (4.7%)	7.41	1204/33,681 (3.6%)	7.32
2011	2934/62,577 (4.7%)	7.49	1121/33,881 (3.3%)	7.55
2012	2100/59,526 (3.5%)	6.94	929/32,776 (2.8%)	6.75
2013	2840/57,520 (4.9%)	6.85	1134/31,349 (3.6%)	8.37
2014	2482/56,002 (4.4%)	7.06	888/29,773 (3.0%)	7.54
2015	1999/52,866 (3.8%)	7.23	772/27,907 (2.8%)	7.80
2016	1322/52,486 (2.5%)	5.52	499/27,781 (1.8%)	5.65
2017	1821/53,979 (3.4%)	4.73	559/26,803 (2.1%)	5.87
	4.0%	**6.65**	**2.9%**	**7.10**

Table 4
Results of pooled-analyses (n = 50; Table S1) and distribution fitting for clinical variables. Based on previous literature the beta-general, PERT, lognormal, logistic, normal and pareto distributions were all tested for fit.

Variable	N	Range	Distribution	Parameters	Goodness of fit *p*
Attack rate	13[a]	Min – 6.7%	Logistic	$\mu = 0.224$ $s = 0.095$	0.87
	22[b]	Max – 64%	Normal	$\mu = 0.243$	0.85
	15[c]	Min – 3.1%	Normal	$\sigma = 0.169$	0.78
		Max – 92%		$\mu = 0.243$	
		Min – 5%		$\sigma = 0.169$	
		Max – 63%			
HUS rate[a]	8	Min – 0.3%	Normal	$\mu = 0.077$	0.948
		Max – 27.2%		$\sigma = 0.027$	
Hospitalization rate	5[a]	Min – 7.5%	Normal	0.013	0.999
	2[b]	Max – 35.7%		0.030	
	2[c]	Deterministic			
		Deterministic			
Mortality rate[a]	4[a]	Min – 0.3%	Logistic	$\mu = 0.023$ $s = 0.011$	0.95
		Max – 6.3%			

[a] STEC data.
[b] Norovirus data.
[c] Giardia data.

an appropriate fit for *E. coli* loading among private wells located in both consolidated ($r = 0.753$, $p = 6.81$) and unconsolidated ($r = 0.739$, $p = 7.29$) aquifers, respectively (Figs. S3 and S4).

2.2.5. Contamination duration

An understanding of microbial survival in private groundwater sources and the subsurface environment is critical for the development of increasingly accurate waterborne infection risk assessments. Using the WWTD-WWIS dataset, the authors examined the die-off rate (CFU/100 mL per day decline) and subsequent duration of *E. coli* contamination in private well water in Ontario, and the methodology is explained in depth by: Latchmore et al. (2022). Further, the probability of contamination event overlap, defined as one or more contamination events occurring sequentially resulting in an event characterized by increased duration and a point increase in concentration, in Ontario private well water was calculated. A new *E. coli* loading variable was created if contamination event overlap were to occur (Latchmore et al., 2022). Briefly, private well water samples from the WWTD-WWIS dataset were analyzed relative to extracted "contamination sequences", defined as two or more *E. coli* positive samples in series within one calendar year, and delineated by aquifer type (consolidated/unconsolidated). Thus, all contamination sequences are source-specific, with each sequence pertaining to one well and one calendar year and compiled independently for both aquifer types. Extracted contamination sequences were used to assess contamination duration, defined as number of days (per annum) with *E. coli* > 0 CFU/100 mL, for individual private wells.

The *E. coli* die-off rate (per day decline of *E. coli* concentration [CFU/100 mL]), defined as the average *E. coli* concentration divided by the number of days from mean contamination concentration to zero CFU/100 mL (negative test result), was separately calculated for wells associated with consolidated and unconsolidated aquifers. Study findings indicate a median *E. coli* die-off rate of 0.38 CFU/100 mL per day and 0.64 CFU/100 mL per day, for private wells located in unconsolidated and consolidated aquifers, respectively (Latchmore et al., 2022). equating to first-order die-off rates of 0.0054 h^{-1} to 0.0089 h^{-1}. Contamination events associated with unconsolidated aquifers were significantly longer (days/annum) compared to consolidated aquifers ($t = 82.10$; $p < 0.0001$). Contamination frequency (section 2.2.3), employed in concurrence with calculated mean concentration and mean time to zero (Table 2) predict mean durations of 21 (CI: 1.92–80.7; min 0 max 347) and 16 (CI: 3.31–53; min 0 max 208) "annual contamination days" among wells located in consolidated and unconsolidated aquifers, respectively, based on one contamination event. Thus, while contamination events are shorter in consolidated aquifers, they experience more contamination days per year. The Anderson-Darling and Kolmogorov-Smirnov statistics indicate the two parameter Weibull distribution provides a statistically appropriate fit for contamination duration in unconsolidated aquifers, while the log-normal distribution provides a statistically appropriate fit in consolidated aquifers (Figs. S6 and S7).

2.2.6. Private well water consumption

A province-wide online survey was employed between May and August 2018 to investigate levels of awareness, attitudes, risk perceptions and beliefs among private well users in Ontario (Lavallee et al., 2021b) (n = 1162). As part of this work, 81.5% of respondents (n = 947) reported their daily well water consumption (i.e., tap water). Results indicated a mean daily well water consumption rate of 1132 mL/day (SD = 649 mL/day). To develop a discrete mean value (and continuous distribution for use in QMRA), deterministic midpoint values were assigned to each consumption range based on an assumption of within-range normality and subsequently distributed as previously described by Hynds et al. (2012). Goodness of fit tests (e.g., Anderson-Darling and Kolmogorov- Smirnov) indicate the log-normal ($p < 0.001$), exponential ($p < 0.001$) and normal distributions all provide a similar fit for total daily well water consumption (Figure A.5). However, the log-normal and normal distributions significantly overestimate water consumption within the upper quartile and therefore the exponential distribution was employed (Lavallee et al., 2021a).

Consumption estimates for vulnerable sub-populations (i.e., children <10 years old) were more difficult to ascertain given the age distribution of the province-wide survey was predominantly individuals over the age of 25 years old (1161/1169; 99.5%). Therefore, a distribution was developed using previously published estimates from Pintar et al. (2009) in concurrence with the province-wide survey data. More specifically, Pintar et al. (2009) reported that children (<10 years of age) consume approximately 20% less well water than adults. Therefore, using the private well water consumption estimates for adults reported by Lavallee et al. (2021a) [1132 mL/day], daily private well water consumption for children was estimated by applying a 20% decrease to the adult consumption distribution. Thus, the consumption estimate for children was characterised by a mean daily well water consumption rate of 905.6 mL/day (SD 519 mL/day). Goodness of fit indicate an exponential distribution ($p < 0.001$) provides an appropriate fit for daily well water consumption for children (rate = 0.00111).

2.2.7. Pathogen contribution

Previous studies have identified positive correlations between *E. coli* in private groundwater systems and the presence and concentration of

bacterial pathogens ($r = 0.636$, $p = 0.02$; Hynds et al., 2014a) and viruses ($r = 0.33$, $p < 0.001$; Fout et al., 2017). Further, it is difficult to monitor drinking water for all fecal pathogens due to limitations in currently available technologies and the large sample volumes required for detection (e.g., up to 100 L) (Cabral, 2010; Felleiter et al., 2020). Therefore, the presence and concentration of enteric pathogens were extrapolated from total *E. coli* CFU per 100 mL. In the context of the current risk assessment, pathogen contribution is defined as the likely ratio of laboratory confirmed *E. coli* to enteric pathogens in private well water in Ontario. The current study employed a uniform distribution of the most recent point estimates of pathogen occurrence (0.014–0.019; Table 1) utilized in QMRA of waterborne infection and pathogen occurrence studies to account for variability and uncertainty throughout the studies (Murphy et al., 2016; Allen et al., 2017; Burch et al., 2021). More specifically, Murphy et al. (2016) utilized a point estimate of 1.9% (7/371) derived from a pooled analysis of previously published data on pathogen occurrence. Similarly, Burch et al. (2021) utilized a point estimate of 1.4% (2/138) obtained from a study which examined enteric pathogen presence in private well water in northeastern Wisconsin (Borchardt et al., 2021). Lastly, Allen et al., 2017 identified an estimate of 1.7% (2/118) obtained from an Ontario, Canada based study assessing the presence of enteric viruses in private and municipal well water.

2.2.8. Dose-response parameters

The overall purpose of the dose-response assessment is to establish a relationship between the level of pathogen exposure and the probability of an adverse human health reaction (Haas et al., 2014). The human response after exposure to a waterborne pathogen is highly variable based on multiple underlying factors including pathogen (strain) virulence, immune status of the individual and magnitude of exposure (Buchanan et al., 2000).

2.2.8.1. Shiga toxin producing E. coli (STEC). The Beta-Poisson (BP) model is a frequently used, single-hit dose-response model and has previously been applied to shiga-toxin producing *E. coli* (Haas et al., 2000; Hynds et al., 2014; Murphy et al., 2016). Utilizing data from previous human outbreaks, Teunis et al. (2008) developed a hierarchical BP dose-response model on which current estimates are based. More specifically, in the current study, the 95th percentile predictive parameters ($\alpha = 398.9$, $\beta = 3.96 \times 10^4$) were employed, where ED is the dose and α and β are parameters of the BP distribution (Equation (2a)).

2.2.8.2. Giardia. The exponential dose response model (Equation (2b)) is the most frequently used for depicting *Giardia* exposure in waterborne QMRA (Balderrama-Carmona et al., 2017). The exponential dose-response curve was used with $r = 0.0199$ (Rose and Slifko, 1999; Teunis and Havelaar, 2002; Balderrama-Carmona et al., 2014).

2.2.8.3. Norovirus. For individuals >10 years, the modified BP model (Equation (2a)) has been identified as the most appropriate fit for norovirus and has been utilized in previous waterborne QMRA, allowing for comparability of QMRA estimates across studies (Teunis et al., 2008b; Murphy et al., 2016). More specifically, Teunis et al., 2008b, estimated that approximately half (50%) of all norovirus genome copies are infectious, with each infectious virus capable of causing an infection. The BP model was used with the parameters ($\alpha = 0.04$, $\beta = 0.05$; Equation (2a)).

Given that young children are particularly susceptible to this disease, requiring medical attention more frequently than any other age group, a fractional Poisson model was identified as being most appropriate for children (<10 years of age) (Messner et al., 2014). The fractional Poisson model is recommended when the pathogen(s) of interest are more virulent and/or for pathogen(s) that likely provoke an acute immune response in exposed individuals, such as norovirus (Messner et al., 2014). Further, given the likelihood of low doses, and the susceptibility of children, the fractional Poisson was the preferred method where P is the fractional Poisson parameter ($P = 0.72$; Equation (2c)).

2.2.9. Pooled - analyses

To accurately calculate risk estimates several pathogen-specific clinical variables are necessary including attack rate, secondary infection rate and hospitalization rate. Thus, fifty published academic peer-reviewed articles relating to waterborne *STEC*, *Giardia* and norovirus outbreaks and events were reviewed and analyzed with specific variables extracted (Table 4; Table S1). Kolmogorov-Smirnov tests were employed to assess goodness-of-fit between parametric distributions and empirical metadata for development of statistical (input) distributions (Delignetter-Muller and Dutang, 2015) (Table 5) (see Table 6 and Table 7).

3. Results

3.1. Exposure assessment

QMRA simulations predict that 97.5% of adult private well water users who drink from *E. coli* contaminated sources consume 0–10 *E. coli* CFU/day. This translates into a predicted mean daily consumption, during a contamination event, of 6 CFU/day and 4 CFU/day for those served by private wells located in consolidated and unconsolidated aquifers, respectively. The maximum likely rate of ingestion found at very high *E. coli* concentrations (e.g., >70 CFU/day) in concurrence with maximum daily well water consumption (>3500 mL/day) was 80 CFU/day and 50 CFU/day in consolidated and unconsolidated aquifers, respectively. Moreover, QMRA simulations predict that 97.5% of children (<10 years of age) consume 0–8 CFU/day with a predicted mean daily consumption of 3 CFU/day and 2 CFU/day for those served by private wells located in consolidated and unconsolidated aquifers, respectively. The maximum likely rate of ingestion found at very high *E. coli* concentrations (e.g., >70 CFU/day) in concurrence with maximum daily well water consumption (>3500 mL/day) was 58 CFU/day and 39 CFU/day in consolidated and unconsolidated aquifers, respectively.

3.2. Hazard characterization

During a contamination event, both daily and annual probabilities of

Table 5
Daily and annual probability of infection of STEC, *Giardia* and norovirus attributable to private well water in Ontario.

	Daily Probability of Infection (PD*inf*)		Annual Probability of Infection (PA*inf*)	
	Adults	Children	Adults	Children
STEC	4.1×10^{-3} (SD 3.8×10^{-2})[a] 3.8×10^{-3} (SD 3.7×10^{-2})[b]	5.2×10^{-3} (SD 4.1×10^{-2})[a] 4.4×10^{-3} (SD 3.7×10^{-2})[b]	1.30×10^{-2} (SD 2.5×10^{-2})[a] 1.36×10^{-2} (SD 2.4×10^{-2})[b]	1.4×10^{-2} (1.9×10^{-2})[a] 1.5×10^{-2} (2.1×10^{-2})[b]
Norovirus	1.5×10^{-2} (SD 9.1×10^{-2})[a] 1.1×10^{-2} (SD 8.2×10^{-2})[b]	2.3×10^{-2} (SD 1.2×10^{-1})[a] 1.6×10^{-2} (SD 1.0×10^{-1})[b]	7.9×10^{-2} (SD 5.6×10^{-2})[a] 8.1×10^{-2} (SD 5.6×10^{-2})[b]	3.6×10^{-1} (2.5×10^{-1})[a] 3.7×10^{-1} (2.6×10^{-1})[b]
Giardia	6.2×10^{-3}[a] (SD 5.2×10^{-2})[a] 4.9×10^{-3} (SD 4.6×10^{-2})[b]	8.0×10^{-3} (SD 5.7×10^{-2})[a] 6.7×10^{-3} (SD 5.3×10^{-2})[b]	2.2×10^{-2} (SD 4.6×10^{-2})[a] 2.6×10^{-2} (SD 4.0×10^{-2})[b]	2.7×10^{-2} (3.6×10^{-2})[a] 2.9×10^{-2} (4.0×10^{-2})[b]

[a] consolidated.
[b] unconsolidated.

Table 6
Predicted illnesses per annum attributable to STEC, *Giardia* and norovirus from untreated private well water in Ontario for adults and children (95% confidence interval).

	Annual Illnesses		Crude Incidence Rates	
	Adults	Children	Adults	Children
STEC	417 (341–493)[a] 160 (148–290)[b]	49 (35–55)[a] 20 (17–23)[b]	63.4/ 100,000[a] 56.8/ 100,000[b]	75.3/ 100,000[a] 71.8/ 100,000[b]
Giardia	731 (614–858)[a] 311 (270–350)[b]	99 (85–111)[a] 45 (39–50)[b]	111.2/ 100,000[a] 110.4/ 100,000[b]	152.3/ 100,000[a] 134.4/ 100,000[b]
Norovirus	1870 (1717–2020)[a] 728 (684–769)[b]	298 (289–306)[a] 95 (90–122)[b]	284.5/ 100,000[a] 258.4/ 100,000[b]	458.4/ 100,000[a] 341.1/ 100,000[b]
Total Illness	4217	607	449.1/ 100,000	653.6/ 100,000

STEC Public Health Ontario 2020: 132 cases, 0.9 rate per 100,000, 49 hospitalizations, 3 deaths.
Giardia Public Health Ontario 2020: 834 cases, 5.7 rate per 100,000, 4 hospitalizations, 0 deaths.
[a] consolidated.
[b] unconsolidated.

infection, from pathogen contaminated private well water for adults and children were calculated from Equation (2)*a-d* (Table 1). Overall, the highest daily and annual probabilities of infection within both hydrogeological settings and sub-populations (i.e., adults and children under the age of 10) were attributed to norovirus followed by *Giardia* and STEC. Among adults, the predicted mean daily likelihood of symptomatic infection due to a contamination event in contaminated private wells located in consolidated aquifers were 1.5×10^{-2} (0.015; SD = 9.1×10^{-2}), 6.2×10^{-3} (SD = 5.2×10^{-2}) and 4.1×10^{-3} (SD = 3.8×10^{-2}), for norovirus, *Giardia* and STEC, respectively. The mean annual probability of infection ranged from 1.3×10^{-2} (0.013; SD = 2.5×10^{-2}) for STEC to 7.9×10^{-2} (0.079; SD = 5.6×10^{-2}) for norovirus. Among children, the daily mean probabilities of infection were higher with daily risks of 2.3×10^{-2} (0.023; SD = 1.2×10^{-1}), 8.0×10^{-3} (SD = 5.7×10^{-2}) and 5.2×10^{-3} (SD = 4.1×10^{-2}) attributed to norovirus, *Giardia*, and STEC, respectively. Among the <10-year sub-population, mean simulated annual probabilities of infection ranged from 1.4×10^{-2} (0.014; SD = 1.9×10^{-2}) to 3.6×10^{-1} (0.36; SD = 2.5×10^{-1}) for STEC and norovirus, respectively. Likewise, in unconsolidated aquifers, the highest mean daily and annual probability of infection was associated with norovirus, followed by *Giardia* and STEC (Table 1). More specifically, daily probability of infection ranged from 3.8×10^{-3} (SD = 3.7×10^{-2}) to 1.1×10^{-2} (0.011; SD = 8.2×10^{-2}) for adults and from 4.4×10^{-3} (SD = 3.7×10^{-2}) to 1.6×10^{-2} (0.016; SD = 1.0×10^{-1}) for children. Annual probability of infection ranged from 1.36×10^{-2} (0.0136; SD = 2.4×10^{-2}) to 8.1×10^{-2} (0.081; SD = 5.6×10^{-2}) for adults and from 1.5×10^{-2} (0.015; SD = 2.1×10^{-2}) to 3.7×10^{-1} (0.37; SD = 2.6×10^{-1}) among children (Table 2).

3.3. Risk characterization

Province-wide, QMRA simulations predict a total of 4823 (crude incidence rates (CIR) among private well users = 467.8/100,000) cases of illness annually across all three pathogens due to consumption of contaminated private well water. The majority of cases are associated with norovirus for both adults and children, accounting for approximately 62% (2991/4823) of total cases, with 2598 and 393 cases of illness due to primary infections among adults and children, respectively (Table 2). *Giardia* infection accounted for 24.6% (1186/4823) of cases annually, with 1042 and 144 cases of illness among adults and children, respectively. Lastly, STEC infection accounted for 13.4% (646/4823) of total predicted illness with 577 and 69 cases among adults and children. These estimates equate to pooled age-specific crude incidence rates of 449.1/100,000 and 653.6/100,000 for adults and children for all three infections (Table 2).

Contaminated private wells located in consolidated aquifers were associated with more illness per year for all three pathogens in both age groups (i.e., adults and children) compared to contaminated private wells located in unconsolidated aquifers (Table 2). More specifically, for contaminated private wells located in consolidated aquifers QMRA simulations predict 3018 illness per year while unconsolidated aquifers were associated with 1199 illnesses per year in the adult population. In the pediatric simulations (<10 years of age), approximately 447 and 160 illness per year are predicted to occur as the result of contaminated private wells located in consolidated and unconsolidated aquifers, respectively.

Based on meta-analyses of previously reported hospitalization rates, the QMRA simulations predicts a total of 132 hospitalizations (CI: 22–163) per year due to primary infections attributable to contaminated private well water consumption in Ontario. By pathogen, STEC infection accounted for 64.4% (87/135) of all hospitalizations with 59 and 28 visits per year for adults and children, followed by norovirus (25.9%; 35/135) and *Giardia* (9.6%; 13/135) (Table 3). Based on previously reported hemolytic uremic syndrome (HUS) rates, the QMRA simulation predicts a total of 17 cases of HUS per year, with 11 and 6 among adults and children, respectively. Subsequently, approximately two to three HUS-related deaths per year due to exposure to contaminated private well water are predicted to occur in Ontario (Table 3).

3.4. Sensitivity and scenario analysis

Sensitivity analysis was undertaken for all twelve models (three

Table 7
Predicted HUS rates, hospitalization rates and mortality per annum attributable to STEC, *Giardia* and norovirus from untreated private well water in Ontario for adults and children (95% confidence interval).

	HUS cases*		Hospitalization		Mortality	
	Adults	Children	Adults	Children	Adults	Children
STEC	8 (6–11)[a] 3 (2–5)[b]	4 (<1–5)[a] 2 (<1–3)[b]	40 (80–111)[a] 19 (24–61)[b]	20 (2–15)[a] 8 (4–10)[b]	1.87 (0.99–2.6)[a] 0.892 (0.49–1.012)[b]	1 (0.09–0.65)[a] 0 (<1–1)[b]
Giardia	NA		7 (4–12)[a] 3 (<1–6)	2 (<1–3)[a] 1 (0–1)	N/A	
Norovirus	NA		15 (26–60)[a] 5 (9–20)[b]	10 (7–15)[a] 5 (2–5)[b]	N/A	
Total	11	6	89	46	3	1

STEC Public Health Ontario 2020: 132 cases, 0.9 rate per 100,000, 49 hospitalizations, 3 deaths.
Giardia Public Health Ontario 2020: 834 cases, 5.7 rate per 100,000, 4 hospitalizations, 0 deaths.
[a] consolidated.
[b] unconsolidated.

pathogens x two hydrogeological settings [i.e., consolidated, and unconsolidated aquifers] x two age categories [i.e., adults and children <10]) using Spearman's rank order correlation with respect to predicted annual illness on all parameters in the models (Table S2). Findings indicate frequency of contamination (Fc) as the model parameter with the greatest overall impact on all twelve model predictions (correlation coefficient ranging from 0.655 to 0.659), followed by *E. coli* loading (C_{ecoli}) and pathogen contribution (P1) (Table S2). Consumption volume had the lowest level of model output sensitivity in all models.

The current QMRA model assumes 100% treatment efficacy for individuals who treat their well water with a system that is theoretically effective against microbial contamination. However, given the high levels of uncertainty and variability associated with treatment efficacy (Hynds et al., 2014; Owens et al., 2020) scenario analysis was

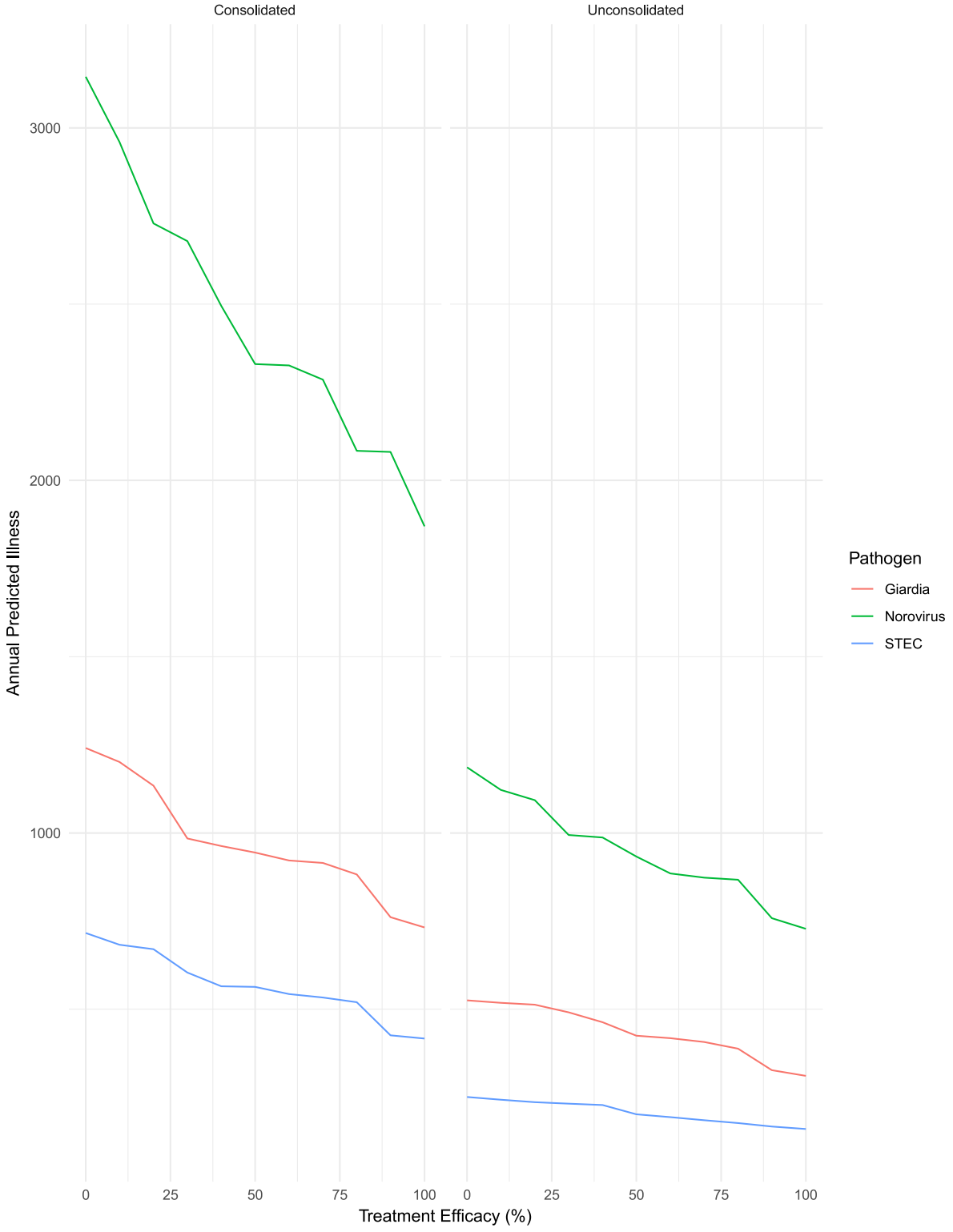

Fig. 2. Results of the scenario analysis of treatment efficacy (%) on number of predicted illnesses per year attributable to norovirus, *Giardia* and Shiga-toxin producing *E. coli* in the non-pediatric QMRA model in consolidated and unconsolidated aquifers.

undertaken to assess the potential of a private well water treatment system completely or partially failing (e.g., range of 0%–100% treatment efficacy), and the corresponding effect on predicted annual illness in all twelve models (Figs. 2 and 3). In the non-pediatric model, results indicate that total annual illness may increase from 4217 to 7064 cases per year for all three pathogens in the case of total treatment failure (Fig. 2). In the pediatric model total annual illness increased from 607 to 926 cases per year for total treatment failure (Fig. 3). Alternatively, with 50% treatment efficacy, total annual illness would increase to 5397 and 738 in the non-pediatric and pediatric models, respectively.

4. Discussion

An understanding of the spatio-temporal patterns of human health

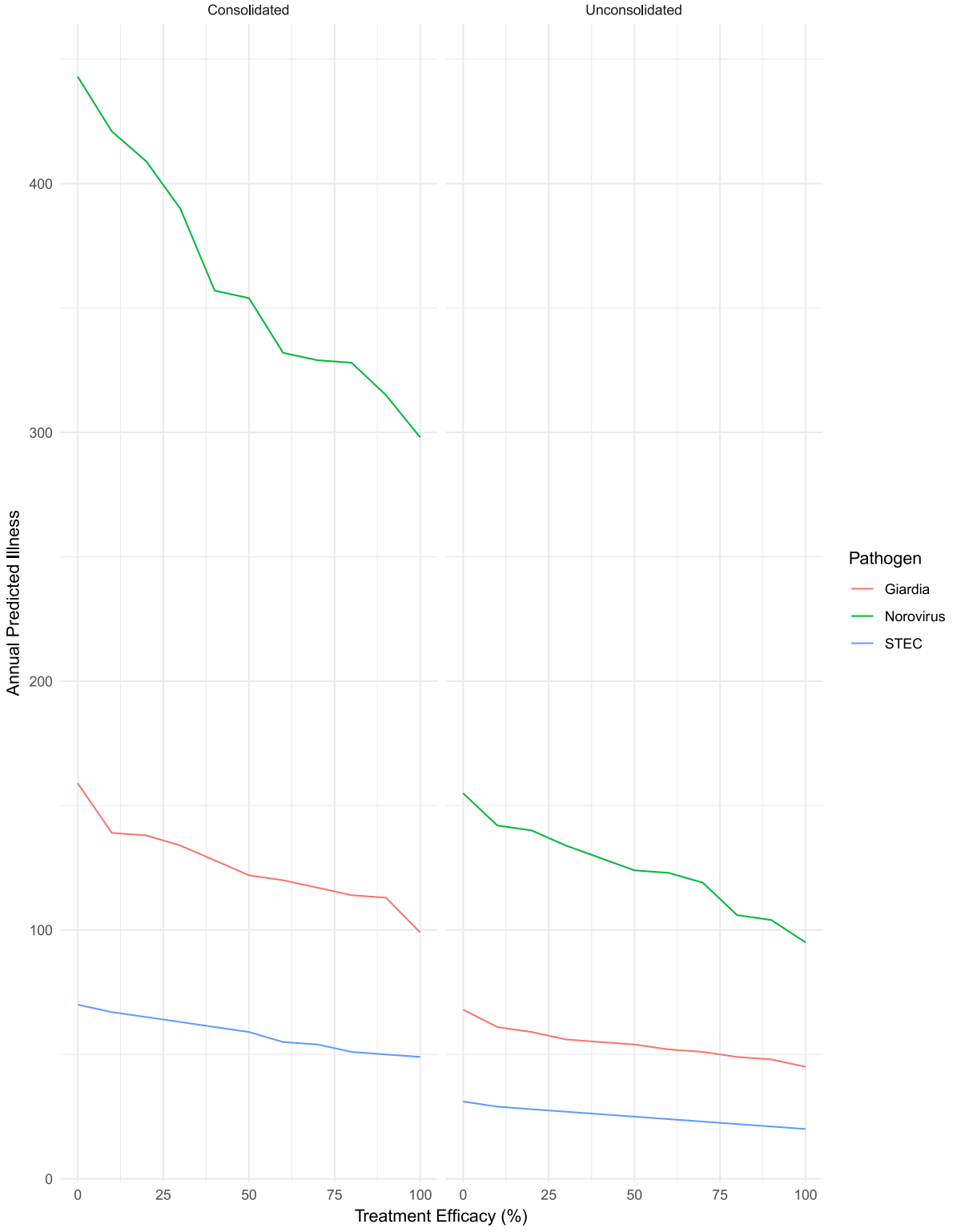

Fig. 3. Results of the scenario analysis of treatment efficacy (%) on number of predicted illnesses per year attributable to norovirus, *Giardia* and Shiga-toxin producing *E. coli* in the pediatric QMRA model in consolidated and unconsolidated aquifers.

risk associated with private water wells is critical for improving water quality and public health surveillance. Most failures in private drinking water management are identified only after an outbreak has occurred, causing severe illness, and costing both private (e.g., households) and public systems (e.g., local public health authorities, hospitals) millions of dollars (Vicente and Christoffersen, 2006; Chyzheuskaya et al., 2017; Gilpin et al., 2020; Collier et al., 2021). For example, an outbreak of *E. coli* O157:H7 and *Campylobacter jejuni* in the small rural community of Walkerton, Ontario resulted in 2300 people symptomatically ill, seven deaths, and a directly measurable cost estimated at more than CAN$155 million (Majowicz et al., 2005; Meinhardt, 2006). It is important to note that the Walkerton outbreak was in a municipal well water system where cost information was available. Costing data for private well water systems is not available, further highlighting the gaps in knowledge regarding private wells, including in public health management and subsequent interventions. Moreover, the Walkerton outbreak was fundamental in bringing the gaps in rural and remote water quality management to the forefront of public health policies and services, as there is no justification for permitting lower public health standards (e.g., water quality and quantity) for rural and remote residents of Ontario than those residing in Urban areas (Majowicz et al., 2005). Due to the unregulated nature of private water wells and the lack of public health surveillance, QMRA likely represents the most robust planning tool for public health officials in terms of mitigating waterborne illness. The current study employed a hydrogeologically delineated QMRA using a large spatio-temporal groundwater quality dataset (>700,000 samples) and multiple spatially-specific variables (e.g., hydrogeological setting, consumption volumes) to provide an increasingly accurate estimation of the human health burden associated with private water wells.

4.1. Waterborne AGI rates

Developed simulations predict provincial incidence rates of 62.6, 114.9 and 1162 cases/100,000 private well users per year for STEC, *Giardia* and norovirus, respectively. In Ontario in 2019, the incidence rates for STEC and *Giardia* (attributable to all sources) were 1.6 and 8.9 cases/100,000 people across the entire population, based on laboratory-confirmed cases notified through the iPHIS monitored by Public Health Ontario (Ontario Agency for Health Protection and Promotion, 2020). Norovirus is not a reportable disease and, therefore, cases are not documented provincially. Extrapolating these estimates to the entire Ontario population, the current simulated waterborne incidence rates would be 569 (62.6/11% [private well water population]) and 1044 (114.9/11% [private well water population]) cases/100,000 per people per year for STEC and *Giardia*, respectively. Consequently, the extrapolations from the current study for STEC and *Giardia* exceed those reported provincially for all transmission sources by more than an order of magnitude; therefore, the health risk attributable to private drinking water is undoubtedly significantly underestimated based on provincial estimates (OAHPP, 2020). Previous studies have attempted to quantify AGI underreporting rates in Ontario and estimated that for each reported case of enteric illness, the number in the community is approximately 313 (Majowicz et al., 2005). Based on these estimates, the provincially reported STEC and *Giardia* rates (attributable to all sources) would be approximately 281.7 and 1784.1 cases per 100, 000 per person per year, thus the current study estimates, and extrapolations are conceivable.

Provincial AGI rates are likely lower than current study estimates for several reasons. Cases of waterborne infection are frequently mild or self-limiting, with cases tending to be sporadic in nature (i.e., one-off infection and/or limited to a single household) due to the inherent nature of the transmission source. Consequently, individuals do not typically seek medical care nor receive a laboratory-confirmed diagnosis which is required to be provincially documented, if indeed the disease is reportable (Hrudey and Hrudey, 2007; Haagsma et al., 2013; Haas et al., 2014). A recent province-wide survey of private well users across Ontario indicated that 15% (168/1120) of respondents reported that at least one household member had presented with gastrointestinal symptoms in the 12-month period prior to surveying, of which just 13.7% had a clinically confirmed diagnosis (Lavallee et al., 2021a; Lavallee et al., 2022), suggesting an underreporting rate of approximately 86% (145/168). Applying this level of underreporting to the estimated AGI cases found in the current study (89/646 STEC; 162/1297 *Giardia*), simulated waterborne incidence rates that would be identified by the province attributable to private well water would be approximately 0.61 and 1.12 cases/100,000 per people per year for STEC and *Giardia*, respectively. Thus, given these theoretical estimates and comparing with actual reported provincial incidence rates for all sources in 2019 (1.6 and 8.9 cases/100,000 people per year), the hypothesis that the public health risk attributable to private wells is significantly underestimated and undocumented is further supported. Accessing a physician may be difficult for rural residents given the distance to hospital/clinic, wait times for general practitioners and the self-limiting nature of the infection (Shah et al., 2020).

Overall, 135 hospitalizations (87 STEC; 13 *Giardia*), 17 cases of hemolytic uremic syndrome (HUS) and three HUS related deaths per year due to infections attributed to contaminated private well water are predicted based on presented QMRA simulations. Provincially, during 2019, 62 and 14 hospitalizations were reported for STEC and *Giardia*, respectively, with zero STEC related deaths (OAHPP, 2020). Accordingly, current findings again highlight likely significant levels of underreporting and/or misdiagnosis occurring provincially. Previous studies have identified that waterborne illnesses are commonly misdiagnosed by the medical community unless a larger outbreak is happening (Meinhardt 2006; Haagsma et al., 2013). Additionally, there are several clinical and administrative procedures that must occur for an individual infection to be documented, with an etiologic agent and potential source identified, to the local Public Health Authority (Fig. S7). Consequently, given the sporadic nature of waterborne illnesses as they relate to private drinking water wells, and the process of confirming an individual infection, it is likely that some hospitalizations and deaths occurring in the province may be attributed to other etiological agents, co-infections and/or sources (e.g., foodborne, person to person contact). Low levels of provincially reported AGI, hospitalizations, and mortality rates attributable to private water wells may create a false sense of security with respect to waterborne transmission of disease, resulting in decreased household/domestic monitoring and public health measures, leaving private well water users increasingly exposed (Hooks et al., 2019).

Study findings indicate a cumulative annual risk estimate of 4.6×10^{-3} (i.e., illnesses per person per year) from all twelve models. Comparatively, on a per pathogen basis, Murphy et al., (2016), reported the daily probability infection to norovirus, *Giardia* and STEC was 43.9%, 0.053% and 0.0082%, for all of Canada, compared to 1.5% (norovirus), 0.62% (Giardia) and 0.38% (STEC) examined in the current study for Ontario. Conversely, Burch et al., 2021 did not identify norovirus, *Giardia* or STEC in their waterborne QMRA, highlighting the importance of regionally specific risk estimates as contamination source and occurrence differ based on region. The estimates provided in the current study are based on one of the largest available spatiotemporal groundwater quality datasets (702, 861 samples from 239,244 wells) over a seven-year period and contains spatially tailored model inputs (e.g., consumption volume, contamination duration). For example, contamination duration distributions utilized in the current QMRA remove the presumption of there being an equal probability of contamination 365 days per year, which has been employed in previous QMRAs (e.g., Murphy et al., 2016). Thus, the current overall risk assessment represents a more realistic estimate of overall illness associated with private water wells given the large sample used to create model inputs distributions. Finally, the uncertainty associated with all model inputs makes comparisons difficult and results must be interpreted with caution (Haas et al., 2014; Burch et al., 2021). Nonetheless,

the United States Environmental Protection Agency acceptable target (for public water systems) is 1×10^{-4} infections per person per year (Mara et al., 2007), which the overall (4.6×10^{-3}), consolidated aquifer (4.9×10^{-3}) and unconsolidated aquifer (4.4×10^{-3}) risk estimates all exceed by over an order of magnitude.

4.2. Scenario analysis: treatment efficacy

Findings indicate that approximately 34% of individuals who treat their well water, use a treatment system effective against microbial contamination. Scenario analyses indicate that in the (unlikely) event of total treatment failure among this population, total mean annual illness nearly doubled (4217 to 7064 cases per year for all twelve models), highlighting the importance of effective water treatment in reducing infectious health risks attributable to private well water in Ontario. Despite the recognized risk associated with untreated well water, many private well users do not employ a treatment system effective against microbial contaminants, or improperly maintain the systems, rendering them ineffective. (Kreutzwiser et al., 2011; Flanagan et al., 2015; Malecki et al., 2017; Seliga et al., 2022). It is important to note that utilizing a water treatment system is entirely voluntary and can be costly to the well owner, however, a doubling of illness due to failing treatment systems would lead to a significant increase in direct and indirect costs across healthcare systems and households. To reduce the risk associated with private well water to the acceptable target threshold, it is recommended that private well owners are provided with a comprehensive testing program that is reflective of their local groundwater environment and regularly engage in well water stewardship behaviours (e.g., routine testing, well maintenance and efficacious treatment). However, private well owners experiencing frequent and on-going contamination may require a treatment system that is effective against microbial contamination.

4.3. Study limitations

In the current study, and the majority of groundwater related QMRAs, the pathogen contribution input distribution represents a notable limitation, as the authors were unable to directly analyze the *E. coli* to pathogen ratio across the study area and were thus compelled to rely on previously published estimates which may misrepresent the overall human health risk. This is true of most published QMRAs. If possible, future waterborne QMRAs should be accompanied by a study of pathogen ratio sourced from specific or representative study locations to provide an increasingly accurate depiction of pathogen occurrence in private drinking water wells. Further, to model contamination duration, the current study employed distributions based solely on *E. coli* as an indicator of fecal contamination (Latchmore et al., 2022). However, there is variability and uncertainty in these estimates given varying survival times of viruses and protozoa in untreated groundwater due to differences in microbial characteristics, mechanisms, and resistance to treatment; therefore, waterborne AGI estimates produced in this study and similar QMRAs should be interpreted with caution. Finally, given the age demographic of the province-wide survey and limited Census data, it was not possible to separately examine the human health impact private water wells have on the elderly (>65-year-old), immunocompromised and pregnant women. It is speculated that the reported hospitalizations, HUS rates, and mortality rates from the current study are likely acquired by those of vulnerable sub-populations, but without age-specific data it is difficult to draw definitive conclusions. Future QMRAs should seek to acquire age-specific data on vulnerable sub-populations within the study location.

4.4. Recommendations

Sensitivity analysis of input variables found that all twelve models display similar patterns. The input variables of most importance were contamination frequency (r = 0.655 to 0.659) and *E. coli* loading (r = 0.357 to 0.420). Hynds et al. (2014b) previously identified the same variables to be most significant for a QMRA model of private well water in Ireland. Therefore, the recommendation put forth by Hynds et al., (2014) of improved groundwater protection is echoed here; while the frequency and concentration of private well water contamination varies across the globe, the primary mechanisms driving contamination are relatively analogous. Public health interventions should focus on improving well water stewardship behaviours (e.g., well maintenance and construction, treatment and testing) to mitigate the human health risk associated with localized contamination pathways. A previous study of private well owners in Ontario has identified three statistically distinct sub-groups (i.e., clusters) of private well owners based on well water stewardship, and exposure via well water consumption (Lavallee et al., 2022). Future work is ongoing to introduce these "socio-cognitive clusters" to create distinct "well user type" risk profiles to better understand how and to what extent human behaviour impacts well water susceptibility and human infection.

5. Conclusion

QMRA is a powerful mathematical tool for estimating the risk of exposure to, and subsequent infection from, waterborne pathogens in contaminated private well water supplies. Based on QMRA simulations, consumption of contaminated private well water in Ontario is responsible for approximately 4823 AGI cases annually. By pathogen, waterborne AGI is attributable to norovirus (62%; 2991/4823), *Giardia* (24.6%; 1186/4823) and STEC (13.4%; 646/4823). Further, while public health professionals can potentially quantify the health risk associated with private well water, the responsibility of mitigating the risk rests with well owners, even in the presence of evidence-based public health guidance. Thus, private well owners must be provided with the appropriate knowledge of their systems, the risk they pose and the tools to strengthen their own behaviours and stewardship actions. However, a one-size-fits all approach will not suffice, due to varying levels of knowledge, attitudes, and risk perception among private well owners. Therefore, the authors consider that levels of risk should be identified for subsets of private well owners themselves, as the potential health threat cannot be mitigated unless private well user socio-cognitive profile is identified and modified. Further, study findings highlight the likely significant underreporting of waterborne AGI rates at the provincial level which may skew public health interventions and programs that are effective in monitoring and minimizing the human health risk associated with private well water.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.114077.

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Assessment of exposure to pesticide mixtures in five European countries by a harmonized urinary suspect screening approach

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ABSTRACT

Humans are exposed to a mixture of pesticides through diet as well as through the environment. We conducted a suspect-screening based study to describe the probability of (concomitant) exposure to a set of pesticide profiles in five European countries (Latvia, Hungary, Czech Republic, Spain and the Netherlands). We explored whether living in an agricultural area (compared to living in a peri-urban area), being a a child (compared to being an adult), and the season in which the urine sample was collected had an impact on the probability of detection of pesticides (-metabolites).

In total 2088 urine samples were collected from 1050 participants (525 parent-child pairs) and analyzed through harmonized suspect screening by five different laboratories. Fourty pesticide biomarkers (either pesticide metabolites or the parent pesticides as such) relating to 29 pesticides were identified at high levels of confidence in samples across all study sites. Most frequently detected were biomarkers related to the parent pesticides acetamiprid and chlorpropham. Other biomarkers with high detection rates in at least four countries related to the parent pesticides boscalid, fludioxonil, pirimiphos-methyl, pyrimethanil, clothianidin, fluazifop and propamocarb. In 84% of the samples at least two different pesticides were detected. The median number of

Abbreviations: HBM, Human Biomonitoring; HBM4EU, European Human Biomonitoring Initiative; LC-HRMS, Liquid chromatography coupled to High Resolution Mass Spectrometry; SPECIMEn, Survey on PEstiCIde Mixtures in Europe; SS, Suspect Screening.

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detected pesticides in the urine samples was 3, and the maximum was 13 pesticides detected in a single sample. The most frequently co-occurring substances were acetamiprid with chlorpropham (in 62 urine samples), and acetamiprid with tebuconazole (30 samples). Some variation in the probability of detection of pesticides (-metabolites) was observed with living in an agricultural area or season of urine sampling, though no consistent patterns were observed. We did observe differences in the probability of detection of a pesticide (metabolite) among children compared to adults, suggesting a different exposure and/or elimination patterns between adults and children.

This survey demonstrates the feasibility of conducting a harmonized pan-European sample collection, combined with suspect screening to provide insight in the presence of exposure to pesticide mixtures in the European population, including agricultural areas. Future improvements could come from improved (harmonized) quantification of pesticide levels.

1. Introduction

Humans are typically exposed to pesticides through multiple sources, including diet, occupational or environmental exposures (Damalas and Eleftherohorinos, 2011; Deziel et al., 2015). Growing evidence indicates that living in an agricultural area where pesticides are applied contributes to higher exposure than residents living away from agricultural areas (Dereumeaux et al., 2020; Figueiredo et al., 2021; Teysseire et al., 2020, 2021). Determinants contributing to this increased exposure include proximity to agricultural fields where pesticides are applied, crop acreage around the home, and season (Dereumeaux et al., 2020; Teysseire et al., 2021). Pesticide exposure has been linked to various short-term and chronic health effects such as respiratory or neurological development issues (Kim et al., 2017; Ntzani et al., 2013). Therefore a comprehensive characterization of the exposure to real-life mixtures of pesticides, which includes the contribution of living close to agricultural areas where pesticides are applied, is essential for human health risk assessment.

Most non-occupational pesticide exposure studies focus on selected sets of targeted pesticides for human biomonitoring (HBM), often based on *a priori* selected biomarkers related to e.g. the spraying activities in a certain area, the health outcome of interest, or practical considerations such as the commercial availability of standards (Dereumeaux et al., 2020; Teysseire et al., 2021). Currently, HBM for urinary pesticide biomarkers by targeted methods is limited to mostly pyrethroids and non-specific markers of organophosphorus pesticides. However, in real-life pesticide exposure often is already a mixture of multiple co-occurring compounds with repeated exposure timeframes (Crépet et al., 2019). With more than 450 active pesticides currently approved (plus 50 more currently pending) for use in the European Union (EU Database Pest, 2022), there is a growing need for information on the co-occurrence of these compounds in the human body. HBM of pesticides in urine is a useful method to assess the aggregate exposure of pesticides from various exposure sources and routes, by measuring the parent pesticide and/or the corresponding biotransformation products (Bonvallot et al., 2021). However, as the list of registered pesticides is long and they occur often highly metabolized in urine, a large number of targeted assays would be required to assess presence of all urinary pesticides and their metabolites in each sample. This is currently not feasible since many human urinary biomarkers of exposure (typically phase I/II metabolites) are often unknown, and the analytical reference standards are not readily available. Suspect screening (SS) approaches based on full scan High Resolution Mass Spectrometry (HRMS) emerge as an innovative way to assess the presence of a broad range of exposure markers and better capture the complexity of pesticide mixtures (Andra et al., 2017; Pourchet et al., 2020; Huber et al., 2022).

The study presented here, the Survey on PEstiCIde Mixtures in Europe (SPECIMEn), aimed to generate new pesticide exposure data in a harmonized pan-European setting (as part of the European Human Biomonitoring Initiative HBM4EU, www.hbm4eu.eu). This was done by analyzing 2088 urine samples collected in five countries through a multi-laboratory high-throughput SS approach. This study aimed at exploring co-occurrence (probability of exposure) of pesticide biomarkers across Europe and within each participating country. It also aimed at assessing differences of exposure patterns by location (living close to agricultural fields *versus* non-agricultural areas), seasons (differences in spraying activities), as well as age groups (adults *versus* children, of which the latter are more sensitive to health effects and usually have higher internal exposure levels, due to e.g. a higher food intake/kg body weight (Eskenazi et al., 1999; Sapbamrer and Hongsibsong, 2019). The study design therefore provides insight into local contributions, based on a broad combination of pesticides. Higher detection frequencies of pesticide markers might be expected for those pesticides applied on local crops during the spraying season in residents living close to the agricultural fields.

2. Material and methods

2.1. Sampling strategy

To create geographical coverage across Europe, study sites from five countries were included to provide insight into variations of pesticide exposure patterns across Europe, namely the Czech Republic, Hungary, Latvia, Spain and the Netherlands. Within each country, urine samples were collected simultaneously at two locations: agricultural and non-agricultural areas. Each address in the agricultural area was located within 250 m from an agricultural field where pesticides were typically applied, mainly focusing on tree-crops or so-called 'overhead cultures' (except Latvia where tree-crops were hardly grown). These 'overhead-cultures' will result in potentially higher exposure concentrations in the air due to machine-drawn air blast or a hand-held overhead spray, which are more prone to drift (Willenbockel et al., 2022). The crop types differed slightly between countries due to e.g. differences in climate. A detailed description of the area selection in all five country can be found in Supplementary Material F. In summary, Spain focused on residential areas close to citrus fruits, Czech Republic on apples, vineyards, peach, plums and apricots, Hungary on apples, the Netherlands on apples and pears, and Latvia mostly on winter and summer rapeseed, summer wheat and barley. Non-agricultural areas were defined as sub-urban areas at least 500 m away from any agricultural fields.

Per country at each agricultural and non-agricultural area, 50 parent-child pairs (50 households) were included (total of 100 parent-child pairs per country). Each parent-child pair was composed of one child aged 6–11 years at the time of inclusion, accompanied by one of their parents or legal guardians living in the same household. Adults who worked in the agricultural sector (i.e. farmers) were excluded from recruitment, since the sample size was too limited to distinguish occupational exposures. The same selection criteria were used in all five countries.

A minimum of 100 parent-child pairs per country (200 individuals) provided a first morning void urine sample, and completed a harmonized questionnaire. The admission of the questionnaire, sample collection procedures and timing of sampling was coordinated, and sampling materials such as cups and tubes were bought in bulk to avoid any batch differences. All collected urine samples were stored and transported refrigerated (at 4 °C), until samples were aliquoted and

stored at −80 °C (within 48 h of sample collection). Samples were transported to the laboratory of analysis after each season.

All households were visited twice: the first visit was made in winter 2019/2020 (season 1), the second in summer 2020 (season 2). The specific sampling dates (Supplementary Material A) differed slightly between study sites, partly due to differences in spraying season due to climate and the type of crop grown on the field. The sampling of the second season was slightly delayed (end of summer) due to the COVID-19 pandemic and accompanied uncertainties. The recruitment strategy differed between the study sites, a detailed description of the recruitment strategy per country can be found in Supplementary Material F. In summary, the Hungarian partner involved local public health officers to get in touch with the participants, while others sent out letters (the Czech Republic and the Netherlands), contacted colleagues as study participants (Spain and the Netherlands), conducted an online campaign (the Czech Republic and the Netherlands), and/or contacted participants through schools (Spain and Latvia). A detailed questionnaire was completed during the first season by the parent, and a subset of questions was asked again during the second season (Supplementary Material B). The joint questionnaire was developed in English, and subsequently translated to the local languages. The questionnaire covered personal and household characteristics, activities up to three days prior to sampling, potential pesticide exposure scenarios (occupational, usage of products containing pesticides), and the food consumption pattern of the day prior to sampling (origin of consumed foods as well as a food frequency table for food consumption 24 h prior to sampling).

All partner countries acquired approval from the appropriate local medical ethical committees, and written informed consent was obtained from all participants (parents and children separately). A description of the ethical approval procedure per country can be found in supplementary material F. A harmonized informed consent form was used for all participants, which was evaluated by an internal HBM4EU review board.

2.2. Suspect screening approach

A SS methodology was applied to analyze the urine samples, of which a detailed description can be found in Huber et al. (2022). Briefly, the applied analytical workflow from sample preparation, instrumental analysis, and data processing was conducted under harmonized conditions in five different laboratories across Europe, in the Netherlands, Germany, France, the Czech Republic and Spain (Vitale et al., 2022). Each laboratory analyzed approximately 400 urine samples originating from one of the five SPECIMEn study sites. Samples were analyzed after each season, and potential batch effects were addressed (Huber et al., 2022). The suspect database generation, MS data analysis and confirmation procedures were performed in a centralized way. Several consolidated quality assurance/quality control (QA/QC) dispositions, parameters and criteria were first implemented to ensure the consistency of the results obtained across the different participating laboratories as well as to document the applied method performances (Vitale et al., 2022). The applied analytical workflow was described in detail by Huber et al. (2022) and consists of i) SPE cleanup/concentration (5-fold) of the urine after pH adjustment, ii) measurement of the extracts by full scan liquid chromatography coupled to HRMS (LC-HRMS), iii) data pre-processing and analysis, iv) prioritization of putative detects, v) generation of a list of representative samples for follow up identification experiments using tandem mass spectrometry (MS/MS), and vi) final confirmation of putative detects by spectral comparison with reference standards either purchased/synthesized or generated *in vitro* by human liver S9 incubations. The curated suspect list of pesticides may include multiple metabolites originating from the same parent compound, resulting in a final datafile with potentially several metabolites that reflect exposure of the same parent compound. This redundancy is considered enhancing confidence. In the case of SPECIMEn, this list focused on pesticides and one aggregated list of known and predicted pesticide metabolites from all five laboratories was used as suspect database. 'Fully identified' were those with the highest level of confidence: Schymanski level 1 if a reference standard material is commercially available, or Schymanski 2 by diagnostic evidence acquired by human liver S9 incubation experiments (Schymanski et al., 2014). Biomarkers which were identified at a lower tier will end up in lower confidence levels, reflecting the level of uncertainty about the identity of that feature. In the context of the present paper, only biomarkers identified with confidence levels 1 and 2 were considered.

2.3. Statistical analysis

In line with the basic principle of the SS approach, the data generated in SPECIMEn are 'semi-quantitative', i.e. quantitative signal intensities for each representative spectrometric mass are reported per sample, yet these intensities cannot be considered as urinary pesticide concentrations and are not standardized across laboratories. The data was analysed by dichotomizing the intensities into 'detected' versus 'non-detected', which allows comparisons across study sites as well as inclusion of biomarkers with low detection rates in the statistical analysis.

The detection rate was calculated as the number of samples in which a particular biomarker was detected and identified with confidence levels 1 and 2 over the total number of samples collected, expressed in percentage. Based on the parent pesticides (if multiple metabolites and the parent pesticide were measured, these were considered as one), the patterns of co-occurrences were explored. First, the total number of pesticides per urine sample was evaluated. Secondly, with the usage of an UpSet plot it was evaluated which parent pesticide combinations co-occurred and how frequent. Thirdly, the correlation pattern in the total set of parent pesticides was evaluated for each study site with a weighted correlation network using the *IsingFit* R package v0.3.1 (van Borkulo et al., 2015). This package estimates the network based on the Ising model: combining L1-regularized logistic regression with EBIC model selection (gamma 0.25). On this network a clustering algorithm was applied (*walktrap)*, to detect communities of closely related features indicated by different colours in the network (Pons and Latapy, 2005).

To assess the influence of co-variates, logistic mixed effects regression models were applied, with participant ID and household ID as random effects. Our main model includes fixed effects for season (season 1/season 2), location (agricultural/non-agricultural) and age category (child/adult). We assessed the sensitivity for further adjustment for potential confounding by including body mass index (BMI) level, education of the parent, consumption of homegrown foods (yearly average percentage), and a summary indicator for pesticide usage in an extended model. The pesticide usage indicator indicates whether pesticide containing products were used up to three days prior to sampling either for human use, in the garden, indoors and/or for professional use. The estimates for season, location and age groups were transformed to Odds Ratios (OR) with 95% Confidence Intervals (CI) for both the main and extended models.

3. Results

3.1. Population characteristics

The description of the study population for the five study sites of the SPECIMEn study is provided in Table 1. In total 2088 urine samples were collected, which were equally spread across the five study sites and areas. The loss to follow-up of individuals between seasons was low, varying from 0.9 to 2.9%. Reasons for loss to follow-up were loss of contact, divorce and/or move to another location. The adult samples mainly originated from the mothers, while gender was equally divided across the children's samples. The mean age of the adults was comparable across all study sites, varying from 38 to 44 years. The mean BMI (self-reported) of the adults originating from Latvia and Hungary was

Table 1
Descriptive characteristics of the SPECIMEn study participants by study site and location.

Study site	ES[a]		LV[a]		HU[a]		CZ[a]		NL[a]	
Area	Agricultural	Non-Agricultural	Agricultural	Non-Agricultural	Agricultural	Non-Agricultural	Agricultural	Non-Agricultural	Agricultural	Non-Agricultural
Adult-child pairs[b], n	52	53	50	51	51	52	51	60	55	50
Urine samples, n	206	212	200	202	201	208	204	238	219	198
Season 1	104	106	100	102	102	104	102	120	110	100
Season 2	102	106	100	100	99	104	102	118	109	98
Gender, female, %										
Adults	50	87	90	82	94	85	71	60	71	66
Children	54	49	58	47	49	52	43	43	53	46
Mean age, years										
Adults	44	44	40	39	38	40	41	42	42	42
Children	8.2	8.7	8.9	8.4	9.7	9.2	8.8	9.1	8.6	8.6
Mean BMI										
Adults	25	24	26	26	26	26	24	24	24	23
Children	17	17	17	17	18	19	16	16	16	16.0
Educational level adult, %										
No or only primary education	0	0	2.0	0	40	5.8	0	1.7	1.8	0
Secondary education	7.8	17	30	12	28	20	2.0	3.3	5.5	2.0
Tertiary education (post-secondary)	25	17	8.0	7.8	23	26	26	10.0	18	18
University studies (BSc, MSc, PhD)	67	66	60	77	8.0	48	71	83	71	76
Don't Know/ NA	0	0	0	3.9	0	0	2.0	1.7	3.6	4.1
Usage of pesticide (-products) up to 3 days prior to sampling[c], n households										
Season 1	9	5	4	7	2	6	4	2	1	6
Season 2	27	8	12	8	22	7	14	12	10	4
Seasonal homegrown vegetables, fruit and/or herbs consumption, % of total consumption										
Winter	6.7	1.1	30	22	23	4.5	13	10	2.0	0.1
Spring	10	3.4	28	19	21	9.4	22	12	4.8	2.2
Summer	12	8.0	63	44	41	25	64	51	15	7.8
Autumn	8.9	6.0	63	45	39	17	45	40	8.3	4.5

[a] ES: Spain, LV: Latvia, HU: Hungary, CZ: Czech Republic, NL: the Netherlands.
[b] Number of individuals included in season 1.
[c] Summary indicator which includes: pesticides for human use, use indoors, use in garden, and professional use. For specification of the categories see Supplementary Material B.

slightly higher compared to the adults from other study sites. Most of the participants did not smoke, although in the agricultural areas of Spain and Hungary there was a substantial group of current smokers 35% and 45%, respectively (Supplementary Material B). Based on the total household income categories, participants of agricultural areas mostly earned less money than those living in non-agricultural areas. In all areas except the agricultural area in Hungary, the majority of the participants had a university education level. In Spain and Hungary, about half of the households in agricultural areas used pesticide products during summer season, which includes the use of consumer products, usage indoors, in the garden and/or professional use. These different categories are presented separately in Supplementary Material B. Overall, the homegrown food consumption percentage was higher in households in agricultural areas than those in non-agricultural areas, mostly during summer.

3.2. Annotations and detection rates

The application and harmonization of the SS approach was performed on 2088 urine samples using the method described in detail in Huber et al. (2022). A total number of 498 tentative annotations of pesticide biomarkers was obtained and prioritized, of which 40 pesticide biomarkers were annotated with confidence level 1 or 2 (Table 2). These 40 related to a total of 29 parent pesticides. In addition to these 40, 54 other pesticide biomarkers (either pesticide metabolites or the parent pesticides as such) were detected with a lower confidence level (Schymanski levels 3–5) which are detailed in Supplementary Material C. These 54 are not further described in this paper and not used in the analyses.

For each annotated exposure marker (confidence levels 1 and 2), the overall detection rate per study site was calculated (Table 2). Overall, biomarkers were generally detected below 25% of the samples. The results evidenced a significant variability between study sites, with Latvia having generally the lowest number of detects and Spain the highest one. Overall, the metabolites related to the parent pesticides acetamiprid (N-demethylated metabolite) and chlorpropham (4-HSA metabolite) were most frequently detected in samples of all study sites. Other biomarkers that had detection rates of at least 10% (including both locations and both season) relate to the parent pesticides boscalid (not in Hungary), chlorpyrifos (only in Spain and Czech Republic), clothianidin (not in Latvia), cyprodinil (not in Latvia and Hungary), flonicamid (not in Latvia and Czech Republic), fluazifop (not in Latvia), fludioxonil (not in Hungary), imazalil (only in Spain and Latvia), imidacloprid (only in Spain), pirimiphos-methyl (not in Hungary), propamocarb (not in Latvia), pyrimethanil (not in Hungary), tebuconazole (not in Latvia), and thiamethoxam (only in Spain and Hungary). Biomarkers that were detected at low frequencies (<10%) across all study sites include 2,4-dichlorophenoxy acetic acid (2,4-D), ametoctradin,

Table 2
Annotated pesticide biomarkers with Schymanski confidence levels 1 and 2 (p = 40) and their overall detection frequency (%) per study site (Schymanski et al., 2014).

ID	Pesticide type[a]	Parent pesticide	Pesticide (metabolite) annotation[b]	Confidence level[c]	Overall Detection Frequency (%)				
					ES[d]	LV[d]	HU[d]	CZ[d]	NL[d]
P1	H	2,4-Dichlorophenoxyacetic acid	Parent	1	4.1	0	2.2	2.7	0
P2_a	I	Acetamiprid	-CH2	1	99	33	94	98	93
P3_a	F	Ametoctradin	-C2H6 +2O	1	5.0	2.7	1.2	4.8	2.9
P5_a	F	Boscalid	+O + SO3 [e]	2	36	18	3.9	23	33
P5_b			+O + SO3 [f]	2	7.2	0	0	0.5	0.2
P6	I	Chlorantraniliprole	+O	2	3.8	0.3	0.2	0	0.2
P8_a	H, GR	Chlorpropham	+O + SO3 (4-HSA)	1	56	32	31	34	75
P9_a	I	Chlorpyrifos (methyl)	TCPy	1	1.7	0	0.2	0.2	0.2
P9_b			-CH2	1	36	0	6.9	21.7	6.5
P10	H	Clopyralid	Parent	1	1.0	0	0	1.4	0.7
P11_a	I	Clothianidin (can come from thiamethoxam)	Parent	1	34	1.7	22	25	20
P11_b			-NO2 +H	1	0.5	0	0.2	0	0.2
P11_c			-CH2	2	21	0.8	9.8	6.6	3.1
P12_a	I	Cypermethrin, cyfluthrin, permethrin, transfluthrin	DCCA	1	0.5	0	0	0	0
P13_a	F	Cyprodinil	+O + SO3	2	14	7.7	2.7	10	26
P18_a	I	Flonicamid	Parent	1	1.7	0.8	2.0	2.7	5.7
P18_b			-C2HN	2	15	0.3	27	0.2	57
P19_a	H	Fluazifop	Parent [e]	1	20	2.5	11	18	21
P19_b			Parent [f]	1	8.1	1.5	4.9	5.2	8.2
P20	F	Fludioxonil	+O + C6H8O6	2	16	15	2.0	14	27
P21_a	F	Fluopyram	+O + SO3	2	3.6	0.5	0.2	1.1	1.0
P21_b			+O + C6H8O6	2	2.4	0.8	0.5	3.2	4.8
P21_c			-2H	2	11	6.7	0.5	3.4	3.1
P22_a	I	Flupyradifurone	Parent	1	2.6	0.3	0.5	0.7	2.2
P25_a	I, Ac	Fluvalinate	-C14H9NO	2	1.0	0	0.7	0.2	0
P27_a	F	Imazalil	+C6H8O6	2	19	11	8.3	4.5	4.6
P28_a	I	Imidacloprid	-NO2 +H	1	17	1.7	4.2	0.7	9.4
P32_a	F	Penconazole	+O + C6H8O6	2	6.5	1.7	2.2	2.0	2.4
P34_a	I, Ac	Pirimiphos-methyl	-CH2	1	85	10	6.6	24	48
P35_a	F	Propamocarb	Parent	1	9.6	1	11	5.0	23
P35_b			+O	2	21	5.5	18	12	43
P37	H	Propyzamide	+H2O3	2	8.6	0	0.5	0.9	1.0
P38_a	F	Pyrimethanil	+O + SO3	2	27	14	4.9	22	32
P38_b			+O	2	0.7	0	2.7	0	0.5
P40_a	F	Tebuconazole	-2H +2O	2	71	5.5	25	52	36
P41_a	F	Thiabendazole	+O + C6H8O6	2	0	0.8	0.2	0	0.5
P42_a	I	Thiacloprid	+O	2	8.4	0.8	2.9	7.9	4.6
P43_a	I	Thiamethoxam	Parent	1	0.7	0	2.4	0	0.5
P43_b			-NO2 +H	1	23	0	15	0	0.2
P46_a	F	Trifloxystrobin	-CH2 -CH2	2	0.7	0.5	0	3.6	3.8

[a] H: Herbicide, F: Fungicide, I: Insecticide, GR: Plant Growth Regulator, Ac: Acaricide.
[b] Metabolite annotation: "-CH2" means the molecular formula of the metabolite is that of the parent minus CH2 (corresponding to demethylation). Similarly, "+O" means the metabolite is the parent compound plus one oxygen atom (hydroxylation). "+SO3" and "+C6H8O6" indicate sulfation and glucuronidation, respectively.
[c] Schymanski confidence level, ranging from 1 to 5, (Schymanski et al., 2014).
[d] ES: Spain, LV: Latvia, HU: Hungary, CZ: Czech Republic, NL: the Netherlands.
[e] Positive precursor ion.
[f] Negative precursor ion.

chlorantraniliprole, clopyralid, fluopyram, flupyradifurone, fluvalinate, penconazole, propyzamide, thiabendazole, thiacloprid, trifloxystrobin, as well as the metabolite permethric acid (DCCA) (originated from parent pesticides cypermethrin, cyfluthrin, permethrin or transfluthrin).

3.3. Co-occurrence of pesticides

In order to assess how many pesticides were co-occurring within the same individual at a single time point, the number of detected parent pesticides per urine sample are presented in Fig. 1. In line with the detection ratios, the lowest number of detected pesticides were in samples originating from Latvia, with mostly less than 3 co-occurring pesticides per urine sample. Samples originating from Spain showed the highest numbers of co-occurring pesticides, with a median value of 7. In the majority of the samples the number of parent pesticides per samples typically ranged from 2 to 5. The maximum number of different pesticides detected in the same urine sample was 13, which was the case for two samples. The samples with no (n = 100) or only one (n = 225) detected pesticide add up to 16% of the total amount of samples, indicating that in a majority of the samples from the SPECIMEn study at least two different parent pesticides were detected.

The next step was to evaluate which pesticides were co-occurring in each urine sample, for which the most frequently (in 5 or more urine samples) co-occurrent pesticides or mixtures are presented in an UpSet plot in Fig. 2. These most frequent co-occurrences consisted of 44 different combinations based on 14 different pesticides. The majority of co-occurrences consisted of 2 or 3 pesticides, with minimal overlap across all study sites. The most common co-occurrence was acetamiprid with chlorpropham, detected in 62 samples although this combination was not detected in any sample originating from Spain. The second most frequently co-occurring pesticides were acetamiprid with tebuconazole, however this combination was not seen in the Netherlands. The only co-occurrence combination detected in all five study sites was acetamiprid with pirimiphos-methyl. The less frequent the co-occurrent pesticides the more variation in combinations were seen, which was even more pronounced when detected in just 2, 3 or 4 urine samples (see Supplementary Material D for the extended UpSet plot).

The stability of the co-occurrences at each study site can be evaluated with correlation networks, which are presented in Fig. 3A–E. Similar to the findings of Fig. 2, mostly small groups (two to four biomarkers) of

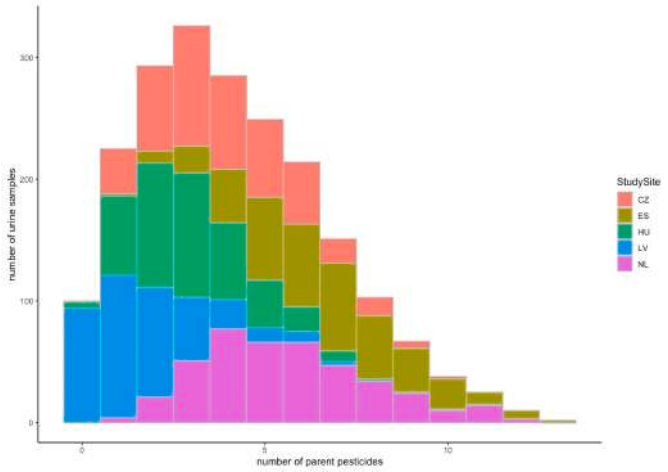

Fig. 1. Number of parent pesticides (p = 29) detected per urine sample (n = 2088), with the five different study sites indicated in different colors (CZ=Czech Republic, ES = Spain, HU=Hungary, LV = Latvia, NL=Netherlands). Multiple metabolites and/or parent compounds related to the same parent pesticide were considered as one.

co-occurrent pesticides were found. Consistent across all study sites was the positive relation between cyprodinil (P13) and fludioxonil (P20), both fungicides, although sometimes together with other pesticides and/or part of a different community. Also, in both Spain and the Czech Republic, imazalil (P27) was related to pyrimethanil (P38), which are both fungicides. Finally, in Spain and Hungary chlorpyrifos-methyl (P9) was related to pirimiphos-methyl (P34), which are both insecticides. Interestingly, the relations of acetamiprid (P2) with chlorpropham (P8) or tebuconazole (P40) were not detected in the networks.

3.4. Changes in occurrence of pesticides by location, season, age category

To explore the differences in occurrence of the pesticide biomarkers by location, season, and age category, logistic mixed effects models were constructed. The main model includes the covariates for location, season and age category, the extended model was also corrected for pesticide usage (self-reported), BMI, level of education and homegrown food consumption. Results of the models of the biomarkers detected in at least four study sites are shown in Table 3, the full table with estimates for all exposure markers associated with confidence levels 1 and 2 can be found in Supplementary Material E.

In Spain, no effect of location was detected in the models, except for clothianidin which was less frequently detected in agricultural areas compared to non-agricultural areas. Chlorpropham, chlorpyrifos, clothianidin, fluazifop, fludioxonil, imazalil, imidacloprid, pyrimethanil, and tebuconazole were most frequently detected during the first sampling season. These effects were not influenced by inclusion of the additional predictors in the extended model. Between the group of parents and children in Spain, the biomarkers related to boscalid, and cyprodinil were most frequently detected among parents, while chlorpropham, chlorpyrifos, chlothianidin, pirimiphos-methyl, tebuconazole, and thiacloprid were more frequently detected among children. The extended models confirmed most of these effects (not for clothianidin and cyprodinil).

In Latvia, propamocarb was the only biomarker more frequently detected at the agricultural area. Acetamiprid, fluopyram, imazalil, and propamocarb were more frequently detected in the first season (winter), while pyrimethanil and tebuconazole were more frequently detected during the second season (summer). Only the effects related to propamocarb and pyrimethanil were confirmed with the extended models. Chlorpropham, pirimiphos-methyl, and propamocarb were more frequently detected among the Latvian children compared to adults, while imazalil was more frequently detected within Latvian parents (not in extended model).

In Hungary, both biomarkers related to clothianidin were more frequently detected at the agricultural areas. On the other hand, chlopyrifos, pirimiphos-methyl, propamocarb, tebuconazole, and thiacloprid were most frequently detected at the non-agricultural areas. Chlorpyrifos, chlothianidin, pirimiphos-methyl, propamocarb, and tebuconazole were most frequently detected during the second season. While, in contrary, chlorpropham and imazalil were most frequently detected during the first season. Acetamiprid, chlorpropham, chlorpyrifos, chlothianidin, fluazifop, pirimiphos-methyl, propamocarb, and tebuconazole, were most frequently detected among the Hungarian children. Of which chlorpropham, fluazifop, pirimiphos-methyl, propamocarb and tebuconazole were confirmed in both models.

In the Czech Republic, the metabolite of ametoctradin was more frequently detected at the agricultural areas, although this effect disappeared in the extended model. The biomarkers related to cyprodinil and fludioxonil were more frequently detected at the non-agricultural locations (only cyprodinil confirmed with the extended model). The chlorpropham metabolite (4-HSA) was more frequently detected during the second season. While the biomarkers related to ametoctradin, imazalil, and pyrimethanil showed an opposite effect, and were more frequently detected during the first season. Of these three, only the effect of pyrimethanil was confirmed with the extended model. Seven different biomarkers were found to be more detected among children compared to adults: boscalid, chlorpropham, chlorpyrifos, flonicamid, pirimiphos-methyl, tebuconazole, and thiacloprid. The extended model confirmed the effects seen for chlorpropham and tebuconazole.

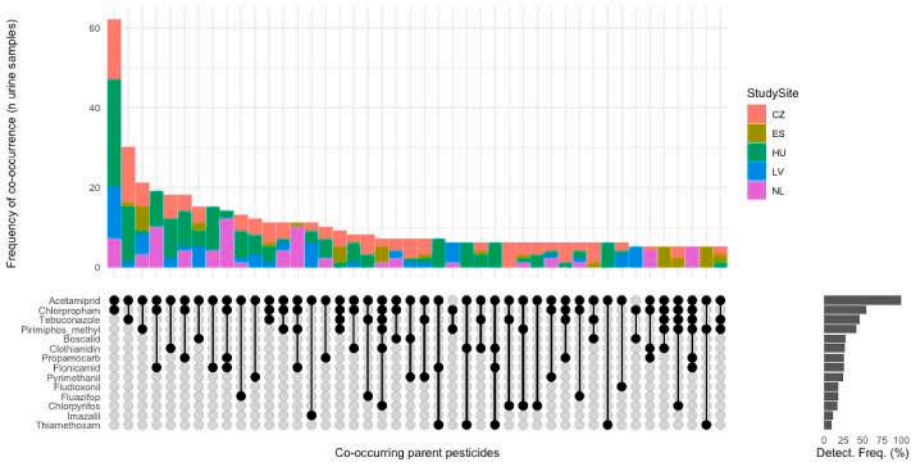

Fig. 2. Frequency (number of urine samples, n = 2088) of co-occurrent parent pesticides; the most frequent (in 5 or more urine samples) co-occurrences are shown. Different study sites are indicated by colors (CZ=Czech Republic, ES = Spain, HU=Hungary, LV = Latvia, NL=Netherlands), the detection frequency (%) of the listed parent pesticides is given on the right. Pesticides are co-occurring in the same sample when both have a black connected dot. Multiple metabolites and/or parent compounds related to the same parent pesticide were considered as one.

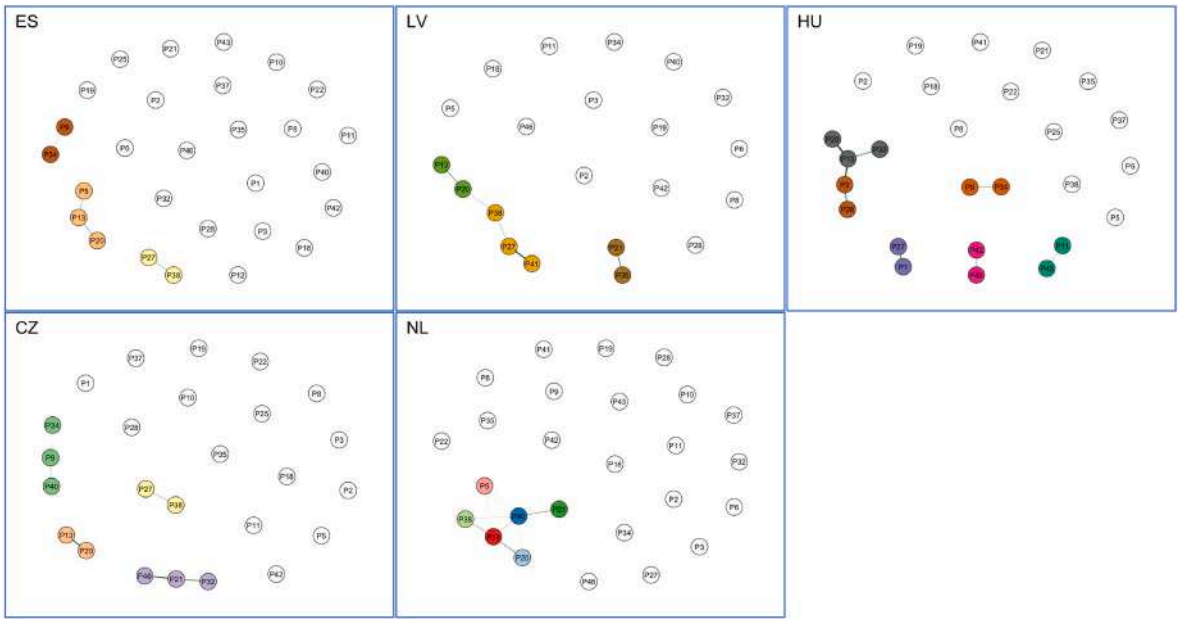

Fig. 3. Weighted correlation networks per study site based on the parent pesticides. Relationships between markers are indicated by a line (green = positive, red = negative). The colours indicate the different communities or groups of more closely related markers. **ES)** Spain (p = 28), **LV)** Latvia (p = 21), **HU)** Hungary (p = 26), **CZ)** Czech Republic (p = 25), **NL)** the Netherlands (p = 26). See Table 2 for a description of the used ID numbers for each pesticide. Multiple metabolites and/or parent compounds related to the same parent pesticide were considered as one.

Finally, in the Netherlands, the metabolites of chlorpropham were most frequently detected at agricultural areas. While biomarkers related to cyprodinil and pyrimethanil had higest detection frequencies at the non-agricultural areas. Chlorpropham, fluazifop, and thiacloprid were more frequently detected during the second season, while acetamiprid, chlorpyrifos, clothianidin, imazalil, and pyrimethanil had highest detection rates during the first season. The biomarkers related to ametoctradin, cyprodinil, flonicamid, fludioxonil, pirimiphos-methyl, and tebuconazole were more frequently detected among children. Of these, the effects seen for pirimiphos and tebuconazole were confirmed in the extended models. While on the other hand, propamocarb was more frequently detected among adults (only in extended model).

Overall, almost no biomarkers were more frequently detected in both agriculture areas and (summer) season 2. Only exceptions were chlorpropham (4-HSA metabolite) in the Netherlands, and clothianidin (parent compound and the N-demethylated metabolite) in Hungary.

4. Discussion

This study reports on the co-occurrence patterns of 40 different pesticide biomarkers at study sites from five European countries, and identifies whether proximity to agrigultural fields, season, and age category impacted the probability of detection of these biomarkers. The developed application of a harmonized SS methodology allowed screening for 1000s of suspects (pesticides and their known/predicted phase I/II metabolites), and enabled detection of many pesticides/metabolites at different levels of confidence in urine. As such, this study should be seen as the first step towards a more complete assessment of the pesticide mixture exposure in the general European population.

4.1. Detected pesticides and the impact of location, season and age category

The most frequently detected biomarkers across all study sites were related to the parent pesticides acetamiprid and chlorpropham. Acetamiprid is a neonicotinoid (insecticide), is approved in the EU and commonly used on fruit trees such as apples, pears and citrus, but also on e.g. potatoes and rapeseed (Allema et al., 2017; EU Database Pest, 2022). All study sites included agricultural areas where these crops are grown. However since we did not find a difference between areas for acetamiprid, this high detection frequency is likely due to other factors such as diet. For Latvia and the Netherlands, acetamiprid was less frequently detected during the second season (summer), arguing that additional exploration is needed on for example the change of diet between seasons. Chlorpropham is a plant growth regulator and herbicide, commonly used on e.g. onions and potatoes to prevent sprouting. In the Netherlands only, chlorpropham had a higher probability of detection in the summer season, which is consistent with an earlier study on flower bulb fields in the Netherlands (Gooijer et al., 2019; Oerlemans et al., 2021). Although chlorpropham has no longer been approved as pesticide since 2019 in the EU, still high probabilities of detection were seen in both seasons (EU Database Pest, 2022). This is not unexpected due to periods of grace until October 2020, which overlaps with both sampling periods of the current study. Interestingly, in Spain and Hungary chlorpropham was more frequently detected during the first season, while in Czech Republic and the Netherlands highest frequencies were seen in the second season. Chlorpropham also had higher probabilities of detection in children compared to adults, which could be related to food consumption: children have a larger food intake per kg of bodyweight; also, biological elimination mechanisms may differ between children and adults (Arena et al., 2017).

Also, high detection rates in SPECIMEn were found for the biomarkers related to pirimiphos-methyl and tebuconazole, which are in good agreement with other targeted studies (Norén et al., 2020; Yusà et al., 2022). For these and other highly detected pesticides, no consistent effect across all countries of season or location was found, in contrast with expectations based on previous findings (Dereumeaux et al., 2020; Teysseire et al., 2020). Differences in study sites might occur due to different crop types. Detected differences are most likely influenced by a set of other covariates not included in the current regression models, such as diet. Dietary habits of participants may differ between the countries, locations within countries, seasons and age groups. Also, there might be differences in percentage of consumption of imported foods, and percentage of homegrown food consumption. These aspects make the variety of exposure due to diet complex and subject to many changes; therefore future work needs to focus on the actual consumed

Table 3
Results of logistic mixed effects models, main and extended. Results are presented as Odds Ratios (OR) with 95% confidence intervals (CI). Significance levels based on p-value: '***' <0.001, '**' <0.01, '*' <0.05. Random effects are household and participant ID. Main model includes the predictors: location, season, and age category. Extended model includes additional predictors for pesticide usage, BMI, level of education and homegrown food consumption. Results are shown of features detected in at least 4 study sites.

ID	Parent pesticide	Category	SP		LV		HU		CZ		NL	
			Main OR (95% CI)[a]	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)
P2_a	Acetamiprid	Agricultural vs Non-Agricultural[b]	NA[c]	NA[d]	1.0 (0.6; 1.6)	1.1 (0.7; 1.9)	1.2 (0.1; 2.7)	1.4 (0.5; 3.5)	0.5 (0.1; 2.2)	0.6 (0.1; 3.0)	2.0 (0.3; 14)	2.4 (0.9; 6.2)
		Season 2 vs 1[b]	0.5 (0.1; 2.7)	0.5 (0.1; 2.7)	0.6 (0.4; 1.0)	0.6 (0.4; 1.0) *	1.2 (0.5; 2.7)	1.3 (0.5; 3.1)	0.6 (0.1; 2.5)	0.8 (0.2; 3.9)	0.2 (0.0; 0.8) *	0.5 (0.2; 1.2)
		Parent vs Child[b]	0.2 (0.0; 1.7)	0.2 (0.0; 2.8)	0.8 (0.5; 1.3)	0.7 (0.4; 1.5)	0.4 (0.2; 1.0) *	0.5 (0.2; 1.5)	1.0 (0.2; 4.1)	12 (1.0; 149)	1.3 (0.2; 9.0)	1.7 (0.3; 8.0)
P3_a	Ametoctradin	Agri. vs Non-Agri.	0.3 (0.0; 2.8)	0.4 (0.1; 1.0)	1.8 (0.5; 6.3)	1.3 (0.3; 5.3)	1.6 (0.3; 9.5)	NA[d]	3.2 (1.1; 9.5) *	3.0 (1.0; 9.4)	0.8 (0.03; 20)	NA[d]
		Season 2 vs 1	0.4 (0.1; 1.5)	0.6 (0.2; 1.4)	0.6 (0.2; 2.0)	0.5 (0.2; 1.9)	0.7 (0.1; 4.1)	NA[d]	0.4 (0.1; 1.0) *	0.4 (0.1; 1.1)	0.3 (0.1; 1.6)	NA[d]
		Parent vs Child	2.1 (0.3; 17)	3.0 (0.9; 10)	0.8 (0.2; 2.8)	2.3 (0.3; 15)	4.0 (0.4; 37)	NA[d]	0.6 (0.2; 1.5)	0.2 (0.0; 1.3)	0.1 (0.02; 0.9) *	NA[d]
P5_a	Boscalid	Agri. vs Non-Agri.	1.0 (0.6; 1.9)	1.0 (0.5; 1.8)	1.2 (0.5; 2.6)	1.4 (0.6; 3.1)	0.8 (0.2; 2.2)	0.5 (0.1; 1.5)	1.4 (0.8; 2.5)	1.4 (0.8; 2.7)	0.6 (0.3; 1.0)	0.5 (0.3; 1.0)
		Season 2 vs 1	0.7 (0.4; 1.0)	0.6 (0.4; 1.0)	0.9 (0.5; 1.6)	0.9 (0.5; 1.6)	0.6 (0.2; 1.7)	0.6 (0.2; 1.9)	0.7 (0.4; 1.2)	0.8 (0.5; 1.3)	1.4 (0.9; 2.2)	1.4 (0.9; 2.2)
		Parent vs Child	2.9 (1.8; 4.6) ***	2.5 (1.3; 4.9) **	1.3 (0.7; 2.4)	1.0 (0.4; 2.6)	2.1 (1.0; 9.9)	2.4 (0.6; 9.3)	2.1 (1.3; 3.5) **	2.5 (1.0; 6.1)	1.4 (0.9; 2.1)	1.1 (0.4; 2.6)
P8_a	Chlorpropham	Agri. vs Non-Agri.	0.7 (0.4; 1.3)	0.7 (0.4; 1.3)	1.3 (0.7; 2.7)	1.2 (0.6; 2.5)	1.3 (0.7; 2.5)	1.5 (0.7; 3.2)	1.0 (0.6; 2.0)	1.0 (0.5; 2.0)	2.1 (1.1; 3.9) *	2.1 (1.1; 4.1) *
		Season 2 vs 1	0.4 (0.3; 0.7) ***	0.4 (0.3; 0.7) ***	1.6 (1.0; 2.6)	1.5 (0.9; 2.4)	0.5 (0.3; 0.8) **	0.5 (0.3; 0.9) *	2.1 (1.3; 3.3) **	1.9 (1.2; 3.1) *[e]	2.8 (1.7; 4.7) ***	2.7 (1.6; 4.6) ***
		Parent vs Child	0.4 (0.2; 0.6) ***	0.3 (0.2; 0.6) ***	0.3 (0.2; 0.6) ***	0.4 (0.2; 1.0) *	0.5 (0.3; 0.7) **	0.4 (0.2; 0.7) **	0.4 (0.2; 0.6) ***	0.3 (0.1; 0.8) *	0.6 (0.4; 1.1)	0.5 (0.2; 1.2)
P9_a	Chlorpyrifos (/methyl)	Agri. vs Non-Agri.	0.8 (0.5; 1.3)	0.8 (0.5; 1.3)	ND[f]	ND[f]	0.2 (0.1; 0.7) *	0.3 (0.1; 1.0)	1.3 (0.7; 2.4)	1.3 (0.7; 2.4)	1.2 (0.4; 3.2)	1.2 (0.4; 3.3)
		Season 2 vs 1	0.2 (0.1; 0.4) ***	0.2 (0.1; 0.4) ***	ND[f]	ND[f]	2.5 (1.0; 6.1) *	2.7 (1.1; 6.5) *	0.6 (0.4; 1.0)	0.6 (0.4; 1.0)	0.5 (0.2; 1.1)	0.4 (0.1; 1.0) *
		Parent vs Child	0.5 (0.3; 0.7) ***	0.4 (0.2; 0.7) **	ND[f]	ND[f]	0.5 (0.2; 1.1)	0.2 (0.1; 0.8) *	0.5 (0.3; 0.7) **	0.7 (0.3; 1.7)	0.8 (0.3; 1.8)	0.9 (0.2; 5.2)
P11_a	Clothianidin (can come from thiamethoxam)	Agri. vs Non-Agri.	0.5 (0.3; 0.8) **	0.4 (0.3; 0.7) ***	1.4 (0.3; 6.3)	0.9 (0.2; 4.6)	2.8 (1.6; 4.7) ***	2.8 (1.5; 5.1) **	1.3 (0.8; 2.2)	1.4 (0.8; 2.5)	1.3 (0.7; 2.3)	1.4 (0.8; 2.7)
			0.6 (0.4; 0.9) **	0.5 (0.3; 0.8) **	6.3 (0.7; 53)	5.7 (0.7; 50)	3.1 (1.8; 5.2) ***	3.5 (2.0; 6.1) ***	0.6 (0.4; 1.0)	0.7 (0.4; 1.1)	0.6 (0.4; 1.0)	0.6 (0.4; 1.0)
		Season 2 vs 1	0.6 (0.5; 0.9) *	0.6 (0.3; 1.0)	0.2 (0.0; 1.4)	0.3 (0.0; 5.7)	0.6 (0.4; 1.0)	0.4 (0.2; 0.7) **	1.0 (0.6; 1.5)	1.1 (0.5; 2.7)	0.9 (0.5; 1.5)	1.7 (0.6; 4.6)
P11_c			1.4 (0.8; 2.8)	1.4 (0.7; 2.8)	ND[f]	ND[f]	4.4 (1.8; 11) ***	5.5 (2.1; 14) ***	1.3 (0.6; 2.7)	1.3 (0.6; 2.8)	1.5 (0.0; 38)	1.4 (0.7; 2.6)
		Parent vs Child	0.9 (0.5; 1.5)	0.8 (0.5; 1.4)	ND[f]	ND[f]	1.9 (0.9; 3.9)	2.5 (1.2; 5.5) *	0.5 (0.2; 1.1)	0.5 (0.2; 1.1)	0.02 (0.0; 0.3) **	0.6 (0.4; 1.0)[e and g]

(*continued on next page*)

Table 3 (*continued*)

ID	Parent pesticide	Category	SP		LV		HU		CZ		NL	
			Main OR (95% CI)[a]	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)
			0.7 (0.4; 1.3)	0.8 (0.4; 1.8)	ND[f]	ND[f]	0.8 (0.4; 1.5)	0.3 (0.1; 0.8) *	0.9 (0.4; 2.0)	1.2 (0.3; 4.6)	1.6 (0.0; 42)	1.8 (0.7; 4.9)
P13_a	Cyprodinil	Agri. vs Non-Agri.	1.4 (0.7; 2.7)	1.4 (0.7; 2.7)	1.1 (0.4; 2.8)	1.1 (0.4; 2.9)	1.9 (0.1; 44)	1.8 (0.1; 61)	0.3 (0.1; 0.6) **	0.3 (0.1; 0.6) **	0.6 (0.3; 0.9) *	0.5 (0.3; 0.9) *
		Season 2 vs 1	1.2 (0.7; 2.1)	1.2 (0.6; 2.2)	0.7 (0.3; 1.5)	0.7 (0.3; 1.5)	0.3 (0.1; 2.0)	0.4 (0.1; 2.9)	0.9 (0.5; 1.6)	0.9 (0.5; 1.7)	0.8 (0.5; 1.2)	0.8 (0.5; 1.2)
		Parent vs Child	2.0 (1.6; 5.6) ***	2.3 (1.0; 5.4)	0.9 (0.4; 2.1)	1.5 (0.5; 5.1)	6.2 (0.9; 40)	2.9 (0.3; 26)	0.8 (0.4; 1.5)	1.0 (0.3; 3.0)	0.5 (0.3; 0.8) **	1.2 (0.5; 2.8)
P18_a	Flonicamid	Agri. vs Non-Agri.	2.6 (0.5; 14)	2.6 (0.4; 15)	ND[f]	ND[f]	1.0 (0.3; 4.3)	2.3 (0.5; 11)	2.7 (0.1; 84)	NA[d]	0.4 (0.1; 1.5)	0.4 (0.1; 1.3)
		Season 2 vs 1	1.4 (0.3; 6.3)	1.4 (0.3; 7.1) [e]	ND[f]	ND[f]	3.1 (0.6; 16)	3.8 (0.7; 20)	1.0 (0.2; 5.3)	NA[d]	1.6 (0.6; 4.0)	1.5 (0.6; 3.8)
		Parent vs Child	6.2 (0.7; 52)	7.8 (0.6; 96)	ND[f]	ND[f]	0.3 (0.1; 1.6)	0.2 (0.0; 1.5)	0.1 (0.0; 0.4) **	NA[d]	0.3 (0.1; 0.8) *	0.3 (0.0; 2.3)
P19_a	Fluazifop	Agri. vs Non-Agri.	1.0 (0.6; 1.9)	1.1 (0.6; 2.1)	4.2 (0.9; 20)	3.1 (0.6; 16)	1.1 (0.5; 2.2)	0.9 (0.4; 2.1)	1.1 (0.6, 2.1)	1.3 (0.7; 2.4)	1.4 (0.7; 3.0)	1.5 (0.7; 3.1)
			0.5 (0.3; 0.8) **	0.5 (0.3; 0.9) *	0.7 (0.2; 2.4)	0.6 (0.1; 2.3)	1.1 (0.6; 2.0)	0.9 (0.4; 1.7)	0.9 (0.5; 1.4)	0.8 (0.5; 1.4)	1.0 (0.6; 1.6)	1.0 (0.6; 1.7)
		Season 2 vs 1	1.0 (0.6; 1.7)	0.7 (0.3; 1.5)	1.0 (0.3; 3.5)	1.2 (0.2; 7.8)	0.6 (0.3; 1.2)	0.4 (0.2; 1.0) *	0.7 (0.4; 1.2)	0.5 (0.2; 1.3)[e]	0.7 (0.4; 1.2)	0.5 (0.2; 1.5)
P19_b			1.6 (0.7; 3.6)	1.6 (0.6; 4.1)	5.2 (0.6; 45)	4.6 (0.5; 44)	2.1 (0.3; 17)	2.4 (0.8; 7.2)	0.9 (0.3; 2.3)	0.9 (0.3; 2.5)[e]	1.4 (0.2; 10)	3.1 (0.3; 29)
		Parent vs Child	0.8 (0.4; 1.6)	0.7 (0.3; 1.6)	0.5 (0.1; 2.7)	0.4 (0.1; 2.6)	1.4 (0.1; 4.5)	1.0 (0.3; 2.6)	2.5 (1.0; 6.3)	2.4 (0.9; 6.3)	7.2 (1.6; 32) **	4.5 (1.1; 17) *
			1.0 (0.5; 2.1)	0.4 (0.1; 1.2)	0.5 (0.1; 2.7)	1.9 (0.1; 29)	0.2 (0.0; 0.9) *	0.3 (0.1; 0.9) *	1.3 (0.6; 3.2)	1.0 (0.2; 4.6)	0.6 (0.1; 4.1)	0.0 (0.0; 2.9)
P20	Fludioxonil	Agri. vs Non-Agri.	1.1 (0.6; 2.1)	1.0 (0.5; 2.0)	0.8 (0.4; 1.7)	0.9 (0.4; 1.9)	0.6 (0.1; 2.6)	0.9 (0.2; 4.8)	0.5 (0.3; 0.9) *	0.6 (0.3; 1.1)	0.9 (0.5; 1.6)	0.8 (0.5; 1.4)
		Season 2 vs 1	0.5 (0.3; 0.9) *	0.5 (0.3; 0.9) *	0.8 (0.4; 1.4)	0.8 (0.4; 1.4)	1.7 (0.4; 7.2)	1.7 (0.4; 7.5)	0.7 (0.4; 1.2)	0.7 (0.4; 1.2)	0.6 (0.3; 0.9) *	0.6 (0.4; 0.9) *
		Parent vs Child	1.5 (0.8; 2.8)	0.9 (0.4; 2.2)	1.1 (0.6; 2.1)	0.8 (0.3; 2.1)	1.0 (0.2; 4.0)	0.9 (0.2; 5.5)	0.7 (0.4; 1.2)	1.1 (0.4; 3.1)	0.5 (0.3; 0.9) *	0.8 (0.3; 1.8)
P21_c	Fluopyram	Agri. vs Non-Agri.	1.4 (0.6; 3.4)	1.5 (0.6; 4.0)	0.6 (0.1; 4.3)	0.7 (0.2; 2.2)	ND[f]	ND[f]	1.3 (0.2; 10)	1.9 (0.2; 20)	1.0 (0.1; 22)	NA[d]
		Season 2 vs 1	1.0 (0.5; 1.9)	1.1 (0.5; 2.2)	0.2 (0.0; 0.8) *	0.4 (0.2; 1.1)	ND[f]	ND[f]	1.8 (0.5; 6.4)	1.8 (0.5; 7.2)	4.3 (0.5; 36)	NA[d]
		Parent vs Child	1.5 (0.8; 3.0)	0.9 (0.3; 2.6)	0.9 (0.1; 6.1)	1.1 (0.3; 4.3)	ND[f]	ND[f]	0.8 (0.2; 2.8)	1.0 (0.1; 12)	0.5 (0.2; 12)	NA[d]
P27_a	Imazalil	Agri. vs Non-Agri.	1.0 (0.5; 2.0)	1.1 (0.6; 2.2)	1.7 (0.6; 4.9)	0.7 (0.2; 2.2)	0.6 (0.2; 2.0)	0.7 (0.2; 2.3)	0.9 (0.1; 11)	1.0 (0.4; 2.6)	1.6 (0.6; 4.2)	1.6 (0.6; 4.1)
		Season 2 vs 1	0.2 (0.1; 0.3) ***	0.2 (0.1; 0.4) ***	0.4 (0.2; 0.8) *	0.4 (0.2; 1.1)	0.2 (0.1; 0.5) ***	0.2 (0.1; 0.5) **	0.1 (0.0; 0.6) *	0.4 (0.2; 1.2) [e]	0.3 (0.1; 1.0) *	0.4 (0.1; 1.0) [e]
		Parent vs Child	1.1 (0.7; 2.0)	0.8 (0.4; 1.9)	2.4 (1.1; 5.2) *	1.1 (0.3; 4.3)	0.5 (0.2; 1.2)	0.3 (0.1; 1.1)	2.9 (0.2; 44)	3.1 (0.6; 15)	1.4 (0.6; 3.6)	2.2 (0.4; 11)
P28_a	Imidacloprid	Agri. vs Non-Agri.	1.5 (0.7; 3.1)	1.2 (0.6; 2.6)	1.4 (0.3; 6.2)	1.1 (0.2; 5.7)	0.9 (0.2; 3.3)	1.0 (0.3; 3.7)	ND[f]	ND[f]	1.2 (0.6; 2.5)	1.4 (0.6; 2.9)

(*continued on next page*)

Table 3 (*continued*)

ID	Parent pesticide	Category	SP		LV		HU		CZ		NL	
			Main OR (95% CI)[a]	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)
		Season 2 vs 1	0.6 (0.3; 1.0) *	0.5 (0.3; 1.0) *	0.2 (0.0; 1.4)	0.2 (0.0; 1.4)	0.9 (0.3; 2.5)	1.1 (0.4; 3.0)	ND[f]	ND[f]	1.1 (0.5; 2.1)	1.1 (0.5; 2.1)
		Parent vs Child	1.4 (0.8; 2.5)	1.8 (0.8; 4.2)	1.4 (0.3; 6.1)	0.7 (0.1; 6.2)	0.9 (0.3; 2.4)	0.5 (0.1; 2.0)	ND[f]	ND[f]	0.8 (0.4; 1.7)	0.9 (0.2; 3.3)
P32_a	Penconazole	Agri. vs Non-Agri.	0.7 (0.3; 1.7)	0.8 (0.3; 1.9)	1.4 (0.3; 6.1)	1.6 (0.3; 7.7)	0.5 (0.1; 2.1)	0.7 (0.0; 38)	2.4 (0.6; 9.7)	2.2 (0.5; 9.3)	0.9 (0.3; 3.2)	0.8 (0.2; 3.0)
		Season 2 vs 1	1.1 (0.5; 2.5)	1.2 (0.5; 2.6)	0.8 (0.2; 3.4)	0.8 (0.2; 3.4) [g]	3.6 (0.7; 18)	9.5 (1.0; 93)	0.5 (0.1; 2.0)	0.6 (0.1; 2.5)[e,g]	1.0 (0.3; 3.6)	1.0 (0.3; 3.6) [g]
		Parent vs Child	2.2 (0.9; 5.0)	2.9 (0.9; 9.0)	0.8 (0.2; 3.4)	0.6 (0.1; 6.0)	1.3 (0.3; 4.8)	0.7 (0.0; 12)	2.0 (0.5; 8.3)	2.9 (0.3; 30)	1.5 (0.4; 5.5)	0.5 (0.1; 4.5)
P34_a	Pirimiphos-methyl	Agri. vs Non-Agri.	0.7 (0.3; 1.4)	1.1 (0.5; 2.5)	1.0 (0.5; 1.9)	1.5 (0.3; 7.4)	0.1 (0.02; 0.4) ***	0.2 (0.1; 0.8) *	1.5 (0.8; 2.7)	1.3 (0.7; 2.5)	1.4 (0.8; 2.7)	1.4 (0.8; 2.7)
		Season 2 vs 1	0.8 (0.4; 1.4)	1.0 (0.5; 2.0)	1.5 (0.8; 2.9)	0.8 (0.2; 3.8)	3.6 (1.4; 9.4) **	4.1 (1.5; 11) **	0.6 (0.4; 1.0)	0.6 (0.4; 1.1)	0.7 (0.4; 1.0)	0.7 (0.4; 1.0)
		Parent vs Child	0.4 (0.2; 0.9) *	0.2 (0.1; 0.7) *	0.4 (0.2; 0.9) *	0.6 (0.1; 5.4)	0.2 (0.1; 0.5) **	0.1 (0.0; 0.3) ***	0.5 (0.3; 0.8) **	0.6 (0.2; 1.5)	0.3 (0.2; 0.5) ***	0.3 (0.1; 0.8) *
P35_a	Propamocarb	Agri. vs Non-Agri.	1.3 (0.5; 3.1)	1.0 (0.4; 2.4)	ND[f]	ND[f]	0.5 (0.2; 1.0)	0.5 (0.2; 1.2)	0.5 (0.1; 4.5)	0.5 (0.2; 1.9)	1.6 (0.8; 3.0)	1.9 (1.0; 3.6)
			1.9 (0.9; 4.1)	1.7 (0.8; 3.5)	ND[f]	ND[f]	1.1 (0.6; 2.1)	1.1 (0.6; 2.2)	1.5 (0.4; 6.0)	1.4 (0.5; 3.5) [e]	1.4 (0.9; 2.4)	1.5 (0.9; 2.5)
		Season 2 vs 1	1.1 (0.5; 2.5)	1.2 (0.4; 3.4)	ND[f]	ND[f]	0.4 (0.2; 1.0) *	0.3 (0.1; 0.9) *	0.5 (0.1; 3.9)	1.5 (0.2; 10)	1.0 (0.6; 1.6)	3.1 (1.1; 8.5) *
P35_b			1.3 (0.7; 2.4)	1.2 (0.7; 2.2)	4.2 (1.1; 16) *	4.0 (1.0; 17)	0.5 (0.3; 0.9) *	0.5 (0.2; 1.0) *	0.7 (0.3; 1.9)	0.8 (0.3; 2.1)	1.2 (0.7; 1.9)	1.3 (0.8; 2.2)
		Parent vs Child	1.2 (0.7; 1.9)	1.1 (0.7; 1.9)	0.3 (0.1; 0.9) *	0.3 (0.1; 0.8) *	2.1 (1.2; 3.6) *	2.3 (1.3; 4.1) **	1.4 (0.7; 2.6)	1.4 (0.7; 2.8)	1.0 (0.6; 1.5)	1.0 (0.7; 1.5)
			1.1 (0.7; 1.9)	1.1 (0.5; 2.3)	0.4 (0.1; 1.1)	0.2 (0.0; 1.0) *	0.5 (0.3; 0.9) *	0.4 (0.2; 0.8) *	0.5 (0.3; 1.0)	0.9 (0.2; 3.3)	1.2 (0.8; 1.8)	3.2 (1.4; 7.3) **
P38_a	Pyrimethanil	Agri. vs Non-Agri.	0.8 (0.5; 1.3)	0.8 (0.5; 1.3)	1.2 (0.6; 2.4)	1.1 (0.5; 2.3)	0.4 (0.1; 2.0)	0.3 (0.0; 3.1)	0.9 (0.5; 1.7)	1.0 (0.5; 1.8)	0.6 (0.4; 1.0) *	0.6 (0.4; 0.9) *
		Season 2 vs 1	0.6 (0.4; 1.0) *	0.7 (0.4; 1.0)	2.1 (1.1; 3.8) *	2.1 (1.1; 3.8) *	1.3 (0.5; 3.8)	1.6 (0.5; 5.2)	0.4 (0.3; 0.7) **	0.5 (0.3; 0.8) **	0.6 (0.4; 1.0) *	0.6 (0.4; 1.0) *
		Parent vs Child	0.8 (0.5; 1.2)	0.7 (0.3; 1.3)	1.0 (0.6; 1.8)	1.3 (0.5; 3.2)	2.3 (0.8; 7.2)	1.7 (0.8; 7.4)	1.4 (0.9; 2.3)	2.1 (0.9; 5.3)	0.8 (0.5; 1.2)	1.1 (0.5; 2.4)
P40_a	Tebuconazole	Agri. vs Non-Agri.	0.9 (0.5; 1.7)	0.7 (0.4; 1.4)	1.5 (0.4; 5.3)	1.5 (0.6; 4.0)	0.5 (0.3; 1.0)	0.5 (0.2; 1.0) *	1.0 (0.6; 1.5)	1.1 (0.7; 1.7)	0.8 (0.4; 1.5)	0.8 (0.4; 1.6)
		Season 2 vs 1	0.5 (0.3; 0.9) *	0.5 (0.3; 0.8) **	3.3 (1.1; 9.3) *	2.8 (1.1; 7.4)	2.1 (1.2; 3.5) **	2.2 (1.3; 3.8) **	0.7 (0.5; 1.0)	0.7 (0.4; 1.0)	0.6 (0.4; 1.0)	0.6 (0.4; 1.1)
		Parent vs Child	0.3 (0.2; 0.4) ***	0.2 (0.1; 0.4) ***	0.8 (0.3; 2.1)	2.9 (0.7; 12)	0.3 (0.2; 0.5) ***	0.5 (0.2; 0.9) *	0.2 (0.2; 0.4) ***	0.4 (0.2; 0.7) **	0.1 (0.1; 0.2) ***	0.2 (0.1; 0.5) ***
P42_a	Thiacloprid	Agri. vs Non-Agri.	1.6 (0.6; 4.4)	1.6 (0.6; 4.5)	ND[f]	ND[f]	0.1 (0.0; 0.7) *	0.1 (0.0; 0.5) *	1.6 (0.6; 4.7)	2.1 (0.8; 5.8)	0.4 (0.1; 3.5)	0.5 (0.1; 4.0)
		Season 2 vs 1	0.9 (0.4; 2.0)	1.1 (0.5; 2.4)	ND[f]	ND[f]	3.2 (0.8; 12)	3.5 (0.9; 14)[g]	0.9 (0.4; 2.0)	1.2 (0.6; 2.7)	6.5 (1.5; 29) *	6.3 (1.3; 30) *[e]
		Parent vs Child	0.4 (0.2; 1.0) *	0.3 (0.1; 1.2)	ND[f]	ND[f]	0.3 (0.1; 1.2)	0.6 (0.1; 3.3)	0.3 (0.1; 0.7) **	0.2 (0.1; 1.1)	0.6 (0.2; 2.1)	2.4 (0.2; 27)

[a] OR: Odds Ratio, CI: Confidence Interval.
[b] First mentioned is the reference category.
[c] 100% detected in one of the categories, no estimate could be provided.
[d] Due to low detection rate no extended model possible.
[e] Model not corrected for level of Education, separation issue.
[f] ND: Not detected or low detection rate (<1%), no model possible.
[g] Model not corrected for Pesticide usage, separation issue.

diet and their pesticide residue levels versus the suspect screening patterns. For example, the consumption of organic foods has been linked to lower exposure concentrations of several pesticides such as organophosphates and pyrethroids (Baudry et al., 2019; Hyland et al., 2019).

As a final remark on the detected pesticides, the SS methodology is only recently being applied in large scale studies to assess exposure to pesticides, and only a few HBM studies have previously applied SS approaches to complement for example targeted monitoring programs (Gerona et al., 2018; Pellizzari et al., 2019; Plassmann et al., 2015; Wang et al., 2018). Within a cohort of approximately 300 pregnant women in France, Bonvallot et al. (2021) performed a large targeted pesticide exposure study which was extended with the application of suspect screening. This SS approach resulted in the most frequent detection of the parent pesticides azoxystrobin, fenpropimorph, phenmedipham, fluazifop(/butyl) and chlorpyrifos. From these, only the metabolites of fluazifop(/butyl) and chlorpyrifos overlapped and were also detected in the samples of the SPECIMEn study. This is due to among others differences in the suspect database, for example fenpropimorph was not included in our current study because it didn't contain Cl, Br or PO3 (Huber et al., 2022). Another interesting point is the difference in detection frequency between the TCPy and -CH2 biomarkers of chlorpyrifos (methyl) in Spain and Czech Republic. TCPy can originate from both parent compounds chlorpyrifos and methyl-chlorpyrifos. -CH2 is not a human metabolite of chlorpyrifos, and its detection is likely due to exposure through diet. Also, a higher sensitivity for -CH2 compared to TCPy at an individual instrument level might have contributed to this difference.

4.2. Co-occurrence

To explore the exposure to pesticide mixtures in the general population, it was assessed which parent pesticides co-occurred in the same urine sample. With the current work we were able to assess the probability of detection of 29 different parent pesticides simultaneously. In a large majority of the samples (84%) two or more different pesticides were detected. Our findings confirm the presence of mixtures and the necessity of assessing co-occurrent exposures, which is a topic of high concern in risk assessment (European Commission, 2020; Socianu et al., 2022; Luijten et al., 2022). The number of co-occurring pesticides typically ranged from 2 to 5, with a maximum of 13 different pesticides (2 urine samples). These two urine samples originate both from the Spanish non-agricultural area, one from a child of the first season, the other of an adult of the second season. Both individuals had a lower number of co-occurring pesticides during the other season, resepectively 8 and 10 pesticides.

Based on the 14 most frequent co-occurrent pesticides, 44 different combinations could be made, resulting in highly individualized exposure profiles. The most common combination of acetamiprid with chlorpropham, occurred in just 3% (n = 62) of the urine samples. Also, assessment of the co-occurrence patterns at country level (network analysis), did not result in strong relations and hardly any overlap across countries was seen. The underlying correlations between these probabilities of detection were also low, generally below 0.3. These results indicate that, qualitatively, pesticide mixtures might be highly variable between individuals. Nevertheless, the combined exposures may still pose a concern in terms of public health, especially when the different components of a chemical mixture share modes of action underlying toxicity (Rotter et al., 2018). Acetamiprid and chlorpropham seem to induce different toxicological effects (Arena et al., 2017; EFSA, 2016). Acetamiprid has been reported to mainly target the liver (EFSA, 2016), where it may cause, at least in rodents, oxidative stress leading to mitochondrial dysfunction (EL-Hak et al., 2022; S. Li et al., 2021). Exposure to chlorpropham rather leads to adverse effects on the hematopoietic system (Arena et al., 2017; Fujitani et al., 2000, 2004). Hemotoxicity such as hemolytic anemia, however, is considered to be due to oxidative stress (Rokushima et al., 2007; Sivilotti, 2004). Chlorpropham belongs to the family of carbamates, which have been reported to induce oxidative stress in occupationally exposed workers (Saad-Hussein et al., 2022). The other frequently observed combination of co-occurring substances involved acetamiprid and tebuconazole. Tebuconazole is a fungicide that mainly affects the liver and the adrenals (EFSA, 2014). Additionally, it has been reported to induce oxidative stress in the liver and endocrine disruption including anti-androgenic effects (Taxvig et al., 2007; Yang et al., 2018). Follow-up studies involving a larger number of participants and targeted biomarkers for these substances are needed to better assess the composition of the relevant mixtures and associated health risks.

4.3. Strengths and limitations

With the uniform design of our study a comparison could be made across Europe between agricultural and non-agricultural areas, seasons, and adults and children. Close collaborations with partners from all five countries resulted in the harmonized data collection, with little loss to follow up. The collection of the urine samples required a minimal invasive protocol, reducing the burden of citizens to participate in this survey, and opening up possibilities for scaling-up studies in future endeavours. A novel SS approach was harmonized and standardized across laboratories, with extensive QA/QC procedures (Vitale et al., 2022). Such harmonization is crucial to compare SS data and results coming from different laboratories and countries, a situation that is often unavoidable in large-scale studies. The applied SS approach allows for a relatively cost-effective way of providing semi-quantitative measurements of a large number of pesticides. A clear strength of the SPECIMEn study is that information is obtained on (putative) internal exposure to pesticides not or hardly monitored before, and on simultaneous exposure to multiple pesticides. Across countries, different pesticides targeted to different controls on different crops are likely to have been applied at the time of sampling, of which the variation is covered with the SS approach. As such, this study should be seen as the first step towards a more complete assessment of the pesticide mixtures that the general European population is exposed to. Further in-depth screening of the collected data and further methodological developments will increase the number of biomarkers that can be detected in the collected urine samples. This allows an increasingly more complete coverage of all pesticides that are present in these samples as well as the detection of other biomarkers that might potentially interact with the pesticide mixture. Also, future more quantitative analysis of signal intensities will allow for a semi-quantitative interpretation, both in co-occurrence patterns and in the role of determinants of pesticide levels.

Although the current study yields many new insights and perspectives on pesticide occurrence and mixtures, several limitations need to be addressed. From an analytical methodology point of view, the suspect screening approach is less sensitive than targeted methods (Pourchet et al., 2020), and the data mining was biased towards halogenated pesticides (Huber et al., 2022). Despite harmonized methods between

the involved laboratories, differences in sensitivity between the instruments used by the labs did occur, potentially introducing variability between countries which should be interpreted with care (Vitale et al., 2022, Huber et al., 2022). Importantly, data generated by the SS approach applied in the SPECIMEn can currently not be related to urinary pesticide concentration levels in the traditional quantitative way as in targeted analysis, but rather as semi-quantitative intensities as indicators of exposure.

With regards to the sample collection, it should be kept in mind that samples of the second season were collected during the COVID-19 pandemic, while the first sample collection was not affected by the pandemic. Activity patterns or diet of participants might have been altered, and differences between seasons should be interpreted with caution. Also, the different seasons cannot be interpreted as 'non-spraying' and 'spraying', since the timing of the actual spraying activities (and spraying techniques) most likely differed between countries and crop types. Since the applied study design was not timed with an actual spraying activity, the detected exposures might be an underestimation as compared to what has been reported in the literature (Dereumeaux et al., 2020; Teysseire et al., 2020). Agricultural areas were selected based on national databases on land-use (see Supplementary Material F for a description of the area selection per country), due to which the application of pesticides during the time of sampling could not be confirmed. Within SPECIMEn, only first morning void urines were collected. Due to the rapid excretion of many pesticides, the detected pesticides in the morning voids likely do not reflect the total daily exposure (A. J. Li et al., 2019; Scher et al., 2007). Finally, with respect to the performed logistic regression models, no correction for multiple testing was performed, since we wanted to detect any possible effects, accepting the risk of false-positive results. The inclusion of both location and season could have lead to an over-correction, especially since no difference between seasons at the non-agricultural locations would be expected due to any spraying activity (although diet might still differ between the seasons).

5. Conclusions

The current survey demonstrates the feasibility of conducting a harmonized pan-European sample collection combined with suspect screening (SS) to provide insight in the co-occurrence of pesticide mixtures in European agricultural areas. The application of a novel LC-HRMS based SS approach harmonized between different laboratories, resulted in detection of 40 biomarkers related to 29 parent pesticides with high levels of confidence. Some effects of living close to agricultural fields or season were detected, but these effects were not common at a European level. This study is a first step in addressing pesticide mixture exposure under real-life conditions. Combined with a suspect screening approach, this approach is a promising strategy for pesticide mixture risk assessment in the European population, that can guide the prioritization of pesticide (metabolites) to be measured using quantitative targeted methods.

Credit author statement

Conceptualization and design (IO, JV, EL, RV, JA); Investigation (IO, JV, EL, PČ, LŠ, OM, TS, SK, IM, ZM, LA, OP, SF, CC, SP)); Analytical methodology (JA, CH, AL, OP, SF, MK, LD, KW, RN, HM, CM, JK, BG, NL); Formal analysis (IO, JV); Writing - Original Draft (IO, JV, EL); Writing – Review and Editing (all authors); Visualization (IO); Supervision (ML, RV); Project administration (IO, JV, EL); All authors read and approved the final manuscript.

Declaration of competing interest

Declarations of interest: none.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.114105.

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Association between antinuclear antibody positivity and chemical exposure among pregnant Japanese women: A cross-sectional study based on the Japan environment and Children's study

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ABSTRACT

Antinuclear antibodies (ANAs) are detected in healthy individuals, they are more prevalent in women than in men. Pregnant women are immunologically unique, but epidemiological data on ANA positivity in them remain limited. The exposure received from the mother during the fetal period impacts the future health of the fetus and has thus received increased attention in recent years. Thus, we investigated the association between ANA positivity and chemical exposure among pregnant Japanese women, registered in the Japan Environment and Children's Study (JECS). ANA titers were assessed by indirect immunofluorescence with HEp-2 cells at a cutoff dilution of 1:40. Sociodemographic and other data were obtained in the JECS from a self-administered questionnaire. We analyzed 1,235 Japanese women in their first trimester of pregnancy. The ANA prevalence was 17.2%. Among ANA-positive women, a speckled pattern was the most common (95.3%), followed by a homogeneous pattern (72.3%). Exposure to chemicals more than once a week significantly increased the probability of ANA positivity (kerosene, petroleum, benzene, or gasoline: adjusted odds ratio [AOR], 2.11; 95% confidence interval [95% CI], 1.03–4.34; chlorine bleach or germicide: AOR, 1.97; 95% CI, 1.10–3.54; organic solvents: AOR, 5.34; 95% CI, 1.40–20.36; and photocopying machines or laser printers: AOR, 1.73; 95% CI, 1.17–2.54). ANA positivity was associated with exposure to several chemicals in Japanese women. Our exploratory results suggested that ANAs as potential markers of chemical exposure warrant further research.

1. Introduction

Antinuclear antibodies (ANAs) target nuclear and cytoplasmic proteins, nucleic acids, and their complexes. They are important biomarkers produced before the onset of a disease and are associated with certain autoimmune diseases, such as systemic lupus erythematosus, systemic sclerosis, and mixed connective tissue disease (Pashnina et al., 2021; Firestein and Kelley, 2013). Hence ANAs are widely used in the diagnosis and classification of autoimmune diseases. However, even healthy individuals test positive for ANA, although at low titers (Tan et al., 1997; Fernandez et al., 2003).

ANA positivity may be associated with exposure to various xenobiotics, such as environmental chemicals (Dinse et al., 2020), silica (Zaghi et al., 2010; Pfau et al., 2004), mercury (Somers et al., 2015), lead and other metals (Scammell et al., 2020), triclosan (Dinse et al., 2016), agricultural chemicals, methyl bromide, petroleum oils, insecticides,

Abbreviations: ANA, antinuclear antibody; JECS, Japan Environment and Children's Study; IQR, interquartile range; AOR, adjusted odds ratio; CI, confidence interval; VOC, volatile organic compound.

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and organochlorine compounds (Parks et al., 2019). In Japan, a report did not show a significant association between ANA positivity and environmental chemicals in healthy individuals (Kuroda, 2015). Air, water, food, and household products contain a variety of chemicals. There is a constant exposure to these through ingestion, inhalation, and dermal absorption in our daily lives (Nakayama et al., 2018). Exposome, which is the sum of environmental factors that humans are exposed to during their lifetime, has recently attracted research attention (The National Institute for Occupational Safety and Health, 2014). It can be expected that differences in susceptibility to toxic chemicals are affected by various interrelated factors, such as immunogenetic, socioeconomic, lifestyle, and environmental factors (Laio et al., 2019). A variety of parental environmental factors affect children's health and risk of chronic diseases, as suggested in the developmental origins of health and disease theory (Barker and Thornburg, 2013; Fleming et al., 2018). The Hokkaido cohort study in Japan also suggested that chemical exposure in the fetal stage affects the long-term health of the child (Kishi et al., 2021). In fact, Japanese pregnant women are exposed to a variety of chemicals (Iwai-Shimada et al., 2018), and countermeasures are required.

ANAs are more prevalent in women than in men, owing to estrogen and other sex hormones (Whitacre, 2001); immune-related genes on the X chromosome; fetal microchimerism; reproductive factors (Pennell et al., 2012); and lifestyle factors, such as cosmetics and hair dye (Oliver and Silman, 2009). Pregnant women are immunologically unique because they have both immunological tolerance to the genetically foreign fetus and immune responses to pathogens, such as bacteria and viruses (Rowe et al., 2012). ANAs have been studied in relation to some complications of pregnancy, such as unexplained infertility (Tinneberg and Gasbarrini, 2013; Deroux et al., 2017), miscarriage (Ticconi et al., 2016; Cavalcante et al., 2020), infertility owing to placental insufficiency (Chen et al., 2020; Sakthiswary et al., 2015), and perinatal death (Kiuttu et al., 1994). However, data on the association between ANA positivity and chemical exposure in healthy pregnant women are lacking. The influence of race, genetic background, geography, and lifestyle on ANA production has been suggested. However, the research on ANAs is largely from Europe and the United States, and epidemiological data from Japan are scarce.

Thus, this study aimed to obtain baseline data on the prevalence of ANAs among pregnant Japanese women and investigate the association between ANA positivity and chemical exposure.

2. Materials and methods

2.1. Ethical considerations

This study was approved by the Institutional Review Board on Epidemiological Studies of the Ministry of the Environment, ethics committees of all participating institutions (approval number: 100,910,001). This study was also approved by the ethics committee of Kumamoto University Graduate School of Life Sciences (approval number: 590). The participants provided written informed consent after being explained that their participation in this study was voluntary, that they could withdraw at any time, and that there would be no disadvantage due to nonparticipation.

2.2. Study design and participants

This cross-sectional study enrolled pregnant women from the Kumamoto University regional center of the Japan Environment and Children's Study (JECS). This study was conducted as an adjunct study of the JECS by the Ministry of the Environment, Government of Japan. The JECS aims to elucidate the effects of environmental factors on children's growth and development from the fetal stage. The JECS protocol has been described previously (Kawamoto et al., 2014; Michikawa et al., 2018). As a part of the JECS in Kumamoto, 3,082 women in their first trimester of pregnancy were recruited between January 2011 and March 2014 and surplus serum samples were collected from 1,353 women who consented to participate.

2.3. Data collection

We collected data on age, height before pregnancy (cm), weight before pregnancy (kg), previous pregnancy, previous delivery, smoking habits of pregnant women and their husband/partner, drinking habits, occupation, and frequency of chemical exposure from the JECS dataset: jecs-ta-20190930.

The data on age, previous pregnancy, and previous delivery were recorded from medical records and notes kept by physicians, midwives/nurses, and/or research coordinators. The data on health and living conditions during the first trimester of pregnancy were collected through self-administered questionnaires filled out by the pregnant women. The occupations were defined or categorized based on the Japanese Standard Classification of Occupations (Ministry of Internal Affairs and Communications in Japan, 2009): Professional and engineer, Service, Clerical, Sales, Manufacturing process, Agriculture, Forestry, and Fisheries, Management, Carrying, Cleaning, Packaging, and Security. We recorded the frequency of using or handling the following chemicals during work for more than half a day: kerosene, petroleum, benzene, gasoline, chlorine bleach and germicide (with the warning "Do not mix. Hazardous."), medical sterilizing disinfectants, permanent markers, water-based paint or inkjet printers, organic solvents (e.g., paint thinner, solvents used for examination/analysis/extraction, dry-cleaning detergent, stain-removing agent, paint coating, nail polish remover), photocopying machines, laser printers, engine oil, formalin, formaldehyde, anticancer drugs (not including those received by the participants for their treatment), insecticide, herbicide, and hair dyes (hair coloring). The term "machine" in this study simply refers to transport mechanisms that provide exposures to the chemicals used by these machines; this definition is in accordance with similar previous JECS studies (Ooka et al., 2021; Adachi et al., 2019). For example, the exposure to chemical substances such as emit dust and VOCs emitted by photocopying machines while operating them.

The frequency of chemical exposure was recorded as "never," "1–3 times a month," "1–6 times a week," or "daily." Referring to previous studies, the "1–6 times a week" and "daily" options in the questionnaire were summed to "more than once a week" (Ooka et al., 2021; Adachi et al., 2019).

2.4. ANA testing

ANA titers were assessed by indirect immunofluorescence with HEp-2 cells according to a standard protocol at Fujirebio Inc. (Tokyo, Japan). ANA positivity was defined as an antibody titer $\geq$40. For ANA-positive samples, the cell staining pattern was also recorded. The surplus serum samples collected during the first trimester were used for ANA testing.

2.5. Statistical analyses

Continuous and categorical variables are presented as median (interquartile range [IQR]) and number (percentage), respectively. The approximate normality was confirmed for continuous variables using the Kolmogorov–Smirnov test. Continuous variables were analyzed using the Mann–Whitney *U* test, whereas categorical variables were analyzed using the chi-square test. Multivariate analysis was performed using logistic regression analysis, and odds ratios and adjusted odds ratios (AORs) with 95% confidence intervals (CI) were calculated. The adjustment variables were age, body mass index, previous delivery, smoking, and occupation. However, the history of alcohol use and partner's smoking history were excluded from the adjustment variables due to multicollinearity with the pregnant women's smoking history. All

Table 1
Characteristics of study, overall and stratified by ANA positivity

	Total	ANA positive	ANA negative	*P* value
	n = 1,235	*n* = 213	*n* = 1,022	
Age (years)	30.0 (26–34)	31.0 (27–35)	30.0 (26–34)	**0.04**
Height (cm)	158.0 (154.0–161.0)	157.5 (154.0–161.0)	158.0 (154.0–161.0)	0.54
Weight (kg)	52.0 (47.0–58.0)	53.0 (47.0–58.0)	52.0 (47.0–59.0)	0.88
BMI (kg/m^2)	20.8 (19.3–23.2)	20.7 (19.4–22.8)	20.9 (19.2–23.3)	0.57
Previous pregnancy				
Primigravida	286 (23.2)	50 (23.5)	236 (23.1)	0.90
Multigravida	949 (76.8)	163 (76.5)	786 (76.9)	
Previous delivery				
Nulliparous	406 (32.9)	72 (33.8)	334 (32.7)	0.75
Parous	829 (67.1)	141 (66.2)	688 (67.3)	
Smoking				
Never	727 (58.9)	133 (62.4)	594 (58.1)	0.36
Former	253 (20.5)	45 (21.1)	208 (20.4)	
Stopped	187 (15.1)	24 (11.3)	163 (15.9)	
Current	68 (5.5)	11 (5.2)	57 (5.6)	
Partner smoking, *n*=1,212				
Never	271 (22.4)	60 (28.4)	211 (21.1)	0.09
Former	227 (18.7)	40 (19.0)	187 (18.7)	
Stopped	24 (2.0)	5 (2.4)	19 (1.9)	
Current	690 (56.9)	106 (50.2)	584 (58.3)	
Drinking				
Never	434 (35.1)	84 (39.4)	350 (34.2)	0.35
Stopped	648 (52.5)	105 (49.3)	543 (53.1)	
Current	153 (12.4)	24 (11.3)	129 (12.6)	
Occupation, *n*=1,230				
Unemployed	200 (16.3)	40 (18.9)	160 (15.7)	0.26
Employed	1,030 (83.7)	172 (81.1)	858 (84.3)	
Professional and engineer	350 (34.0)	46 (26.7)	304 (35.4)	
Service	286 (27.8)	43 (25.0)	243 (28.3)	
Clerical	239 (23.2)	60 (34.9)	179 (20.9)	
Sales	70 (6.8)	11 (6.4)	59 (6.9)	
Manufacturing process	51 (5.0)	7 (4.1)	44 (5.1)	
Agriculture, Forestry, Fisheries	18 (1.7)	3 (1.7)	15 (1.7)	
Management	10 (1.0)	1 (0.6)	9 (1.0)	
Carrying, Cleaning, Packaging	4 (0.4)	1 (0.6)	3 (0.3)	
Security	2 (0.2)	0 (0.0)	2 (0.2)	

Abbreviations: ANA, antinuclear antibody; IQR, interquartile range; BMI, body mass index.
Continuous variables are shown as Median (IQR) and categorical variables as n (%).
The *p* values were calculated using the Mann–Whitney *U* test and the chi-square test for continuous and categorical variables, respectively.
BMI was calculated as weight (kg) divided by height (m) squared.
"Former" smoking: previously smoked but quit before noticing their current pregnancy.
"Stopped" smoking: previously smoked but quit after noticing their current pregnancy.
"Stopped" drinking: previously drank but quit.
"Unemployed": full-time homemakers, students, or unemployed women.
Occupation, *n*=1,230 (5 women on maternity leave or with unknown occupations were excluded).

Table 2
ANA titers and staining patterns

	n	%
ANA titer (*n* =1,235)		
<40	1,022	82.8
1:40	172	13.9
1:80	34	2.8
1:160	6	0.5
1:1280	1	0.1
ANA positive total	213	17.2
ANA staining pattern (*n* =213)		
Speckled	203	95.3
Homogeneous	154	72.3
Nucleolar	16	7.5
Cytoplasmic	5	2.3
Discrete speckled	2	0.9
Peripheral	0	0.0
Others	8	3.8

Abbreviations: ANA: antinuclear antibody
ANA was tested using indirect immunofluorescence with HEp-2 cells.
ANA positive was defined as antibody titer ≥40.

statistical analyses were performed using IBM Statistical Package for Social Sciences (SPSS, version 27; IBM, NY, USA). A *P* value < 0.05 was considered statistically significant.

3. Results

After excluding 118 participants with missing data, 1,235 pregnant women in their first trimester (mean 13.3 weeks, standard deviation 3.5) were included in the analysis. Table 1 shows the characteristics of women stratified according to ANA positivity. The ANA-positive participants were significantly older than ANA-negative participants (median 31.0 [IQR, 27–35] versus median 30.0 [IQR, 26–34], $p < 0.05$). ANA positivity was not significantly associated with body size, previous pregnancy, previous delivery, smoking habits, drinking habits, or employment. Overall, 1,030 (83.7%) pregnant women were employed. Most of the participants were professional and engineer ($n = 350$, 34.0%), service ($n = 286$, 27.8%), or clerical ($n = 239$, 23.2%).

The ANA titers and staining patterns are summarized in Table 2. Overall, 213 (17.2%) pregnant women were ANA positive (antibody titer ≥40), of whom 172 (80.8%) were positive at a cutoff of 1:40, 34

Table 3
Association between chemical exposure and ANA positivity ($n = 1,235$)

Chemical	Exposure	ANA positive	Crude OR	Adjusted OR
	n (%)	*n* (%)	95% CI	95% CI
Kerosene, petroleum, benzene, gasoline				
Never	1,080 (87.4)	178 (16.5)	ref.	ref.
1–3 times a month	112 (9.1)	24 (21.4)	1.38 (0.86–2.23)	1.44 (0.88–2.35)
More than once a week	43 (3.5)	11 (25.6)	1.74 (0.86–3.52)	**2.11 (1.03–4.34)**
Chlorine bleach, germicide				
Never	1,014 (82.1)	161 (15.9)	ref.	ref.
1–3 times a month	146 (11.8)	35 (24.0)	**1.67 (1.10–2.53)**	**1.68 (1.10–2.57)**
More than once a week	75 (6.1)	17 (22.7)	1.55 (0.88–2.74)	**1.97 (1.10–3.54)**
Medical sterilizing disinfectants				
Never	1,064 (86.2)	184 (17.3)	ref.	ref.
1–3 times a month	41 (3.3)	8 (19.5)	1.16 (0.53–2.55)	1.54 (0.69–3.45)
More than once a week	130 (10.5)	21 (16.2)	0.92 (0.56–1.51)	1.17 (0.69–2.00)
Permanent markers				
Never	783 (63.4)	136 (17.4)	ref.	ref.
1–3 times a month	217 (17.6)	30 (13.8)	0.76 (0.50–1.17)	0.79 (0.52–1.22)
More than once a week	235 (19.0)	47 (20.0)	1.19 (0.82–1.72)	1.32 (0.90–1.93)
Water-based paint, inkjet printer				
Never	961 (77.8)	159 (16.5)	ref.	ref.
1–3 times a month	130 (10.5)	20 (15.4)	0.92 (0.55–1.52)	0.96 (0.57–1.60)
More than once a week	144 (11.7)	34 (23.6)	**1.56 (1.02–2.37)**	1.52 (0.97–2.36)
Organic solvents				
Never	1,163 (94.2)	193 (16.6)	ref.	ref.
1–3 times a month	62 (5.0)	16 (25.8)	1.75 (0.97–3.15)	**2.01 (1.10–3.71)**
More than once a week	10 (0.8)	4 (40.0)	3.35 (0.94–11.99)	**5.34 (1.40–20.36)**
Photo copying machines, laser printers				
Never	837 (67.8)	123 (14.7)	ref.	ref.
1–3 times a month	112 (9.1)	25 (22.3)	**1.67 (1.03–2.71)**	**1.79 (1.09–2.93)**
More than once a week	286 (23.2)	65 (22.7)	**1.71 (1.22–2.39)**	**1.73 (1.17-2.54)**
Engine oil				
Never	1,212 (98.1)	210 (17.3)	ref.	ref.
1–3 times a month	18 (1.5)	2 (11.1)	0.60 (0.14–2.61)	0.63 (0.14–2.80)
More than once a week	5 (0.4)	1 (20.0)	1.19 (0.13–10.73)	1.68 (0.18–15.63)
Formalin, formaldehyde				
Never	1,227 (99.4)	211 (17.2)	ref.	ref.
1–3 times a month	5 (0.4)	2 (40.0)	3.21 (0.53–19.33)	4.32 (0.70–26.71)
More than once a week	3 (0.2)	0 (0.0)	No data	No data
Anticancer drug				
Never	1,220 (98.8)	210 (17.2)	ref.	ref.
1–3 times a month	13 (1.1)	2 (15.4)	0.87 (0.19–3.97)	1.21 (0.26–5.62)
More than once a week	2 (0.2)	1 (50.0)	4.81 (0.30–77.20)	7.20 (0.44–118.63)
Insecticide				
Never	1,139 (92.2)	193 (16.9)	ref.	ref.
1–3 times a month	87 (7.0)	20 (23.0)	1.46 (0.87–2.47)	1.57 (0.92–2.67)
More than once a week	9 (0.7)	0 (0.0)	No data	No data
Herbicide				
Never	1,223 (99.0)	210 (17.2)	ref.	ref.
1–3 times a month	12 (1.0)	3 (25.0)	1.61 (0.43–5.99)	1.52 (0.40–5.81)
More than once a week	0 (0.0)	0 (0.0)	No data	No data
Hair dyes				
Never	1,171 (94.8)	197 (16.8)	ref.	ref.
1–3 times a month	56 (4.5)	13 (23.2)	1.50 (0.79–2.83)	1.55 (0.81–3.00)
More than once a week	8 (0.6)	3 (37.5)	2.97 (0.70–12.52)	4.02 (0.90–17.93)

Abbreviations: OR, odds ratio; CI, confidence interval.
ORs were calculated using logistic regression analysis.
The adjustment variables were age, BMI, previous delivery, smoking, and occupation.
The adjustment variables did not include drinking and partner's smoking because of multicollinearity.
Exposure was defined as using or handling the materials during work for more than half a day.
Organic solvents included paint thinner, solvents used for examination/analysis/extraction, dry-cleaning detergent, stain-removing agent, paint coating, and nail polish remover.
The values in bold text are statistically significant.

(16.0%) at 1:80, 6 (2.8%) at 1:160, and 1 (0.5%) at 1:1280. The most common staining pattern was speckled (n = 203, 95.3%) followed by homogeneous (n = 154, 72.3%). All homogeneous pattern–positive participants also had a speckled pattern.

Table 3 shows the association between the frequency of chemical exposure and ANA positivity during the first trimester of pregnancy. The most common exposure was of permanent markers, followed by photocopying machines or laser printers. When the exposure frequency was more than once a week, the AORs of ANA positivity were significantly high for exposure to kerosene, petroleum, benzene, or gasoline (AOR, 2.11; 95% CI, 1.03–4.34); chlorine bleach or germicide (AOR, 1.97; 95% CI, 1.10–3.54); organic solvents (AOR, 5.34; 95% CI, 1.40–20.36); and photocopying machines or laser printers (AOR, 1.73; 95% CI, 1.17–2.54). For organic solvent exposures more than once a week, only four ANA-positive pregnant women were found, and the confidence interval was large.

4. Discussion

This study suggested that ANA positivity in Japanese pregnant women in their first trimester of pregnancy was associated with the exposure to chemicals, including fuels such as kerosene, chlorine bleach or germicide, organic solvents, and photocopying machines or laser printers.

This study is subject to selection bias because it is based on a population in one region of Japan. However, the specificity was low, and we considered the trend to be similar to the results for pregnant women in Japan. First, the age of the participants was similar to the mean age of pregnant women in Japan (Ministry of Health, Labour and Welfare in Japan, 2020). Second, the height and weight of the participants were comparable to the average height and weight of Japanese women aged 15–45 years (Ministry of Health, Labour and Welfare in Japan, 2019). Third, the most common occupations in this study were consistent with the top occupations among Japanese women: professionals and technicians, service workers, and clerical workers (Statistics Bureau, Ministry of Internal Affairs and Communications in Japan, 2021). Furthermore, the present data, shown in Table 3 for chemical exposure, showed high concordance with the JECS national data (Iwai-Shimada et al., 2018) (kappa coefficient = 0.898, $p < 0.001$). In general, regional differences in chemical exposure frequency are expected due to geographical and industrial factors. The data in this study is not specific characteristic of the region studied, and we considered that they could indicate Japanese nationwide trends.

We obtained epidemiological data on ANA positivity among pregnant Japanese women. This study was conducted among pregnant women from the general population, and positivity with low titers was expected in most cases. Thus, we decided to use a cutoff dilution of 1:40, which has a low specificity but high sensitivity. The prevalence of ANAs was 17.2%, and 80.8% of the women had a low serum dilution of 1:40. As hypothesized, this was consistent with reports that ANA titers are generally low in healthy individuals (Marin et al., 2009). Previous Japanese studies using an ANA titer of 1:40 as a cutoff found a prevalence of 34.6% among women aged 21–80 years (Hayashi et al., 2001), 19.9% among men and women with a mean age of 32 ± 9 years (Watanabe et al., 2004), and 31.7% among women with a median age of 53 years (Hayashi et al., 2008). According to previous studies, the prevalence of ANAs is 35.4% in participants aged 12–72 years in Mexico (Marin et al., 2009), 14.6% in participants aged 18–60 years in Brazil (Fernandez et al., 2003), and 31.7% in participants aged 21–60 years in the United States, Europe, Australia, Japan, and Canada (Tan et al., 1997). The ANA test has not been standardized, and there are differences in the prevalence and patterns of ANA positivity among laboratories (Tan et al., 1997). Moreover, the rate of ANA positivity varies with age, sex, and race. A number of other complex factors are predicted, and a simple comparison is difficult to perform. In this study, we could not determine whether pregnancy affected ANA positivity, but some previous studies have shown higher positivity rates than the present results. One reason for this may be that the participants of this study were young, with an average age of 30.0 years. Speckled and homogeneous staining patterns, which were common in this study, have been reported to be often detected even in healthy individuals (Pashnina et al., 2021). Further long-term research is required to elucidate the clinical significance of ANA positivity in healthy individuals.

The study found that pregnant women were exposed to multiple chemicals, regardless of whether they were employed. Exposure to some of these chemicals was associated with ANA positivity. Most of the chemicals that showed significant associations with ANA positivity were sources of volatile organic compounds (VOCs). VOCs are organic compounds that are volatile and gaseous in the atmosphere. Exposure to them is common in any setting. Their effects on organisms are manifold, and they can induce inflammation, tissue damage, and changes in hormone and enzyme levels (Barragán-Martínez et al., 2012; Maiyoh et al., 2015). Photocopying machines and other equipment emit dust and VOCs during operation, and there are concerns about their health effects such as inflammation, immune reactions, and cancer (Guo et al., 2020; Nandan et al., 2020; Brown, 1999). Silica, used as an external additive in toners, is associated with autoantibody production and systemic autoimmune disease development (Parks et al., 2002; Aminian et al., 2009). Chlorine bleach may cause exposure-related damage, resulting in asthma, other respiratory diseases, and contact dermatitis (Chia Shi Zhe et al., 2016; Weber et al., 2016). In general, exposure to chemicals is influenced by occupation. As occupational exposures differ by biological and social characteristics between the sexes (Messing, 2000), the influence of sex must be considered (Eng A et al., 2011; Biswas et al., 2021). For example, women have a higher exposure to disinfectants, hair coloring, and textile dust than men (Eng A et al., 2011). Specifically, frequent hair coloring has serious adverse effects on fetuses (Ooka et al., 2021). In 2003, the United Nations recommended the Globally Harmonized System of Classification and Labelling of Chemicals. In Japan, the law defines chemical substances such as lead, mercury, manganese, and polychlorinated biphenyls that affect pregnancy, childbirth, and lactation functions and prohibits women from working in certain occupations. With the recent advancement of women in society, women are engaged in diverse occupations, and some women are employed in unregulated workplaces (Ooka et al., 2021). In Japan, women still perform most of the household chores, regardless of whether they are employed (Gender Equality Bureau and Cabinet Office, 2019). Therefore, when considering women's health, exposure at both the home and the workplace should be considered. In this study, no information was obtained for exposures less than once a month. Recent studies have pointed out that even short-term exposure to very small amounts of chemicals may cause health problems after a long period of time (Kahn et al., 2020); possibly, the cumulative long term effect of a single chemical exposure, even in minute amounts, may be significant. In the future, we would like to research more on trace exposure and combined exposure.

We did not observe significant associations of ANA positivity with participant characteristics other than age. Smoking is a risk factor for autoimmune diseases; it causes oxidative stress and enhances the production of many proinflammatory cytokines (Klareskog et al., 2007; Arnson et al., 2010). However, a consensus regarding the relationship between ANA positivity and smoking is lacking (Dinse et al., 2020; Bonarius et al., 2011; Satoh et al., 2012). We found that pregnant women and their fetuses are highly exposed to secondhand smoke. Thus, it is important to provide guidance on smoking cessation for pregnant women and their families and individuals living in their immediate environment.

Our study has some limitations. First, this study did not include a comparison group as it is a baseline cross-sectional observational study. Second, caution must be exercised in how ANA positivity is captured. The cutoff values for ANA positivity varied among studies, making objective comparisons impossible. In addition, ANA diseases, such as

systemic lupus erythematosus (SLE), systemic sclerosis (SSc), and mixed connective tissue disease (MCTD) (Kavanaugh et al., 2000), are common in women of childbearing age; however, we could not obtain reliable data regarding their diagnoses. Our study population may have included a small number of patients with ANA-related diseases. Third, some sample sizes for chemical exposure frequencies were very small, and the analyses results may not be reliable. For example, engine oil, formalin or formaldehyde, and anticancer drug in the "more than once a week" category. Most of the statistically significant results could exclude the effect of the small sample size; however, the confidence interval for organic solvents was very wide and must be interpreted with caution. Finally, the data on chemical exposure were extracted from self-administered surveys. It is possible that there were underestimations due to poor awareness of exposure and overestimations owing to misconceptions. Because quantitative evaluation using biological samples was not conducted, the half-life, amount of exposure, or effects of combined exposure could not be examined. Furthermore, even unemployed pregnant women often responded to the frequency of exposure to "occupational" chemicals. However, since the survey asked about the frequency of exposure for more than half a day, there is a possibility that the chemical exposure in this survey is steady or continuous exposure, comparatively at work or home.

Despite multiple limitations, our results suggested that ANAs are potential markers of chemical exposure. This study provides a useful base for further robust research, including comparisons with a group of healthy non-pregnant women of the same age and longitudinal changes in ANAs.

5. Conclusions

ANAs were present in 17.2% of Japanese women in their first trimester of pregnancy, and 80.8% of them had a low serum dilution of 1:40. ANA positivity was associated with exposure to several chemicals for more than half a day once a week.

Submission declaration

The study has not been published previously. All authors have read and approved the final version of the manuscript and explicitly by the responsible authorities where the work was carried out.

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Availability of data and material

Data cannot be shared for privacy or ethical reasons. In Japan, it is prohibited by the Act on the Protection of Personal Information (Act No. 57 of May 30, 2003, amendment on 9 September 2015) to publicly deposit data containing personal information. The Ethical Guidelines for Medical and Health Research Involving Human Subjects enforced by the Japan Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labour and Welfare also restrict the open sharing of epidemiological data. All inquiries about access to data should be sent to the following: jecs-en@nies.go.jp. The person responsible for handling inquiries sent to this e-mail address is Dr. Shoji F. Nakayama, JECS Programme Office, National Institute for Environmental Studies.

Declaration of competing interest

All authors declare that they have no competing interests.

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Associations of early life phthalate exposures with adolescent lipid levels and insulin resistance: The HOME Study

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ABSTRACT

Background: Early-life phthalate exposures may disrupt metabolic processes; however few prospective studies have assessed whether these associations extend to cardiometabolic outcomes during adolescence.
Methods: Among 183 mother-adolescent pairs in a prospective cohort study that enrolled pregnant women in Cincinnati, OH (2003–2006), we quantified nine phthalate metabolites in spot urine samples collected twice from mothers during pregnancy and up to seven times from children. At age 12 years, we assessed triglycerides, high-density (HDL) and low-density (LDL) lipoprotein cholesterol, insulin, and glucose from fasting serum samples and calculated homeostatic model assessment of insulin resistance (HOMA-IR). Using multiple informant models, we estimated covariate-adjusted associations between urinary phthalate concentrations at each time period and cardiometabolic biomarkers at age 12 years, including modification by child sex.
Results: Although most associations were weak or null, monoethyl phthalate (MEP), mono-n-butyl phthalate (MnBP), mono-isobutyl phthalate (MiBP), and monobenzyl phthalate (MBzP) concentrations were generally associated with lower LDL at age 12 years. A 10-fold increase in 4- and 12-year MEP was associated with −15.3 mg/dL (95% CI: 27.5, −3.13 mg/dL) and −11.8 mg/dL (−22.0, −1.51 mg/dL) lower LDL, respectively. Discrepant associations were observed in females versus males: a 10-fold increase in 3-year MEP concentrations was associated with 12.0 mg/dL (95% CI: 7.11, 31.1 mg/dL) higher LDL levels in males and −30.4 mg/dL (95% CI: 50.9, −9.8 mg/dL) lower LDL levels in females. Some urinary phthalate concentrations were cross-sectionally associated with HOMA-IR.
Conclusions: Early-life phthalate biomarker concentrations may be inversely associated with LDL during early adolescence in an exposure-period and sex-dependent manner.

1. Introduction

The prevalence of metabolic disorders is increasing among children and adolescents (Ogden et al., 2016; Reisinger et al., 2021). Dyslipidemia during the adolescent period, characterized by high concentrations of total cholesterol and low-density lipoprotein cholesterol (LDL), is a risk factor for atherosclerosis and may contribute to coronary heart disease during adulthood (Haney et al., 2007; Lozano et al., 2016). Similarly, adolescents with increased insulin resistance are more likely to develop obesity, type 2 diabetes mellitus, and coronary heart disease

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as adults (Levy-Marchal et al., 2010; Morrison et al., 2008; Tagi et al., 2019). Laboratory studies suggest that phthalates may influence cardiometabolic health through modification of peroxisome proliferator-activated receptors (PPARs) or adipokine production (Desvergne et al., 2009; Millar 2013; Pereira-Fernandes et al., 2013; Philips et al., 2017; Taxvig et al., 2012).

Phthalates are the most commonly used plasticizers, scent retainers, and emollients in the world (Huang et al., 2021). Of particular concern are exposures occurring during periods of heightened susceptibility, such as gestation and early life (Braun 2017; Heindel et al., 2017; Lawler et al., 2015). While some phthalates, such as di (2-ethylhexyl) phthalate (DEHP), are prohibited for use in children's consumer products and toys (Consumer Product Safety Improvement Act 2008), phthalate exposures are ubiquitous in pregnant women and children. Many phthalate metabolites have been detected in over 95% of urine samples in the United States, including among pregnant women and children (Centers for Disease Control and Prevention, 2022). Phthalate exposures may alter lipid and glucose metabolism (Heindel et al., 2017).

Three studies have examined associations of gestational phthalate exposures with dyslipidemia in childhood (Perng et al., 2017; Sol et al., 2020; Vafeiadi et al., 2018). Sol et al. found associations with higher non-fasting triglycerides at age 9 years (only among males), while other studies have observed no associations during pregnancy among children at age 4 years (Vafeiadi et al., 2018) or ages 8–14 years (Perng et al., 2017). Only two studies have examined associations between childhood phthalate exposures and dyslipidemia; both found that urinary phthalate metabolite concentrations were associated with higher triglyceride and lower high-density lipoproteins (HDL) among males and females at 9 years of age (Han et al., 2019; Silva et al., 2021).

Four prospective studies have examined either pregnancy (Sol et al., 2020; Watkins et al., 2016) or postnatal (Han et al., 2019; Silva et al., 2021) phthalate exposure biomarkers and childhood measures of insulin resistance. Pregnancy phthalate exposure biomarkers were associated with lower insulin (Watkins et al., 2016) or glucose levels (Sol et al., 2020) among males, but not females. Childhood phthalate exposure biomarkers were associated with increased homeostatic model assessment for insulin resistance (HOMA-IR) in one study (Han et al., 2019) but not another (Silva et al., 2021). Few studies, all cross-sectional, have assessed insulin resistance among adolescents; these studies reported that exposures to some phthalates, particularly high molecular weight phthalates, are associated with increased insulin resistance (Attina and Trasande 2015; Kataria et al., 2017; Kim et al., 2018; Smerieri et al., 2015; Trasande et al., 2013).

Studies assessing early life phthalate exposures in relation to lipid levels and insulin resistance are sparse. Few studies have assessed phthalate exposures prospectively or serially during childhood to determine the relative importance of pregnancy and postnatal exposures to outcomes measured in adolescence. The objective of this study was to investigate associations of maternal gestational and childhood urinary phthalate concentrations with serum lipid concentrations and biomarkers of insulin resistance at age 12 years. We aimed to identify potential periods of heightened susceptibility and explore differences by child's sex.

2. Materials and methods

2.1. Study participants

The Health Outcomes and Measures of the Environment (HOME) Study is an ongoing prospective pregnancy and birth cohort study that enrolled pregnant women from the Cincinnati, Ohio area between 2003 and 2006. Cohort details have been described previously (Braun et al. 2017, 2020). Briefly, women were eligible to participate in the cohort if they were 16 ± 3 weeks of gestation, spoke English, were age 18 years or older, lived in a home built before 1978, and had no history of HIV infection or other medical conditions. Of the 1263 women that were eligible, 468 (37%) agreed to participate in our study. Of these, 67 pregnant women dropped out before delivery leaving 401 women who delivered 389 singletons, 9 sets of twins, and 3 stillbirths (Fig. S1) (Braun et al., 2017; Etzel et al., 2022). Follow-up assessments were completed at post-natal ages 4-weeks, 1, 2, 3, 4, 5, 8, and 12 years and follow-up rates ranged from 48% to 94% (Braun et al. 2017, 2020).

After study protocols were explained by trained research assistants, women provided written consent for themselves and their children, and adolescents provided written assent at age 12 years. The Cincinnati Children's Hospital Medical Center (CCHMC) and participating delivery hospitals' Institutional Review Boards (IRB) approved the HOME Study. The Centers for Disease Control and Prevention (CDC) relied on CCHMC IRB.

2.2. Pregnancy and early childhood phthalate exposure assessment

At an average of 16.0 (min-max: 10.4–22.6) and 26.5 (min-max: 19.1–34.6) weeks of gestation, women provided two spot urine samples. Children provided up to seven spot urine samples during follow-up visits at ages 1, 2, 3, 4, 5, 8, and 12 years (Table S1). For children who were not toilet trained, urine samples were collected in surgical inserts placed into a diaper. Children who were being toilet-trained had urine samples collected using a training toilet lined with inserts. Pregnant women and children who were toilet-trained provided urine samples in polypropylene specimen cups. After collection, all urine samples were stored at −20 °C before being shipped frozen overnight to the CDC for analyses. Urine samples were analyzed using isotope dilution high-performance liquid chromatography coupled with tandem mass spectrometry (Silva et al., 2004) for the following urinary phthalate metabolites: monoethyl phthalate (MEP), mono-n-butyl phthalate (MnBP), mono-isobutyl phthalate (MiBP), mono (3-carboxypropyl) phthalate (MCPP), monobenzyl phthalate (MBzP), and four metabolites of DEHP: mono (2-ethylhexyl) phthalate (MEHP), mono (2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono (2-ethyl-5-oxohexyl) phthalate (MEOHP), and mono (2-ethyl-5-carboxypentyl) phthalate (MECPP). Limits of detection (LOD) ranged from 0.2 to 1.2 ng/mL depending on the metabolite, and urinary phthalate concentrations that were below the LOD were assigned a value of $LOD/\sqrt{2}$ (Silva et al., 2004).

Diaper inserts were contaminated with DEHP, DnBP, and DiBP phthalates, therefore urinary concentrations of MEHP, MnBP, and MiBP could not be quantified at 1–3 years of age because these metabolites were detected in the diaper inserts (Watkins et al., 2014a). Therefore, we used the three oxidative DEHP metabolites (MEOHP, MEHHP, MECPP) that were measured in all maternal and child samples to calculate the molar sum of maternal and childhood DEHP metabolites ($\sum$DEHP). We calculated the molar sum by dividing each of the three metabolites by its molar mass, summing the concentrations, and then multiplying the sum by the molar weight of MECPP to put concentrations on the original scale.

2.3. Adolescent lipid level and insulin resistance outcomes

Participants provided an overnight fasting serum sample via venipuncture at the 12-year follow-up visit. We measured serum triglycerides, high- and low-density lipoproteins, glucose, and insulin using immunoassays. Trained laboratory technicians in the Cincinnati Children's Hospital Medical Center NIH-funded Clinical Translational Research Center Core Laboratory conducted all assays. Triglyceride concentrations were right-skewed and thus $\log_2$-transformed for analysis. Model estimates were reported as the percent change of 12-year triglycerides per 10-fold increase in prenatal urinary phthalate metabolites. Homeostatic model assessment for insulin resistance (HOMA-IR) was calculated using the following standard formula: HOMA-IR = [fasting insulin (mIU/L) x fasting glucose (mg/dL)]/405 (Matthews et al., 1985). Because HOMA-IR was strongly correlated with insulin concentrations (Pearson's $\rho = 0.99$), we used HOMA-IR as our measure

of insulin resistance.

2.4. Covariates

To ascertain demographic and other maternal and child characteristics, trained research assistants administered a standardized interview to the mothers during the second and third trimesters of pregnancy and each child follow-up visit. Serum cotinine concentrations were measured at 16 and 26 weeks of pregnancy as previously described (Bernert et al., 1997; Braun et al., 2010) to measure tobacco smoke exposure during gestation. Research staff reviewed medical charts to ascertain additional pregnancy and delivery information. Height and weight were measured at each child follow-up visit. While children were dressed in undergarments, weight was obtained to the nearest 0.01 kg using a calibrated ScaleTronix 5002 scale (Hill-Rom Inc, Chicago, IL). Height was measured to the nearest 0.1 cm using either a wall-mounted 226 Hite-Rite stadiometer (Ayrton Corporation, Prior Lake, MI) or a Harpenden stadiometer (Holtain Ltd, Crymych, UK). Trained research assistants obtained measurements in triplicate; the average of the three measurements was used. Maternal and child height and weight were used to calculate body mass index (BMI) (kg/m^2). We calculated children's age- and sex-standardized BMI z-scores using CDC reference data from U.S. children (Kuczmarski et al., 2000). We standardized maternal mid-pregnancy BMI measured at the first visit at 16 weeks gestation to our study population for unit consistency in regression models. At the 12-year follow-up visit, child physical activity was measured using the Physical Activity Questionnaire for Older Children (PAQ-C), a self-administered detailed 7-day recall instrument that measures physical activity among children between the ages of 8 and 14 years (Kowalski et al., 2004). Additionally, three 24-h dietary recalls (2 weekdays and 1 weekend day) were collected at the 12-year visit. Food recalls were analyzed using the Nutrition Data Systems for Research software and foods database (University of Minnesota, MN). We calculated Healthy Eating Index (HEI) composite scores as a measure of dietary quality (Guenther et al., 2013).

We identified potential confounders that may be associated with both urinary phthalate biomarker concentrations and 12-year lipid levels and insulin resistance outcomes using a directed acyclic graph (DAG) (Fig. S2). Our final adjusted models included child sex assigned at birth and the following maternal baseline characteristics: maternal age at delivery, pregnancy weight gain, mid-pregnancy BMI, average cotinine concentration during pregnancy (ng/mL), race/ethnicity, prenatal fruit and vegetable consumption ("Monthly", "Weekly", "Daily"), and prenatal vitamin use ("Never", "Weekly/Monthly", or "Daily"). We also included two time-varying covariates: childhood BMI and household income. In addition to potential confounders, we adjusted for PAQ-C composite scores at age 12 years to improve precision of our estimates.

2.5. Statistical analyses

Of the 256 mother-child pairs who returned for the follow-up visit at age 12 years, we excluded twins (n = 7 sets), those missing maternal phthalate measures from pregnancy (n = 3), those who did not complete the blood draw at the age 12 years visit (n = 16), and those missing maternal covariates during pregnancy [anthropometry (n = 38); serum cotinine (n = 2)]. Therefore, our final analytic dataset comprised 183 mothers and their children (Fig. S1). All 183 participants in our study had phthalates metabolites measured at least once during gestation and at age 12 years (Table S2).

To account for urinary dilution, we used covariate-adjusted standardization (O'Brien et al., 2016). Briefly, we multiplied urinary phthalate biomarker concentrations by the ratio of observed to predicted creatinine concentrations as described by O'Brien et al. Our creatinine prediction model for pregnant women included pregnancy complications (gestational diabetes and hypertension), maternal race/-ethnicity, family income, maternal age, height, and mid-gestation body mass index (BMI) (kg/m^2). We included child's sex assigned at birth, height, BMI, age, family income, and race/ethnicity in our child creatinine prediction models. Due to their non-normal distributions, covariate-adjusted creatinine standardized urinary phthalate biomarker concentrations were $\log_{10}$ transformed to reduce the influence of outlying values. For women who provided more than one sample during pregnancy, we averaged the two $\log_{10}$-transformed covariate-adjusted creatinine standardized urinary phthalate biomarker concentrations for analysis.

We used multiple informant models to examine potential periods of susceptibility for associations of pregnancy and repeated childhood urinary phthalate concentrations with adolescent lipid levels and insulin resistance. The multiple informant model approach has been described previously (Sanchez et al., 2011) and applied in the HOME Study (Jackson-Browne et al., 2018; Shoaff et al., 2017; Stacy et al., 2017). Briefly, multiple informant models use generalized estimating equations (GEEs) for parameter estimation with embedded separate linear regression models for each exposure period. Separately for each urinary phthalate biomarker, we used the multiple informant model to jointly estimate the difference in serum lipid levels and insulin resistance per 10-fold increase in phthalate biomarker concentrations at each time period (i.e., gestation and age 1, 2, 3, 4, 5, 8, and 12 years). Associations of phthalate biomarker concentrations at each time period with lipid levels and insulin resistance are not co-adjusted, however, the multiple informant model allows for a statistical test to determine if associations differ by time period. We considered associations to be significantly different by any exposure period when the phthalate biomarker x exposure period product term p-value was $\leq$0.10.

2.6. Secondary analyses

We assessed effect measure modification (EMM) by child sex using sex-stratified multiple informant models. To generate a statistical test of EMM by sex, we also fit multiple informant models with a phthalate biomarker x exposure period x child sex three-way interaction term. We considered associations to be significantly different by any exposure period or child sex when the phthalate biomarker x exposure period x child sex product term p-value was $\leq$0.10.

2.7. Sensitivity analyses

Diet is a predictor of phthalate exposures (Buckley et al., 2019b) as well as dyslipidemia and insulin resistance (Funtikova et al., 2015). Although we did not collect detailed maternal or child diet data before age 12 years, we conducted a sensitivity analysis adjusting for the child's HEI composite score at the 12-year follow-up visit.

We used SAS software, version 9.4 (SAS Institute, Inc., Cary, North Carolina). and RStudio, version 1.2.509 (RStudio: Integrated Development Environment for R) for all analyses.

3. Results

3.1. Overview of the study sample

Over half of the women in our study were non-Hispanic White (59%), aged 26–35 years at delivery (61%), and reported taking prenatal vitamins daily (73%). About one third had a mid-pregnancy BMI between 25 and 30 kg/m^2 (36%) and an average household income during pregnancy of $40,000–80,000 (34%). About half reported consuming fruit and vegetables weekly during pregnancy (51%) (Table 1). Characteristics were similar among women included in our study sample and those with complete prenatal covariates at enrollment (Table S3).

Children attended the 12-year study visit at an average of 12.3 ± 0.7 years of age. At the study visit, children had a mean ± SD PAQ composite score of 1.5 ± 0.6 and HEI composite score of 44.8 ± 10.6 (Table 2). HDL, LDL, and triglyceride concentrations at age 12 years

Table 1
Characteristics of women in the HOME Study, 2003–2006 (N = 183).

	N (%)
Maternal Age at Delivery	
18–25	44 (24)
> 25–35	111 (61)
> 35	28 (15)
Maternal Race/Ethnicity	
Non-Hispanic White	108 (59)
Non-Hispanic Black	64 (35)
Other	11 (6)
Household Income During Pregnancy	
> $80 K	49 (27)
$40–80 K	62 (34)
$20–40 K	28 (15)
< $20 K	44 (24)
Maternal Weight Gain during Pregnancy (lbs) (mean, SD)	21.0 (10.1)
Maternal Mid-Pregnancy BMI (kg/m^2)	
<25	74 (40)
25–30	65 (36)
>30	44 (24)
Tobacco Smoke Exposure during Pregnancy (≥3 ng/mL)	16 (9)
Prenatal Vitamin Use	
Never	19 (10)
Weekly/Monthly	31 (17)
Daily	133 (73)
Prenatal Fruit and Vegetable Consumption	
Monthly	9 (5)
Weekly	94 (51)
Daily	80 (44)

[1] Smoke exposure based on prenatal serum cotinine concentrations; >3.0 ng/mL was considered active smoking exposure.

Table 2
Characteristics of 183 HOME Study participants at the 12-year follow-up visit by child sex, mean (SD).

Characteristics	Overall (N = 183)	Males (N = 88)	Females (N = 95)
Age (years)	12.3 (0.7)	12.4 (0.7)	12.3 (0.6)
Triglycerides [median (min-max)] (mg/dL)	74.6 (25.1–487.8)	74.0 (33.2–487.8)	76.3 (25.1–239.3)
High Density Lipoprotein (HDL) (mg/dL)	52.9 (11.8)	53.8 (13.0)	52.0 (10.6)
Low Density Lipoprotein (LDL) (mg/dL)	86.8 (25.5)	88.2 (23.0)	85.6 (27.7)
Glucose (mg/dL)	91.9 (6.3)	93.5 (6.7)	90.3 (5.5)
Insulin (mIU/L)	14.7 (9.8)	11.5 (7.1)	17.7 (11.0)
HOMA-IR	3.4 (2.3)	2.7 (1.7)	4.0 (2.5)
PAQ Composite Score	1.5 (0.64)	1.6 (0.72)	1.4 (0.54)
HEI Composite Score[a]	44.8 (10.6)	44.7 (10.6)	45.0 (10.9)

[a] 1 male and 1 female were missing HEI composite scores at age 12 years.

were similar among males and females (Table 2). Measures of insulin resistance at age 12 years were significantly different by child's sex ($p < 0.01$) (Table 2). Males had higher glucose (93.5 ± 6.7 mg/dL vs. 90.3 ± 5.5 mg/dL) and lower fasting insulin (11.5 ± 7.1 mIU/L vs. 17.7 ± 11.0 mIU/L) and lower HOMA-IR (2.7 ± 1.7 vs. 4.0 ± 2.5) than females (Table 2). Lipid, insulin, and glucose levels were weakly to moderately correlated (Pearson's $\rho = 0.08$–0.43).

Generally, median creatinine-standardized urinary concentrations of MEP and ∑DEHP were higher than other urinary phthalate concentrations (Table S4). Urinary MEP and MnBP concentrations were the highest during pregnancy, MCPP, MBzP, and ∑DEHP concentrations were highest at the 3-year visit, and MiBP concentrations were highest at the 8-year visit (Table S4). Median concentrations of all urinary phthalate biomarkers were lower at age 12 years than during early childhood (Table S4).

3.2. Phthalate biomarkers and 12-year lipid levels

In adjusted models, urinary MEP, MBzP, and ∑DEHP concentrations between the ages of 2–5 years were associated with lower triglycerides at age 12 years whereas associations were null or positive at other exposure periods (phthalate biomarker x exposure period interaction p-value >0.11) (Figs. 1–2; Table S5). For example, a 10-fold increase in 2-year MBzP and ∑DEHP concentrations was associated with −20.3% (95% CI: 33.4, −4.7%) and −25.5% (95% CI: 41.1, −5.8%) lower triglyceride concentrations at age 12 years, respectively (Fig. 2; Table S5). Estimates for other exposure periods and phthalate biomarkers were mostly null (phthalate biomarker x exposure period p-values >0.11; Table S5).

Some associations of MEP and MiBP with HDL varied by exposure period (Fig. 1; Table S6). Gestational and early childhood MEP and MiBP urinary biomarker concentrations were associated with lower levels of HDL at age 12 years but 8-year MEP and MiBP urinary biomarker concentrations were positively associated with 12-year HDL levels (Fig. 1; Table S6). For example, a 10-fold increase in gestational urinary MEP concentrations was associated with −3.57 mg/dL (95% CI: 7.15, 0.01 mg/dL) lower 12-year HDL levels whereas a 10-fold increase in 8-year MEP was associated with 6.19 mg/dL (95% CI: 1.34, 11.04 mg/dL) higher HDL levels at age 12 years (Fig. 1; Table S6).

Urinary MEP, MCPP, MBzP, and ∑DEHP concentrations were generally associated with lower levels of LDL although associations varied by exposure period (phthalate biomarker x exposure period interaction p-values <0.18) (Figs. 1–2; Table S7). For example, a 10-fold increase in 4 and 12-year MEP was associated with −15.3 mg/dL (95% CI: 27.5, −3.13 mg/dL) and −11.8 mg/dL (95% CI: 22.0, −1.51 mg/dL) lower LDL levels at age 12 years, respectively (Fig. 1; Table S7). Similarly, a 10-fold increase in 2-year and 3-year urinary MBzP concentrations were associated with −10.5 mg/dL (95% CI: 21.0, 0.01 mg/dL) and −11.5 mg/dL (95% CI: 21.4, −1.52 mg/dL) lower LDL levels at age 12 years, respectively (Fig. 2; Table S7). However, we also observed higher LDL levels at age 12 years per 10-fold increase in 1-year MBzP concentrations (β: 10.5 mg/dL; 95% CI: 0.44, 21.5 mg/dL) (Fig. 2; Table S7).

3.3. Phthalate biomarkers and 12-year insulin resistance

In general, we found that prenatal and childhood phthalate biomarkers were not associated with measures of insulin resistance or glucose at age 12 years; estimates were mostly null with little evidence of differential associations by exposure period (Figs. 1–2; Tables S8–S10). However, we found that some 12-year phthalate biomarker concentrations were associated with increased insulin resistance. For example, a 10-fold increase in 12-year MnBP, MiBP, and MCPP was associated with 0.58-point (95% CI: 0.01, 1.15), 0.68-point (95% CI: 0.08, 1.27), and 0.77-point (95% CI: 0.09, 1.46) higher HOMA-IR at age 12 years, respectively (Table S8). Associations were similar with insulin (Table S9).

3.4. Exploratory analyses

Some associations of urinary phthalate biomarker concentrations with HDL and LDL varied by child sex, with associations being generally null or positive among males but null or negative among females. Associations between urinary MCPP and ∑DEHP concentrations and HDL at age 12 years varied by exposure period and child sex (Table S6). For example, a 10-fold increase in pregnancy ∑DEHP was associated with 7.11 mg/dL (95% CI: 0.98, 13.25 mg/dL) higher HDL among males and −4.17 mg/dL (95% CI: 9.54, 1.12 mg/dL) lower HDL among females at age 12 years (Table S6). Associations between urinary MEP, MiBP, and MBzP and LDL at age 12 years also varied by child sex (Table S7). For example, a 10-fold increase in 3-year MEP concentrations was associated with 12.0 mg/dL (95% CI: 7.11, 31.1 mg/dL) higher LDL levels among

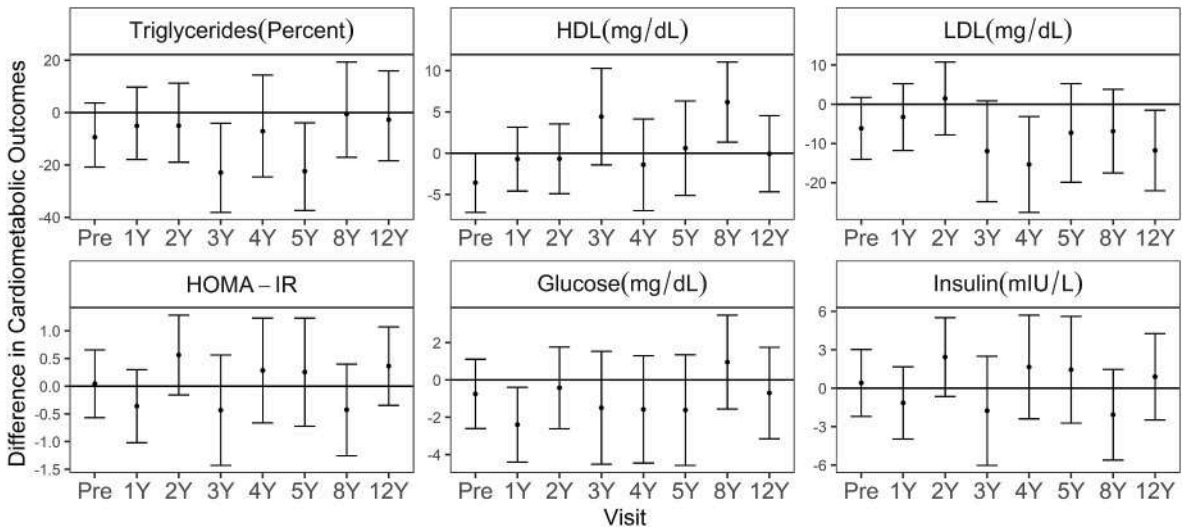

Fig. 1. Adjusted differences in cardiometabolic outcomes at age 12 years per 10-fold increase in prenatal and childhood urinary monoethyl phthalate (MEP) concentrations.
Differences and 95% confidence intervals (CI) estimated in multiple informants models adjusted for maternal race/ethnicity, smoking during pregnancy, pregnancy weight gain, prenatal vitamin use, prenatal fruit and vegetable consumption; BMI z-score and income level at each visit; and child's sex, and physical activity at age 12 years.

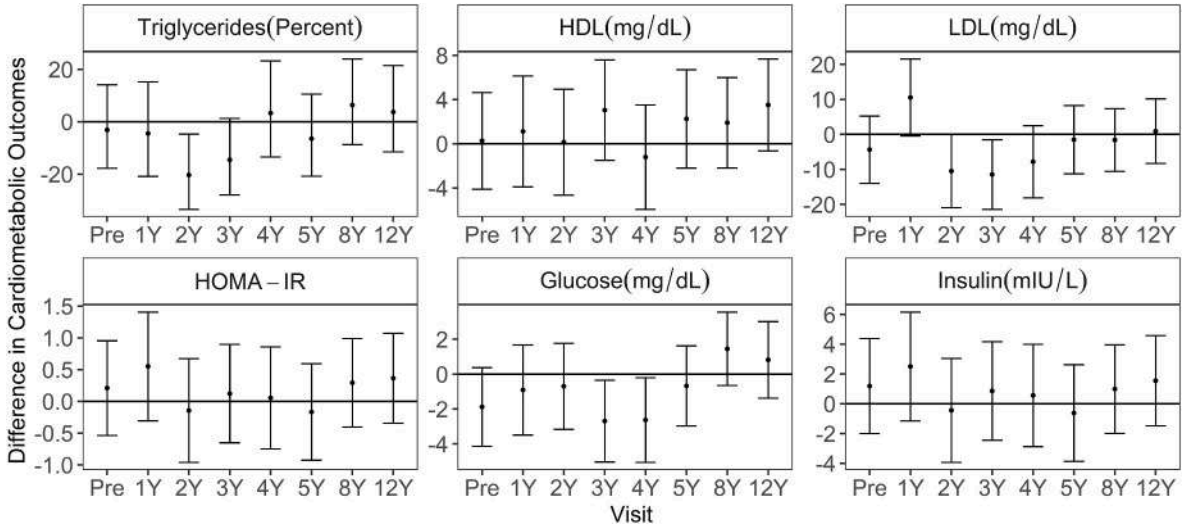

Fig. 2. Adjusted differences in cardiometabolic outcomes at age 12 years per 10-fold increase in prenatal and childhood urinary monobenzyl phthalate (MBzP) concentrations.
Differences and 95% confidence intervals (CI) estimated in multiple informants models adjusted for maternal race/ethnicity, smoking during pregnancy, pregnancy weight gain, prenatal vitamin use, prenatal fruit and vegetable consumption; BMI z-score and income level at each visit; and child's sex, and physical activity at age 12 years.

males and −30.4 mg/dL (95% CI: 50.9, −9.83 mg/dL) lower LDL levels among females (Table S7). Associations of phthalate biomarker concentrations with triglycerides, HOMA-IR, insulin, and glucose did not vary by child sex (Table S5; S8–S10).

3.5. Sensitivity analyses

Estimates did not change appreciably after adjustment for child's HEI composite score at age 12 years (<5% change in estimates) (Tables S11–S12).

4. Discussion

In this prospective pregnancy and birth cohort, most associations between phthalate metabolites and lipid levels were weak or null. However, some gestational and early childhood MEP, MiBP, and MnBP concentrations were associated with lower LDL and HDL concentrations. Additionally, many associations varied by child sex with pregnancy and childhood phthalate concentrations being associated with lower LDL among females but not males. Early-life urinary phthalate concentrations were not associated with insulin resistance at age 12 years, but some phthalates were associated with higher HOMA-IR at age 12 years in cross-sectional analyses.

Three prior studies have examined gestational phthalate exposures and lipid levels during childhood (Perng et al., 2017; Sol et al., 2020; Vafeiadi et al., 2018). In the Generation R Study, total phthalic acid concentrations were associated with higher non-fasting triglyceride concentrations among males, but not females at 10 years (N = 757) (Sol

et al., 2020). In contrast, Vafeiadi et al. found no associations between gestational phthalate concentrations and lipid levels at age 4 years among 500 mother-child pairs from Crete, Greece. Similarly, Perng et al. found no associations between gestational phthalate exposures and fasting lipid profiles among 248 Mexican youth ages 8–14 years (Perng et al., 2017; Vafeiadi et al., 2018). Consistent with these latter two studies, we found that phthalate exposure biomarkers were not associated with lower triglycerides, HDL, and LDL. As adolescents may be more vulnerable to hormonal changes during puberty, particularly females who begin puberty at a younger age, differences between study findings may be due to the age that lipid levels were assessed. These previous studies measured lipid levels during early to middle childhood and not the during the adolescent period (Sol et al., 2020; Vafeiadi et al., 2018). Additionally, Sol et al., 2020 and Vafeiadi et al., (2018) measured non-fasting lipid levels where Perng et al., (2017) and our study used fasting lipid levels, which is recommended as triglycerides in particular vary among a fasted and non-fasted state (National Heart Lung and Blood Institute 2012).

Two previous studies examined childhood phthalate exposures and childhood lipid biomarkers (Han et al., 2019; Silva et al., 2021). Han et al. found positive associations between MEHP, MEOHP, and mono-isononyl phthalate (MiNP) urinary concentrations at age 3–5 years and fasting triglyceride levels at age 7–9 years in 164 children from Seoul, South Korea (Han et al., 2019). Among 471 Dutch children, low molecular weight phthalates and DEHP urinary metabolite concentrations at age 6 years were associated with lower non-fasting HDL and higher non-fasting triglyceride levels at age 10 years, and results did not vary by child sex (Silva et al., 2021). In our study, we observed sex-differences in associations of childhood phthalate biomarker concentrations with lipid levels, though this was metabolite-specific. Silva et al. found no differences by child sex and Han et al. did not assess differences by child sex. Similar to differences in results with pregnancy associations, inconsistencies with results may be attributed to the timing of lipid measurements during childhood or adolescence as well as fasting state. Additionally, while phthalate concentrations during childhood were similar between our study and Silva et al. concentrations were much higher among the children from Seoul, South Korea. For example, in our study, median 5-year MnBP was 27.6 ng/mL compared to 141.49 ng/mL in Han et al., (2019).

In our study, we found no prospective associations between gestational or childhood phthalate concentrations with insulin and HOMA-IR. However, we observed some cross-sectional associations between 12-year phthalate concentrations and these measures. Among 250 mother-child pairs from Mexico City, *in utero* exposure to MEP was associated with lower insulin secretion among males at 8–17 years of age, but not females; their results varied by pubertal status (Watkins et al., 2016). Although not statistically significant, gestational MEP concentrations were associated with lower insulin levels among both males and females in our study. Sol et al. observed an increase in prenatal high molecular weight phthalates was associated with lower glucose concentrations among males but not females (Sol et al., 2020). We observed similar patterns with $\sum$DEHP concentrations and glucose levels in our study, however confidence intervals were wide.

Two prospective studies examined childhood phthalate exposures and childhood measures of insulin resistance (Han et al., 2019; Silva et al., 2021). Consistent with our study, Han et al. found positive associations between MEHP, MEOHP, and MiNP urinary concentrations and HOMA-IR, however, they did not assess associations by child sex (Han et al., 2019). In contrast, Silva et al. found no associations between urinary phthalate metabolites and insulin or glucose levels at 10 years of age and these results did not vary by child sex (Silva et al., 2021). Puberty is a period of heightened hormonal activity and changes. As such, adolescents may be more vulnerable to endocrine-disrupting chemicals, especially females, who often enter pubarche at an earlier age. Consequently, differences between study findings may be due to the age that insulin resistance was assessed. Whereas Han et al. and Silva et al. measured insulin sensitivity during middle childhood, we measured insulin sensitivity during the early adolescent period (Han et al., 2019; Silva et al., 2021).

Evidence from experimental studies suggests that gestational and childhood phthalate exposures may influence lipid levels and insulin resistance through multiple mechanisms (Grun and Blumberg 2009; Heindel et al., 2015). First, several phthalates have weak estrogenic activity and strong anti-androgenic activity, which could influence sex-specific cardiometabolic outcomes (Borch et al., 2006; Harris et al., 1997; Jobling et al., 1995; Sohoni and Sumpter 1998). Second, phthalates have been shown to modify PPARs (Desvergne et al., 2009; Pereira-Fernandes et al., 2013; Taxvig et al., 2012), which are important for the regulation of lipid and carbohydrate metabolism and anti-inflammatory processes (Millar 2013; Philips et al., 2017). Next, *in vivo* animal studies as well as human epidemiologic studies have shown that phthalates are associated with oxidative stress markers, which are in-turn associated with chronic inflammation (Ferguson et al., 2014). Additionally, animal studies have shown that gestational phthalate exposures in rats induces endothelial and cholesterol dysfunction (Lee et al., 2016; Rahmani et al., 2016). Taken together, these potential mechanisms suggest that phthalates may disrupt carbohydrate and lipid metabolism and promote inflammation and endothelial dysfunction, which may ultimately lead to the development of dyslipidemia and insulin resistance during adolescence persisting into adulthood (Philips et al., 2017). Finally, the strong anti-androgenic activity of phthalates may explain sex-specific associations with insulin resistance observed in our study (Heindel et al., 2017).

High levels of LDL are considered a risk factor for cardiovascular disease among adults, since LDL contributes to vascular inflammation and atherosclerotic plaque development and progression (Budoff 2016). While our study showed that an increase in urinary phthalate biomarker concentrations were generally associated with lower LDL levels among adolescents, particularly among females, this may be due to sexually dimorphic changes occurring during physical growth and maturation along with the fact that participants in our study were in various stages of pubertal development. Though previous epidemiological research suggests that lipid levels (particularly triglyceride and non-HDL cholesterol) are significantly lower among adolescents that have reached maturity or advanced puberty, compared to those who are prepubescent or in early pubarche (Schienkiewitz et al., 2019), we did not adjust for puberty in our models since it may be a mediator between phthalate exposures and cardiometabolic health biomarker (Berger et al., 2018; Sears and Braun 2020; Watkins et al. 2014b, 2017).

Our study has several notable strengths and limitations. First, we had repeated phthalate biomarker measurements during pregnancy and childhood, that allowed us to formally assess the influence of both gestational and childhood phthalate exposures on adolescent insulin resistance (Buckley et al., 2019a; Sanchez et al., 2011). Using multiple measurements of phthalates during pregnancy and childhood is also important due to the low within person variability of phthalates. In the HOME Study, intraclass correlation coefficients range from 0.09 to 0.38 (Watkins et al., 2014a). However, there is the potential for selection bias via loss to follow-up in our study due to its longitudinal nature and relatively long follow-up. For some follow-up visits, women who returned for follow-up visits were more likely to be non-Hispanic White, well-educated, and have higher household income compared to those who did not return for follow-up visits (Braun et al., 2017). In addition, due to our modest sample size we had relatively low power, particularly to detect differences by child sex or investigate the role of the pubertal transition during adolescence. Future studies are needed to examine how puberty may be a potential mediator between phthalate exposures and dyslipidemia and insulin resistance.

Finally, we collected a rich set of covariates during pregnancy and childhood that allowed us to adjust for a variety of potential confounders. Still, residual confounding remains a potential limitation. Diet is a major source of phthalate exposures (Buckley et al., 2019b) and lipid

levels are also associated with dietary intake (Ruiz et al., 2019). We lacked a detailed or standardized measure of gestational or early childhood diet, but we adjusted for fruit and vegetable consumption during pregnancy and other measured confounders that may block the backdoor paths related to dietary intake. In addition, associations were similar to main findings in our sensitivity analyses adjusting for HEI composite scores at age 12 years.

5. Conclusion

We found some urinary phthalate biomarker concentrations, particularly of low-molecular weight phthalates, were associated with lower levels of HDL and LDL during adolescence; some associations varied by exposure period and child sex. Phthalate biomarkers were not associated with insulin resistance among adolescents, though some cross-sectional associations with insulin resistance were found with 12-year phthalate measures. Further research in larger cohorts with longer follow-up is needed to confirm our findings, particularly sex-specific effects, and to determine long-term implications.

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Disclosure summary

TME, JRK, XW, NTM, AMC, KMC, AC, KY, HJK, and JPB have nothing to declare. Dr. Braun's institution was financially compensated for his services as an expert witness for plaintiffs in litigation related to PFAS-contaminated drinking water; these funds were not paid to JMB directly. Dr. Lanphear served as an expert witness in cases related to childhood lead poisoning, but he was not personally compensated.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the CDC. Use of trade names is for identification only and does not imply endorsement by the CDC, the Public Health Service, or the US Department of Health and Human Services.

Data availability

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.114102.

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Associations of maternal gestational urinary environmental phenols concentrations with bone mineral density among 12-year-old children in the HOME Study

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ABSTRACT

Background: Early life environmental exposures may affect bone mass accrual in childhood, but only one study has assessed the role of environmental phenols on child bone health.
Methods: We used data from 223 mother-child dyads enrolled in the Health Outcomes and Measures of the Environment (HOME) Study (Cincinnati, OH; 2003–2006). We quantified benzophenone-3, bisphenol A (BPA), 2,5-dichlorophenol, and triclosan in maternal urine collected at 16- and 26-weeks gestation and calculated the average of creatinine-adjusted concentrations. We performed dual x-ray absorptiometry at age 12 years and calculated Z-scores for whole body (less head), total hip, femoral neck, and 1/3rd distal radius bone mineral content (BMC) and areal bone mineral density (aBMD) as well as ultra-distal radius aBMD and spine BMC and bone mineral apparent density (BMAD). We estimated covariate-adjusted associations per doubling of maternal urinary environmental phenol concentrations in linear regression models. We also examined effect measure modification by child's sex and estimated associations of the environmental phenol mixture with BMC and aBMD using quantile g-computation.
Results: We observed generally null associations for all analytes and bone measures. Yet, in adjusted models, higher urinary 2,5-dichlorophenol concentrations were associated with higher 1/3rd distal radius BMC (β: 0.09; 95% CI: 0.02, 0.17) and aBMD (β: 0.09; 95% CI: 0.02, 0.17) Z-scores in the overall sample. In sex-stratified analyses, the magnitude of the BMC association was positive for females (β: 0.16; 95% CI: 0.06, 0.26) and null for males (β: 0.02; 95% CI: 0.08, 0.13). The environmental phenol mixture was associated with greater 1/3rd distal radius BMC and aBMD Z-scores in both sexes, which was mostly driven by benzophenone-3 in males and 2,5-dichlorophenol in females.
Conclusion: In this prospective cohort study, we observed generally null associations for environmental phenols with BMC and aBMD at age 12 years. While there was a positive association of 2,5-dichlorophenol concentrations during fetal development with distal radius BMC and aBMD at age 12 years, future studies utilizing methods

Abbreviations: BPA, bisphenol A; BMD, Bone mineral density; BMI, Bone Mass Index; CDC, The Centers for Disease Control and Prevention; DAG, Directed acyclic graph; DXA, Dual-energy X-ray absorptiometry; GM, Geometric Mean; HOME, Health Outcomes and Measures of the Environment; NHANES, National Health and Nutrition Examination Survey.

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capable of differentiating cortical and trabecular bone are needed to elucidate potential mechanisms and implications for bone strength and microarchitecture.

1. Introduction

Bone mass accrual during early childhood is critical for lifelong bone health (Weaver et al., 2016). Bone mass accretion begins during fetal growth, increases slowly during childhood, and bone mass accretion velocity peaks during puberty (Bailey et al., 1999; Weaver et al., 2016). Although bone accrual continues after puberty, 95% of adult bone mass is achieved within four years following the peak velocity (Baxter-Jones et al., 2011). Low bone mineral density (BMD) during childhood is linked with lower peak bone mass in young adulthood (Wren et al., 2014), with low peak bone mass being a major factor in the development of bone fractures and osteoporosis in older adults (Bonjour et al., 1994). Osteoporosis, characterized by low BMD (T-score < -2), affects more than 10 million adults over age 50 years (10.3%) in the USA (Wright et al., 2014). Thus, determining whether environmental exposures affect optimal bone accrual in early life has long-lasting implications for reducing risk of fractures and osteoporosis later in life.

Environmental phenols (including bactericides, benzophenones, and bisphenols) are ubiquitous endocrine-disrupting chemicals found in personal care products, plastics, and cleaning supplies (Diamanti-Kandarakis et al., 2009). Although environmental phenols are non-persistent chemicals excreted within 24 h (Völkel et al., 2002; Weatherly and Gosse, 2017), the ubiquity of exposure sources leads to ongoing exposures that may have a profound health effect (Jeon et al., 2008). Environmental phenols have been shown to affect skeletal formation and remodeling (Agas et al., 2013, 2018; Chin et al., 2018; Smith et al., 2017; Thent et al., 2018; Turan, 2021; Vitku et al., 2018). While the exact mechanisms are not known, environmental phenols may disrupt bone metabolism through binding with estrogen receptors α and β, or by inducing inflammation and oxidative stress (Chin et al., 2018; Yang et al., 2009).

Most human epidemiologic studies of environmental phenols and bone health have been cross-sectional and among adult populations, and they have reported inconsistent associations (Cai et al., 2019; Vitku et al., 2018; Wang et al., 2020). One cross-sectional study of postmenopausal women using 2005–2010 National Health and Nutrition Examination Survey (NHANES) data found a positive association of urinary 2,5-dicholorophenol concentrations with greater femoral neck areal BMD (aBMD) as well as an association of urinary triclosan concentrations with lower femoral neck aBMD (Wang et al., 2020). Similarly, Cai et al. (2019) reported an association of higher urinary triclosan concentrations with lower total femur, intertrochanteric region (i.e., where the thigh and hip muscles attach to the femur), and lumbar spine aBMD among women aged $\geq$20 years in 2005–2010 NHANES. Additionally, women in the highest tertile of triclosan concentration had elevated prevalence of osteoporosis compared with women in the lowest tertile (Cai et al., 2019).

The developing fetus may be particularly susceptible to endocrine-disrupting chemicals because many of them, including environmental phenols, cross the placenta (Balakrishnan et al., 2010; Schönfelder et al., 2002). Further, given their abilities to disrupt key hormones involved in ossification (i.e., bone formation) and remodeling/mineralization, such as estrogen and thyroid stimulating hormone (Aker et al., 2019; Delitala et al., 2020; Versonnen et al., 2003; Völkel et al., 2002), fetal exposure to environmental phenols may impact skeletal health in later life. Despite this, we are aware of only one epidemiologic study focused on gestational exposures to environmental phenols and bone health in children (van Zwol-Janssens et al., 2020). Among 1362 offspring enrolled in the Generation R cohort, no associations were observed between maternal urinary bisphenol A (BPA) concentrations in the first, second, or third trimester and children's whole body aBMD at age 6 or 10 years (van Zwol-Janssens et al., 2020). However, no population-based studies of children have assessed relations of other phenolic compounds such as benzophenone-3, triclosan, and 2,5-dichlorophenol with bone health in children, nor has any study examined phenol mixtures, and no studies have evaluated other skeletal sites.

Our goal was to examine associations of maternal urinary concentrations of four phenolic compounds (benzophenone-3, BPA, 2,5-dichlorophenol, triclosan) during pregnancy with bone mineral content (BMC) and aBMD at six skeletal sites among adolescents in the Health Outcomes and Measures of the Environment (HOME) Study. We also evaluated effect measure modification by child's sex and estimated effects of the environmental phenol mixture on bone health outcomes.

2. Material and methods

2.1. Study population

We used data from the HOME Study, a prospective pregnancy and birth cohort study conducted in greater Cincinnati, Ohio from March 2003–January 2006. Detailed information regarding this cohort has been described previously (Braun et al., 2017, 2020). Pregnant women were eligible if they were aged 18 years or older; HIV negative; not receiving seizure, thyroid or chemotherapy/radiation medications; and living in a home built prior to 1978. Of the 1263 eligible participants, 468 were initially enrolled and 398 delivered live-born singleton infants (389 remained enrolled until delivery, 9 dropped out prior to delivery but re-enrolled at a later visit). In this study, we include 223 singleton children with at least one maternal urinary environmental phenol measure during pregnancy and bone outcome measurements at age 12 years.

The institutional review boards (IRBs) of Cincinnati Children's Hospital Medical Center (CCHMC), and the participating hospitals, reviewed the study. The Centers for Disease Control and Prevention (CDC) deferred to CCHMC IRB as the IRB of record. Participants provided written informed consent. At the 12-year follow-up visit, children also provided informed assent.

2.2. Urinary biomarker quantification

Urine samples were collected in polyethylene containers and stored at $-20\circ$C before shipping them overnight on dry ice to the CDC Division of Laboratory Sciences. We quantified concentrations of benzophenone-3, BPA, 2,5-dichlorophenol, and triclosan (hereafter referred to collectively as "environmental phenols") in maternal urine samples collected at 16 weeks gestation (range: 11.3–21.4) and 26 weeks gestation (range: 21.6–34.6) using high-performance liquid chromatography–isotope-dilution tandem mass spectrometry (Ye et al., 2005a). We quantified at least one environmental phenol biomarker for 222 women at the 16-week visit and 207 women at the 26-week visit. One urine sample collected at the 16-week visit did not have a quantifiable creatinine concentration and was excluded from further analyses. Creatinine was measured by an enzyme reaction using a Roche Hitachi 912 chemistry analyzer (Roche Hitachi, Basel, Switzerland). Additional methodological details regarding the quantification of environmental phenols have been published previously (Ye et al., 2005a, b).

2.3. Outcome assessment

During the 12-year follow-up visit, trained research staff measured children's standing height to the nearest 0.1 cm, in triplicate, with an Ayrton Stadiometer Model S100 (Prior Lake, MN). Technicians

performed dual energy x-ray absorptiometry (DXA) scans of the whole-body, lumbar spine, hip, and radius with a Hologic Horizon densitometer (Marlborough, MA). We assessed the long-term calibration stability of our DXA scanner by daily scanning of the Hologic anthropomorphic spine phantom. Scans were analyzed using Apex 5.5 software to estimate BMC (g) and areal BMD (aBMD; g/cm^2) = BMC (g)/projected area (cm^2) for the whole body (less head), total hip, femoral neck, 1/3rd distal radius, ultra-distal radius, and lumbar spine. We excluded the head from whole body measures since the proportion of BMC contained in the skull varies relative to a child's body size in an inconsistent manner (Taylor et al., 1997). Whereas BMC is a measure of bone mass, BMD is a measure of bone density, and is less affected by bone size (Zemel et al., 2010). We also calculated spine bone mineral apparent density (BMAD = BMC/bone area$^{1.5}$) as an estimated measure of spine volumetric BMD (g/cm^3) not affected by height (Kindler et al., 2019). For all skeletal sites, we calculated Z-scores using reference ranges from the Bone Mineral Density in Childhood Study (Kindler et al., 2019, 2020a; Zemel et al., 2011). We calculated height-for-age adjusted age-, sex-, and race-specific whole body (excluding head), total hip, femoral neck, and 1/3rd distal radius BMC and aBMD Z-scores as well as lumbar spine BMC Z-scores. Because DXA provides 2-dimensional scans of bone, it does not account for bone depth, and therefore resulting BMC and BMD Z-scores may be confounded due to short or tall stature. We used height-for-age Z-scores to account for this artifact (Zemel et al., 2010). We also calculated age-, sex-, and race-specific ultra-distal radius aBMD and spine BMAD Z-scores, which were not height-for-age adjusted as these outcomes are weakly correlated with height-for-age (Kindler et al., 2019, 2020b).

2.4. Covariates

We identified and selected covariates for our adjusted regression models based on a directed acyclic graph (DAG) (Supplemental Material Fig. S1). Maternal characteristics included race and ethnicity, mid-pregnancy body mass index (BMI), height, household income level, age at delivery, prenatal vitamin use, average maternal blood lead concentrations during pregnancy, average serum cotinine concentrations during pregnancy, and self-reported frequency of fruit and vegetable consumption during pregnancy ($\geq$4 days per week vs. $<$ 4 days per week). We also adjusted for child's age in months at follow-up and sex assigned at birth. We collected sociodemographic characteristics using standardized, computer-assisted interviews and medical chart reviews by trained research assistants. Lead was measured via inductively coupled plasma mass spectrometry (Jones et al., 2017) in whole blood samples collected at up to three time points (16 weeks gestation, 26 weeks gestation, and delivery) and stored at −80 °C until shipment on dry ice to the CDC for analysis. We adjusted for lead exposure using the average of maternal blood lead concentrations as a measure of gestational lead exposure because the HOME Study was initially designed as a randomized trial of lead and injury hazard controls to reduce children's blood lead levels (Braun et al., 2017).

2.5. Statistical methods

We singly-imputed biomarker concentrations below the limit of detection (LOD) using LOD/ $\sqrt{2}$ (Hornung and Reed, 1990). We calculated Spearman's rank correlations between environmental phenol biomarkers using average biomarker concentrations during pregnancy, and also using concentrations from individual timepoints during pregnancy. After visually examining distributions, we $\log_2$-transformed concentration to reduce the influence of outliers and because of positive-skewness of their distributions. We applied covariate-adjusted creatinine standardization to adjust for the influence of urine dilution on environmental phenol concentrations (O'Brien et al., 2016) as described in detail previously (Kuiper et al., 2022). Briefly, we fitted a linear model of urinary creatinine values (at 16 weeks or 26 weeks) based on maternal age, maternal mid-pregnancy BMI, maternal height, maternal race/ethnicity, household income, hypertension status, and gestational diabetes status; then, we multiplied each biomarker concentration by the ratio of fitted to observed creatinine values, resulting in covariate-adjusted creatinine-standardized biomarker concentrations.

We assessed associations between creatinine-standardized urinary environmental phenol biomarker concentrations with bone outcome Z-scores by constructing linear regression models with and without covariates. Adjusted models included midpoint of reported household income range category (continuous), age at delivery (continuous), prenatal vitamin use (dichotomous: ever, never), average maternal lead concentration (continuous), self-reported frequency of fruit and vegetable consumption during pregnancy (dichotomous: $\geq$4 days per week, $<$4 days per week), self-reported maternal race and/or ethnicity (dichotomous: non-Hispanic White; non-Hispanic Black or any other race or ethnicity), maternal mid-pregnancy BMI (continuous), maternal height (cm; continuous), child's sex assigned at birth (dichotomous: male, female), and child age at follow-up (continuous). Our primary models used the average of available $\log_2$-transformed concentrations from 16- and 26-weeks gestation because repeated measures can reduce exposure measurement error for non-persistent chemicals (Calafat et al., 2015). For this averaged variable, we used a single concentration if only one time point was available ($n = 16$ with only 16-week and $n = 1$ with only 26-week concentrations). As an exploratory analysis, we evaluated potential periods of heightened susceptibility to exposure by estimating associations for each timepoint (16- or 26-weeks of gestation) in separate linear regression models. We used an alpha level of 0.05 for hypothesis testing and full information maximum likelihood to account for missing covariate information.

Environmental phenols, which are endocrine disrupting chemicals, might have sexually-dimorphic relations with bone mineral accrual and strength. Thus, we evaluated sex as an effect measure modifier by stratifying models by child's sex and calculating p-values for the difference in sex-specific estimates via two-sample Z-tests (Buckley et al., 2017). For analyses of effect measure modification, we used an alpha level of 0.1 as indication of effect measure modification by child's sex. While there is no standard alpha threshold for evaluation of effect measure modification, we chose a less conservative value since we were likely underpowered to detect underlying sex-differences in associations.

We used quantile-based g-computation to estimate the overall effect of the environmental phenol mixture and individual contributions of each biomarker in the mixture to the overall effect. This method categorizes the continuous biomarker concentrations into quantiles to estimate the overall effect of increasing all biomarkers by one quantile and defines weights for each biomarker as a measure of their contributions to the overall associations with bone outcomes (Keil et al., 2020). With this model, we estimated 1) differences (95% confidence interval, CI) in outcome Z-score per quartile increase in all environmental phenols (ψ), 2) differences in bone outcome Z-score per quartile increase in environmental phenols with estimated effects in either the positive direction or the negative direction (i.e., directional scaled effects), and 3) weights for the percent contribution of each environmental phenol to the overall mixture effect. We also assessed the potential for effect measure modification of the overall mixture effect by child's sex via inclusion of a product term between ψ and child's sex. For these mixture models, we restricted analysis to participants with all biomarkers quantified (n = 212), and we accounted for missing covariate data using a single stochastic imputation to address potential bias due to missing covariate data. We fit overall models using the R package *qgcomp* and obtained sex-specific estimates using *qgcompint*.

We conducted all single-biomarker analyses using Stata (version 15.1, College Station, TX: StataCorp LP) and all mixtures analyses using R (version 4.0.2, R Core Team, 2020).

3. Results

3.1. Study sample characteristics

Most mothers were between 25 and 35 years of age (60%), more than half were non-Hispanic White (57%), and 59% had an annual household income more than $40,000 at baseline (Table 1). We observed no systematic differences in sociodemographics between the study sample (n = 223) and full sample of mothers with singleton pregnancies (n = 398) (Table 1). The average child age at follow-up was 12.4 (0.7) years (Table 2). Geometric mean maternal urinary concentrations were 23.0 ng/mL for benzophenone-3, 2.1 ng/mL for BPA, 8.0 ng/mL for 2,5-dichlorophenol, and 19 ng/mL for triclosan (Table 3). Spearman's rank correlations between environmental phenols were low for most pairwise combinations, except for 2,5-dichlorophenol and BPA (ρ = 0.42) (Supplemental Material Fig. S2). Correlations between concentrations of the same biomarker measured at 16- and 26-weeks of gestation ranged from 0.27 for BPA to 0.71 for 2,5-dichlorophenol (Supplemental Material Fig. S3).

Table 1
Baseline characteristics of HOME Study participants included in the study sample and full sample. All statistics are n (%) unless otherwise stated.

Variables	Study sample,n = 223[a]	Full sample,n = 398
Maternal age		
<25 years	59 (26)	100 (25)
25–35 years	133 (60)	236 (59)
>35 years	31 (14)	62 (16)
Maternal mid-pregnancy BMI (kg/m^2)		
< 18.5 (underweight)	3 (1)	3 (1)
18.5 to < 25 (normal weight)	85 (41)	153 (41)
25 to < 30 (overweight)	69 (33)	125 (34)
≥ 30 (obese)	51 (25)	88 (24)
Missing	15	29
Maternal height (cm)		
< 152.4 (<5 feet)	7 (3)	15 (4)
152.4 to < 167.6 (≥5 feet <5.5 feet)	133 (64)	230 (62)
167.6 to < 182.9 (≥5.5 feet <6 feet)	64 (31)	120 (33)
≥ 182.9 (≥6 feet)	4 (2)	4 (1)
Missing	15	29
Maternal race/ethnicity		
Non-Hispanic White	127 (57)	241 (61)
Non-Hispanic Black	84 (38)	129 (33)
Other	12 (5)	25 (6)
Missing	0	3
Household income		
< $20,000	57 (26)	88 (22)
$20,000 – < $40,000	35 (16)	67 (17)
$40,000 – < $80,000	77 (35)	140 (35)
≥ $80,000	54 (24)	103 (26)
Parity		
Nulliparous	90 (42)	171 (44)
Primiparous	72 (33)	124 (32)
Multiparous	54 (25)	92 (24)
Missing	7	11
Prenatal vitamin use		
Never used	27 (13)	46 (12)
Ever used	188 (87)	340 (88)
Missing	8	12
Fruit and vegetable consumption during pregnancy		
< 4 days per week	50 (23)	81 (21)
≥ 4 days per week	165 (77)	305 (79)
Missing	8	12
Maternal blood lead concentration during pregnancy (μg/dL)[b]		
< 0.50	39 (18)	59 (15)
≥ 0.50 – < 1.00	154 (69)	283 (71)
≥ 1.00	30 (13)	56 (14)

Abbreviations: BMI = body mass index; cm = centimeter; dL = deciliter; mL = milliliter; ng = nanogram; μg = microgram.
[a] Participants with at least one urinary environmental phenol biomarker quantified during gestation.
[b] Average of up to three timepoints: 16 weeks gestation, 26 weeks gestation, and delivery.

Table 2
Characteristics of HOME Study participants in the study sample at follow-up. Statistics are mean (SD) unless otherwise stated.

Variable	Overall (n = 223)	Males (n = 99)	Females (n = 124)
Age at visit (years)	12.4 (0.70)	12.4 (0.74)	12.4 (0.67)
Pubertal stage[a] (*n*, %)			
1	23 (10)	17 (17)	6 (5)
2	56 (25)	31 (31)	25 (20)
3	65 (29)	26 (26)	39 (32)
4	45 (20)	16 (16)	29 (24)
5	33 (15)	9 (9)	24 (20)
Missing	1	0	1
BMC measures (g)			
Whole body (excluding head)	1228 (325)	1174 (328)	1272 (318)
Total hip	25.2 (6.69)	25.6 (7.62)	24.8 (5.86)
Femoral neck	3.69 (0.78)	3.68 (0.77)	3.70 (0.80)
1/3rd Distal radius	1.45 (0.28)	1.42 (0.27)	1.47 (0.28)
Spine	38.9 (11.8)	35.7 (11.1)	41.5 (11.8)
BMC Z-score[b]			
Whole body (excluding head)	−0.05 (0.82)	−0.16 (0.78)	0.04 (0.85)
Total hip	−0.08 (0.92)	−0.12 (0.94)	−0.06 (0.90)
Femoral neck	0.23 (0.97)	0.13 (0.93)	0.31 (0.99)
1/3rd Distal radius	0.10 (0.98)	−0.02 (0.93)	0.19 (1.01)
Spine	0.09 (0.86)	0.06 (0.87)	0.12 (0.85)
aBMD measures (g/cm^2)			
Whole body (excluding head)	0.826 (0.094)	0.809 (0.092)	0.841 (0.093)
Total hip	0.853 (0.137)	0.838 (0.130)	0.866 (0.142)
Femoral neck	0.798 (0.136)	0.782 (0.122)	0.810 (0.145)
1/3rd Distal radius	0.603 (0.064)	0.592 (0.062)	0.612 (0.063)
Ultra-distal radius	0.350 (0.056)	0.347 (0.047)	0.352 (0.063)
Spine (BMAD, g/cm^3)	0.228 (0.036)	0.209 (0.026)	0.244 (0.036)
aBMD Z-score[b]			
Whole body (excluding head)	−0.38 (0.90)	−0.43 (0.89)	−0.34 (0.92)
Total hip	−0.02 (1.07)	−0.06 (1.10)	0.02 (1.05)
Femoral neck	−0.02 (1.07)	−0.10 (1.10)	0.05 (1.04)
1/3rd Distal radius	0.23 (0.96)	0.22 (0.91)	0.23 (1.00)
Ultra-distal radius[c]	0.28 (1.17)	0.18 (1.00)	0.36 (1.29)
Spine (BMAD)[c]	0.33 (1.02)	0.32 (1.02)	0.34 (1.02)

Abbreviations: cm = centimeter; g = grams; kg = kilograms; aBMD = areal bone mineral density; BMAD = bone mineral apparent density; BMC = bone mineral content.
[a] Tanner stage self-assessment.
[b] Height-for-age adjusted age-, sex-, and population ancestry-specific Z-scores.
[c] Age-, sex-, and population ancestry-specific Z-score.

3.2. Multivariable linear regression

In unadjusted (Supplemental Material Table S1) and adjusted (Supplemental Material Table S3) analyses of the overall study sample, a doubling in average 2,5-dichlorophenol concentration was associated with greater BMC and aBMD Z-scores for most bone sites. Associations were greatest in magnitude for 2,5-dichlorophenol with aBMD (β = 0.09; 95% CI: 0.02, 0.17) and BMC (β = 0.09; 95% CI: 0.02, 0.17) at the 1/3rd distal radius (Fig. 1; Supplemental Material Table S3).

Table 3
Covariate-adjusted, creatinine-standardized[a] maternal gestational urinary environmental phenol concentrations (ng/mL), 2003–2006.

	Visit	Sample size	LOD	N (%) > LOD[b]	GM (GSD)	25th Percentile	50th Percentile	75th Percentile	95th Percentile
Benzophenone-3	16 Weeks	190	0.4	188 (99)	22.4 (6.3)	5.63	16.9	72.5	703
	26 Weeks	197	0.4	196 (99)	23.1 (6.1)	5.90	15.7	76.4	646
	Average[c]	215	NA	NA	23.0 (5.3)	5.95	18.2	70.3	569
BPA	16 Weeks	221	0.4	203 (92)	2.14 (2.7)	1.06	1.91	3.79	15.5
	26 Weeks	207	0.4	189 (91)	1.98 (2.7)	1.01	1.68	3.08	9.13
	Average[c]	223	NA	NA	2.10 (2.2)	1.15	1.92	3.34	7.15
2,5-Dichlorophenol	16 Weeks	217	0.2	216 (99)	9.03 (4.5)	3.12	6.41	22.0	193
	26 Weeks	177	0.2	176 (99)	5.84 (4.7)	2.01	4.16	14.0	135
	Average[c]	219	NA	NA	7.99 (4.2)	2.71	5.71	20.3	136
Triclosan	16 Weeks	218	2.3	206 (95)	21.8 (4.8)	6.26	17.7	57.6	459
	26 Weeks	204	2.3	178 (87)	15.8 (4.7)	5.10	12.5	38.3	447
	Average[c]	222	NA	NA	18.7 (3.8)	6.79	16.9	42.1	237

Abbreviations: BPA = bisphenol A; GM = geometric mean; GSD = geometric standard deviation; LOD = limit of detection; NA = not applicable; ng/mL = nanogram per milliliter.

[a] Raw analyte concentrations were multiplied by the ratio of fitted to observed creatinine concentration. Fitted creatinine was estimated from a linear regression model that included maternal age, maternal mid-pregnancy BMI, maternal height, maternal race/ethnicity, household income, hypertension status, and gestational diabetes status.

[b] Concentrations < LOD replaced by LOD/$\sqrt{2}$.

[c] Average of 16- and 26-week measures or a single measure if only one is available.

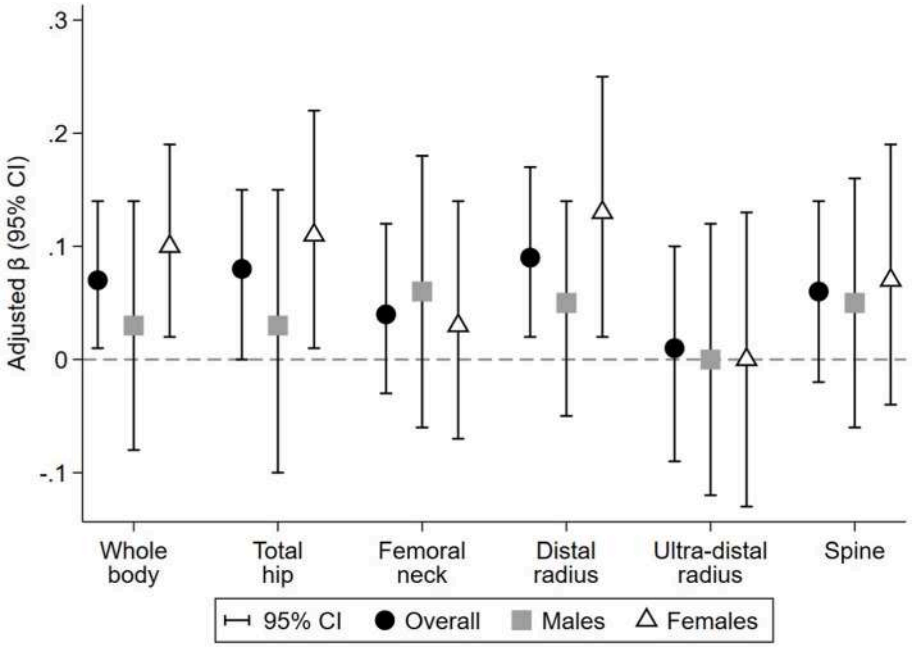

Fig. 1. Adjusted associations of average covariate-adjusted, creatinine-standardized[a] maternal gestational urinary 2,5-dichlorophenol concentration (ng/mL) with BMD Z-scores. Difference (95% confidence interval) in outcome Z-score per doubling in environmental phenol concentration estimated in separate linear regression models adjusted for maternal age at delivery, mid-gestation BMI, height, race/ethnicity, household income, prenatal vitamin use, average blood lead concentration during pregnancy, average serum cotinine concentration during pregnancy, fruit and vegetable consumption during pregnancy, and age at follow-up. Overall models additionally adjusted for child's sex. All outcomes are aBMD Z-scores, except spine which is BMAD Z-score. aBMD, areal bone mineral density; BMAD, bone mineral apparent density. [a] Urinary dilution was accounted for using covariate-adjusted, creatinine-standardization. Raw analyte concentrations were multiplied by the ratio of fitted to observed creatinine concentration. Fitted creatinine was estimated from a linear regression model that included maternal age, maternal mid-pregnancy BMI, maternal height, maternal race/ethnicity, household income, hypertension status, and gestational diabetes status.

Associations with other environmental phenols were generally null, though associations for BPA and triclosan were often positively associated with BMC and aBMD Z-scores but with wide confidence intervals (Supplemental Material Table S3). In our exploratory analysis of periods of susceptibility, all associations were comparable for concentrations measured during 16- and 26-weeks of gestation (Supplemental Material Table S4).

We found limited evidence of effect measure modification by child's sex in unadjusted (Supplemental Material Tables S1 and S2) and adjusted (Supplemental Material Table S5) analyses. Only 2,5-dichlorophenol and triclosan showed evidence of differential associations between sexes, and only at the 1/3rd distal radius and total hip, respectively. A doubling of average 2,5-dichlorophenol concentration was associated with higher 1/3rd distal radius BMC Z-scores among females (β = 0.16; 95% CI: 0.06, 0.26), but not males (β = 0.02, 95% CI = −0.08, 0.13) (Supplemental Material Table S5). Conversely, a doubling in average triclosan concentration was associated with lower total hip aBMD (β = −0.05; 95% CI: 0.13, 0.03) and BMC (β = −0.06; 95% CI: 0.13, 0.02) Z-scores among females, whereas among males triclosan was associated with higher aBMD (β = 0.07; 95% CI: 0.02, 0.16) Z-scores (Supplemental Material Table S5).

3.3. Quantile g-computation

Similar to the single-biomarker models, we observed the strongest association of the environmental phenol mixture with aBMD and BMC at the 1/3rd distal radius. For the overall mixture effect (ψ), a quartile increase in the mixture was associated with a 0.16 (95% CI: 0.04, 0.37) greater aBMD and 0.23 (95% CI: 0.02, 0.44) greater BMC Z-score at the 1/3rd distal radius (Table 4). Associations of the environmental phenol mixture with aBMD and BMC were weaker and not statistically significant for other skeletal sites (Table 4). While associations with BMC and aBMD were generally comparable between males and females, the contributions of individual biomarkers to the overall mixture effect often differed by sex (Supplemental Material Table S6 and Table S7). For example, among males, the overall positive association for the mixture

Table 4
Adjusted associations of the maternal urinary environmental phenol mixture with bone outcome Z-scores at age 12 years estimated using quantile g-computation, overall and by child sex.

Outcome	Overall (n = 212)	Males (n = 93)	Females (n = 119)
BMD Z-score			
Whole-body aBMD	0.11 (−0.06, 0.29)	0.07 (−0.21, 0.35)	0.14 (−0.08, 0.36)
Total hip aBMD	0.07 (−0.13, 0.28)	0.12 (−0.23, 0.47)	0.05 (−0.20, 0.30)
Femoral neck aBMD	0.05 (−0.15, 0.25)	0.09 (−0.23, 0.40)	0.04 (−0.20, 0.28)
1/3rd Distal radius aBMD	0.16 (−0.04, 0.37)	0.21 (−0.11, 0.52)	0.13 (−0.11, 0.37)
Ultra-distal radius aBMD	0.03 (−0.22, 0.25)	0.02 (−0.33, 0.37)	0.06 (−0.23, 0.36)
Spine BMAD	−0.03 (−0.24, 0.18)	−0.13 (−0.42, 0.17)	0.03 (−0.25, 0.31)
BMC Z-score			
Whole-body BMC	0.08 (−0.08, 0.23)	0.09 (−0.16, 0.34)	0.07 (−0.12, 0.26)
Total hip BMC	0.06 (−0.12, 0.24)	0.16 (−0.12, 0.44)	−0.01 (−0.23, 0.22)
Femoral neck BMC	0.12 (−0.07, 0.31)	0.13 (−0.16, 0.42)	0.11 (−0.13, 0.35)
1/3rd Distal radius BMC	0.23 (0.02, 0.44)	0.24 (−0.10, 0.58)	0.22 (−0.04, 0.47)
Spine BMC	0.07 (−0.10, 0.24)	−0.08 (−0.36, 0.20)	0.16 (−0.05, 0.38)

Note: Difference (95% confidence interval) in outcome Z-score per quartile increase in all average covariate-adjusted, creatinine-standardized[a] maternal gestational urinary environmental phenols estimated using quantile g-computation. Adjusted for maternal age at delivery, mid-gestation BMI, height, race/ethnicity, household income, prenatal vitamin use, average blood lead concentration during pregnancy, average serum cotinine concentration during pregnancy, fruit and vegetable consumption during pregnancy, and child's age at follow-up. Overall models were additionally adjusted for child's sex. Missing covariate information accounted for using single stochastic imputation by chained equations. BMC, bone mineral content; aBMD, areal bone mineral density; BMAD, bone mineral apparent density.

[a] Raw analyte concentrations were multiplied by the ratio of fitted to observed creatinine concentration. Fitted creatinine was estimated from a linear regression model that included maternal age, maternal mid-pregnancy BMI, maternal height, maternal race/ethnicity, household income, hypertension status, and gestational diabetes status.

with 1/3rd distal radius aBMD was driven primarily by benzophenone-3 (positive weight = 56.2%); whereas among females, the overall positive mixture effect was driven by 2,5-dichlorophenol (positive weight = 100%) (Supplemental Material Table S6).

4. Discussion

In this prospective cohort, we observed mostly null associations of urinary environmental phenol concentrations during fetal development with BMC and aBMD Z-scores at six skeletal sites at age 12 years. However, we did find that higher concentrations of 2,5-dichlorophenol and the environmental phenol mixture were associated with higher BMC and aBMD Z-scores, particularly at the 1/3rd distal radius and among females. The mixture effect was primarily driven by benzophenone-3 among males and 2,5-dichlorophenol among females. Magnitudes of the mixture associations were as high as 0.24 standard deviation per quartile increase in all environmental phenols. To place this into context, vigorous physical activity (each additional hour per day) was associated with a 0.05 greater whole body (less head) BMC and total hip aBMD Z-score in the Bone Mineral Density in Childhood Study (Mitchell et al., 2016). Additionally, a standard deviation decrease in either BMC or aBMD has been associated with a 30–40% increase in odds of forearm fracture in children (Kalkwarf et al., 2011).

Compared with other studies of gestational environmental phenols exposures during the same general time-period, median uncorrected (i.e., not adjusted for urinary dilution) average pregnancy concentrations in our study were similar for BPA (1.90 ng/mL) and triclosan (15.1 ng/mL) (Berger et al., 2021; Buckley et al., 2018; van Zwol-Janssens et al., 2020; Wolff et al., 2008). The median concentration of 2,5-dichlorophenol (6.15 ng/mL) was comparable to those reported in a French cohort of mother-child dyads (EDEN cohort) (Philippat et al., 2014) but also lower than those reported in the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) study (Berger et al., 2021) and the Children's Environmental Health Study (Wolff et al., 2008). The median concentration of benzophenone-3 (22.4 ng/mL) was both lower (Wolff et al., 2008) and higher (Berger et al., 2021; Philippat et al., 2014) compared to these other studies.

To date, only one epidemiologic study assessed the associations between fetal exposure to environmental phenols and child bone health (van Zwol-Janssens et al., 2020). Among 1362 participants of the Generation R Study, maternal urinary BPA concentrations in pregnant women were not associated with their children's BMD or area-adjusted BMC at age 6 and 10 years, similar to null findings observed in our study. Importantly, van Zwol-Janssens et al. only examined exposure to bisphenols, and no previous studies have explored associations of maternal urinary 2,5-dichlorophenol, triclosan, and benzophenone-3 concentrations or their mixture with child bone health.

2,5-dichlorophenol has been used as an intermediate in dye production, pharmaceuticals, and agricultural products (Ye et al., 2005b) and is also a metabolite of paradichlorobenzene (also known as 1, 4-dichlorobenzene), a disinfectant chemical used in products such as mothballs and deodorizing agents and with demonstrated clastogenic activity *in vitro* (Mohtashamipur et al., 1987). In our study, we observed a positive association between 2,5-dichlorophenol and 1/3rd distal radius bone mineral content and density among females, but not males, at age 12 years. We are not aware of any other studies evaluating associations with gestational urinary 2,5-dichlorophenol concentrations. This sex-difference might be due to sexually-dimorphic endocrine-disrupting actions of 2,5-dichlorophenol (Brouwer et al., 1999; Völkel et al., 2002), potentially by affecting pubertal timing and development (Bigambo et al., 2020). A previous systematic review and meta-analysis of four environmental phenols in 4737 children from nine studies, showed that 2,5-dichlorophenol was the only chemical associated with the risk of early puberty (OR: 1.13; 95% CI: 1.06, 1.20) (Bigambo et al., 2020). Relatedly, three prior studies have found that greater urinary childhood 2,5-dichlorophenol concentrations were associated with earlier menarche among girls (Binder et al., 2018; Buttke Danielle et al., 2012; Wolff et al., 2017). Mechanisms of 2, 5-dichlorophenol may also be independent of puberty; 2,5-dichlorophenol exhibits estrogenic activity *in vitro* (Versonnen et al., 2003), and estrogen regulates bone formation and inhibit bone resorption (Khosla et al., 2012). Moreover, short-term exposure to estrogen during fetal development is positively associated with peak bone mass in animal models (Migliaccio et al., 1996, 2000).

Experimental studies have shown that gestational exposures to BPA may adversely affect bone development (Huang et al., 2021; Hwang et al., 2013; Kanno et al., 2004; Lejonklou et al., 2016; Lind et al., 2019; Pelch et al., 2012; Toda et al., 2002). However, our study and van Zwol-Janssens et al. (2020) did not find evidence to support an association. Similarly, four prior epidemiologic studies have investigated associations of BPA exposure on BMD among adult populations, with mostly null findings (Gu et al., 2022; Kim et al., 2012; Wang et al., 2020; Zhao et al., 2012).

In a study investigating the effect of triclosan on sea urchin embryos, exposure to triclosan (1 μM) decreased the expression of several genes related to skeletal development, including bone morphogenic proteins, a group of growth factors that are critical to chondrogenesis and osteogenesis (Hwang et al., 2017). In our study, though associations were imprecise, we observed sex differences in associations of triclosan with

total hip bone mineral content and density. Specifically, higher triclosan concentrations were associated with lower total hip aBMD and BMC Z-scores among females but lower Z-scores among males. We are not aware of other epidemiologic studies evaluating gestational exposures to triclosan with offspring skeletal development, though two cross-sectional studies of adults observed that greater triclosan exposure was associated with lower BMD in the total femur, intertrochanteric region of the femur, femoral neck, and lumbar spine as well as greater odds of osteoporosis, among females (Cai et al., 2019; Wang et al., 2020).

As with 2,5-dichlorophenol and triclosan, no studies have evaluated the impact of gestational exposure to benzophenone-3, an ultraviolet filter used in sunscreen and other products, on offspring bone outcomes. We are aware of only one experimental study of benzophenone-3 and bone relevant outcomes, an *in vitro* study that evaluated the effects of benzophenone-3 exposure (0.1 nM–10 μM) on rat calvarial osteoblast-like cells (Ziolkowska et al., 2006). This study found significantly reduced proliferative activity among cells treated with the highest dose of benzophenone-3; though, this was suspected to be from cytotoxicity independent from endocrine-disrupting mechanisms (Ziolkowska et al., 2006).

Since fractures in children and adolescents most frequently occur at the forearm (containing two long bones, the ulna and the radius), our findings suggests that fetal exposure to certain environmental phenols could affect fracture risk (Jones et al., 2002; Kalkwarf et al., 2011; Korup et al., 2022). Still, greater bone content and density does not necessarily equate to greater bone strength. Additionally, any deviations from normal-for-age bone development may be considered as potentially adverse for health.

Lack of consistent associations across skeletal sites in our study may reflect differences in microarchitecture, composition of bone (i.e., dense cortical vs. spongy trabecular bone), and distribution of mineral within the bone at these sites. As such, environmental chemical exposures may not necessarily be expected to exert effects equally on all skeletal sites. In our study, 2,5-dichlorophenol was almost universally associated with higher bone mineral content and density at all skeletal sites. This suggests that perhaps its parent compound, paradichlorobenzene, is able to affect both cortical and trabecular bone. However, DXA is not able to differentiate bone composition and would instead require the use of peripheral quantitative computed tomography. Taken together, we found little evidence that gestational exposure to environmental phenols affected skeletal development in offspring.

This study contributes to the few studies of environmental phenols and bone health in early adolescence. Our study has several strengths, including the measurement of BMC and BMD at multiple skeletal sites, adjustment for many potential confounders, and consideration of chemical mixtures. We quantified phenols concentrations in two spot urine samples (16 weeks and 26 weeks gestation). Environmental phenols have short biological half-lives (e.g., <24 h) (Völkel et al., 2002; Weatherly and Gosse, 2017) and reliance on only two urinary measurements during pregnancy may result in bias from exposure misclassification (Perrier et al., 2016). However, using the average of available concentrations during pregnancy may better approximate of usual exposure to these compounds (or their parent compounds) during gestation. Additionally, while our study focused on frequently detected environmental phenols (or their metabolites), other compounds may impact bone development. Future studies should consider a wider array of phenolic compounds, especially increasingly utilized replacements for legacy compounds, e.g., bisphenol F and bisphenol S (Lehmler et al., 2018; van Zwol-Janssens et al., 2020). Our relatively modest sample size of 223 mother-and-child pairs limited our statistical power, especially for sex-specific associations. Additionally, we conducted multiple hypothesis tests given the array of exposures and outcomes evaluated. As such, our findings could be due to chance, though we have focused on patterns of associations, broadly, as opposed to individual statistical tests. While there was loss to follow-up in our cohort, baseline characteristics of sample participants were similar to the full cohort. Lastly, childhood exposures and other postnatal factors may also be important for early adolescent bone health. Future studies should assess exposures occurring across childhood and possible modification by postnatal factors, e.g., physical activity, diet, and breastfeeding.

5. Conclusions

In this prospective study, gestational environmental phenol exposures were not consistently associated with measures of early adolescent bone health. 1,4-paradichlorobenzene (the precursor of 2,5-dichlorophenol) and the environmental phenol mixture were associated with greater distal radius bone mineral content and density at age 12 years, especially among females. Yet, further studies on exposures and bone outcomes, particularly those utilizing methods capable of differentiating cortical and trabecular bone, are needed to confirm these findings and further determine effects of 1,4-paradichlorobenzene on cortical and trabecular bone strength and microarchitecture.

Declaration of competing financial interests

Dr. Braun and his institution were financially compensated for his services as an expert witness for plaintiffs in litigation related to PFAS-contaminated drinking water. Dr. Lanphear served as an expert witness in cases related to childhood lead poisoning, but he was not personally compensated. The other authors declare they have no actual or potential competing financial interests.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention. Use of trade names is for identification only and does not imply endorsement by the CDC, the Public Health Service, or the US Department of Health and Human Services.

Data sharing

Data from the HOME Study is available upon request. The HOME Study principal investigators have actively engaged in collaborative data-sharing projects. We welcome new collaborations with other investigators. Investigators interested in HOME Study data can explore options at the following location: https://homestudy.research.cchmc.org/and use the available link to contact the investigators to discuss collaborative opportunities. The Data Sharing Committee meets regularly to review proposed research projects and ensure they do not overlap with extant projects and are an efficient use of scarce resources (e.g., biological samples). Funds to support staff efforts in the assembly of data sets are required.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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Associations of metal mixtures in the meconium with birth outcomes in northern Taiwan

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ABSTRACT

Previous studies investigated prenatal exposure to neurotoxic metals in relation to birth anthropometrics. However, limited information has been developed on associations with birth outcomes of fetal exposure to metal mixtures using the meconium as a biomarker. The purpose of this study was to evaluate relationships of the combined effects of mercury (Hg), lead (Pb), cadmium (Cd), and arsenic (As) concentrations in the meconium on birth outcomes (i.e., birth weight, birth length, and head circumference). This cross-sectional study was conducted in northern Taiwan between January 2007 and December 2009. We collected 526 meconium samples within the first 24 h after birth to measure the in utero mixed-metal exposure determined using inductively coupled plasma/mass spectrometry (ICP-MS). We used a multivariable regression and Bayesian kernel machine regression (BKMR) to estimate associations of the combined effects and identify important mixture components with growth impairments. Our results revealed Hg, Pb, Cd, and As concentrations in the meconium and enhanced the quantity of research on meconium analyses. The overall effects of Hg, Pb, Cd, and As concentrations in the meconium as prenatal exposure biomarkers were negatively associated with birth growth. Fetal exposure to Hg and Pb was correlated with decreased birth weights. Hg and Pb concentrations in the meconium were linearly inversely related to the birth weight, birth length, and head circumference. Effects of fetal exposure to As and Cd on birth outcomes were not obvious. A significant increasing relationship was detected between Hg concentrations in the meconium and maternal fish consumption during pregnancy. Higher Pb concentrations in the meconium were observed among infants of mothers who consumed Chinese herbal medicines. Reducing maternal fish consumption and Chinese herbal medicine consumption during pregnancy could limit infant exposure to metals.

1. Introduction

Prenatal exposure to toxic environmental metals can interfere with fetal growth and development. Fetal exposure to lead (Pb) can increase the risks of a preterm delivery, a low birth weight, and mental developmental impairment (Falcón et al., 2003). A longitudinal study indicated that Pb concentrations in the meconium were negatively associated with the birth length (Gundacker et al., 2010). Prenatal Pb exposure was associated with maternal age and the consumption of nutritional supplements (Al-Saleh et al., 2008). A birth-cohort study suggested that mercury (Hg) concentrations in cord blood were directly related to a poor birth weight, and indicated a direct path from fish consumption to cord blood Hg (Kim et al., 2017). Low-level erythrocyte cadmium (Cd) and Hg concentrations were inversely correlated with worse birth anthropometrics in a prospective birth-cohort in Sweden (Gustin et al., 2020). Low-level maternal arsenic (As) exposure during pregnancy appeared to decrease the birth weight and head and chest circumferences as determined from an analysis of 1578 mother-infant pairs recruited in Bangladesh (Rahman et al., 2009). However, few studies have evaluated the joint effects of fetal exposure to metal mixtures on birth outcomes. A birth cohort study conducted in Bangladesh indicated that mixed metal levels in cord blood were associated with reduced newborn anthropometrics (Lee et al., 2021). A significant negative relationship of a mixture of chromium, copper, molybdenum,

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and zinc in the meconium with the birth weight was shown in a cross-sectional study (Pavilonis et al., 2022).

The meconium has been applied in several studies as a biomarker of fetal exposure to heavy metals (McDermott et al., 2020; Ostrea et al., 2006; Pavilonis et al., 2022). The meconium is an accumulated material mixture formed by the fetus during the second and third trimesters of pregnancy and excreted by the newborn within the first 24–48 h (Michelsen et al., 2021; Ortega et al., 2006). Measurement of prenatal exposure using the meconium compared to maternal blood and urine or cord blood was more useful for assessing long-term fetal exposure to environmental toxicants in utero due to the meconium representing the accumulation of materials over approximately 6 months (Barr et al., 2007; Michelsen-Correa et al., 2021). Previous studies used a meconium analysis to evaluate fetal exposure to metals. A survey conducted in a community with high Hg contamination revealed a higher prevalence of Hg in the meconium compared to maternal fluid compartments such as blood and breast milk. Their results also showed that Hg concentrations in the meconium were positively correlated with those in maternal blood and in cord blood (Ramirez et al., 2000). An analysis of the meconium was effectively applied to measure fetal exposure to metals in a mining-exposed environment (McDermott et al., 2020) and in an urban area (Pavilonis et al., 2022).

The purpose of this study was to establish distributions of Hg, Pb, Cd, and As concentrations in the meconium among a large population living in urban areas of northern Taiwan and evaluate potential related factors. The combined effects of fetal exposure to a metal mixture on birth outcomes (i.e., birth weight, birth length, and head circumference) were evaluated. The importance of metal exposure variables on birth outcomes was also assessed.

2. Materials and methods

2.1. Study population

In total, 526 mother-infant pairs living in northern Taiwan were recruited between January 2007 and December 2009. Mothers were enrolled in the third trimester (after 24 weeks) at an outpatient clinic, and they delivered their babies at either Taipei Mackay Memorial Hospital or at its Hsinchu branch, both in northern Taiwan. All of the subjects had single live births with uncomplicated pregnancies and deliveries. Uncomplicated pregnancies and deliveries refer to a composite normotensive pregnancy defined as the absence of pregnancy complications (e.g., multiple pregnancies, gestational diabetes, preeclampsia, stillbirth, preterm birth, and other complications) (Chappell et al., 2013; Relph et al., 2021). After obtaining informed consent, the birth outcomes of their babies and the sociodemographic characteristics of the mothers were collected using self-reported questionnaires. Meconium samples were collected from the first bowel movement of newborns within the first 24 h after birth and obtained along with the questionnaires. This work was approved by the Institutional Review Board of Taipei Medical University (P940030) and Taipei Mackay Memorial Hospital (MMH-I-S-596). Written informed consent was prepared for both participating mothers and infants.

2.2. As, Cd, Hg, and Pb levels in the meconium

Meconium samples were collected within the first 24 h after the birth of the child and placed in a freezer at −20 °C before analysis. We lyophilized the meconium sample and then approximately 30 mg of a lyophilized sample was subsequently supplemented with activated alumina, sodium carbonate, and calcium hydroxide. The Hg concentration in the meconium was analyzed using Hg cold-vapor atomic absorption spectroscopy (Nippon Instruments Mercury/MA-2000SC). Approximately 0.5 g of a lyophilized meconium sample was added to 5 mL of 69% nitric acid and wet-digested at 100 °C for 2 h. Pb, Cd, and As concentrations in the meconium were determined using inductively coupled plasma/mass spectrometry (ICP-MS; Thermo X-series II). A certificated reference material for trace elements, 1566 b Oyster Tissue, was used to confirm the precision and accuracy of the meconium analyses. The precision and accuracy of measurement were 91.95–96.7% and 97.9%–99.8%, respectively. Detection limits (calculated as three times the standard deviation (SD) of the blank concentration) of Hg, Pb, Cd, and As in the meconium were 0.23, 0.08, 0.23, and 0.12 ng/g, respectively.

2.3. Measurement of birth outcomes

Fetal birth size indicators were measured at the time of delivery by well-trained field staff at Taipei Mackay Memorial Hospital or its Hsinchu branch. The birth length was measured to the nearest 0.1 cm using an infantometer with the infant's knees fully extended and soles of the infant's feet resting against the bottom of the measuring device. Birth weight was obtained to the nearest 10 g using a digital scale. Head circumference was recorded with a standard measuring tape to an accuracy of 0.1 cm. Birth outcome measures were transformed to z-scores which were standardized to the mean and SD of the study infants for each week of gestational age. Therefore, associations of metal exposures and birth outcome z-scores were assessed using regression models without adjusting for gestational age.

2.4. Covariates

Neonatal demographic characteristic data on gender and gestational age as well as maternal sociodemographic characteristics data on educational level, age at delivery, reproductive history, and smoking habit, amalgam fillings, Chinese herbal medicine consumption, and fish consumption during the pregnancy were collected by a structured questionnaire.

2.5. Statistical analyses

Continuous variables are presented as the mean ± SD, and categorical variables are presented as counts and percentages. Metal concentrations in meconium samples were described in the form of the geometric mean (GM) ± geometric SD (GSD) or median and interquartile range (IQR). Metal concentrations in meconium samples were logarithm-transformed to reduce their skewness for the correlation and regression analyses. Bivariate associations of metal exposures with birth outcome indicators were analyzed with Pearson correlation coefficients. Associations between covariates and prenatal metal exposures were assessed using Pearson and Spearman correlation coefficients.

A multivariable regression was applied to evaluate associations between fetal exposure to metal mixtures and birth outcome indicators. Selection of covariates as a priori variables were referenced from previous studies and plausible correlations with birth outcomes (Lee et al., 2021; Santri et al., 2021). Covariates with $p < 0.2$ in the univariate regression analysis were further included in the multivariable model analysis. The final multivariable models were adjusted for covariates including maternal body-mass index (BMI) at delivery, neonatal gender, and maternal smoking during pregnancy. We assessed effects of single-metal concentrations in the meconium on birth outcomes in the single-metal model adjusted for covariates (model 1). Also, the mixed metal model adjusted for covariates was used to evaluate effects of mixed metal concentrations in the meconium on birth outcomes (model 2). The variance inflation factor (VIF) was used to evaluate the multicollinearity of the linear regression models.

A Bayesian kernel machine regression (BKMR) was performed to evaluate the joint effects of prenatal mixed metal exposure on birth outcome indicators using the "bkmr" R package (Bobb et al., 2014, 2018). The BKMR uses a Gaussian kernel function to estimate nonlinear and non-additive exposure-response relationships, and capture interactions among mixture components. The BKMR model was applied as

$Y_i = h(Hg_M, Pb_M, Cd_M, As_M) + \beta^T Z_i + e_i$, where Y refers to birth outcome indicators (birth weight, birth length, or head circumference), h() is an exposure-response function that accommodates nonlinearity and interactions among components of the metal mixture, Hg_M, Pb_M, Cd_M, and As_M are metal concentrations in the meconium, and Z_i and β^T are covariates and their coefficients, respectively. We implemented a component-wise variable selection process to obtain posterior inclusion probabilities (PIPs), which can measure the importance of individual exposure variables to birth outcomes. Exposure variables with a PIP of >0.5 are usually considered of significant importance (Bobb et al., 2018). Assessments of the joint effects and interactions of metal exposures were fit using a Markov chain Monte Carlo (MCMC) algorithm with 50,000 iterations. All statistical analyses were conducted using SAS (vers. 9.4, SAS Institute, Cary, NC, USA). The BKMR analysis was performed using R software (vers. 4.1.2; R Foundation for Statistical Computing).

3. Results

3.1. Demographic characteristics

Demographic characteristics of the maternal-child pairs are shown in Table 1. The average age of mothers at delivery was 30.7 ± 4.6 years, and the mean BMI at delivery was 26.7 ± 3.6 kg/m^2. This birth was the first fetal parity of 58% of mothers, and 65.9% had a college degree or above. Very few mothers had smoked (n = 5) or had amalgam fillings during the pregnancy (n = 15); 4.8% reported Chinese herbal medicine consumption during the pregnancy; and 76.8% of the mothers had consumed fewer than two meals/week of fish during the pregnancy. Of the infants, the average gestational age was 38.7 ± 1.4 weeks, and 51.1% were boys. The average birth outcomes of newborn babies were 3151 ± 445 g for the birth weight, 49.7 ± 3.5 cm for the birth length, and 33.4 ± 1.4 cm for the birth head circumference.

Table 1
Demographic characteristics of the maternal-children pairs (N = 526).

Characteristic	Mean ± SD or n (%)
Maternal characteristics	
Age at delivery (years)	30.7 ± 4.6
BMI at delivery (kg/m^2)	26.7 ± 3.6
Fetal parity	
1	304 (58.0)
≥2	222 (42.0)
Education	
High school or lower	174 (34.1)
College or above	352 (65.9)
Smoking during pregnancy	
Yes	5 (1.0)
No	521 (99.0)
Amalgam fillings during pregnancy	
Yes	15 (2.9)
No	511 (97.1)
Chinese herbal medicine consumption during pregnancy	
Yes	25 (4.8)
No	501 (95.2)
Fish consumption during pregnancy	
<2 meal/week	404 (76.8)
≥2 meal/week	122 (23.2)
Monthly family income (USD) (n = 296)	
≤1033	33 (11.2)
1033–2414	105 (35.5)
≥2414	158 (53.3)
Neonatal characteristics	
Gestation (weeks)	38.7 ± 1.4
Males	267 (51.1)
Birth weight (g)	3151.7 ± 445.5
Birth length (cm)	49.7 ± 3.5
Head circumference (cm)	33.4 ± 1.4
Z-scores for birth weight	0.02 ± 0.04
Z-scores for birth length	−0.04 ± 0.004
Z-scores for head circumference	0.09 ± 0.04

Abbreviations: SD, standard deviation; BMI, body-mass index.

3.2. Fetal exposure to mixed metals

Distributions of fetal exposures to Hg, Pb, Cd, and As concentrations in the meconium are shown in Table 2. The GM ± GSD of Hg, Pb, Cd, and As levels were 76.98 ± 1.84, 18.48 ± 2.73, 8.56 ± 4.00, and 33.80 ± 2.11 ng/g dry weight (d.w.), respectively. A correlation matrix for Hg, Pb, Cd, and As concentrations in the meconium is shown in Fig. S1. There was a significant moderate correlation between As and Pb concentrations in the meconium (r = 0.51, p < 0.0001). Hg concentrations in the meconium were significantly positively associated with As concentrations in the meconium (r = 0.16, p = 0.007) and maternal fish consumption during pregnancy (r = 0.12, p = 0.02). Infants of mothers who consumed Chinese herbal medicines had higher Pb concentrations in the meconium (with a median value of 26.3 ng/g) compared to infants whose mothers had not consumed Chinese herbal medicines (with a median value of 19.5 ng/g), but the association was not statistically significant (Fig. S2).

3.3. Multivariable regression analyses

A multivariable analysis was used to evaluate associations of fetal exposure to a single-metal and to mixed metals with birth outcomes (Table 3). There were two models of individual metal exposure including the single-metal model inclusion of one metal exposure which was adjusted for covariates and the mixed metal model inclusion of all other metal exposure which was adjusted for covariates. In the single-metal model, there were no associations between each metal exposure and birth outcomes. In the mixed-metal model with additional adjustments for the maternal BMI at delivery, neonatal gender, and maternal smoking during the pregnancy, an increase in the Hg concentration of 10 ng/g in the meconium was associated with a 0.47-g decline in the birth weight (β = −0.47, 95% confidence interval (CI): 0.87 to −0.06). An increase in log Pb concentrations in the meconium was associated with a 0.34-g decrease in the birth weight (β = −0.34, 95% CI: 0.61 to −0.06). No significant correlations were estimated for mixed-metal exposure with the birth length or head circumference.

3.4. BKMR analyses

The joint effects of fetal exposure to the metal mixture on birth outcomes estimated by the BKMR model are shown in Fig. 1. We found negative associations of the metal mixture with the birth weight, birth length, and head circumference, with an especially stronger association of the metal mixture with birth weight (Fig. 1A). The potential nonlinearity of the single-metal exposure-response functions when all the other mixed metals were fixed at their median concentrations is shown in Fig. 2. The curves indicated that Hg and Pb concentrations in the meconium were linearly inversely associated with the birth weight,

Table 2
Metal concentrations in the meconium.

Metal (ng/g dry weight)	GM ± GSD	Mean ± SD	Median	IQR
Mercury (Hg)	76.98 ± 1.84	92.64 ± 71.28	82.06	60.70–104.82
Lead (Pb)	18.48 ± 2.73	27.46 ± 27.72	19.50	11.26–35.26
Cadmium (Cd)	8.56 ± 4.00	26.21 ± 54.30	7.13	3.71–14.71
Arsenic (As)	33.80 ± 2.11	45.94 ± 59.90	36.72	23.52–51.99

Abbreviations: GM, geometric mean; GSD, geometric standard deviation; IQR, interquartile range.

Table 3
Multivariable regression models analyzing associations between prenatal metal exposure and birth outcomes.

Metal (log transformed)	Birth size z-scores					
	Birth weight		Birth length		Head circumference	
	β (95% CI)	*p*	β (95% CI)	*p*	β (95% CI)	*p*
Hg						
Model 1	−0.12 (−0.48, 0.24)	0.49	−0.005 (−0.04, 0.03)	0.78	−0.31 (−0.66, 0.05)	0.09
Model 2	**−0.47 (-0.87, -0.06)**	**0.02**	−0.02 (−0.06, 0.02)	0.25	−0.25 (−0.66, 0.16)	0.23
Pb						
Model 1	−0.15 (−0.39, 0.08)	0.21	−0.008 (−0.03, 0.01)	0.49	−0.18 (−0.41, 0.06)	0.15
Model 2	**−0.34 (-0.61, -0.06)**	**0.02**	−0.01 (−0.04, 0.01)	0.39	−0.24 (−0.52, 0.04)	0.09
Cd						
Model 1	0.02 (−0.15, 0.19)	0.83	−0.003 (−0.02, 0.01)	0.73	0.05 (−0.12, 0.22)	0.54
Model 2	0.04 (−0.14, 0.22)	0.64	−0.002 (−0.02, 0.02)	0.86	0.11 (−0.08, 0.29)	0.25
As						
Model 1	0.18 (−0.14, 0.49)	0.28	0.002 (−0.03, 0.03)	0.92	−0.06 (−0.38, 0.26)	0.69
Model 2	0.39 (0.01, 0.77)	0.05	0.01 (−0.02, 0.05)	0.51	0.05 (−0.34, 0.44)	0.78

Abbreviations: CI, confidence interval; BMI, body-mass index.
Model 1: the single-metal model included one metal concentration (log-transformed) in the meconium and was adjusted for maternal BMI at delivery, neonatal gender, and maternal smoking during the pregnancy.
Model 2: the mixed-metal model included model 1 additionally adjusted for all other (log-transformed) metal concentrations.

birth length, and head circumference. The potential nonlinearity of As and Cd exposure-response functions was not obvious. PIPs of the BKMR model revealed that Hg concentrations in the meconium made important contributions to the birth weight, and Pb concentrations in the meconium made important contributions to the birth weight, birth length, and head circumference due to their PIPs being >0.5 (Table S1). Fig. S3 shows differences in birth outcomes for single-metal exposure at the 75th percentile compared to the 25th percentile, when all remaining metals were fixed at their 25th, 50th, or 75th percentiles. Hg and Pb concentrations in the meconium were negatively associated with birth outcomes. The bivariate exposure-response functions for every two-metal exposure on birth outcomes are displayed in Fig. S4. Similar slopes of the curves indicated that no potential interactions between prenatal As, Cd, Hg, and Pb exposure existed.

4. Discussion

To our best knowledge, this is the first study to evaluate associations of fetal exposure to mixed metals using the meconium as a prenatal exposure biomarker with birth outcomes (i.e., birth weight, birth length, and head circumference). We showed the combined effects of Hg, Pb, Cd, and As concentrations in the meconium on worsening birth outcomes using a BKMR analysis. Fetal exposure to Hg and Pb were negatively correlated with birth weights. Inverse linear relationships of Hg and Pb concentrations in the meconium were detected with birth weight, birth length, and head circumference.

The median Hg concentration in the meconium in our study was 82.06 ng/g d.w., which was higher than previous results reported in Split and Dalmatian County in Croatia (35.7 ng/g d.w.; Knezović et al., 2016) and in China (34.4 ng/g d.w.; Peng et al., 2015). Due to higher fish consumption by Taiwanese (Kao et al., 2022), elevated Hg levels in the meconium were found in our study. Moreover, we found that maternal fish consumption during pregnancy was significantly associated with Hg concentrations in the meconium ($r = 0.12$, $p = 0.02$), which was consistent with previous observations (Knezović et al., 2016; Trdin et al., 2019). Fish consumption by pregnant women may contribute to Hg exposure by their infants. The higher Hg concentration in the meconium in our study compared to previous results reported from Slovenia (11.1 ng/g wet weight; Trdin et al., 2019) were due to the method of drying meconium samples prior to analyses in our study.

Our analysis showed that the median Pb concentration in the meconium was 19.50 ng/g d.w., which was comparable to those found in the USA (22.2 ng/g d.w.; Pavilonis et al., 2022), in Turkey (14.9 ng/g wet weight; Turker et al., 2013), and in Austria (15.5 ng/g wet weight; Gundacker et al., 2010), whereas our result was much lower than those in other studies conducted in a highly contaminated environment or an industrial city (Ostrea et al., 2002; Turker et al., 2006). Higher Pb concentrations in the meconium (a median value of 26.3 ng/g d.w.) were found among infants of mothers who consumed Chinese herbal medicines compared to those mothers who did not consume Chinese herbal medicines (a median value of 19.5 ng/g d.w.) in our analysis. However, the numbers of mothers who consumed Chinese herbal medicines was too small to observe statistical significance. A previous study in Taiwan indicated significant effects of traditional Chinese herbal consumption among mothers on their infants' exposure to Pb (Chien et al., 2006).

Cd and As concentrations in the meconium in our results were comparable to those reported in China (Peng et al., 2015), but much lower than those reported in highly contaminated environments in the Philippines (Ostrea et al., 2002) and in Turkey (Turker et al., 2006). The method of freeze-drying the meconium samples prior to analyses was applied in our study, whereas a method of without freeze-drying samples prior to analyses was used in the previous survey (McDermott et al., 2020). Therefore, fetal exposure to heavy metals in our study in an urban area were lower than the results conducted in a mining community in the USA (McDermott et al., 2020). It was revealed that the contribution of mining contamination to fetal metal exposure was greater than that by metropolitan pollution. Other factors such as lifestyle (Hinwood et al., 2013; White et al., 2018) and dietary habits (Mantha et al., 2017; Wei et al., 2019) may influence As and Cd exposures of mothers and their babies. Infants of mothers who consumed Chinese herbal medicines had a median As concentration in the meconium of 43.3 ng/g, which was higher than those of infants whose mothers did not consume Chinese herbal medicines (with a median value of 36.7 ng/g) in our results. However, the numbers of participants who consumed Chinese herbal medicines was too small to obtain statistical significance. Ortega et al., (2006) revealed that an increasing concentration of toxicants from the meconium collected in the first 10 h, between 11 and 20 h, and afterwards until 48 h of age. In our study, meconium samples were collected at the time of the first bowel movement of a newborn within the first 24 h after birth to reduce the effect of the sampling time on concentrations.

Few studies have analyzed the joint effects of metal mixtures in the meconium on birth outcomes. We applied a BKMR model to evaluate associations of metal mixtures with birth size, and found combined effects of Hg, Pb, Cd, and As concentrations in the meconium on worsening birth outcomes, which was consistent with results of a birth cohort study in Bangladesh which indicated that metal mixtures in cord blood were associated with reduced newborn anthropometrics (Lee et al., 2021). Our analysis also revealed that fetal exposure to Hg and Pb was negatively correlated with the birth weight, which was similar to results of a cross-sectional study conducted on 113 full-term newborns in New York City, which indicated an inverse correlation between Pb concentrations in the meconium and birth weights (Pavilonis et al., 2022), and

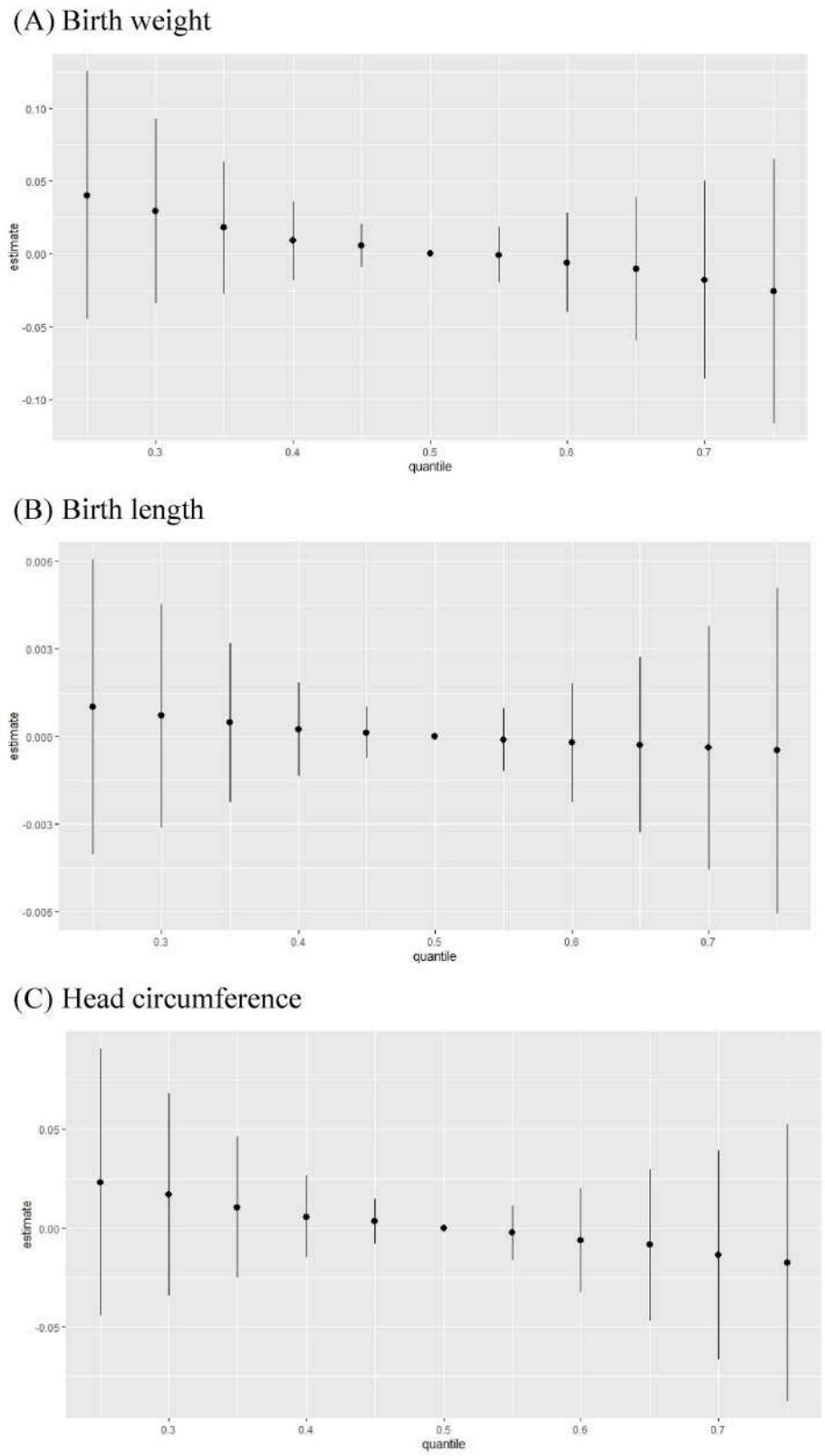

Fig. 1. Joint effects (estimates and 95% confidence intervals) of prenatal mixed metal exposure on birth outcomes as estimated by the Bayesian kernel machine regression (BKMR) adjusted for maternal body-mass index (BMI) at the start of the pregnancy, neonatal gender, and maternal smoking during the pregnancy. (A) Birth weight; (B) birth length; and (C) head circumference.

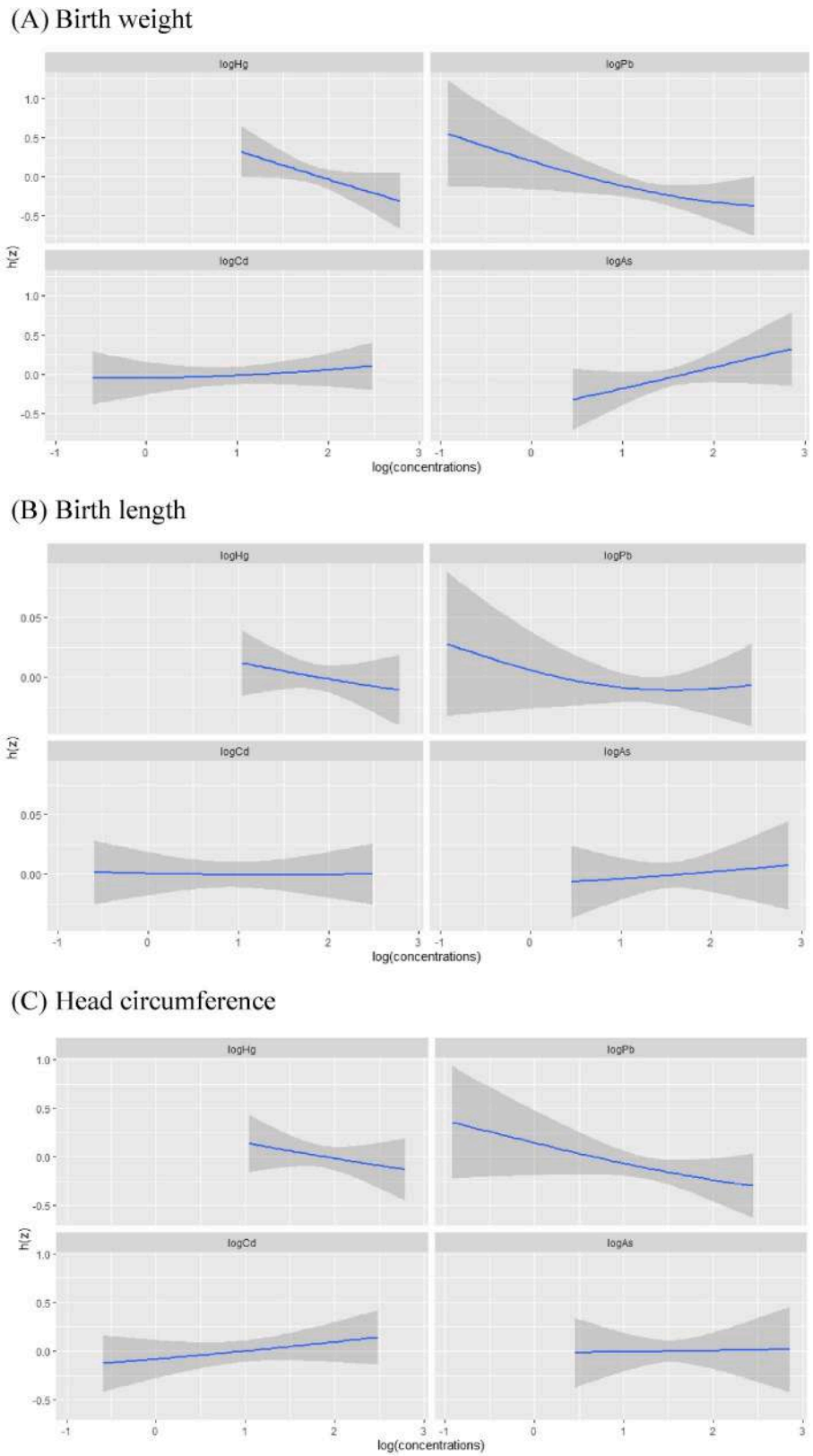

Fig. 2. Univariate exposure-response functions (95% confidence intervals) of relationships between outcomes and each metal exposure when all other metals were fixed at their median concentrations. (A) Birth weight; (B) birth length; (C) and head circumference.

to results of a birth cohort study showing a relationship between cord blood Hg concentrations and lower birth weights (Kim et al., 2017). Using the meconium and maternal blood as biomarkers, a low level of prenatal Pb exposure was found to have led to lower birth weights in an Austrian study (Gundacker et al., 2010). A Swedish prospective birth-cohort survey suggested that low-level maternal erythrocyte Hg (median: 1.5 μg/kg) and Cd concentrations (median: 0.29 μg/kg) were correlated with decreased birth weights and birth lengths (Gustin et al., 2020). Hg and Pb can induce oxidative stress, disruption of placental functions, impaired nutrient transportation, and endocrine disruption, which may be associated with fetal growth impairments (Baldewsingh et al., 2020; Caserta et al., 2013). An epidemiological cohort study indicated that third-trimester oxidative stress was associated with decreased birth weights (Arogbokun et al., 2021). In our study, fetal exposure to Hg and Pb was negatively correlated with the birth weight. Hg and Pb concentrations in the meconium were linearly inversely related with the birth weight, birth length, and head circumference. Fish consumption by pregnant women was a major component of fetal exposure to Hg. Higher Pb concentrations in the meconium were observed among infants of mothers who consumed Chinese herbal medicines. Reducing maternal fish consumption and Chinese herbal medicine consumption during pregnancy can decrease fetal exposure to metals.

There are some strengths of our study. First, this study investigated a mixture of Hg, Pb, Cd, and As in the meconium in relation to fetal birth outcomes. Second, we strengthened research on the meconium as an effective biomarker for evaluating fetal exposure to metals and provided distributions of Hg, Pb, Cd, and As concentrations in the meconium. Third, we explored non-additive exposure-response relationships and interactions between fetal exposure to mixed metals and impairments of birth outcomes. However, there are several limitations to this study. First, although we collected data on fish consumption, we did not obtain information on other dietary categories or nutritional supplements such as supplementary vitamin D, calcium, omega-3, and multiple micronutrients which were previously found to be related to decreased risks of fetal birth impairments (Kinshella et al., 2021). Second, we had no information on local land use characteristics which were previously found to be associated with birth anthropometrics (Santri et al., 2021). Third, we measured total concentrations of each metal rather than individual forms. For instance, methyl-Hg has a lipophilic characteristic, which can readily penetrate the placenta and interfere with fetal growth (Karagas et al., 2012); Furthermore, a family's socioeconomic status may influence fetal exposure to heavy metals (Kao et al., 2022; Marshall et al., 2020). Hence, a higher proportion of our study population had college or higher levels of education and higher family income compared to the general population of Taiwan, which may have limited the generalizability to the larger population.

5. Conclusions

Results of this study revealed Hg, Pb, Cd, and As concentrations in the meconium and enhanced the quantity of research on meconium analyses. The combined effects of Hg, Pb, Cd, and As concentrations in the meconium as a prenatal exposure biomarker were associated with birth growth impairments. Our results enhance the quantity of research on measuring fetal exposures to metal mixtures using the meconium as a biomarker. Furthermore, we provided joint effects of mixed metals on birth outcomes and potential nonlinear relationships of each metal concentration in the meconium with birth outcomes.

Declaration of competing interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.114092.

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Biomonitoring of DEET and DCBA in Canadian children following typical protective insect repellent use

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ABSTRACT

N,N-diethyl-m-toluamide (DEET) is an ingredient found in many consumer insect repellents and its use is recommended to Canadians by government agencies, including Health Canada, for protection against insect bites including mosquitos and ticks. The majority of research on DEET exposure and toxicokinetics in humans has focused on adult populations with little information from vulnerable populations, including children. We aimed to fill this knowledge gap by examining real-world exposure data for DEET and its metabolite 3-diethylcarbamoyl benzoic acid (DCBA) in a sample population of Canadian children. We conducted a 24-h observational exposure human biomonitoring study at three overnight summer camps in Ontario, Canada through July and August 2019. Participating children aged 7–13 years provided multiple spot urine samples over a 24-h period and completed a journal to document insect repellent use and factors that could influence absorption of DEET. Children were instructed to use insect repellent as they usually would while attending a summer camp. Exposure was quantified using the information from the participant's journal and the change in the mass of their insect repellent containers over the course of the study. A total of 389 urine samples were collected from 124 children. Among participants using insect repellent, urinary levels of DEET were elevated between 2 and 8 h post-application and decreased thereafter but remained qualitatively higher than concentrations in participants who did not use insect repellent on the study day, even at 18–22 h post-application. DCBA was the predominant metabolite of DEET exposure in urine. DCBA was elevated between 8 and 14 h post-application, and declined thereafter, but not to the level observed among those who did not use insect repellent on the study day. Children who used more insect repellent, or used higher concentration insect repellent (10%–30% DEET) excreted higher levels of DEET and DCBA. Excreted DEET and DCBA accounted for 0.001% (median) and 1.3% (median) of the estimated applied DEET, respectively. Children did not reach an undetectable level of DEET or DCBA in urine, even among those not using insect repellent during the study day, indicating a potentially complex multi-route exposure to insect repellents in a real world scenario. This work provides targeted biomonitoring data for children intentionally using DEET-based insect repellents for normal protective use, and will support the risk re-evaluation of DEET by Health Canada.

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1. Introduction

Government agencies recommend using insect repellents containing *N*,*N*-diethyl-*meta*-toluamide (DEET) to protect against biting insects and vector borne diseases including Lyme disease and West Nile virus (Government of Canada (GOC), 2020). In Canada, insect repellents approved for sale are available with concentrations of up to 30% DEET. Health Canada, a part of the Canadian federal government, provides guidelines on insect repellent use for children under 12 years of age; these guidelines are more restrictive than those for adults (Government of Canada, 2020). DEET has consistently been one of the top ten active ingredients in the domestic sector use category of pest control products over the last five years. More than 100,000 kg of the active ingredient DEET was sold in Canada in 2018 (Health Canada, 2020).

DEET exposure occurs seasonally with the majority of the use occurring in the spring and summer months during peak biting insect season (Bernot et al., 2013; Singh and Suthar, 2021). National biomonitoring programs such as the Canadian Health Measures Survey (CHMS) or the National Health, Nutrition and Environment Survey (NHANES) collect samples throughout the year, resulting in an averaging of annual exposure for the general population of Canada and the United States, respectively, without restrictions to a specific time period of exposure (Health Canada, 2019; United States Centers for Disease Control and Prevention, 2021). Targeted biomonitoring studies allow for the identification of intentional use patterns and an understanding of resulting exposures.

Four previous studies assessed biomarkers of DEET exposure in children (Arcury et al., 2007; Tian and Yiin, 2014; Calafat et al., 2016; Heffernan et al., 2016); however, results of these studies may not be reflective of real-life exposure to DEET. Arcury et al. (2007) analysed the first morning void of 60 children of farm workers, aged 1–6 years. This study was designed to detect incidental exposure via paraoccupational (e.g., parental occupation as farm workers), residential and environmental factors, rather than intentional application. In both Calafat et al. (2016) and Heffernan et al. (2016), data were derived from urine samples collected through NHANES, a larger biomonitoring surveillance initiative, and routine medical urine sampling in Australia, respectively. Neither of these sampling strategies can identify intentional application. Additionally, Heffernan et al. (2016) amalgamated urine into pooled samples to do a preliminary analysis of Australian background exposure to a range of pesticides and related compounds, including DEET, obscuring any individual-level variability. Tian and Yiin (2014) performed a controlled exposure study on 17 children aged 5–7 years, which provided a first look at the relationship between the application and excretion of DEET-based insect repellents in a controlled experimental setting. To date, no study has considered the real-world scenario of a population of children who are actively using insect repellent.

As no Canadian data are available on the use of DEET-based insect repellents by children, we designed a targeted biomonitoring study to address these knowledge gaps (Gibson et al., 2022). The purpose of this study is to generate human biomonitoring data for Canadian children following exposure to DEET through individual intentional use of insect repellents in a summer camp setting, to describe insect repellent usage patterns in a group of Canadian children attending summer camp, and to examine factors that may influence DEET absorption. Children attending overnight summer camps were invited to participate in this real-world exposure observational study. Participants applied insect repellents as they would normally, as a part of their camp routine.

2. Material and methods

2.1. Study design and sampling description

In 2019, a 24-h observational human biomonitoring study on the use of and exposure to DEET-based insect repellents in Canadian children was conducted at three overnight summer camps in Ontario, Canada. Complete details for the study design and laboratory analysis has been described elsewhere (Gibson et al., 2022). The project and all materials associated with the project met ethical requirements for research involving humans by the Government of Canada's Health Canada and Public Health Agency of Canada's Research Ethics Board (REB). The project has retained a Certificate of Ethics Review under Project File Number REB 2018-019H since November 2018.

Through partnerships with the overnight summer camps, children between the ages of 7 and 13 years were recruited. Parents provided written informed consent for the children on drop-off day or prior to arrival, and children provided written informed assent on arrival to participate in the study. Children were instructed to use insect repellent as they would normally to protect themselves against biting insects.

Parents completed a questionnaire detailing the age, biological sex at birth (male/female), and health information of the child participants as well as household socio-economic information (parent education, annual income), and family insect repellent habits.

At the campsites, the child's height (cm), and weight (kg), as well as the mass (g) and product information of their insect repellent were recorded to facilitate dose exposure estimates. Children who did not have their own DEET-based insect repellent were provided with an insect repellent conforming to Health Canada guidelines for children under the age of 12, containing 7% DEET (OFF!® familycare®, SC Johnson).

Over the course of a 24-h sampling period, up to six urine samples were collected from each participating child. Samples were stored at approximately 4 °C and were delivered to the Health Canada Pesticide Laboratory for analysis at the end of each 24-h sampling period.

At the time of each urine sample collection, children completed an activity journal with the assistance of study staff. This journal tracked daily behaviour (i.e., swimming, showering, and activities leading to sweating) and details on insect repellent application. Children indicated if, when, and where on their body insect repellent was applied, whether hand sanitizer and/or sunscreen had been used in concert with insect repellent application, and hand washing habits post-insect repellent application.

At the end of the 24-h study period, the final insect repellent mass (g) was recorded and children were asked if any urinations had not been sampled during the 24-h study period, or if they spilled or shared their repellent, or used anyone else's repellent.

Three field quality assurance and quality control (QA/QC) spike samples were created three times (morning, afternoon, and evening) over the course of the 24-h sampling period. These samples, as well as field blanks, were stored, shipped, and analysed with the urine samples collected.

2.2. Analytical methods of urine samples

Chemical analysis of urine samples was performed by the Health Canada Pesticide Laboratory for DEET, N,N-diethyl-m-(hydroxymethyl) benzamide (DHMB), 3-diethylcarbamoyl benzoic acid (DCBA) after β-glucuronidase deconjugation and solid-phase extraction, by LC-MS/MS (Shimadzu UPLC-AB Sciex QTRAP 6500+), and urine specific gravity (SG) was measured using an Atago PAL-10S handheld refractometer. DHMB is not reported here due to unreliable QA/QC sample recoveries (Gibson et al., 2022). Creatinine (CR) analysis was done at a Health Canada Health Products and Foods Branch laboratory using the colorimetric end-point Jaffe kinetic method, and Janovsky complex absorbance was read at 510 nm using a Horiba Medical ABX Pentra 400 chemistry autoanalyser.

2.3. Definitions and methods of calculating exposure

As children were free to apply their insect repellent and participate in urine collection at any time throughout the study (i.e., as they would normally at overnight summer camp), it was not possible to standardize

the timing of the insect repellent application and urine collection times. Therefore, the results of the urine sampling were contextualized with the insect repellent usage. We defined three time lags to account for absorption, distribution and metabolism after application of insect repellent, in relation to the time that the urine sample was collected. Further details on the development of these time lags is available elsewhere (Gibson et al., 2022).

Briefly, we incorporated a lag time between insect repellent application and urine sampling to sort the urine sample data into different time intervals representing time since insect repellent application (e.g., 0 - ≤6 h, >6 - ≤12 h, etc.). The lags applied to these time intervals were 0, 2, and 4 h, named lag0, lag2, and lag4, respectively. During this time period, an insect repellent application was not expected to impact the amount of DEET and metabolites measured in urine. Fig. 1 shows a model of how urine samples were separated into time intervals based on time since insect repellent application using lag2 as the example, and shows how the lag time affected urine sample classification into the study group or the reference groups. The reference group consisted of urine samples where: 1) repellent was not used prior to the urine sample during the study day period (at lag0); 2) the only repellent application prior to urine sampling was within the lag time (during which it would not be expected to influence the urine sample) (i.e., lag2 and lag4); and 3) urine samples from children who did not apply repellent at all during the 24-h period of the study. Within this reference group, sub-groups were created for urine samples where the previous application of repellent that was reported occurred "yesterday", "1–2 days ago", "last week" or "never".

Dose exposure estimates were calculated for each time a child applied repellent, based on the reported areas of application to the body. Using the change in mass (g) of the insect repellent containers over the course of the 24-h study day and the percentage of DEET in solution, we constructed an estimate of the amount of DEET applied for each reported application based on the skin surface area exposed. It was assumed that 100% of the change in the mass of the insect repellent container was applied to the skin and was available to be absorbed. For a full description of the method used, see the Supplemental Material. Based on the dose exposure estimates, we created tertiles of lower range, mid-range and upper range dose exposure groups for analysis. Cumulative dose exposure was also calculated as the sum of the DEET per application for all DEET applications prior to the urine sampling time, minus those not included due to the applied lag since exposure (i.e., lag0, 2 or 4). Based on this calculation, participants were separated into tertiles of low, mid-, and upper cumulative dose exposure groups for analysis.

DCBA was expressed as a DEET equivalent, by dividing the grams of the metabolite found in urine by the molecular mass of the metabolite and multiplying by the molecular mass of the parent compound:

$$DEET_{equivalent(DCBA)} = \frac{DCBA(g)}{221.25} \times 191.27$$

The total applied DEET for a participant was compared to the total excreted DEET or DEET equivalent to determine the percentage of applied DEET excreted via urine.

The ratio of the geometric means of DEET and DCBA was also calculated to identify the proportions of DEET and DCBA as excretion products:

$$Ratio\ (\%) = GM\left(\frac{DEET}{DCBA}\right)$$

2.4. Statistical analysis

We analysed DEET and DCBA concentrations standardized by measured specific gravity (SG), as well as by measured creatinine (CR) (Gibson et al., 2022).

Intra-class correlation coefficients (ICC) were calculated using a one-way random effects model to estimate the between and within-subject variability of the multiple spot urine samples collected within a 24 h period.

Linear mixed effect models were used to account for multiple urine samples from the same participants. "Participant" was treated as a random effect. Time since repellent application, dose exposure, demographics (i.e., age, sex), and camp behaviours (i.e., type of spray/lotion, application behaviour) were treated as fixed effects. We investigated whether the urine concentrations of DEET and DCBA differed according to the following variables: age, sex, type of repellent (lotion/pump versus aerosol spray), percentage of DEET in repellent (>10%, ≤10%), number of times repellent was applied (1, 2, 3 or more times), as well as number of repellent applications expected to affect the urine sample, washing/showering on study day, sweating on study day, swimming on study day, use of hand sanitizer or sunscreen after applying DEET product, washing hands after applying DEET product. A variable was created to consider the number of repellent applications preceding the application counted as affecting a urine sample, with groups of "Number of prior applications: no sprays" and "Number of prior applications: 1+ sprays". We also investigated whether there was a difference between voids throughout the study day and morning voids taken at the end of the study. Concentrations of DEET and DCBA at different time intervals were compared to the reference group. The study sample concentrations were also compared to urine concentrations detected in the final study day time interval ending at 28 h. Incomplete data collection on the change in mass (g) of the insect repellent containers were treated as unknown DEET exposure samples.

The above analysis was re-run for cumulative dose exposure for the study samples. A sensitivity analysis for the study samples analysis was conducted where we included the unknown DEET exposure samples (Suppl. Mat.). This was completed in order to compare the DEET and DCBA levels in urine for this unknown dose exposure group to the other dose exposure groups (low, medium and high), and to understand the potential impact of these samples on the overall results.

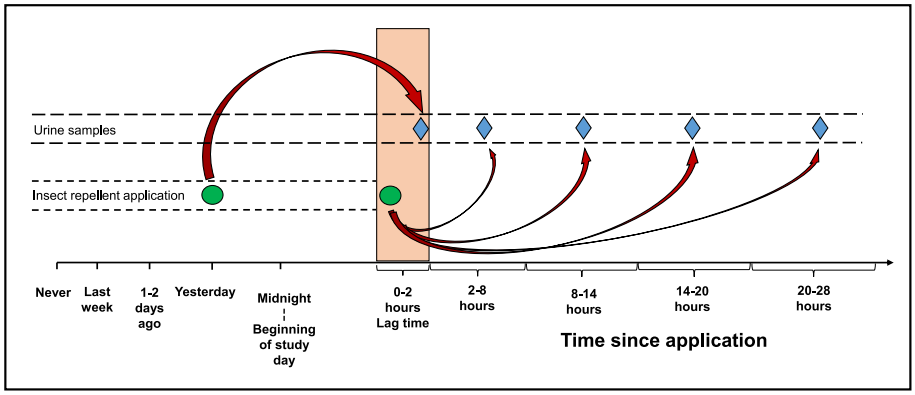

Fig. 1. Model showing an example of the time context between urine samples and insect repellent applications, based on a lag2 time difference. Green circles indicate insect repellent application reports. Blue diamonds indicate urine sampling times. Red arrows show the insect repellent association. The orange box shows the 2 h time lag applied to the first urine sample, during which a recent insect repellent application occurs. This associates the first urine sample with the insect repellent application of yesterday, and all following urine samples are associated with the insect repellent application occurring on the sample day [See Fig. 1 file attachment]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

In all models, the interaction between time since application and dose exposure was assessed in order to verify that the dose response relationship with respect to time was similar in all dose groups. Where the interaction was not significant ($p > 0.05$), it was dropped from further analysis. If the interaction was significant (i.e., the dose relationship with respect to time was not parallel between dose groups, $p < 0.05$), then the relationship between the variable of interest and DEET/DCBA was examined at each time point and dose exposure group.

Analyses were conducted using SAS Enterprise Guide 7.1. Statistical significance was specified as ($p < 0.05$). Where multiple pair wise comparisons were required to compare groups to a reference group, Dunnett's pairwise test correction was applied. In all other cases, Bonferroni corrections were applied for pair-wise comparisons.

3. Results

Of the 126 children recruited, 124 children provided at least one urine sample during the 24-h period of the study (mean = 3.1 urine samples; range = 0–6 urine samples). All analyses are based on this sample of 124 children. The median age of the children was 11 years (range 7–13 years). A total of 389 urine samples were collected and analysed, with an almost equal number of urine samples from female (n = 192, 49.4%) and male children. Children reported not collecting 129 urine samples (mean = 1.07 urine samples; range = 0–5 urine samples).

Our primary model was based on SG standardized results and the lag2 time period because it was considered the most biologically relevant lag period (Moody et al., 1989; Selim et al., 1995; Yiin et al., 2015). The results from the analyses including lag0 and lag4, as well as the CR standardized results are provided in the Supplemental Material.

The SG standardized urine ICC of DCBA was higher than the uncorrected or CR standardized values, whereas for DEET, the uncorrected ICC was higher. In all three urine values (uncorrected, SG- or CR-standardized) of DCBA, ICCs were moderate to high, ranging from 0.55 to 0.65. This indicates the covariance structure within individual's 24-h urine samples needs to be accounted for in the modelling (i.e., random coefficient model).

3.1. Insect repellent use

The majority of children (80.6%) used a lotion or pump container for insect repellent with the remaining children using an aerosol style spray can (Table 1). DEET levels ranged from 5% to 30%, but the majority of children used an insect repellent with DEET levels under 10% (62.1%). During the 24-h study period, most children (61.3%) applied insect repellent once or twice (Table 1). Twenty-six children did not apply repellent during the study, and 22 children applied repellent three or more times. Most frequent areas of insect repellent application reported by children were legs (85.2%), arms (82.4%), hands (59.7%), and neck/ears (53.4%), whereas parents reported family habits of insect repellent use on arms (66.4%), legs (64%), and neck/ears (62.4%).

Table 1
Descriptive characteristics and activity monitoring results among children attending overnight summer camp (n = 124).

Variable	Group	n	%
Sex at birth	Female	53	42.7
	Male	71	57.3
Age (years)	Missing	1	0.8
	7–8	12	9.6
	9	19	15.3
	10	21	16.9
	11	24	19.4
	12	27	21.8
	13	20	16.1
Repellent Type	Lotion/pump	100	80.6
	Aerosol spray	24	19.4
% DEET in repellent	≤10%	77	62.1
	>10%	47	37.9
Number of applications	0	26	21
	1	40	32.3
	2	36	29
	3 or more times	22	17.7
Did you wash/shower today	No	117	94.4
	Yes	7	5.6
Did you sweat today	No	54	43.6
	Yes	70	56.4
Did you swim today	No	35	28.2
	Yes	89	71.8

The mean overall application rate of DEET applied was 0.03 g/kg body weight (range: 0.0002–0.4 g/kg body weight). When examining the timing of insect repellent application through the 24-h study period, children reported application in every hour between 7:00 and 23:00 on the study days. However, there were two clusters of more common application times, between 7:00 and 9:00 and between 18:00 and 21:00. The majority of children did not wash or shower on the study days (94.4%). However, the majority of children went swimming (71.8%) and reported sweating during the course of the study day (56.4%) (Table 1).

3.2. Estimated DEET application

The estimated amount of DEET applied per application (DEET per application) ranged from 0 to 12.0 g, with a median of 0.24 g and 95th percentile of 3.1 g. Fourteen participants did not return to weigh their insect repellent container at the end of the study, resulting in 25 urine samples without a dose exposure estimate, which were classified as "Unknown". Twelve urine samples, from seven individuals, are in the top 5th percentile with a DEET per application greater than 3.0g. The total amount of DEET used for these 12 samples, determined by the change in mass of the insect repellent container used and the DEET percentage in the insect repellent, ranged from 3.1g to 22.3g, and DEET concentrations in the insect repellent used ranged from 15% to 30%. Forty individuals used repellents with DEET concentrations between 25% and 30%, but approximately half (52.5%) of these individuals had DEET per applications of less than 3.0g, and for 13 individuals (32.5%) DEET use data were incomplete. Eleven participants used more than 20g of repellent during the 24-h period of the study, with DEET concentrations in the insect repellent ranging from 7 to 30%, but only the seven abovementioned participants achieved DEET per applications in excess of 3.0g. For all three lags (0, 2, and 4 h), the dose exposure groups were defined as low range (0 - <0.17g), mid-range (0.17 - <0.40g) and upper range (0.40–12.05g).

At lag2, the geometric mean SG-standardized DEET and DCBA levels in urine were only significantly different in the upper range estimated dose exposure group compared to the low range estimated dose exposure, although participants in the mid-range group had twice the geometric mean DEET concentrations of the low-range group (Table 2). For lag0 and lag4, see Suppl. Mat. Table 1.

3.3. Reference group samples

Urine samples that were redefined as reference group samples under lag2 conditions were compared to the original lag0 reference groups. For samples standardized by SG, these lag2 additional reference samples were statistically similar to the reference groups of "yesterday" and "1–2 days ago", and were considered to have statistically higher levels of DEET and DCBA compared to the "last week" and "never" reference groups. Age was treated as a continuous variable, and increasing age was associated with increasing lag2 levels of DCBA in the reference groups after adjusting for time difference in the models.

3.4. Distribution of geometric means

Table 3 presents the geometric means and corresponding 95% confidence intervals (CI) of SG-standardized DEET and DCBA levels in the

Table 2
Lag2 urinary DEET and DCBA geometric means (95% CI) by estimated dose exposure group (g) standardized by SG (μg/L) and adjusted for time difference, as well as the ratio of the geometric means of excreted DEET and DCBA.

	Estimated DEET exposure			
	Low range	**Mid-range**	**Upper range**	**Unknown dose**
	(<0.17g)	**(0.17–0.4g)**	**(0.4–12.0g)**	
	(n = 52)	**(n = 50)**	**(n = 53)**	**(n = 19)**
DEET (μg/L)	5.6 (3.4, 8.9)	11.0 (7.6, 15.8)	28.3** (19.2, 41.6)	5.9 (2.6, 13.3)
DCBA (μg/L)	8130 (4787, 13808)	12401 (8003, 19217)	38678** (24770, 60396)	6877 (2414, 19592)
Ratio % DEET: DCBA	0.077 (0.061, 0.097)	0.088 (0.063, 0.122)	0.071 (0.057, 0.088)	0.086 (0.062, 0.119)

** - Significantly different from "Baseline:Yesterday", $p < 0.01$.
DEET: N,N-diethyl-m-toluamide; DCBA: 3-diethylcarbamoyl benzoic acid.

different time intervals by lag2. For SG standardized lag0 and lag4 data and creatinine-standardized data, see Supplemental Material Tables 2 and 3 For DEET, geometric mean concentrations were highest within the first time interval and decreased with increasing time since insect repellent application. DCBA urine concentrations increased into the >8-≤14 h time interval, and decreased thereafter. Both the first and second time interval DCBA concentrations were significantly higher than concentrations within the "Yesterday" group. Children "Never" exposed to insect repellent had significantly lower levels of DEET and DCBA compared to those who were exposed to insect repellent "Yesterday" ($p < 0.05$) (Table 3, Tables 2–3 Suppl. Mat). Concentrations in the reference groups "Yesterday", "1–2 days ago", and "Last week" were statistically similar ($p > 0.05$), but they were not combined because it was observed that these groups were decreasing across those intervals by a factor of 1.5–2. Across time intervals (0–28 h), regardless of lag time used, urine samples with a shorter time since application had significantly higher DEET and DCBA concentrations, compared to urine samples where the most recent application was "Yesterday".

3.5. Demographics and behaviours

We did not observe large differences in urinary concentrations of

Table 3
Geometric means and 95% confidence interval (CI) of DEET and DCBA (μg/L) in urine samples (n, number of urine samples in the time interval) standardized for urine dilution by SG, by time since insect repellent application using the lag2 adjustment. p-values are Bonferroni corrected for multiple comparisons.

	Time since application (hours), lag2							
Analyte	>2-≤8	>8-≤14	>14-≤20	>20-≤28	Yesterday	1–2 days ago	Last week	Never
(μg/L)	(n = 87)	(n = 56)	(n = 20)	(n = 11)	(n = 90)	(n = 58)	(n = 29)	(n = 38)
DEET	12.4** (8.7, 17.7)	10.4 (7.3, 14.9)	9.4 (5.1, 17.4)	8.4 (4.0, 17.4)	7.6 (5.2, 11.0)	4.3 (2.7, 6.9)	2.4 (1.0, 5.7)	1.6** (0.8, 2.9)
DCBA	12102** (8217, 17823)	16635** (11105, 24920)	14522* (7177, 29383)	12406 (4996, 30804)	8140 (5504, 12039)	4576 (2678, 7817)	3207 (985, 10443)	1966** (968, 3997)

* Significantly difference from Reference group "Yesterday", $p < 0.05$.
** Significantly different from Reference group "Yesterday", $p < 0.01$.
DEET: N,N-diethyl-m-toluamide; DCBA: 3-diethylcarbamoyl benzoic acid.

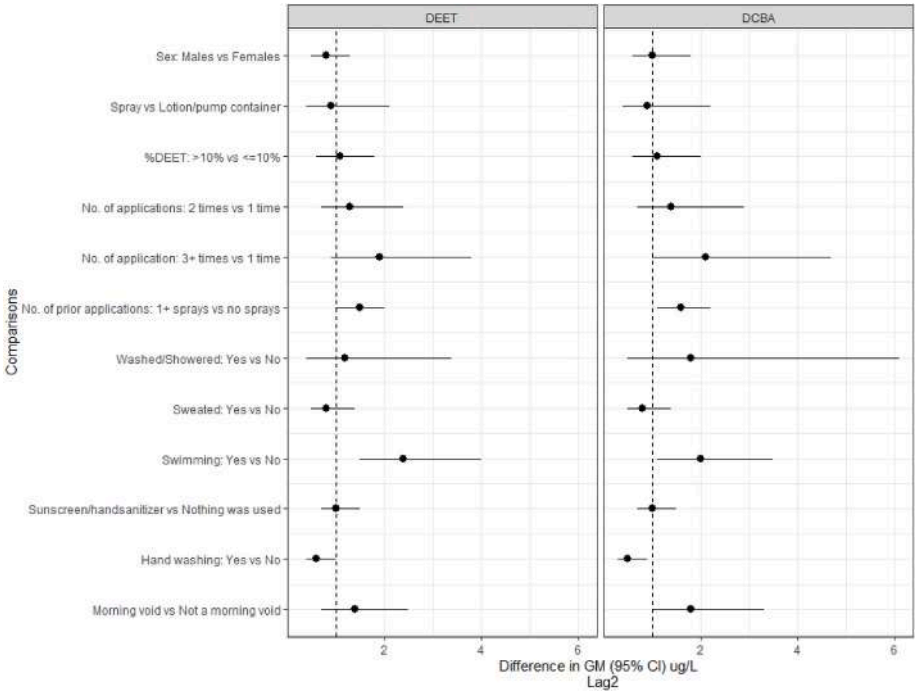

Fig. 2. Comparison of geometric means (95% confidence interval CI) between levels of a variable to reference group for DEET and DCBA at lag2. When comparing geometric means, the difference is compared to 1, since the difference between geometric means is the ratio of the log means [See Fig. 2 file attachment.].

DEET or DCBA (adjusting for time difference and dose exposure) for males vs. females, age, insect repellent applicator type (aerosol vs. lotion/pump), insect repellent concentrations (≤10% vs. >10% DEET), washing/showering, sweating, and the use of hand sanitizer/sunscreen (Fig. 2). For lag0 and lag4 data, see Suppl. Mat. Fig. S1.

3.6. Number of applications

At lag2, the geometric mean concentrations of DEET and DCBA in urine were statistically similar when not adjusting for time difference and dose exposure, regardless of the number of times participants applied bug repellent on their body (i.e., 1, 2 or 3+ times). However, levels of SG-standardized DCBA were visually higher among those who applied the repellent three or more times during the 24-h study period (Fig. 2).

Adjusted geometric means of both DEET and DCBA were approximately 1.5 times higher ($p < 0.05$, for both DEET and DCBA) among the "Number of prior applications: 1+ sprays" group, who applied repellent one or more times in addition to the application that was counted to have affected the urine sample, as compared to the "Number of prior applications: no sprays" group who had no preceding repellent applications before a urine sample (Fig. 2).

3.6.1. Swimming

At lag2, on average, those who reported swimming during the 24-h period had adjusted geometric means of both DEET and DCBA concentrations approximately 2.4 (95% CI: 1.5–4) and 2.0 (95% CI: 1.1–3.5) times higher ($p < 0.05$, in both cases) than those who did not swim, respectively (Fig. 2). This was also true for the relationship between the unadjusted geometric means of both DEET and DCBA and those who reported swimming during the 24-h period of the study.

3.7. Hand washing

Hand washers had significantly lower DCBA (0.5 times lower (95% CI: 0.3–0.9)) compared to those who did not report washing their hands after applying insect repellent (Fig. 2). At low range dose exposure levels (<0.17g), significantly higher levels of DEET were observed among those who did not wash their hands compared to those who did wash their hands, but this difference disappeared in the mid- and upper range dose exposure groups.

3.8. Morning voids

Twenty percent of the urine samples collected were morning voids at the end of the study period. At lag2, adjusted geometric means of DCBA, but not DEET, were significantly higher (1.8 times higher (95% CI: 1.0–3.3)) in morning voids compared to study day voids (Fig. 2).

3.9. Time difference and cumulative dose

The estimated cumulative dose ranged from 0 to 22.3g, with a median of 0.5g. The lower, medium, and upper cumulative dose exposure tertiles were defined as lower (0 - <0.3g), mid- (0.3 – <0.8g) and upper (0.8–22.3g) (Table 4; lag0 and lag4 Suppl. Mat. Table 4). Participants in the medium and upper cumulative dose groups had concentrations of DEET and DCBA that were 3–6 times higher than the low cumulative dose group ($p < 0.01$).

Fig. 3 presents the change in geometric means of DEET and DCBA over time for lag2 at each cumulative dose exposure group. The separation of the three cumulative dose exposure groups is evident. The study day time interval geometric means were statistically compared to the geometric mean for the final study day time interval. At lag2, both SG and CR standardized Overall DEET levels (Suppl. Mat. Fig. S3) in the first time interval were significantly higher compared to levels in the last time interval (>20-≤28 h). These differences were not detected in any individual cumulative dose group, due to small sample size in each dose group and a lack of statistical power. The peak urine geometric mean in the upper cumulative dose group during the third time interval for both SG standardized DEET and DCBA differs from previous observations of the peak concentration occurring in the second time interval. Comparison of the concentrations by study day time for lag0, lag4, as well as creatinine standardized urine samples, are in the Supplemental Materials Figs. S2 and S3.

Table 4
Urinary DEET and DCBA geometric means (95% CI) by estimated cumulative dose group (g) standardized by SG (μg/L) and time since last exposure at lag2.

Analyte (μg/L)	Cumulative Dose		
	Low	**Medium**	**Upper**
	(<0.3g)	**(0.3–0.8g)**	**(0.8–22.3g)**
	(n = 55)	**(n = 57)**	**(n = 43)**
DEET	5.8	14.4**	30.9**
	(3.9, 8.8)	(10.2, 20.2)	(20.2, 47.2)
DCBA	6854	19268**	41260**
	(4190, 11214)	(13903, 26702)	(24150, 70490)

* Significantly different from low cumulative dose group, $p < 0.05$.
** Significantly different from low cumulative dose group, $p < 0.01$.
DEET: N,N-diethyl-m-toluamide; DCBA: 3-diethylcarbamoyl benzoic acid.

We investigated associations between behavioural relationships, i.e., hand washing, swimming, etc., and the cumulative dose exposure groups. Results were similar to those reported for the dose exposure groups (data not shown).

3.10. Excretion of estimated applied DEET

The participants excreted very little of the parent compound, DEET, as compared to the estimated applied DEET. Based on the change in the mass of the insect repellent containers, of the total estimated applied DEET, 0.001% (0.00001–0.12%) was excreted as DEET (median (range)). Applied DEET was excreted mainly as DCBA, calculated as 1.3% (0.01–117%) of the estimated applied DEET.

4. Discussion

The purpose of this study was to generate biomonitoring data that represented the real life, intentional protective use of DEET-based insect repellents by Canadian children attending overnight summer camp. We found that DEET and DCBA were elevated over a 24-h period in a monotonic dose-response fashion with increasing dose exposure estimates. The concentrations of DCBA metabolite and DEET parent compound excreted in urine represent a small proportion of the estimated total DEET applied, suggesting that the commercial preparations of DEET used in this study were not highly absorbed by the children. However, children did not reach an undetectable level of DEET or DCBA in urine within the 24-h study period.

The DEET concentrations detected in urine in our study was extremely low overall, when compared to DCBA excretion or the estimated DEET application. It was observed by Wu et al. (1979), with one adult human male volunteer applying 10.4g of DEET from a commercial preparation over 75% his skin surface, that approximately 10–14% of the applied DEET was present as unchanged parent compound in the urine in the first hour post-application. This was reduced to 2% by the fourth hour post-application, yet DEET was detected for 18 h post-exposure. In our study, the highest geometric mean concentrations of DEET were found within urine samples collected during the first 6 h after insect repellent application in the lag0 scenario, although the concentrations of the unchanged parent compound did not approach the proportion observed by Wu et al. (1979). This finding demonstrates direct excretion of the parent compound by the children.

Applying a 2-h lag between insect repellent application and urine

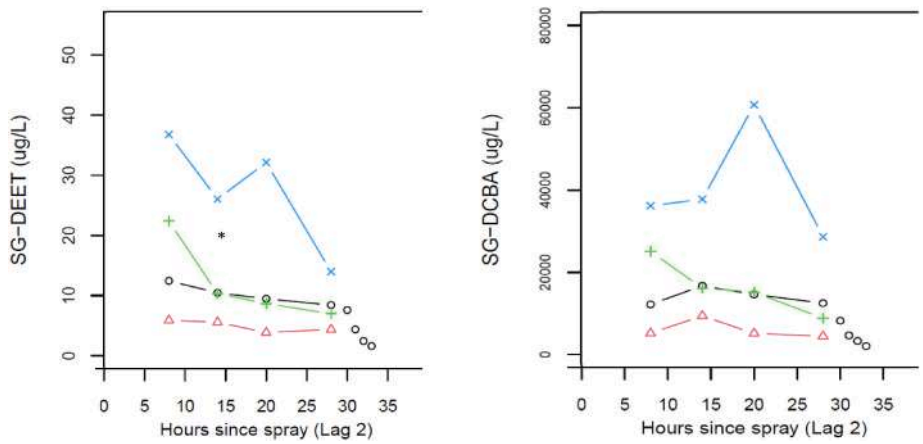

Fig. 3. Geometric mean DEET and DCBA urine concentrations (SG standardized) for cumulative dose exposure groups by time since insect repellent application at lag2.
Legend: ∘ Overall; Δ Low exposure (<0.30g); + Medium exposure (0.30g to < 0.80g); x Upper exposure (0.80g–22.3g); * All dose groups, significantly different from the final time interval, $p < 0.05$.

sampling appeared to be the most biologically relevant time window to take into account absorption, distribution, and metabolism of DEET before excretion. We detected some DCBA within the first 6 h after application, indicating metabolism of the parent compound was occurring. Also, geometric means of DCBA increased from zero hours to the maximum observed in the 8–14 h interval, after which the geometric mean concentrations decline with time since insect repellent application, in alignment with data from adults (Selim et al., 1995).

The unchanged DEET excreted accounted for only 0.001% (median) of the estimated total applied DEET, and excreted DCBA was calculated as 1.3% (median) of the estimated total applied DEET. This excretion is low, compared to the amount of DEET applied. Studies in adults have provided estimates of absorption ranging from 3.8% to 17% after DEET was applied to the skin (Blomquist and Thorsell, 1977; Feldman and Maibach, 1970; Selim et al., 1995). Other routes of excretion for DEET can include the feces. Selim et al. (1995) found that applied DEET was predominantly excreted via urine by the human participants following a dermal dose of [^{14}C] radiolabeled DEET, with only 0.02–0.08% of the applied dose excreted via the feces. Similarly, Schoenig et al. (1995) detected a small amount of the administered [^{14}C] radiolabeled DEET excreted via feces in rats (4–7%) as compared to the urine (74–78%). In this study, it is likely that a small proportion of the DEET absorbed by participants was excreted via the feces; however fecal concentrations were not measured, and no calculations were attempted to estimate that proportion. Due to the conservative nature of the construction of the dose estimates (Suppl. Mat.), it is probable that the amount of DEET assumed to have been applied to exposed skin is an overestimate, as there would be loss of sprayed insect repellent to the environment or clothing. The DCBA % excretion is quite low, even compared to the lowest estimate in adults. It is possible that the carrier of the DEET prevented greater absorption, as newer formulations could have been designed to reduce systemic absorption (Qiu et al., 1997, 1998; Ross and Shah, 2000; Kaushik et al., 2010). This potential difference was not tested and would require a follow up study in more controlled conditions to confirm. Finally, there is a potential for evaporation of DEET from the skin (Spencer et al., 1979; Reifenrath et al., 1989; Bhatt, 2006; Santhanam et al., 2005) reducing the amount of DEET available for absorption. In this study, evaporation could be influenced by children participating in outdoor activities, potentially increasing airflow over the insect repellent covered skin surface. The impact of increased airflow on DEET absorption would also require a follow up study in more controlled conditions to confirm.

Previous research on DEET exposure and toxicokinetics has focused on adult populations with little evidence from vulnerable populations, including children. Tian and Yiin (2014) examined a controlled exposure in children 5–7 years old, as well as adults 23–25 years old, and detected a difference in the proportions of DEET and metabolites excreted in urine between children and adults. In our study, the DEET: DCBA ratio ranged from 0.071 to 0.088%, indicating that DCBA was the main compound excreted and DEET was a very minor component of excretion. We calculated dose exposure estimates incorporating the change in mass of the insect repellent container, the frequency of use, the reported body areas where insect repellent was applied, and the child's estimated body surface area. The biomarkers DEET and DCBA were elevated in a monotonic dose-response fashion with increasing dose exposure estimates.

Relative to urine samples impacted by insect repellent on the previous day only, we observed that among participants using insect repellent, urinary levels of unchanged DEET were elevated between 2 and 8 h post-application and decreased thereafter. However, these concentrations remained qualitatively higher even at 18–22 h post-application. Similarly, DCBA was generally elevated between 8 and 14 h post-application, and declined thereafter, but did not decline to the level observed among those who used insect repellent on the previous day only. This is in line with research in adults, with peak plasma concentrations of DEET occurring 8–9 h after dermal application (Selim et al., 1995); however, a direct comparison of our results with this study is complicated by the use of different matrices. In a study using [^{14}C] radiolabelled DEET in 12 healthy adult male volunteers, most of the absorbed dose of dermally applied 15% DEET in ethanol was excreted in urine within 12 h of application, with nearly complete excretion after 24 h (Selim et al., 1995). Our findings in children do not align with these previous studies. While concentrations declined considerably over a 24-h period, excretion was not complete. Of consideration is that 47% of children in our study applied insect repellent more than once compared to controlled studies using a single exposure. Multiple applications likely impact the amount of time required to reach complete excretion.

Urine concentrations in the reference groups, where self-reported DEET application occurred prior to study onset, were lower than urine concentrations from the study day. However, they still show evidence of DEET excretion as they were higher than the urine concentrations within the reference group that reported never having applied insect repellent. It is worth noting that even children who reported never using a DEET-based insect repellent prior to the urine collection had detectable levels of DEET and DCBA. This suggests low-level background exposure to DEET throughout the study period, although these levels were nearly 10-times lower than in urine samples affected by intentional application.

Significant differences were detected between the low range dose

exposure group and the upper range dose exposure group urine concentrations of DEET and DCBA (Table 2). While forty participants used insect repellents with DEET concentrations between 25% and 30%, only seven participants had DEET per applications in excess of 3.0g. Thus, it was not only the concentration of the insect repellent that led to upper range dose exposures, but a combination of higher concentration product and amount used. These participants used more DEET, and therefore excreted higher quantities of the parent compound DEET and its metabolite, DCBA, after those dose exposures.

We did not determine any highly influential reported behaviours on urine concentrations of DEET and DCBA. The use of an aerosol spray container was significantly related to increased DEET and DCBA urine concentrations at lag0, but the significance was lost when the 2-h lag was applied to the data. This could potentially indicate an inhalation exposure from the aerosol spray container that would be absorbed and excreted more quickly than via dermal exposure. This is not possible to determine with the current data. Similarly, the use of products containing $\leq$10% DEET were significantly related to higher levels of DEET and DCBA in urine at lag0, but not at lag2. The low number of children who washed or showered during the course of the study limited our ability to detect the significance of this behaviour. The investigation of the impact of perspiration on DEET absorption was based on a re-wetting or wash-in effect that was considered to be a possibility to re-initiate absorption of DEET (Moody and Maibach, 2006; Rodriguez and Maibach, 2016). No significant relationships were observed with this behaviour. Swimming was thought to either provide a washing-off type benefit, in reducing the amount of DEET absorbed by removing it from the skin, or provide a re-wetting, wash-in effect. At lag2, swimming behaviour was associated with significantly higher levels of DEET and DCBA in urine. This finding was also seen in lag0, where swimming was significantly related to increased DEET levels in urine, and with increased DCBA levels in the low range dose exposure group ($<$0.17g). The upper range dose exposure group ($\geq$0.4g) showed the inverse relationship, where those who engaged in swimming behaviour had lower levels of DCBA compared to those who did not swim. This relationship was not significant, but it could suggest an interplay between dose exposure, and the ability for swimming to act as either an absorption reducer or an absorption enhancer. The data requested on swimming behaviour did not require the children to report the time or duration of their swimming, although they did identify whether the swimming occurred in the morning, afternoon, or evening. This was not specific enough to relate the swimming and the timing of insect repellent application or urine sampling for a more granular analysis.

At the summer camps, while hand washing stations were available, hand sanitizer was often used in place of washing hands with soap and water. As hand sanitizer is commonly ethyl alcohol based, it was a potential absorption enhancer (Stinecipher and Shah, 1997). Additionally, prior work on sunscreen and insect repellent products indicated a potential concern about the combination enhancing the absorption of DEET (Pont et al., 2004; Ross et al., 2004; Wang and Gu, 2007; Chen et al., 2010; Yiin et al., 2015; Rodriguez and Maibach, 2016). In this study, no significant relationships were found in association with hand sanitizer and sunscreen use and DEET or DCBA concentrations in urine, although lag0 urine concentrations were overall slightly higher among those who reported using hand sanitizer and/or sunscreen. Those participants who reported not washing their hands after insect repellent application showed higher concentrations of DEET and DCBA at lag0, lag2 and lag4. This could suggest an oral exposure component if insect repellent on hands was transferred to food during eating.

At lag2, DCBA was significantly higher in morning voids as compared to study day voids. There was a cluster of common insect repellent use between the hours of 18:00 and 21:00, placing morning voids between the hours of 6:00 and 9:00 in the 9–15 h post-exposure time interval. The 8–14 h time interval was observed to be the peak of DCBA concentrations in urine (Table 3). Therefore, the significantly higher morning voids were most likely to be a result of the time elapsed since the evening insect repellent application.

The overall number of applications of insect repellent were not related to increased urine concentrations of DEET and DCBA. However, when categorized into "one or more preceding repellent applications" and "no preceding repellent applications", the adjusted geometric mean concentrations of DEET and DCBA in urine were significantly higher among those who applied repellent one or more times in addition to the application that was counted to have affected the urine sample. It was anticipated that the metabolism and excretion time would allow for overlapping applications of insect repellent through the course of the 24-h study period, given the extended period of time for DEET excretion in adults (e.g., Selim et al., 1995). Thus, the impact of multiple repellent applications on increasing urine concentrations of DEET and DCBA is to be expected.

The geometric mean urine concentrations of creatinine-standardized DEET and DCBA from this study are higher than creatinine-standardized DEET and DCBA measurements reported for children 6–11 years of age in NHANES (2013–2014) (United States Centers for Disease Control and Prevention, 2021). The geometric mean of urinary DEET concentrations could not be calculated for 6–11 year olds due to the high percentage of samples with concentrations below the limits of detection. The 95th percentile urinary DEET concentration for the 2013–2014 NHANES 6–11 year olds was 0.666 (CI: $<$LOD-1.17) μg/g creatinine. The lowest geometric mean for our study was in the "Never" reference group with a geometric mean urinary DEET concentration of 1.3 (CI: 0.7–2.4) μg/g creatinine, which is still twice what was reported for the NHANES 95th percentile for 2013–2014.

The geometric mean urinary DCBA concentrations reported for the same age group was highest in the 2015–2016 cycle of NHANES (7.29 (CI: 4.99–10.7) μg/g creatinine). In our study, the lowest geometric mean, calculated from lag0 creatinine-standardized DCBA, was also from the "Never" reference group (1720 (CI: 840–3521) μg/g creatinine). The 95th percentile reported for 6–11 year olds in the 2015–2016 cycle of NHANES was 312 (CI: 81.7–529) μg/g creatinine. Calafat et al. (2016) detected a urine concentration comparable to the present study, where one individual's maximum urine DCBA concentration was measured at 30,400 μg/L; however, it is not known whether this was from a child or an adult. Our study's peak geometric mean concentration of DCBA in lag0 was 20,278 (CI: 13,371–30,752) μg/g creatinine.

These differences in exposure likely stem from the surveillance nature of NHANES as compared to targeted biomonitoring. NHANES represents an overview of the baseline concentrations of chemicals present in the general American population. By contrast, our results after typical protective use, and therefore known exposure, demonstrate the value of targeted biomonitoring of children actively and intentionally using DEET-based insect repellents. The only values that approach similarity with NHANES are the "Never" reference group, who self-reported not using DEET based insect repellents at all prior to, or during, the study. As shown in this comparison, intentional use results in much higher excretion concentrations than those found in general population surveys where DEET exposure may only be incidental.

4.1. Strengths and limitations

Our sample population families tend to skew higher than the average Ontario population in education and household income (Government of Canada, 2019). Our study represents a targeted population of Canadian children with self-led, intentional use of DEET-based insect repellent to protect against biting insects.

Since the study involved free-living conditions, there were many uncontrolled factors. The timing of the insect repellent application and urine collection was not controlled, making the analysis of the data complex. We have approached the data analysis in such a way that it puts the urine samples into context with the insect repellent usage (Gibson et al., 2022) based on the information we collected. The activity journal was a functional tool for recording insect repellent use and camp

behaviours, but it is likely that children forgot to record some of their insect repellent applications. While this would not impact the urine concentrations, it could shift them from one dose exposure category to another. Misclassification would be directional in this context, resulting in children being classified as lower dose when in actuality their use pattern would fit into the mid-range or upper range, as it is unlikely that children would record extra, fictional insect repellent applications to the journal. This directional misclassification would bias the results towards the null. It is known that an average of one urine sample per child were not collected during the course of the study day, resulting in incomplete urine series from many of the children. This was likely due to activities taking place away from central sampling locations, overnight urinations, inconvenience, or reduced participant interest while participating in summer camp activities.

Additional limitations of the study have been outlined above, including the assumptions, conservative estimates, and decisions made in the course of the analyses, all of which can introduce error.

5. Conclusions

This study provides results from the first field-based, observational exposure human biomonitoring study on DEET usage and exposure in children. The biomarkers DEET and DCBA were elevated over a 24-h period in a monotonic dose-response fashion with increasing dose exposure estimates. DEET excretion in children occurred over the course of 24 h, but children did not reach undetectable levels of DEET and DCBA in urine. Even children reporting their last DEET use as occurring several days prior to the study day showed evidence of DEET metabolism and excretion, although those concentrations were significantly lower than children who reported active DEET use during the study period. We recommend that future studies on DEET exposure and toxicokinetics examine urinary DEET and DCBA concentrations at least 2 h post-application; we found splitting subsequent time intervals into 6-h increments to be an efficient model. We also recommend repeated sampling of the exposed population over the course of at least 24 h, to capture the excretion of DEET more fully.

Declaration of competing interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.114093.

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Detections of organophosphate and pyrethroid insecticide metabolites in urine and sweat obtained from women during infrared sauna and exercise: A pilot crossover study

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ABSTRACT

Synthetic pesticides such as organophosphates and pyrethroids are commonly used worldwide yet the metabolic and long-term human health effects of these environmental exposures are unclear. Urinary detections of metabolites involving both classes of insecticides have been documented in various global populations. However, reports documenting similar detections in human sweat are sparse. In this study, the concentrations of four insecticide metabolites were measured using liquid chromatography coupled with tandem mass spectrometry in repeated sweat and urine collections (n = 85) from 10 women undergoing three interventions (control, infrared sauna and indoor bicycling) within a single-blinded randomised crossover trial. The Friedman test with *post-hoc* two-way analysis of variance, the related-samples Wilcoxon signed rank test and the Spearman's rank-order correlation test were used to analyse the results. Organophosphate metabolites were detected in 84.6% (22/26) and pyrethroids in 26.9% (7/26) of the collected sweat samples (pooled per individual, per intervention). Urinary concentrations of three of the four metabolites marginally increased after infrared sauna bathing: 3,5,6-trichloro-2-pyridinol (z = 2.395, p = 0.017); 3-phenoxybenzoic acid (z = 2.599, p = 0.009); and *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (z = 2.090, p = 0.037). Urinary 3-phenoxybenzoic acid also increased after exercise (z = 2.073, p = 0.038) and demonstrated the most temporal variability (days to weeks) of any of the urinary metabolites. Definitive sweat/urine correlations were not demonstrated. These results indicate metabolites from organophosphate and pyrethroid pesticides can be detected in human sweat and this raises intriguing questions about perspiration and its role in the metabolism and excretion of synthetic pesticides.

1. Introduction

With the banning of dichlorodiphenyltrichloroethane (DDT, an organochlorine pesticide) in the 1970s, the uses of synthetic insecticides such as organophosphates (OP) and pyrethroids (PYR) grew exponentially (Soltaninejad and Shadnia, 2014). These insecticides play an important role in controlling the spread of vector-borne diseases such as malaria or dengue fever and assisting agricultural practices, yet our understanding of the metabolism and human health effects of these exposures is still emerging. The ubiquity of global exposures to these pesticides, whether via diet or other environmental pathways such as inhalation or dermal contact, is reflected in the wealth of reports documenting urinary metabolites of OP and/or PYR in various worldwide populations, often 80–100% of sampling (CDC, 2019; Dereumeaux et al., 2018; Heffernan et al., 2016; Li and Kannan, 2018; Qi et al., 2012).

Recent studies suggest some of these insecticides have endocrine disrupting properties impacting neurodevelopment in the foetus,

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List of abbreviations

OP	organophosphate
PYR	pyrethroid
PNP	*para*-nitrophenol
TCPY	3,5,6-trichloro-2-pyridinol
3-PBA	3-phenoxybenzoic acid
***trans*-DCCA**	*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid
LC-MS/MS	liquid chromatography with tandem mass spectrometry
GC-MS	gas chromatography with mass spectrometry
LOD	limit of detection
QA/QC	quality assurance/quality control
IR	infrared sauna
EX	exercise
CTRL	control
BMI	body mass index
USG	urine specific gravity
SSG	sweat specific gravity
SD	standard deviation
IQR	interquartile range

neurobehavioral markers in young children, male spermatogenesis/fertility, onset of female puberty, development of endometriosis in women and disrupted thyroid hormone levels (Collotta et al., 2013; Li et al., 2020; McKinlay et al., 2008; van der Plaat et al., 2018; Ye et al., 2017). This hints at complex multisystem metabolism with multiorgan impacts. Sampling of other human excretions (in addition to urine, the current gold standard) could help to elucidate additional metabolic and excretion pathways for these insecticides.

Human sweat is an excretion from the largest organ of the body, yet its roles are less understood in excretion metabolism. It can be sampled non-invasively and its analysis with newer metabolomics/proteomics laboratory platforms, in comparison to urine and blood, is identifying unexpected metabolic content (Hussain et al., 2017; Luque de Castro, 2016; Serag et al., 2021). To our knowledge, OP or PYR metabolites have not been documented in human sweat, although respective detections of other chemical classes of insecticides (*e.g.*, organochlorines) have been reported (Genuis et al., 2013, 2016; Kapka-Skrzypczak et al., 2015).

With urine as the gold standard biofluid for measuring these insecticide exposures, this pilot study aims to explore the role of perspiration in the human metabolism and excretion of OP and PYR insecticides, by comparatively measuring the concentrations of key metabolites in the repeated sweat and urine collections from participants while infrared sauna bathing and indoor bicycling, as compared to a control intervention. By way of design, shorter term (within hours) and longer term (within days to weeks) variability in urinary excretion of these

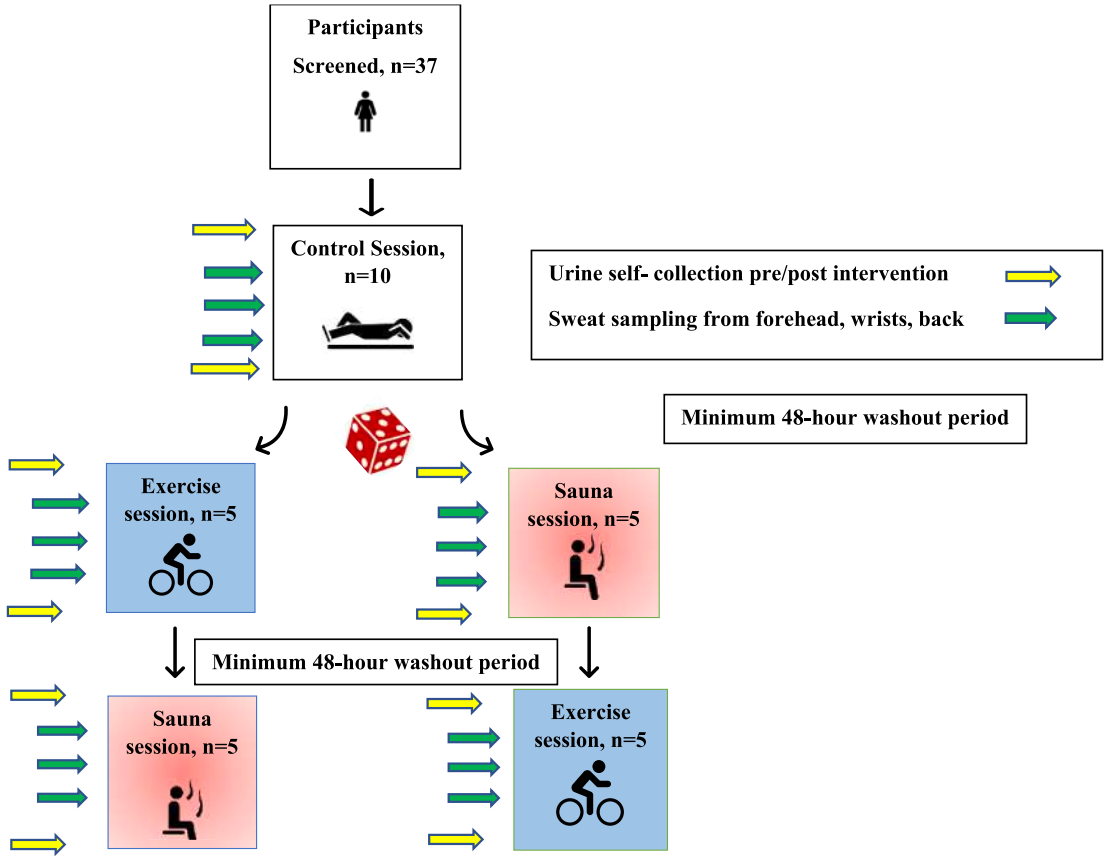


Fig. 1. Crossover design of study with urine and sweat sampling
Study participants were screened by telephone/email. They were introduced to the study location and protocols on their 1st visit to the research gym for their control session. During the 1st visit, they were provided with the randomised sequence for their 2nd and 3rd visit interventions. Urine self-collections were performed pre/post interventions. Sweat was collected from the forehead, forearms and back of each participant, twice during control visit and 3 times during sauna and exercise visits.

metabolites around these interventions were also assessed.

2. Materials and methods

2.1. Study design

A randomised, controlled, single-blinded crossover trial was conducted following Consolidated Standards of Reporting Trials (CONSORT) guidelines (Dwan et al., 2019; Schulz et al., 2010) (Fig. 1). Each participant visited the research gym on three separate occasions. The first for everyone was a control visit, at which baseline collections of urine and synthetic sweat (see section 2.5.2 Sweat) were obtained. During this visit, participants were randomly assigned an order to attend the follow up exercise and sauna visits (random draws.com/au/random-sequence-generator), to ensure balanced allocations. Urine and sweat samples were collected from August 2019 to March 2020, at the Queensland Academy of Sport/Queensland Sport and Athletics Centre in Brisbane, Australia. These samples were collected as part of a larger study assessing women and their thermal and cardiovascular responses to infrared sauna and exercise. Further design considerations (e.g., conducting only one visit per day, scheduling visits at least 48 h apart, etc.) are included in the separately published results of the physiological findings (Hussain et al., 2022).

2.2. Study population

Ten premenopausal, non-pregnant women were recruited from the general population. All participants were chosen to be $\geq$ 18 years old, non-smokers, non-elite exercisers, non-frequent sauna bathers, not diagnosed with any medical disorders, not taking any medications regularly (except hormonal contraceptives), and generally considered healthy, within normal BMI range ($<$30.0 and $\geq$ 18.5kg/m^2). Study participants were pre-screened by telephone/email.

2.3. Ethics

All subjects provided written informed consent before participation. Study procedures were conducted in concordance with ethics committee approvals granted by RMIT University (approval no. 21191) and The University of Queensland (approval no. 31342). This study was registered with the Australian New Zealand Clinical Trials Registry (ANZCTR no. 12618000679280).

2.4. Experimental protocol

For 24 h prior to each visit, the participants were advised to abstain from the use of over-the-counter medicines and the application of topical skin preparations. As instructed, participants fasted overnight, showered in the mornings without soap/lotions and remained fasting until the assigned interventional visit was completed. All experimental visits occurred in the mornings and were completed within 3 h. An indoor hygrometer (ThermoPro, TP-50, Guangdong, China) was used to monitor room temperature (temp) and relative humidity (RH) throughout visits. Participants were encouraged to drink water made accessible throughout experimental sessions.

The control (CTRL) intervention involved engaging in a quiet activity for three 15-min sessions. The room temp was adjusted to participant comfort (~25 °C). For the exercise (EX) intervention, participants engaged in three 15-min sessions of 'moderate intensity' aerobic exercise (Norton et al., 2010) on a standard bicycle ergometer, with 5-min breaks at room temp (~25 °C) between sessions. The infrared sauna (IR) intervention utilised a full-spectrum infrared cabin (Clearlight Jacuzzi™ Sanctuary 2 Unit, Berkeley, CA, U.S.A.), maintained at 60 °C, $<$20% RH for the three 15-min sessions. The 5-min cool-downs between sessions were experienced at the same room temp (~25 °C) as the CTRL and EX breaks.

2.5. Sample collections

Matched collections of two urine samples (pre/post intervention) and one pooled sweat specimen (collected from wrists, forehead and back) were obtained from participants during each of their 3 interventional visits.

2.5.1. Urine

Urine samples were self-collected in sterile polypropylene urine specimen containers (Techno Plas™ 70-ml) by each participant within 30 min of starting and completing each intervention. After collection, urine samples were weighed, and an aliquot of 5 mL was removed to perform manual dipstick urinalysis (Combur-10 Roche Cobas® urine test strips) of specific gravity, pH, leukocytes, nitrite, protein, glucose, ketones, urobilinogen, bilirubin and blood. Samples were stored on ice until transport on the same day to the laboratory, where they were then stored at −20 °C until analysis (maximum storage time at −20 °C = 13 months).

2.5.2. Sweat

As many as feasible, up to 6 sweat samples per visit, were harvested from the skin surfaces of forehead, inner forearms and the lower back of participants during the 5-min breaks between sessions of intervention. A micropipette with disposable tips was utilised for the sweat collection. The procedural steps involved with sweat collection are further detailed in Appendix A1. For the control intervention, the skin sampling - referred to as 'synthetic sweat' collection - was devised to help monitor for potential external contaminations; this consisted of ultra-purified H_20 (*i.e.*, Milli-Q water) pipetted and re-pipetted onto/off the corresponding skin surfaces. All sweat samples were immediately placed on ice and transported on the same day of collection for storage at the laboratory freezer (−20 °C), until analysis was performed (maximum storage time at −20 °C = 13 months).

2.6. Chemical analysis

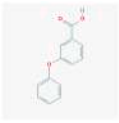
The de-identified urine and sweat samples were analysed by the Queensland Alliance for Environmental Health Sciences (QAEHS) laboratory to determine concentrations of the four insecticide metabolites using LC-MS/MS (Table 1). Analytical methods and QA/QC procedures for urine have been published elsewhere (Li et al., 2019) and the specific applications for this current study, including the methods adapted for sweat samples, are provided in Appendix A2.

Briefly, 50uL of internal standard mixture (TCPY-$^{13}C_3$, *trans*-DCCA-$^{13}C_2D_1$, 3-PBA-$^{13}C_6$, and PNP-D_4 at 100ng/mL in MeOH) was added to 1.0mL urine and 500uL sweat prior to enzyme hydrolysis to deconjugate the insecticide metabolites from glucuronides and sulphates prior to solid phase extraction. The extracts were injected onto a liquid chromatography (Shimadzu, Nexera 2 UHPLC system, Kyoto, Japan) coupled with a tandem mass spectrometer (SCIEX QTRAP® 6500+, Ontario, Canada). The instrument was operated in negative ionisation mode with multiple reaction monitoring of the insecticides. Identification of the analytical responses was confirmed using a combination of signal to noise ratio, relative retention time to a specific internal standard, and response ratio for the two transitions monitored. Analyte concentrations were quantified from their relative response to a specific internal standard against the slope of a seven-point calibration curve, with the concentration of target compounds ranging from 0.05 to 50ng/mL. Analytical accuracy ranged from 91% to 110%, depending on the chemical. Analytical variation was $<$9% for all the compounds assessed by low and high positive control samples, and $\leq$15% as assessed by duplicates of real samples.

2.6.1. Normalisation of specimen concentrations

Urine specimen concentrations were normalised with respective urine specific gravity (USG) measurements, obtained with a digital

Table 1
Insecticide metabolites selected for analysis.

Abbreviation	Biomarker	Structure[a]	Parent compounds[a]	Chemical group[a]
PNP	*Para*-nitrophenol		Parathion; parathion-methyl; ethyl parathion, O-ethyl-O-(4-nitrophenyl) phenylphosphonothioate; nitrobenzene	Organophosphate
TCPY	3,5,6-Trichloro-2-pyridinol		Chlorpyrifos; chlorpyrifos-methyl; triclopyr	Organophosphate
3-PBA	3-Phenoxybenzoic acid		Cyhalothrin; cypermethrin; deltamethrin; fenpropathrin; permethrin; tralomethrin	Pyrethroid
Trans-DCCA	*Trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid		Permethrin; cypermethrin; cyfluthrin	Pyrethroid

[a] Information was sourced from the National Centre for Biotechnology Information/PubChem, USA: https://pubchem.ncbi.nlm.nih.gov and the Centers for Disease Control and Prevention: https://www.cdc.gov/biomonitoring/methyl_eethyl_parathion_biomonitoringsummary.html, accessed November 6, 2020 and November 3, 2022, respectively.

refractometer (UG-α; ATAGO CO., LTD., Japan) which was calibrated using MilliQ water before each use. Then the Levine-Fahy equation was applied to correct for solute effects, as detailed in Appendix A3. (Cone et al., 2009).

Since normalisation methods to account for sample volume and content variations in human sweat samples are still evolving and not yet standardised, the units of sweat specimen concentrations were adjusted using sweat specific gravity (SSG) measurements, obtained with the same digital refractometer, as detailed in Appendix A3.

The laboratory modeling algorithm NDExpo (https://expostats.ca/site/app-local/NDExpo) was utilised to determine numeric values used in statistical analysis when specimen concentrations were detected below the limit of detection (LOD).

2.7. Statistical analysis

Data were compiled with Microsoft Excel (Office 365) and statistically analysed using IBM SPSS Statistics 26.0 (SPSS, Chicago, IL). Datasets were assessed for normality using Kurtosis/Skewness values, Shapiro-Wilk testing and plotted histograms. These tests indicated the concentrations of the insecticide metabolites (PNP, TCPY, 3-PBA and *trans*-DCCA) in urine and sweat samples did not have normal distributions. The Friedman test was selected to analyse the repeated measures (three baseline and three post intervention urine toxicant concentrations, and three pools of sweat toxicant concentrations) across the experimental interventions (CTRL, IR and EX). In the event of significant interaction ($\alpha = 0.05$), *post-hoc* testing with related-samples Friedman's two-way analysis of variance by ranks using Bonferroni adjustment (SPSS v26.0, 2019) were performed to report the multiple pairwise differences between the interventions.

Changes in urinary toxicant concentration from pre to post intervention (by intervention) were further evaluated independently (within each toxicant-intervention dataset) using the related-samples Wilcoxon signed rank test. Correlations between urine and sweat toxicant concentrations were explored with inspection of scatterplots and, if indicated, the Spearman's rank-order correlation test. Statistical data are reported as means ± standard deviation (SD) or medians with 25th - 75th interquartile range (IQR), depending on the distribution of the dataset.

3. Results

3.1. Participant characteristics

Ten women enrolled and all participants completed their three experimental visits in their allocated order, with none dropping out of the study. Baseline demographic and clinical characteristics of participants are presented in Table 2.

Adjustment of indoor environmental settings resulted in mean room temp 25.4 ± 0.9 °C, mean RH 50 ± 10% across all the participants/interventions. The women presented and departed the study visits in states of adequate hydration (USG <1.025) (Kenefick and Cheuvront, 2012) with pre/post USG median (IQR) of crossover group I = 1.005 (0.005)/1.005 (0.005); and of crossover group II = 1.000 (0.000)/1.008 (0.008).

The volumes and sweat specific gravity (SSG) of synthetic and intervention-induced sweat recovered from individuals significantly varied across the interventions: $X^2(2) = 9.333$, p = 0.009 (for sweat

Table 2
Demographic and clinical characteristics of participants by crossover group and entire cohort (total).

	Crossover Sequence for Experimental Visits						
Characteristic	Group I: Control visit, followed by IR, then EX (n = 5)			Group II: Control visit, followed by EX, then IR (n = 5)			Total (n = 10)
Age, yrs	39.8 ± 8.7			33.0 ± 9.8			36.4 ± 9.4
Weight, kg	58.6 ± 9.9			53.8 ± 3.0			56.2 ± 7.3
Height, cm	167.1 ± 5.0			163.7 ± 3.7			165.4 ± 4.5
Body mass index, kg/m^2	21.1 ± 3.7			20.1 ± 1.8			20.6 ± 2.8
Intervention	**C**	**IR**	**EX**	**C**	**EX**	**IR**	
Washout time, no. of days after preceding interventional visit	N/A	3–14	7–28	N/A	2–8	2–42	

Values are means ± SD. IR = infrared sauna; C = control; EX = exercise; N/A, not applicable.

volumes) and $X^2(2) = 10.333$, p = 0.006 (for SSG). Sweat volumes in median (IQR) were 5.4 mL (1.7 mL) for control, 1.3mL (1.2mL) for infrared, and 0.7mL (1.2mL) for exercise. Median (IQR) of SSG: 1.0000 (0.0001) g/cm^3 for control, 1.0053 (0.0024) g/cm^3 for sauna and 1.0059 (0.0033) g/cm^3 for exercise. *Post-hoc* analysis revealed significant pairwise differences between control and exercise (but not between control/sauna or exercise/sauna) with both measurements: p = 0.012 (for higher 'synthetic sweat' volumes with control) and p = 0.004 (for higher SSG with exercise).

3.2. Missing samples

A total of 59 urine samples were analysed for toxicant content from the 10 participants collecting 2 samples (pre/post) from each of the 30 interventional visits. One urine collection was missing due to an adverse event (vasovagal episode) experienced by one participant after completing the exercise intervention.

Due to small volumes of recovered sweat, multiple vials of collected sweat from each participant per interventional visit were pooled together. This resulted in 26 pooled sweat samples analysed for toxicant content, collected from the 30 interventional visits. Four pooled samples were missing due to insufficient sweat produced during the intervention (all involving exercise) or evaporative losses and/or cracked glass vials during the transport/freezing/thawing process. Therefore, 6 of the 10 participants had matched sweat samples (to urine) involving all 3 interventions for complete comparative analysis.

3.3. PNP

Of the data for PNP, 55.2% of the urine samples and 84.6% of the sweat samples had concentrations above the limits of detection (LOD). Table 3 presents the LOD (in ng/mL) and detection frequencies for each metabolite in both urine and sweat.

There were no statistical differences in the baseline urine concentrations of PNP obtained across control, sauna and exercise activities, $X^2(2) = 0.200$, p = 0.905. Changes in post-intervention urine concentrations of PNP were detected across interventions: $X^2(2) = 6.222$, p = 0.045, with median (IQR) values of 1.0 (1.3) ng/mL-SG$_{norm}$ (sauna), 0.87 (1.9) ng/mL-SG$_{norm}$ (exercise) and 0.58 (0.37) ng/mL-SG$_{norm}$ (control), (Table 4 and Fig. 2). However, *post-hoc* testing did not confirm statistical significance for any of the pairwise comparisons: z = −1.111, p = 0.055 (sauna/control); z = - 0.889, p = 0.178 (exercise/control); and z = 0.222, p = 1.000 (exercise/sauna). Furthermore, the additional testing run separately within each intervention-specific dataset failed to detect any pre *vs* post urine PNP differences.

Differences were detected in sweat-PNP concentrations across the interventions, $X^2(2) = 6.333$, p = 0.042 (Fig. 3) but again, no pairwise differences were found with *post-hoc* testing. Median (IQR) concentrations of sweat PNP were 0.65 (0.14) ng/g-SG$_{adj}$ (sauna), 0.63 (0.24) ng/g-SG$_{adj}$ (exercise) and 0.29 (0.27) ng/g-SG$_{adj}$ (control), (Fig. 3).

Inspections of sweat PNP concentrations (n = 26) compared to matched pre/post intervention urine PNP concentrations (involving 9 participants) on scatterplots suggested low possibility of correlation. Further testing failed to demonstrate statistical correlation between sweat/urine PNP concentrations, either pre intervention: $r_s(24) = 0.066$, p = 0.749 or post intervention $r_s(24) = 0.355$, p = 0.075 (Fig.A4.1-Appendix).

Table 3
Limits of detection (LOD) and frequency of detection (FREQ) in samples of urine and sweat, by metabolite.

Metabolite	Urine -LOD[a]	Urine -FREQ	Sweat - LOD[a]	Sweat –FREQ
PNP	0.10	32/59 (55.2%)	0.23	22/26 (84.6%)
TCPY	0.072	59/59 (100%)	0.16	3/26 (11.5%)
3-PBA	0.0040	59/59 (100%)	0.0090	7/26 (27%)
trans-DCCA	0.023	18/59 (31%)	0.052	1/26 (3.8%)

[a] All units of LOD concentration in ng/mL.

Table 4
Urine and sweat concentrations of metabolites, by intervention – median (IQR).

Metabolite/ Intervention	Urine - pre (ng/mL–SG$_{norm}$)	Urine - post (ng/mL–SG$_{norm}$)	Sweat (ng/g-SG$_{adj}$)
PNP			
Control, n = 10	0.70 (0.86)	0.58 (0.37)	0.29 (0.27)
Infrared, n = 10	0.63 (0.80)	1.0 (1.3)	0.65 (0.14)
Exercise, n = 10	0.81 (0.98)	0.87 (1.9), n = 9	0.63 (0.24), n = 6
Total	0.73 (0.99), n = 30	0.84 (1.3), n = 29	0.56 (0.37), n = 26
TCPY			
Control, n = 10	3.8 (1.7)	4.4 (2.0)	0.080 (8.0 × 10^{-6})
Infrared, n = 10	5.8 (10.5)*	9.3 (11.9)*	0.080 (2.0 × 10^{-4})
Exercise, n = 10	4.3 (9.2)	7.4 (16.0), n = 9	0.080 (0.15), n = 6
Total	4.4 (5.3), n = 30	5.4 (8.4), n = 29	0.080 (4.1 × 10^{-4}), n = 26
3-PBA			
Control, n = 10	0.59 (1.1)	0.89 (1.5)	0.0045 (0.0000)
Infrared, n = 10	0.46 (0.80)**	1.4 (1.4)**	0.0045 (0.015)
Exercise, n = 10	1.0 (3.1)**	1.5 (4.0),**n = 9	0.046 (0.098), n = 6
Total	0.74 (1.1), n = 30	1.0 (1.6), n = 29	0.0045 (0.029), n = 26
trans-DCCA			
Control, n = 10	0.45 (0.86)	0.47 (0.65)	0.054,$^{\infty}$ n = 1.
Infrared, n = 10	0.35 (0.56)$^{\#}$	0.96 (1.7)$^{\#}$	N/A
Exercise, n = 10	0.51 (0.58)	0.77 (2.2), n = 9	N/A
Total	0.40 (0.66), n = 30	0.57 (1.2), n = 29	0.054,$^{\infty}$ n = 1.

SG$_{norm}$ = urine specific gravity-normalised; SG$_{adj}$ = sweat specific gravity-adjusted.
*Significant difference between infrared urine TCPY pre and post concentrations (z = 2.395, p = 0.017) using related samples Wilcoxon Signed Rank Test.
**Significant difference between infrared sauna bathing urine PBA-pre and -post concentrations (z = 2.599, p = 0.009) and exercise urine PBA-pre- and -post concentrations (z = 2.073, p = 0.038) using related samples Wilcoxon Signed Rank Test.
#Significant difference between infrared urine *trans*-DCCA pre/post concentrations (z = 2.090, p = 0.037) using related samples Wilcoxon Signed Rank Test.
$^{\infty}$Only one sweat sample (#10-control) had detectable *trans*-DCCA > LOD; no IQR applicable.

3.4. TCPY

All urine samples (100%) had detectable levels of TCPY (Table 3). No significant differences were demonstrated in the urine concentrations of TCPY across the three interventions either pre: $X^2(2) = 3.800$, p = 0.150; or post: $X^2(2) = 2.667$, p = 0.264. However, urine pre/post intervention changes were detected independently within-intervention, but only with the sauna subset of TCPY concentration medians (IQR): pre 5.8 (10.5)/post 9.3 (11.9) ng/mL–SG$_{norm}$, z = 2.395, p = 0.017, and not with respective pre/post medians of exercise or control (Fig. 2, Fig.A4.2-Appendix). Few participants (3 of 10) had sufficiently detectable sweat TCPY concentrations, precluding any meaningful correlation analysis with urine levels.

3.5. 3-PBA

All urine samples (100%) had detectable levels of 3-PBA; conversely, only 27% of the sweat samples had concentrations above the LOD (Table 3). Differences in the urinary concentrations of 3-PBA were detected across all interventions: $X^2(2) = 6.200$, p = 0.045 (pre) and $X^2(2) = 8.222$, p = 0.016 (post). Median (IQR) urine concentrations of 3-PBA were the highest with exercise, in both pre and post datasets: 1.0 (3.1) ng/mL-SG$_{norm}$-pre and 1.5 (4.0) ng/mL-SG$_{norm}$-post (Table 4).

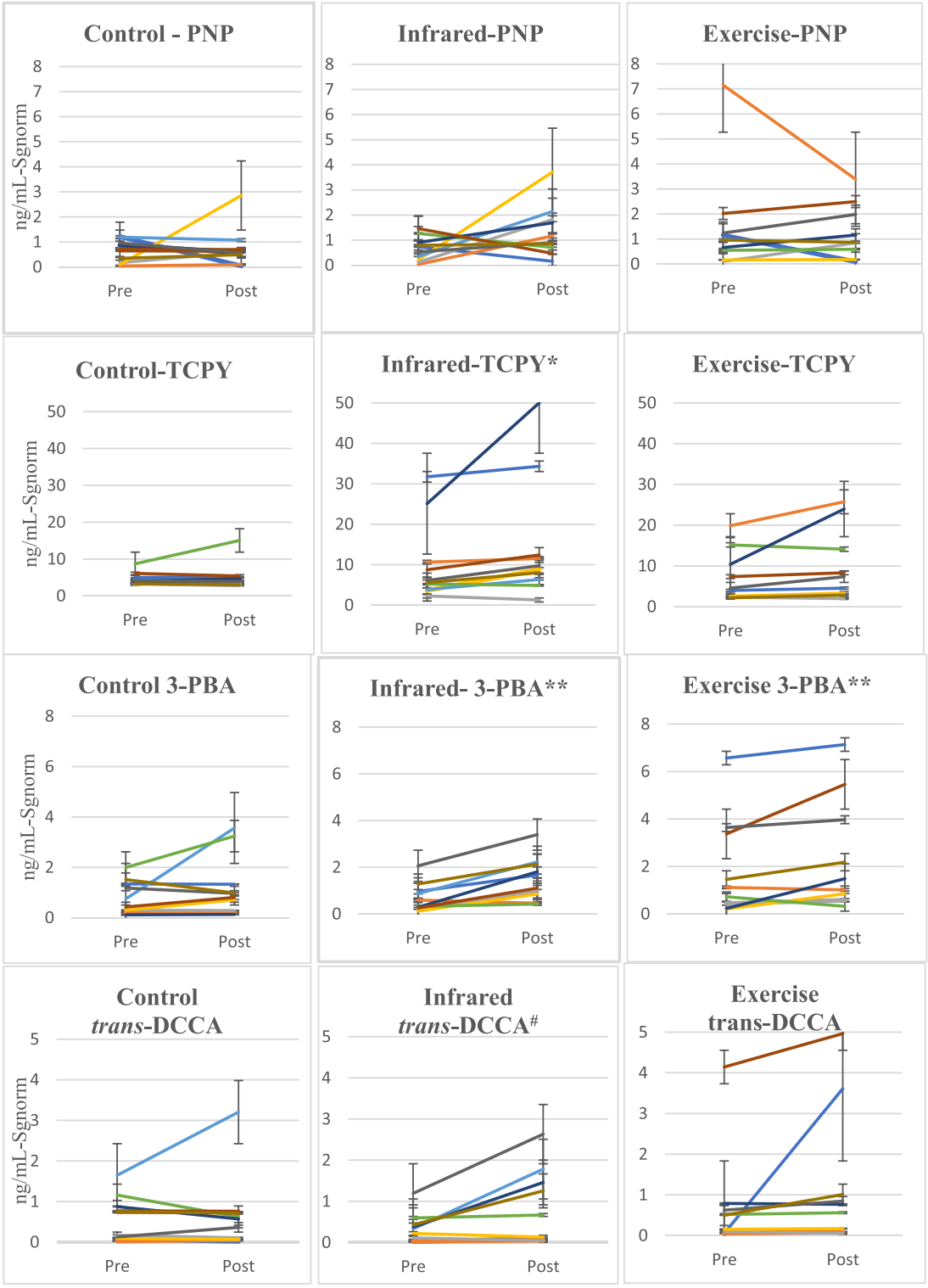


Fig. 2. Urine Concentrations of PNP, TCPY, 3-PBA and *trans*-DCCA.
Urine concentrations of metabolites, pre and post intervention, displayed by metabolite and by intervention. Results are colour-coded by individual participant, consistent across the different metabolites and interventions.
Error bars indicate standard error.
*, **, # Indicates statistical differences pre/post, as detailed in legend of Table 4. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

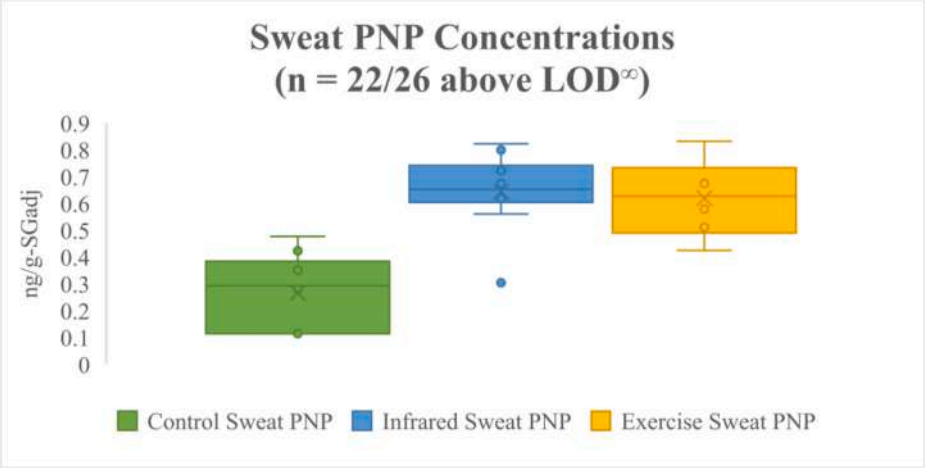


Fig. 3. Sweat concentrations of PNP, compared across interventions.
Median, average and quartiles displayed in standard box and whisker plot.
∞LOD = limit of detection.
Error bars indicate standard error.

Post-hoc testing confirmed pairwise differences between exercise and sauna at baseline: 3-PBA urine concentrations (EX 1.0 (3.1) *vs* IR 0.46 (0.80) ng/mL-SG_{norm}, z = −1.100, p = 0.042); yet an inconsistent pairwise difference (between exercise and control) at post-intervention: (EX 1.5 (4.0) *vs* CTRL 0.89 (1.5) ng/mL-SG_{norm}, z = −1.222, p = 0.029) (Table 4).

With sweat, no significant differences in 3-PBA concentration could be demonstrated between interventions: $X^2(2) = 2.333$, p = 0.311, despite a trend of higher median values of sweat 3-PBA concentrations with the exercise intervention (Fig.A4.3-Appendix). Inspections of matched sweat-to-urine 3-PBA concentrations (involving 9 participants) on scatterplots revealed possible correlations, particularly in the subset of sweat 3-PBA results from the exercise intervention. Further testing confirmed statistical correlation between sweat and urine 3-PBA pre intervention concentrations, but paradoxically not with the post intervention results: $r_s(24) = 0.524$, p = 0.006 (pre) vs $r_s(24) = 0.367$, p = 0.065 (post). Further testing on the subset of matched sweat-urine pre/post 3-PBA results (involving 6 participants) from only the exercise intervention revealed higher correlations: $r_s(4) = 0.992$, p < 0.0001 (pre) and $r_s(4) = 0.915$, p = 0.011 (post).

3.6. *trans*-DCCA

Of the urine samples, only 31% had detectable *trans*-DCCA concentrations and only one of the 26 sweat samples had a detectable level, precluding any correlation analysis. No significant changes in the urine concentration of *trans*-DCCA were detected in the pre or post intervention datasets when comparing across interventions: $X^2(2) = 0.800$, p = 0.670 (pre) and X^2 (2) = 4.222, p = 0.121 (post). Independent analysis within only the urine sauna dataset revealed a significant increase in pre-to-post medians {pre 0.35 (0.56)/post 0.96 (1.7) ng/mL–SG_{norm}, z = 2.090, p = 0.037} but not within respective datasets of exercise or control (Table 4, Fig. 2, Fig.A4.4-Appendix).

4. Discussion

4.1. Summary of findings

Urinary concentrations of TCPY, 3-PBA and *trans*-DCCA significantly increased in the women after sauna bathing, with 3-PBA urine levels also increasing after exercise. Urinary 3-PBA concentrations were significantly higher after exercise compared to control levels. 3-PBA was the only metabolite that displayed significant temporal variation (over days to weeks) in the urinary baseline measurements.

OP and PYR insecticide metabolites were detected in human sweat, with evidence of PNP in 84.6% (22/26) and 3-PBA in 26.9% (7/26) of the total 26 samples. Correlations between urine-sweat toxicant concentrations were not significantly appreciated, with the possible exception of 3-PBA. However, the 3-PBA urine-sweat correlations were based upon a suboptimal proportion of the sweat samples with detectable levels.

4.2. Comparative changes in urine OP, PYR metabolite concentrations by intervention

Our results are commensurate with the literature pertaining to urine OP/PYR biomonitoring (Li and Kannan, 2018), (Bravo et al., 2020), (Wielgomas and Piskunowicz, 2013), (Dereumeaux et al., 2018; Le Grand et al., 2012) and comparative studies are detailed in Appendix A5. Within-subject differences in the urine toxicant concentrations between activities were most pronounced with 3-PBA yet it was confounding that both the pre (baseline) and the post interventional datasets had significant but inconsistent pairwise differences (Fig. 2). With the pre intervention urine 3-PBA concentrations, the significant pairwise differences (in medians) were between exercise and infrared sauna, but not when comparing with control. Whereas with the post-intervention 3-PBA urine concentrations, the significant pairwise differences were instead between exercise and control, but not when comparing either to the infrared sauna. These findings suggest interesting possibilities.

Firstly, higher day-to-day variations in baseline 3-PBA urine concentrations, as compared to the other toxicants measured in this study, could explain some of these results. This is supported by the findings of a U.S. research study (Morgan et al., 2016) that investigated the temporal variability of urine PYR metabolites, reporting 3-PBA concentrations having large variability over 24 h, 1 week and 6 weeks in 50 adults, with the lowest levels detected in early morning samples. Nonetheless, these findings contradict a smaller study from Poland (Wielgomas, 2013) suggesting that urine 3-PBA levels were stable in 7 individuals over 7 days, evaluating first morning voids, spot samples and 24-hr urine samples.

Secondly, 3-PBA may be mobilised via different metabolic pathways with passive heat *vs* exercise. Also, these findings may reflect compensatory hydration state/urine concentrating effects from differing rates and amounts of sweating. It was recorded that most participants sweated earlier and more profusely (noted by the observing researcher) with the infrared sauna as compared to exercise, which supports this idea. However, urine 3-PBA concentrations tended to increase as a result of all the interventions (Fig. 2 and Fig.A4.3), as was statistically demonstrated with the sauna (p = 0.009) and exercise (p = 0.038) pre/post interventional concentrations.

In fact, a trend of urine toxicant concentrations increasing (post-

intervention > pre-intervention levels) after all the interventions (including control) was noted with all the toxicants. It was statistically demonstrated for TCPY, 3-PBA and *trans*-DCCA within the sauna intervention and in the case of 3-PBA, within the exercise intervention as well. This trend may reflect circadian-based effects, as all the interventions and sample collections were conducted at the same time of day (mornings). This was also implied by the study with temporal findings already mentioned, whereby the lowest levels of urine 3-PBA were detected in the mornings (Morgan et al., 2016).

Another recent study measuring temporal urinary levels of different environmental toxicants (OP flame retardants), also conducted in 10 adults, reported similar confounding findings to ours, concluding the differences in the inter- and intra-individual variance were compound specific and related to the nature of the exposure (*e.g.*, diet *vs* air *vs* topical, *etc.*) as well as the individual toxicokinetic properties of the toxicant (Bastiaensen et al., 2021). In our study, varying volumes of oral fluids consumed before study visits, hormonal fluctuations (anti-diuretic hormone, aldosterone, renin, fasting state hormonal influences, *etc.*), various degrees of post-exercise and emotional stress levels may also complicate the interpretation of trends.

4.3. OP, PYR metabolite concentrations in sweat

Measurable concentrations of PNP and 3-PBA were detected in participants' sweat samples without significant correlation with respective urinary concentrations. This finding is curious and suggests the metabolism through sweat may not be linearly dependent on blood/serum/plasma levels and/or liver/kidney detoxification mechanisms. Although this marks the first reporting of OP and PYR metabolites detected in human sweat, these results pose more questions than answers. Without definitively knowing the duration or route of toxicant exposure in these participants, this finding could also point to the skin surface as a residual reservoir from prior exposure(s). Also, these findings could reflect environmental contaminations during the sweat collection process, despite our best efforts to avoid this.

Nevertheless, PNP was the best detected metabolite (84.6% of pooled samples) and every participant had at least one of their pooled sweat samples with concentrations of PNP that were either similar or greater to corresponding concentrations in urine (Fig.A4.1). This hints at PNP exposures and/or metabolism involving the skin and/or sweat glands differently or to a greater degree than the other toxicants and highlights the need for further studies to reproduce and elaborate on these findings.

4.4. Study strengths and limitations

The main strength of this study was its novel crossover design, which incorporated a control intervention instead of a control group. This allowed for temporal and interventional comparisons of these OP and PYR metabolites in urine and sweat to be analysed with the individual participant serving as their own control. This highlighted intra-individual over inter-individual variations with the metabolism of these toxicants, of which much is still not known.

An obvious limitation of the study was the small sample size, with COVID-19 pandemic restrictions prematurely closing recruitment and data collection. Sweat-related limitations were also encountered. There were issues standardising concentrations to account for differences in sweat volumes/dilution effects. Although specific gravity measurements were employed to address this issue, methods for standardising sweat for metabolomics-related laboratory analysis are still being developed (Hussain et al., 2017). This is especially true with exercise or thermally-induced sweat as opposed to chemically-induced (with pilocarpine) sweat, which has evolved standardisation techniques, not without controversy, specific to its use for sweat chloride testing to diagnose cystic fibrosis (Collie et al., 2014). Although great strides were made to control external contaminations during the sweat collection procedures (including the collection of individualised synthetic sweat and censoring the data with LOD based on these control and additional blank collections), such interferences cannot be entirely excluded.

5. Conclusions

To our knowledge, this is the first reporting of OP and PYR metabolite detections in human sweat. Even though urine/sweat correlations were not demonstrated, these preliminary findings pave the way for sweat analysis to play a growing role in biomonitoring of insecticides and other classes of environmental contaminants. Further studies are required to assess the reliability and implications of these results.

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Author contributions

JH, MC, RG, XW and JFM conceptualised the study idea. JH conducted the data collections, statistical analyses and wrote the original manuscript draft. XW and YL coordinated all the laboratory-based analyses. All authors provided interpretation, reviewed and edited the manuscript.

Declarations of competing interest

All authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.114091.

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Environmental contamination with highly resistant microorganisms after relocating to a new hospital building with 100% single-occupancy rooms: A prospective observational before-and-after study with a three-year follow-up

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ABSTRACT

Introduction: Inanimate surfaces within hospitals can be a source of transmission for highly resistant microorganisms (HRMO). While many hospitals are transitioning to single-occupancy rooms, the effect of single-occupancy rooms on environmental contamination is still unknown. We aimed to determine differences in environmental contamination with HRMO between an old hospital building with mainly multiple-occupancy rooms and a new hospital building with 100% single-occupancy rooms, and the environmental contamination in the new hospital building during three years after relocating.

Methods: Environmental samples were taken twice in the old hospital, and fifteen times over a three-year period in the new hospital. Replicate Organism Direct Agar Contact-plates (RODACs) were used to determine colony forming units (CFU). Cotton swabs premoistened with PBS were used to determine presence of methicillin-resistant *Staphylococcus aureus,* carbapenemase-producing *Pseudomonas aeruginosa,* highly resistant Enterobacterales, carbapenem-resistant *Acinetobacter baumannii,* and vancomycin-resistant *Enterococcus faecium*. All identified isolates were subjected to whole genome sequencing (WGS) using Illumina technology.

Results: In total, 4993 hospital sites were sampled, 724 in the old and 4269 in the new hospital. CFU counts fluctuated during the follow-up period in the new hospital building, with lower CFU counts observed two- and three years after relocating, which was during the COVID-19 pandemic. The CFU counts in the new building were equal to or surpassed the CFU counts in the old hospital building. In the old hospital building, 24 (3.3%) sample sites were positive for 49 HRMO isolates, compared to five (0.1%) sample sites for seven HRMO isolates in the new building ($P < 0.001$). In the old hospital, 89.8% of HRMO were identified from the sink plug. In the new hospital, 71.4% of HRMO were identified from the shower drain, and no HRMO were found in sinks.

Discussion: Our results indicate that relocating to a new hospital building with 100% single-occupancy rooms significantly decreases HRMO in the environment. Given that environmental contamination is an important source for healthcare associated infections, this finding should be taken into account when considering hospital designs for renovations or the construction of hospitals.

1. Introduction

Inanimate surfaces in hospitals, especially in patient rooms and bathrooms, can be a reservoir for pathogenic and possibly highly resistant microorganisms (HRMO) (Weber et al., 2013). From these environmental reservoirs, microorganisms can be transmitted to patients. Depending on the species, microorganisms are able to survive in the

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List of abbreviations

Aztreonam broth	Tryptic soy broth with aztreonam 75 mg/L
CFU	Colony forming units
CPE	Carbapenemase-producing Enterobacterales
CP-PA	Carbapenemase-producing *Pseudomonas aeruginosa*
CR-AB	Carbapenem-resistant *Acinetobacter baumannii*
Erasmus MC	Erasmus MC University Medical Center, Rotterdam, the Netherlands
ESBL	Extended-spectrum β-lactamase
ESBL-E	Extended-spectrum β-lactamase-producing Enterobacterales
FCW	Facility care worker
HAI	Healthcare-associated infections
HRMO	Highly resistant microorganisms
ICU	Intensive Care Unit
IPC	Infection prevention and control
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight mass spectrometry
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
PBS	Phosphate buffered saline
RODAC	Replicate Organism Direct Agar Contact-plates
TSA	Trypticase Soy Agar
TSB	Tryptic Soy Broth
Vancomycin broth	Tryptic soy broth with vancomycin 50 mg/L
VIM-PA	VIM-positive *Pseudomonas aeruginosa*
VRE	Vancomycin-resistant *Enterococcus faecium*
WGS	Whole genome sequencing

environment for long periods of time, ranging from a few hours up to several months or even years (Kramer et al., 2006; Suleyman et al., 2018). Environmental contamination of patient rooms can therefore be a prolonged source of pathogens. A review of 1561 published outbreaks has identified that the hospital environment was the source in almost one fifth of the studied outbreaks (Gastmeier et al., 2006). Furthermore, various studies have shown that when the previous room occupant was colonized or infected with an HRMO, subsequent patients had an increased risk for acquisition of that microorganism (Mitchell et al., 2015; Wu et al., 2019). This illustrates that transmission via the environment also occurs in non-outbreak settings. Additionally, Chen et al. showed transmission from the environment to patients and vice versa for methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci, and *Clostridioides difficile* (Chen et al., 2019). These findings all highlight the importance of achieving a microbiologically safe hospital environment for patients. Cleaning is a key component for this, but hospital design, disinfection practices, and surface composition should be taken into account as well.

New hospital designs nowadays frequently consist of either mainly or only single-occupancy rooms. Research indicates that single-occupancy rooms are an important infection prevention and control (IPC) measure, and are part of aiming for a healing environment in general (Schreuder et al., 2016; Stiller et al., 2016). Transitioning from multiple-occupancy rooms to single-occupancy rooms eliminates the risk of acquiring a microorganism from infected or colonized roommates (*i.e.* via direct or indirect contact), but not from prior room occupants (*i.e.* indirect contact). Currently, literature about the effect of single-occupancy rooms on environmental contamination is lacking.

On May 18, 2018, the Erasmus MC University Medical Center, Rotterdam, the Netherlands (Erasmus MC), relocated from an old hospital building with mainly multiple-occupancy rooms and shared bathrooms to a newly constructed hospital building with 100% single-occupancy rooms with private bathrooms. This provided a unique opportunity to study differences in environmental contamination between multiple- and single-occupancy rooms. We aimed to determine differences in environmental contamination between multiple-occupancy rooms and single-occupancy rooms in a non-outbreak setting, by determining the overall number of colony forming units (CFU) and the presence of HRMO on different locations in patient rooms and bathrooms. Second, we aimed to determine changes in environmental contamination of the newly constructed hospital over a three-year follow up-period. Third, we aimed to determine if there was persistent contamination of surfaces over time by using whole genome sequencing (WGS), and to identify clusters.

2. Methods

2.1. Study design

This prospective observational before-and-after study was performed in the Erasmus MC, a university hospital in Rotterdam, the Netherlands. Environmental sampling was performed between April 2018, and May 2021. The relocation to the new hospital building took place during the study period, at May 18, 2018. Samples were taken at two moments in the old hospital building; two weeks and one week before relocating (Fig. 1). In the new hospital building, samples were taken at 15 different moments; two weeks, one week and one day before relocating patients, and one day, one week, two weeks, one, three, six, nine, 12, 15, 18, 24, and 36 months after relocating patients (Fig. 1).

2.2. Study setting

2.2.1. Old hospital building

The old hospital building of the Erasmus MC opened in 1961, consisted of 1200 beds, and had mainly two- and four-patient rooms and shared bathrooms. Exceptions were the adult Intensive Care Unit (ICU), which consisted of only single-occupancy rooms; the isolation ward, which consisted of single-occupancy rooms with anterooms and private bathrooms, and three hematology wards, which consisted of mainly single-occupancy rooms with anterooms and private bathrooms. Additionally, hematology ward I had one three-patient room, hematology ward II had two two-patient rooms, and hematology ward III had two two-patient and two three-patient rooms, all with attached bathrooms. Two of the hematology wards were located at another location in Rotterdam; the Erasmus MC Cancer Institute, location Daniel den Hoed. The Cancer Institute also relocated to the new hospital building on May 18, 2018.

In the old hospital building, 10 two-person rooms, 15 four-person rooms, four isolation rooms with anteroom, three hematology rooms with anteroom, 10 ICU rooms, of which two with anteroom, and nine bathrooms were sampled. Two hematology rooms were located at the Cancer Institute. Of the sampled bathrooms, one belonged to a hematology room and one to an isolation room. In Supplementary file 1, the medical specialty corresponding to the sampled patient rooms and bathrooms is described.

2.2.2. New hospital building

The new hospital building consisted of 503 single-occupancy rooms with private bathrooms, 22 isolation rooms with anterooms and private bathrooms, and 56 single-occupancy adult ICU rooms. While isolation rooms in the old hospital building where located at one ward, isolation rooms in the new building were located at multiple wards in the hospital

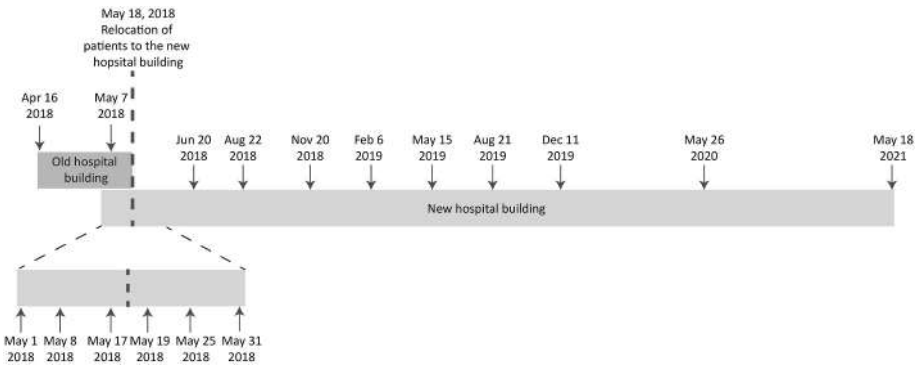

Fig. 1. Timeline of the study. Arrows indicate the sampling moments in the old and the new hospital building.

building.

In the new hospital building, 30 single-occupancy rooms, of which three hematology and four isolation rooms, all with anterooms; 10 ICU rooms, of which two with anteroom; and 10 bathrooms were sampled. Bathrooms sampled in the new building belonged to eight included single-occupancy rooms, one included hematology room, and one included isolation room (Supplementary file 1). Rooms were selected before the start of sampling and the same rooms were sampled during each sampling moment, unless it was not possible to enter the room (*e.g.* patient was in a clinically unstable condition or was admitted with an indication for isolation in a normal patient room). In these circumstances, a nearby patient room was sampled.

2.3. Sample sites

Sample sites in patient rooms were the nightstand, table, wall, sink, and the top and bottom of the sink plug (Supplementary file 2). When multiple nightstands or tables were present in a patient room, all were sampled. In four-person rooms, two locations on the wall were sampled. Sample sites in bathrooms were the toilet seat, shower chair, shower drain, door handle on the inside of the bathroom, the sink, and the top and bottom of the sink plug (Supplementary file 2). Sink plugs were installed in 2013 in six wards, including the ICU, as an IPC measure, to prevent splashing of water from the sink drain. In the old building, sink plugs were not present in 31 sinks. When not present, the top of the sink drain was sampled, which was considered the same sample site as the bottom of the sink plug for analyses. In the new hospital building, a sink plug was present in all sinks, with the exception of one sampled bathroom sink, where the top of the sink drain was sampled. In rooms with an anteroom (*e.g.* hematology and isolation rooms), the sink was located in the anteroom instead of in the patient room. Furthermore, in both the old and the new hospital building, two ICU rooms had a sink in the anteroom and a sink in the patient rooms. For these rooms, both the sink and sink plug in the anteroom, as well as the sink and sink plug in the patient room were sampled.

2.4. Sampling methods

To determine the total number of CFUs, Replicate Organism Direct Agar Contact-plates (RODAC) with Trypticase Soy Agar (TSA) with Lecithin and Polysorbate 80 (Bruker Daltonics, Bremen, Germany) were used. Of all sample sites, one RODAC per sampling moment was taken, with the exception of the bottom of the sink plug. Since it was not feasible to sample the bottom of the sink plug with a RODAC, CFU counts were not determined for this location. The RODACs were pressed firmly on surfaces for about 10 s, according to standard practice. For the door handle and the top of the sink plug, the RODAC was carefully rotated over the surface, to ensure that the whole RODAC came in contact with the surface. Sterile cotton swabs (BSN medical, Almere, the Netherlands) were used to determine the presence of MRSA, vancomycin-resistant *Enterococcus faecium* (VRE), extended-spectrum β-lactamase (ESBL)-producing Enterobacterales (ESBL-E), carbapenemase-producing Enterobacterales (CPE), carbapenemase-producing *Pseudomonas aeruginosa* (CP-PA), and carbapenem-resistant *Acinetobacter baumannii* (CR-AB). For each sampling site, two swabs were pre-wetted with phosphate buffered saline (PBS) before sampling a standardized surface of 100 cm^2 (Supplementary file 2). During sampling, swabs were rotated and moved in multiple directions as predefined in our sampling protocol (Supplementary file 2). Due to the specific shapes of door handles, shower drains and the top and bottom of the sink plug, no standardized surface of 100 cm^2 was sampled. Instead, the complete surfaces were sampled, while the swab was rotated and moved in multiple directions according to our protocol. Directly after sampling, in random order, one swab was placed in a tryptic soy broth (TSB) with aztreonam 75 mg/L (aztreonam broth) and one swab in TSB with vancomycin 50 mg/L (vancomycin broth).

2.5. Microbiological methods

RODACs were incubated twice overnight at 35 °C, after which CFUs were counted. When more than 100 colonies were counted, this was reported as >100 CFU. Both the vancomycin and the aztreonam broth were incubated for 24 h at 35 °C.

On the incubated aztreonam broth, a *vanA, vanB, mecA/mecC* PCR was performed using established procedures. When the *vanA/B* PCR was positive, a *Brilliance*™ VRE (Oxoid, Basingstoke, UK), was inoculated and incubated twice overnight at 35 °C. All suspected *Enterococcus* spp. colonies were identified to species level using Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight mass spectrometry (MALDI-TOF) (Bruker Daltonics, Bremen, Germany) running the MBT Compass Library, Revision E; MBT 7854 MSP Library and MBT Compass Library, Revision F MBT 8468 MSP Library. For *E. faecium* isolates, an additional *vanA* and *vanB* PCR was performed. When the *mecA/mecC* PCR was positive, a TSA plate with 5% sheep blood (blood agar [Becton Dickinson, New Jersey, USA]) and a BBL™ CHROMagar ™ MRSA II* (Becton Dickinson, New Jersey, USA) were inoculated and incubated twice overnight at 35 °C. All morphologically suspected *S. aureus* isolates were identified using MALDI-TOF. A cefoxitin disk diffusion was performed on a Mueller Hinton agar (Becton Dickinson, New Jersey, USA). A growth inhibition zone of <22 mm was considered resistant and confirmatory for MRSA.

From the incubated vancomycin broth, a CHROMID® CARBA SMART Agar (bioMérieux, Marcy-l'Etoile, France), and an ESBL plate (Oxoid, Basingstoke, UK) were inoculated and incubated twice overnight at 35 °C. All morphologically different colonies were identified to species level using MALDI-TOF. For *P. aeruginosa, A. baumannii*, and Enterobacterales isolates growing on the CARB side of the CHROMID® CARBA SMART agar, a PCR was performed to detect bla_{VIM}, bla_{IMP}, bla_{NDM}, bla_{KPC} and $bla_{OXA\text{-}48\text{-}like}$ genes using established procedures. For isolates growing on the OXA side, an OXA-48-like PCR was performed. When the PCR was negative, a CIM test was performed for *P. aeruginosa*

and Enterobacterales, and an antimicrobial susceptibility test with VITEK®2 (bioMérieux) for *A. baumannii*. Colonies growing on the ESBL plate were identified to species level using MALDI-TOF. Antimicrobial susceptibility was determined with VITEK®2, and a combination disk-diffusion method (ESBL + AmpC Screen Kit; Rosco Diagnostica, Taastrup, Denmark) was performed to phenotypically confirm the presence of an ESBL. A CIM test was performed when the presence of a carbapenemase was suspected as well.

Antibiotic susceptibility results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (European Committee on Antimicrobial Susceptibility Testing: Clinical breakpoints, 2017). All identified MRSA, VRE, ESBL-E, CPE, CP-PA and CR-AB isolates were stored at −80 °C.

2.5.1. Whole genome sequencing

WGS was performed for all identified isolates. DNA was extracted using the MagNA pure 96 platform (Roche Applied Science, Mannheim, Germany) and shipped to Novogene (HongKong, China) for sequencing. Genomic DNA was fragmented by shearing to a size of ~350 bp. Libraries were prepared using the NEBNext® DNA Library Prep kit (New England Biolabs, Ipswich, MA, USA) and subjected to 150 bp paired-end sequencing generating >100 × coverage using Illumina. Incidental, samples were sequenced using the in-house platform. Library preparation was conducted with the Illumina DNA Prep (Illumina, San Diego, CA, United States). Sequencing was conducted using the iSeq 100 System (Illumina) generating 150 bp paired-end reads. *De novo* genomic assemblies were generated using CLC Genomics Workbench v21 (Qiagen, Hilden, Germany). Presence of antibiotic resistance genes was analyzed using the web-based interface of the Comprehensive Antimicrobial Resistance Database (CARD - https://card.mcmaster.ca/accessed on July 4.2022). The analysis was restricted to include perfect and strict hits (Alcock et al., 2020; Jia et al., 2017). Plasmid replicon types were detected using the online Plasmidfinder software v2.1 (https://cge.food.dtu.dk/services/PlasmidFinder accessed on November 16, 2022/) with default settings (Carattoli et al., 2014) Identification confirmation was performed using the Type strain genome server (TYGS) (https://tygs.dsmz.de)/(Meier-Kolthoff and Göker, 2019). For *Enterobacter* spp. and *P. aeruginosa*, conventional Multi Locus Sequence Types (MLST) and core-genome MLST (cgMLST) or whole-genome MLST analysis (wgMLST) was performed using the available schemes available in BioNumerics (Applied Maths, St-Martens-Latem, Belgium) and for *K. pneumoniae* and *E. faecium* using the schemes available in SeqSphere (Ridom, Munster, Germany). For *Citrobacter freundii* an *ad hoc* wgMLST scheme was created in SeqSphere using the cgMLST Target Definer v1.5 with the genomic sequence of the Type strain (ATCC 8090, accession nr. CP049015.1) as seed genome and 24 NCBI Refseq genomes as penetration query genomes. Genomes improperly assigned to *C. freundii* and plasmid based genes were excluded. The resulting scheme consisted of 3162 core genes and 1142 accessory genes. The sequence data for this study has been deposited in BioProject ID: PRJNA904531.

2.5.2. Cleaning protocol

In both hospital buildings, the same external company was hired for environmental cleaning of hospital surfaces. Both in the old and new hospital building, rooms were cleaned daily with microfiber cloths dampened with water, unless disinfection was indicated. Sinks were part of this daily cleaning routine and the protocol for sink cleaning remained unchanged during the study period. To ensure quality, internal and external audits were performed regularly. After a patient in the old building was discharged, the nightstand and bed were removed to be cleaned, but no additional cleaning measures were taken besides daily cleaning. In the new hospital building, the whole room was cleaned before a new patient could be admitted to the room. Additionally, cleaning staff received extra training after relocating. Also, in the new building, facility care workers (FCW) were introduced. Several cleaning tasks were transferred from the cleaning staff to the FCW. In general, when no disinfection was indicated, the cleaning staff was responsible for the cleaning of the built in furniture, where the FCW was responsible for the cleaning of the other equipment and furniture in the room.

2.6. Statistical analyses

The different patient rooms were categorized in 1) general patient rooms (*i.e.* two- and four-person rooms in the old hospital building, and single-occupancy rooms on general wards in the new hospital building), 2) ICU rooms, 3) rooms with an anteroom (*i.e.* isolation rooms and hematology rooms), and 4) bathrooms. CFU counts per RODAC were converted into CFU counts per square cm (cm^2), by dividing the CFU counts by the surface of the RODAC. CFU counts per cm^2 were presented as medians. Differences between the sample moments in the old hospital building and between the two hospital buildings were analyzed using the Mann-Whitney-U test, differences within the new hospital building were analyzed with the Wilcoxon Signed-Ranks Test. Presence of HRMO was defined as yes/no, and presented with numbers and percentages, and analyzed with chi-squared analyses. $P < 0.05$ was considered statistically significant. IBM Statistical Package for the Social Sciences Solutions (SPSS) version 25 (IBM Corp., Armonk, New York, USA) was used for all analyses.

3. Results

3.1. Colony forming units over time

In total, 4993 sample sites were sampled, 724 in the old building and 4269 in the new building. RODACs were taken from 4211 out of 4993 (84.3%), 673 out of 724 (93.0%) sample sites in the old hospital building, and 3536 out of 4269 (82.8%) in the new hospital building. For nine (0.2%) sample sites the RODAC went missing in the laboratory, and the other 773 (15.5%) sample sites were bottom of sink plugs, where no RODACs were taken according to our sampling protocol. The highest median number of CFUs per cm^2 was identified from the shower drain (3.95 CFUs per cm^2), and the lowest from the wall (0.04 CFUs per cm^2).

The observed CFU counts per cm^2 at both sampling moments in the old hospital building are presented in Supplementary file 3. The CFU counts determined one month before relocating to the new hospital building were used as the reference for the old hospital building (Table 1). Before relocating patients to the new hospital building, we observed significantly lower CFU counts ($P < 0.05$, Table 1) for almost all locations in single-occupancy rooms and bathrooms compared to the old hospital building, but not for ICU rooms and for rooms with an anteroom (Table 1). After relocating patients, we observed an overall build-up in CFU counts during the first three months to a median of 0.47 CFU per cm^2, and fluctuating CFU counts after this moment (Fig. 2). The CFU counts in the new building were equal to or surpassed the median number of CFU counts in the old building within nine months for single-occupancy rooms, within 18 months for ICU rooms, within one month for rooms with anteroom, and within three months for bathrooms (Table 1). For the single-occupancy rooms, we observed significantly lower CFU counts ($P < 0.05$, Table 1) six months after relocating for all locations, while we observed significantly higher CFU counts ($P < 0.05$, Table 1) nine months after relocating. For the bathrooms, we noticed significantly lower CFU counts up to one month after relocation (Table 1). For the ICU rooms, the sink did not reach the same median number of CFU counts as in the old building, and we observed significantly lower CFU counts for the sink throughout the three year follow-up period (Table 1). At the two sampling moments during the COVID-19 pandemic (May 2020 and May 2021), we observed significantly lower CFU counts ($P < 0.05$, Table 1) in single-occupancy rooms, but not in other room types (Fig. 2, Table 1).

Table 1
The median CFU count per cm^2 determined in the new hospital building compared to the median CFU count per cm^2 determined in the old hospital building one month before relocating.

Room type	Sample site	Old hospital building	Two weeks before	One week before	One day before	One day after	One week after	Two weeks after	One month after	Three months after	Six months after [a]	Nine months after	12 months after	15 months after	18 months after	24 months after	36 months after
Single-occupancy room [b]	Overall	0.74	0.08 ↓89%	0.08 ↓89%	0.12 ↓84%	0.20 ↓73%	0.12 ↓84%	0.16 ↓78%	0.23 ↓69%	0.51 ↓31%	0.08 ↓89%	1.13 ↑52%	0.55 ↓26%	0.31 ↓58%	0.63 ↓15%	0.16 ↓78%	0.12 ↓84%
	Nightstand	1.46	0.20 ↓86%	0.23 ↓84%	0.39 ↓73%	0.66 ↓55%	0.59 ↓60%	1.05 ↓28%	0.86 ↓41%	1.37 ↓6%	0.41 ↓72%	3.40 ↑133%	1.09 ↓25%	0.94 ↓36%	2.19 ↑50%	0.70 ↓52%	0.43 ↓71%
	Table	1.29	0.04 ↓97%	0.12 ↓91%	0.10 ↓92%	0.20 ↓84%	0.39 ↓70%	0.51 ↓60%	0.66 ↓49%	0.78 ↓40%	0.47 ↓64%	3.13 ↑143%	0.78 ↓40%	0.31 ↓76%	2.19 ↑70%	0.27 ↓79%	0.39 ↓70%
	Wall	0.12	0.00 ↓100%	0.00 ↓100%	0.00 ↓100%	0.00 ↓100%	0.04 ↓67%	0.00 ↓100%	0.00 ↓100%	0.04 ↓67%	0.04 ↓67%	0.47 ↑292%	0.12 0%	0.00 ↓100%	0.08 ↓33%	0.04 ↓67%	0.00 ↓100%
	Sink	0.31	0.12 ↓61%	0.04 ↓87%	0.12 ↓61%	0.20 ↓35%	0.08 ↓74%	0.08 ↓74%	0.27 ↓13%	0.39 ↑26%	0.04 ↓87%	0.59 ↑90%	0.31 0%	0.59 ↑90%	0.27 ↓13%	0.16 ↓48%	0.23 ↓26%
	Top of sink plug	0.39	0.00 ↓100%	0.04 ↓90%	0.12 ↓69%	0.20 ↓49%	0.39 0%	0.27 ↓31%	0.12 ↓69%	0.63 ↑62%	0.00 ↓100%	0.23 ↓41%	0.63 ↑62%	0.59 ↑51%	0.63 ↑62%	0.08 ↓79%	0.12 ↓69%
ICU room	Overall	0.25	0.04 ↓84%	0.12 ↓62%	0.18 ↓28%	0.18 ↓28%	0.20 ↓20%	0.08 ↓68%	0.08 ↓68%	0.12 ↓62%	0.21 ↓16%	0.08 ↓68%	0.14 ↓44%	0.12 ↓62%	0.33 ↑32%	0.10 ↓60%	0.08 ↓68%
	Nightstand	0.25	-	-	0.57 ↑128%	0.55 ↑120%	0.59 ↑136%	0.08 ↓68%	0.12 ↓52%	0.23 ↓8%	0.59 ↑136%	0.47 ↑88%	0.23 ↓8%	0.35 ↑40%	0.63 ↑152%	0.55 ↑120%	0.53 ↑112%
	Wall	0.06	0.00 ↓100%	0.04 ↓33%	0.00 ↓100%	0.00 ↓100%	0.04 ↓33%	0.00 ↓100%	0.02 ↓66%	0.02 ↓66%	0.04 ↓33%	0.04 ↓33%	0.00 ↓100%	0.06 0%	0.27 ↑350%	0.10 ↑67%	0.04 ↓33%
	Sink	1.56	0.18 ↓88%	0.20 ↓87%	0.33 ↓79%	0.45 ↓71%	0.20 ↓87%	0.21 ↓87%	0.21 ↓87%	0.47 ↓70%	0.29 ↓81%	0.14 ↓91%	0.20 ↓87%	0.16 ↓90%	0.23 ↓85%	0.06 ↓96%	0.12 ↓92%
	Top of sink plug	0.41	0.02 ↓95%	0.10 ↓76%	0.14 ↓66%	0.06 ↓85%	0.31 ↓24%	0.16 ↓61%	0.08 ↓80%	0.68 ↑66%	0.47 ↑15%	0.00 ↓100%	0.16 ↓61%	0.08 ↓80%	0.39 ↓5%	0.00 ↓100%	0.04 ↓90%
Room with anteroom	Overall	0.23	0.20 ↓13%	0.12 ↓48%	0.47 ↑104%	0.04 ↓83%	0.12 ↓48%	0.12 ↓48%	0.23 0%	0.16 ↓30%	0.16 ↓30%	0.18 ↓22%	0.16 ↓30%	0.23 0%	0.39 ↑70%	0.16 ↓30%	0.20 ↓13%
	Nightstand	0.51	0.47 ↓8%	0.27 ↓47%	0.35 ↓15%	0.63 ↑24%	0.39 ↓24%	1.56 ↑206%	1.17 ↑129%	1.56 ↑206%	0.59 ↑16%	1.29 ↑153%	0.27 ↓47%	0.39 ↓24%	1.17 ↑129%	1.88 ↑269%	1.84 ↑261%
	Table	0.35	0.20 ↓43%	0.20 ↓43%	0.47 ↑34%	1.37 ↑291%	0.31 ↓11%	0.59 ↑69%	0.27 ↓23%	0.35 0%	2.73 ↑680%	1.48 ↑323%	0.55 ↑57%	1.25 ↑257%	2.46 ↑603%	0.51 ↑46%	0.55 ↑57%
	Wall	0.04	0.12 ↑200%	0.04 0%	0.00 ↓100%	0.00 ↓100%	0.00 ↓100%	0.00 ↓100%	0.00 ↓100%	0.04 0%	0.00 ↓100%	0.16 ↑300%	0.00 ↓100%	0.04 0%	0.08 ↑100%	0.00 ↓100%	0.12 ↑200%
Bathroom	Sink	0.23	0.16 ↓30%	0.12 ↓48%	0.47 ↑104%	0.00 ↓100%	0.04 ↓83%	0.08 ↓65%	0.16 ↓30%	0.04 ↓83%	0.04 ↓83%	0.04 ↓83%	0.08 ↓65%	0.23 0%	0.12 ↓48%	0.08 ↓65%	0.04 ↓83%
	Top of sink plug	0.20	0.20 0%	0.08 ↓60%	0.04 ↓80%	0.04 ↓80%	0.20 0%	0.08 ↓60%	0.27 ↑35%	0.35 ↑75%	0.08 ↓60%	0.08 ↓60%	0.04 ↓80%	0.08 ↓60%	0.39 ↑95%	0.08 ↓60%	0.08 ↓60%
	Overall	1.76	0.08 ↓95%	0.08 ↓95%	0.10 ↓94%	0.39 ↓78%	0.51 ↓71%	0.55 ↓69%	1.02 ↓42%	1.76 0%	1.13 ↓36%	3.81 ↑116%	1.52 ↓14%	3.24 ↑84%	3.40 ↑104%	1.64 ↓7%	1.31 ↓26%
	Toilet seat	1.56	0.10 ↓94%	0.16 ↓90%	0.12 ↓26%	0.27 ↓83%	0.98 ↓37%	1.15 ↓62%	1.04 ↓33%	1.52 ↓3%	2.58 ↑65%	3.05 ↑96%	1.09 ↓30%	2.25 ↑44%	1.66 ↑6%	2.13 ↑37%	1.27 ↓19%
	Shower chair	2.11	0.04 ↓98%	0.12 ↓94%	0.59 ↓72%	3.52 ↑67%	1.17 ↓45%	1.89 ↓10%	2.30 ↑9%	2.56 ↑21%	0.59 ↓72%	3.95 ↑87%	1.07 ↓49%	3.95 ↑87%	3.40 ↑61%	1.97 ↓7%	3.14 ↑49%
	Shower drain	3.95	0.57 ↓86%	0.06 ↓98%	0.12 ↓97%	3.13 ↓21%	0.68 ↓83%	1.35 ↓66%	3.95 0%	3.95 0%	3.95 0%	3.95 0%	3.95 0%	3.95 0%	3.95 0%	3.95 0%	3.95 0%
	Door handle	2.07	0.08 ↓96%	0.10 ↓95%	0.08 ↓96%	0.20 ↓90%	0.25 ↓88%	0.57 ↓72%	0.25 ↓88%	0.86 ↓58%	0.45 ↓78%	2.77 ↑34%	1.33 ↓36%	0.82 ↓60%	1.66 ↓20%	0.47 ↓77%	0.20 ↓90%
	Sink	0.94	0.06 ↓94%	0.14 ↓85%	0.04 ↓96%	0.31 ↓67%	0.20 ↓79%	0.10 ↓89%	0.66 ↓30%	1.17 ↑24%	0.57 ↓39%	3.40 ↑262%	1.04 ↑11%	2.25 ↑139%	2.27 ↑141%	0.80 ↓15%	0.61 ↓35%
	Top of sink plug	1.76	0.04 ↓98%	0.04 ↓98%	0.00 ↓100%	0.47 ↓73%	0.27 ↓85%	0.04 ↓98%	0.37 ↓79%	1.56 ↓11%	0.41 ↓77%	3.67 ↑109%	0.66 ↓62%	2.64 ↑50%	0.51 ↓71%	1.68 ↓5%	0.82 ↓53%

Arrows and percentages indicate the change in CFU counts compared to the old hospital. For example, an overall CFU/cm^2 count of 0.08 in single-occupancy rooms two weeks before relocating is a decrease of 89% compared to a CFU count/cm^3 of 0.74 in the old building. This is a significant reduction, indicated by the color of the cell. Green cells indicate a significant decrease in CFU counts, red cells indicate a significant increase in CFU counts. Light green/light red 0.05<P<0.01, Green/Red 0.01≤P<0.001, Dark green/dark red P≤0.001. Before; before relocating patients, After; after relocating patients. Abbreviation: CFU colony forming units. [a] The RODACs for the single-occupancy rooms and bathrooms were accidentally incubated for 24 hours instead of 48 hours [b] Single-occupancy rooms were compared with the combined median CFU counts per cm^2 of two- and four patient rooms in the old hospital building.

3.2. Presence of highly resistant microorganisms in the environment

In the old building, 49 HRMO isolates were identified from 24 of the 724 (3.3%) sampled sites (Table 2). Thirty-seven out of 49 (75.5%) isolates were identified from patient rooms, not the ante-room or bathroom, and 44 out of 49 (89.8%) isolates were identified on the top or bottom of the sink plug (Table 2). In the new building, seven HRMO isolates were identified from five of the 4269 (0.1%) sampled sites, a significant decrease compared to the old building ($P < 0.001$) (Table 2). All seven isolates were identified in the patient bathroom, five (71.4%) were identified from the shower drain (Table 2). In the new building, no HRMO were identified from the top or bottom of sink plugs (Table 2).

In the old hospital building, 16 ESBL-E isolates were identified on 15 sample sites (eight *Enterobacter* spp., five *Citrobacter* spp., three *Klebsiella* spp.), 24 CP-PA isolates on 13 sample sites, and nine CPE isolates on five sample sites (four *C. freundii* isolates on three locations and five *Enterobacter* spp. isolates on three locations) (Table 2). In the new building, we identified three VRE isolates on three sample sites, three CPE isolates on one location (*E. hormaechei*) and one ESBL-E isolate on one sample site (*K. pneumoniae*) (Table 2). The three VRE positive locations were all identified in the same bathroom, one week after relocating. In both hospital buildings, no MRSA and CR-AB were detected.

WGS was performed on all strains. Unfortunately, due to human error, we were unable to link the results of the WGS of isolates identified in the old hospital building to the locations where the isolates were found. Details of the analysis of the isolates were shown in Supplementary file 4. Most noteworthy, in CP-PA isolates a bla_{VIM-2} gene was detected, whereas in carbapenem-resistant *C. freundii* it involved a bla_{KPC-2} gene and in carbapenem-resistant *Enterobacter* spp. a bla_{OXA-48} gene was detected. AmpC type beta-lactamase genes (*e.g.* bla_{CMY} and bla_{DHA}) were most often found in *C. freundii* (6 out of 8 isolates). In this relatively small collection of isolates, seven isolates (two *C. freundii* and five *E. asburiae*) contained an *mcr-9* variant gene, but this involved several clonally related isolates. Upon clone correction this involved 3 strains. Three *mcr*-9 positive isolates had an minimum inhibitory concentration (MIC) of 0.5 μg/mL, and four strains had an MIC of 8 μg/mL, as measured by Vitek 2. No other *mcr* genes were detected. In isolates that were considered to be genetically closely related, variation in the

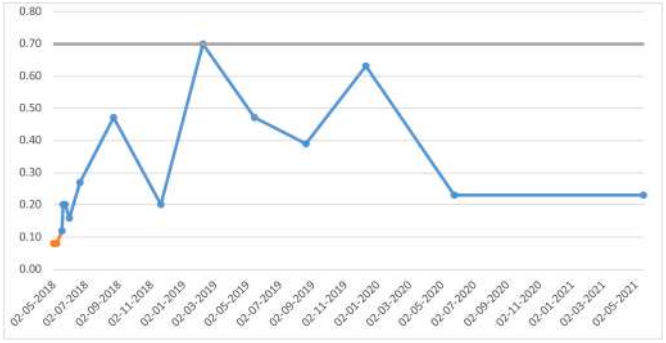

Fig. 2. Overall median CFU count per cm^2 determined over time in the new hospital building and the CFU count per cm^2 determined in the old hospital building one month before relocating as a reference. Orange line; CFU count in the new hospital building before relocating patients. Blue line; CFU count in the new hospital building after relocating patients. Grey line; CFU count observed one month before relocating patients in the old building, as reference value. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

presence of AMR genes was detected.

4. Discussion

The relocation to the new hospital building with 100% single-occupancy rooms with private bathrooms resulted in a significant reduction of environmental contamination with HRMO during the three-year follow-up period. We observed lower CFU counts up to three months after relocating, with fluctuating CFU counts after that moment. Two- and three years after relocating, during the COVID-19 pandemic, CFU counts in single-occupancy rooms were significantly lower compared to the multiple-occupancy rooms in the old hospital building.

Our findings should be considered in the broader context of the relocation. Besides the transition to 100% single-occupancy rooms, the introduction of a final cleaning after discharge of a patient in the new building might be associated with the reduction in environmental contamination with HRMO. Such a final cleaning is, however, more feasible in a single-occupancy room compared to a multiple-occupancy room. A second explanation for the higher number of HRMO identified in the old building is the number of VIM-positive *Pseudomonas aeruginosa* (VIM-PA) that was identified. The presence of VIM-PA in the old

Table 2
Number of sample sites positive for highly resistant microorganisms, and the number of resistant isolates detected on the sites during both sampling moments in the old hospital building and all sampling moments in the new hospital building.

Old hospital building							New hospital building						
Room type	Sample site	Positive sample sites (%)	ESBL-E	CPE	CP-PA	VRE	Room type	Sample site	Positive sample sites (%)	ESBL-E	CPE	CP-PA	VRE
Two- and four patient room	Nightstand (n = 149)	–	–	–	–	–	Single-occupancy room	Nightstand (n = 315)	–	–	–	–	–
	Table (n = 79)	–	–	–	–	–		Table (n = 324)	–	–	–	–	–
	Wall (n = 79)	–	–	–	–	–		Wall (n = 324)	–	–	–	–	–
	Sink (n = 50)	–	–	–	–	–		Sink (n = 324)	–	–	–	–	–
	Top of sink plug (n = 20)	1 (5.0)	1	–	–	–		Top of sink plug (n = 322)	–	–	–	–	–
	Bottom of sink plug (n = 50)	4 (8.0)	5	–	–	–		Bottom of sink plug (n = 324)	–	–	–	–	–
ICU room	Nightstand (n = 20)	–	–	–	–	–	ICU room	Nightstand (n = 128)	–	–	–	–	–
	Wall (n = 20)	–	–	–	–	–		Wall (n = 150)	–	–	–	–	–
	Sink (n = 24)	1 (4.2)	1	–	–	–		Sink (n = 181)	–	–	–	–	–
	Top of sink plug (n = 24)	3 (8.3)	1	–	4	–		Top of sink plug (n = 181)	–	–	–	–	–
	Bottom of sink plug (n = 24)	11 (45.8)	3	4	20	–		Bottom of sink plug (n = 181)	–	–	–	–	–
Room with anteroom	Nightstand (n = 14)	–	–	–	–	–	Room with anteroom	Nightstand (n = 88)	–	–	–	–	–
	Table (n = 14)	–	–	–	–	–		Table (n = 93)	–	–	–	–	–
	Wall (n = 14)	–	–	–	–	–		Wall (n = 95)	–	–	–	–	–
	Sink (n = 14)	–	–	–	–	–		Sink (n = 95)	–	–	–	–	–
	Top of sink plug (n = 0)	–	–	–	–	–		Top of sink plug (n = 95)	–	–	–	–	–
	Bottom of sink plug (n = 14)	1 (7.1)	1	2	–	–		Bottom of sink plug (n = 95)	–	–	–	–	–
Shared bathroom	Toilet seat (n = 18)	–	–	–	–	–	Private bathroom	Toilet seat (n = 138)	1 (0.7)	–	–	–	1
	Shower chair (n = 17)	–	–	–	–	–		Shower chair (n = 138)	1 (0.7)	–	–	–	1
	Shower drain (n = 17)	2 (11.8)	2	3	–	–		Shower drain (n = 138)	3 (2.2)	1	3	–	1
	Door handle (n = 18)	–	–	–	–	–		Door handle (n = 138)	–	–	–	–	–
	Sink (n = 20)	–	–	–	–	–		Sink (n = 138)	–	–	–	–	–
	Top of sink plug (n = 5)	–	–	–	–	–		Top of sink plug (n = 126)	–	–	–	–	–
	Bottom of sink plug (n = 20)	1 (5.0)	2	–	–	–		Bottom of sink plug (n = 138)	–	–	–	–	–
Total	Sample sites (n = 724)	24 (3.3%)	16	9	24	–	Total	Sample sites (n = 4269)	5 (0.1%)	1	3	–	3

Abbreviations: CPE, carbapenemase-producing Enterobacterales; CP-PA, carbapenemase-producing *Pseudomonas aeruginosa*; ESBL-E, extended-spectrum β-lactamase-producing Enterobacterales; VRE, vancomycin-resistant *E. faecium*; ICU, Intensive Care Unit.

building was known since 2010, as a long-lasting multi-ward outbreak with the ICU as most affected ward (Van der Bij et al., 2011). A persistent presence of VIM-PA in the sink drains of the ICU was then identified, which is reflected by the results of our study (Pham et al., 2022; Pirzadian et al., 2020; Pirzadian et al., 2022; Van der Bij et al., 2012; Van der Bij et al., 2011; Voor In 't Holt et al., 2018). To contain this reservoir, a bundle of 'water free' patient care was introduced in the ICU in 2011 (Pham et al., 2022). This was discontinued in the ICU in the new building, although for bathing of patients pre-packed washcloths remained instead of water and soap. After relocating to the new hospital building, VIM-PA did not colonize the sink drains within the time frame of this study. All *P. aeruginosa* isolates identified in our study all belonged to the outbreak strain (ST111) (Pirzadian et al., 2020). When we analyzed the difference in environmental contamination with HRMO between the old and the new hospital building without the VIM-PA strains, there were still significantly less HRMO identified in the new hospital building ($P < 0.001$).

Sinks and sink drains are known and important reservoirs for HRMO, and often play a role in outbreaks (Decker and Palmore, 2013; Kizny Gordon et al., 2017). Where in the old building 89.8% of HRMO isolates were identified from sink plugs, in the new building, no HRMO were identified from this location. This difference cannot be explained by a change in material. In both the old and the new building, drains and drain plugs were made of stainless steel. When we exclude sink plugs from the comparison between the old hospital and the new hospital building, the difference in environmental contamination is no longer statistically significant ($P = 0.06$), although this could also be explained by a lack of statistical power. However, for our hospital's new building, the decision was made to keep sinks in the ICU patient rooms, as a facility for healthcare workers to wash their hands and arms in case of unexpected contact with body fluids of the patient, or for specific microorganisms that are less susceptible to alcohol-based hand rub. Thus, these potential reservoirs of HRMO were present in the new building, but over a period of three years of patient care, we showed that they did not emerge as reservoir for HRMO again.

Overall, the contamination rates with HRMO in both hospital buildings were low, especially when compared to other studies, where they showed contamination of HRMO in up to 55% of rooms (Chen et al., 2019; Mody et al., 2019; Shams et al., 2016; Tanner et al., 2021). An important explanation for these low contamination rates is the difference in prevalence of HRMO. Most studies have been conducted in the United States of America, where the prevalence of HRMO carriage among patients is higher than in the Netherlands, with consequently higher environmental contamination rates (CDC, 2019; De Greeff and Mouton, 2017; Gupta et al., 2019). Secondly, an explanation for the low contamination rates could be the chosen sample method. Based on our selection of sampled surfaces, we decided to sample with premoistened cotton swabs. While this method has some disadvantages, such as difficulty to standardize, they also come with several important advantages (Rawlinson et al., 2019). Cotton swabs have high recovery rates on wet surfaces, similar or better recovery rates compared to other sampling methods, and they can be used on all surfaces, including surfaces that are more difficult to sample such as door handles (Moore and Griffith, 2007; Rawlinson et al., 2019; Rose et al., 2004). Additionally, since the swabs were directly placed in a selective broth, we were able to identify HRMO in low concentrations. A third explanation could be that, while other studies focused mostly on "high-touch" surfaces (*e.g.* bed rails, call buttons) we sampled built-in surfaces, with the exception of the nightstand (Chen et al., 2019; Mody et al., 2019; Shams et al., 2016; Tanner et al., 2021). These locations might be less frequently contaminated, but since these surfaces are used by all room occupants, they are potentially a better indicator of differences between multiple-occupancy and single-occupancy rooms. Interestingly, no sink or shower drains were sampled in the other studies, while we identified almost all HRMO on these surfaces, and not on "dry" surfaces (*i.e.* nightstands, tables). Notwithstanding, the contamination rates observed in our study are low, even after considering the low prevalence of HRMO in the Netherlands and our chosen sample methods. Thus, it is likely that other factors, such as our cleaning protocol, have contributed to these low rates.

There are several explanations for the fluctuations over the three year follow-up period in CFU counts per cm^2. As expected, the CFU counts in single-occupancy rooms and bathrooms were significantly lower before transferring patients to the new hospital building. However, this was not observed for the ICU rooms or rooms with an anteroom. One explanation for this is the fact that, while the construction of the single-occupancy rooms was mostly finished during the sampling moments, construction of the ICU rooms and rooms with anterooms was still ongoing. Consequently, more construction workers were present in these rooms, leading to relatively higher contamination levels. The fluctuations in CFU counts during the three years most likely reflected the use of the rooms. CFU counts were compared with the CFU counts determined in the old hospital building one month before relocating patients, since we believed that this was more representative for the contamination than the values determined one week before relocating patients. One week before relocating, the number of admissions to the hospital was lower, to prepare for the transfer of patients, and thus locations were used less frequently. We did not correct for use or nonuse of the bathroom by the patient. It is unclear why the CFU counts nine months after opening were higher in single-occupancy rooms. There were no changes in sampling or lab protocol that could explain the increase, and on later sampling moments, this increase in CFU counts was not observed again. A possible explanation is that there were changes in indoor temperature, or in humidity, which can impact the bacterial load (Klassert et al., 2021). However, since we did not measure this, we cannot be sure about this. The final two sampling moments took place during the COVID-19 pandemic. The lower CFU count could be explained by enhanced cleaning and increased disinfection rates with 1000 ppm chlorine. Only four of the included single-occupancy rooms were dedicated for suspected COVID-19 patients, and two of the included isolation rooms were dedicated for COVID-19-care.

Other studies have suggested a cutoff value for the number of CFU for hand contact surfaces in the healthcare environment. Dancer et al. suggested 5 CFU/cm^2, however, due to our cutoff value of 100 CFU per RODAC, which translates to a maximum of 3.95 CFU/cm^2, we were unable to determine if this criteria was exceeded (Dancer, 2004). Griffith et al. suggested <2.5 CFU/cm^2 as a cutoff value, a value that they found was practicable for all sites after disinfection (Griffith et al., 2000; Malik et al., 2003). Nonetheless, CFU counts are not helpful to determine if a source is contaminated with HRMO. While we did not determine the correlation between CFU counts and HRMO presence, other studies have not shown a correlation between CFU counts and HRMO presence (Al-Hamad and Maxwell, 2008; Widmer et al., 2019).

WGS was performed on all identified isolates. No persistent contamination over time was identified in the new hospital building. Remarkably, in isolates that were considered to be genetically closely related, variation in the presence of AMR genes was detected. We believe this to be the result of plasmid gain/loss in strains of otherwise identical genetic background. Plasmid gain/loss as possible explanation for these observations fell beyond the scope of this study. Another interesting result is that one *K. pneumoniae* strain was of ST16 (Supplement 4). This strain is an important emergent lineage of *K. pneumoniae*, has caused multiple outbreaks within European hospitals, and is known to carry multiple carbapenem resistance genes (Boff et al., 2021; Espinal et al., 2019). However, the strain identified in the old hospital did not carry any gene encoding carbapenem resistance. Another interesting finding is that seven *E. hormaechei* strains, both from the old and the new hospital building, were of ST78 (Supplement 4). ST78 isolates are successful One Health clones, that are considered high risk and are of global interest (Cardoso et al., 2022). Additionally, nosocomial infections with this ST, both in Europe and Asia, are increasingly reported (Gomez-Simmonds et al., 2018; Villa et al., 2019). The ST78 isolates we identified from the hospital environment were CPE

and carried bla_{OXA-48}. As far as we know, these strains have not yet lead to nosocomial infection in our patients, but it is important to monitor presence of this strain.

4.1. Strengths and limitations

The main strength of this study is that we sampled the old and the new hospital building, with identical sampling methods and sampling locations. A second strength of our study is the follow-up period of three years in the new building. This follow-up period not only provided us with the opportunity to look at a situation where environmental contamination had developed, but also provided time for that contamination to build up further. Thirdly, we did not focus on environmental contamination with one type of HRMO, but looked at the presence of MRSA, ESBL-E, CPE, CP-PA, CR-AB, and VRE. Finally, we sampled a large number of rooms, on different wards, including isolation-, hematology-, and ICU rooms.

A limitation of our study is that we were not able to sample every room at every sample moment. When a patient was cared for in isolation, in a non-isolation room, we did not sample this room, but we sampled a nearby room instead. During the next sampling moment, the original room was sampled again. Secondly, it is likely that our study shows an underestimation of the environmental contamination. This could be due to our chosen sampling method or the selected sample sites. On the other hand, every sample method or selection of sampled surfaces will inherently introduce bias, and hence, it is unlikely that other studies have not shown an underestimation of the contamination rates. Thirdly, we only determine presence of HRMO, and not the abundance in which they were present. However, since the concentration of nosocomial pathogens is generally low, they are often only detectable with broth enrichment, which makes determining the abundance impossible (Otter et al., 2011). Fourth, we did not correct for the timing and compliance of cleaning or disinfection. During the three-year follow-up, rooms were sampled 15 times, and at different time points during the day. Some rooms were sampled directly after daily or final cleaning, while other rooms were sampled before cleaning. Since rooms were located throughout the hospital and thus cleaned at different moments, and we looked at the median CFU counts, we believe that our results are representative for the environmental contamination of our hospital. Finally, we did not determine how our results correlate with the incidence of healthcare-associated infections (HAI).

5. Conclusion

We observed significantly less HRMO in the single-occupancy rooms in the new hospital building over the three-year follow up, while CFUs were not impacted. This finding shows that, with regard to environmental contamination, single-occupancy rooms are favorable over multiple-occupancy rooms. These finding should be taken into account when considering hospital designs for renovations or the construction of hospitals. Future research should focus on the effect of changes in environmental contamination on the incidence of HAI. Additionally, the effect of single-occupancy rooms on environmental contamination in countries with higher HRMO prevalence should be determined. Finally, the impact of transitioning to single-occupancy rooms on other environmental aspects, such as the microbiome, should be studied further.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Author contributions

Conceived and designed the study: MV, JS, AV, MB, JH, DG. Collecting data: AS, AV. Analyzed the data: AS, AV, CK. Wrote the paper: AS, AV. All authors read and approved the final manuscript.

Declaration of competing interest

AS, JS, CK, DG, MB, JH, AV, and MV declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.114106.

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Estimating the restraint of SARS-CoV-2 spread using a conventional medical air-cleaning device: Based on an experiment in a typical dental clinical setting

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ABSTRACT

Objectives: Droplets or aerosols loaded with SARS-CoV-2 can be released during breathing, coughing, or sneezing from COVID-19-infected persons. To investigate whether the most commonly applied air-cleaning device in dental clinics, the oral spray suction machine (OSSM), can provide protection to healthcare providers working in clinics against exposure to bioaerosols during dental treatment.

Method: In this study, we measured and characterized the temporal and spatial variations in bioaerosol concentration and deposition with and without the use of the OSSM using an experimental design in a dental clinic setting. *Serratia marcescens* (a bacterium) and *ΦX174 phage* (a virus) were used as tracers. The air sampling points were sampled using an Anderson six-stage sampler, and the surface-deposition sampling points were sampled using the natural sedimentation method. The Computational Fluid Dynamics method was adopted to simulate and visualize the effect of the OSSM on the concentration spatial distribution.

Results: During dental treatment, the peak exposure concentration increased by up to 2–3 orders of magnitude (PFU/m^3) for healthcare workers. Meanwhile, OSSM could lower the mean bioaerosol exposure concentration from 58.84 PFU/m^3 to 4.10 PFU/m^3 for a healthcare worker, thereby inhibiting droplet and airborne transmission. In terms of deposition, OSSM significantly reduced the bioaerosol surface concentration from 28.1 PFU/m^3 to 2.5 PFU/m^3 for a surface, effectively preventing fomite transmission.

Conclusion: The use of OSSM showed the potential to restraint the spread of bioaerosols in clinical settings. Our study demonstrates that OSSM use in dental clinics can reduce the exposure concentrations of bioaerosols for healthcare workers during dental treatment and is beneficial for minimizing the risk of infectious diseases such as COVID-19.

1. Introduction

Since December 2019, the coronavirus disease 2019 (COVID-19) has spread rapidly worldwide, causing enormous economic and social impacts (Mousavi et al., 2021; Salian et al., 2021). The sudden emergence of SARS-CoV-2 variants, such as Delta and Omicron, has further intensified the global prevention and control situation (Dai and Zhao, 2022a; Suzuki et al., 2022). As many healthcare systems approach capacity and capability limits, suppressing the spread of COVID-19 has become a globally sought-after and increasingly important goal (Dai and Zhao, 2022b). SARS-CoV-2 can be transmitted through droplets, contact with contaminated surfaces, and aerosols (Desai et al., 2021; Zhang et al., 2021; Zhao et al., 2020). SARS-CoV-2 is released in virus-carrying droplets and aerosols during respiratory activities such as breathing, speaking, and coughing (Anand, 2020; Dong et al., 2022; D'Orazio et al., 2021). Infection can occur through inhalation or direct deposition onto mucous membranes when exposed to full-size distributed droplets or aerosols at a close range (<2 m). Airborne transmission occurs at long

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distances (>2 m) by inhaling small aerosols suspended in the air. Fomite transmission occurs through contact with contaminated surfaces and subsequent contact with mucous membranes (Castaño et al., 2021). Owing to the highly infectious nature of SARS-CoV-2 (Mutesa et al., 2021), healthcare workers (HCWs) are at significant risk of infection during the treatment of patients with COVID-19, especially in dental clinics. In addition, the use of tools and instruments, such as high-speed turbine dental handpieces, ultrasonic scaling machines, and ultrasonic bone knives, during dental treatment intensifies the generation of bioaerosols, which are mixed with patients' blood, saliva, and various microorganisms (Cristina et al., 2008). A study showed that 16 species of bacteria and 23 species of fungi were detected in the air in dental treatment areas (Zemouri et al., 2017). Bioaerosols containing pathogens can spread infectious diseases, such as ophthalmic infections, tuberculosis, and hepatitis B (Kim et al., 2015; T. Liu et al., 2021). Studies have confirmed that the saliva of persons infected with COVID-19 contains SARS-CoV-2 (Huang et al., 2021). Therefore, dental clinics can be at a high risk of COVID-19 transmission when receiving COVID-19 patients. It is critical to understand the distribution of bioaerosols from patients in dental clinics and develop effective intervention strategies for protection against infectious diseases, including COVID-19.

In dental treatment, devices, such as rubber barriers, extraoral suction machines, and air purifiers, are often used to inhibit the spread of bioaerosols. Balanta-Melo et al. (2020) studied the effectiveness of a rubber barrier and high-volume suction device using a dummy simulating a patient for dental treatment. The experimental results confirmed that a rubber barrier combined with a high-volume suction device could significantly reduce dental aerosol particles. Chen et al. (2010) studied the migration patterns of indoor bacterial aerosols in a dental clinic after using air purifiers and found that air purifiers placed close to the patient's head could reduce the number of aerosol particles reaching the respiratory area of HCWs. Lloro et al. (2021) used an ATP fluorescent labeling method to evaluate the effectiveness of using perioral suction devices during dental scaling to reduce biological contamination and found that perioral suction devices could substantially reduce airborne contamination throughout the dental clinic environment. The oral spray suction machine (OSSM) is the most common air-cleaning device used in dental clinics, but its effectiveness in inhibiting the spread of bioaerosols is unknown.

To investigate the influence of OSSM use on reducing the transmission of pathogens such as SARS-CoV-2, two kinds of bioaerosols (*Serratia marcescens* and *ΦX174 phage*) were used as tracers to simulate the two conditions of using or not using an OSSM during dental treatment and to explore the effect of using an OSSM on the spatial distribution and deposition of bioaerosols during the dental treatment process. Air and surface deposition sampling were performed in the surgical area. The effect of OSSM adoption on pathogen transmission was also evaluated. The results will contribute to an in-depth understanding of the significance of medical devices for epidemic control, to efficiently support epidemic prevention and control in dental clinics.

2. Method

2.1. Description of the dental clinic, ventilation parameters, and bioaerosol

The study dental clinic, located in the Fifth Medical Center of the People's Liberation Army of China in Beijing, has dimensions of 4.5 m × 3.5 m × 3.5 m and an installed upper-supply and upper-return ventilation system. The dental clinic has four tables (one movable and three immovable), a wall cabinet, a dental chair, and dental consultation equipment. The room temperature was 28 °C, and the humidity in the room was 20%. The air change rate in the dental clinic was 5 ACH. During the dental treatment, the dentist and nurse were on the right and left sides of the patient, respectively. The mobile table was beside the dentist, which was convenient for the dentist to take and place the surgical tools. Additionally, an OSSM was provided with a suction port in front of the patient's breathing zone. *Serratia marcescens* (ATCC 8039) and *ΦX174 phage* (ATCCl3706-B1) were used as model pathogens in the experiments of bioaerosol release from the patient during dental treatment.

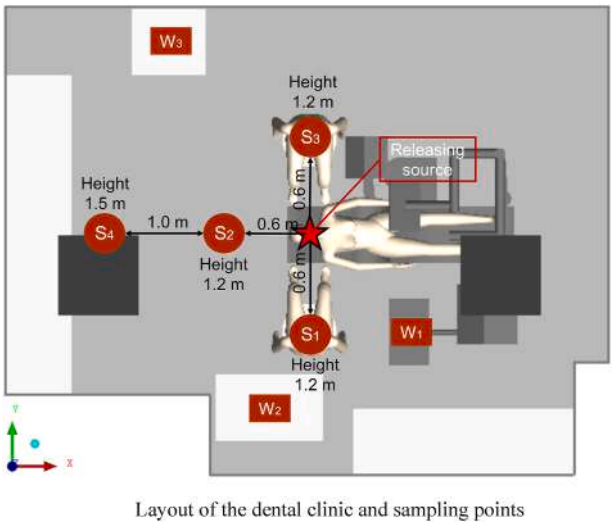

Fig. 1. Room layout and sampling points.

2.2. Experimental procedure

The experiments were carried out with and without the OSSM. The simulated dental treatment lasted for 30 min. Under the OSSM condition, the OSSM was turned on at the beginning of the dental treatment and turned off after the treatment. The air entering the OSSM was treated and returned to the room to clean the air.

Fig. 1 shows the layout of the sampling points in the dental clinic. Four air sampling points (S_1, S_2, S_3, and S_4) were set up, and surface deposition samples were collected on three key tables (W_1, W_2, and W_3) in the operation area used to place the surgical equipment. The patient lay on a chair, and the position of the patient's mouth was 0.9 m perpendicular to the ground. The aerosol generator was fixed at this point for bioaerosol release. The release rate of the aerosol generator was constant and set as the real emission rate of bioaerosols from a patient during dental surgery (Micik et al., 1969). Aerosol production lasted 30 min, releasing 1.25×10^9 CFU(PFU) microorganisms into the room. In the aerosol droplet experiments, volatile and nonvolatile substances were mixed to prepare artificial saliva to ensure realistic simulations (Chao and Wan, 2006; Leung et al., 2013). Considering the volatility of droplet aerosols, artificial saliva containing non-volatile components with a mass concentration of 8% was used as the aerosol generation material, and its evaporation rate was close to that of real saliva (Wang et al., 2022). The released solution was formulated using a bacterial strain and phosphate-buffered saline (PBS).

Simultaneously, four Andersen six-stage samplers were placed at S_1–S_4 for air sampling, and 28.3 L/min was set as the flow rate. S_1, S_2, and S_3 were 0.6 m from the release source, at a height of 1.2 m above the ground. The HCWs operated within this range most of the time. S_1 was located at the height of the dentist's nose, and S_3 was located at the height of the nurse's nose. S_4 was located 1.0 m behind S_2 and at a height of 1.5 m. Three open Petri dishes (φ90) were placed on the surface of W_1–W_3 for natural sedimentation sampling. W_1 was the equipment tray fixed on the dental chair closest to the patient's mouth. W_2 and W_3 were two tables around the HCWs at a certain distance from the patient. The distances from the patients on W_1, W_2, and W_3 were 102 cm, 134 cm, and 181 cm, respectively. The three surfaces are frequently touched by

Table 1
Sampling time point and sampling duration.

No.	1	2	3	4	5	6	7	8
Sampling point (min)	0	10	20	30	45	60	90	120
Sampling duration (min)	1	1	1	1	5	5	5	10

HCWs, and bioaerosol deposits on the surface would contaminate the equipment and increase the infection risk of the HCWs. The original release concentration of the solution was 2.5×10^8 CFU(PFU)/ml. The dental treatment time was 30 min, and a previous study showed that the bacterial number in the treatment area might gradually decrease 1–2 h after the operation (An et al., 2020; Shivakumar et al., 2007). Hence, the duration of the entire process in this study was set to 120 min. The time sampling points and sampling durations of each point are listed in (Table 1). Because the first 30 min was the time of bioaerosol release and the concentration of bioaerosols was high, a shorter sampling duration was set. With time, part of the indoor bioaerosol was discharged, part of the indoor bioaerosol was deposited, and the concentration of indoor bioaerosols decreased. Therefore, a longer sampling duration was used to ensure that the number of samples at each sampling time point was within the scientific counting range.

Before each experiment, a control experiment was conducted. The room was sterilized the day prior to each experiment. As a result, the other species in the room had little effect on the experiment. The data in this study were calculated to account for controlled experimental results.

2.3. Sample culture

The Petri dishes sampled at S_1–S_4 and W_1–W_3 were cultured for 24 h. The culture temperature of *Serratia marcescens* was set at 30 °C, and the *ΦX174 phage* was cultured on a double-layer nutrient AGAR plate in a 37 °C incubator. Escherichia coli (13706) was used as the host bacterium for phage culture. The upper host bacterial suspension was poured into the sampled plate. At the end of the culture period, the visible culturable colonies on the Petri dish were counted.

2.4. Data analysis

First, the culturable bioaerosol concentration in the space was calculated using Eq. (1) after counting the number of bacterial colonies:

$$C\left(CFU\,/\,m^3\right) = \frac{\sum_{i=1}^{6} P_{r_i} \times 1000}{T \times F} \tag{1}$$

where C is the concentration of culturable microorganisms in the air, P_{ri} is the corrected number of colonies at the i level, T is the sampling time (min), and F is the sampling flow rate (L/min, taken as 28.3 L/min in this study). The bioaerosol release rate was set as the real emission rate of bioaerosols from a patient during dental surgery for both *Serratia marcescens* and *ΦX174 Phage* (3.7×10^4 CFU(PFU)/min) (Micik et al., 1969), which made the absolute value of concentration meaningful. When sampling with the Andersen six-stage sampler, colony coverage from bioaerosols hitting the plate through the same sieve may result in a smaller number of colonies than the actual number. Therefore, it is necessary to revise the number of samples obtained by counting (Hu, 1998). The revised equation is shown in Eq. (2).

$$P_{r_i} = N_i\left(\frac{1}{N_i} + \frac{1}{N_i - 1} + \frac{1}{N_i - 2} + \cdots \frac{1}{N_i - r_i + 1}\right) \tag{2}$$

where P_{ri} is the corrected number of class i colonies; N_i is the i-level standard sieve number of the sampler; r_i is the number of class i colonies.

Because only four spatial points and three surface points were sampled in the dental clinic, to evaluate the transmission of bioaerosols more comprehensively, we adopted a numerical simulation method (Z. Liu et al., 2021). The RNG k–ε turbulence model was used for the numerical simulation of the flow field, whose control equations included continuity, momentum, energy, turbulent kinetic energy k, and turbulent dissipation rate ε, as shown in Eq. (3). The RNG k–ε turbulence model performs well in simulating indoor airflow with a low-Reynolds-number two-equation model; thus, it has been widely used (Küçüktopcu and Cemek, 2019; Kwon et al., 2015). In this study, the airflow of a dental clinic with a low-velocity air supply belongs to a low-Reynolds-number flow; thus, the RNG k–ε model was adopted.

$$\frac{\partial(\rho\varphi)}{\partial t} + \nabla\cdot(\rho\varphi V) = \nabla\cdot(\Gamma_\varphi \nabla\varphi) + S_\varphi \tag{3}$$

where ρ is the air density, V is the air velocity vector, φ is the three velocity components, Γ_φ is the effective diffusion coefficient of φ, and S_φ is the source term.

The Discrete Phase Model (DPM) model based on the Lagrange framework was used to track bioaerosol particles, which could solve the motion trajectory of a single particle, as shown in Eq. (4):

$$\frac{du_{\mathrm{pi}}}{dt} = \frac{18\mu}{\rho_{\mathrm{pi}} d_{\mathrm{pi}}^2}\frac{C_{\mathrm{D}}Re}{24}\left(u - u_{\mathrm{pi}}\right) + \frac{g_x\left(\rho_{\mathrm{pi}} - \rho\right)}{\rho_{\mathrm{pi}}} + F_{ai} \tag{4}$$

where μ is the molecular velocity of air, d_{p} is the diameter of the particle, C_{D} is the drag coefficient, u is the velocity of the particle, u_{p} is the airflow velocity, ρ_{p} is the density of a single particle, ρ is the density of air, and F_{ai} is the additional force per unit particle mass.

The simulated airflow velocity and boundary conditions of bioaerosol release were obtained from the experimental values. An Air Velocity Meter (TSI-9965) was used to measure the airflow velocity at the air inlet and inlet of the OSSM. The airflow velocity at the air inlet was 0.2 m/s, the air supply temperature was 28 °C, and the airflow velocity at the inlet of the OSSM was 1.0 m/s. The release rate of bioaerosols was 1.2 m/s. It is generally accepted that the aerodynamic diameter can reflect a series of bioaerosol characteristics. This parameter has also been used in previous studies (Li et al., 2016; McDonagh and Byrne, 2014). Therefore, the particle sizes of *Serratia marcescens* and *ΦX174 phage* in this study were also the median particle sizes measured by APS 3321, which were 0.791 μm and 0.783 μm, respectively. The number of bioaerosol particles used in the simulation was also converted from the tested values to ensure a good agreement between the experimental and numerical simulation results.

The deposition rate is adequate to describe the bioaerosol deposition characteristics on the surfaces, which was calculated using Eq. (5):

$$d = \frac{P}{TA} \times 100\% \tag{5}$$

where d is the deposition rate of culturable microorganisms on the surface, P is the corrected number of colonies at the i level, T is the sampling time (min), and A is the sampling area of the Petri dishes (m^2, 0.006 m^2 was used in this study). The concentration on the surface, C_s, after the experiment was calculated as follows:

$$C_s = \int d(t)dt \tag{6}$$

The rate of the total amount of bioaerosols deposited on the surface was calculated using Eq. (7):

$$\theta_i = \frac{C_{si}A_i}{ST} \times 100\% \tag{7}$$

where θ_i is the deposition rate of surface i, C_{si} is the bioaerosol deposition concentration on surface i, A is the sampling area of surface i, and ST is the total amount of released bioaerosols. This parameter measures the dimensionless deposition rate of a surface.

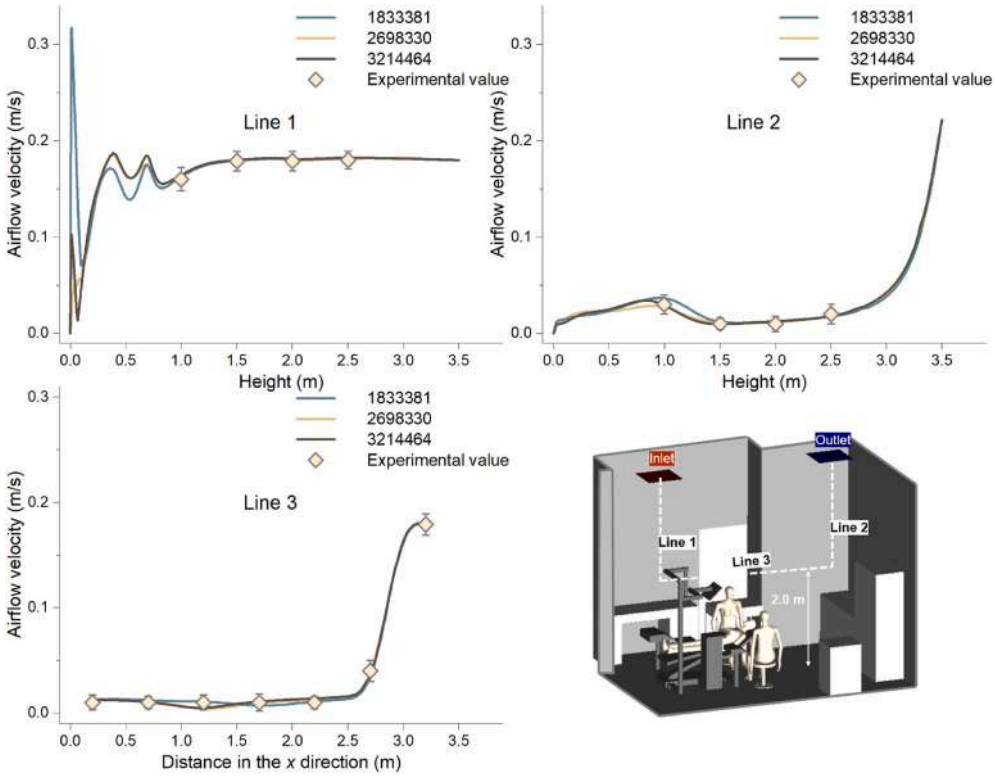

Fig. 2. Grid independence verification at Line 1, Line 2, and Line 3.

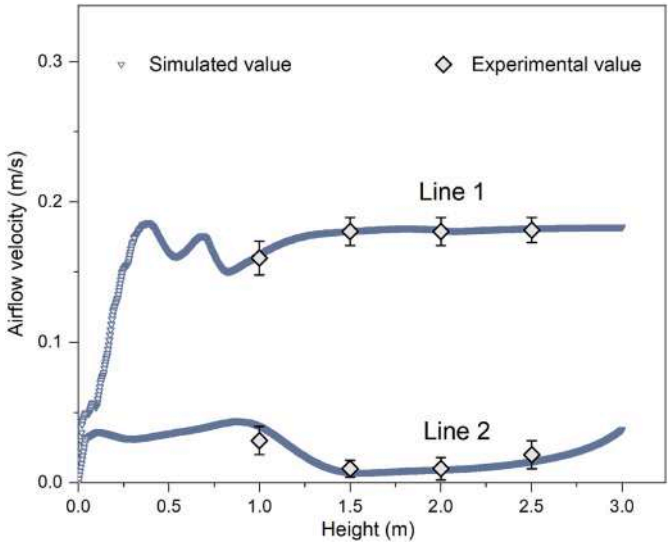

Fig. 3. Flow filed verification.

2.5. Verification of the numerical simulation model

We recently assessed the accuracy of a case without OSSM (Z. Liu et al., 2021). In this study, we verified the accuracy of the simulation using the OSSM. In the numerical simulation, the grid structure and density of the model would affect the simulation results of the flow field (Li et al., 2015). Therefore, to build a reasonable grid, the quality of the grid was verified and three grids were established: 1833381, 2698330, and 3214464. A flow-field simulation was performed for the models with three grid sizes. The airflow velocities at vertical Line 1 directly below the inlet, vertical Line 2 directly below the outlet, and Line 3 (along the *x*-direction) with a height of 2.0 m were selected for validation. The numerically simulated velocity on the three lines was compared with the velocity tested experimentally for validation (Fig. 2). As shown in Fig. 2, the computational simulation velocity of the three grids was in fair agreement with the tested value, but the velocity of the 1833381 grids fluctuated significantly compared with the other two grids. The simulation results for the 2698330 and 3214464 grids agreed well with the tested value. Thus, the 2698330 grid was selected in this study, considering the computational speed.

The simulated airflow field must be verified to obtain accurate numerical simulation results. The simulation velocities at different heights on vertical Line 1 below the air supply outlet and vertical Line 2 below the air exhaust outlet were compared with the experimental results. Fig. 3 shows that the numerical simulation results were in good agreement with the experimental values.

3. Results

3.1. Bioaerosol transmission in a dental clinic setting

First, we explored the distribution of bioaerosols in the dental clinic without an OSSM. Fig. 4 shows the overall dynamic spatiotemporal distribution of bioaerosol (*ΦX174 phage*) diffusion without OSSM. The distribution of bioaerosols was limited beside the patient during the first minute and was uniformly distributed around the clinic with airflow at 45 min. The number of bioaerosols in the air was at the maximal value at 20–30 min due to the ongoing dental treatment procedure. In contrast, after 30 min, that is, after the end of the treatment, the number of bioaerosols in the air gradually decreased owing to ventilation exclusion and bioaerosol deposition. At 120 min, the majority of the bioaerosols were evacuated, and the remainder floated in the upper part of the dental clinic.

The bioaerosol (*ΦX174 phage*) concentrations at the four key locations (S_1, S_2, S_3, and S_4) were monitored throughout the sampling period (Fig. 5). The bioaerosol concentrations of S_1–S_4 also showed a trend of increasing and decreasing with time, and the peak exposure

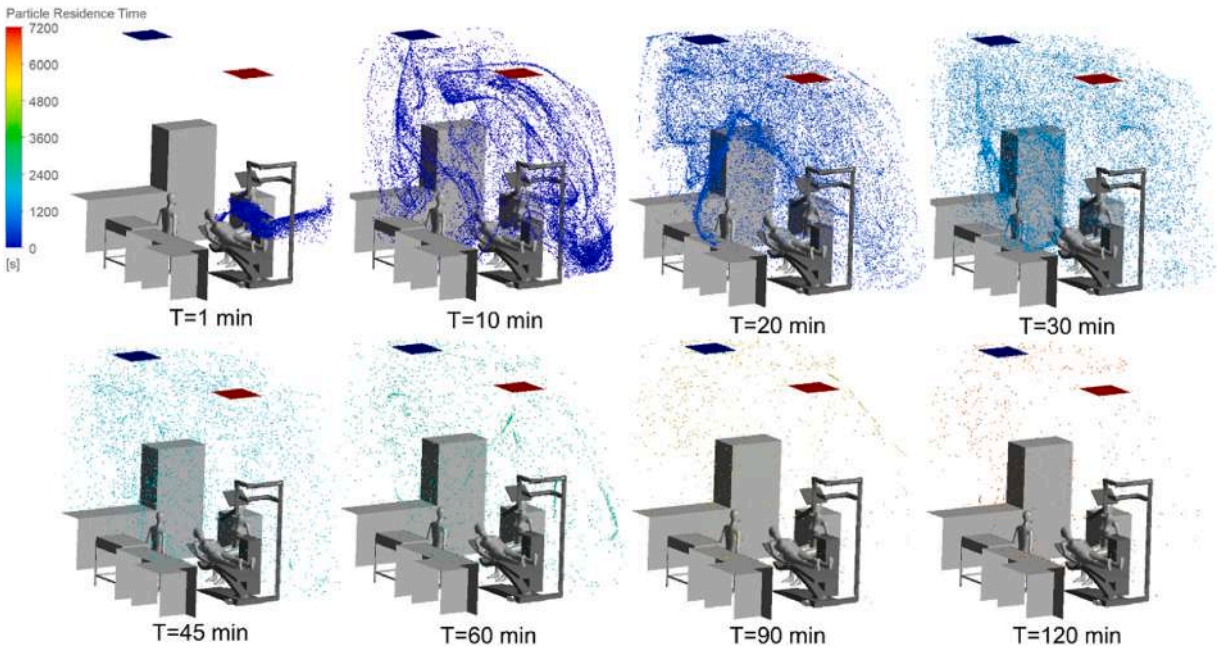

Fig. 4. Spatial and temporal distribution of the bioaerosol (*ΦX174 phage*) without OSSM. (Legends indicate particle residence time).

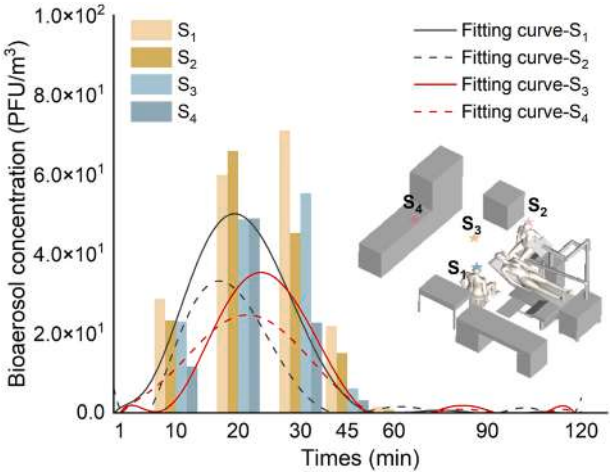

Fig. 5. Spatiotemporal distribution of bioaerosol (*ΦX174 phage*).

concentration of bioaerosols occurred during the 20–30-min period. The peak bioaerosol concentrations were 75.19 PFU/m^3, 69.08 PFU/m^3, 55.22 PFU/m^3, and 48.87 PFU/m^3 at S_1–S_4, respectively. The bioaerosol concentrations steadily decreased below 1 PFU/m^3 after 90 min, and 99% of the bioaerosol was evacuated from the air at 90 min. Furthermore, because of the close proximity of S_1 and S_2 to the patient, the bioaerosol concentrations in S_1 and S_2 were higher than those in S_3 and S_4.

3.2. Influence of OSSM on spatial concentration of bioaerosols

We further investigated the effect of the OSSM on the airborne transmission of bioaerosols. Before the study of bioaerosol transmission, the indoor velocity field was first analyzed. The study on the airflow diffusion trajectory would serve as the basis for subsequent bioaerosol transmission because bioaerosols are small in size and light in weight, and would move along with the airflow diffusion trajectory. The OSSM was adopted and opened during the treatment procedure in the first 30 min. The OSSM had no noticeable effect on the airflow field in the entire dental clinic but only significantly influenced the airflow in the small space near the OSSM. This means that the OSSM had no evident impact on the clinic's operation and comfort of patients and HCWs.

Fig. 6 shows the overall dynamic spatiotemporal distribution of bioaerosol diffusion with the OSSM. The results showed that the trend in the bioaerosol concentration in the respiratory area of the dentist over time with the OSSM was similar to that without the OSSM throughout the process. The bioaerosol concentration gradually increased over time after the onset of the dental procedure and peaked at 20–30 min (Fig. 6). After the dental surgery (at 30 min), the bioaerosol concentration gradually decreased. However, the concentration in the presence of OSSM was significantly lower than that without OSSM.

Fig. 7 shows the trend of simulated and experimental concentration of the four air sampling points from S_1 to S_4 with the OSSM. The simulation was consistent with the measured concentration, thus proving the correctness of the numerical simulation method and the feasibility of subsequent simulation calculation. In addition, the two kinds of bioaerosols showed similar concentration variation trends. The results showed that the bioaerosol concentrations of S_1–S_4 tended to increase and then decrease with time for both *Serratia marcescens* and *ΦX174 phage*.

Fig. 8 compares the measured bioaerosol concentrations with or without the OSSM at the sampling points. The results showed that OSSM could significantly reduce the bioaerosol concentration. The bioaerosol concentrations with the OSSM were about an order of magnitude lower than those without the OSSM. For example, the mean bioaerosol concentration decreased from 58.84 PFU/m^3 (without OSSM) to 4.10 PFU/m^3 (with OSSM) at 20 min at S_1 for *ΦX174 phage* (Fig. 8(b)). Regarding the other sampling points, we also found a similar inhibitory effect on the transmission of bioaerosol, indicating the effectiveness of the OSSM in removing bioaerosols from the dental clinic.

3.3. Influence of OSSM on the deposition of bioaerosols

Finally, we investigated the deposition of bioaerosols with and without the OSSM on the three surfaces most commonly used in the dental clinic. Fig. 9 shows the deposition rates of the three surfaces.

The trend of the deposition rate was similar to that of the spatial concentration. The surface deposition rates were initially lower, then increased to a maximum value, and finally gradually decreased (Fig. 9).

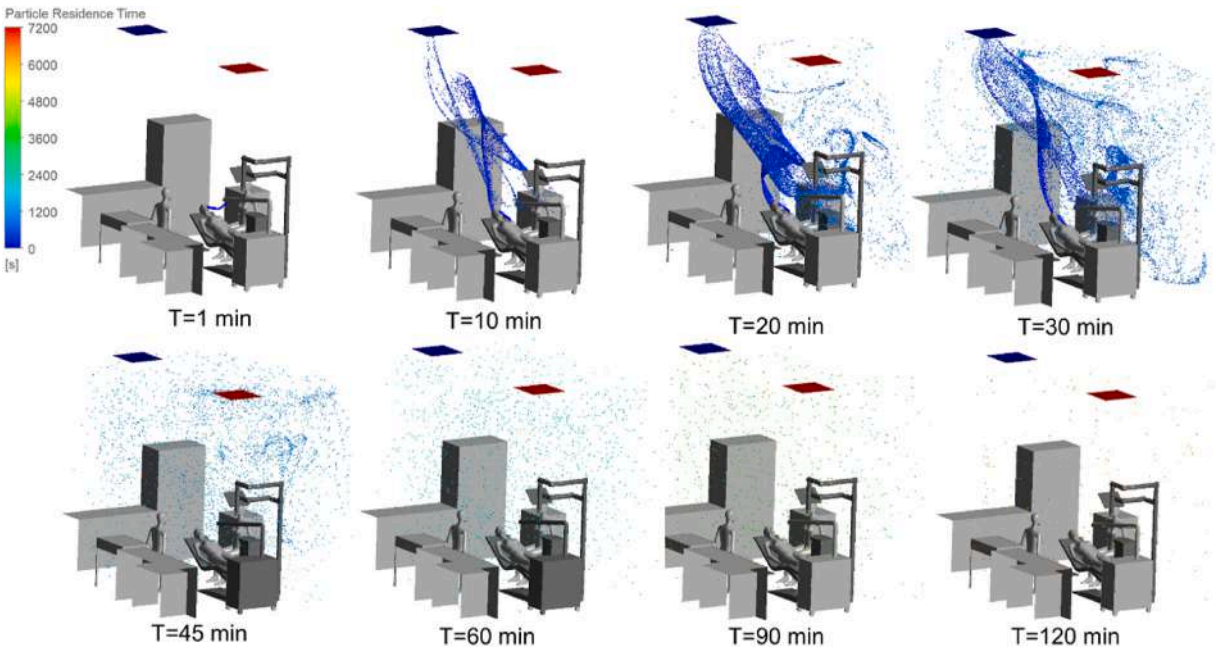

Fig. 6. Spatial and temporal distribution of the bioaerosol (*ΦX174 phage*) with OSSM. (Legends indicate particle residence time).

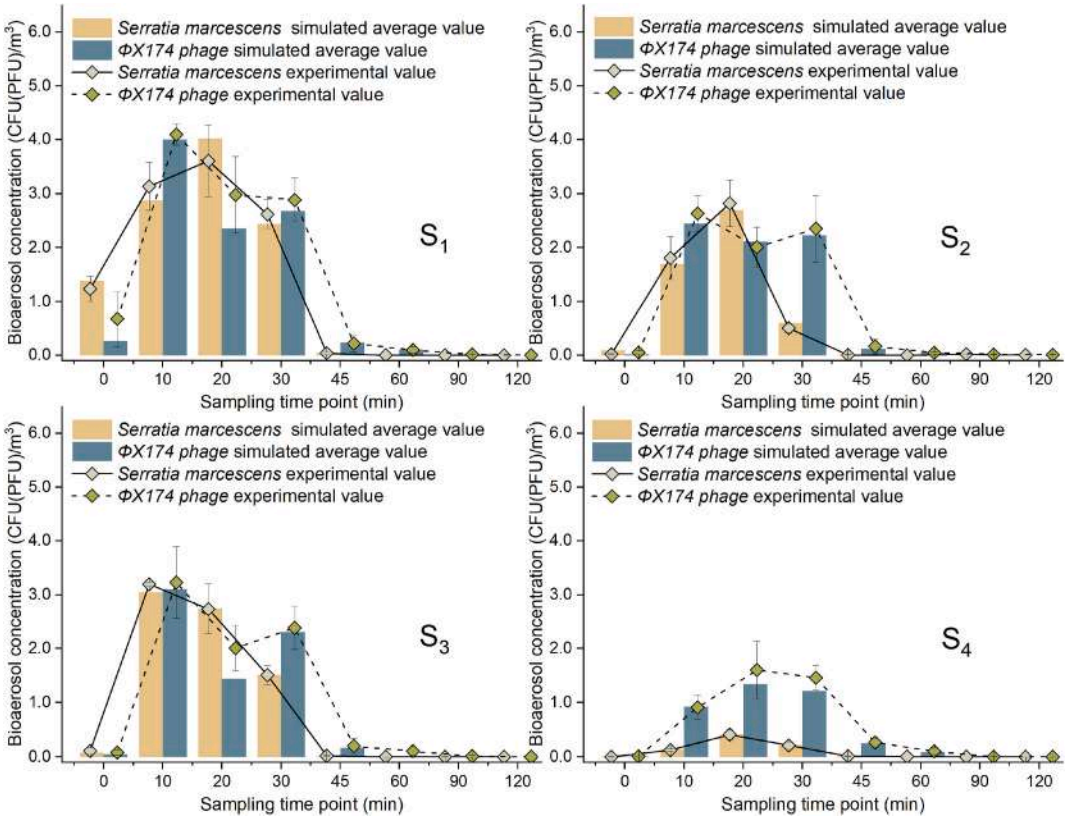

Fig. 7. Simulated and experimental concentration verification at S_1–S_4.

Without the OSSM, the W_1, W_2, and W_3 concentrations (*ΦX174 phage*) related to the dental treatment were 437.4 PFU/m^2, 28.6 PFU/m^2, and 28.1 PFU/m^2, respectively. The concentrations on W_2 and W_3 were significantly lower than that on W_1 because of the farther distances. With the OSSM, the W_1, W_2, and W_3 accumulated concentrations (*ΦX174 phage*) were 3.5 PFU/m^2, 1.1 PFU/m^2, and 2.5 PFU/m^2, respectively, which was a 91%–99% reduction.

The ratios of the total amount of bioaerosols deposited (θ_i) on the surface were also compared. Without the OSSM, the θ_1–θ_3 values were 0.47%, 0.08%, and 0.054% on the 0.15-m^2, 0.4-m^2, and 0.27-m^2 surfaces, respectively. A considerable portion of the bioaerosols was deposited on the surfaces. However, with the OSSM, θ_1 decreased to less than 0.03%, and θ_2 and θ_3 were close to 0. The OSSM eliminated most of the released bioaerosols and reduced the bioaerosol ratio on the

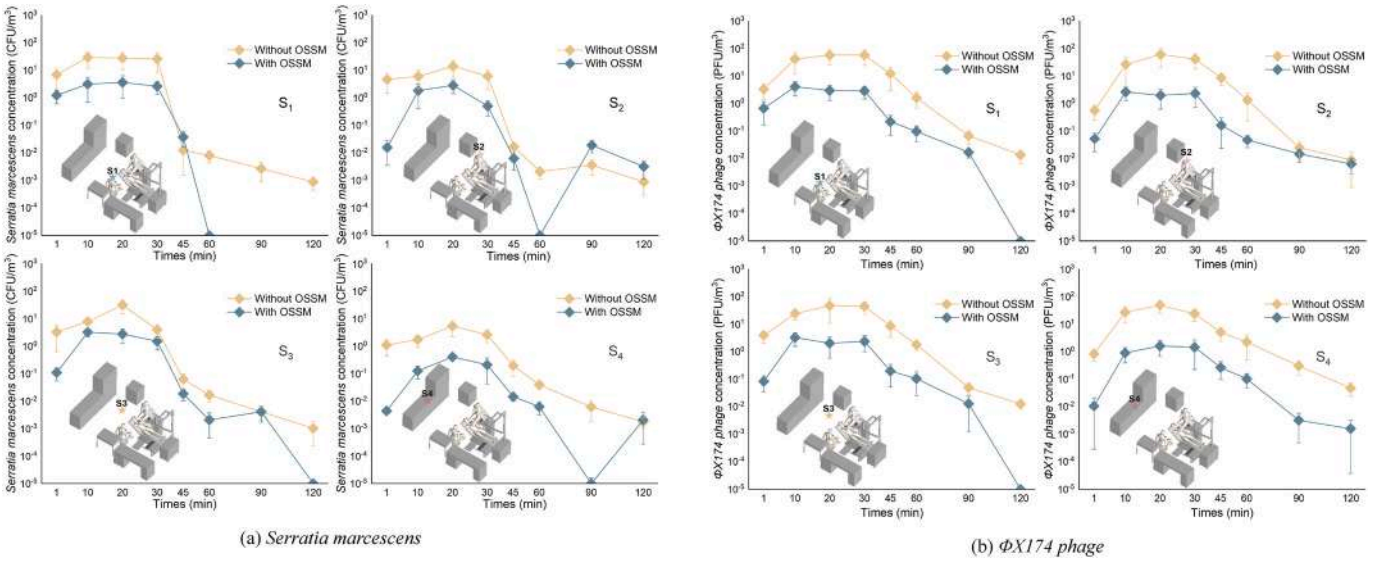

Fig. 8. Experimental bioaerosol concentrations at sampling point S_1–S_4.

surfaces.

4. Discussion

The transmission of bioaerosols in the dental clinic was studied by both experimental research and numerical simulation, which is the most typical method to investigate bioaerosol concentrations (Komperda et al., 2021; Liu et al., 2022; Polednik, 2021). A previous study reported that dental treatment causes a considerable increase in airborne bioaerosol concentrations in dental clinics, although the analyses were not comprehensive (Dutil et al., 2008). Infected individuals can produce viruses containing large short-range droplets and small airborne droplet nuclei by breathing, coughing, or sneezing, and transmission between patients and HCWs is possible (Castaño et al., 2021). In this study, the spatial and temporal quantitative distributions of bioaerosols during dental treatment were investigated and visualized. The patients' peak bioaerosol concentration value increased by up to 2–3 orders of magnitude (CFU/m^3). A previous study showed similar results: the median bacterial concentration increased from 78 to 166 CFU/m^3 before and during the dental treatment procedure, and then fell to 110 CFU/m^3 when the dental treatment procedure was completed, which was determined by measuring bacterial concentrations in the air during dental treatment (Pasquarella et al., 2012). Similarly, it was discovered that, during dental treatment in a dental clinic, the airborne bacterial concentration increased from 176 CFU/cm^3 to 372 CFU/cm^3, which is a 2.1-fold increase (Polednik, 2021). Furthermore, dentists closer to patients were more likely to be exposed to bacteria emitted from the patient's mouth during oral scaling treatment (Chuang et al., 2014). The spread of airborne bacteria at various distances and directions during scaling treatments in patients with periodontitis has been previously reported. Airborne bacteria reached up to 150 cm horizontally and 115 cm vertically from the mouth, and the most contaminated area was 50 cm horizontally away from the mouth cavity (Chuang et al., 2014; Horsophonphong et al., 2021). As described in the results of this study and previous research, HCWs face the potential risk of exposure when performing dental treatment.

Furthermore, it is essential to observe the deposition of bioaerosols onto surfaces. These settled bioaerosols may be touched and transported by hand and transferred into mucous membranes through the eyes, nose, and mouth, whey they can cause infection, known as fomite transmission (Mutesa et al., 2021). Therefore, the deposition of bioaerosols in clinics cannot be ignored (Z. Liu et al., 2021). Cristina et al. (2008) evaluated surface contamination in a dental clinic by measuring the hemoglobin (Hb) concentration on surfaces and found that they were contaminated with blood particulates with a mean concentration of 1.56 μL/m^2. In addition, all surface samples were 100% positive for Hb in 80% of the monitored compartments. Knowledge of the influence of the OSSM on fomite transmission is also fundamental to developing effective intervention strategies for infectious diseases. The OSSM is the most commonly used air-cleaning device in dental clinics. According to the results of this study on the deposition of bioaerosols, without OSSM, the W_1, W_2, and W_3 concentrations (*ΦX174 phage*) related to the dental treatment were 437.4 PFU/m^2, 28.6 PFU/m^2, and 28.1 PFU/m^2, respectively. The spatial bioaerosol concentration can be reduced by 93% using the OSSM, effectively preventing droplet and airborne transmission. With the OSSM, bioaerosol deposition can be reduced by 91%, effectively preventing fomite transmission. This was of the same order of magnitude in the operating room (Napoli et al., 2012) and in other dental clinics (Decraene et al., 2008). The OSSM could play a significant role in reducing bioaerosol deposition and controlling fomite transmission in dental clinics.

5. Conclusions

This study used experiments to investigate the effects of using the OSSM on the spatial concentration distribution and deposition of bioaerosols in a typical dental clinic. Our study provides evidence that OSSM use in dental clinics can effectively reduce the exposure levels of bioaerosols for healthcare workers during dental treatment, which can be of clinical importance to minimize the risk of infectious diseases such as COVID-19.

Author contribution statement

Zhijian Liu, conceptualization, methodology, project administration; Peiwen Zhang, software, investigation, formal analysis, writing (original draft, review and editing); Haiyang Liu, formal analysis, writing (original draft, review and editing); Junzhou He, formal analysis, writing (original draft, review and editing); Yabin Li, funding acquisition, investigation; Guangpen Yao, software, investigation; Jia Liu, investigation; Meng Lv, investigation; Wenhui Yang, investigation, writing (review and editing). All authors read and approved the final manuscript.

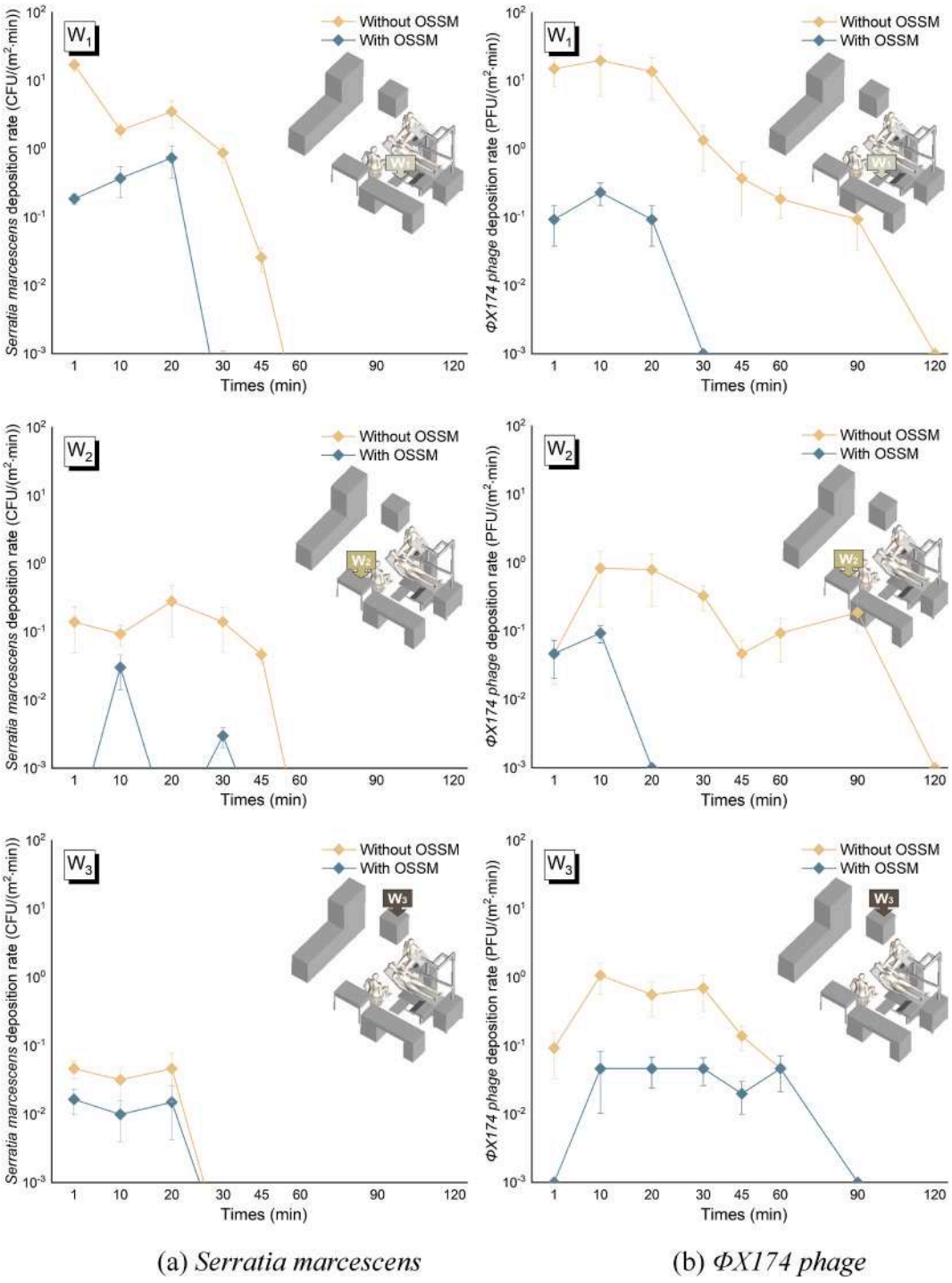

Fig. 9. Experimental bioaerosol deposition rates with and without OSSM at W_1, W_2, and W_3.

Declaration of competing interest

The authors declare no competing interest.

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Exposure to polycyclic aromatic hydrocarbons assessed by biomonitoring of firefighters during fire operations in Germany

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ABSTRACT

Background: Firefighters are exposed to a variety of hazardous substances including carcinogens such as polycyclic aromatic hydrocarbons (PAH) during firefighting. In order to minimize the uptake of such substances into the body, firefighters wear personal protective equipment. Only few data exist from real-life firefighting missions and under common although highly variable exposure scenarios such as fighting fires in residential buildings, outdoor, and vehicle fires. The aim of this study is to assess the levels of 1-Hydroxypyrene (1-OHP) as marker for incorporated PAH during firefighting operations in Germany using biomonitoring methods.
Methods: We analyzed urine samples for 1-OHP from 77 firefighters who reported firefighting operations (with and without creatinine adjustment). Urine samples were collected before (baseline) and, where applicable, after firefighting operations at three time points subsequent (2–4, 6–8, and 12 h).
Results: Compared to the baseline measurements, mean 1-OHP concentrations after firefighting missions were doubled (0.14 vs. 0.31 μg/L urine, 0.13 μg/g vs. 0.27 μg/g creatinine) and this increase was observed 2–4 h after firefighting. Firefighting in residential buildings (N = 54) and of outdoor and vehicle fires (N = 17) occurred most frequently, whereas blazes, vegetation fires, and fires in underground facilities (N = 6) were rarely encountered. For residential building fires, a 3-fold increase in mean 1-OPH concentrations was observed, whereas no increase could be observed for outdoor and vehicle fires. The highest increase was observed for firefighters with interior attack missions (0.11 μg/L vs. 0.48 μg/L 1-OHP) despite the use of self-contained breathing apparatus (SCBA). During the suppression of outdoor or vehicle fires using SCBA, again, no increase was observed. Although PAH are taken up during certain firefighting missions, the 1-OHP levels almost entirely remained (in 64 of the 77 reported missions) within the normal range of the German general population, i.e., below the reference levels (95th percentiles) of smokers (0.73 μg/g creatinine) and non-smokers (0.30 μg/g creatine).
Conclusion: Under study conditions, properly applied protective clothing and wearing of SCBA led to a significant reduction of PAH exposure levels. But there are individual situations in which PAH are increasingly incorporated since the incorporation depends on several factors and can be extremely variable. In contrast to many workplaces with high occupational exposure levels, firefighters are not exposed to PAH on a daily basis. Nevertheless, the possibility of an individual increased cancer risk for a particular firefighter cannot completely be ruled out.

1. Introduction

In Germany, there are approximately 40,000 full-time and 1.3 million volunteer firefighters who are exposed to a variety of hazards during firefighting including carcinogenic hazardous substances such as, among others, benzene, cadmium, asbestos, silica, and polycyclic

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aromatic hydrocarbons (PAH) (IARC 2010). Fire smoke is a complex mixture of hazardous substances, the composition of which depends on many factors (burned material, ventilation, oxygen supply, etc.). Therefore, firefighters are instructed to wear personal protective equipment (PPE), that is designed for protection of the body from heat stress as well as flames, but it also protects from gaseous and particulate substances Where appropriate, self-contained breathing apparatus (SCBA) are used during fire operations. Based on the most recent available data, IARC re-evaluated the carcinogenic hazard associated with occupational exposure as a firefighter and, most importantly, commented on the degree of evidence for particular cancer sites in humans (Demers et al., 2022). IARC now classifies occupational exposure as a firefighter as "carcinogenic to humans (Group 1) based on sufficient evidence for cancer in humans". According to IARC, there was "sufficient evidence" in humans for mesothelioma and cancer of the bladder, whereas there was only “limited evidence” for colon, prostate, and testicular cancer, melanoma of the skin, and non-Hodgkin lymphoma. Furthermore “strong” mechanistic evidences like “is genotoxic”, “induces epigenetic alterations”, “induces oxidative stress”, “induces chronic inflammation”, and “modulates receptor-mediated effects” with key characteristics for human carcinogenetic were reported. This re-evaluation was done after in 2007, the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) classified firefighter work as possibly carcinogenic to humans (Group 2B) (IARC 2010). In more recent meta-analyses (Jalilian et al., 2019; Casjens et al., 2020), an increase of the overall cancer incidence and mortality for specific cancer sites such as melanoma of the skin, prostate cancer and mesothelioma were reported. Nonetheless, the underlying studies showed great heterogeneity. In addition, time- and country-specific effects were also observed (Casjens et al., 2020).

Since fires are often accidental, unannounced events with different intensities, most epidemiological studies lack information on actual exposure and, therefore, it is difficult to establish a link between a defined substance or mixture of substances and cancer risk. Moreover, each member of the firefighting team has to perform a defined function or activity in firefighting. Consequently, the exposure situations can be different depending on an individuals’ function at the same event, with probably the highest exposure during interior attacks for firefighting in urban areas during residential firefighting. In contrast to this, during wildland firefighting or special operations the use of personal protective equipment (PPE) applied for internal attack is not useful. Therefore, different special lighter PPE for wildland fire are used (Oliveira et al., 2016). Because urban firefighters can use SCBA to protect themselves from inhalation of smoke and other hazardous substances released during fires, personal protective devices are covered by pollutants and improper removal of such devices may also cause exposures, e.g., via contamination of the skin. In addition, other potential sources for exposure are by contaminated skin areas, gear penetration before or after donning the SCBA. Biomonitoring is a suitable method for measuring hazardous substances absorbed by humans. This method can be used to estimate the internal exposure, regardless of whether they enter the body via the respiratory tract, the digestive tract, or the skin. The majority of biomonitoring studies on hazardous substances (e.g. PAH, carbonmonoxide, volatile organic compounds or phenols) during fire operations were conducted in training situations or experimental settings (Rosting and Olsen 2020; Banks et al., 2021; Rossbach, 2020; Fent et al., 2019; Feunekes et al., 1997; Moen and Ovrebø, 1997). An overview of published studies is given in a recent review (Engelsman et al., 2020). Also a few studies observed firefighters during wild land firefighting (Oliveira et al., 2016; Adetona et al., 2017; Cherry et al. 2019, 2021). In contrast, three studies were published on biomonitoring results of firefighters during urban real-life fire operations, two in Canada (Caux et al., 2002; Keir et al., 2017) and one in the United States (Hoppe-Jones et al., 2021). Another study was conducted in Denmark, but did not observe an increase of the internal exposure after fire suppression (Andersen et al., 2018). In Germany, no comparable study has been published yet.

One of the most studied group of substances that occur in fires are PAH which are formed during the incomplete combustion of carbon-containing material (wood, plastic, etc.) and can also adhere to smoke particles. PAH consist of various individual compounds. Although several of those such as anthracene and pyrene are not known to cause cancer in humans, the mixture also frequently contains carcinogenic compounds such as naphthalene and benzo[a]pyrene (BaP). Therefore, the entire class of PAH is considered carcinogenic to humans independent from its individual composition. Biomonitoring PAH exposure is usually carried out by measuring 1-hydroxypyrene (1-OHP), a metabolite of pyrene, in urine. Although pyrene itself is not known to be carcinogenic itself, 1-OHP in urine is a well-known, robust and established parameter from an analytical point of view since the first biomonitoring method for 1-OHP became available (Jongeneelen 1997, 2014). 1-OHP has been used extensively in studying PAH exposure in multiple occupational and environmental settings in Germany (Marczynski et al., 2009; Becker, 1998; Wilhelm et al., 2008). Therefore, due to decades of experience, background levels of 1-OHP in the general population (smokers and non-smokers) are known which, in turn, can be used to assess exposures that occur on top of environmental exposures, e.g. at the workplace. This knowledge makes it possible to compare exposure levels of firefighters to those of other occupations and the general population. Most importantly, there is also sufficient knowledge on the relationship between 1-OHP in urine and an increased risk of DNA damage in PAH-exposed industrial workers (ACGIH 2017; Jongeneelen 2014).

The aim of this study was to assess and interpret PAH-exposures among firefighters in Germany during live firefighting operations by biomonitoring of 1-OHP. The results were also used to further improve primary prevention measures in everyday operations.

2. Material and methods

2.1. Study setting

Between January 2018 and July 2020, members of the departments of the German cities Bochum, Hamburg, and Berlin were invited to participate in this cross-sectional study with repeated measurements. Firefighters were followed until the first fire operation on which they provided urine samples and therefore participated only once in the study. Both, professional firefighters and volunteer firefighters were included. Besides active firefighters, also employees of the respiratory protection and hose workshop and of a training facility were included. In this analysis we report only results on firefighters in duty.

Information sessions were held at selected fire stations and the study was presented to the firefighters. All fire stations were situated in urban areas. Participating firefighters could make an appointment with the fire department physician. At this baseline visit, firefighters were asked to provide written informed consent. Urine and blood samples were collected and frozen at −20 °C. The time and day of the baseline measurements were also recorded if a firefighter fought fires in the days prior sampling. A self-administered questionnaire was applied with questions about smoking habits, diet, and employment as firefighter. Afterwards, firefighters were given the study materials, including urine containers and an additional questionnaire to be filled after real firefighting operations. This questionnaire included questions, among others, on the density and color of smoke, the use of personal protective equipment such as SCBA, and the presence or absence of hygiene measures after firefighting. The following operation types were given in the questionnaire: Residential building fire, blaze with massive smoke deployment, firefighting in underground facilities, vegetation fire, vehicle or other outdoor fire, and operation with special conditions. The aim of the study was to investigate different tasks during the operation. This concerns captains, who are responsible for the operation and act as supervisors of the operation, attack teams with SCBA during internal

attack, and attack teams working outside the danger zone. Also engine operators working around the fire truck and may be exposed to fire smoke and diesel engine emissions. The study was approved by the ethics committee of the Ruhr University Bochum, Germany (reference number 17–6071, 5th September 2017).

2.2. Urine collection and analysis

After a fire operation, participating firefighters were asked to provide spot urine samples at three different time periods (2–4 h, 6–8 h, and 12 h) after the mission to cover the respiratory as well as dermal exposure paths. In the time in between no urine was sampled. Samples were stored at −20 °C at the fire station. Sample collection was documented on a sample run sheet by firefighters themselves. Baseline and post-fire urines were regularly picked up and transported frozen.

For the analysis of 1-OHP, urine samples were thawed and aliquoted. Urinary creatinine was determined by contract analysis (Jaffe method, L.u.P. GmbH Labor und Praxisservice, Bochum). 1-OHP was determined after liquid/liquid extraction and derivatization with a silylating agent (N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide) by gas chromatography, tandem mass spectrometry, and negative chemical ionization (GC–NCI–MS/MS) as previously described (Gaudreau et al., 2016). The limit of quantification (LOQ) was 0.05 μg 1-OHP per liter urine.

2.3. Statistical analysis

We calculated descriptive statistics to characterize urinary 1-OHP measurements for each of the four sampling dates. Measurements below the limit of quantification were included in the calculations at half the LOQ (1/2 LOQ = 0.025 μg/L). Two post-fire samples of two firefighters were missing, and in another sample of a third firefighter there were interferences, so 1-OHP was not measurable. We used the multiple imputation procedure Proc MI of the statistical software SAS to impute these three measurement values.

1-OHP values were evaluated with and without adjustment for creatinine. Creatinine-adjustment was only performed if the creatinine value was between 0.3 g/L and 4.0 g/L with a value between 0.3 g/L and 3.0 g/L representing a normally hydrated adult person (Bader et al., 2020). Because this study involved participants who are generally fitter and have an above average higher muscle mass, creatinine values up to 4.0 g/L were accepted for adjusting volume-related 1-OHP-levels. Values outside this range and below the LOQ for 1-OHP were excluded for creatinine-adjustment to avoid bias in the results due to artificially introduced lower variability. Creatinine levels were missing for two urine samples and, again, we used multiple imputation to estimate the missing values.

The non-parametric Wilcoxon matched-pairs signed-rank test was used to compare the medians of the 1-OHP concentrations after firefighting to them at baseline. It was not used for the creatinine adjusted 1-OHP concentrations, because the adjustment were only be done for a subset of measurements and test can only be performed on subjects which data are available at all time points.

Analyses were stratified by fire operation scenarios, role in firefighting, and smoking status. If a firefighter had more than one fire operation during a shift or different roles, the operation scenario or role with the highest expected exposure to smoke was chosen. There were three firefighters who were deployed in two fire operations during one shift. Both fire scenarios were fires in residential buildings.

To account for the longitudinal nature of the study data we applied Generalized Estimating Equations (GEE) models to the 1-OHP concentrations as dependent variable. As independent variables the different fire operation scenarios and the role of the firefighters during firefighting were used. Two separate models for each of the two variables were computed and a third with both. All models were adjusted for smoking status. We used a log link and the normal distribution with an exchangeable working correlation matrix. Estimates and 95% confidence intervals (95% CI) were reported. The software package SAS, version 9.4 (SAS Institute Inc., Cary, NC, USA), was used for the analysis.

2.4. Result interpretation

BEI® levels are health-based values and do not distinguish between smokers and non-smokers. Although there are fine lines between safe and dangerous exposures, BEI® levels represent conditions to which nearly all workers may be repeatedly exposed without adverse health effects (ACGIH 2017). In case of PAH exposures, the BEI® is based on the relationship between 1-OHP in urine and different genotoxic endpoints that, although having no clear link with the development of cancer, minimize the risk of genetic effects and disease. In case of PAH, the BEI® is also not a fixed value and should be adjusted for the specific pyrene/BaP ratio in the PAH mixture of the workplace under consideration. If this ratio is not known (which is the case for fire smoke or different types of fires), ACGIH suggests to use the default value of 2.5 μg 1-OHP/L urine as prudent guidance which, for preventive reasons, assumes a high yield of carcinogenic BaP in the smokes.

As a second evaluation standard, we used the reference values of 1-OHP in smokers (0.73 μg/g creatinine, 1.03 μg/L) and non-smokers (0.30 μg/g, 0.53 μg/L) of the German general population not occupationally exposed to PAH. These reference levels are based on the 1998 Environmental Survey in Germany (Becker, 1998; Wilhelm et al., 2008) and, in contrast to BEI® values, are statistically derived (95th percentiles) and not health-based.

3. Results

In total, 209 firefighters agreed to participate in the study and provided baseline urine samples and questionnaires. Due to changing positions between the shift, e.g., shift one internal attack team, shift two paramedic, and shift three engine operator, it was possible to include 77 subjects who reported firefighting operations and who provided up to three spot-urine post-fire urine samples and questionnaires at the end of the study. We report on these 77 firefighters, all other are not further investigated. Table 1 depicts the characteristics of these 77 participants with operations. The mean age of the professional firefighters was seven years higher than that of the volunteers, and professional firefighters, on average, served four years longer. Among volunteer firefighters, the proportion of never smokers was almost three-quarters, whereas 54% of the professional firefighters had never smoked. The proportion of former smokers was different in both groups (professional 21.4% vs. volunteer firefighter 4.8%). In total, 25% of the study population were currently smoking.

At baseline, the proportion of 1-OHP measurements below the LOQ was 41.6%, at the first and second post-exposure sampling 16.9%, and at the third sampling 18.2%. The proportion of creatinine levels outside the reference range was 5.2% at baseline, 3.9% at first sampling, 6.5% at

Table 1
Characteristics of the study population (N = 77).

Characteristics	Fire brigade		Total (N = 77)
	Professional (N = 56)	Volunteer (N = 21)	
Sex (N)			
Female	1	–	1
Male	55	21	76
Age (Mean, Min-Max)	36.6 (22.7–56.7)	29.7 (20.3–41.3)	34.7 (20.3–56.7)
Years in fire department (Mean, Min-Max)	13.6 (1–33)	9.6 (1–23)	12.5 (1–33)
Smoking status (N, %)			
Never smoker	30 (53.6)	15 (71.4)	45 (58.4)
Former smoker	12 (21.4)	1 (4.8)	13 (16.9)
Current smoker	14 (25.0)	5 (23.8)	19 (24.7)

second sampling and 1.3% at third sampling. All creatinine levels outside the reference range were lower than 0.3 g/L except for two samples at the first sampling which were higher than 4.0 g/L creatinine. At baseline, for a single firefighter (smoker), the creatine adjusted 1-OHP measurement was already above the evaluation standard and also the unadjusted 1-OHP measurement above the BEI® (2.5 μg/L).

Table 2 summarizes the 1-OHP measurements before and after firefighting. Compared to the baseline measurements, mean concentrations were approximately doubled, regardless of creatinine adjustment. The standard deviations were after the fire events higher than at the baseline regardless creatinine adjustment. This increase was lower for median concentrations of the creatinine-adjusted measurements than for the unadjusted volume-related levels. The elevated median concentrations were statistically significant at each time point. While mean and median concentrations after the fire events remained below both the reference levels for smokers (0.73 μg/g) and non-smokers (0.3 μg/g) and also the BEI®, we nevertheless observed levels in 13 subjects that exceeded these standards. The 95th percentiles of the 1-OHP concentrations were below the reference levels at baseline and exceeded them after firefighing. At the first sampling, four measurements were above one of the standards, at the second sampling ten measurements, and at the third sampling eight measurements. At each sampling point after firefighting, one sample was above the BEI® from one subject (Fig. 1; firefighter #2). This firefighter had the highest 1–OHP creatinine concentration at all sampling points after firefighting, but the 3rd sampling concentration of this firefighter was missing and were imputated. He had completed two subsequent missions, a residential building fire and afterwards a blaze. He was involved in the interior attack using SCBA and also in a fire dampening-down operation wearing SCBA. These operations lasted 4 h. The firefighter with the highest creatinine adjusted 1-OHP measurement (4.27 μg/g creatinine) was deployed at a residential building fire outside the area of immediate danger and without SCBA for 30 min (Fig. 1, firefighter #8).

In total, 14 out of the 77 firefighters with a mission had measurements above the reference levels for smokers (0.73 μg/g) and non-smokers (0.3 μg/g), 13 of them after the fire mission, which is shown in Fig. 1. In four cases, the exceedances were seen after the first sampling. In the majority of cases, exceedances were at the second and third sampling. One of these 13 firefighters was a smoker. Thus, non-smokers were more affected, but also represented the largest group of participants. Fig. 2 shows the 1-OHP concentrations stratified by smoking status. Smokers started from higher levels of 1-OHP concentrations than non-smokers and ex-smokers, and a link between 1-OHP in urine with the fire mission is apparent regardless of smoking status.

Fifty-four of all studied events (70.1%) were residential building fires followed by outdoor or vehicle fires (N = 17), blazes (N = 4), vegetation fires (N = 1), and underground fires (N = 1). Due to low numbers, we combined the last three fire scenarios into one group. Table 3 depicts the biomonitoring results by scenario. An approximately threefold increase was observed for the mean 1-OHP concentration, whether or not adjusted for creatinine, for residential building fires. Outdoor or vehicle fires did not show a mean increase of the concentrations. Only for residential building fires a statistically significant change in median concentrations were observed. The increase of the mean during the first sampling of the 1-OHP concentrations in other scenarios was mainly caused by a single firefighter (Fig. 1, firefighter #4) who was a captain during a fire in an underground facility and had led an interior attack without using SCBA.

The summary of the 1-OHP measurements by role during firefighting are depicted in Table 4. The highest mean and median concentration of 1-OHP independent from creatinine adjustment were observed for firefighters with interior attack wearing SCBA, with a threefold to fourfold increase from baseline levels. Captains, who did not wear SCBA and were on duty on different of the investigated scenarios, also showed an increase in their 1-OHP concentrations, but the increase was less pronounced. During the suppression of outdoor or vehicle fires with SCBA use, no increase in 1-OHP concentrations was observed. The twofold increase of the mean 1-OHP concentration for other roles was mainly caused by a single firefighter, who was 30 min outside the danger zone of a residential fire without using SCBA. For the creatinine-adjusted concentration value, the corresponding increase was sixfold.

In Table 5 the results of the modelling of the 1-OHP concentrations using GEE regressions are depicted. Compared to firefighters at residual building fires, firefighters at outdoor or vehicle fires and other scenarios showed a higher association with 1-OHP concentrations (Model 3). In the model whoch includes only the fire operation scenarios the other scenarios instead showed a decreased association compared to the scenarios residential building (Model 1). For the roles during firefighting other roles as firefighter showed a lower association followed by outdoor or vehicle fires with SCBA and captains (Model 3). In all models current smoking is associated with higher 1-OHP concentrations. None of the estimates of all models are statistically significant.

4. Discussion

We examined 77 firefighters before and after fire operations using biological monitoring. We assessed 1-OHP concentrations in urine as an exposure marker for PAH incorporated during fire operations. Professional and voluntary firefighters of three cities in Germany were included in this study. The 1-OHP concentrations at baseline correspond to what would be expected in a non-occupationally exposed general population. We observed an approximately twofold increase of the 1-OHP mean concentration after the fire operations compared to the baseline measurements. For residential building fires, the mean 1-OHP concentration increased threefold and outdoor or vehicle fires showed the highest association. The same difference was observed for interior attacks despite the use of SCBA and here the highest associations between role during firefighting and 1-OHP concentration were found. In addition, fire captains showed higher concentrations after a mission.

In the case of PAH and 1-OHP in urine, a binding limit value in

Table 2
Summary of urinary 1-OHP measurements before and after firefighting (N = 77).

Parameter	1-OHP (μg/L)						P-value‡	1-OHP (μg/g creatinine)					
	Sample size	Mean	SD	Median	95th percentile	Range		Sample size	Mean	SD	Median	95th percentile	Range
Baseline	77	0.14	0.39	0.07	0.36	LOQ* – 3.41		45	0.13	0.16	0.09	0.26	0.03–1.10
1st sampling	77	0.31	0.51	0.15	0.99	LOQ – 3.55	<0.0001	62	0.18	0.31	0.11	0.38	0.03–2.23
2nd sampling	77	0.32	0.67	0.13	1.28	LOQ – 5.41	<0.0001	62	0.22	0.31	0.12	0.61	0.04–2.07
3rd sampling	77	0.31	0.60	0.14	0.94	LOQ – 4.75	<0.0001	63	0.27	0.59	0.11	0.58	0.04–4.27

SD = standard deviation; * Below limit of quantification; Values below LOQ are excluded for creatinine correction; Baseline: before firefighting; 1st sampling 2–4 h after firefighting; 2nd sampling 6–8 h after firefighting; 3rd sampling >12 h after firefighting; ‡ P-values of the Wilcoxon matched-pairs signed-rank test in contrast to baseline sampling.

	Firefighter													
	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12	#13	#14
Baseline	X													
1st sampling		X	X	X	X									
2nd sampling		X		X	X	X	X	X	X	X	X	X		
3rd sampling		X						X	X	X	X	X	X	X

Fig. 1. Pattern of the creatinine adjusted 1-OHP concentrations (µg/g creatinine) of the 14 firefighters with measurements above the corresponding evaluation standard (0.3 µg/g creatinine for non-smokers and 0.73 µg/g creatinine for smokers).

Europe and the USA does not exist. In order to assess an increased health risk associated with PAH exposure, a guidance value (BEI®) of the US-ACGIH based on health effects in humans was applied (ACGIH 2017). In addition to their carcinogenic potential, PAH exposure has been linked to other health effects like cardiopulmonary mortality (Kim et al. 2013). As mentioned previously, at levels of the BEI® for 1-OHP in urine (2,5 µg/L) or above, studies in PAH exposed workers in various industrial settings found increased DNA damage due to exposure to PAH compared to non-exposed controls. These workers were usually exposed continuously, i.e., day by day and during their work life rather than during single events such as urban firefighting, which do not last an entire shift and occur not during each shift. But multiple missions, or seasonal boosts for firefighters are possible. Individually spot urine samples were collected after a fire. Assuming no other relevant exposure outside these assignments, the collected urine samples can be considered as post-shift samples and it seems appropriate to apply the BEI® for a health-based interpretation of the 1-OHP levels in urine. It is important to note that BEI® does not differentiate between smokers and non-smokers. In addition, damage to DNA is not equivalent to a possible subsequent diagnosis of cancer. In particular, this is true for one-time/short-term exceedances in 1-OHP levels. Duration and frequency of an excess exposure over the entire working life need to be considered. In our study, one single firefighter showed 1-OHP concentrations above the BEI® after a firefighting operation. This value may be explained by two subsequent firefighting missions that this firefighter had to undertake during a single work shift. Since this was only a single event, it cannot be generalized. Although this situation does not occur frequently, it should be considered that subsequent missions in a single shift should be avoided.

Another way to interpret internal 1-OHP exposure is to compare biomonitoring concentrations with those of the non-exposed general population. Due to potential country- and region-specific differences in natural PAH sources and life-style habits, the respective national reference levels should be used, here those of Germany. Since PAH, and thus pyrene, are also present in cigarette smoke, consequently, the reference value for 1-OHP in the urine of non-smokers is per se lower than that of smokers. In this context, it is important to note, that reference values are statistically derived values and have no relation to health effects. The exceedance of a reference value can only be interpreted as an increased exposure compared to the general population, but is not necessarily associated with a relevant increased health risk.

The 1-OHP concentrations offer insight into the PAH-exposure of firefighters after real firefighting missions. Generalization of these results is not possible since each firefighting operation is different, depending on the fire material, ventilation, building structure and many other factors. Therefore, our study cannot offer a selection of representative fire operations in Germany. Nevertheless, our study shows the range of exposure to PAH from various fire operations which - based on a twofold difference in 1-OHP-levels between pre- and post-firefighting missions - must be considered small from a biological perspective. The twofold difference was also shown in another study, in which 1-OHP excretion in a training container depended on the fire material (Fent et al., 2019).

Predominantly, fires in residential buildings and outdoor fires were documented in our investigation (N = 71). In 49 of these operations, firefighters wore a SCBA, and33 of these SCBA-operations were interior attack missions. We were able to take samples from 14 captains, whereas engine operators and responders without SCBA hardly participated. In our study, firefighters were exposed to the highest levels of PAH during direct firefighting. Our study indicates that PAH may also absorbed by a firefighter when using PPE.

A more detailed evaluation of the cases, in which elevated concentrations were measured, reveals that these were generally operations involving heavy smoke development. Even the regular wearing of SCBA could not completely prevent absorption of PAH, but at least reduce it to such an extent that concentrations above the BEI® associated with health effects could only be observed in one single case. Captains who entered the scene without SCBA after firefighting had higher internal exposures. Therefore, correctly applied PPE including especially SCBA, during the entrance of the scene after fighting the fire down can additionally minimize the uptake of the fire-smoke components, whereas most of the firefighters stay in the range of the occupational unexposed general population. The results of the internal attack teams suggest that the uptake of PAH most likely occurred via the skin, since inhalation uptake through the PPE would only have been possible after leaving the fire site and after removal of the SCBA. Another possible route of uptake - again most likely via the skin - may be contact with contaminated clothing or other objects after leaving the fire scene. This can happen, when firefighters are removing their PPE and PPE penetration of PAH, or even when removing the SCBA in areas considered or perceived as safe respectively smoke free (Andersen et al., 2018). For example, firefighters, although wearing SCBA, who were exposed directly to the fire smoke (e.g. during interior attacks), showed higher 1-OHP concentrations.

Published studies of training sessions (Banks et al., 2021; Rossbach, 2020; Fent et al., 2019; Feunekes et al., 1997; Moen and Ovrebø, 1997) or real-fire operations (Caux et al., 2002; Keir et al., 2017; Hoppe-Jones et al., 2021) yielded similar results to our study.

In Toronto, 43 firefighters were sampled after their fire assignment (Caux et al., 2002),predominantly residential and building fires and responders were directly assigned to fire suppression or attended search and rescue missions. At baseline, median value was below the limit of detection. A doubling of the median 1-OHP concentration from 0.11 (directly after firefighting) to 0.22 µmol/mol creatinine (0–4 h after the operation) was observed. The median 8–12 h after firefighting was 0.10 µmol/mol creatinine. Caux et al. used a different metric, but the magnitude of the metabolized pyrene levels after firefighting was comparable to our study.

In Ottawa, a study of real-life fire operations in an urban setting was conducted and fires were residential or commercial building (Keir et al., 2017). There, urine samples were obtained from 16 male responders who had a total of 19 fire calls. The fold-change of 1-OHP was higher than in our study, but the maximum values observed for 1-OHP were lower. As we have seen from our results, each mission is different and therefore will result in different PAH exposure levels. Also, the sampling strategy was different in Ottawa. Urine samples were collected (pooled urine) over an 18-h period after deployment, and biomarkers of exposure such as 1-OHP were determined in the pooled samples. We collected spot urine at different sampling times. This may also be a reason of higher 1-OHP concentration in our study with spot urine samples, but a lower fold-increase because in our study urine samples between the sampling timepoints were missing and peak concentrations

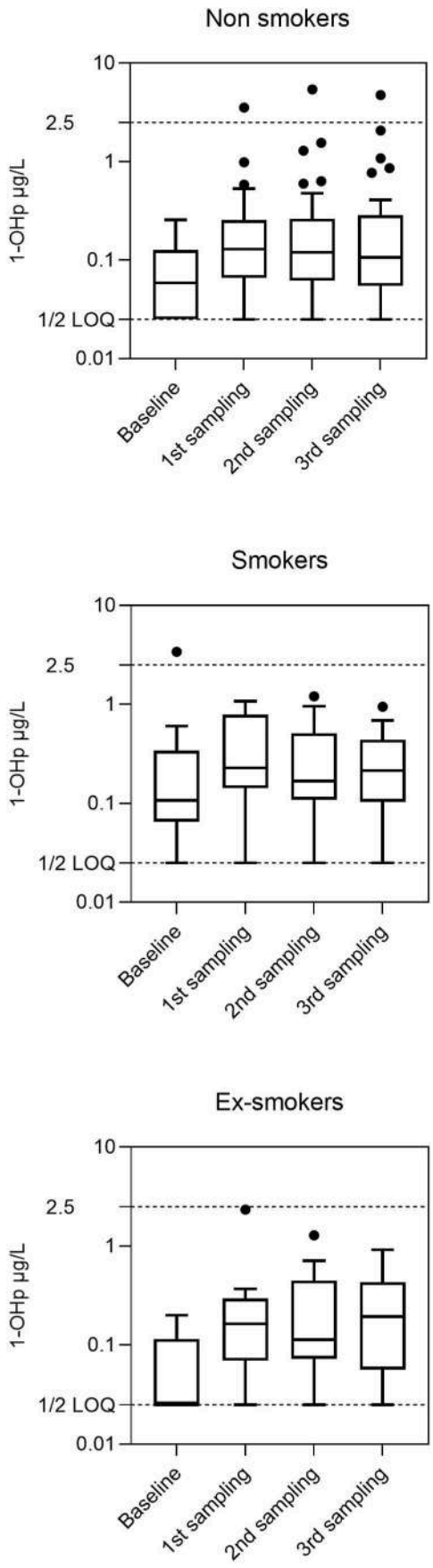

Fig. 2. 1-OHP concentrations (µg/L) stratified by smoking status before and after firefighting (N = 77).

could also be missing. Overall, however, the results of the two studies are comparable.

The third study using biomonitoring in real fire scenarios was conducted in Tucson, Arizona, from 2015 to 2017 (Hoppe-Jones et al., 2021). In total, 141 firefighters provided urine samples prior and after firefighting operations. A total of 15 fire incidents were sampled, including 11 house fires, one apartment fire, and three other building fires. Depending on the function during the mission, a 1.6–2.2-fold change in 1-OHP concentration was observed 2–4 h post-fire thus our results, again, were comparable to the US-study. In addition, as seen here, the highest fold-change was seen in direct firefighting operations.

Overall, we see consistent results between our findings and those of the three previous studies from Canada and the United States. Although differences in 1-OHP concentrations between studies are smaller than within the studies, both intra- and inter-study differences can be considered negligible from a biological point of view and an approximately 2-3-fold difference in 1-OHP levels between pre- and post-firefighting missions is consistent in all studies. Different burned materials, ventilation, oxygen supply emit different amounts of PAH and also personal behavior of the firefighters determined the amount of absorbed PAH in each individual fire operation, additional to life-style factors and smoking habits. Due to these results, we did not observe big differences between the amount of ingested pyrene in these four studies. All studies showed that PAH may enter the body during fire operations and that, in a few individual cases and under unfavorable conditions, health-relevant assessment values such as the BEI®, may also be exceeded. However, this depends on a wide variety of factors, including the extent of fire smoke development (visibility, height of the room) and the location of the operation (indoor attack).

The strengths of this study are the uniform protocol to assess 1-OHP and potential influencing factors during firefighting such as fire scenarios. In addition, with 77 participants, we recruited a large collective for a biomonitoring firefighter study with, in contrast to the majority of previous published studies, with actual firefighting missions. These real fire events do not reflect all worst-case scenarios, but reflect a wide range of real-life exposures caused by the different fire events.

Limitations of the study are that the study population is not a random sample of German firefighters. The study size was too small to be representative, but the study gives insights into the heterogenous exposure situation of firefighters and possible exposure scenarios. Not all participants had a fire call during the study period and despite intensive efforts, more fire operations could not be included. A recruitment bias is possible, if firefighters were too busy to provide urine samples after fire events because of a subsequent mission. Another limitation is that, due to the accidental nature of fire events, we did not have a chance to sample baseline urine directly before the firefighting operation and that daily PAH intake is influenced by additional factors such as diet rather than smoking only. Whether the observed two-fold increase in 1-OHP levels after firefighting missions is solely due to PAH uptake from firefighting or possibly from insufficient hygiene after the firefighting mission (e.g., when removing protective devices or contaminated protective clothing) remains unknown. Additionally, we may miss the peak exposure due to collected spot urine samples and not a full urine collection after the operation. Also, the creatinine excretion can change with physical exercise, and renal function, which is a factor that might be more relevant for firefighters than other occupations.

Our study suggests that the skin is an uptake route for PAH in firefighters because a significant increase in internal exposures was found in responders who wore SCBA during operations (including indoor and outdoor attacks). If SCBA is used during outdoor and vehicle fires, no increase in internal dose was observed. Caux et al. pointed out that when SCBA is worn, the 1-OHP concentration, compared to industrial workplaces with PAH exposure, remains low and the activity of a firefighter and the respective exposure to PAH is far lower when compared to an industrial worker (Caux et al., 2002), e.g., in coke-manufacturing, production of refractory materials, or production of graphite electrodes

Table 3
Summary of urinary 1-OHP measurements by fire operation scenarios (N = 77).

Parameter	1-OHP (µg/L)					P-value‡	1-OHP (µg/g creatinine)#				
	Sample size	Mean	SD	Median	Range		Sample size	Mean	SD	Median	Range
Residential building											
Baseline	54	0.10	0.10	0.06	LOQ* – 0.38		32	0.10	0.06	0.09	0.03–0.30
1st sampling	54	0.29	0.50	0.15	LOQ – 3.55	<0.0001	43	0.18	0.33	0.11	0.03–2.23
2nd sampling	54	0.37	0.78	0.13	LOQ – 5.41	<0.0001	45	0.25	0.35	0.13	0.04–2.07
3rd sampling	54	0.36	0.70	0.16	LOQ – 4.75	<0.0001	46	0.30	0.68	0.13	0.04–4.27
Outdoor or vehicle fire											
Baseline	17	0.31	0.81	0.10	LOQ – 3.41		12	0.20	0.30	0.10	0.04–1.10
1st sampling	17	0.29	0.32	0.15	LOQ – 1.07	0.0833	15	0.13	0.11	0.08	0.05–0.38
2nd sampling	17	0.22	0.24	0.15	LOQ – 0.96	0.2769	14	0.19	0.12	0.09	0.04–0.58
3rd sampling	17	0.23	0.28	0.10	LOQ – 1.08	0.6788	14	0.19	0.19	0.10	0.04–0.59
Other scenarios†											
Baseline	6	0.04	0.03	LOQ	LOQ – 0.10		1	0.05	–	0.05	–
1st sampling	6	0.52	0.91	0.11	LOQ – 2.34	0.1875	4	0.40	0.48	0.21	0.07–1.11
2nd sampling	6	0.16	0.19	0.07	LOQ – 0.42	0.2500	4	0.20	0.13	0.19	0.07–0.36
3rd sampling	6	0.13	0.15	0.08	LOQ – 0.40	0.2500	3	0.15	0.10	0.10	0.08–0.26

SD = standard deviation; † Four blazes, one vegetation fire, and one fire in underground facilities; * Below limit of quantification; # Values below LOQ are excluded for creatinine correction; Baseline: before firefighting; 1st sampling 2–4 h after firefighting; 2nd sampling 6–8 h after firefighting; 3rd sampling >12 h after firefighting; ‡ P-values of the Wilcoxon matched-pairs signed-rank test in contrast to baseline sampling.

Table 4
Summary of urinary 1-OHP measurements by role during firefighting (N = 77).

Parameter	1-OHP (µg/L)					P-value‡	1-OHP (µg/g creatinine)#				
	Sample size	Mean	SD	Median	Range		Sample size	Mean	SD	Median	Range
Interior attack with SCBA											
Baseline	33	0.11	0.10	0.07	LOQ* – 0.38		20	0.11	0.06	0.10	0.05–0.26
1st sampling	33	0.34	0.61	0.19	LOQ – 3.55	<0.0001	27	0.22	0.41	0.12	0.04–2.23
2nd sampling	33	0.48	0.95	0.17	LOQ – 5.41	<0.0001	29	0.29	0.38	0.17	0.04–2.07
3rd sampling	33	0.43	0.82	0.20	LOQ – 4.75	<0.0001	28	0.26	0.38	0.18	0.05–2.05
Outdoor or vehicle fire with SCBA											
Baseline	16	0.31	0.83	0.11	LOQ – 3.41		11	0.18	0.30	0.11	0.04–1.10
1st sampling	16	0.26	0.30	0.14	LOQ – 1.07	0.1354	14	0.11	0.09	0.08	0.05–0.38
2nd sampling	16	0.23	0.25	0.15	LOQ – 0.96	0.0906	13	0.15	0.13	0.10	0.06–0.48
3rd sampling	16	0.23	0.29	0.09	LOQ – 1.08	0.6257	13	0.16	0.18	0.09	0.04–0.58
Captain											
Baseline	14	0.08	0.09	0.04	LOQ – 0.34		7	0.10	0.05	0.09	0.05–0.21
1st sampling	14	0.42	0.61	0.19	LOQ – 2.34	0.0012	12	0.22	0.30	0.11	0.06–1.11
2nd sampling	14	0.18	0.16	0.10	LOQ – 0.51	0.0024	13	0.15	0.10	0.13	0.05–0.36
3rd sampling	14	0.20	0.14	0.16	0.07–0.52	0.0012	14	0.15	0.22	0.08	0.04–0.87
Other roles†											
Baseline	14	0.10	0.15	0.04	LOQ – 0.60		7	0.11	0.13	0.05	0.03–0.39
1st sampling	14	0.16	0.24	0.06	LOQ – 0.79	0.1230	9	0.12	0.09	0.11	0.03–0.29
2nd sampling	14	0.17	0.40	0.07	LOQ – 1.55	0.5703	7	0.25	0.43	0.11	0.04–1.23
3rd sampling	14	0.24	0.54	0.06	LOQ – 2.07	0.4961	8	0.67	1.46	0.09	0.05–4.27

SD = standard deviation; † three engine operators, five firefighters with SCBA during damping down operations, two firefighters with SCBA during external attack, three firefighter outside the danger area without SCBA, and one fire fighter during vegetation fire w/o SCBA; * Below limit of quantification; # Values below LOQ are excluded for creatinine correction; Baseline: before firefighting; 1st sampling 2–4 h after firefighting; 2nd sampling 6–8 h after firefighting; 3rd sampling >12 h after firefighting; SCBA: Self-contained breathing apparatus; ‡ P-values of the Wilcoxon matched-pairs signed-rank test in contrast to baseline sampling.

(Marczynski et al., 2009). In addition, exposure in firefighters does not occur on a daily basis throughout the entire working life. Accordingly, the resulting health risk may also be considerably lower than for (industrial) employees exposed to PAH.

5. Conclusions

Within the study, it could be shown that firefighters, especially captains, who entered the scene without SCBA after firefighting and firefighters during interior attack, had higher internal exposures and uptake of PAH. During interior attack SCBA were used and therefore exposure occurred most likely via the skin. Therefore, functional protective clothing, and wearing SCBA is important to reduce the intake of PAH. But there are individual situations during firefighting in which the concentrations exceeded currently existing guidance levels such as reference levels or the BEI®. Therefore, the possibility of an individually increased cancer risk from firefighting cannot be ruled out. Because each fire is different and exposure depends on many factors, we recommend to study additional firefighting scenarios. Although guidance levels for exposure- and health-based interpretation are missing, we also recommend to study biomarkers of carcinogenic PAH in urine such as metabolites of BaP (according to Barbeau, 2018) to gain, similar to 1-OHP, 'field experience' in the use of such markers and to foster the development of respective guidance values.

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Declaration of competing interest

DT, SK, HUK, TW, TB, TBe as staff of the Institute for Prevention and Occupational Medicine (IPA), are employed by the study's main

Table 5
GEE Models of urinary 1-OPH concentrations (µg/L) adjusted for fire operation scenarios, role during firefighting, and smoking status (N = 77).

Parameter	Model 1[a]		Model 2[b]		Model 3[c]	
	Estimate	95% CI	Estimate	95% CI	Estimate	95% CI
Intercept	−1.42	−2.09–−0.75	−1.19	−1.99–−0.38	−1.19	−2.03–−0.34
Smoking						
Non (reference)	0.00	–	0.00	–	0.00	–
Current	0.33	−0.39–1.06	0.23	−0.56–1.02	0.22	−0.64–1.08
Fire operation scenarios						
Residential building (reference)	0.00	–			0.00	–
Outdoor or vehicle fire	0.09	−0.71–0.88			0.43	−0.34–1.20
Other scenarios[†]	−1.23	−1.34–1.08			0.13	−0.94–1.19
Role during firefighting						
Interior attack with SCBA (reference)			0.00		0.00	–
Outdoor or vehicle fire with SCBA			−0.15	−1.14–0.83	−0.57	−2.03–0.88
Captain			−0.37	−1.15–0.41	−0.41	−1.13–0.32
Other roles[‡]			−0.66	−1.69–0.37	−0.72	−1.71–0.29

† Four blazes, one vegetation fire, and one fire in underground facilities; ‡ three engine operators, five firefighters with SCBA during damping down operations, two firefighters with SCBA during external attack, three firefighter outside the danger area without SCBA, and one fire fighter during vegetation fire w/o SCBA; a GEE model adjusted for smoking and fire operation scenarios; b GEE model adjusted for smoking and role during firefighting; c GEE model adjusted for smoking, fire operation scenarios, and role during firefighting.

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Face mask performance related to potentially infectious aerosol particles, breathing mode and facial leakage

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ABSTRACT

During the COVID 19 pandemic, wearing certified Respiratory Protective Devices (RPDs) provided important means of protection against direct and indirect infections caused by virus-laden aerosols. Assessing the RPD performance associated with infection prevention in standardised certification tests, however, faces drawbacks, such as the representativeness of the test aerosols used, the protection of third parties during exhalation or the effect of facial leaks. To address these drawbacks, we designed a novel test bench to measure RPD performance, namely the number based total efficiency, size-segregated fractional filtration efficiency and net pressure loss, for 11 types of certified surgical masks and Filtering Face Pieces dependent on breathing mode and facial fit. To be representative for the context of potentially infectious particles, we use a test aerosol based on artificial saliva that is in its size distribution similar to exhaled aerosols. In inhalation mode excluding facial leaks, all investigated samples deposit by count more than 85% of artificial saliva particles, which suggests a high efficiency of certified RPD filter media related to these particles. In exhalation mode most RPDs tend to have similar efficiencies but lower pressure losses. This deviation tends to be significant primarily for the RPDs with thin filter layers like surgical masks or Filtering Face Pieces containing nanofibers and may depend on the RPDs shape. Both the filtration efficiency and pressure loss are strongly inter-dependent and significantly lower when RPDs are naturally fitted including facial leaks, leading to a wide efficiency range of approximately 30–85%. The results indicate a much greater influence of the facial fit than the filter material itself. Furthermore, RPDs tend be more effective in self-protection than in third-party protection, which is inversely correlated to pressure loss. Comparing different types of RPDs, the pressure loss partially differs at similar filtration efficiencies, which points out the influence of the material and the filter area on pressure loss.

1. Introduction

Pathogen dissemination through aerosol particles emitted by the respiratory system best explains several super-spreading events during the COVID-19 pandemic (Katelaris et al., 2021; Kutter et al., 2021; Lu et al., 2020; Zhang et al., 2020) and is therefore in the focus of SARS-CoV-2 transmission. Particles that are formed and expelled through the respiratory system, for example when talking, coughing or breathing, may differ in size and number based on several factors such as the individual's physiology, health condition or activity (Archer et al., 2022; Morawska et al., 2009; Schwarz et al., 2010). In SARS-CoV-2 infected persons, these particles may act as vehicles for pathogens (Gutmann et al., 2022; Ma et al., 2021) and thus are determinant for the definition of protective measures. Present studies suggest that the mode of the exhaled particle size distribution most likely is in the order of 0.1–0.5 μm (Scheuch, 2020) allowing for the particles to stay airborne over several hours in indoor environments. Contrary to breathing, talking or coughing produces larger particles from the submicronic and small micrometre range to particles larger than 50 μm (Alsved et al., 2020; Asadi et al., 2019). Exposure to these respiratory-emitted particles leads to two possible routes of infection. On the one hand, particles may be transported directly from an infected person to a susceptible host (direct route of infection), whereby the probability of larger particles reaching a susceptible host decreases with the distance due to the particles' settling velocity. Airborne transmission, on the other hand, only occurs indoors, where the smaller fraction of respiratory-emitted particles may accumulate in the indoor air with increasing durations of stay, numbers of persons present and their activity. Since particle transport is still possible after an infected person has left the room, airborne transmission may also be referred to as an indirect infection route (Brlek

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et al., 2020; Cai et al., 2020).

To reduce the risk of both direct and indirect infections, infection control measures such as ventilation, air purifying technologies or the wearing of Respiratory Protective Devices (RPDs) were discussed and introduced during the COVID-19 pandemic in many areas of public life, such as schools, kindergartens, offices, public buildings, hospitals or the transportation sector. While ventilation and air purifying technologies may affect mostly the indirect route of infection (Nardell, 2021), the wearing of RPDs counteracts both direct and indirect infections by reducing the number of inhaled as well as exhaled particles and thus potentially provides an effective means of protecting oneself (self--protection) and others (third-party protection) (Asadi et al., 2020). Since certified RPDs, in particular surgical masks (DIN EN 14683:2019-10) and filtering face piece respirators such as FFP (DIN EN 149:2009-08), N95 (NIOSH approved 42 CFR 84) or KN95 (GB 2626–2006) are subjected to standardised test procedures, requirements for the separation performance are defined. Filtering Face Pieces according to DIN EN 149:2009-08 are categorized into three classes, with class FFP2 requiring a mass-based total efficiency of at least 94% and the total inward leakage not exceeding 11%. Test procedures use aerosols containing submicronic solid-phase sodium chloride or liquid-phase paraffin oil particles with a broad range allowed for the geometric standard deviation (Zoller et al., 2021) that partly overlap the size range of potentially infectious aerosols (Penner et al., 2022). As the test procedure originates from occupational health and safety, the focus is on self-protection against occupational pollutants, with third-party protection not being considered. Surgical masks according to DIN EN 14683:2019-10, on the other hand, are designated to protect others from infectious droplets during medical procedures. In certification, the number-based total filtration efficiency of the filter medium is determined by the use of infectious particles from a bacterial suspension with a median diameter of 3 μm that are one order of magnitude larger than exhaled virus-laden aerosol particles from the respiratory tract. As with all filtration processes, however, the efficiency of the separation mechanisms is highly dependent on the particle size and particle characteristics (Hinds and Zhu, 2022; Lee and Liu, 1982). To effectively remove respiratory particles from both the inhaled and exhaled air, RPD filter media need to be highly efficient with respect to particles in the relevant size range and with similar properties to infectious particles such as shape, charge and density. Furthermore, the overall efficiency is dependent on the face-to-mask seal, whereby leakage flows can cause unfiltered breathing air to be inhaled or exhaled that bypasses the filter medium (Koh et al., 2021; Pan et al., 2021). As a result, the performance related to potentially infectious particles considering the nature and size of exhaled aerosol particles and also the filtration performance associated with facial leakages in both self-protection and third-party protection may be a drawback of certification procedures for evaluating the RPD performance in the COVID-19 pandemic context.

Several studies with a focus on RPD performance related to infection protection have already been conducted. Studies involving submicronic particle collectives to determine total filtration efficiencies of certified RPDs show that the certified filter media are highly effective even when considered on a number basis. Rengasamy et al. (2014) reported penetration rates of less than 1% for sealed respirators and less than 10% for surgical masks at a flow rate of 40 l/min using a NaCl aerosol. Bagheri et al. (2021) suggested similar penetration rates for FFP2 masks with dolomite dust, which are all below 6%, but have found a higher variance in the penetration rates of different surgical masks. This includes several masks with penetration rates below 12%, as well as up to 75%. Other work (Bałazy et al., 2006; Grinshpun et al., 2009; Zangmeister et al., 2020) similarly shows that penetrations are distributed over a wider area in surgical masks than in respirators. When looking at fractional efficiencies, the most penetrating particle size (MPPS) varies for certified RPDs and is typically in the order of 30–300 nm, with the upper bound being more relevant for surgical masks (Bagheri et al., 2021; Bałazy et al., 2006; Grinshpun et al., 2009; Zangmeister et al., 2020). RPDs that have not been sealed in the test procedure show that facial leakage sharply decreases the overall efficiency. Grinshpun et al. (2009) found that the total inward leakage is particle size dependent from 7 to 20 times greater than the penetration through the filter medium for respirators and size independent from 4.8 to 5.8 times greater for surgical masks. Various studies point to facial leakages lead to similar filtration efficiencies for both respirators and surgical masks, independent of the initial efficiency of the filter medium (Grinshpun et al., 2009; Li et al., 2006; Rengasamy et al., 2014). When looking at the total outward leakage, which is relevant for the effectivity in third-party protection, however, only a few studies were conducted. Koh et al. (2021) and Pan et al. (2021) indicate that both inward and outward leakages are similar in respirators. For surgical masks, however, they indicate that the outward leakage exceeds the inward leakage.

Despite the clear evidence that the filter media used in certified RPDs is efficient for submicronic particles, to date little is known about how the filtration performance is modulated by facial leaks on both the self-protection and third-party protection. Questions on how the filtration performance is influenced by the real use case in the context of infection prevention remain unanswered. For example, how is the filtration efficiency affected when using a test aerosol representative for exhaled aerosols? Does the certification or characteristics of the RPD influence the filtration performance in the real use case considering facial leaks? How is the pressure loss, as a measure for the breathing resistance, effected by facial leaks or the flow direction? To answer these questions, the aim of this work is to determine performance parameters, namely the fractional filtration efficiency, number based total efficiency and net pressure loss, for different RPD classes and characteristics under conditions representative for infection prevention in the COVID-19 pandemic context. This includes the

- set-up of a test bench to determine the performance under representative test conditions.
- selection of a suitable fluid for particle generation related to respiratory-emitted aerosols and the evaluation of the test aerosol by comparison to human exhaled particles.
- determination of RPD performance as a result of fractional filtration efficiency, representative number based total efficiency and net pressure loss.
- comparison of performance parameters dependent on flow direction in terms of self- and third-party protection, as well as dependent on facial fit considering facial leakages.

First, in Sec. 2, the basic transport mechanisms of filtration and the equations of the performance parameters are presented. Then, in Sec. 3, the experimental setup, the test procedure and the materials used are described. In Sec. 4, the results are presented and discussed. This includes the validation of the test aerosol with human exhaled particles, as well as the screening of different RPDs. Finally, in Sec. 5, a conclusion of this work is drawn and an outlook on future work is given.

2. Theory

Aerosol particles may be removed from the gas phase by porous media when they reach the inner surface of a filter through various transport mechanisms, namely Brownian diffusion, direct interception, inertial impaction and electrostatic attraction (see Fig. 1).

Transport mechanisms are strongly dependent on particle size and flow velocity. Brownian diffusion is the dominating mechanism for small particle sizes and low flow velocities, whereby the particle motion is governed by a superordinated chaotic movement. Thus, particles do not follow the streamlines exactly and may randomly hit filter fibres. Brownian diffusion may be significant for the separation of the smaller particle fraction in the size of the infectious SARS-CoV-2. Particles that follow exactly the streamlines may be removed by direct interception, if the streamline passes within the particle radius on a filter fibre. Inertial

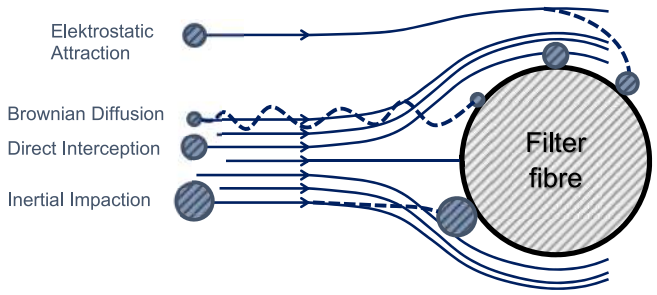

Fig. 1. Schematic illustration of the transport mechanisms in depth filtration.

impaction is mainly dominant on larger particles that, due to their inertia, are deflected of their streamline by its redirection around a fibre. With mask leakage, also the total leakage flow can be accelerated and redirected at the mask-to-face seal, potentially allowing inertial impaction to effect the deposition of larger particles in the case of unsealed RPDs (Hinds and Kraske, 1987). The interaction of the three transport mechanisms typically results in a most penetrating particle size (MPPS), which represents the least effectively separated particle size and is thus a characteristic of the respective filter medium. In air filtration, the MPPS is empirically around 0.3 μm and therefore in the size range of exhaled aerosol particles. In order to increase the removal probability of small particles, filter media, such as media based on nanofibres, aim to increase the efficiency at the MPPS through small pore sizes. However, materials based on synthetic melt blown fibres are most commonly used in RPDs. Meltblown fibres are electrostatically charged due to their manufacturing process and therefore able to attract particles of the opposite charge by electrostatic attraction. This may be advantageous in increasing the efficiency at the MPPS without reducing permeability and thus, increasing pressure loss.

To evaluate filtration performance dependent on particle size as well as to determine the MPPS, the fractional filtration efficiency is an elementary parameter. The fractional filtration efficiency is defined according to Eq. 1

$$E_{C_n}(x_i) = \frac{dC_{n,upstream}(x_i) - dC_{n,downstream}(x_i)}{dC_{n,upstream}(x_i)} \tag{1}$$

and represents the measurable difference in particle concentration of discretised particle size intervals in the raw gas (upstream of the filter) and clean gas (downstream of the filter) related to the raw gas particle concentration.

When only considering total particle concentrations, the total filtration efficiency is obtained according to Eq. (2).

$$E_{C_n} = \frac{C_{n,upstream} - C_{n,downstream}}{C_{n,upstream}} \tag{2}$$

Compared to the fractional filtration efficiency, the total filtration efficiency depends on the particle size distribution and the metric with which particle concentrations are measured (Zoller et al., 2021). Therefore, test aerosols with a size distribution similar to that of potentially infectious aerosol particles are a pre-requisite to evaluate the protective effect of RPDs. With mass concentrations, larger particles have a higher relative importance for the overall efficiency than with number concentrations. However, since particularly the small particles are relevant in the context of disease transmission, we only use efficiencies based on number concentrations.

To evaluate the wearing comfort of RPDs, the second performance parameter is the pressure loss, which is given in porous media by the Darcy equation if the flow is creeping, i.e. if the Reynolds number is smaller than 1:

$$\frac{\Delta p}{H} = \frac{\eta \bullet \bar{v}_f}{B} \tag{3}$$

The pressure loss Δp related to the layer thickness H depends on the dynamic viscosity η, the filter velocity $\bar{v}_f$ and the permeability$B$, which is a material constant depending on the fiber diameter and porosity. The net differential pressure Δp_{net} of RPDs is determined similar to the procedure described in DIN EN 13274-3:2002-03

$$\Delta p_{net} = \Delta p_F - \Delta p_H \tag{4}$$

where Δp_F is the measured differential pressure with the RPD mounted to a test head. Δp_H takes into account pipe friction losses, changes in cross-section and diversions due to the measuring apparatus, which is determined from a second measurement.

3. Material and methods

In this section, the materials and methods used for testing RPDs are described. First, the mask test bench is presented in detail with focus on both the experimental set-up and the test procedure. Subsequently, the materials used and the considered RPDs are described.

3.1. Mask test bench

Fig. 2 first illustrates the experimental test set-up to determine filtration-specific performance parameters of RPDs. Essential components are an aerosol generator (1), a test head (2), a volume flow-controlled fan (3) and measuring devices for the fractional particle number concentration (4a) and the differential pressure (4 b).

The primary function of the aerosol generator (AGK 2000, Palas GmbH) (1) is to produce test aerosol particles from a feeding liquid by the use of a two-substance nozzle and compressed air in order to mimic infectious particles from the respiratory system. Test aerosol particles are injected at the beginning of the test bench tubing and thereby diluted with ambient air to obtain a dry aerosol in measurable concentration. The total volume flow is controlled and generated by a radial fan mounted on the suction side. For flow control, an ultrasonic flowmeter is used to contactless measure the pressure- and temperature-compensated volumetric flow without influencing the flow profile and thus interfering particle sampling. The different RPDs under consideration are mounted to an additively manufactured head within a measuring cell. The measuring cell allows the test head to be mounted in such a way that it can be either flowed through from the outside to the inside (third-party protection) or vice versa from the inside to the outside (self-protection). In order to be representative towards facial leaks the dimensions of this test head are similar to ISO/TS 16976-2:2015-04 and represent an average Central European head size. Differential pressure is measured by the use of static ring pressure taps upstream and downstream the measuring cell with two differential pressure sensors in the range of 250 Pa and 1250 Pa, respectively. Particle concentrations are measured in both the raw and clean gas using an optical particle counter (Promo 3000, Palas GmbH). Therefore, an intrument-specific sampling volume flow of 5 l/min is taken isokinetically upstream and downstream the measuring cell. The particle concentration is determined by scattered light using an optical particle sensor with a measuring range of 10^6 P/cm^3 (WELAS 2070).

3.2. Test procedure

A total of four different test scenarios are considered. First, RPDs are attached to the test head by their existing head or ear loops. This intends to mimic the natural fit with leakage flows through the mask-to-face seal may influence the RPD performance. Second, to exclude facial leakage, RPDs are firmly attached to the test head by the use of a sealing compound. This provides the performance of RPDs if they would perfectly fit to a wearer's face, which partly is a comparable configuration to standardised certification procedures. Both modes of attachment are looked at separately for two flow directions, inhalation and exhalation,

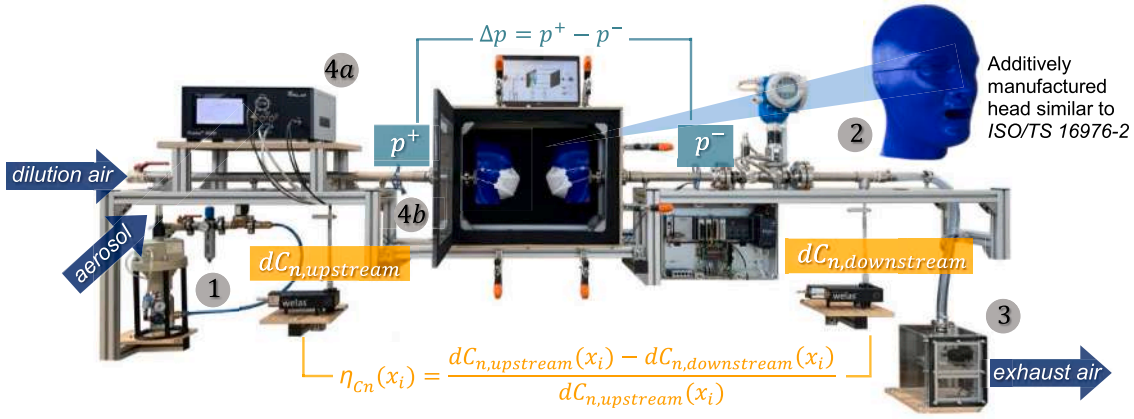

Fig. 2. Experimental test set-up for the determination of mask performance parameters (fractional filtration efficiency and net pressure loss).

by varying the position of the test head in the measuring cell. As a result, the mask performance for perfect and imperfect fitting RPDs can be ascertained distinctive for the flow directions of inhalation and exhalation in self-protection and third-party protection. Here, third-party protection only represents the efficiency of particle removal in the expiratory volume flow, while self-protection represents particle reduction in the inspiratory volume flow.

To prepare a measurement, first the test head is fitted with an RPD and then installed in the measuring cell according to the considered test scenario. A constant volume flow of 95 l/min is then applied to represent most unfavourable conditions. Thus, 95 l/min is an unrealistically high flow rate for breathing, it is also used in DIN EN 149:2009-08 DIN EN 149 as an inspiratory flow rate and intends to mimic the peak condition during sinusoidal breathing at 30 l/min according to DIN EN 13274–3. As a result, the determined mask performance is representative only for the peak condition of the breathing cycle. After preparation, tests are carried out under room air conditions ($p = 10^5$ Pa, $T = 25$ °C, $\varphi =$ 30–45%). Absolute pressure and temperature are measured online and used for volume flow compensation with regard to small fluctuations in ambient conditions. After equilibration, the pressure loss of the unloaded RPD is measured over a time interval of 30 s. The net pressure loss is then determined according to Eq. (4), subtracting the reference pressure loss of the test head and measuring cell in this configuration. After the pressure loss measurement is finished, test aerosol is injected into the tesdslbt tubing. In the first 300 s, the raw gas concentration is determined. Here, the loading time of 300 s is necessary to equilibrate the particle concentration in the measuring cell. The total number concentration in the raw gas is approx. 50,000 P/l, thus background particle concentration of 20 P/l is three orders of magnitude lower and is therefore neglected. After a steady-state particle concentration has been established, the clean gas concentration is determined during the next 300 s. To determine the fractional efficiency according to Eq. (1), the last 60 s of the raw gas measurement and the first 60 s of the clean gas measurement are used.

3.3. Artificial saliva

Aiming on a representative test aerosol to respiratory-emitted particles, a saliva substitute solution (apomix® Speichelersatzlösung SR) is used as a feeding liquid for aerosol generation. Saliva substitutes are often used to moisten the oral mucosa in patients with xerostomia and therefore intended to imitate certain properties of human saliva, such as viscosity (Łysik et al., 2019). In saliva substitutes, the viscosity is mainly influenced by either the additive carboxymethylcellulose (CMC) or mucin (Foglio-Bonda et al., 2022). Other components include electrolytes such as sodium chloride, potassium chloride, calcium chloride and magnesium chloride. Moreover, water, sorbitol and substances that serve as pH buffers and for preservation are contained.

3.4. RPDs

Certified surgical masks and filtering face pieces were selected for a screening in four different test scenarios, as described. The selected RPDs are listed in Fig. 3 and are categorized into five groups based on in their shape and characteristics. Four fish-shaped masks were considered, with two based on meltblown filter media (FFP2_3; FFP3_1) and two based on nanofibres (FFP2_1*; FFP2_2*). Further, duckbill-shaped (FFP2_4; FFP2_5) and classical axe-shaped filtering face pieces (FFP2_6; FFP2_7) all based on meltblown filter media were selected. In addition, two medical masks (SM_1; SM_2) as well as a reusable fabric mask with a nanofilter insert (FFP2_8* (R)) were screened.

4. Results and discussion

Prior to the actual measurements, the particle size distribution of the test aerosol was investigated and compared to exhaled aerosols (Sec. 4.1) in order to evaluate its representativeness for respiratory-emitted aerosols. Thereafter, the actual RPD screening was done on five new mask samples in each configuration with the test bench and test procedure described in Section 3. Performance parameters, namely the net pressure loss, the number based total efficiency and the fractional filtration efficiency were determined, aiming at a differentiated distinction between flow direction (third-party/self-protection) and fitting (including/excluding facial leakage) (Sec. 4.2).

4.1. Test aerosol

Test aerosols for determining the filtration performance of RPDs can be generated from various liquids such as those used in certification, for example sodium chloride solutions and liquid paraffin oil (DIN EN 13274-7:2019-09; DIN EN 149:2009-08), or biogenic solutions containing viable bacteria (DIN EN 14683:2019-10). Unlike the norms, the focus of the RPD screening is to determine the mask performance based on a representative test aerosol that mimics respiratory emitted particles. Representative in this context means that the characteristic of droplets and the size distribution are similar between exhaled and technically generated particles. Therefore, we use a saliva substitute solution as a feeding liquid for technical aerosol generation (see Sec. 3.3). To evaluate representativeness, the particle size distribution of the technically generated aerosol from artificial saliva is compared to an exhaled aerosol optically measured in Penner et al. (2022) and compared in Fig. 4. Since the total number of exhaled particles is several orders of magnitude lower than of technically generated aerosols, a different optical sensor with a lower measuring range was used for the

Fig. 3. Selected meltblown based and nanofibre* based RPDs for performance screening.

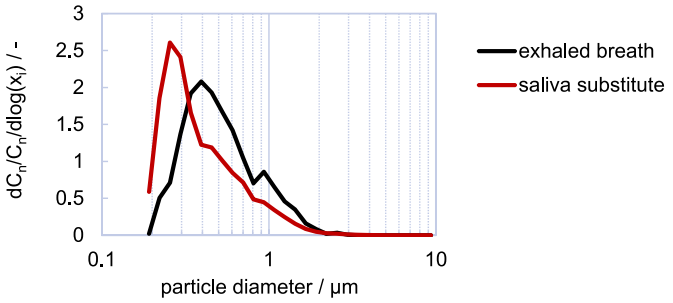

Fig. 4. Comparison of the particle size distributions of the technically generated aerosol from saliva substitute solution and human exhaled aerosols of 13 subjects normalized to the total particle concentration and logarithmic bin size (Penner et al., 2022).

exhalation measurement. To allow for comparison, the particle number concentration of each particle size interval (dC_n) is normalized to the total particle number concentration (C_n) as well as the logarithmic bin size ($\Delta\log(x_i)$) of the optical particle counter.

The size distribution of exhalation measurements is presented as the mean of 21 measurements of 13 test persons and compared to a single measurement of the saliva substitute solution that is technically dispersed with the aerosol generator. The results show that both aerosols contain particles in a similar size range that are mainly smaller than 2 µm. However, in the technically generated aerosol, the mode of the size distribution is close to the metrological boundary of the optical particle counter in the range of 0.2 µm. Here, counting errors may occur, which suggests that the actual concentration at the mode may be even higher. The mode for exhaled particles, on the other hand, is in the range of 0.4 µm and thus the exhaled size distribution contains relatively larger particles. Thus, the technical generation principle is based on a two-substance nozzle with larger particles partly being removed by a cyclone, the used aerosol generator does not mimic the generation mechanism of particles in the human lungs, which may explain the slight differences in both distributions. Another reason may be the test conditions for both set-ups, with the exhaled particle size distribution determined undiluted at an air humidity of approx. 90% due to the low particle concentration. Although the time required for exhaled particles to evaporate is very short due to their small size and the associated high surface tension (Gregson et al., 2022; Walker et al., 2021), incomplete evaporation cannot be ruled out in this set-up due to the high humidity. Technical aerosol, on the other hand, is diluted to a total flow of 95 l/min, which may result in a faster evaporation of the water content and thus to a smaller particle size. On the whole, the overall differences are minor; moreover, saliva substitute solution represents a more comparable fluid in terms of its composition and properties in the context of infection protection and is therefore used for the RPD screening.

4.2. Screening of RPDs in new condition

RPDs act as particle sinks for the inhaled and exhaled air and thus potentially provide an effective means of protecting oneself and others from direct and indirect infections. The filtration performance, however, may differ in self-protection and third-party protection for both perfect and natural fitted RPDs that partly allow for unfiltered breathing air to pass at the mask-to-face seal. The RPD screening aims to provide the performance related to this dependency on flow direction and facial leakage by the use of a representative test aerosol (Sec. 4.1) and a newly conceived test bench (Sec. 3.1). Therefore, 11 surgical masks and Filtering Face Pieces (Sec. 3.3) are tested at a steady-state volume flow of 95 l/min, which aims to represent the peak volume flow occurring during sinusoidal breathing at 30 l/min. For each type of RPD, the fractional filtration efficiency, number based total efficiency and the net pressure loss (Sec. 2) are determined using five new RPD samples. Fig. 5 illustrates the averaged fractional filtration efficiencies.

The diagrams aligned vertically differ in whether RPDs were sealed or naturally fitted to the test head. When the RPDs were sealed, thus were “perfectly fitted”, 7 out of 11 masks exceed an efficiency of 95% at each particle size, which indicates a good filtration performance related to aerosol particles from saliva substitute. Surgical mask SM_1 is similar efficient compared to meltblown based Filtering Face Pieces, while the second surgical mask (SM_2) shows a lower efficiency that is still above 85% at the MPPS. RPDs containing nanofibres (FFP2_1*, FFP2_2*, FFP2_8*(R)) appear to have lower efficiencies of approx. 75% (disposable) and 85% (reusable) at the MPPS. For sealed nanofibre-based RPDs, however, the fractional filtration efficiency curves deviate significantly in self-protection and third-party protection, thus the differences cannot be explained solely by a lower efficiency of nanofibre-based filter media but may also be the result of a more complicated sealing of these materials to the test head with the sealing compound used.

When RPDs were naturally fitted and facial leakage is expected to occur, the fractional efficiency curve of each RPD type is significantly lower than in its sealed installation variant. This confirms the expectation in general. Moreover, it can be observed that the efficiency curves deviate over a wider range of approx. 20%–90%, which suggests a significant influence of facial leakage on filtration performance dependent on the RPD fit. To take a closer look, diagrams aligned horizontally differ only in inhalation and exhalation mode, which is intended to represent self-protection and third-party protection. Naturally fitted RPDs in self-protection, generally, tend to have a higher efficiency than their equivalent in third-party protection. This difference is most pronounced in the case of surgical masks, nanofibre-based RPDs and two of the meltblown-based FFP masks, with these masks depositing partly twice as the amount in self-protection as in third-party protection. The RPD models FFP2_3, FFP2_7 and FFP3_1, however, deviate in their filtration performance in both modes only slightly. As a result, this indicates that the efficiency of an RPD may strongly differ between inhalation and exhalation dependent on its properties to minimize facial leakage, which is discussed in the context of Fig. 7 in more detail.

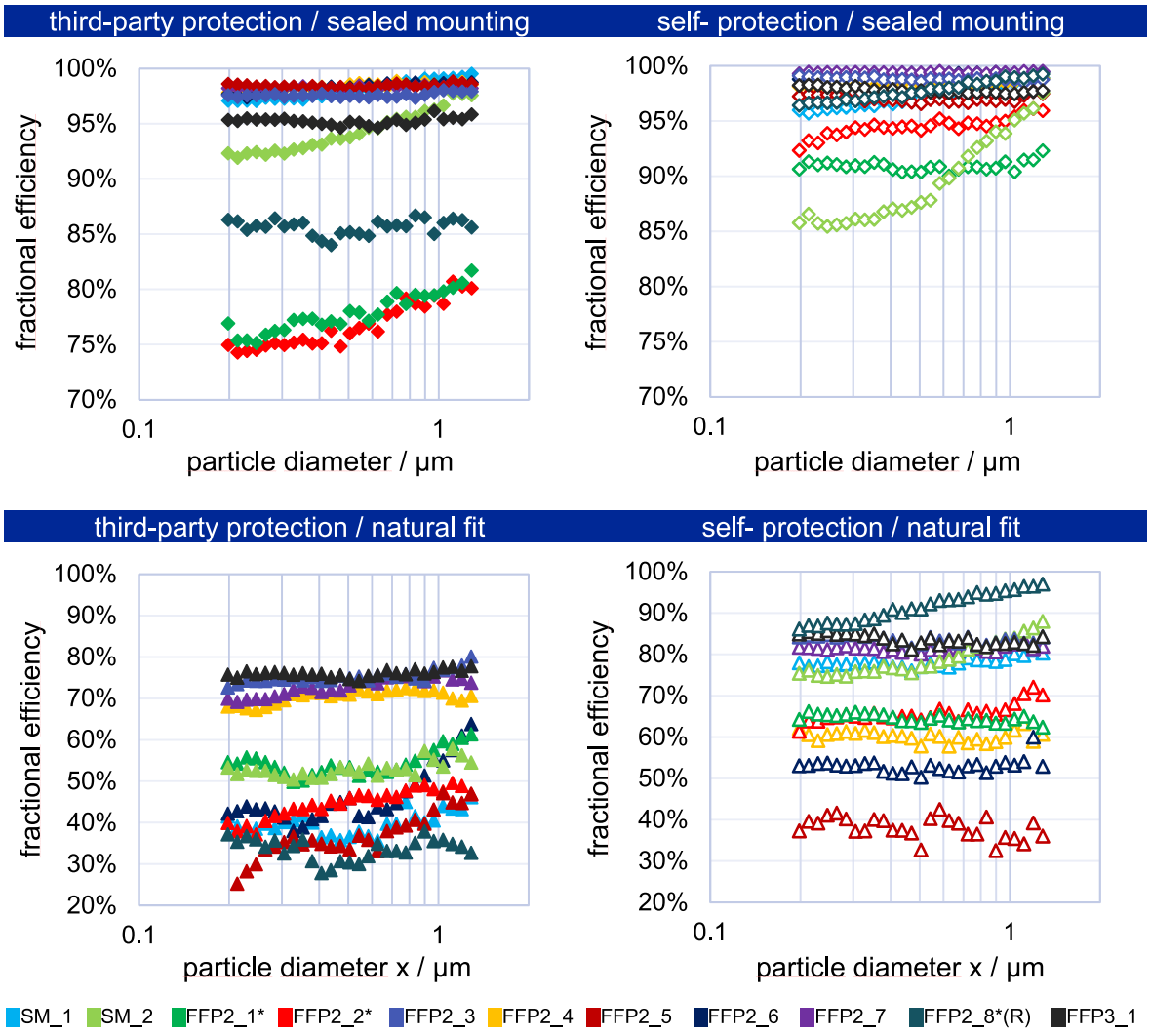

Fig. 5. Fractional separation efficiencies of selected RPDs at 95 l/min using artificial saliva in sealed installation excluding leakage, as well as in natural installation including leakage, differentiated in self-protection and third-party protection. Each fractional filtration efficiency curve is the mean of five measurements on five new RPD samples.

With the sealed installation, there are fewer differences between the two flow directions compared to naturally attached RPDs. Deviating filtration efficiency curves can be seen in the surgical masks and nanofibre-based RPDs, which, in addition to the directionality of the facial leakage, also indicates a directionality of the filter material on filtration performance. Since these mask materials are generally thinner and less rigid, they may be more easily drawn to the test head in the inhalation mode, thus reducing the effective filter area and increasing the specific load. As described in Section 2, the transport mechanisms of particles to the inner surface of the filter material are dependent on the flow velocity, which would well explain the observed differences here. Assuming further that the distance between an RPD and a wearers face is very small, so that the time required for complete evaporation of the water content of the particles during exhalation is insufficient, larger particle sizes could be relevant for third-party protection. In this case, RPDs with increasing efficiency over particle size, such as the SM_1 and SM_2 surgical masks and the FFP2_1* and FFP2_2* nanofibre-based masks, would be more efficient in a real application.

Pressure loss, as the second key performance parameter, is an indicator of breathing resistance and thus crucial for the wearing comfort. RPDs with a low pressure loss impair breathing less and are thus desirable especially for vulnerable individuals with pre-existing conditions of the respiratory system or low tidal volumes. As with the filtration efficiency, also the pressure loss may depend on facial leakage and the direction of flow for different mask characteristics. In order to view both performance parameters side-by-side, the number-based total filtration efficiencies determined from the fractional efficiencies are illustrated above the net pressure loss in Fig. 6. Each point is the mean of five measurements on five new RPD samples, with the error bars representing the standard deviation. The total filtration efficiency in sealed installation shows for most RPDs again that the requirement of DIN EN 149 for a lower penetration than 6% is fulfilled, if the total efficiency is determined on a number basis and with a representative test aerosol of saliva substitute solution, both in third-party and self-protection. Naturally fitted masks, on the other hand, vary in the range of 30% and 85%, thus the efficiency is significantly decreased due to facial leakage.

A comparison of the different RPDs in sealed installation shows that the pressure loss varies over a wide range, with the surgical masks at the lower bound of approximately 30 Pa–90 Pa. FFP masks, for example FFP2_5 and FFP2_7, tend to highly differ in pressure loss although the filtration efficiency is similar. In general, these observed differences can simply be explained by different effective filter areas, material thicknesses and permeabilities. When comparing naturally fitted RPDs, on the contrary, the pressure loss is strongly reduced, resulting from the effect of facial leakage. RPDs with a sharp decrease in pressure loss,

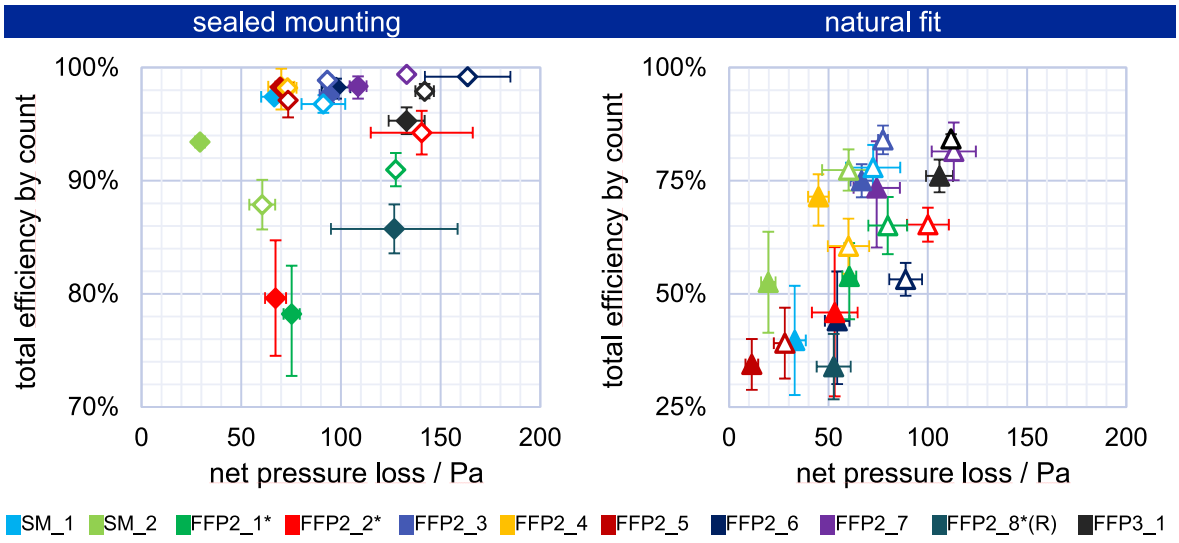

Fig. 6. Comparison of the number-based total separation efficiency with the net pressure loss @ 95 l/min. Open symbols represent measuring points in self-protection, filled symbols represent the third-party protection. Each point is the mean of five measurements on five new mask samples, with the error bars representing the standard deviation of the fivefold determination.

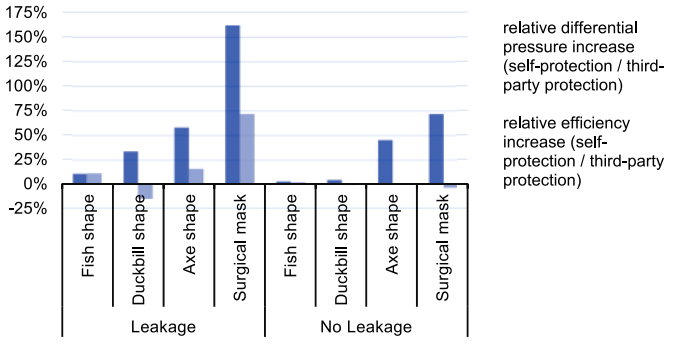

Fig. 7. Illustration of the relative change of the performance parameters in self-protection in relation to third-party protection. A relative value of 100% would mean that twice the value was measured in self-protection as in third-party protection.

compared to its sealed fit, also show a sharp decrease in total filtration efficiency, suggesting that both performance parameters are affected in a mutually dependent manner. Nevertheless, when comparing different RPD types in the natural fit, such as FFP2_3 and FFP2_7, for example, then similar efficiencies but different pressure losses can be observed. Despite the strong influence of facial leakage, this demonstrates the still existing dependency on filter area and filter material. By looking at the dependency of pressure loss on flow direction in Fig. 6, with open symbols representing self-protection and filled symbols representing third-party protection, RPDs in the inhalation mode exhibit the highest pressure losses in both sealed and non-sealed installation. This suggests a greater resistance when inhaling than when exhaling. Comparing the surgical mask SM_2 and the Filtering Face Piece FFP3_1 in both breathing modes, it is also evident that this difference in pressure loss but also filtration efficiency between inhalation and exhalation is significantly greater with the surgical mask.

In order to take a closer look at how breathing mode-based differences result for different mask types, Fig. 7 aims at a relative comparison. Here, the performance parameters of self-protection are related to those of third-party protection, subdivided into the mask groups described in Section 3.

As already seen on the basis of the fractional filtration efficiencies in sealed installation, the filtration efficiency is similar in both breathing modes when avoiding facial leakage. However, axe-shaped FFP and surgical masks have a higher pressure loss during inhalation, which may be due to a deformation of the mask caused by the direction of flow. In axe-shaped masks, both halves of the mask may contact each other due to the negative pressure during inhalation, while medical masks may touch the test head due to their flexible material. Both would lead to a reduction in filter area, increasing the flow velocity at constant volume flow, which in turn leads to a higher pressure loss based on Equation (3). As discussed above, relatively higher filtration efficiencies but also pressure losses are determined in self-protection in the natural fit including facial leaks. Subdivided into the different RPD shapes, this difference is found to be most pronounced for surgical masks. Fish-shaped FFP masks show the smallest differences, while duckbill-shaped masks and axe-shaped masks are in between. A closer look at the measured pressure loss of fish-shaped RPDs shows that the pressure loss increase in self-protection related to third-party protection is also differently pronounced within this subgroup. FFP2_1 and FFP2_2 with nanofibre materials show a higher relative pressure loss increase on inhalation than FFP2_3 and FFP3_1 based on meltblown filter media, highlighting the still existing influence of the mask material. Mask shape and filter material most likely affect the extent to which RPDs are being drawn to the test head on inhalation due to the negative pressure. The reduction of leakage areas may be advantageous for self-protection, but the minimization of leakage areas also increases the pressure loss.

5. Conclusions

This work focused on a screening of certified surgical and FFP masks used in the COVID-19 pandemic context with respect to respiratory-emitted particles. To this end, we presented a novel experimental set-up that allows the determination of mask performance parameters, namely the fractional filtration efficiency and the net pressure loss, as a function of the flow direction (self and third-party protection) and of the facial fit (sealed and natural fit) using a test aerosol based on artificial saliva. The particle size distributions of exhaled breath and the test aerosol were compared in exhalation mode. Measurements show that they are in a similar size range up to 0.4 µm with most particles smaller than 2 µm. The results of the mask screening in sealed fitting show that both the FFP and surgical masks examined feature a high filtration efficiency with regard to artificial saliva and, with a few exceptions, would meet total number-based efficiencies of 94% related to the requirements in DIN EN 149 using artificial saliva. The filtration efficiencies of the

sealed fit are similar in both flow directions, but higher pressure losses are found in self-protection. One reason for this might be a reduction in filter area during inhalation, which presumably results from the drawing of masks with less stiff and thinner materials against the test head or, especially in the case of axe-shaped masks, might be the result of two mask surfaces being merged. In natural fitting, facial leakage significantly decreases both the filtration efficiency and pressure loss for each mask model tested. Here, the total efficiencies between different masks are in the order of 30%–85%, whereas the pressure loss appears to decrease in an inter-dependent manner with filtration efficiency. As a result, we conclude that the mask performance is more influenced by the mask fit and sealing material qualities than the filtration-specific properties of the filter media. As far as the flow direction is concerned, the filtration efficiency and pressure loss tend to be lower in third-party protection than in self-protection. This can similarly be caused by a drawing to the test head during inhalation, which might reduce the size of leakage areas between test face and RPD. Here, the relative change of the performance parameters may be influenced by thin and less stiff materials that favour such drawing to the test head, but may also be influenced by different RPD shapes (fish-, duckbill-, axe-shape).

One can conclude from our study that, considering naturally fitted masks, in addition to filtration-specific material properties, the flow direction, the dimensional stability, the mask shape as well as the sealing material properties influence the RPD performance significantly. However, these properties may be influenced also through humid and particle-laden breath, which is why future work needs to focus on the influence of wearing time on RPD performance. In addition, the influence of RPD shape indicates potential for optimisation, especially for the development of well-separating masks with reduced pressure losses that are suitable for infection prevention even for high-risk patients with restricted tidal volume.

Data availability statement

Data are available from the corresponding author upon reasonable request.

Declaration of competing interest

The authors declare no conflict of interest.

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Gestational exposure to organophosphate esters and adiposity measures of children up to 6 years: Effect modification by breastfeeding

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ABSTRACT

Organophosphate esters (OPEs) are synthetic chemicals used in various commercial products. Accumulating evidence has shown that they may act as metabolic disruptors. However, no study has investigated the long-term effects of gestational OPEs exposure on childhood adiposity. Breast milk represents the optimal nutritional form of feeding for infants and may protect against the adverse effects of gestational OPEs exposure on offspring development. Using data from the Shanghai-Minhang birth cohort study, we investigated the associations of gestational OPEs exposure with adiposity measures in children up to 6 years of age, and whether breastfeeding could modify these associations. A total of 733 mother-child pairs with available data on OPE concentrations and child anthropometry were included. Eight OPE metabolites were assessed in maternal urine samples collected at 12–16 weeks of pregnancy. Information on children's weight, height, arm circumference, and waist circumference was collected at birth and 0.5, 1, 4, and 6 years of age. Weight-for-age and body mass index-for-age z scores were calculated. The duration of children's breastfeeding was categorized as ≤4 months or >4 months. The generalized estimate equation and Bayesian Kernel Machine Regression models were used to examine the associations of OPEs exposure with children's adiposity measures. Selected OPEs exposure was associated with higher children's adiposity measures. Particularly, we found stronger associations of bis(1-chloro-2-propyl) phosphate (BCIPP), bis(2-chloroethyl) phosphate (BCEP), bis(1,3-dichloro-2-propyl) phosphate (BDCPP), and di-o-cresyl phosphate and di-p-cresyl phosphate (DCP) with higher adiposity measures in children breastfed for ≤4 months, while little evidence of associations was found among those breastfed for >4 months. Our study suggested that gestational OPEs exposure could alter children's adiposity measures, but the potential effects were attenuated if children were breastfed for >4 months.

1. Introduction

Organophosphate esters (OPEs) are a class of synthetic chemicals and extensively used as flame retardants (FRs) and plasticizers in furniture (polyurethane foam), textiles, electronics, building materials, and baby products (nursing pillows and car seats) (Cooper et al., 2016; Du et al., 2019; Hoffman et al., 2015). Due to the progressive phase-out of polychlorinated biphenyls and polybrominated diphenyl ethers, OPEs

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have been among the most commonly used alternative FRs in recent years. Global consumption of OPEs accounted for 11% of the total consumption of FRs in 2008 and increasingly over 30% in 2017 (Wang et al., 2020). Most OPEs are additives and are not chemically bound to the products in which they are used; thus, they can easily migrate into the surrounding environment (Hou et al., 2016). Humans can be exposed to OPEs through ingestion (contaminated foodstuff and dust), inhalation (dust), and skin contact (Mäkinen et al., 2009; Poma et al., 2017; Yang et al., 2014). Recent studies have reported the ubiquitous detection of OPEs in human samples, such as blood, urine, and breast milk (Kim et al., 2014; Wang et al., 2021; Zhao et al., 2016). Particularly, OPEs have been detected in human deciduae and chronic villi, suggesting that OPEs may undergo maternal-fetal transfer during pregnancy (Zhao et al., 2017), which raises concerns about the adverse effects of gestational OPEs exposure.

Gestational OPEs exposure may have adverse effects on the growth and development of offspring, especially with regard to the risk of obesity (Crawford et al., 2020; Wang et al., 2019). OPEs may act as metabolic disruptors given their potential to disrupt lipid and glucose metabolism (Wade et al., 2019; Wang et al., 2019) and sex steroid activities (Liu et al., 2013) in animals, and thyroid functions in humans (Preston et al., 2017); thus, may disrupt energy homeostasis. Animal studies have revealed that perinatal OPEs exposure can result in increased body mass, fat mass, fasting leptin levels, and total energy intake in rats (Green et al., 2017; Moser et al., 2015; Patisaul et al., 2013). Several epidemiological studies investigating the associations of gestational OPEs exposure with birth size (Feng et al., 2016; Hoffman et al., 2018; Kuiper et al., 2020; Luo et al. 2020, 2021) have yielded inconsistent results. Gestational exposure to individual OPE metabolites or their mixtures was shown to be associated with lower birth weight and the risk of being large-for-gestational age (Bommarito et al., 2021; Luo et al. 2020, 2021). Another study found that gestational OPEs exposure could affect infants' weight and waist circumference (WC) during the first 6 weeks of life, and the effects were sex-specific (Crawford et al., 2020). However, null associations of OPEs exposure with birth weight have also been reported (Crawford et al., 2020; Hoffman et al., 2018; Kuiper et al., 2020). To our knowledge, no studies have investigated the long-term effects of gestational OPEs exposure on childhood adiposity measures.

Maternal nutritional resources play an important role in the growth and development of offspring in early life. Accumulating evidence suggested that the effects of gestational environmental pollutants exposure may be modified by maternal dietary factors (e.g. fruit and vegetable intake, fat intake, fish consumption, and vitamin D intake) (Gennings et al., 2020; Guxens et al., 2012; Somm et al., 2009). Breast milk represents the optimal nutritional form of feeding for infants and contains high levels of macronutrients, micronutrients, and bioactive factors, which may reduce oxidative stress and inflammation that may be associated with the development of obesity (Andreas et al., 2015). Additionally, breastfeeding is a key factor that may drive the transition of infants' gut microbiota in the first months of life, which can modulate energy homeostasis and adiposity (Petraroli et al., 2021). Furthermore, previous studies have shown that gestational OPEs exposure can induce oxidative stress (Wang et al., 2015) and disturb gut microbiota (Wang et al., 2019) in animals. Thus, breastfeeding may confer developmental benefits and act as a potential effect modifier. While previous studies have shown that adequate breastfeeding may protect against the adverse impacts of gestational environmental pollutants exposure on children's health (Guxens et al., 2012; Lertxundi et al., 2015), no study has investigated the potential effect modification by breastfeeding on the associations of OPEs exposure with childhood obesity.

This study aimed to investigate the associations of gestational OPEs exposure with adiposity measures in children from birth to 6 years of age, and the potential effect modification by breastfeeding on these associations in a birth cohort study.

2. Method

2.1. Study population

The participants were mother and child pairs from the Shanghai-Minhang birth cohort study which was designed to investigate the adverse effects of environmental pollutants exposure on the growth and development of offspring. A total of 1, 292 pregnant women were recruited from April to December 2012 at the Minhang Maternal and Child Health Hospital, Shanghai, China. The detailed eligibility criteria have been described elsewhere (Ji et al., 2019). Briefly, eligible pregnant women were at 12–16 weeks of gestation, were residents of Shanghai, had no history of major chronic diseases, were intended to deliver in the study hospital, and were willing to attend the scheduled interviews during pregnancy and after delivery.

We included 1, 225 mother-child pairs after excluding twin births (n = 8), abortion or stillbirths (n = 31), and deliveries in other hospitals because of pregnancy complications (n = 28). At recruitment, a structured questionnaire was administered to obtain information about maternal characteristics, lifestyle factors, medical history, and reproductive history. A total of 1195 single-spot urine samples were collected from the pregnant women. Follow-up visits were then conducted to obtain anthropometric measurements of 1,191, 765, 632, 627, and 541 children at birth and 0.5, 1, 4, and 6 years of age. The OPE concentrations were measured in 733 samples. The conditions for measurement were enough urine volume, and available information at delivery and at least one follow-up visit. In total, the study finally included 733, 589, 498, 486, 413 mother-child pairs at birth and 0.5-, 1-, 4-, and 6-years visits, respectively (Fig. S1). Information on breastfeeding duration was collected at the 0.5-year visit.

All mothers gave informed consent for themselves and their children at recruitment and each follow-up visit. The study protocol was approved by the ethical committee of the Shanghai Institute for Biomedical and Pharmaceutical Technologies.

2.2. Exposure measurement

OPEs are quickly metabolized to their diesters and monoesters and excreted in the urine (Kuiper et al., 2020). Thus, urinary OPE metabolite concentrations were used as an indicator of exposure as they have previously been shown to be relatively stable across pregnancy (Romano et al., 2017). The spot urine samples were frozen at −80 °C until they were shipped on dry ice to Jinan University for OPEs assay. Eight specific OPE metabolites were assessed in this study: bis(1-chloro-2-propyl) phosphate (BCIPP), bis(2-chloroethyl) phosphate (BCEP), bis(1, 3-dichloro-2-propyl) phosphate (BDCPP), diphenyl phosphate (DPP), di-o-cresyl phosphate (DoCP) and di-p-cresyl phosphate (DpCP), bis (2-butoxyethyl) phosphate (BBOEP), bis(2-ethylexyl) phosphate (BEHP), and dibutyl phosphate (DBP). Because it was difficult to separately quantify the DoCP and DpCP (two metabolites of tricresyl phosphate), their total concentrations of them were presented (DCP).

Approximately 1 mL of urine was thawed and spiked with 0.1 mL of ammonium acetate buffer (1 M, pH = 4.5), 50 μL of β-glucuronidase (≥100,000 units/mL, *Escherichia coli* K12, 2 μL/mL in water), and isotopically labeled internal standards (including BCIPP-d_{12}, BDCPP-d_{10}, BEHP-d_{34}, DoCP-d_{14}, DBP-d_{18}, and DPP-d_{10}, BBOEP-d_{18}). The mixture was thoroughly mixed and incubated overnight at 37 °C and then cleaned through an Oasis WAX column (3 cc/60 mg, Waters Inc.) pre-washed in sequence with 6 mL methanol (1% ammonia), 6 mL acetonitrile, and 6 mL water (0.2% formic acid). After sample loading, the cartridge was washed with 3 mL of a mixture of water and methanol (95:5, v/v) containing 0.2% formic acid, and then the targets were eluted out with 2 mL of methanol and 3 mL of methanol containing 0.1% ammonia. The final extract was concentrated and determined on ultra-performance liquid chromatography coupled to an AB Sciex 5500 triple quadrupole mass spectrometer (Toronto, Canada).

Detailed information on the instrumental analysis and quality assurance/control procedures was introduced in our earlier study (Xie et al., 2021). Briefly, several procedures were conducted to ensure data quality. Matrix spiking tests were conducted to evaluate the recoveries of the added standards from the sample treatment. Matrix effect tests were conducted to assess the interference of the sample matrix with the data analysis. A procedural blank was processed along with every ten samples. We also conducted blind analysis of duplicated samples and evaluated laboratory contamination. The limits of detection (LOD), defined as an analyte response five times the standard deviation of the noise, were determined for BCIPP (0.015 ng/mL), BCEP (0.110 ng/mL), BDCPP (0.042 ng/mL), DPP (0.004 ng/mL), DCP (0.002 ng/mL), BBOEP (0.015 ng/mL), BEHP (0.003 ng/mL), and DBP (0.020 ng/mL), respectively. Urinary creatinine levels were measured and adjusted in expressing urine OPE metabolite concentrations (corrected for creatinine by division) to address the variability due to urinary dilution.

2.3. Follow-up visits and measurement of child anthropometry

The weight-for-age (WAZ) and body mass index-for-age (BMIz) z scores, arm circumference (AC), and WC of children were used as the primary outcomes in this study. The relevant information was collected when children were at birth and 0.5, 1, 4, and 6 years of age. Specifically, information on the child's biological sex assigned at birth, birth weight, birth length, AC, and WC at birth was extracted from the medical birth records. After birth, child's weight, length, AC, and WC were obtained from the routine physical examination records at 0.5- and 1-year visits and were measured using standard protocols by trained interviewers at 4-year and 6-year visit. Weight was measured using a digital scale, with the child wearing light clothes and no shoes. Length was measured with the child stretching out their trunk and legs and in the recumbent position using an infant length board when the child was 1 year old and younger; height was measured with the child standing upright against the wall and with no shoes and head covering on using a uniform tape when the child was older than 1 year. The child's AC was measured by placing a flexible tape at the mid-point between the tip of the upper right arm and the elbow. The child's WC was measured around the abdomen at the level of the umbilicus after normal expiration using a flexible tape. All the measures were reported to the nearest 0.1 cm (for height and length) or 0.1 kg (for weight). WAZ and BMIz were calculated according to the 2006 World Health Organization Child Growth Standards (WHO 2006).

2.4. Covariates

Potential covariates were considered if the factors were related to children's adiposity measures based on the previous literature (Hoffman et al., 2018; Luo et al., 2021). The covariates were included in the models when they changed the coefficients of the associations of OPE metabolite concentrations with adiposity measures by more than 10%, or when they were associated with outcomes ($p < 0.20$). The following covariates were included: maternal age (<25, 25–29, 30–34, and ≥35 years), maternal education level (high school and below/college and above), household income per capita (<4000 CNY/month, 4000–8000 CNY/month, and >8000 CNY/month), maternal prepregnancy body mass index (BMI) (<18.5, 18.5–24.0, and ≥24.0 kg/m^2), maternal prepregnancy passive smoking (yes/no), parity (nulliparous/parous), gestational age (<37/≥37 weeks), delivery mode (vaginal/cesarean), gestational weight gain (<10, 10–20, and ≥20 kg), and child's biological sex (male/female). Additionally, the child's age at follow-up (years) was included in the models investigating AC and WC.

2.5. Statistical analyses

2.5.1. Main analyses

The demographic characteristics of the included and excluded mother-child pairs were presented using counts (percentage). The OPE metabolite concentrations were natural logarithms (ln) transformed to better approximate normal distributions. Values of OPE metabolite concentrations below the LOD were substituted with LOD/ $\sqrt{2}$ (Davis et al., 1991). Additionally, we calculated the sum of molar concentrations of chlorinated OPE metabolites (∑Cl-OPEs, including BCIPP, BCEP, and BDCPP), nonchlorinated OPE metabolites (∑NCl-OPEs, including DPP, DCP, BBOEP, BEHP, and DBP), and all the 8 OPE metabolites (∑OPEs). The distributions of OPE metabolite concentrations in maternal urine were described using geometric means (GM) and percentiles. Pearson correlation coefficients were calculated to determine the correlations between different OPE metabolite concentrations (ln-transformed).

This study was based on complete-case analyses, which were confined to the cases with no missing values. The primary analyses longitudinally examined the associations of gestational OPEs exposure with children's adiposity measures (WAZ, BMIz, AC, and WC) using linear regression models and generalized estimate equation (GEE) models. Autoregressive working correlation matrices were determined and used in the models according to the quasi information criterion. Interaction terms between OPE metabolite concentrations (continuous) and child ages (categorical) were also included into the GEE models to examine whether the effects of gestational OPEs exposure differed over time. As the majority (87.50%) of the interaction terms were not statistically significant ($p > 0.10$) (Table S1), estimates based on GEE models were provided in the present study. Nevertheless, the associations of OPE metabolites with children's adiposity measures at each time point in linear regression models were shown in Table S1.

Effects modification by breastfeeding and child's sex on associations of OPEs with adiposity measures were assessed using stratified analyses and by including interaction terms into GEE models. Breastfeeding was defined as receiving any breast milk, regardless of the supplementation with any other food or liquids. The duration of breastfeeding was categorized as ≤4 months and >4 months, with a ratio of sample sizes of the two strata of nearly 1:4.

In addition to single-exposure models, we conducted Bayesian Kernel Machine Regression (BKMR) models to elucidate the effects of OPE metabolite mixtures and individual OPE metabolites as part of the mixture. Using a gaussian kernel function, BKMR can estimate the nonlinear and non-additive relationship between a multiple-pollutant mixture and health outcomes and fit with a random effect to address repeated measures (Bobb et al., 2015). We implemented BKMR using ln-transformed and scaled (centered and standardized) OPE metabolite concentrations, and controlled for the same covariates as in the GEE models. This method can also account for the collinearity of exposure variables by including a hierarchical variable selection procedure that yields group- and conditional-posterior inclusion probabilities (groupPIP and condPIP) to indicate the probability that a mixture group or a specific exposure was selected into the model after iterations (Bobb et al., 2018). The values of groupPIP and condPIP range from 0 to 1, and a threshold of 0.5 was used to draw inference on variable importance. Therefore, we grouped chlorinated OPE metabolites into group 1 and nonchlorinated OPE metabolites into group 2 and computed groupPIP and condPIP after 10,000 iterations. The fit and convergence of the BKMR models were monitored by visually inspecting the trace plots of the model parameters. To estimate the effect of the OPE metabolite mixture, we calculated the difference in each adiposity measure (95% credible interval (CrI)) by comparing the effects of all eight OPE metabolite concentrations concurrently fixed at the same percentile (ranging from 25th to 75th with 5 percentile intervals) to the effects of all eight OPE metabolite concentrations fixed at the 25th percentile. To evaluate the effect of individual OPE metabolites as part of the mixture, we calculated the difference in each adiposity measure (95% CrI) for a change in individual OPE metabolite concentration between the 25th and 75th percentiles, with the other metabolite concentrations were concurrently fixed at the 25th, 50th, and 75th percentile, respectively.

2.5.2. Complementary and sensitivity analyses

We further tested the effect modification by child's biological sex or breastfeeding by timepoints (0.5-, 1-, 4- and 6-year visits) using linear regression models, as several interaction terms (12.5%) between child age and OPEs exposure were significant in the GEE models. The main analyses were repeated in children born at term to examine whether preterm birth could affect our results. Considering gestational age may cause potential collider-stratification bias (Wilcox et al., 2011), we reconducted our analyses without adjusting for gestational age. We reconstructed the GEE models using OPE metabolite concentrations without creatine correction as independent variables and urinary creatinine level (ln-transformed) as a covariate (O'Brien et al., 2016). As sampling time (morning or afternoon) and sampling season may influence the urinary creatinine levels (Sallsten and Barregard 2021) and OPE concentrations of the participants (Kuiper et al., 2020), we further repeated our analyses in participants whose urine samples were collected in the morning (8:00 a.m.-12:00 a.m.) and whose urine samples were collected in the summer season (June, July, and August), respectively. Maternal diets during pregnancy (intake of red meat, daily or less than daily; fresh vegetable and fruits, daily or less than daily) was additionally adjusted in the models, as they may affect offspring's body weight. We further tested the effect modification by breastfeeding using GEE models, in which breastfeeding duration was recategorized into two groups using 6 months as a cut-off (≤6 months/> 6 months, with a sample size ratio of nearly 1:2), based on WHO's recommendations (WHO 2011).

All analyses were performed using SAS 9.4 (SAS Institute Inc, Cary, NC, USA), except that the BKMR models were conducted in R 4.0.4 (R Development Core Team) using "bkmr" package (Bobb et al., 2018). *P*-values <0.05 and <0.10 were considered statistically significant for the main analyses and interaction terms, respectively.

3. Results

3.1. Participant characteristics

Table 1 shows the comparisons of demographic characteristics between the included and excluded mother-child pairs. A total of 733 mother-child pairs were included in the study. Compared with the excluded mothers, those included in the present analyses were more likely to be nulliparous, aged 25–34 years, less likely to gain <10 kg weights during gestations, and with children more likely to be males and with long gestational weeks (Table 1). The other characteristics were comparable between the included and the excluded mothers. The characteristics of the included mother-child pairs were similar across the five follow-up visits (Table S2).

3.2. OPE metabolite concentrations

Table 2 presents the detection rates and the distribution profiles of crude (wet-weight) and creatinine-corrected OPE metabolite concentrations in the urine. Among these metabolites, DCP (99.32%) and DBP (99.05%) were detected in nearly all samples, while BDCPP, BCIPP, BCEP, DPP, BBOEP, and BEHP had detection rates ranging from 69.44% to 91.41%. BCEP had the highest geometric mean concentration (0.36 μg/g creatinine), followed by DBP (0.24 μg/g creatinine) and DPP (0.21 μg/g creatinine). The eight OPE metabolites were weakly or moderately correlated with each other (r_s: 0.03–0.47) (Table S3).

3.3. Gestational OPEs exposure and adiposity measures

There was a general profile of BDCPP, DCP, BBOEP, ∑Cl-OPEs, and ∑OPEs exposure associated with higher adiposity measures in GEE models, with statistical significances found between DCP and WAZ (β = 0.08, 95% CI: 0.02, 0.14) (Fig. 1). Certain evidence arose that breastfeeding could modify the associations of OPEs exposure with adiposity measures in children up to 6 years. Specifically, when introducing interaction terms into the GEE models, we observed statistically significant effect modifications by breastfeeding on the associations of OPEs with WAZ (BCIPP, BCEP, BDCPP, DCP, ∑Cl-OPEs, ∑NCl-OPEs, ∑OPEs), BMIz (BCIPP, ∑Cl-OPEs, ∑OPEs), AC (BCIPP, BDCPP, DCP, ∑Cl-OPEs, ∑NCl-OPEs, ∑OPEs), and WC (BCIPP, ∑Cl-OPEs, ∑OPEs) (Table 3). After stratifying the analyses by breastfeeding status, a consistent pattern for each OPE metabolite exposure associated with higher adiposity measures was observed in children who were breastfed for ≤4 months, but little evidence of an association was found in children who were breastfed for >4 months. Specifically, for children breastfed for ≤4 months, statistical significances were found between the OPEs and WAZ (BCIPP, BCEP, BDCPP, DCP, ∑Cl-OPEs, ∑NCl-OPEs, and ∑OPEs), BMIz (BCIPP, BDCPP, DCP, ∑Cl-OPEs, ∑NCl-OPEs, ∑OPEs), AC (BCIPP, BDCPP, ∑Cl-OPEs, ∑NCl-OPEs, ∑OPEs), and WC (BCIPP, BCEP, BDCPP, ∑Cl-OPEs, ∑OPEs) (Table 3). We did not observe a significant effect modification by sex, except for the BCIPP-

Table 1
Maternal urinary concentrations of OPEs by mother and child characteristics (n = 733).

Characteristic	The excluded (n = 492)	The included (n = 733)
	N (%)	N (%)
Maternal characteristics		
Age (years) **		
<25	76 (15.45)	73 (9.96)
25–29	259 (52.64)	404 (55.12)
30–34	136 (27.64)	221 (30.15)
≥35	21 (4.27)	35 (4.77)
Prepregnancy BMI		
<18.5	104 (21.49)	140 (19.47)
18.5–24	338 (69.83)	523 (72.74)
≥24	42 (8.68)	56 (7.79)
Education		
High school or below	120 (24.44)	174 (23.77)
College or above	371 (75.56)	558 (76.23)
Passive smoking before gestation		
Yes	196 (40.00)	298 (40.77)
No	294 (60.00)	433 (59.23)
Parity**		
Nulliparous	397 (81.19)	631 (86.8)
Multiparous	92 (18.81)	96 (13.20)
Household income per capita (CNY/month)		
<4000	118 (24.38)	135 (18.62)
4000–8000	188 (38.84)	301 (41.52)
>8000	178 (36.78)	289 (39.86)
Delivery mode		
Vaginal	173 (48.19)	350 (47.81)
Cesarean	186 (51.81)	382 (52.19)
Gestational weight gain (kg) **		
<10	36 (8.91)	36 (5.01)
10–20	287 (71.04)	523 (72.74)
≥20	81 (20.05)	160 (22.25)
Children's characteristics		
Gestational age (weeks) **		
<37	32 (6.5)	14 (1.91)
≥37	460 (93.5)	719 (98.09)
Biological sex **		
Male	249 (50.92)	418 (57.03)
Female	240 (49.08)	315 (42.97)
Breastfeeding		
≤4 months	66 (24.90)	140 (19.86)
>4 months	199 (75.09)	565 (80.14)

Missing values: the included: prepregnancy BMI (n = 14), education (n = 1), passive smoking before gestation (n = 2), parity (n = 6), household income per capita (n = 8), delivery mode (n = 1), gestational weight gain (n = 14), and breastfeeding (n = 161); the excluded: prepregnancy BMI (n = 8), education (n = 1), passive smoking before gestation (n = 2), parity (n = 3), household income per capita (n = 8), delivery mode (n = 133), gestational weight gain (n = 88), sex (n = 3), and breastfeeding (n = 28). **p < 0.05.

Table 2
Distributions and variability of urine OPE metabolite concentrations with and without creatinine-corrected[a].

	N	Undetected	LOD	>LOD (%)	Min	P5	P25	P50	P75	P95	Max	GM (GSD)
OPE metabolites (ng/ml[b], μmol/L[c])												
BCIPP	733	224	0.015	69.44	<LOD	<LOD	<LOD	0.19	0.39	1.29	21.40	0.11 (5.61)
BCEP	733	149	0.110	79.67	<LOD	<LOD	0.14	0.29	0.54	1.89	23.90	0.30 (2.83)
BDCPP	733	63	0.042	91.41	<LOD	<LOD	0.06	0.11	0.25	0.68	9.40	0.12 (2.76)
DPP	733	75	0.004	89.77	<LOD	<LOD	0.12	0.22	0.42	1.21	11.66	0.17 (4.97)
DCP	733	5	0.002	99.32	<LOD	0.01	0.03	0.05	0.08	0.19	0.47	0.05 (2.47)
BBOEP	733	128	0.015	82.54	<LOD	<LOD	0.02	0.06	0.10	0.21	0.93	0.05 (2.65)
BEHP	733	104	0.003	85.81	<LOD	<LOD	0.08	0.20	0.38	1.03	12.72	0.12 (6.66)
DBP	733	7	0.020	99.05	<LOD	0.06	0.12	0.19	0.34	0.92	13.47	0.20 (2.42)
ΣCl-OPEs[d]	733				0.47	0.65	1.65	2.92	5.26	14.26	108.70	3.02 (2.47)
ΣNCl-OPEs[e]	733				0.47	1.52	2.44	3.60	5.29	10.72	65.36	3.71 (1.86)
ΣOPEs[f]	733				0.94	3.08	5.08	7.10	10.55	23.08	113.79	7.51 (1.88)
Creatinine-corrected OPE metabolites (μg/g[b], μmol/g[c])												
BCIPP	733				0.00	0.01	0.03	0.20	0.48	1.63	85.60	0.13 (5.75)
BCEP	733				0.02	0.06	0.17	0.33	0.70	2.75	49.02	0.36 (3.18)
BDCPP	733				0.01	0.03	0.08	0.14	0.29	0.96	9.77	0.15 (2.85)
DPP	733				0.00	0.01	0.13	0.23	0.51	1.60	9.55	0.21 (4.49)
DCP	733				0.00	0.01	0.03	0.05	0.09	0.20	0.62	0.05 (2.25)
BBOEP	733				0.00	0.01	0.03	0.06	0.12	0.33	1.66	0.06 (3.00)
BEHP	733				0.00	0.00	0.06	0.21	0.67	3.44	55.50	0.14 (10.29)
DBP	733				0.01	0.05	0.11	0.22	0.48	1.89	68.66	0.24 (3.12)
ΣCl-OPEs[d]	733				0.31	0.81	1.90	3.48	6.94	20.73	348.17	3.62 (2.67)
ΣNCl-OPEs[e]	733				0.39	1.17	2.20	3.93	7.98	27.14	390.43	4.45 (2.65)
ΣOPEs[f]	733				1.13	2.31	4.88	8.42	14.87	41.51	400.84	9.00 (2.44)

[a] Values below LOD were replaced with LOD/ $\sqrt{2}$ to calculate geometric mean (GM) and geometric standard deviation (GSD).
[b] For individual OPE metabolite concentration.
[c] For molar sum of OPE metabolite concentrations.
[d] Molar sum of BCEP, BCIPP, BDCPP.
[e] Molar sum of DPP, DCP, BBOEP, BEHP, DBP.
[f] Molar sum of all OPE metabolites.

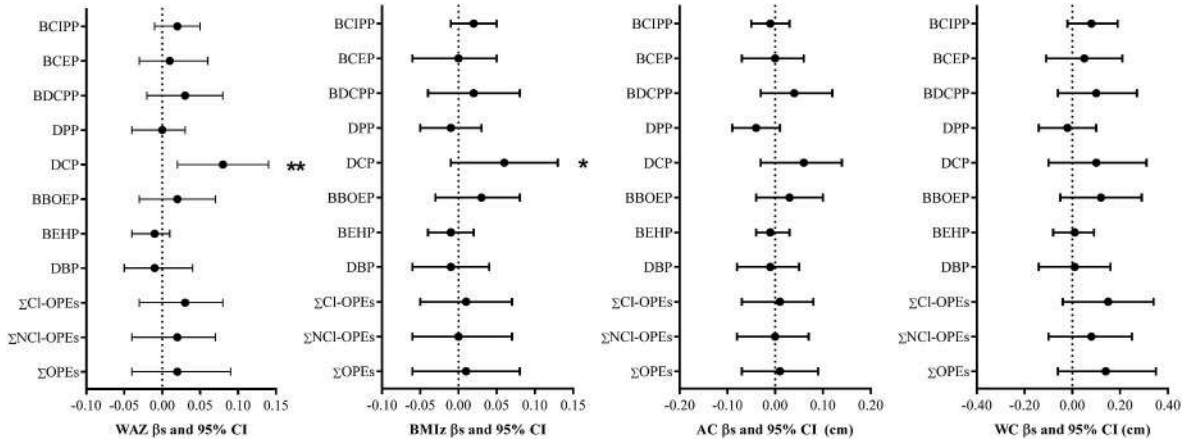

Fig. 1. Association of ln-transformed OPE metabolite concentrations (creatinine-corrected) with repeated adiposity measures of children up to 6 years using GEE models (n = 733). Dots indicate estimates, and vertical lines indicate 95% CI. All models were adjusted for maternal age, maternal education level, household income per capita, maternal prepregnancy BMI, maternal prepregnancy passive smoking, parity, gestational age, delivery mode, gestational weight gain, and child's biological sex. Child's age at follow-up were additionally adjusted in models investigating AC and WC. *P < 0.1, **P < 0.05.

WAZ association (*p*-value for interaction = 0.07). However, in the stratified analyses, we found statistical significances between BCIPP and DCP and WAZ in males (Table S4).

The results from BKMR models supported the results observed in the single-exposure models, as well as the effect modification by breastfeeding. For all children, OPE metabolite mixture exposure tended to be associated with higher adiposity measures. Compared with their 25th percentile, the OPEs metabolite mixture was statistically significantly associated with higher WAZ when they were above their 45th percentile, and with higher WC when they were above their 25th percentile (Fig. 2). Consistent with the GEE models, BDCPP, DCP, and BBOEP exposure also tended to be associated with higher adiposity measures. BEHP exposure was associated with decreased WAZ (Fig. 2). The result from the stratified analyses by breastfeeding using BKMR models were consistent with those from the analyses using GEE models. For children breastfed for ≤4 months, there was an increasing trend for each adiposity measure as all the OPE metabolite concentrations increased by 5 percentile intervals. Compared with all OPEs concentrations fixed at their 25th percentile, we observed statistically significantly higher WAZ, BMIz, and AC when all the OPE metabolites were above their 30th percentile, and WC when all the OPE metabolites were above their 45th percentile (Fig. 3). For each adiposity measure, the BKMR models identified group 1 (chlorinated OPE metabolite group) as the predominant contributor to the overall effects of the OPE metabolite mixture. Within group 1, BCIPP contributed the most to the overall effects on WAZ, BMIz, and WC, while BDCPP contributed the most to the overall

Table 3
Association of ln-transformed OPE metabolite concentrations (creatinine-corrected) with repeated adiposity measures in children up to 6 years stratified by breastfeeding status using GEE models (short breastfeeding, n = 140; long breastfeeding, n = 565)[a].

	WAZ			BMIz			AC[b]			WC[b]		
	≤4 months	>4 months	p-value for interaction	≤4 months	>4 months	p-Value for interaction	≤4 months	>4 months	p-Value	≤4 months	>4 months	p-value for interaction
BCIPP	0.14 (0.09,0.19)**	0.00 (-0.03,0.04)	**0.00**	0.13 (0.06,0.20)**	−0.01 (-0.05,0.03)	**0.01**	0.12 (0.04,0.21)**	−0.03 (-0.08,0.02)	**0.01**	0.41 (0.21,0.61)**	0.01 (-0.11,0.13)	**0.01**
BCEP	0.14 (0.04,0.23)**	0.00 (-0.05,0.05)	**0.04**	0.09 (-0.02,0.21)	−0.02 (-0.08,0.04)	0.16	0.12 (0.00,0.25)*	−0.02 (-0.10,0.05)	0.12	0.44 (0.05,0.84)**	−0.02 (-0.19,0.16)	0.15
BDCPP	0.15 (0.04,0.26)**	0.01 (-0.05,0.07)	**0.08**	0.14 (0.02,0.26)**	0.01 (-0.06,0.07)	0.27	0.25 (0.11,0.38)**	0.00 (-0.09,0.08)	**0.01**	0.41 (0.09,0.74)**	0.02 (-0.18,0.22)	0.12
DPP	0.04 (-0.02,0.10)	−0.01 (-0.05,0.03)	0.19	0.03 (-0.05,0.11)	−0.02 (-0.07,0.03)	0.18	0.00 (-0.08,0.08)	−0.06 (-0.13,0.00)*	0.11	0.06 (-0.16,0.28)	−0.05 (-0.19,0.10)	0.33
DCP	0.19 (0.09,0.29)**	0.06 (-0.02,0.13)	**0.05**	0.14 (0.00,0.28)**	0.04 (-0.05,0.13)	0.21	0.20 (0.06,0.34)	0.02 (-0.09,0.12)	**0.05**	0.27 (-0.11,0.64)	0.02 (-0.23,0.27)	0.28
BBOEP	0.09 (-0.01,0.19)*	0.00 (-0.06,0.06)	0.10	0.07 (-0.04,0.18)	0.02 (-0.04,0.08)	0.32	0.06 (-0.08,0.20)	0.01 (-0.07,0.09)	0.38	0.22 (-0.14,0.58)	0.08 (-0.12,0.28)	0.53
BEHP	0.02 (-0.04,0.07)	−0.01 (-0.03,0.02)	0.48	0.00 (-0.06,0.06)	0.00 (-0.03,0.03)	0.76	0.03 (-0.05,0.10)	0.00 (-0.04,0.03)	0.57	0.07 (-0.12,0.25)	0.02 (-0.08,0.11)	0.89
DBP	0.05 (-0.04,0.14)	−0.01 (-0.06,0.05)	0.38	0.06 (-0.06,0.18)	−0.02 (-0.07,0.04)	0.38	0.07 (-0.04,0.18)	−0.03 (-0.11,0.05)	0.17	0.15 (-0.14,0.44)	−0.02 (-0.20,0.15)	0.44
∑Cl-OPEs	0.28 (0.17,0.39)**	−0.01 (-0.07,0.05)	**0.00**	0.24 (0.10,0.37)**	−0.03 (-0.10,0.04)	**0.00**	0.27 (0.12,0.42)**	−0.04 (-0.13,0.04)	**0.00**	0.86 (0.42,1.31)**	0.01 (-0.20,0.22)	**0.01**
∑NCl-OPEs	0.13 (0.03,0.23)**	0.01 (-0.05,0.07)	**0.06**	0.13 (0.00,0.26)**	−0.01 (-0.07,0.06)	0.12	0.16 (0.03,0.29)**	−0.04 (-0.13,0.04)	**0.01**	0.37 (0.00,0.75)*	0.02 (-0.18,0.23)	0.15
∑OPEs	0.24 (0.13,0.36)**	0.00 (-0.07,0.07)	**0.00**	0.22 (0.08,0.36)**	−0.02 (-0.10,0.05)	**0.01**	0.26 (0.11,0.41)**	−0.04 (-0.14,0.05)	**0.00**	0.78 (0.30,1.27)**	0.03 (-0.20,0.25)	**0.02**

**p < 0.05, *p < 0.10.

[a] All models were adjusted for maternal age, maternal education level, household income per capita, maternal prepregnancy BMI, maternal prepregnancy passive smoking, parity, gestational age, delivery mode, gestational weight gain, and child's biological sex.

[b] Additionally adjusted for child's age at follow-up.

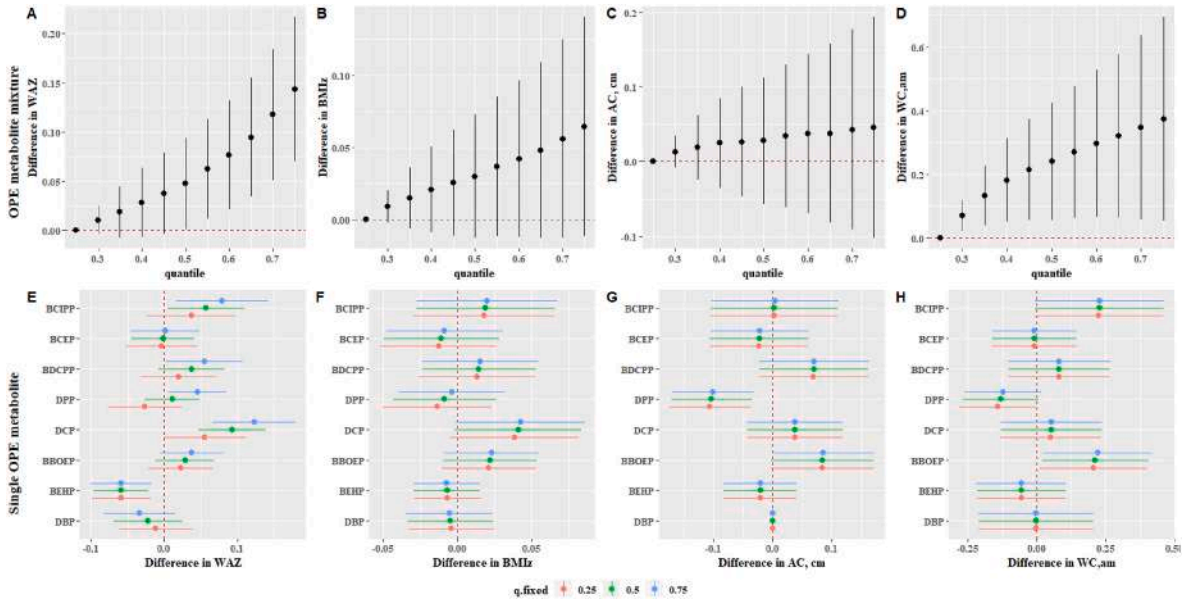

Fig. 2. Association of ln-transformed OPE metabolite concentrations (creatinine-corrected) with repeated adiposity measures of children up to 6 years using BKMR models (n = 733). Dots indicate estimates, and vertical lines indicate 95% CI. All models were adjusted for maternal age, maternal education level, household income per capita, maternal prepregnancy BMI, maternal prepregnancy passive smoking, parity, gestational age, delivery mode, gestational weight gain, and child's gender. Child's age at follow-up were additionally adjusted in models investigating AC and WC.

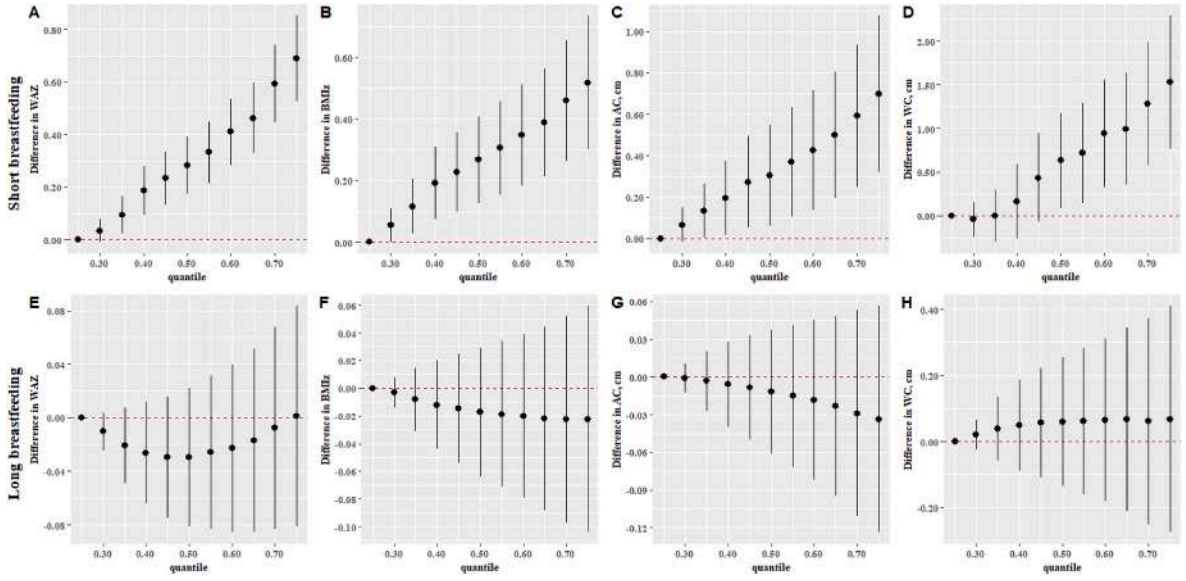

Fig. 3. Overall effects of OPE metabolite concentrations (creatinine-corrected) on adiposity measures of children by breastfeeding status from BKMR models (n = 140). This figure shows the estimated percent change (95% CrI) in each adiposity measure when comparing the effects of chemical exposure all fixed at particular percentile and the effects of chemical exposure all fixed at their 25th percentile. All models were adjusted for maternal age, maternal education level, household income per capita, maternal prepregnancy BMI, maternal prepregnancy passive smoking, parity, gestational age, delivery mode, gestational weight gain, and child's biological sex. Child's age at follow-up were additionally adjusted in models investigating AC and WC.

effects on AC (Table S5). For the effects of individual OPE metabolite as part of the mixture, the BKMR models yielded largely similar results with the GEE models. BCIPP, BCEP, DCP, and BBOEP tended to be associated with higher adiposity measures, with statistically significant increases found in BCIPP with WAZ, BMIz, and WC, and DCP with WAZ (Fig. 4, Table S5). For children with breastfeeding >4 months, non-significant overall associations of OPE metabolites with each adiposity measure was found (Fig. 3, Table S6).

3.4. Complementary and sensitivity analyses

We further test the effect modification by breastfeeding by outcome timepoints, and the results largely supported our primary findings (Table S7). The other analyses to test the robustness of our results were conducted in children breastfed for ≤4 months using GEE models. Among children born at term, the results were essentially unchanged (Table S8). After excluding gestational age from the models, the estimated effects remained largely unchanged (Table S9). When urinary creatine levels were used as a covariate (ln-transformed), the effects of all eight OPE metabolites on adiposity measures were consistent with

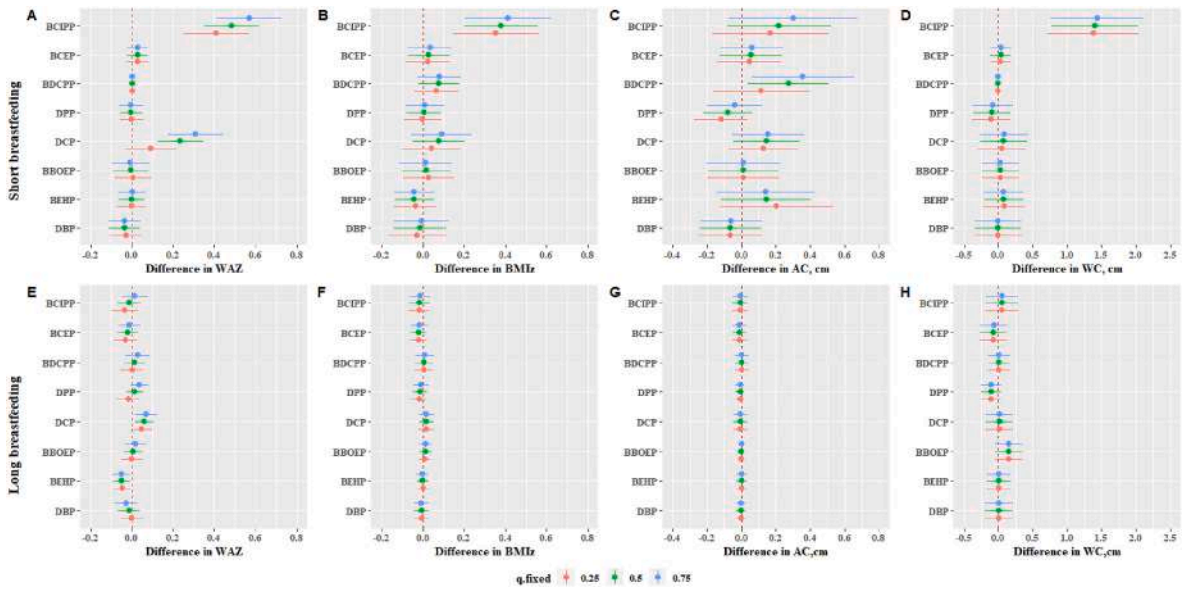

Fig. 4. Associations of individual OPE metabolite concentration (creatinine-corrected) with adiposity measures of children by breastfeeding status from BKMR models. This figure shows the estimated percent change (95% CrI) in each adiposity measure for an IQR change in the ln-transformed concentration of each individual metabolite when all the other metabolites concurrently fixed at their 25th, 50th, 75th percentile. All models were adjusted for maternal age, maternal education level, household income per capita, maternal prepregnancy BMI, maternal prepregnancy passive smoking, parity, gestational age, delivery mode, gestational weight gain, and child's biological sex. Child's age at follow-up were additionally adjusted in models investigating AC and WC.

our main findings (Table S10). When the analyses were restricted to the participants with urine samples collected in the morning and in the summer, respectively, larger estimated effects were observed (Table S11 and Table S12). Further adjustment for maternal diet (intake of red meat and fresh vegetable and fruits) during pregnancy did not markedly change our results (data not shown). After recategorizing breastfeeding duration using 6 months as a cut-off and repeating the analyses, similar effect modification by breastfeeding was found (Table S13). We further investigated the associations of OPEs exposure with adiposity measures using OPE metabolite concentrations as categorized variables based on quartiles with GEE models, and the results were consistent with those of the primary analyses (data not shown).

4. Discussion

To our knowledge, this study is the first to investigate the long-term effects of gestational OPEs exposure on adiposity measures (WAZ, BMIz, AC, and WC) in children up to 6 years. We found that selected OPEs exposure were associated with higher adiposity measures, and, particularly, stronger associations were observed among those with breastfeeding for $\leq$4 months. The BKMR models further supported the findings from GEE models and identified chlorinated OPE metabolites (BCIPP and BDCPP) as important individual exposures driving the effect estimates.

BDCPP and DPP were the most commonly examined OPE metabolites in previous studies (Table S14). The detection rates and concentrations of these metabolites varied largely in pregnant women from different regions or countries, indicating region-specific exposure profiles. Previous studies have reported BDCPP and DPP detection rates ranging from 17% to 100% and from 79.4% to 100%, respectively (Castorina et al., 2017; Chen et al., 2019; Feng et al., 2016). Our study showed relatively higher detection rates for these two metabolites (91.41% and 89.77% respectively for BDCPP and DPP, respectively), suggesting wide exposure to their parent compounds. On the other hand, the crude median concentrations of BDCPP varied from 0.08 μg/L to 1.41 μg/L, while those of DPP varied from 0.29 μg/L to 2.94 μg/L (Castorina et al., 2017; Ingle et al., 2019; Kosarac et al., 2016; Liu et al., 2021). Our study population may represent relatively low OPE levels, as the crude median concentrations of BDCPP (0.11 μg/L) and DPP (0.22 μg/L) in our study were approximately 3–15 times lower than those in North America (Castorina et al., 2017; Ingle et al., 2019; Kosarac et al., 2016) but comparable to those reported in China (BDCPP, 0.08 μg/L; DPP, 0.29 μg/L) (Liu et al., 2021).

Although no studies have investigated the effects of gestational OPEs exposure on childhood adiposity measures, previous epidemiological studies have examined the associations of gestational OPEs exposure with birth size. Birth size is an indicator of health status across the life course, with even small changes predicting the increased risk of obesity in later life (Jones-Smith et al., 2013; Khera et al., 2019). Particularly, Kuiper et al. reported that BCIPP and BDCPP concentrations were associated with a higher ponderal index and lower insulin and leptin levels, suggesting the disruption of glucose and lipid metabolism due to gestational OPE exposure in infants (Kuiper et al., 2020). Additionally, some studies found that exposure to individual OPE metabolites (BDCPP, DPP, and BBOEP) or a mixture of them were associated with lower birth weight and the risk of large-for-gestational age (Bommarito et al., 2021; Hoffman et al., 2018; Luo et al. 2020, 2021), while other studies with small sample sizes ($n < 80$ for the most studies) showed null associations (Crawford et al., 2020; Feng et al., 2016; Hoffman et al., 2018; Kuiper et al., 2020). These studies on gestational OPEs exposure and birth size suggested that OPEs exposure may play a role in the gestational programming of obesity in offspring. In our study, gestational exposure to selected OPE metabolites were associated with higher adiposity measures. The interaction terms between sex and OPE metabolites were generally non-significant. However, we found that associations of BCIPP and DCP with higher adiposity measures were more pronounced in males, which was consistent with a previous study (Crawford et al., 2020). Therefore, future studies are needed to clarify the sex-specific effects of gestational OPEs exposure.

The potential biological mechanisms of the effects of OPEs on adiposity measure were not clearly understand, but previous studies may provide some clues. One hypothesis is that OPEs could disrupt the endocrine homeostasis in the body, including sex steroid hormones, thyroid hormones, and insulin-like growth factor (IGF) (Kojima et al., 2013; Preston et al., 2017; Zeng et al., 2018). All sex steroid hormones, thyroid hormones, and IGF-1 play important roles in the gestational

programming of obesity, such as preadipocyte differentiation, adipogenesis, and lipid accumulation (Moreno-Mendez et al., 2020). In *vitro* and animal studies, Tris (1,3-dichloro-2-propyl) phosphate (TDCPP, the parent compound of BDCPP) and triphenyl phosphate (TPP, the parent compound of DPP) exposure could affect estrogen levels and body weight through binding to estrogen receptors (ER) or influencing the expression of hypothalamic-pituitary-gonadal axis-related genes (Kojima et al., 2013; Krumm et al., 2018; Liu et al., 2013). Similarly, previous studies have suggested the effects of gestational TDCPP and tris (2-butoxyethyl) phosphate (TBOEP, the parent compound of BBOEP) exposure on thyroid homeostasis by influencing regulatory circuits of the hypothalamic-pituitary-thyroid axis (Ma et al., 2016; Wang et al., 2015). Additionally, the dysregulation of genes involved in the growth hormone/IGF axis may also contribute to the altered body mass in zebrafish following OPEs exposure (Zhu et al., 2017). Another possible mechanism may be via the elevation of inflammation responses, including peroxisome proliferator-activated receptors (PPARs) levels and oxidative responses. Evidence supported that TPP had PPARs agonist activities, and could initiate PPARs-driven transcriptions, inducing mature adipocyte differentiation and lipid accumulation (Pillai et al., 2014). Furthermore, oxidative stress could stimulate the hyperplasia and hypertrophy of adipocytes and lipogenesis and has been recognized as a detrimental factor in the etiology of obesity (Pérez-Torres et al., 2021). Increased oxidative stress (reactive oxygen species) in offspring might also contribute to the altered body weight in offspring gestationally exposed to TDCPP (Wang et al., 2015). Besides, studies have also found that gestational TPP exposure perturbed offspring's composition and metabolites of the gut microbiome, which may result in metabolic dysfunction and the development of obesity (Wang et al., 2019).

Breastfeeding is an important source of postnatal OPEs exposure during the first year of life (Hammel et al., 2020), and thus may act as a confounder. Thus, we first additionally adjusted for breastfeeding in our models, but the results did not markedly change (data not shown). On the other hand, postnatal OPE concentrations might be highly correlated with gestational OPE concentrations (Gibson et al., 2019), and we expected stronger associations among children who were breastfed for a longer time when conducting stratified analysis by breastfeeding duration. However, we found stronger positive associations of each OPE exposure with adiposity measures in children breastfed for $\leq$4 months, and little evidence of an association in children breastfed for >4 months in both GEE and BKMR models. Our finding suggested that breastfeeding might act as a modifier on the associations of gestational OPEs exposure with childhood adiposity measures. Previous studies have also shown that breastfeeding could protect against the adverse effects of gestational exposure to risk factors (maternal obesity, gestational diabetes, and environmental chemicals) on the growth and development of offspring (Bider-Canfield et al., 2017; Lertxundi et al., 2015; Sauder et al., 2019). However, the underlying mechanism supporting the effects remains unclear. The macronutrients, micronutrients, and bioactive substances in breast milk, together with the breastfeeding practice itself, may play a role. For example, the antioxidant nutrients, such as long-chain polyunsaturated fatty acids and antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase et al.), in breast milk may reduce oxidative stress which participates in the development of obesity (Castillo-Castañeda et al., 2019; Rodriguez-Palmero et al., 1999). Moreover, breastfeeding plays an important role in driving the transition toward the adult-like gut microbiota of infants in the first months of life, which have been demonstrated to modulate energy homeostasis and adiposity through various mechanisms (Petraroli et al., 2021). Additionally, breast milk leptin also contributes to the infants' circulating leptin levels, thus affecting body weight regulation. However, further studies are needed to elucidate the potential mechanisms.

The present study had several strengths. Based on the prospective design and repeated adiposity measures from birth to 6 years, we were able to investigate the long-term effects of gestational OPEs exposure on children's adiposity measures. In the present study, we measured four adiposity measures, including WAZ, BMIz, AC, and WC, which could identify the altered distribution of body fats from different aspects (Balkau et al., 2007; Shi et al., 2020). The combined usage of these four outcomes helped us to capture a more complete picture of the effects of gestational OPEs exposure on the development of obesity in children. The consistent patterns found across these measures further strengthened our findings. Another strength is that we applied BKMR to evaluate the overall effects of gestational OPEs exposure on children's adiposity measures and to identify the key OPEs that might drive these results. Overall, results from the BKMR models further supported our findings from the GEE models. The chlorinated OPE metabolites, BCIPP and BDCPP were identified as the important contributors to the overall associations. We also collected information about an extensive array of maternal and children's characteristics allowing for adjustment for potential confounders in the analyses.

We acknowledged some limitations in this study. Firstly, loss to follow-up may raise concerns about selection bias. The characteristics of the included participants differed slightly from those of the excluded, including maternal age, parity, gestational weight gain, and the child's gestational age and sex. However, these characteristics were not associated with OPE metabolite concentrations ($p > 0.10$). Thus, our results were less likely to be affected by selection bias. Secondly, we characterized gestational OPEs exposure based on a single-spot urine sample. OPEs have short half-lives varying from hours to days (Bui et al., 2017; Hou et al., 2016). Therefore, the representativeness of the single urine collection for the actual exposure levels may be limited. However, several studies in pregnant women reported moderate reproducibility of OPEs over one week (Hoffman et al., 2014), or even across three trimesters (Romano et al., 2017). Furthermore, the exposure misclassifications due to the variability of OPE concentrations were expected to be non-differential; thus, the associations would be biased toward the null. Thirdly, the hydroxylated OPE metabolites were not measured in our study due to the limited funding. Hydroxylated metabolites exposure may also influence the offspring development (Luo et al., 2021). Thus, residual confounding by hydroxylated metabolites may be a concern. Another limitation is the multiple comparisons conducted in our analyses, which may raise the concerns for chance findings due to type I errors. However, as these outcomes are inter-correlated, it may be not appropriate to adjust p-values for multiple comparisons (Barnett et al., 2022; Feise 2002). Furthermore, consistent patterns for OPE metabolites were found across adiposity measures, which may be less prone to the issues of multiple comparisons. Thus, we decided not to adjust for multiple comparisons and instead base on the strength and consistency of the associations observed across the outcomes. Another consideration is residual confounding due to urinary dilution. Our primary analyses corrected for variation in urinary dilution by dividing the OPE metabolite concentrations by the urinary creatinine concentrations. However, this method may bias the results toward the null when there exist some factors which may affect the creatinine, exposure and outcomes (O'Brien et al., 2016). This might also partially explain our larger estimates of the association of OPEs with adiposity measures after adjusting for sample collection time. Thus, other methods that can deal with the variation in urinary dilution, such as covariate-adjusted standardization (O'Brien et al., 2016), is warranted. Finally, although we accounted for the co-exposure to multiple OPEs through BKMR models, concern still exists regarding the potential residual confounding rising from other environmental chemicals. Future studies are warranted to verify our findings.

5. Conclusion

In this longitudinal follow-up study, we concluded that gestational OPEs exposure could alter children's adiposity measures. More importantly, this study also suggested that breastfeeding could attenuate the

adverse effects of OPEs exposure on children's adiposity measures. The magnitudes of the associations of OPEs with adiposity measures in children breastfed for ≤4 months may be insufficient to have clinical implications at the individual levels, but do have important public health implications given the ubiquitous exposure of OPEs.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.114089.

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Gestational organophosphate ester exposure and preschool attention-deficit/hyperactivity disorder in the Norwegian Mother, Father, and Child cohort study

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ABSTRACT

Background: Attention-deficit/hyperactivity-disorder (ADHD) is a leading neurodevelopmental disorder in children worldwide; however, few modifiable risk factors have been identified. Organophosphate esters (OPEs) are ubiquitous chemical compounds that are increasingly prevalent as a replacement for other regulated chemicals. Current research has linked OPEs to neurodevelopmental deficits. The purpose of this study was to assess gestational OPE exposure on clinically-assessed ADHD in children at age 3 years.

Methods: In this nested case-control study within the Norwegian Mother, Father, and Child Cohort study, we evaluated the impact of OPE exposure at 17 weeks' gestation on preschool-age ADHD. Between 2007 and 2011, 260 ADHD cases were identified using the Preschool Age Psychiatric Assessment and compared to a birth-year-stratified control group of 549 children. We categorized bis(2-butoxyethyl) phosphate (BBOEP) and bis(1,3-dichloro-2-propyl) phosphate (BDCIPP) as values < limit of detection (LOD) (BBOEP N = 386, BDCIPP N = 632), ≥LOD but < limit of quantification (LOQ) (BBOEP N = 413; BDCIPP N = 75), or above LOQ (BBOEP N = 70; BDCIPP N = 102). Diphenyl phosphate (DPhP) and di-n-butyl phosphate (DnBP) were categorized as quartiles and also modeled with a log10 linear term. We estimated multivariable adjusted odds ratios (ORs) using logistic regression and examined modification by sex using an augmented product term approach.

Results: Mothers in the 3rd DnBP quartile had 1.71 times the odds of having a child with ADHD compared to the 1st quartile (95%CI: 1.13, 2.58); a similar trend was observed for log10 DnBP and ADHD. Mothers with BDCIPP ≥ LOD but < LOQ had 1.39 times the odds of having a child with ADHD compared to those with BDCIPP < LOD (95%CI: 0.83, 2.31). Girls had lower odds of ADHD with increasing BBOEP exposure (log10 OR: 0.55 (95%CI: 0.37, 0.93), however boys had a weakly increased odds (log10 OR: 1.25 (95%CI: 0.74, 2.11) p-interaction = 0.01].

Conclusions: We found modest increased odds of preschool ADHD with higher DnBP and BDCIPP exposure.

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Abbreviations

ADHD	Attention-deficit/hyperactivity-disorder
BBOEP	Bis(2-butoxyethyl) phosphate
BDCIPP	Bis(1,3-dichloro-2-propyl) phosphate
DnBP	Di-n-butyl phosphate
DPhP	Diphenyl phosphate
EMM	Effect measure modification
LOD	Limit of detection
LOQ	Limit of quantification
MoBa	The Norwegian Mother, Father, and Child Cohort Study
OPE	Organophosphate ester
PAPA	Preschool Age Psychiatric Assessment PBDEs Polybrominated diphenyl esters
PCBs	Polychlorinated biphenyls
TBOEP	Tris (2-butoxethly) phosphate
TDCIPP	Tris (1,3-dichloro-2-propyl) phosphate
TnBP	Tri-n-butyl phosphate
TPhP	Triphenyl phosphate

1. Introduction

Organophosphate esters (OPEs) are ubiquitous chemicals, generally used as flame retardants and/or plasticizers (van der Veen and de Boer, 2012), found in a multitude of everyday items such as household furniture (van der Veen and de Boer, 2012), electronics (van der Veen and de Boer, 2012), baby products (Stapleton et al., 2011), personal care products (Ingle et al., 2019a, 2020a), and nail polish (Ingle et al., 2019a, 2020a; Mendelsohn et al., 2016). OPE usage has substantially increased over time as a replacement for other regulated chemicals such as polybrominated diphenyl esters (PBDEs) and polychlorinated biphenyls (PCBs), that have been previously phased out due to their potential toxicity, ability to bioaccumulate, and environmental persistence (van der Veen and de Boer, 2012; Morelle, 2016; European Union (EU), 2001). Although OPEs do not bioaccumulate, they can leach into the surrounding environment where they are capable of entering the human body through inhalation, dermal absorption, and/or ingestion of contaminated food or water (van der Veen and de Boer, 2012).

There has been growing concern regarding OPEs due to their ubiquitous distribution and potential for adverse human health effects. Recent epidemiological studies have found associations between OPEs and adverse neurodevelopmental- (Lipscomb et al., 2017; Castorina et al., 2017; Doherty et al., 2018, 2019; Choi et al., 2021a), thyroid-based- (Hoffman et al., 2017; Preston et al., 2017), reproductive- (Carignan et al., 2018a; Meeker et al., 2009, 2013a), respiratory- (Bamai et al., 2018; Araki et al., 2014, 2018), and dermal- (Araki et al., 2018) endpoints; however, some studies have found no association (Deziel et al., 2018; Canbaz et al., 2016). OPE exposure during the gestational period is of significant interest because OPEs can cross the placental barrier (Ding et al., 2016; Wang et al., 2013) and may accumulate in the placenta (Ding et al., 2016) and/or amniotic fluid (Bai et al., 2019).

Attention-deficit/hyperactivity disorder (ADHD) is a prevalent neurodevelopmental disorder commonly diagnosed in childhood, but symptoms of ADHD may emerge as early as the preschool period (AAo, 2019). Estimates of the persistence of ADHD from preschool through childhood vary (Overgaard et al., 2022a; Riddle et al., 2013; Lahey et al., 2016), however early identification and treatment of ADHD may have benefits, due to the substantial adverse individual (de Zeeuw et al., 2017; Galera et al., 2009; Rokeach and Wiener, 2018; Sarkis, 2014; Quintero et al., 2017; Lee et al., 2016a) and economic (Sciberras et al., 2017; Gupte-Singh et al., 2017; Le et al., 2014; Quintero et al., 2018; Cadman et al., 2012; Kotsopoulos et al., 2013) burdens associated with this disorder.

Several studies suggest gestational OPE exposure is associated with risk of ADHD or ADHD-like behaviors (Castorina et al., 2017; Doherty et al., 2019; Choi et al., 2021a). The only study to date to evaluate this association with ADHD as a clinical diagnosis was conducted by Choi (2021) et al. using a nested case-control study, also within the Norwegian Mother, Father, and Child cohort (MoBa) (Choi et al., 2021a). Choi et al. examined gestational OPE concentrations in relation to a diagnostic code registration of ADHD among children in the Norwegian National Patient Registry (NPR) (Choi et al., 2021a). Choi et al. reported that higher gestational concentrations of diphenyl phosphate (DPhP) and bis(1,3-dichloro-2-propyl) phosphate (BDCIPP) were associated with elevated risk of childhood ADHD registration. While preschool ADHD is correlated with childhood ADHD, it is not perfectly predictive (Overgaard et al., 2022a; Riddle et al., 2013; Lahey et al., 2016). Furthermore, diagnostic registrations of ADHD may be affected by variability in clinical practice, over-representation of more severe and disruptive cases, and an under-representation of girls. To date, all the studies we identified that evaluated associations of individual OPEs on ADHD, assessed and found evidence of an association between BDCIPP and ADHD or ADHD-like symptoms. Similarly, the majority of such studies have found evidence of associations with DPhP. Fewer consistent patterns exist for other OPEs, and, overall, there is a lack of agreement as to the existence of sex-specific effects (Castorina et al., 2017; Doherty et al., 2019; Choi et al., 2021a), with Choi et al. observing larger adverse associations among girls (Choi et al., 2021a) and three other studies observing no appreciable sex-related effect measure modification (EMM) (Castorina et al., 2017; Doherty et al., 2019). As such, the purpose of this study is to examine whether gestational OPE exposure may impact early manifestation of ADHD, specifically in relation to preschoolers.

A potential causal link between OPEs and ADHD could result in public health interventions with a meaningful impact through regulatory changes. As such, this study has the potential to identify or dismiss a modifiable cause of one of the leading neurodevelopmental disorders in preschoolers worldwide. To address this important question, we leverage a nested Preschool ADHD Substudy of MoBa, in which a high-quality standardized on-site clinical evaluation of all children was conducted, to determine the potential impact of OPEs on preschool ADHD. To evaluate potential sex differences in associations, we examined sex-related modification of the OPE-ADHD association in preschoolers.

2. Material and methods

2.1. Study population

MoBa is an ongoing, prospective population-based cohort study of pregnant Norwegian-speaking women enrolled between 1999 and 2008 (Magnus, 2007; Magnus et al., 2016). Women consented to participation at their first ultrasound appointment (~17 weeks' gestation) and, upon enrollment, blood and urine samples were collected. Questionnaires were completed by mothers at 17-, 22-, and 30-weeks' gestation as well as longitudinally after birth (Magnus et al., 2016). Of the original 227,702 pregnancies invited, 40.7% (N = 112,908 pregnancies; 95,200 women) agreed to participate, of which ~107,000 children are still actively enrolled (Magnus et al., 2016; Kamai et al., 2021).

This ADHD Substudy of MoBa consisted of children born between April 2004 and January 2008 that were the result of a singleton pregnancy, resided within close proximity to Oslo, did not meet criteria for the Autism Birth Cohort study (Stoltenberg et al., 2010), and with completed 36-month postnatal questionnaire (Engel et al., 2018a). Included in the 36-month questionnaire were 11 questions aimed at identifying children displaying ADHD-like behaviors (Overgaard et al., 2022b; Biele et al., 2022). These questions included 5 from the Diagnostic and Statistical Manual of Mental Disorders, Version 4, Text Revision (Association, 2000) (DSM-IV-TR; easily distracted, difficulty

waiting his/her turn, difficulty sustaining attention, talks excessively, and does not seem to listen) and 6 from the Child Behavior Checklist/1.5–5 (Achenbach and Rescorla, 2010) (can't concentrate, can't sit still, can't stand waiting, demands must be met immediately, gets into everything, and quickly shifts activities) (Biele et al., 2022). From these questions, we developed a quantitative index, referred to as the "sampling screener" (Biele et al., 2022). Children from the ADHD eligible population that scored in the 90th percentile or higher on this screener were automatically invited to participate in an on-site clinical evaluation (N = 2798) as well as a small random sample of children from the ADHD eligible population (N = 654). Of the original 3452 pregnancies invited, 1195 (34.6% participation rate) agreed to participate, 870 of which had a maternal urine sample available. This substudy of children that underwent clinical examination is referred to as the MoBa ADHD Substudy. Children that participated in the clinical evaluation were between 3.1 and 3.8 years of age (Ahmad and Warriner, 2001; Skogan et al., 2015). A flow diagram outlining selection into our current study can be viewed in Supplemental Fig. 1. The original study design was a case-cohort design, but we excluded cases from the referent sub-cohort in our study. As such, the current study design is a case-control design; control selection is discussed in more detail in the section 'selection of controls'.

2.2. Selection of preschool ADHD cases

In contrast to the study by Choi et al. who used primarily childhood ADHD cases as the study outcome (i.e. children ages 6–11 years), we utilized children with preschool ADHD defined as children ages 2–5 years (all children in this study were 3 years old) (Choi et al., 2021a; Surén et al., 2012). Preschool ADHD differs from childhood ADHD in relation to symptom occurrence (proportion of hyperactive vs inattentive cases) and symptom severity, with both occurrence and severity declining between preschool and late childhood, particularly in relation to hyperactivity (Overgaard et al., 2022a; Lahey et al., 2005, 2016; Leopold et al., 2016; Curchack-Lichtin et al., 2014). As such, preschool ADHD is predictive but not perfectly correlated with childhood ADHD (Overgaard et al., 2022a; Riddle et al., 2013; Lahey et al., 2016).

In our study, preschool ADHD status was determined using the Preschool Age Psychiatric Assessment (PAPA) (Egger and Angold, 2004). PAPA is an interviewer-based psychiatric instrument, validated in children 2–5 years, and provides information on the magnitude and frequency of neurodevelopmental symptoms set forth in the DSM-IV-TR (Association, 2000; Egger and Angold, 2004; Egger et al., 2006). PAPA is not equivalent to a clinical diagnosis as this requires a more in-depth evaluation from multiple sources. In this study, trained graduate psychology students conducted standardized and structured psychiatric interviews using PAPA with a caregiver (generally the mother) under the supervision of a child psychologist or psychiatrist; all parents of children who participated in the 1-day clinical exam were interviewed with PAPA (Rohrer-Baumgartner et al., 2014). Preschool ADHD symptoms were counted as present if they persistent for 3-months or more across at least 2 settings (Egger and Angold, 2004). The inter-rater reliability of PAPA for preschool ADHD symptoms in our study was assessed by having a second blinded rater rescore the audiotapes of 79 interviews and found to be high (total preschool ADHD symptoms interclass correlation coefficient: 0.98) (Øvergaard et al., 2018). At the end of the ADHD section in PAPA, an impairment/impact section was also administered to participants who reported at least one ADHD symptom (Bendiksen et al., 2017). This section consisted of six functional domains (family relationships, friends, learning, play/leisure activities, child's quality of life, and family burden) (Bendiksen et al., 2017). Each functional domain was scored on a 4-point Likert scale (0, 1, 2, or 3 points) for a possible total of 18 points (Bendiksen et al., 2017). Impairment was considered present when the child scored 2 or higher overall (sum score) (Kamai et al., 2021).

In this study, a child was classified as meeting clinical criteria for ADHD if they had 6 or more symptoms (from a possible 9 symptoms) on PAPA and was also scored as having an impairment (Kamai et al., 2021; Egger and Angold, 2004). Children who presented with 6 or more preschool ADHD symptoms but no impairment or 3 to 5 preschool ADHD symptoms and an impairment were classified as having subclinical ADHD (Kamai et al., 2021). For this study, we considered children to have preschool ADHD if they score in the clinical (n = 114) or subclinical (n = 146) range on the PAPA for a total of 260 children hereafter referred to as "preschool ADHD cases" (Kamai et al., 2021; Egger and Angold, 2004).

2.3. Selection of controls

We selected a birth-year stratified random sample of participants, representative of the ADHD eligible population (i.e., representing the exposure distribution that gave rise to the cases), as a comparison group for the preschool ADHD cases (n = 556). This random sample was frequency matched to preschool ADHD cases on birth year and did not undergo clinical evaluation. Because only 7 preschool ADHD cases were also included in the random sample, we treated these children as preschool ADHD cases only, resulting in 549 children in the final control group.

2.4. Measurement of organophosphate ester (OPE) metabolites

Details regarding urine collection and analysis have been previously published (Choi et al., 2021a; Rønningen et al., 2006; Paltiel et al., 2014). Briefly, urine samples were collected ~17 weeks' gestation at which time they were shipped unrefrigerated overnight to the central biorepository in Oslo, Norway for immediate processing (Choi et al., 2021a; Rønningen et al., 2006; Paltiel et al., 2014). Bacterial growth was prevented during shipment through the inclusion of chlorhexidine (Hoppin et al., 2006). The majority of samples were received within one (66%) or two (10%) days of collection. All samples were processed the day of receipt and less than 1% underwent freeze-thaw cycles (Hoppin et al., 2006). Standard operating procedures were derived for comprehensive quality insurance. A laboratory information management system was used to prevent sample identity errors and track specimens.

In this study, we measured 4 OPE metabolites using ultra performance liquid chromatography (UPLC) coupled with quadrupole-time-of-flight (Cequier et al., 2014a; Choi et al., 2021b). These metabolites were di-n-butyl phosphate (DnBP), DPhP, BDCIPP, and bis (2-butoxyethyl) phosphate (BBOEP), which correspond with the parent compounds tri-n-butyl phosphate (TnBP), triphenyl phosphate (TPhP), tris (1,3-dichloro-2-propyl) phosphate (TDCIPP), and tris (2-butoxethly) phosphate (TBOEP) respectively.

Quality control procedures have been detailed previously (Choi et al., 2021b; Engel et al., 2018b). Briefly, 2 in-house control samples and 4–6 quality controlled pooled urine samples were included per analytic batch to assess assay precision and batch-batch variability respectively and cases and controls were randomized to batch. To account for urine dilution, we measured specific-gravity using a pocket refractometer (PAL-10S) from Atago.

2.5. Statistical analysis

Data analysis was based on version 9 of the quality-assured MoBa data files. We selected potential confounders *a priori* from the OPE-ADHD literature and assessed their relationship via direct acyclic graph (DAG) (Supplemental Fig. 2). (Pearl and Robins, 1995) From this DAG, we determined a minimally-sufficient adjustment set (MSS). The covariates in the MSS included maternal fish intake (Sala et al., 2022; Choi et al., 2022), birth year, child sex, family income, maternal age at delivery, and maternal education. From the Medical Birth Registry of Norway, we obtained maternal age at delivery, child sex (male/female), and birth year. We derived information on maternal education (<4-year

college degree, 4-year college degree, post-graduate degree or higher), marital status (married, single/co-habiting), parity (nulliparous/parous), financial difficulty in the past 6 months (yes/no), and maternal smoking during pregnancy (yes/no) and maternal drinking during pregnancy (yes/no) from the 17-gestational-weeks' questionnaire. We determined maternal fish intake from a semiquantitative food frequency questionnaire administered at 22-gestational-weeks' and calculated servings per day for total fish consumption by summing daily, weekly, and monthly intake (Brantsæter et al., 2008). Additionally, we derived a binary indicator of maternal ADHD symptoms via the Adult ADHD Self-Report Scales (ASRS), a validated assessment for adult ADHD symptoms that was completed as part of the 36-month questionnaire (Kessler et al., 2005). As maternal ADHD may confound the OPE-offspring ADHD relationship due to its high heritability (Banerjee et al., 2007; Silberg et al., 2015; Freitag et al., 2010; Faraone et al., 2005) and potential to affect OPE exposure levels through an unknown confounding pathway, we considered maternal ADHD for inclusion in our models in addition to covariates in the MSS. To address concerns regarding positivity, we removed variables that had a minimal impact on the final effect estimates and did not improve model fit for parsimony. Covariates in the final models included total fish consumption, maternal education, financial difficulty, maternal age, and child sex.

As BBOEP and BDCIPP both had more than 50% of their values below the limit of detection (LOD), we categorized these 2 OPEs as $<$ LOD, LOD to $<$ limit of quantification (LOQ), and $\geq$ LOQ because, imputation of these variables in a continuous form could potentially introduce bias (Lubin et al., 2004). Next, we adjusted OPE concentrations for urine dilution using specific-gravity as described by Hauser et al. (2004) et al. (Hauser et al., 2004) For other OPEs, we imputed exposure values below the LOD and missing covariate data from a log-normal distribution using a multivariate imputation by chained equation (MICE) approach ($m = 20$), with exposure data truncated at the LOD, and conditional on outcome, exposure, and other covariates. From this, we obtained summary estimates via Rubin's rules for imputation (Lubin et al., 2004; Rubin, 1987; Harel et al., 2018; Allison, 2001).

We calculated outcome-stratified Spearman correlations between non-imputed and imputed specific-gravity-corrected OPEs to determine correlations between exposure variables. We then determined optimal functional forms of OPEs and covariates from bivariate assessments and multivariable logistic regression models, with an emphasis on minimizing Akaike Information Criterion. Next, we created quartile cut-points for DPhP and DnBP concentrations based on the birth-year-stratified control group to represent the exposure distribution in the study base. We modeled DPhP and DnBP continuously using a log10 transformation and as quartiles to allow for nonlinear and/or non-monotonic trends. To estimate the association between each OPE and preschool ADHD, we calculated odds ratios (ORs) using logistic regression models, adjusted for covariates.

We assessed child sex as a potential effect measure modifier on the multiplicative scale using an augmented product term approach (Buckley et al., 2017). We considered EMM to be present if the p-value for the interaction term between child sex and OPE was at or below the *a priori* threshold of 0.10. All analyses were performed using SAS 9.4 (Cary, NC).

2.6. Sensitivity analyses

To assess the impact of imputing missing covariate data, we reran models without covariate imputation. To account for the potential influence of correlated OPE metabolites, we reran models co-adjusting for the other three main metabolites (one at a time). To investigate any potential batch-effects, we reran models excluding one batch at a time to determine if the exclusion of any single batch meaningfully impacted any of the final associations.

3. Results

Fifty-six percent of preschool ADHD cases were boys; however, child sex was equally distributed in our control group (Table 1). Mothers in our study averaged ~30 years of age at delivery. Mothers of children with preschool ADHD were less likely to be college educated (63% vs 74%) and more likely to be nulliparous (60% vs 49%), smoke during pregnancy (24% vs 14%), and report experiencing more financial difficulty (26% vs 14%) compared to mothers of children in the control group (Table 1). No covariate in our study had more than 10% missingness (Table 1).

DPhP and DnBP were usually detected, with greater than 90% of values above the LOD; as noted above, BBOEP and BDCIPP were less frequently detected (Table 2). Geometric means and percentiles for OPE metabolite measures were comparable between preschool ADHD cases and controls. OPE metabolites were minimally correlated, with Spearman correlations ranging from |0.020| to |0.345| for non-imputed measures (Supplemental Table 1A); imputation had little effect on these correlations (Supplemental Table 1B).

Mothers in the 3rd DnBP quartile had 1.71 times the odds (95%CI: 1.13, 2.58) of having a child with preschool ADHD compared to the 1st quartile; however, across quartiles of DnBP, the trend did not appear monotonic (Fig. 1; Supplemental Table 2). Similarly, increased log10

Table 1
Characteristics of a nested case-control study of preschool ADHD in MoBa, 2004–2007.

	Preschool ADHD group *Mean (SD)* or N (%)	Controls[a] *Mean (SD)* or N (%)
Total N	260	549
Maternal age at delivery (years)	*30.0 (4.05)*	*30.9 (4.23)*
Missing	0 (0.0)	2 (0.4)
Child sex, N		
Male	145 (55.8)	275 (50.1)
Female	115 (44.2)	274 (49.9)
Missing	0 (0.0)	0 (0.0)
Maternal education, N (%)		
Not a college graduate	92 (35.4)	121 (22.0)
College graduate	108 (41.5)	237 (43.2)
Post-college education	56 (21.5)	168 (30.6)
Missing	4 (1.5)	23 (4.2)
Marital status		
Single/Co-inhabiting	146 (56.2)	257 (46.8)
Married	113 (43.5)	287 (52.3)
Missing	1 (0.4)	5 (0.9)
Parity		
Nulliparous	156 (60.0)	270 (49.2)
Parous	104 (40.0)	277 (50.5)
Missing	0 (0.0)	2 (0.4)
Maternal ADHD symptoms	33 (12.7)	21 (3.8)
Missing	2 (0.8)	12 (2.2)
Maternal fish intake (g/day)	*26.2 (18.33)*	*27.4 (19.05)*
Missing	4 (1.5)	7 (1.3)
Any smoking in 1st or 2nd trimester	62 (23.8)	76 (13.8)
Missing	1 (0.4)	6 (1.1)
Any alcohol consumption in 1st or 2nd trimester	32 (12.3)	65 (11.8)
Missing	21 (8.1)	44 (8.0)
Experienced financial difficulty in the past year[b]	67 (25.8)	75 (13.7)
Missing	1 (0.4)	2 (0.4)
Birth year		
2004	26 (10.0)	55 (10.0)
2005	62 (23.9)	131 (23.9)
2006	90 (34.6)	190 (34.6)
2007	82 (31.5)	173 (31.5)
Missing	0 (0.0)	0 (0.0)

[a] Controls are birth-year-stratified to account for changes in exposure or outcome frequency attributable to birth year.
[b] Past year refers to the year before enrollment (around 17 weeks' gestation).

Table 2
Gestational specific gravity-corrected OPE metabolite distribution in a nested case-control study of preschool ADHD in MoBa, 2004–2007.

Exposure	Geometric Mean[a]	Geometric SD[a]	Min	25%	50%	75%	Max	LOD[b]	% ≥ LOD[b]	LOQ[c]	% ≥ LOQ[c]
DPhP (ng/mL)								0.03		0.10	
Preschool ADHD (N = 260)	0.48	2.95	<LOD	0.29	0.49	0.93	37.22		96.5%		92.3%
Controls[d] (N = 549)	0.46	2.93	<LOD	0.26	0.48	0.88	11.16		96.5%		93.4%
DnBP (ng/mL)								0.07		0.20	
Preschool ADHD (N = 260)	0.26	2.18	<LOD	0.17	0.25	0.37	10.93		95.4%		66.5%
Controls[d] (N = 549)	0.25	2.36	<LOD	0.15	0.23	0.38	25.59		92.7%		58.5%
BBOEP (ng/mL)	–	–						0.07		0.20	
Preschool ADHD (N = 260)	0.08	2.01	<LOD	<LOD	<LOD	0.13	0.84		44.2%		12.3%
Controls[d] (N = 549)	0.09	2.13	<LOD	<LOD	<LOD	0.13	3.40		43.7%		14.6%
BDCIPP (ng/mL)								0.17		0.50	
Preschool ADHD (N = 260)	0.17	2.40	<LOD	<LOD	<LOD	<LOD	16.83		20.4%		10.8%
Controls[d] (N = 549)	0.18	2.72	<LOD	<LOD	<LOD	<LOD	96.92		19.5%		12.2%

[a] Values below the limit of detection (LOD) were imputed using LOD/ $\sqrt{2}$ for the geometric mean and standard deviation.
[b] Limit of detection.
[c] Limit of quantification.
[d] Controls are birth-year-stratified to account for changes in exposure or outcome frequency attributable to birth year.

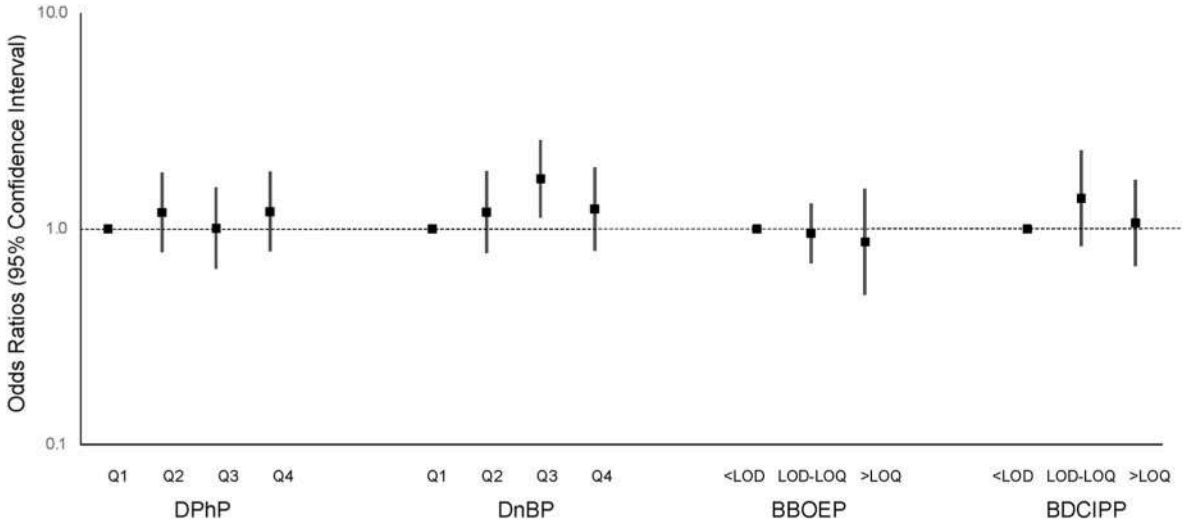

Fig. 1. Associations between quartiles of DPhP and DnBP and categorized measures of BBOEP and BDCIPP, based on their limits of detection and quantification respectively, in relation to preschool ADHD in a nested case-control study of preschool ADHD in MoBa, 2004–2007 (N = 809). All models are from an imputed logistic regression model adjusted for total fish consumption, maternal education, financial difficulty, and maternal age.

DnBP exposure was also association with higher odds of ADHD [OR: 1.30; 95%CI: 0.86, 1.97); Table 3]. Some quantile- or category-specific ORs were elevated, for example BDCIPP measures ≥ LOD but < LOQ had 1.39 times the odds (95%CI: 0.83, 2.31) of having a child with preschool ADHD compared to those with BDCIPP measures < LOD (Fig. 1; Supplemental Table 2). However, we did not observe monotonic patterns of association for any OPE. Results from multiply-imputed models were consistent with those from a complete case analysis (Supplemental Table 2). Furthermore, associations were not meaningfully affected by mutual adjustment for any other OPE metabolite (Supplemental Table 3), and no batch-effects were observed (Supplemental Figs. 3A–3D).

A product term between BBOEP and child sex was statistically significant, suggesting multiplicative EMM (p-interaction 0.01; Table 3). Specifically, girls had lower conditional odds of preschool ADHD with BBOEP exposure above the LOD, whereas the result for boys suggested associations that were null or in the opposite direction. [Girls: log10 OR: 0.55 (95%CI: 0.37, 0.93); Boys: log10 OR: 1.25 (95%CI: 0.74, 2.11); p = 0.01]. No notable EMM was observed for any other OPE.

4. Discussion

Using a nested case-control subset of MoBa, we examined the relationship between gestational OPE exposure and preschool ADHD. We found some evidence that gestational exposure to DnBP and BDCIPP may be associated with increased odds of ADHD, although associations were modest in magnitude and did not follow a monotonic exposure-response trend. We observed evidence of heterogeneity by child sex for BBOEP and preschool ADHD, with girls having lower odds of preschool ADHD with increased exposure, while boys trending in the opposite direction. However, no other OPE association evidenced modification by child sex.

There is a very limited epidemiological literature on gestational exposure to OPEs and child neurodevelopment; however, substantial concern exists for their potential impact on human neurodevelopment due to their ubiquitous distribution and potential to disrupt neurological process (Dishaw et al., 2011; Gu et al., 2018). Studies on PC12 human cells have found OPEs may inhibit DNA synthesis (Dishaw et al., 2011) and/or total acetylcholinesterase activity (Gu et al., 2018), disrupt cell proliferation (Gu et al., 2018), and increase neurodifferentiation into cholinergic and/or dopaminergic phenotypes (Dishaw et al., 2011).

Four epidemiological studies have assessed OPE exposure and ADHD

Table 3
Assessment of multiplicative effect measure modification by child sex for the relationship between gestational OPEs and preschool ADHD in a nested case-control study of preschool ADHD in MoBa, 2004–2007.

Exposure	Combined[a,b,c]	Boys[d,e]	Girls[d,f]	P-value[h]
	Odds Ratio (95% CI)	Odds Ratio (95% CI)	Odds Ratio (95% CI)	
Log10 exposure				
DPhP	1.07 (0.77, 1.48)	0.95 (0.61, 1.49)	1.23 (0.76, 2.00)	0.45
DnBP	1.30 (0.86, 1.97)	1.47 (0.84, 2.57)	1.12 (0.60, 2.11)	0.54
Exposure ≥ LOD[g]				
BBOEP	0.94 (0.69, 1.28)	1.31 (0.86, 2.00)	0.58 (0.37, 0.93)	0.01
BDCIPP	1.19 (0.83, 1.71)	1.25 (0.74, 2.11)	1.07 (0.63, 1.80)	0.67

[h]P-value is derived from organophosphate ester*sex interaction·

[a] Refers to full model without the incorporation of interaction terms.

[b] Combined model contains 260 Preschool ADHD cases and 549 birth-year-stratified controls.

[c] All models are derived from an imputed logistic regression model adjusted for total fish consumption, maternal education, financial difficulty, maternal age, and sex.

[d] Stratum-specific estimates are derived from models that additionally include interaction terms for each included variable using an augmented product term approach to assess effect measure modification by sex.

[e] Model for boys contains 145 Preschool ADHD cases and 275 birth-year-stratified controls.

[f] Model for girls contains 115 Preschool ADHD cases and 274 birth-year-stratified controls.

[g] Limit of detection.

or ADHD-like symptoms, all observing associations with some OPE metabolites (Lipscomb et al., 2017; Castorina et al., 2017; Doherty et al., 2019; Choi et al., 2021a). The only study to investigate behavioral symptoms in the preschool period was by Doherty et al. (2019) et al. (Doherty et al., 2019) They found higher gestational DPhP and BDCIPP exposure was related to more behavioral and externalizing problems in 3 year-old children in North Carolina, with both DPhP and BDCIPP associated with more attention problems and BDCIPP related to greater hyperactivity (Doherty et al., 2019). However, our results were not entirely consistent with their study. We found little evidence of association with DPhP exposure, and only slightly elevated odds of ADHD in relation to BDCIPP; Doherty et al. did not evaluate DnBP or BBOEP so comparisons could not be made for these OPEs. Differences between our study and Doherty et al. may be attributed to differences between time of exposure collection or differences in OPE levels between our study populations. For example, Doherty et al. collected urine samples later in pregnancy, specifically around 27 weeks' gestation, whereas urine samples in MoBa were collected at approximately 17 weeks' gestation. OPEs have low to moderate reliability when measured ~10 weeks' apart (Percy et al., 2020; Carignan et al., 2018b; Zhao et al., 2019; Kuiper et al., 2020; Ingle et al., 2019b, 2020b; Meeker et al., 2013b; Romano et al., 2017), therefore it could be that associations vary due to differences in an underlying susceptible window (de Graaf-Peters and Hadders-Algra, 2006). Furthermore, in general, exposure levels in our study were somewhat lower than Doherty et al., which would likely have impacted our precision and statistical power for tests of association.

Our results should also be interpreted in conjunction with those of Choi (2021) et al., who also used a nested substudy of MoBa but leveraged the NPR for registered ADHD diagnoses mostly in childhood (i.e. ages 6–11 years; 12 preschool ADHD cases in this study overlapped with NPR registered ADHD cases in the Choi et al. study) (Choi et al., 2021a; Surén et al., 2012). Similar to ours, Choi et al. found a weak trend between higher BDCIPP exposure and greater ADHD risk, but no association with BBOEP (Choi et al., 2021a). Choi et al. also observed greater than medium DPhP exposure was associated with higher ADHD risk, although found no associations for DnBP exposure (Choi et al., 2021a). However, it is important to note that the cases included their study were ADHD registrations in the NPR. Because a gold standard for ADHD diagnosis does not exist, medical registry cases may be affected by provider-level differences in diagnostic procedures. Additionally, as children had to be referred to a specialist provider for evaluation, more severe or more disruptive ADHD cases are likely to be referred, whereas primarily inattentive cases, found more often among girls, may be under-represented. As such, the Choi et al. study had fewer ADHD cases among girls as compared to our study [Choi et al.: 28% ADHD cases were girls; our study: 44% ADHD cases were girls]. It is also important to note that while the demographic and behavioral risk factors commonly associated with ADHD in childhood (prenatal smoking, young maternal age, low maternal education, skewed toward male sex) were also found to be over-represented among our preschool ADHD cases, preschool ADHD symptoms do not always persist into childhood (Riddle et al., 2013; Lahey et al., 2016). It has been suggested that the persistence of the preschool ADHD phenotype is ~90% over the course of childhood and adolescence (Riddle et al., 2013; Lahey et al., 2016). Therefore, differences between our results and those of Choi et al. may be attributable to etiological differences between preschool ADHD and childhood ADHD.

Sexually dimorphic differences of the relationship between OPEs and ADHD have been observed in humans, with female-only dysregulation of thyroid hormones(Xu et al., 2015; Liu et al., 2016), and through androgen-based mechanisms (Kojima et al., 2013, 2016; Ren et al., 2018; Reers et al., 2016). The epidemiological findings on EMM of the OPE-ADHD relationship by child sex have been mixed with one study finding larger adverse effects in girls compared to boys (Choi et al., 2021a) and two studies generally observing no associations (Castorina et al., 2017; Doherty et al., 2019). However, all studies to date (including ours) have had low statistical power to assess EMM, which could result in the observed study differences. Although we found a statistically significant sex-related EMM for BBOEP in our study, odds of ADHD were lower with higher exposure to BBOEP among girls, with the opposite pattern found among boys. This was consistent with the only other study on OPEs and ADHD (Choi et al., 2021a) but inconsistent with two other OPE studies evaluating ADHD -related symptoms that observed no appreciable sex-specific effects (Castorina et al., 2017; Doherty et al., 2019). As such, there is a lack of consensus overall as to the presence of sex-related heterogeneity.

Some study limitations should be noted. In our study, OPE exposure was measured using a single spot urine collection at 17 weeks' gestation. This is not ideal as OPEs have relatively short half-lives lasting only 1–5 days (Hou et al., 2016; Lynn et al., 1981) and have been found to have low (DnBP (Percy et al., 2020), DPhP (Percy et al., 2020; Zhao et al., 2019; Kuiper et al., 2020; Ingle et al., 2019b, 2020b; Meeker et al., 2013b; Romano et al., 2017; Hoffman et al., 2014)) to moderate (BDCIPP (Percy et al., 2020; Carignan et al., 2018b; Kuiper et al., 2020; Ingle et al., 2019b; Ingle et al., 2020b; Meeker et al., 2013b; Romano et al., 2017; Hoffman et al., 2014)) reliability when assessed 2–3 months apart (no epidemiological studies on reliability were found for BBOEP). As such, the OPE metabolite measurements in our study may not reflect OPE exposure throughout pregnancy. Although the pregnancy period of susceptibility for OPEs and neurodevelopment is unknown, the second trimester is a particularly sensitive window for brain growth and development, where adverse exposures could be more salient in terms of long-term neurological effects (Vohr et al., 2017; Selevan et al., 2000). However, DPhP is a non-specific metabolite of TPhP and can be produced by several other parent compounds including resorcinol bis (diphenyl phosphate) (Ballesteros-Gomez et al., 2015a), ethylhexyldiphenyl phosphate (Ballesteros-Gomez et al., 2015b), and tert-butylphenyl diphenyl phosphate (Heitkamp et al., 1985). Therefore, DPhP concentrations in our study may in part reflect exposure to another parent compound that could have a different toxicity compared

to TPhP. However, DPhP is the field standard biomarker for TPhP, and most previous studies observed an association between gestational DPhP exposure and ADHD or ADHD-like symptoms (Doherty et al., 2019; Choi et al., 2021a). Additionally, because OPE exposure occurs as a mixture rather than in isolation, the potential for co-pollutant confounding, synergistic and/or antagonistic interactions, and/or cumulative (i.e. joint) effects cannot be dismissed, although it is unlikely that our findings represent underestimation of real effects due to these phenomena (Braun, 2017). In our study we found low correlations between our 4 measured OPEs, and, in a sensitivity analysis, mutual adjustment for other OPEs did not impact any of our estimates. Furthermore, although preschool ADHD is a valid measure (AAo, 2019), it has low continuity with childhood ADHD and is difficult to diagnose as many of the symptoms associated with preschool ADHD are considered normative for this age-group (Overgaard et al., 2022a; Frick and Nigg, 2012). This study attempts to minimize the potential for misclassification of ADHD in the preschool period by only assessing these outcomes using a validated instrument specifically designed for preschoolers, PAPA, consisting of an in-person in-depth standardized interview conducted by trained personnel. As a result, these measures provide us greater confidence in their accuracy compared to the use of other methods such as diagnostic codes. Regardless, outcome misclassification for ADHD would not likely strongly impact effect estimates as the misclassification is not expected to be differential by exposure. Finally, there is the possibility that selection bias may affect our results given that only the cases were assessed via an in-person assessment whereas non-cases were not (Hernán et al., 2004).

Our study has several important strengths. This case-control study was nested within the large and well characterized Norwegian Mother, Father, and Child cohort (MoBa). Our preschool ADHD cases were identified using a high-quality and validated clinical assessment tool for the preschool period, the Preschool Aged Psychiatric Assessment. The clinical assessment was found to have high inter-rater reliability and was conducted under the supervision of a child psychologist or psychiatrist (Overgaard et al., 2018). As a result, this study is less likely to suffer from outcome misclassification compared to studies relying on self-report (Frick and Nigg, 2012). This study also utilized biomarkers of OPEs rather than reliance on external measures such as household dust. Biomarkers inherently integrate exposure from all sources, thus accounting for the diverse potential sources of OPE exposure including cars (Brommer et al., 2012; Zhou et al., 2017), residential housing (Meeker et al., 2013a; Zhou et al., 2017; Ali et al., 2012; Cequier et al., 2014b; He et al., 2016), and office spaces (Brommer et al., 2012; Zhou et al., 2017; He et al., 2016), as well as potential exposure from food (Zhang et al., 2016a; Li et al., 2019; Ding et al., 2018) and water (Li et al., 2014; Kim and Kannan, 2018; Lee et al., 2016b). Therefore, utilization of biomarkers in our study rather than external measures has the potential to contribute to greater exposure validity. Additionally, this study also has a good representation of girls (44%) allowing for greater statistical power to assess EMM of the OPE-ADHD relationship by child sex. Our study also fills an important research gap on the OPEs DnBP and BBOEP as TBOEP and TnBP are frequently reported as two of the highest OPE concentrations in dust (particularly in Europe) and have been found to be associated with developmental toxicity and dysregulation of thyroid hormones (Chupeau et al., 2020; Zhang et al., 2016b; Liu et al., 2017).

5. Conclusions

OPEs are ubiquitous (van der Veen and de Boer, 2012; Stapleton et al., 2011; Ingle et al., 2020a; Ingle et al., 2019a; Mendelsohn et al., 2016) and increasing in usage (van der Veen and de Boer, 2012), although there is a paucity of research on their safety. Our results support a modest and imprecise association between DnBP and BDCIPP with preschool ADHD. Preschool ADHD is a prevalent neurodevelopmental disorder (Lavigne et al., 2009; Wichstrom et al., 2012; Willcutt, 2012) with substantial adverse individual (Mariani and Barkley, 1997; McGoey KEEckert and VanBrakle, 2001) and economic (Sciberras et al., 2017; Gupte-Singh et al., 2017; Le et al., 2014; Quintero et al., 2018; Cadman et al., 2012; Kotsopoulos et al., 2013) consequences. As such, identification of potentially-modifiable risk factors for this common and impactful neurodevelopment disorder is critically needed.

Ethics

The MoBa study was conducted with a license from the Norwegian Data Protection Agency in accordance with guidelines from the Declaration of Helsinki. The MoBa study is currently regulated by the Norwegian Health Registry Act. The Preschool ADHD study was approved by the Regional Committee for Medical and Health Research Ethics South East Norway (ref. nu. 2011/179). Written informed consent was required and obtained for all participants in MoBa. Similarly, additional approval and written informed consent of participants for the clinical evaluation was required and obtained by the Regional Committee for Medical Research Ethics (ref. nu. 2012/985). Data analyses were performed with approval of the UNC Office of Human Research Ethics (ref. nu. 20–2462).

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Data statement

Data from MoBa and the Medical Birth Registry of Norway used in this study are managed by the national health register holders in Norway (Norwegian Institute of Public Health; NIPH) and can be made available to researchers, provided approval from the Regional Committees for Medical and Health Research Ethics (REC), compliance with the European Union General Data Protection Regulation (GDPR) and approval from the data owners. The consent given by the participants does not open for storage of data on an individual level in repositories or journals. Researchers who want access to data sets for replication should apply through helsedata.no. Access to data sets requires approval from REC in Norway and an agreement with MoBa Analytic code used for the present analysis may be obtained from the corresponding author.

Declarations of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.114078.

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HBM4EU feasibility studies: Lessons learned in combining health and human biomonitoring studies

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ABSTRACT

Background: The European Human Biomonitoring Initiative (HBM4EU) is a joint program evaluating humans' exposure to several environmental substances and their potential health effects. One of the main objectives of HBM4EU is to make use of human biomonitoring (HBM) to assess human exposure to chemicals in Europe to better understand the associated health impacts and to improve chemical risk assessment. In parallel to HBM studies, health examination surveys (HESs), nutrition/dietary surveys, and disease specific health surveys are conducted in many European countries. In HESs, information collected by questionnaire(s) is supplemented with physical examinations and analysis of clinical and biological biomarkers in biological samples. HBM and health examination survey (HES) use similar data collection methods and infrastructures hence the feasibility of combining these two is explored in this paper.

Methods: Within HBM4EU, three feasibility studies (in Finland, Germany, and UK/England) were conducted to evaluate opportunities and obstacles of combining HBM and health studies. In this paper we report lessons learned from these feasibility studies.

Results: The Finnish feasibility study called KouBio-KUOPIO study was a new initiative without links to existing studies. The German feasibility study added a HBM module to the first follow-up examination of the LIFE-Adult-Study, a population-based cohort study. The UK feasibility integrates a sustainable HBM module into the Health Survey for England (HSfE), an annual health examination survey. Benefits of combining HBM and HESs include the use of shared infrastructures. Furthermore, participants can receive additional health information from HES, and participation rates tend to be higher due to the potential to obtain personal health information. Preparatory phases including obtaining ethical approval can be time-consuming and complicated. Recruitment of participants and low participation rates are common concerns in survey research and therefore designing user-friendly

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questionnaires with low participant burden is important. Unexpected events such as the COVID-19 pandemic can cause substantial challenges and delays for such studies. Furthermore, experiences from several countries demonstrated that long-term funding for combined studies can be difficult to obtain.
Conclusions: In the future, incorporating HBM modules into existing HESs can provide a feasible and cost-effective method to conduct HBM studies and obtain a wide range of relevant data to support public health policies and research.

1. Introduction

To respond to a variety of public health questions, and guide policy decisions and possible prevention activities, a holistic picture of health determinants and health outcomes of individuals are needed. Information on potential background variables, chemical exposures as well as health determinants and outcomes are crucial to provide a valuable data source. Combining human biomonitoring (HBM) studies and health examination surveys (HESs) is an option to collate these vast datasets in an effective way.

In many European countries, HBM studies, HESs, nutritional/dietary surveys and disease specific health surveys are conducted (Tolonen et al., 2018). HBM studies are focused on obtaining information on chemical exposures and measuring exposure levels as well as biomarkers of effect plus focused questionnaires, while in HESs, information on socio-economic background and lifestyles collected by questionnaire(s) is supplemented with physical examinations such as blood pressure and anthropometric measurements, and analysis of health-related biomarkers such as blood lipids including total, LDL and HDL cholesterol, and blood glucose from biological samples. The European Health Examination Survey (EHES), established in 2010, coordinates the development of national HESs in Europe (EHES, 2021a). Between 2014 and 2021, ten European countries have conducted a national HES, and in many countries regional HES or disease specific surveys or dietary surveys have been carried out (EHES, 2021b). In both HBM studies and HESs, data is collected through fieldwork, which is one of the most expensive and time-consuming components. As the infrastructures required for both types of studies are similar, and there are overlaps in the information gathered, the potential to combine them has been considered in several countries. It is anticipated that this could represent a cost-effective and mutually beneficial way of conducting health and HBM studies and provide data to study exposure-health outcome relationships.

To date, very few studies have been conducted, which have combined HBM and HES despite the seemingly clear benefits of combining these studies. Many HBM studies already include some health questions, however extensive objective health measurements or analysis of health-related biomarkers are still lacking. Recently, extensive combined surveys have been conducted at the national level in countries such as Germany, France, Israel, USA, and Canada (Kolossa-Gehring et al., 2007; Balicco et al., 2017; Berman et al., 2017; Centers for Disease Control and Prevention, 2021; St-Amand et al., 2014.). Although several smaller, regional or disease specific combined studies of HES and HBM have been performed throughout Europe (Tolonen et al., 2021), there is no systematic evaluation of synergies and benefits obtained or challenges encountered in these studies.

The aim of the feasibility studies conducted in HBM4EU was to assess the opportunities and obstacles encountered when combining two similar survey types with intrinsic differences. Previously conducted combined HBM and HESs have mainly been part of well-established national HBM monitoring programs, but these new feasibility studies were setup for countries/regions without prior experience of such combined studies, except for Germany. Therefore, the aim of these specific feasibility studies was to test the operability and viability of the studies and their protocols rather than obtain comprehensive results of chemical exposures and their health effects.

This paper reviews the protocols and outcomes of these feasibility studies and reports the lessons learned.

2. Methods

Feasibility studies were conducted in the framework of HBM4EU. HBM4EU is a joint project of 30 countries and the European Environment Agency (EEA), co-funded by the European Commission (EC) under Horizon 2020. The HBM4EU initiative was a five-year-project running from January 2017 to June 2022, which coordinated and advanced HBM activities in Europe. The initiative generated evidence of the actual exposure of EU residents to chemicals through HBM. HBM4EU aimed to harmonize HBM across Europe to facilitate the assessment of human exposure to chemicals. The aims were to better understand the health impacts, enhance chemical risk assessment, and support chemical policy. In HBM, the body burden of environmental chemicals and their metabolites can be measured in biological matrices such as blood (whole blood, serum, and plasma), urine, hair, and breast milk. HBM offers an aggregated measure of the level of exposure to chemicals and chemical mixtures via various exposure pathways and sources. HBM is an important tool for assessing exposures of the human population to chemicals, and when harmful chemicals are concerned, estimating potential health risks related to the exposure (HBM4EU, 2021; Ormsby et al., 2017; Ganzleben et al., 2017.).

Feasibility studies were selected to represent different settings and age groups in countries/regions which mostly have no prior experience in conducting combined HBM and HES. The minimum requirements for the feasibility studies were defined (Tolonen et al., 2017), and studies were selected through a competitive call process. The three selected studies were from Finland, Germany, and UK/England. Criteria for the studies selected were the following: feasibility studies are conducted with already planned/starting surveys/studies only in countries which do not have experience in combining HBM and health studies in the past. Furthermore, fieldwork should take place in 2019 (Tolonen et al., 2017.). These criteria were used as basis for the Internal Call (IC) process of the HBM4EU published in 2018. Topics for the first Internal Calls (ICs) were selected and approved, and the Management Board (MB) made decision on the outcomes of the IC in 2018 based on the external reviews, Scientific Advisory Board recommendations and detailed discussions. Thus, the following three feasibility studies were selected: Finland, Germany, and UK/England (Tolonen et al., 2019.).

In Finland, a new study among children was organized while in Germany and UK/England, a HBM module was added to an existing HES. The German study focused on adults and used a follow-up cohort study as a basis while the UK/English study was based on a cross-sectional survey among adolescents and adults. For these feasibility studies, the sample size was not chosen to provide nationally or even regionally representative results but to test the feasibility of combining HBM and HES in a real-world setting.

3. Planning and implementation of the feasibility studies

3.1. Finnish Feasibility Study - KouBio-KUOPIO

The Finnish feasibility study called KouBio-KUOPIO study is a new initiative without any links to existing studies. The KouBio-KUOPIO study was originally planned as an extension of the regular school health examinations, which are organized annually for all pupils in

primary school (grades 1–9). In grades one, five, and eight, an extensive health examination is performed by a doctor and for other grades more limited examination is conducted by a nurse. The aim was to recruit 300 pupils from 5th and 6th grade aged 9–13 years from the selected primary schools of Kuopio city area. (THL, 2021.) The schools were chosen based on their willingness to participate, and both new and old school buildings were included. Since some of the chemicals, such as some of the phthalates, have been prohibited in building materials already ten years ago, one of the research questions was to see do the used building materials impact identified exposure levels. The study aimed to assess the extent of school aged children's exposure to phthalates and bisphenols measured in urine samples. Eight volunteer schools were enrolled for the study.

Preparation of the study plan started in November 2018 with outlining the study design including development of the questionnaires and sample collection procedures. Fieldwork commenced in February 2020 and finished in November 2020. Thereafter phthalates and bisphenol A from collected biological samples were analysed and questionnaire data was entered (paper questionnaires) and evaluated.

During the preparatory phase, several obstacles were faced and needed to be solved before the fieldwork could commence. The preparation of the study protocol benefited from the long experience of organizing HESs in Finland. The HBM4EU standardized questionnaire was used in the study (Pack and Fiddicke, 2021). Parents or guardians made the decision whether their child/children would participate and filled in the questionnaires with them or for them. The questionnaire included questions/response alternatives which are not relevant for the Finnish society, culture, and study area. For example, for question 'Is there any of the following facilities within 300 m of your home? ', response alternatives 'A waste incineration plant', 'A site where photovoltaic devices and sollar cells are produced' among few others were removed as those facilites did not exist in the study area. Therefore, the questionnaire was modified by removing irrelevant questions or response alternatives, and only including those suitable for the Finnish society and for this specific feasibility study. There were 40 questions altogether in the questionnaire. The participants had a choice of completing the questionnaire either in pen and paper or online, with most opting for the paper version (61%).

The Finnish feasibility study experienced delays in the preparatory phase due to the lengthy process of obtaining ethical approval. It took seven months to complete the ethical approval process. Since the target group included pupils who were approached through the schools, approval from the City of Kuopio (health board and school board) was required before application for ethical approval could be submitted to the Ethical Board of Kuopio University Hospital. Approval from the city was obtained four months after initial contact. The process with the Ethical Board of Kuopio University Hospital required two revision rounds before final approval. During the revision rounds, substantial changes to the planned study protocol were required. The biggest change had to be made when the Ethical board declined the permission to conduct the study as an extension to the regular school health examinations. They didn't want the HBM study to be associated as a part of the school health examination. Also, collection of blood samples from children and use of movie tickets as incentives were denied by the Ethical board. These requirements changed the entire study protocol so that collection of blood samples was omitted, and urine samples had to be collected and frozen at home and sent to the study team. Other requested modifications to the information notice were mainly due to requirements of EU General Data Protection Regulation (GDPR), i.e., how data can be shared with the non-EU countries. The GDPR caused detailed requirements for the information notice and informed consent and fulfilling them was time consuming and required consultation of the Data Protection Officer.

The original plan was to provide pupils with questionnaires and sample collection kits with personalized identification information (study ID and corresponding bar code stickers linked to the specific pupil). Since the lists of pupils' names were not accessible due to the GDPR, the recruitment strategy had to be modified in the middle of the process. New protocol included additional steps for the coordination team to ensure that each pupil could be identified at the end and linked to written informed consent and possibly also to data from administrative registers. In the new protocol, the number of pupils in each school were estimated based on the information on the web pages of the schools, and suitable number of pseudonymized material packages were prepared. The material package included invitation and information letters, instructions for spot urine sample collection, questionnaire, and informed consent form together with sample collection materials. Identification stickers were placed on all these materials to allow linkage between the different materials.

Although contacts preceding the visits to the schools went well, engaging the pupils to the study proved difficult. There were no incentives or compensations for participation as it was prohibited by the ethics committee. In the Finnish study, the participation rate turned out to be low, approximately 6%, since 66 responses and urine samples were received from 1200 potential participants originally recruited.

The recruitment strategy of the Finnish study was not ideal, which could have affected the participation rate. The schools informed the parents of the pupils about the study, after which information was given and relevant materials were distributed to the pupils during events organized at the schools. These events were attended by several classes at the same time, and they were too large to be interactive. After the presentations, more than half of the pupils tried to leave without the sampling packages. Not all the involved teachers were well informed about the study and hence were unable to properly encourage the pupils to participate. Also, it was difficult to communicate the goals and importance of the study to the parents as there was no direct contact between them and the study team. Furthermore, the use of incentives in the form of, e.g., movie tickets, was not allowed by the ethics committee. As the participation in the study was voluntary, this could have affected the willingness to participate considering the age of the pupils.

Finally, the COVID-19 pandemic affected the realisation of the study. For example, in the first phase of the Finnish feasibility study, two schools were left unvisited when the pandemic hit, and even later these remaining schools could not be visited in person according to the original plan due to visiting restrictions. The study material was distributed to the schools, but a substantial amount of information and motivation remained unavailable because the study team could not personally visit the schools and give information about the study.

If the sampling could have been conducted during health check-ups as originally planned, the participation rate would have been most likely higher. In the check-ups both a health professional (nurse and/or physician) and the parent(s)/guardian(s) of the children would have been present, and information provided to them directly with the possibility to ask further questions. To enhance the recruitment of the pupils, co-operation with the parents and teachers is crucial, and motivating all of them is a key element.

3.2. German Feasibility Study

The German feasibility study was conducted by adding a HBM module to the first follow-up examination of the LIFE-Adult-Study (Loeffler et al., 2015; Engel et al., 2022). The LIFE-Adult-Study is a population-based cohort study, where the baseline examination has been completed for 10.000 randomly selected inhabitants from the city of Leipzig in 2014. The sample was drawn from the population register based on age (40–79 years) and gender (50% men and 50% women). In 2017, the first follow-up of the LIFE-Adult-Study started with despatching questionnaires to all the 10.000 participants. Additionally, between 2018 and 2021 approximately 2.700 (mostly 60 years and older) participants in the LIFE basic examination with a brain magnetic resonance imaging (MRI) at baseline and a consent for re-contacting were invited to the study centre for a comprehensive second

examination programme.

Prior to the feasibility study, a study protocol was compiled and approved by the Ethics Committee at the Medical Faculty of the University Leipzig (201/17-ek).

The LIFE-Adult-Study investigates environmental factors influencing development of civilization diseases (e.g., obesity, diabetes, heart disease, and cancer) in a population-based cohort. Therefore, both the original study protocol and informed consent were broad, and it was possible to accomplish all necessary processes of the feasibility study.

The participants received an invitation letter to participate in the follow-up study, after which appointments were arranged by phone. Persons who did not respond to the first letter of invitation received a second followed by phone contact attempts. If the letters were undeliverable, the residential addresses were corrected by using the registration register, after which the invitation process was repeated for these persons.

The follow-up provided an opportunity to integrate a HBM module into the LIFE-Adult-Study programme. A regular component of the LIFE-Adult-Study is a comprehensive physical examination and questionnaire program for all participants. Many of the questionnaire entities of the HBM module (sociodemographic characteristics, health status, nutrition, and medication) have already been fully covered by the LIFE-Adult-Study. A concept for a HBM module and a strategy for its integration in the LIFE-Adult-Study was developed together with Fraunhofer IBMT. The concept was formulated in accordance with the HBM4EU standard operating procedures (SOPs) (see https://www.hbm4eu.eu/online-library/for all SOPs) and the HBM4EU guidelines for linking HBM and health studies (Tolonen et al., 2022). Therefore, the HBM module was compared with the LIFE questionnaires, and only information, which was not already examined with the LIFE questionnaire, was recorded. The surveys were conducted by means of interviews and self-completed questionnaires, and the questionnaire had to be filled in at the day of the examination at the study centre. About 85% of the participants used the electronic data entry forms, however some older participants preferred a paper version. Additional urine collection (spot urine) at the examination side had to be established for HBM and integrated into the study process as this was not part of the LIFE follow-up. In addition to receiving some personal health data, the study participants were allocated a compensation of expenses of €15.

The HBM-module was successfully tested in a subset of 400 LIFE participants in the follow-up, which visited the study centre in 2019/2020. In the feasibility study, phthalates, arsenic (As), and mercury (Hg) together with their compounds were identified as priority chemicals of interest. The spot urine samples of 400 participants were processed and stored. The team of the LIFE-Adult Outpatient Clinic provided instructions to the study participants for collection of urine samples and was also responsible for appropriate labelling and transportation of the samples to the pre-analytical laboratory. Due to lack of funding, analysis of the urine samples was not conducted immediately but samples are stored and can be used for future analysis.

In Germany, recruitment of the participants was not an issue, as the HBM component was integrated into an already running follow-up of a cohort study with adult participants. On the regular examination day of the LIFE-Adult-Study, participants were asked if they wished to participate in the additional HBM module. Combining the HBM module into the LIFE-Adult cohort study ensured exceptionally high participation rate of 91%. The long-established cohort study enabled the HBM module to be considered as an additional "health check-up", which might have augmented the participation rate. Furthermore, the study population consisted of adults, many of whom had already retired, and therefore had possibly more time available and increased interest in health issues compared to working aged adults and children or adolescents.

The COVID-19 pandemic did not cause substantial delays or obstacles to the German study. The participants were included in the HBM study between November 2019 and August 2020. During the first wave of the pandemic, the study centre was closed from mid-March to early June 2020. Nevertheless, the recruitment of 400 subjects for HMB was successfully accomplished.

3.3. UK/England Feasibility Study

The UK feasibility study is seeking to integrate a sustainable HBM module into the Health Survey for England (HSfE) (Mindell et al., 2012), an annual health examination survey, which commenced in England in 1991. It is regarded as a Gold Standard health survey collecting information about the health of the population in England, and it is an ideal platform for the integration of a HBM module. HSfE is commissioned by National Health Service (NHS) Digital and carried out by the Joint Health Surveys Unit of National Centre for Social Research (NatCen) in co-operation with the Research Department of Epidemiology and Public Health at University College London (UCL).

Approximately 10.000 people (8.000 adults and 2.000 children) are targeted annually for participation in HSfE. It gathers a population representative sample of children 2–15 years and adults (16+ years as defined by HSfE). The survey utilises a multi-stage stratified probability sampling design, and the populations targeted live in private housing; persons living in institutions are not included. In the HSfE, the 1st contact is by interviewer who conducts an interview, administers the questionnaires and schedules time for the nurse visit during which health measurements and collection of biological samples are conducted.

The first step in the development of a HBM programme involves undertaking a pilot study for the integration of a HBM module into HSfE. The HBM module utilises a subset of 300 participants (minimum 200) from the selected HSfE sample population (N = 10.000), and comprises adolescents aged 16–19 years (N = 60) and adults aged 20–49 years (N = 240). Rather than sampling 150 adults and 150 adolescents, the strategy had to be revised due to the small sample size of the 16-19-year age group. Additionally, it is unclear whether participation of this younger age group may prove more challenging than the adults due to lifestyle etc. Before the start of the pilot study, a dress rehearsal was conducted on a very small number of participants in August/September 2021, and only 12 of the 50 participants recruited for HSfE were with the required age band (18–49 years) and therefore eligible to take part in the HBM module. It was noted that during the dress rehearsal no participants completed the additional online survey for the HBM module. Hence a SMS text reminder will be sent during the fieldwork in 2022 to encourage completion of the online questionnaire. The feasibility study will be conducted in June–December 2022 and many lessons are expected to be learned throughout the process.

Due to the COVID-19 pandemic, there were amendments to the timetable for the 2022 programme (originally scheduled for January–December 2022) as fieldwork for 2021 had to be completed in the first half of 2022. Additionally, there had been some difficulties with the supplies of plastic urine containers, so the HBM module would not have been able to commence in January 2022 had there been no amendment to the field schedule. The revised timetable for the fieldwork is June 2022–March 2023.

Blood and urine samples are being collected from participants to determine their personal exposure to four groups of the HBM4EU priority substances: perfluorinated substances (PFAS), flame retardants, bisphenols, and phthalates, as well as metals.

The most effective and appropriate means to select the households for the HBM module was decided by NatCen at the start of the programme however is subject to change if the participants numbers are low. To align with HBM4EU, the aim will be to reach a minimum 200 but ideally 300 participants (adolescents and adults) including two age groups. Adolescents and adults have been selected due to costs constraints and the inherent difficulties that are experienced when trying to gather samples from children. The households selected for the survey are sent a letter to obtain a permission to be interviewed and participate. At the initial visit, the interviewer administers a questionnaire and asks for

an agreement for a second stage visit of a nurse to collect samples and take measurements. Before 2018, all households included in the initial visit were offered a nurse's visit. Now 16 of the 18 addresses are randomly selected for the stage two. Earlier, a token of £10 voucher was given to the participants in HES.

Regarding the questionnaire module, a maximum of 10 additional minutes of interview for the HBM module could be included in the HSfE. Therefore, there can only be a few questions in the main questionnaire, and a more detailed HBM online questionnaire including questions related to the chemicals of interest is provided for the participants to be completed after the nurse's visit.

During the first phase, the integration of a HBM study into a HES will be limited to England, however the aim is that later the programme is expanded to all four UK nations. The integration of a HBM module to HSfE is currently in progress, with the preparatory work regarding study design, required questionnaires and other materials conducted in 2019–2021.

HSfE have validated methods which have been approved by the Research Ethics Committee (REC). In the UK, the new HBM module needs to follow the timelines and protocols of the HSfE when ethical approval is applied. This means that any additional ethical approval documentation for HBM must be submitted in time for the HSfE ethical approval. Basically, every year since the start of the survey (HSfE) it is reviewed by an independent group of experts to ensure the safety, rights, wellbeing, and dignity of the survey participants. Each year the survey asks for an amendment to their ethical approval, which covers the selected topics for that specific year. For the 2022 programme including the HBM module, ethical approval was sought and gained for the collection of the additional information and biological samples. Substances of interest were listed. However, there was some degree of flexibility in the approval granted for metals since there was no need to list the specific metals to be investigated.

The biological samples collected for the HBM module are transferred to UK Health Security Agency (UKHSA) laboratory in Oxfordshire where they are stored and will be analysed in batches. All analyses will be done in-house but inter-laboratory comparisons for some substances will be conducted in other government department laboratories, e.g., Health and Safety Executive (HSE), Environment Agency (EA) and Department for Environment, Food and Rural Affairs (Defra).

4. Lessons learned

4.1. From previous combined HBM and HES studies

Previously conducted studies provide valuable information about operability of combining HBM and HES in different settings. There were several opportunities but also challenges in previously conducted national HBM programmes from Germany (German environmental survey for children (GerES IV)) (Kolossa-Gehring et al., 2007), France (Esteban survey) (Balicco et al., 2017), and Israel (2011 Israel Biomonitoring study and 2015–2016 Israel MABAT Biomonitoring Study) (Berman et al., 2017) combining HBM and HES surveys. In all these three countries, studies were initiated and implemented at the governmental level with ministerial funding. It has been shown in these studies that utilization of existing logistic infrastructure where a HBM module is added to the existing HES will result in significant cost savings, larger sample size, and access to a wide range of detailed nutrition and health-related data. Since HESs usually benefit from a good public awareness, positive image, and high interest among people, this will also be beneficial to a HBM module and help to increase participation rate. Data from combined surveys allow the investigation of links between exposures and health related outcomes (Tolonen et al., 2018; David et al., 2020.).

On the other hand, combined surveys have some challenges such as limitation in the size of questionnaire and other health parameters and analysis of biomarkers. The respondent burden needs to be kept acceptable not to jeopardize participation rates and therefore, only a limited additional number of questions and measurements of interest can be included to the final study protocol. In addition, the number of biological samples, such as blood and urine, which can be collected from each individual is limited, and a balance between available samples and interested biomarkers may need to be established. There may also be restrictions on when data can be published, and the HBM module will be subject to the timetable of the HES if it is the secondary study. If ethical approval and informed consent are obtained separately for HBM and HES modules, there may be lack of flexibility in the data access and use. Communication between teams responsible for the different modules and to the participants may be challenging and requires extra efforts and training in extensive combined HBM and HES surveys (Tolonen et al., 2018.).

4.2. From HBM4EU feasibility studies

The detailed description of the feasibility studies and their outcomes have been published as outcome of the HBM4EU project (Elonheimo et al., 2021). Here we will describe lessons learned during the preparatory and implementation phase.

There were several benefits identified related to adding a HBM module to existing HESs. To establish a completely new survey requires huge effort and is very costly, and therefore adding a HBM module to an existing survey enables using already existing infrastructure and trained staff. However, HESs are conducted among adults in most countries (see https://ehes.info/national/national_hes_status.htm), and only a few countries such as Germany, France and UK/England have HES designed for children/adolescents.

When organizing a study combining HBM and HES, it is important to identify in the beginning whether data is being collected for research or monitoring purposes, or both. This is due to the difference in the reporting periods; research usually requires longer time scales to accommodate thorough statistical analysis of results before reporting whilst monitoring often report basic results quicker with simple descriptive statistics. The purpose for this data collection may also affect ethical approval and funding possibilities, and country specific differences apply.

Preparation of the combined HBM and HESs is time consuming and requires appropriate resource allocation. In contrast, the effort required to establish a permanent population representative HBM study would require a considerably greater effort, time, and skills. The studies may experience significant delays due to long processes and demands of ethical boards and additional external factors, such as the unexpected global pandemic of COVID-19. Enough time, at least one year, should be allocated for study preparation to ensure everything is in order before starting the fieldwork. GDPR can have a challenging impact on conducting HBM studies in minors. This is particularly true in school settings with children as a target group, as experienced in the Finnish KouBio-KUOPIO study. Thus, information regarding study subjects can be difficult to obtain due to strict legislative rules and regulations. However, obtaining permissions for both modules at the same time can save time, costs, and effort, since there is no need to do these procedures separately for two different modules.

Schools are an appealing setting for conducting a biomonitoring study among children due to an easy access to specific age cohorts. They provide an interesting place for research, because in some countries it can be practical to link sample collection to existing school health check-ups. However, there are also challenges. In addition to children and their parents/guardians, there are many other groups of people involved e.g., principals, teachers, and school nurses, possibly also school boards – all of whom need to be persuaded to commit to the study for recruitment to be successful. Furthermore, ethical rules and requirements, national regulations as well as the GDPR issues are rightfully more protective and stricter regarding children. Children are seen as vulnerable groups and additional protective actions are required for them.

Low participation rates are common, and there is no one-fit-for-all

solution for the recruitment. However, it should be recognized, that participation rates have been higher in those HBM studies which have been combined to HESs. Furthermore, participants of the established cohort study easily consider the HBM module as an additional "health check-up", and they are used to participating in examinations. These facts help in recruiting and increasing participation rates. One additional benefit is that combining HBM data with other health data from HESs provides added value and diversity to data content and gathering. Furthermore, one important aspect to consider is, whether a HBM module can be added to an existing HES without compromising the original participation rate of the HES.

Recruitment efforts should be adjusted for each study. Sufficient information for invitees should be given, and teachers and guardians should be motivated when children in the school setting are concerned. Giving a small incentive and sending frequent reminders via SMS, if applicable, could be used to increase participation rates (Smith et al., 2019; Tolonen et al., 2014). Participation rate can also be increased with a targeted communication concept with trained recruiters and non-material incentives like information materials including (Börsch-Supan et al., 2013), e.g., information on personal health-related results (Morrison et al., 2003). Furthermore, a non-response questionnaire can offer important information about the profiles of non-respondents and reasons for non-response.

Lengthy and complicated questionnaires might decrease response rates, and therefore length and content of the questionnaires should be carefully evaluated. HBM4EU has specific standard questionnaires (Pack and Fiddicke, 2021), however their use in national studies can present challenges since they also include concepts such as specific industries or food items, which are not always relevant to a specific study region, and they do not adequately target e.g., country specific exposure routes. Therefore, national, and local adaptations to the questionnaires may be needed. One alternative to be considered could be, that a short obligatory questionnaire with key questions is included as a part of the main study protocol and more extensive, longer questionnaire is offered for survey participants later to be filled out online. For questionnaire administration online surveys are getting more common, however providing a paper and pen alternative especially for older participants is important.

It should be anticipated that invasive blood sampling can be highly expensive. Also, having trained and qualified staff to conduct HBM data collection is important.

One approach that might be beneficial is accessing samples stored in existing biobanks. In Europe, many biobanks already collect and store human samples for biomedical research and various medical applications. Most of them are organized in infrastructures and/or scientific societies like the European, Middle Eastern, and African Society for Biopreservation and Biobanking (ESBB) and the European Research Infrastructure BBMRI-ERIC. In addition to the primary research and application reasons and depending on the informed consent of the sample donor, many of these biobanks might be open for use of samples for further research including HBM. This might save costs and create reciprocal benefits. However, it needs to be clarified whether the available samples meet the requirement criteria for use in HBM regarding representativeness, target groups, and quality of the samples (Lermen et al., 2020.). Furthermore, sample volume available for biobank samples may be an issue especially if multiple exposure biomarkers are planned to be measured.

Long-term funding, analytical capabilities, facilities for sample handling and storage, and data management procedures are needed for sustainable monitoring programmes. However, receiving funding is a common problem. Finally, the policy value of combined HBM and HES studies might be increased by relatively short transition time between the data collection, analysis, and reporting results.

5. Discussion

The common features and known differences in the organization of HBM and HES studies has been reported separately (Tolonen et al., 2018, 2022). We will discuss some of the key elements which were found most challenging in the feasibility studies namely obtaining ethical approval, non-response, and preparation of the survey questionnaire.

Ethical approval is always required for health-research which includes human participants; this must be obtained before approaching research participant and commencing data collection (Gelling, 2016). Procedures for obtaining ethical permissions varies from one country to another however there are shared regulations which are binding for all the EU countries. The most noteworthy is the EU GDPR. GDPR is Europe's data privacy and security law, which assigns obligations not only on European countries but also any other countries/organisations wishing to target or collect data related to people in the EU. (Gdpr.eu, 2021)

National and regional ethics committees can have varying practises concerning ethical approvals and regulatory requirements. In these three feasibility studies, receiving the ethical approvals were more complicated in Finland and UK/England in comparison to Germany. More harmonized interpretation of the GDPR between European countries would be needed. It should be anticipated that ethical processes can take months, and sometimes several update rounds of ethical approvals may be required. Therefore, enough time should be allocated for the preparation of the materials for the ethics committees and possible revisions.

In all the feasibility studies, the preparatory phases including obtaining of ethical approvals were very time consuming and country-specific differences existed. In Finland a completely new HBM module was created to be performed in the school settings, whereas in Germany and the UK/England the HBM modules were linked with existing cohorts or HESs. The study designs of HES and HBM surveys have major operational and infrastructural aspects in common, such as recruitment of participants, questionnaires, and collection of samples and data (David et al., 2020). However, the studies can differ in specific requirements regarding fieldwork, pre-analytical and analytical phases, as well as sample storage, and taking these into account during the study preparation is essential. Furthermore, recruiting a HBM module as a part of HES will reduce recruitment efforts.

Non-response questionnaires, a short questionnaire offered to non-respondents to obtain their background information and reasons for non-response including ideally a couple of key survey content questions, can provide important information for future activities and studies, and these questionnaires should be scrutinized and analysed to gain experience and learn lessons. Even though incentives are not allowed in Finland, sending out SMS-reminders is not prohibited, and reminders can be a cost-effective way to encourage participation in health studies (Karvanen et al., 2019). However, this requires that mobile phone numbers are included in the sampling frame. In both Germany and the UK, small incentives to the participants are allowed, and therefore small vouchers can be provided, which can help in recruiting more participants to take part in the HBM module of the study. Furthermore, recruiting young participants may prove difficult and the success of recruiting the youth may require additional efforts to motivate guardians and teachers as well.

Participation rates of the two feasibility studies already conducted varied substantially. In the Finnish study, the participation rate was very low (6%), whereas in Germany the rate was exceptionally high (91%). Reasons for the low participation rate may be diverse including, e.g., deficiencies in the study protocols, inadequate motivating of participants and teachers, and restrictions in using incentives. High participation rate, on the other hand, can be attributed to successfully linking a HBM module with a well-established cohort study. Also, the age of the participants can play a substantial role regarding the participation rate; it is estimated that older people are probably more willing to participate

in health studies compared to younger populations.

Targeted communication in all phases and with all involved parties is essential to convey information about the study and its importance to enhance participation. The positive impact of incentives on participation rates is also well documented (Jia et al., 2021). One of the benefits of combining HBM modules with existing HESs can be higher participation rates in comparison to HBM modules being conducted separately. Health surveys are usually more attractive for the invitees due to the possibility to obtain information about one's own health. Also, a HBM module can offer interesting information on internal exposure of a participant to environmental chemicals. The communication of HBM results need to be accompanied by information on sources and routes of exposure, relevant toxicological effects, and recommendations for action on how the exposure can be minimized. For this purpose, HBM4EU has produced factsheets (HBM4EU, 2022) for several substance groups to guide citizens on how to change behaviors to minimize exposure.

Questionnaires are a key tool to collect information about background variables such as socio-economic status and possible exposure sources for HBM studies. They are also used extensively in health surveys to collect information on health behaviours, lifestyle factors, diagnosed diseases, use of medications and health care services, and other aspects and determinants of health and wellbeing (Pack and Fiddicke, 2021; González-Alzaga et al., 2022.). Therefore, questionnaires for combined HBM and HESs can be extensive and time consuming. Within all the feasibility studies, the experience was that modifications of the HBM4EU standard questionnaires were necessary to ensure practicability in the light of participant burden and country-specific circumstances. The HBM4EU questionnaire was designed to cover all relevant questions regarding exposure to the prioritised substances and substance groups in HBM4EU and provide standardized questions for these substances that can be used for further studies.

Although standardized questionnaires exist, applying context-specific questionnaires, and adapting the questionnaires to specific countries and populations is essential. Alternatively, a short obligatory questionnaire with key questions and longer voluntary questionnaire(s) to obtain additional information could be used, like the protocol being used for HSfE. It is important to ensure that participants understand the reasons behind the questions. Additionally, the length of the questionnaires should be carefully considered, as questionnaires that are too long might hinder response rates and produce inaccurate data as participants become fatigued and restless. Therefore, piloting of the complete questionnaire in a large enough sample is essential. Also, it is important to provide both pen and paper and online questionnaires for different participating groups.

Combining HBM modules with representative national HESs is an important aspect to consider and develop. The combined studies have not been very common to present but benefits have already been highlighted. There are recently published guidelines for preparation of combined HBM and HES (Tolonen et al., 2022) which should facilitate establishment of new combined surveys in the future. Also, possibilities to link HBM and HES data with other data sources should be considered (Meltzer et al., 2022).

6. Conclusions

Obtaining comprehensive information on individual's chemical exposures and related determinants, and health outcomes requires surveys which combine features from both HBM studies and HESs. This type of combined HBM studies and HESs can be conducted in many ways as demonstrated with the HBM4EU feasibility studies. Even though these feasibility studies were small on sample size, their study protocols were identical which could be used for a national study and therefore, one would expect that experiences obtained from them hold also for large studies. Establishing a new combined study will be technically and logistically more challenging and expensive than adding a HBM module to an existing HES. HES has already in place logistic infrastructure and recruitment procedures etc. which in many cases is a cost-effective method for collecting and evaluating wide range of health-related data. In theory, it would also be possible to add a HES module to existing HBM study if HBM study has large enough sample size for the needs of the HES (Tolonen et al., 2022).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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HBM4EU results support the Chemicals' Strategy for Sustainability and the Zero-Pollution Action Plan

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ABSTRACT

One of the major goals of the European Human Biomonitoring Initiative (HBM4EU) was to bridge the gap between science and policy by consulting both policy makers and national scientists and generating evidence of the actual exposure of residents to chemicals and whether that exposure would be suggest a potential health risk. Residents' perspectives on chemical exposure and risk were also investigated.

HBM4EU's research was designed to answer specific short-term and long-term policy questions at national and European levels, and for its results to directly support regulatory action on chemicals. A strategy was established to prioritise chemicals for analysis in human matrices, with a total of 18 substances/substance groups chosen to be investigated throughout the five-and a -half-year project. HBM4EU produced new evidence of human exposure levels, developed reference values for exposure, investigated determinants of exposure and derived health-based guidance values for those substances. In addition, HBM4EU promoted the use of human biomonitoring data in chemical risk assessment and developed innovative tools and methods linking chemicals to possible health impacts, such as effect biomarkers. Furthermore, HBM4EU advanced understand of effects from combined exposures and methods to identify emerging chemicals. With the aim of supporting policy implementation, science-to-policy workshops were organised, providing opportunities for joint reflection and dialogue on research results. I, and indicators were developed to assess temporal and spatial patterns in the exposure of European population. A sustainable human biomonitoring monitoring framework, producing comparable quality assured data would allow: the evaluation of time trends; the exploration of spatial trends: the evaluation of the influence of socio-economic conditions on chemical exposure. Therefore, such a framework should be included in the European Chemicals' Strategy for Sustainability and the data would support the Zero Pollution Action Plan.

1. Introduction

The European Green Deal aims to protect the health and well-being of residents from environment-related risks, through a just and inclusive transition (European Commission, 2019). The strategy for sustainable use of chemicals is part of the Green Deal. With over 100,000 chemicals circulating in products on the European market, chemicals are found in the bodies of men, women, children of all age groups including new-borns across Europe (Choi et al., 2017; EUROSTAT, 2021; Gennings et al., 2012).

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The Human Biomonitoring Initiative in Europe (HBM4EU) was an effort of 30 countries, plus the European Environment Agency and the European Commission. It was co-funded under Horizon 2020. The HBM4EU project aimed at coordinating and advancing human biomonitoring in Europe. HBM4EU (2017–2022) generated evidence of the actual exposure of residents to chemicals and the possible health effects to support policy making.

HBM4EU has established a European Union-wide human biomonitoring programme to generate knowledge on human internal exposure to chemicals and their potential health impacts., One of the main goals was to provide evidence to support policy measures to ensure chemical safety and improve health in Europe. It developed and implemented a chemical prioritisation strategy that set out the steps to identify substances of priority concern to be the subject of research and surveys and policy needs (Ougier et al., 2021a). The prioritisation strategy identified specific substances or groups of substances, which were chosen to answer specific policy-related questions and needs in support of legislation.

HBM4EU knowledge will be used to assess progress under several key strategies of the European Green Deal. Evidence on human exposure to pesticides, as well as chemicals used in food contact materials and food contaminants, will be used to assess progress towards the objectives of the Farm to Fork Strategy (Farm to Fork Strategy, 2020). Under the Circular Economy Action Plan, implementing circularity creates new pathways through which humans can be exposed to hazardous chemicals in contaminated material flows (European Commission, 2020a). As an example of how human biomonitoring can add value, HBM4EU collaborated with the e-waste recycling industry to assess workers' exposure to hazardous chemicals and identify opportunities to improve occupational health and safety. The Zero Pollution Action Plan aims to create a toxic-free environment and reduce the burden of premature death and disease driven by pollution in Europe, typically more borne by children, the elderly, persons with disabilities, and those living in poorer socio-economic conditions (European Commission, 2021a). Later in 2022, the European Environment Agency will deliver a first assessment using HBM4EU indicators to establish a baseline on population exposure to chemicals against which to measure progress towards zero pollution. Indicators can be used to assess the effectiveness of current EU chemicals regulations and to identify the need for additional action to protect t the environment and human health.

The Chemicals' Strategy for Sustainability (CSS) provides a progressive approach to managing chemical risks, through upstream measures to ban or restrict the most harmful chemicals and allow essential uses only (European Commission, 2020b). The one substance, one assessment approach promoted under the strategy mirrors the reality of human exposure as captured by human biomonitoring, which measures total internal exposure from multiple sources across legislative silos. HBM4EU data has been made openly accessible via the European HBM dashboard. This allows for the visualization of summary statistics from data collections obtained through HBM4EU, where it is possible to look at exposure levels and trends in chemical exposure of European Residents. HBM data was also generated in the HBM4EU Aligned Studies,[1] and exposure levels can be compared with currently available health-based guidance values. The data included in the dashboard were obtained in a standardized and comparable way. Another platform where the HBM4EU metadata and descriptive statistics is included, is IPCHEM, the Information Platform for Chemical Monitoring, available for risk assessors and researchers to use, so multiplying the added value of this new evidence base. HBM4EU work to assess population exposure against health-based guidance values allows regulators to judge the effectiveness of existing risk management measures and identify those substances for which further efforts are needed to reduce exposure, in particular for vulnerable groups (Apel et al., 2020).

Recognising the observation that in some cases a banned chemical is substituted by another with similar or even unknown properties through regrettable substitution, HBM4EU tackled chemicals in groups (Blum et al., 2019; Buekers et al., 2021; Carvaillo et al., 2019; Lemke et al., 2021; Molina-Molina et al., 2019; Rugard et al., 2020; Sackmann et al., 2018; Trasande, 2017). Grouping of substances is advocated under the chemical strategy as a means of speeding up the risk assessment and management process, for example with per- and polyfluoroalkyl substances (PFAS). HBM4EU has shown that humans are simultaneously exposed to many substances of different chemical classes that enter the human body once these substances are introduced into the environment. HBM4EU has made it very clear that innovative tools to identify human exposure to these new chemicals of emerging concern need to be further developed as well as new approaches for addressing combined exposure assessment and risk assessment dealing with potential mixture effects of these chemicals (Reina-Pérez et al., 2022; Rodríguez-Carrillo et al., 2021; Socianu et al., 2022; Vinggaard et al., 2021; Zare Jeddi et al., 2021). Also methods addressing combined exposure assessment should be targeted in order to find out which groups are higher or lower exposed to multiple chemicals (Willey et al., 2021). The newly generated HBM data on biomarkers of exposure and effect will support a science-based derivation of a Mixture Assessment Factor (MAF) proposed under the CSS strategy. A MAF is an additional safety factor, addressing mixture effects potentially caused by unintentional chemical mixtures.

HBM4EU has created a strategy and of scientific excellence across Europe focusing on translating evidence to knowledge for policy making. It built on a foundation of existing human biomonitoring programmes, the EU co-funded EU-projects COPHES and DEMOCOPHES, and initiatives at national level, to make the whole greater than the parts. It was the first EU chemical-based research project that had a unique two-way open dialogue between policymakers and researchers to prioritise chemical substances and research activities in relation to policy demands, leading to input from the policy perspective for use of results. This relationship reduced the gap between science and policy, with the following underlying principles:

- the need for multi- and transdisciplinary cooperation in the context of complexity.
- the need for opportunities to interact and for dialogue between scientific-, policy- and societal stakeholders.
- the creation of mutual ownership, transparency and a well-structured process architecture with attention to a diversity of relevant perspectives.
- a diversity of policy instruments, policy domains and policy levels.

Human biomonitoring (HBM) delivers a new type of knowledge that resonates with residents, who donate samples to learn about their body burden from chemicals in consumer products and the environment. Responding to their concerns, HBM4EU has produced materials to guide residents in how to change behaviours to minimize exposure to hazardous chemicals, in parallel to channelling evidence into regulatory processes to move forward to a zero-pollution environment.

With the closure of HBM4EU in June 2022, research on human biomonitoring and chemical exposure in support of legislation will be carried out under the Horizon Europe Partnership for the Assessment of Risks from Chemicals (PARC) (European Commission, 2022a). PARC builds on HBM4EU and on its legacy, with the added aspect of strengthening the connection between environmental pollution, effects on the environment and on human health. Therefore, it also follows a more systemic perspectives approach, recognising that humans are an integrative part of their environment. This will support understanding interlinkages between the environment, society and the economy, and

[1] The HBM4EU Aligned Studies are a survey aimed at collecting HBM samples and data as harmonized as possible from (national) studies to derive current internal exposure data representative for the European population/citizens across a geographic spread.

understanding how policies could respond to them (EEA, 2020).

2. Material and methods

2.1. Chemical prioritisation

HBM4EU developed a participatory approach to prioritise substances addressing the most important needs of both policy makers and risk assessors at EU level and in the participating countries and of a broad range of stakeholders, including industry and non-governmental organisations (NGOs). This methodology has been widely endorsed, it is transparent and has been published (Ougier et al., 2021a).

In summary, this strategy consisted of three mains steps:

1) Mapping of knowledge gaps and nomination of substances
2) Prioritisation of substances using a scoring system
3) Listing of priority substances reflective of the scoring, as well as of public policy priorities and available resources

For the first step, a survey was done in which relevant ministries and agencies at European and national levels, as well as members of the Stakeholder Forum, which consists of NGOs, industry and trade unions, each nominated up to 5 substances/substance groups of concern. These nominations were collated and subsequently shortened to another list based on the number of nominations. This initial step, prioritised the substances/groups of substances nominated by the EU Policy Board (with the objective of meeting EU knowledge needs for policy support), followed by substances nominated by two or more National Hubs, or by at least one National Hub and one member of the Stakeholder Forum.

For the second step, substances/substance groups were scored against several prioritisation criteria, namely hazardous properties, exposure characteristics, and societal concern. The scores were used to rank the substances/substance groups. The aim was to understand how much information was already available on substances and to identify the need for new evidence, to ensure that HBM4EU addressed knowledge gaps.

For the third step, and in addition to the ranking and categorisation of the substances, the need for new evidence to support policy priorities at European Union (EU) level was a strong factor influencing the final list of substances and substance groups. This need was communicated to the HBM4EU partners by EU officials in an intensive dialogue involving several workshops.

2.2. Indicators

Indicators are measures of progress or activity and can be visualized to enable data to be interpreted in an easy and accessible way by a broader audience be it scientists, policy makers or the general public. HBM-based indicators are lacking, and hence an approach to develop these was done in HBM4EU. This type of indicators is designed to be relevant for policy, society, and health, and support chemical policy making by using HBM data collections.

During HBM4EU, indicators were developed to assess time and spatial trends in the exposure of European residents to chemicals and to get a picture of whether the population would be at risk. HBM4EU first developed an approach to producing European HBM indicators and proposed two types of indicators (Buekers et al., 2018):

1) Result indicators, which are indicators of internal exposure derived directly from biomarker concentrations.
2) Impact indicators, which are indicators of health risk comparing exposure concentrations to health-based guidance values, such as human biomonitoring guidance values (HBM-GV).

Result indicators measure the concentration of a substance in blood or urine and present time and spatial trends of HBM exposure data, using exposure percentiles e.g., P50 values (or median values) to which participants are exposed. It does not give information on the hazard of the chemical, at which level an effect will occur (potency) or of the risk of being exposed. However, quantitative, and qualitative analysis will allow for an evaluation on policy effectiveness as spatial and temporal trends can be assessed. These indicators are descriptive and allow to respond the question "What are current internal exposures?" and are a good way to track policy efficacy "Are the policy measures working?".

Impact indicators, using exposure values at the higher end of the exposure distribution, e.g., P95 values or 95th percentiles, place HBM data in a health risk context by including the respective HB-GV (or HBM-GV where available), which were derived under HBM4EU (Apel et al., 2020). HBM data can be compared with a level below which no adverse health effects are expected, such as the HBM guidance value (HBM-GV). They are used to assess health impacts ("Is the chemical exposure burden of health concern?").

Health-based human biomonitoring guidance values provide benchmark values against which to compare exposure in the general population.

To allow for the interpretation of HBM data in a health risk context, HBM4EU's scientists derived HBM-GVs for the public and for workers for a number of substances (Apel et al., 2022). Despite not having a regulatory basis, these health-based guidance values were widely endorsed after a consultation process involving all HBM4EU partners, with the methodology made available to the scientific community (Lamkarkach et al., 2021). *"The HBM-GVs derived for the general population represent the concentration of a substance or its specific metabolite(s) in human biological media (*e.g.*, urine, blood, hair) at and below which, according to current knowledge, there is no risk of health impairment anticipated, and consequently no need for action"* (Apel et al., 2020). Although no public consultation took place, the derived HBM-GVs were also shared with the EU Policy Board for input. The HBM-GVs were endorsed by the HBM4EU Management Board after wide consultation of European experts through the HBM4EU national hubs and the EU policy board which was composed of several European Commission's Directorate Generals. These guidance values have no regulatory status but are based on actual scientific knowledge.

Based on selection criteria discussed and defined on a first workshop, indicators have been produced for bisphenol A (BPA), bisphenol S (BPS) and per- and polyfluoroalkyl substances (PFAS), which both have high policy and societal relevance, as well as for cadmium, phthalates, and DINCH, a non-phthalate plasticizer, pesticides and aprotic solvents (Gerofke et al., 2023; Lobo Vicente et al., 2022a, 2022b).

Two pan-European harmonized datasets were used: those from the HBM4EU Aligned Studies with sampling between 2014 and 2021 and those from the previous European human biomonitoring DEMOCOPHES project with sampling in 2011_2012 (Den Hond et al., 2015; Gilles et al., 2022, 2021; Govarts and et al., 2022). Both datasets met requirements of adequate sample size, a successful quality analysis and quality control (QA/QC) of the biomarker analyses and a uniform data handling. In the recent HBM4EU Aligned studies, HBM samples and data were collected in a harmonized way from existing (national) studies or newly conducted studies to derive current internal exposure data for the European population/citizens across a geographic spread (Gilles et al., 2021). HBM4EU developed a HBM European Laboratory Network to ensure the delivery of quality reliable and trustworthy analytical results (Esteban López et al., 2021; HBM4EU, 2021). Based on this harmonized and quality-controlled data, initial indicators have been developed according to the developed HBM indicators' strategy (Buekers et al., 2018).

This approach is illustrated with the impact indicators developed for PFAS, BPA and BPS. The PFAS indicator was produced with data from the HBM4EU Aligned Studies in teenagers, the BPA in children with data from DEMOCOPHES, and the BPA and BPS in adults with data from DEMOCOPHES and the HBM4EU Aligned Studies.

PFASs are a group of synthetic chemicals, also called "forever chemicals" as they do not break down in the environment due to their

strong carbon-fluorine bond, and therefore accumulate over time. PFASs are toxic to human health and the environment with specific PFAS already regulated by several legislations and cross-regulation activities.

Bisphenols are synthetic chemicals found in many types of products including plastics, thermal paper (BPA excluded in receipts), can liners, flooring. Bisphenols enter the human body mainly via food intake and by dermal contact (e.g., with paper receipts).

2.3. Science to policy workshop

To support the use of HBM4EU results for policy making, several participatory case studies were organised to facilitate the joint interpretation of HBM4EU-results and their translation into policy options, in co-creation between scientists, policy makers and societal stakeholders. The case studies consisted of several iterative steps, including desk research and bilateral consultations leading to a final workshop. This allowed to gradually develop the case, not only in terms of content but also as a learning process for the various partners involved. Timely preparatory meetings with key partners enabled to better align expectations, refine messages, and increase engagement and shared ownership. This kind of explorative meetings turned out to be fruitful. Three participatory case studies were implemented: a first focusing on science-policy aspects of phthalates and bisphenols (2018); a second on PFAS (2020–2021); and a third on HBM4EU indicators for PFAS, phthalates and cadmium (2021–2022). Each time, a diversity of actors was invited to participate, including HBM4EU researchers, representatives from various DGs of the European Commission, EU agencies, representatives of national HBM studies and national authorities, and – if the research context allowed – societal stakeholders (including NGO's and industry representatives.

The consortium always strived for the presence of both researchers with detailed knowledge about the research activities as well as members of the Management Board (with a broad view on project activities and objectives). Representatives for the policy actors and societal stakeholders were invited through the HBM4EU Policy Board and Stakeholder Forum, and invitations extended with the snowball method for additional suggestions. Open calls for participation were not used to maintain sufficient control over the size and composition of the group, paying particular attention to a diversity of perspectives, while also maintaining a manageable group size for the discussions. A closed meeting also allowed to present preliminary results or to share information in a confidential setting. This way of working can lead to a certain bias in the group of participants, for example with a predominance of participants who see added value in human biomonitoring for policy purposes. This was not seen as a problem for the consultations, as their main purpose was to initiate an open-ended dialogue on policy relevance and how to optimize and facilitate it, in the context of a science-to-policy research project, rather than producing binding final conclusions. A relevant diversity of perspectives was certainly aimed for, based primarily on the diversity of representatives in the project boards, and expanded through the snowball method. Relevance was defined here firstly as a diversity of policy domains, to also think transversally about policy integration, and secondly as a diversity of interests (represented mainly in the stakeholder forum). We also reasoned in terms of contrasting viewpoints and for instance invited national voices next to European guest speakers and panel members.

Workshops were always organised on two days, from noon to noon. The first workshop in 2018 was organized in Brussels on the premises of the Directorate General for Research and Innovation (DG RTD) of the European Commission. The other workshops, in 2021 and 2022, took place online due to the Covid-19 pandemic. The workshop programs always aimed at a combination of presentations and sufficient space for discussion (both in plenary sessions and break-out discussion groups). Presentations from the consortium were thoroughly prepared in working groups, with attention to formulating clear key messages and relevant questions for further discussion. In two out of three cases, representatives of the European Commission and Member States were also asked to prepare a presentation or lead a discussion group.

Reports of the case studies, including process design, conclusions and participants' evaluation are available on the HBM4EU website (Coertjens et al., 2019, 2021, 2022; Crabbé et al., 2022).

2.4. Residents' perspectives and outreach

The inclusion of resident perspectives and perceptions was part of a systematic, transparent, and participatory strategy within HBM4EU. To gather qualitative in-depth understanding on resident's perceptions of chemical exposure, trust, and concerns on human biomonitoring initiatives, HBM4EU ran focus groups between 2018 and 2021 hosted in 11 countries, including Austria, Portugal, Ireland, the UK, Cyprus, Hungary, the Netherlands, Denmark, Israel, North Macedonia and Latvia. Participants were selected through purposive, non-probabilistic sampling, in order to ensure a heterogeneous group of people in terms of ages and educational background, which was considered also to provide heterogenous perspectives about Human Biomonitoring related topics. Participants were invited to take part on the focus groups either through face-to-face invitation, by email or telephone; and a more detailed description of the methodology may be found in the peer-reviewed publications (Matisāne et al., 2022a; Uhl et al., 2021a).

As part of the outreach to residents, non-representative surveys were conducted in countries that hosted the focus groups. This survey was initially used for the focus groups to better understand their awareness and concerns regarding chemical exposure and human biomonitoring. Additionally, a European resident online survey (non-representative), which ran from September 2020 until February 2021, was done.

Since the European resident survey was developed in 2020, it was updated to harvest more EU-wide results including chemical exposure during the COVID-19 pandemic. The survey was then implemented on the HBM4EU website with a specific link to the translated survey for each of the countries. The collaboration of the 30 National Hubs (country representatives) was requested for the dissemination of the survey. The questions in the resident survey may be found in the Supplemental Data section. More details on how the survey was implemented may be found in the literature (Joana Lobo Vicente et al., 2021).

3. Results and discussion

3.1. Chemical prioritisation

The nominations by 32 different entities were collated into a preliminary list of 48 substances/substance groups, which was subsequently shortened to a list of 23 after considering the total number of nominations each substance/substance group received and the nature of the nominating entities.

A stakeholder workshop was held to reflect on the priorities and capture the stakeholders' concern. A top-10 list of substances was voted on by the HBM4EU Stakeholder Forum (Uhl, 2018). The list included pesticides authorised in the EU and metabolites, glyphosate, siloxanes, mercury and mercury compound, arsenic acid and its inorganic metabolites, nanomaterials, lead and its compounds, UV absorbers and filters, diisocyanates and mycotoxins.

A dialogue between the HBM4EU Management Board and the EU Policy Board took place to assess resources available for the project and the alignment with the policy priorities at European level. A final priority list of 9 substances/substance groups for research activities and surveys within the framework of the HBM4EU project was produced and presented to the Governing Board for approval.

After the prioritisation round of 2017, some modifications were introduced. The following prioritisation round initiated in 2019 included substances that were ranked according to the number of nominations, but they were not scored against prioritisation criteria (hazard, exposure probability, health concern) by an expert team. This

process will be finalised in the PARC, the follow-up partnership to HBM4EU.

It was crucial to have the EU Policy Board involved in this process, comprising of several directorate generals of the European Commission as well as several EU Agencies, which facilitated the swift uptake of results by EU institutions. Having this opened channel of communication also facilitated communication between the policymakers and the researchers, in which the former could express their needs for certain results to the researchers to influence policy processes.

3.2. Indicators

3.2.1. Per- and polyfluoroalkyl substances in teenagers (PFASs)

In HBM4EU, 12 PFAS were covered by the QA/QC process and could be analysed in the Aligned Studies: PFOS, PFOA, PFPeA, PFHxA, PFHpA, PFNA, PFDA, PFUnDA, PFDoDA, PFBS, PFHxS and PFHpS. The most detected PFAS in human blood of European teenagers (12–19 years) were perfluorohexane sulfonic acid (PFHxS), perfluorononanoic acid (PFNA), perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS). They were a fraction of the PFAS to which the European population is exposed to. In 2020, the European Food Safety Authority (EFSA) set a new safety threshold for intake (Tolerable Weekly Intake) as sum value of these 4 PFAS i.e., sum of PFOS, PFOA, PFNA and PFHxS of 4.4 ng/kg BW per week, which corresponds to an internal blood level of 6.9 μg/L in women of child bearing age; the sum of PFOS and PFHxS (4.9 μg/L); and the sum of PFOA and PFNA (2.0 μg/L) (Schrenk et al., 2020). These guidance values were based on serum levels in females aged 35 years old and effects on immunity[2] of their new-borns.

In all studies, a fraction of the participating teenagers exceeded the EFSA based guidance value of 6.9 μg/L (Fig. 1). In all studies, except BEA (Spain), P95 values (95% of the participants had biomarker levels below this value, and 5% above) exceeded the EFSA based guidance value (Fig. 2). Fig. 2, also gives an indication of the extent of the exceedance. It is noteworthy to mention that, both indicators complement each other. Fig. 1 shows the percentage of exceedance related to EFSA's health-based guidance value for the sum of the 4 PFASs, whereas Fig. 2 shows the P95 for the single studies thus showing the extent of exceedance above EFSA's guidance value of 6.9 μg/L. For example, in Fig. 1 Slovenia had 7.45% of the teenager population exceeding EFSA's guidance value, whereas in Fig. 2, the P95 values showed that the 5% most exposed teenagers had an extent of exceedance (EE) of 1.12 above the HBM-GV. The EE was obtained by dividing the P95 value with the health-based guidance value, therefore 7.74 μg/L/6.9 μg/L = 1.12.

The indicator, based on internal exposure data from European teenagers, showed that combined exposure to PFOS, PFOA, PFNA and PFHxS of teenagers in the EU exceeds the EFSA health-based guidance value. Exceedances in the different studies and locations ranges from 1.34% up to 23.78% of the participants (Fig. 1) with an extent of exceedance (P95 value/6.9 μg/L) varying from 0.74 to 1.78. The studies conducted in Western and Northern Europe had the most teenagers exceeding the guidance value.

The indicator based on HBM4EU aligned study data, clearly demonstrated that a significant fraction of European teenagers was exposed above the health-based guidance values. In some study sites, 5% of the participants exceeded the health based guidance values by 78%. Despite the fact these HBM-GVs do not reflect regulatory measures, these data support a swift action to decrease the exposure of the EU population to these compounds and to carefully study PFAS substituents exposure and health impacts.

In recent years, policy attention for PFAS has increased strongly at various policy levels (from the local, to the national, European and international level). Policy processes have been initiated for which HBM4EU provided relevant input (HBM4EU, 2022a). This applies to regulatory initiatives, policy evaluation, agenda setting and various other complementary policy instruments. The HBM4EU indicators emphasise the need for reducing human exposure from existing environmental sources and to prevent exposure from new sources. The data supports adaptation of the chemical regulation under the Chemicals Strategy for Sustainability, with a set of actions already laid out for phasing out PFAS use, unless it's essential (European Commission, 2020b). This should add up to existing regulations at EU level.

PFAS are regulated by a number of pieces of legislation and cross-regulation activities. These cover i) implementation of international conventions, actions and agreements, and wider chemicals legislation; ii) consumer products; iii) occupational exposure, and iv) the environment (e.g., emissions to air and water). As an example, PFOS is regulated under the EU's Persistent Organic Pollutants (POPs) Regulation; PFOA, its salts and related compounds are regulated under the Stockholm Convention, and it has been banned under the POPs Regulation since 4 July 2020. Perfluorohexane sulfonic acid (PFHxS), its salts and related compounds as well as perfluorinated carboxylic acids (C9-14 PFCAs) are being considered for inclusion in the Stockholm Convention and consequent global elimination. In addition, the Chemicals Strategy for Sustainability includes a specific focus on the risks posed by PFAS. The links to the current legislation may be found in the HBM4EU's substance web page (HBM4EU, 2022a). For example, PFOA is covered by REACH Annex XVII restriction, SVHC Candidate List (PBT, Repr.), CLH (Carc. 2, Repr. 1B, STOT RE 1, Acute Tox. 4, Eye Dam. 1), and are proposed for inclusion in the Stockholm Convention, (European Commission, 2017, 2008a, 2008b; ECHA, 2018; UNIDO, 2004). For PFOS (perfluorooctane sulphonate) Heptadecafluorooctane-1-sulphonic acid (linear and branched isomers), it is covered by restriction, CLH (Carc. 2, Repr. 1B, Lact., STOT RE 1, Acute Tox. 4, Aquatic Chron. 2), PIC regulation, POP Regulation (EG) No. 757/2010, Stockholm Convention, environmental legislation Seveso (European Commission, 2017, European Commission, 2012, European Commission, 2010, 2008b; ECHA, 2018; European-Parliament, 2012; UNIDO, 2004).

HBM4EU has submitted data on several occasions to feed information to this these different regulatory processes, and PFAS indicators will be included in the work being developed for the Chemicals' Strategy for Sustainability and the Zero-Pollution Assessment. Despite these regulations already implemented, the European population continues to be widely exposed and health risks cannot be excluded.

At the national and local level there is a strong interest of policy makers and risk managers on how to deal with already existing contamination and prevent further exposure of residents. HBM4EU has created a network of experts working on PFAS contaminated sites in Belgium, Denmark, the Netherlands, Hungary, Italy and Sweden. A guidance document was developed and published with recommendations on identification, human biomonitoring and risk communication in PFAS hotspots (De Brouwere et al., 2022).

3.2.2. Bisphenols

Former DEMOCOPHES[3] data already showed that urinary BPA concentrations were quite similar in children and mothers (Covaci et al., 2015; Schindler et al., 2014).

Within the HBM4EU project, an HBM guidance value of 230 μg/L was derived for BPA exposure in adults and 135 μg/L for BPA exposure in children (>3 years) (Ougier et al., 2021b). Below these values no adverse health effects were expected according to current knowledge.

[2] Ability to resist a particular infection.

[3] COPHES/DEMOCOPHES was a project funded through the European Environment and Health Action Plan of 2004 to "develop a coherent approach on human biomonitoring (HBM) in Europe". It targeted the collection of specimens from 120 mother-child-pairs in each of the 17 participating European countries. These specimens were investigated at that time for six biomarkers: mercury in hair; creatinine, cotinine, cadmium, phthalate metabolites and bisphenol A in urine.

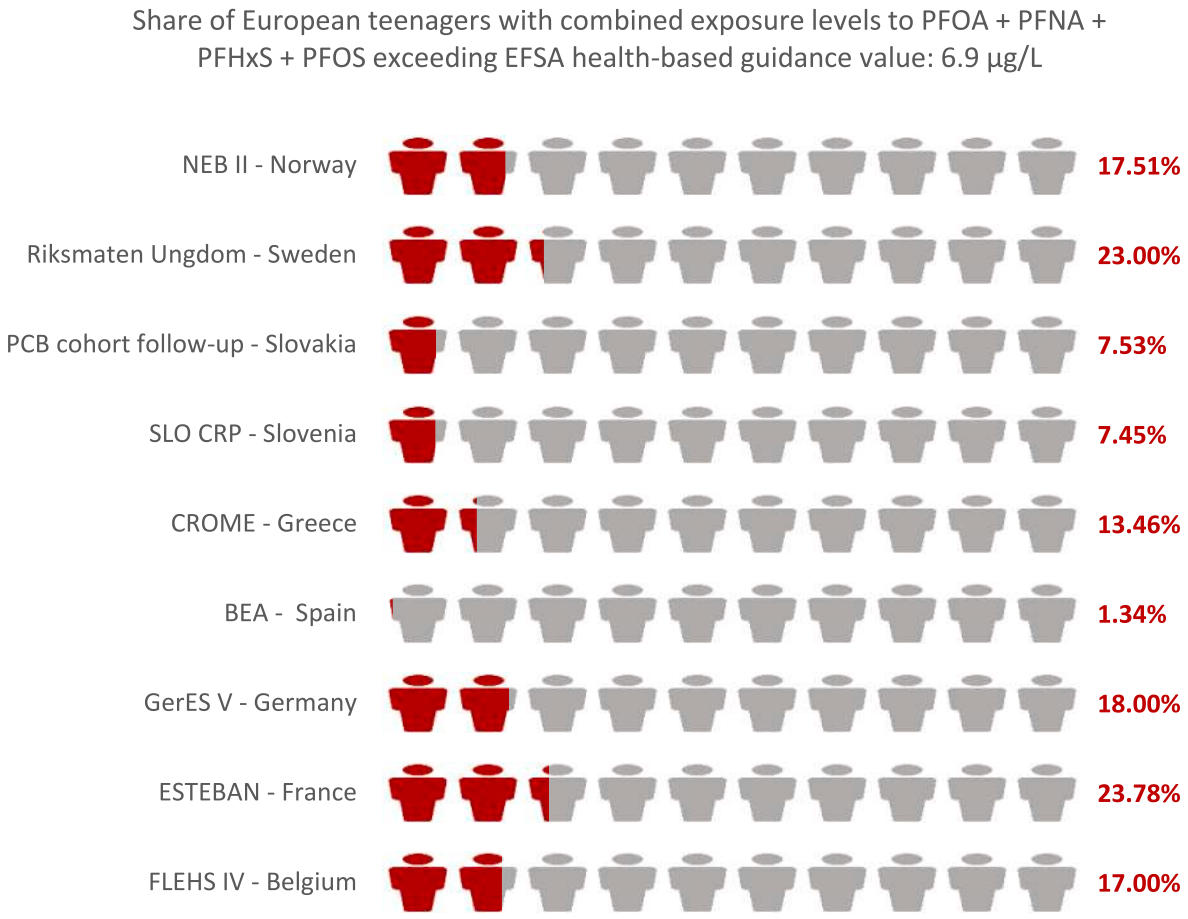

Fig. 1. Share of European teenagers with combined exposure levels to PFOA + PFNA + PFHxS + PFOS exceeding health-based guidance value of EFSA (6.9 µg/L), based on data from the HBM4EU Aligned Studies.

HBM-GV is set at a urinary concentration of total BPA consistent with a steady-state exposure to the temporary TDI of 4 µg/kg bw/day derived by EFSA (2015). A more stringent value was proposed last year. BPA analogues are less studied but data suggest they are also estrogenic (den Braver-Sewradj et al., 2020; Örtl, 2020). BPS is more difficultly removed from the body than BPA, which may lead to relatively higher exposure to a hormonally active substance. For BPS, a HBM-GV of 1 µg/L was derived in HBM4EU, based on animal studies for mammary gland and neurodevelopmental toxicity (Catanese and Vandenberg, 2017; Kolla et al., 2018, 2019; Kolla and Vandenberg, 2019). No reference values have been proposed by EU or non-EU organisations so far. However, there is currently an assessment of BPS ongoing at EU level. Although the safety of BPA substitutes (such as BPS and BPF) is not completely clear at this stage, new text mining/artificial intelligence tools developed in HBM4EU highlighted the health effects of these BPA substituents (Carvaillo et al., 2019; Rugard et al., 2020).

3.2.2.1. BPA in children. This indicator (Fig. 3) illustrated the 95th percentile (P95) BPA values of children from the DEMOCOPHES project (children 5–12 years, 2010–2012, compared to age-dependent HBM-GVs derived before the new EFSA assessment). The observed 95th percentiles in all included EU studies were at least a factor 8 below this HBM-GV derived for children. This will change if EFSA's TDI is updated and the TDI is lowered by a factor of 100,000 (ongoing discussions, pending conclusion). Health concerns remain due to the co-exposure to BPA substitutes with incomplete toxicological data and because of concern for potential mixture effects.

3.2.2.2. BPA in adults. This indicator (Fig. 4) illustrated the 95th percentile (P95) values for urinary BPA compared to the HBM-GVs for adults from specific studies conducted in different geographical areas of Europe. While the observed 95th percentiles in all included EU studies (DEMOCOPHES: 2010–2012; and HBM4EU Aligned Studies in adults: 2014–2021) was below these HBM-GVs, current BPA levels were, however, a reason for concern. These HBM-GVs were based on a tolerable daily intake (TDI) of 4 µg/kg bw/day, set by the European Food Safety Agency (EFSA) in 2015. An increased number of academic studies show adverse effects at current low exposure levels, below the TDI of EFSA (Ougier et al., 2021b). So far, health effects for substitutes BPS and BPF at current exposure levels are still unclear.

In the EU, bisphenol A is regulated under REACH (1907/2006/EC) (European Parliament and European Council, 2006). EU law regulates BPA in plastic materials and articles intended to come into contact with food (European Commission, 2011a), and since 2011, BPA has been banned from infant feeding bottles across Europe (European Commission, 2011b). In 2018, the EU further restricts the use of bisphenol A in certain food-contact materials. A specific migration limit (SML) for BPA in varnishes and coating has been introduced and the specific migration limit (SML) for BPA in the Plastics Regulation has been revised (European Commission, 2018a). Further restrictions are likely to be put in place in the coming years, following the Chemicals' Strategy for Sustainability work, and the Annex XV restriction dossier submission under REACH (European Commission, 2022b).

Several countries have restrictions on the use of BPA in food contact materials and in pacifiers and teething rings (e.g. France, Denmark, Belgium, Austria and Sweden). Occupational exposure limits are also in place in several countries. For a more detailed description of legislation

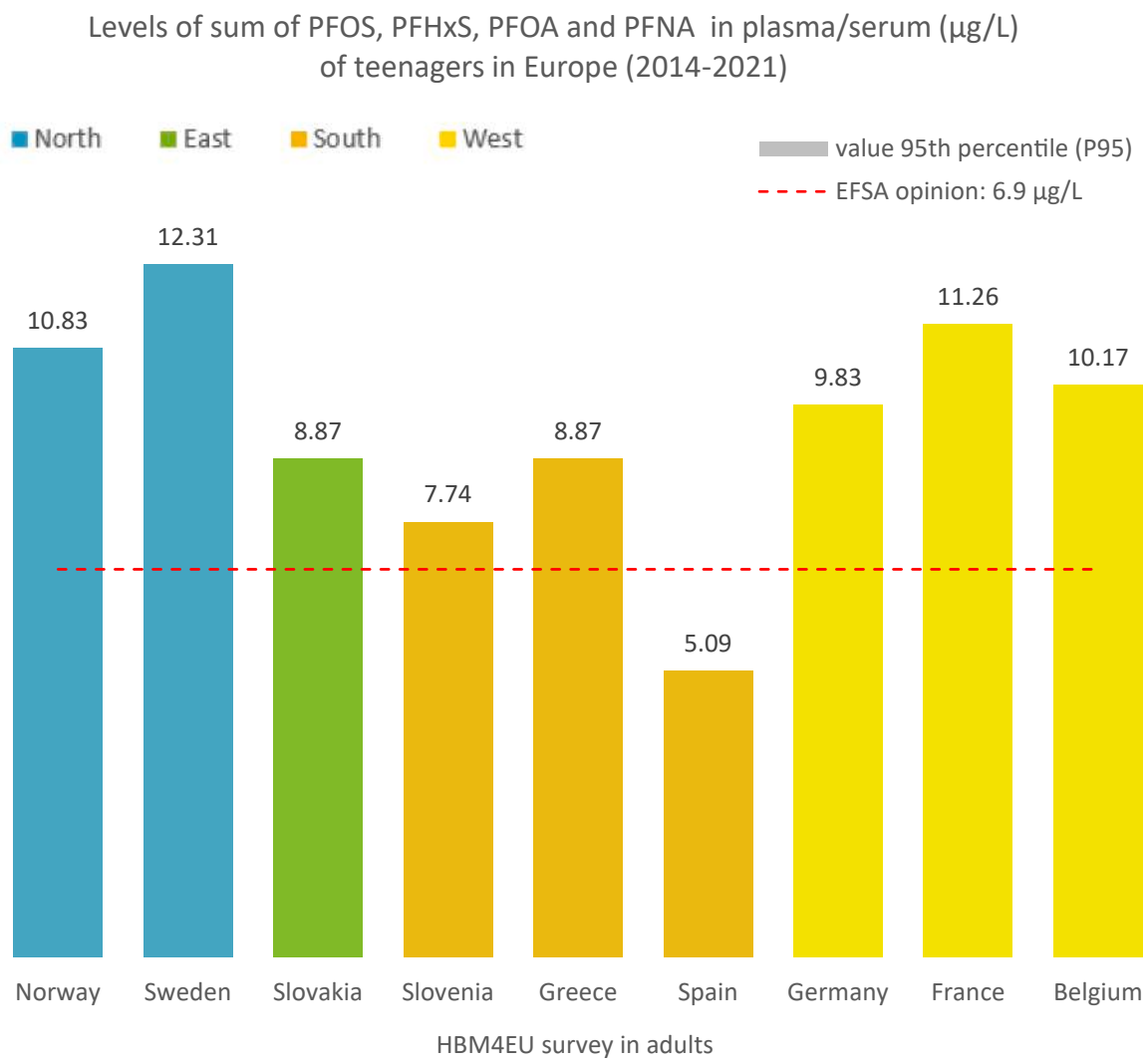

Fig. 2. Indicator showing P95 value of the sum of PFOS, PFHxS, PFOA and PFNA from 9 studies in teenagers (12–18 years) in Europe between 2014 and 2021 compared to EFSA guidance value of 6.9 µg/L. Based on data from the HBM4EU Aligned Studies in teenagers (Norway: NEB II, Sweden: Riksmaten Ungdom, Slovakia: PCB cohort follow-up, Slovenia: SLO CRP, Greece: CROME, Spain: BEA, Germany: GerES V-sub (unweighted), France: ESTEBAN, Belgium: FLEHS IV).

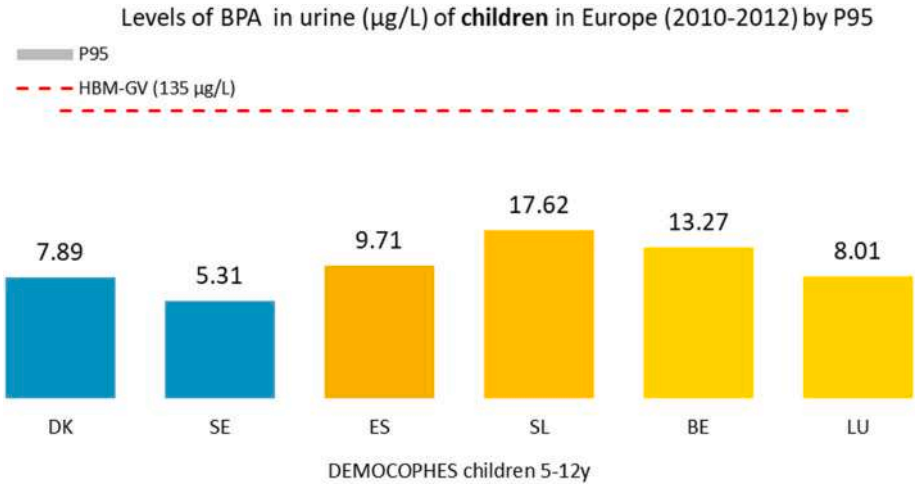

Fig. 3. Impact indicator showing 95th percentile of urinary bisphenol A concentrations of children sampled at 6 different sites in Europe between 2010 and 2012 (DEMOCOPHES project) compared to HBM-GV of 135 µg/L.

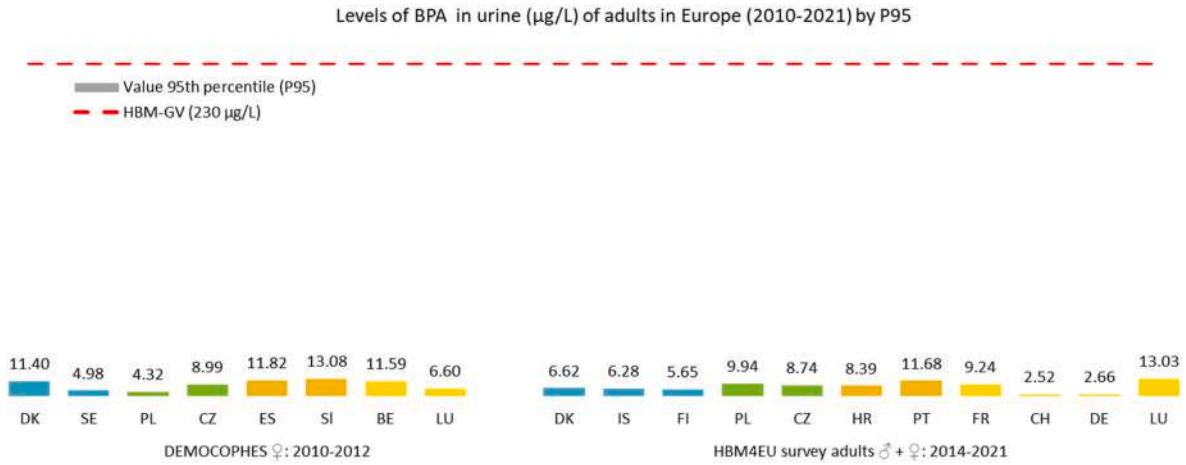

Fig. 4. Impact indicator showing 95th percentile of urinary bisphenol A from 8 studies in adult women (20–59 years) in Europe between 2010 and 2012 (DEMOCOPHES project) and 11 studies in adults (men and women, 20–39 years) in Europe between 2014 and 2021 (HBM4EU Aligned Studies; DK: CPHMINIPUB-parents_DYMS; IS: DIET_HBM, FI: FinHealth; PL: POLAES; CZ: (C)ELSPAC:YA; HR: HBM in adults in Croatia; PT: INSEF-ExpoQuim; FR: ESTEBAN; DE: ESB and LU: Oriscav-Lux2). BPA levels of ESB are measured in 24 h urine samples, all other BPA levels are measured in first morning or random spot urine sample.

at EU-national level, please consult the bisphenol substance page (HBM4EU, 2022b).

HBM4EU results have been provided to different EU-wide consultations including the Chemicals' Strategy for Sustainability, the Zero-Pollution Action Plan, as well as EFSA consultations. These are available in the HBM4EU Science to Policy section (HBM4EU, 2022c). HBM4EU indicator data will also be included in the Zero-Pollution Assessment work due at the end of 2022.

Recently, (December 2021) EFSA's draft opinion proposed lowering the tolerable daily intake (TDI) of BPA from 4 µg/kg of body weight per

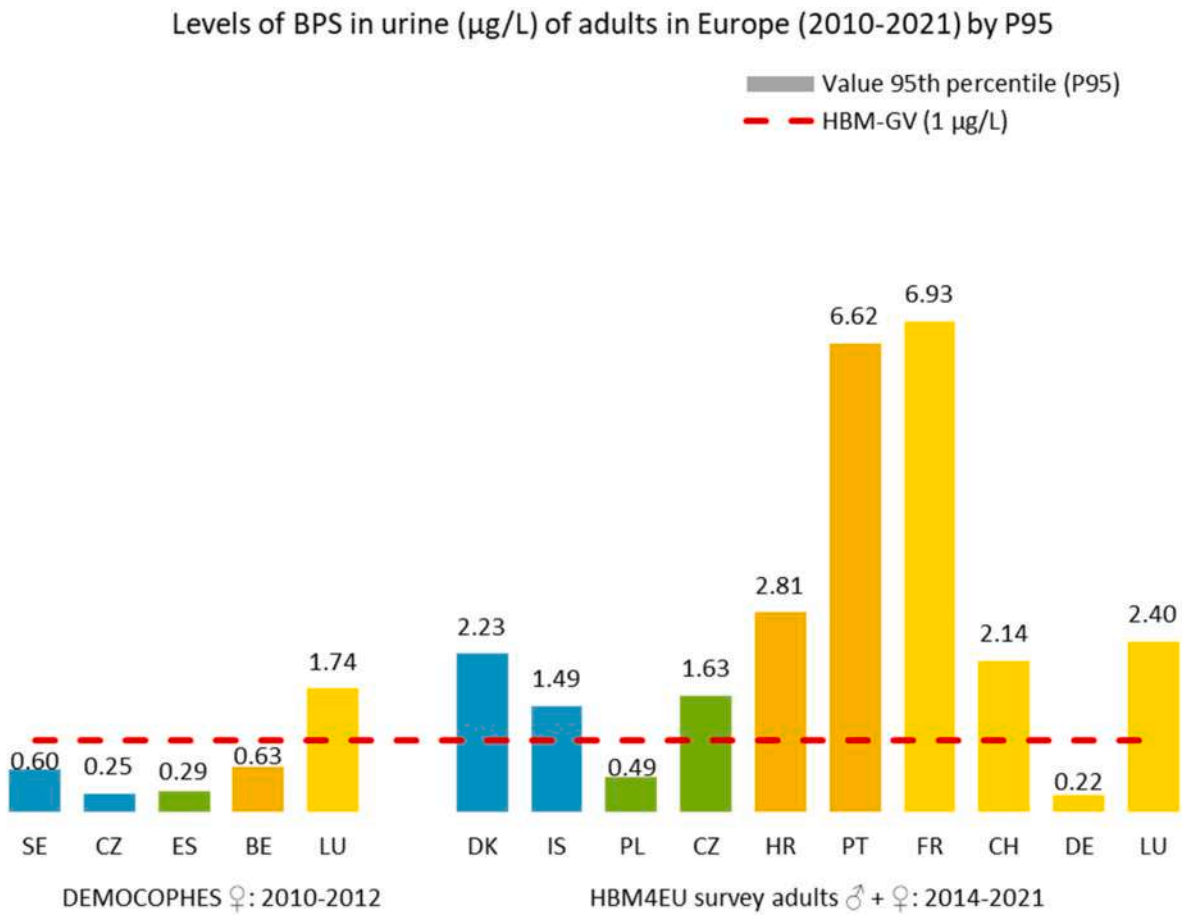

Fig. 5. Impact indicator showing 95th percentile of urinary bisphenol S from 5 studies in adult women (20–59 years) in Europe between 2010 and 2012 (DEMOCOPHES project) and 10 studies in adults (men and women, 20–39 years) in Europe between 2014 and 2021 (HBM4EU Aligned Studies); DK: CPHMINIPUB-parents_DYMS; IS: DIET_HBM, PL: POLAES; CZ: (C)ELSPAC:YA; HR: HBM in adults in Croatia; PT: INSEF-ExpoQuim; FR: ESTEBAN; DE: ESB and LU: Oriscav-Lux2). BPS levels of ESB are measured in 24 h urine samples, all other BPA levels are measured in first morning or random spot urine sample.

day to 0.04 ng/kg body weight per day (European Food Safety Authority, 2021). This is based on immunological parameters instead of on endocrine disrupting chemicals or reprotoxic effects. This value is 100, 000 times lower than the previous one established in 2015. This new TDI value of EFSA was derived taking into consideration new studies on health impacts of BPA; this highlights the importance of such exposure and health research to which HBM4EU has contributed to.

3.2.2.3. BPS in adults. Between 2010 and 2012, only in 1 sampling location the P95 value exceeded the guidance value of 1 μg/L. Between 2014 and 2021, in all sampling locations except POLAES (Poland) and ESB (Germany), the P95 value, exceeded the guidance value of 1 μg/L.

The indicator showed that internal exposure of a fraction of European adults (20–39 years) exceeded the HBM-GV for BPS (Fig. 5). Exceedances in the different studies and locations range from 0.56% up to 19.26% (Fig. 6). The studies with most adults exceeding the HBM-GV were conducted in Southern Europe.

In those sampling sites with >5% of participants exceeding the HBM-GV, the extent of exceedance (P95/2 μg/L) varied from 1.49 to 6.93.

HBM4EU results confirmed that legacy chemicals subject to regulation are in many cases being replaced by substitutes that have entered the human body and that can now be quantified in a large proportion of the EU population. This is a clear case of regrettable substitution, something the European Commission and the Member States are trying to avoid with the implementation of the CSS. To prevent regrettable substitution, it is therefore important to revise the BPS and BPF guidance values following the revision of BPA values by EFSA.

Despite the fact that HBM is an important tool to check policy efficacy, some of the legislation covering PFAS and BPA were put in place whilst HBM4EU samples were being taken, or later and therefore policy efficacy could not always be tracked. However, having indicators that display the chemical exposure of the population in certain periods of time is a good signal in terms of chemical prioritisation for policy making, and will be used as a baseline against which to measure progress in the future.

3.3. Science to policy workshops

Results from the Science to Policy workshops showed a great interest from the side of policy actors and stakeholders into HBM and related scientific results. For the three cases a satisfying diversity of the intended target groups was achieved, and a thoroughly prepared and successful two-day workshop was organized. Evaluation surveys after the workshops showed that most participants were satisfied with the way in which the workshops were organised. Most appreciation went to the opportunities that were offered to provide input and to enter discussion. A big majority of participants indicated that they would use what they have learned in their respective organisations and networks (Dries Coertjens et al., 2022).

The workshops provided an opportunity for an open and in-depth dialogue about HBM4EU results, across the boundaries of science, policy and society, and across policy fields and the interplay of the European and national level. Such processes encourage the further interpretation and evaluation of the statistical and scientific results in terms of policy goals, as the current policy context and specific policy opportunities matter in the formulation of conclusions and key messages. Even in situations where guidance values are available, different interpretations might exist. Thus, these workshops allowed for the identification and transparency on common and divergent views, remaining uncertainties, and obstacles as well as opportunities for policy uptake.

By way of illustration, we summarize here some conclusions from the workshop in 2021 on the HBM4EU results for PFAS, as partly visualized in the previous section on PFAS indicators. These conclusions are, of course, time bound. The measurement results reflect a specific period, just like policy processes that are constantly evolving. Regulatory measures now in place might not have been captured.

In general, all participants seemed to agree that the results clearly support an urgent need for policy action. Concrete opportunities for the use of HBM4EU results were identified, including:

i. The PFAS group restriction that is being prepared and for which HBM4EU results will be important supportive evidence.
ii. Ongoing discussions to set maximal levels in food, as a consequence of EFSA's scientific opinion on for PFAS.
iii. Several legislative changes on the EU agenda with the potential to include limits for PFAS, such as the Water Framework Directive, Groundwater Directive, Environmental Quality Standards Directive, Urban Waste Water Treatment Directive and Sewage Sludge Directive.
iv. Awareness raising of the public and national policy makers, including on the need for preventive health policies, monitoring and remediation, and better enforcement of EU legislation.
v. The current HBM4EU results are also seen as an important baseline to follow up effectiveness of current and future policy measures.

On the other hand, open questions and data needs were identified, including a need to better understand exposure pathways and (local) sources, to act where it is most relevant; to better monitor and study emerging PFAS and PFAS mixtures; and better identification and screening of potential (local) contamination cases as well as exchange of good practices.

A more detailed report is available on the HBM4EU website (Coertjens et al., 2021).

3.4. Residents' perspectives and outreach

The results of the focus groups revealed a level of concern regarding chemical exposure on residents' health and their daily lives (Matisāne et al., 2022b; Uhl et al., 2021b). Residents have an interest in understanding their own chemical body burden and expressed their concern using narratives from their own daily experiences, believing there is a cause–effect relationship between chemical exposure and health. Although the knowledge on human biomonitoring and chemical exposure, varied between participants and different focus groups, residents were aware of potential exposure to chemicals in the environment and how they may enter our bodies. Some identified main exposure pathways to chemicals and made links between sources of exposure and their pathways. For example, car exhaust emissions and car brake dust were linked to chemical exposure through outdoor air. Pesticides used in crops and flavourings, preservatives, and colour additives used in soft drinks production were linked to chemical exposure through food. Environmental reservoirs of antibiotic resistant microorganisms and industrial wastewater discharges were linked to chemical exposure through drinking water.

The mixture effect of combined exposures, a major challenge in the chemical safety field and explored under HBM4EU, is also a concern for residents. Participants were aware that mixtures may influence health, and they suggested that they should be addressed in future human biomonitoring studies.

Another key aspect highlighted by the participants is related to communication. Some highlighted the unintelligibility of the information communicated by scientists and authorities, which is viewed as a barrier to the public understanding of what is being transmitted. Risk information was also pointed out as something that needs to be improved. Furthermore, focus group's participants regarded science as the cornerstone to preventing chemical exposure, allowing scientific information to be better translated into policies and effective protection of human health.

Chemical safety is a matter of public concern. One in four residents

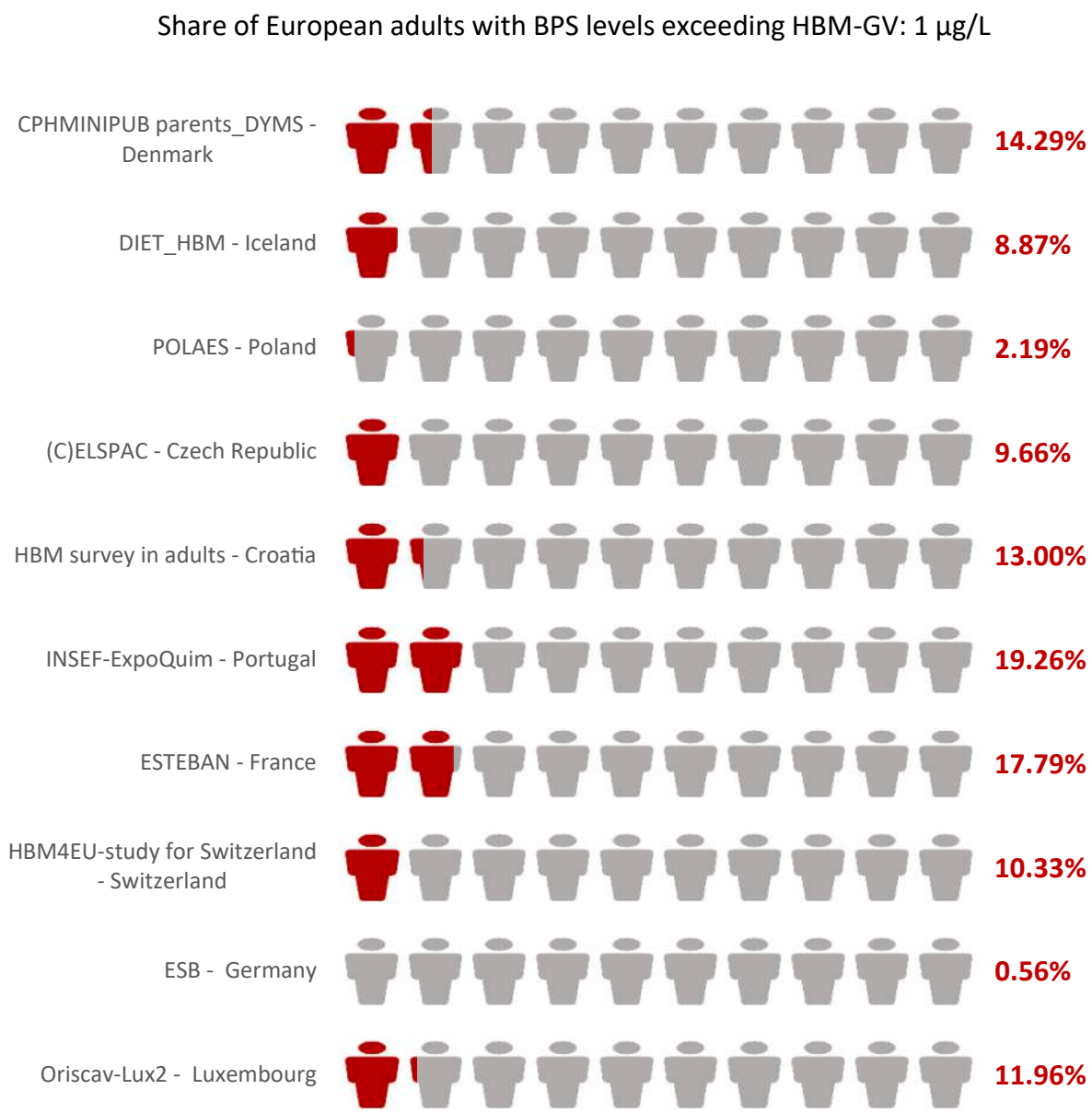

Fig. 6. Share of European adults (20–39 years) with BPS levels exceeding HBM-GV (1 µg/L). Results from the HBM4EU Aligned Studies (2014–2021).

are "very concerned" about exposure to chemicals in their daily life (Matisāne et al., 2022b; Uhl et al., 2021b). Residents want to be sufficiently informed to be able to make targeted decisions. They can choose not to purchase products containing hazardous chemicals and can drive substitution by the competent authorities. Residents also vote and can choose to back parties that promise greater protection for their health and the environment. They feel that they have the right to know what they are exposed to, which chemicals they have in their body and how they should interpret this using the best available science. Informing policy makers and providing science-based information for the public debate is key to this initiative.

Regarding the non-representative resident survey conducted in HBM4EU, it was answered by 5391 residents from 26 of the 30 National Hubs (Joana Lobo Vicente et al., 2021).

For consistency, the same regions as the aligned studies were used, with Israel included in the Southern Europe countries:

- Northern Europe (DK, FI, SE, IS, NO, LV, LT, IE, UK, EE)
- Western Europe (AT, BE, NL, FR, DE, CH, LU)
- Southern Europe (HR, CY, EL, IT, PT, Sl, ES, MK, IL)
- Eastern Europe (CZ, PL, SK, HU).

Based on the above clustering, 30% of the responders resided in "Northern Europe", 33% in "Southern Europe", 12% in "Eastern Europe" and 25% in "Western Europe".

The three main issues that concern the residents in terms of chemical exposure are industrial emissions and pollution, followed by pesticides in food and in the environment, and contaminants in drinking water and food.

European residents were supportive of the use of human biomonitoring as an important and reliable tool on public health policies on chemicals management, that could be used not only at EU level, but also nationally coordinated. These results were also aligned with some of the findings from the HBM4EU focus groups, which included residents' clear articulation on pathways of exposure (Uhl et al., 2021a). Concerning the importance that HBM studies may have, the one sentence most residents totally agreed with was "study the health impacts of chemical exposure", followed by "evaluate chemical exposure of the population" and "the

development of health policy that promote the safe use of chemicals". All these high ranked answers show their opinion on the relevance of HBM studies' contribution to key aspects of health impact and policy.

Although the survey was not representative by design, the results can be used to facilitate decision-making and policy development, and feed into the awareness needs of similar and future projects in Human Biomonitoring. Furthermore, it also brings to light ideas and concepts of residents in shaping collaborative knowledge between residents', experts, scientists, and policy makers on equal terms.

Our results are in line with the Eurobarometer survey on "Chemical Safety", which shows that European residents are concerned by the presence of thousands of chemicals in their environment and in consumer products. According to the Eurobarometer of 2017, around two-thirds of EU residents (65%) are at least a little concerned about being exposed to hazardous chemicals in their daily life, including 26% who are 'very much' concerned. Less than half of respondents (45%) feel well informed about the potential dangers of the chemicals contained in consumer products, and this proportion varies considerably by Member State (Eurobarometer, 2017).

The Eurobarometer survey "Europeans attitudes toward chemicals in consumer products" which assessed the Europeans' attitudes toward chemicals in consumer products, reported that European residents place the greatest trust in the European Union (35%), followed by the national authorities (32%) and the industry (21%) (Eurobarometer, 2010). Public trust towards industry and regulators has been declining since the 1980s, which impairs the risk communication.

Another aspect of resident outreach, was the production of animated videos on specific topics, substance videos, and factsheets and infographics where residents could learn how to reduce their exposure to chemicals, and what type of legislation there is in place to protect them (HBM4EU, 2020).

3.5. Chemicals' Strategy for sustainability

The EU Chemicals Strategy for Sustainability acknowledges the important role chemicals play for human well-being as well as for the green and digital transition in Europe (European Commission, 2020b). It also recognises the urgent need to tackle the health and environmental challenges caused by the most harmful chemicals. Boost innovation for safe and sustainable chemicals and increase protection of human health and the environment against hazardous chemicals is one of the objectives of the Strategy.

To comply with these objectives, the Strategy includes relevant supporting actions:

- Banning the most harmful chemicals from consumer products, such as toys, cosmetics, household items, food contact materials and textiles, unless their use is proven essential for society. Harmful substances include endocrine disruptors, chemicals that affect the immune and respiratory systems, and persistent substances such as per- and polyfluoroalkyl substances (PFAS).
- Minimising and substituting the presence of substances of concern in products, prioritising product categories that affect vulnerable populations and have potential for circular economy;
- Tackling "cocktail effect" i.e., the combination effect of chemicals, by assessing risks from chemicals posed to human health and the environment through daily exposure;
- Establishing a "one substance one assessment" process for the risk and hazard assessment of chemicals
- Promoting the EU's resilience of supply and sustainability of critical chemicals by ensuring that both producers and consumers have access to information on chemical content and safe use (Sustainable Product Policy Initiative).

To support the objectives laid out above, HBM4EU provided data on the priority substances to the consultations on the CSS and the Zero-Pollution Action Plan (ZPAP) and provided input for risk assessment to the HBM4EU EU Policy Board. These included data on bisphenols, PFAS, pesticides and mixtures in general, to name a few, and this will feed directly into the work being developed at EU-level.

Establishing a "one substance one assessment", will support the simplification of coordinating the hazard/risk assessment on chemicals in different legislations by assessing groups of substances instead of individual substances. This will ensure that safety assessments are done in a coordinated manner, that methodologies are harmonized, that decision-making processes are faster and more consistent, as well as reducing the burden on stakeholders.

The indicators generated will be part of the CSS indicator framework and the ZP Assessment which is due in the last quarter of 2022. A report using HBM to understand new chemical exposures in a circular economy has also been produced and explores new pathways through which humans can be exposed to hazardous chemicals as a result of a circular economy (HBM4EU, 2022c).

Another key element of the Strategy is to increase the knowledge base on chemicals and the mention of the importance of human, but also environmental (bio)monitoring. To support this, financial support for EU-wide activities in this field include:

- A research and innovation agenda for chemicals, driven by a EU-level Coordination Group, promoting the regulatory uptake of research findings;
- Fostering multidisciplinary research and digital innovations for advanced tools, methods and models, and data analysis capacities to also move away from animal testing;
- Building an EU early warning and action system for chemicals thus ensuring that EU policies address emerging chemical risks when identified by monitoring and research;
- Developing a framework of indicators to monitor the drivers and impacts of chemical pollution and to measure the effectiveness of chemicals legislation.

HBM4EU results on human exposure to chemicals in products, such as PFAS and bisphenols, highlighted that current human exposure to these substances pose a health risk, and support regulatory action to make products safer. In certain countries, legislations has been put in place after sample collection and hence another sample analysis to check for policy efficacy of new measures would be valuable. Substitutes of the legacy compounds are increasingly detected with scarce knowledge on their potential health effects. Moreover, many of these hazardous chemicals are simultaneously detected in most Europeans without any clear knowledge on the possibility of combined exposure and potential mixture effects. . It has not yet been fully elucidated when and under which circumstances combined exposure leads to a mixture effect.

Evidence of exposure to multiple chemicals at the same time supports efforts to consistently address combined exposure and potential mixture effects in risk assessments, while work to identify chemicals of emerging concern that may pose a health risk through non-target screening provides early warnings of potential risks.

HBM4EU generated scientific knowledge on the exposure of the general population to chemicals and their effects on human health and provided new tools to facilitate the use of these results. These included indicators of chemical exposure, derivation of HBM-GVs, risk assessment analysis, an EU-wide HBM Laboratory Network, and a HBM Dashboard with the data.

3.6. Zero-pollution action plan

The European Union's Zero Pollution Action Plan aims to tackle pollution that causes significant negative impacts on both the environment and human health (European Commission, 2021a). Aligned with the 8th Environment Action Programme, indicators will be part of this process to serve as a political summary to guide policy making, as a way

to summarize and monitor processes, whilst providing information on what has been achieved and the distance to set targets (European Commission, 2022c). It presents a vision for 2050 where pollution is reduced to levels that are no longer harmful to human health and natural ecosystems. The plan aims to deliver on the European Commission's European Green Deal, which recognises that environmental degradation poses an existential threat to Europe and the world (European Commission, 2019).

The use of the terminology 'Zero Pollution' flags the ambitious nature of this action plan and emphasises that systemic changes in key sectors, including transport, energy, agriculture and industry, will be required to deliver on its objectives. The zero-pollution hierarchy (Fig. 7) prioritizes the processes to be used to tackle pollution, with prevention as a first priority, followed by minimising and controlling pollution and finally elimination and remediation of pollution. Previous approaches, such as 'end-of-pipe' treatment of pollution, are now the least favoured option to address pollution.

A key element in delivering on this ambition is the development of a fit-for-purpose "monitoring and outlook framework", proposed by the EU Commission under the Zero Pollution Action Plan (European Environment Agency, 2021). This framework will support delivery of the action plan as follows:

- **Monitoring**: Assess progress in moving towards zero pollution, establish a baseline and measure the distance-to-targets set under the Zero Pollution Action Plan
- **Outlook:** Use future projections based on modelling and forecast approaches to assess the likelihood of achieving the objectives within the 2050 timeframe. This outlook will also identify potential blockers to achieving objectives, considering current and future policies.

The European Commission can then use these assessments to identify policy interventions necessary to deliver zero pollution or to address tensions across policy areas. The monitoring and outlook framework will also feed into the research agenda, identifying areas where new solutions may be required or where new monitoring or modelling techniques are needed in order to develop a more reliable indicator of current status and future outlooks.

The HBM4EU project and the follow-up partnership, PARC, are excellent examples of initiatives to deliver better monitoring data and intelligence to track progress in delivering zero pollution of humans and the environment (ANSES, 2022; European Commission, 2021b).

The role of the European Environment Agency (EEA) is to lead on the development of the 'monitoring' element of the monitoring and outlook framework (European Environment Agency, 2021). The first zero pollution monitoring assessment will be delivered in late 2022, to set a baseline, with the second assessment completed in 2024. EEA will work closely with a range of partners and stakeholders to gather inputs, including the European Environment Information and Observation Network (European Environment Agency, 2022). The assessment will cover the domains of health, ecosystems and production and consumption, based on available indicators and other relevant sources of information from a range of research and knowledge brokers (Fig. 8).

On chemicals and health, EEA will work closely with other HBM4EU partners to showcase HBM4EU knowledge in the baseline assessment report. Robust evidence of European population exposure to chemicals and associated health impacts provides a baseline against which to measure future progress.

As the zero-pollution monitoring report will be an indicator-based assessment, the HBM4EU work to develop indicators of exposure against HBM Guidance Values is particularly valuable.

It is also foreseen that activities planned under the PARC will support future zero pollution monitoring assessments, providing comparable data and analysis to assess the impact of chemicals pollution on human health. Importantly, such data will enable us to map trends in population exposure to chemicals in Europe and tease out the effects of policy interventions on exposure.

3.7. *Bridging science and policy to better protect human health*

To support decision making at European level, HBM4EU generated coherent European-wide datasets on human exposure to chemicals, demanding significant efforts to harmonize methodologies and standardise data collection.

An updated summary mapping relevant HBM4EU results in support of the European Green Deal is available in Table 1.

The HBM4EU Aligned studies have generated new human bio-

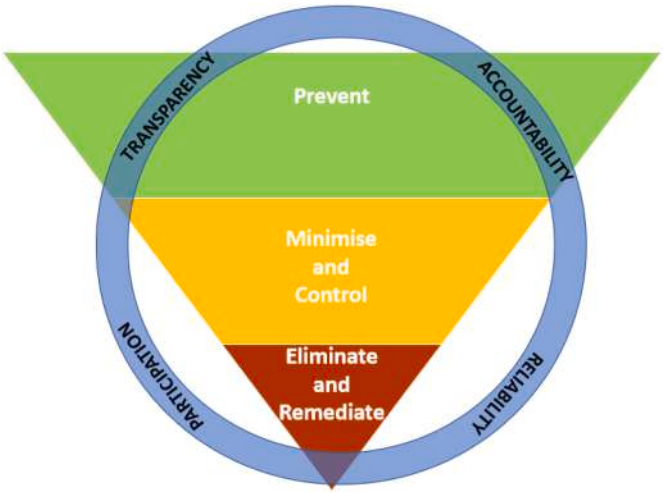

Fig. 7. Zero pollution hierarchy.

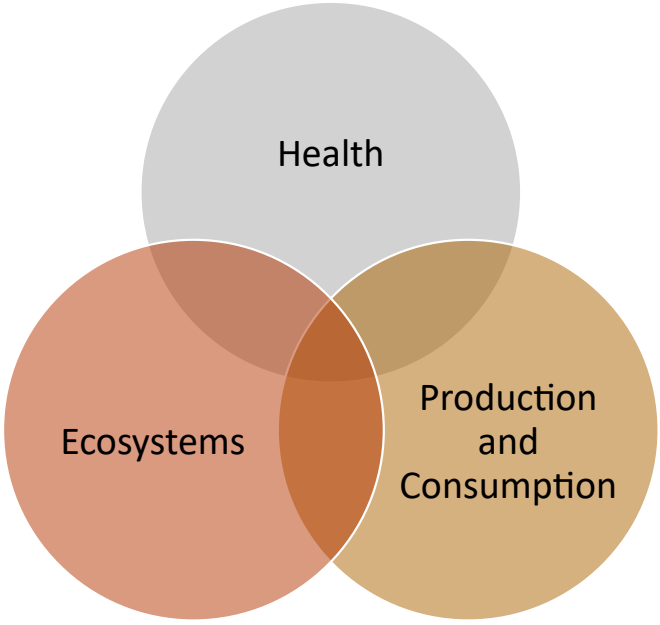

Fig. 8. Domains covered under the zero-pollution assessment.

monitoring data on the current internal exposure of the general population to a selection of HBM4EU priority substances and effect biomarkers in over 10,000 European residents. The data is available in the European Human Biomonitoring Dashboard, which also includes human biomonitoring data from previous studies collated under the HBM4EU project (Gilles et al., 2022, 2021; Govarts and et al., 2022).

Furthermore, aggregate data is included in the Information Platform for Chemical Monitoring (IPCHEM),[4] facilitating the use and reuse of human biomonitoring data in regulatory processes and research (European Commission, 2018b).

A HBM European Laboratory Network was also implemented and it has delivered coherent, robust results on chemical exposure and impacts on health in Europe to support policy making to improve chemical safety for residents (Esteban López et al., 2021; HBM4EU, 2021). HBM4EU has built up scientific capacities for human biomonitoring research across Europe that will continue to serve the public going forward.

HBM4EU has also provided direct input to public consultations on the development of strategies and action plans led by the European Commission under the European Green Deal, the European Chemicals Agency, the European Food Safety Authority, and Secretariat of the Minamata Convention on Mercury at the United Nations Environment Programme (HBM4EU, 2022c). HBM4EU is also supporting regulatory measures addressing priority substances, such as the ongoing proposal to restrict a wide range of PFAS under REACH and the recent EFSA draft opinion on Bisphenol A.

In what concerns chemicals, knowledge has been generated for about 500 out of 100,000 chemical substances on the market, but either little or nothing on most of the others (Fig. 9). Under HBM4EU, work was done on the chemicals of highest concern in terms of risks to health. Research was also done on emerging substances using new screening methods, techniques that HBM4EU sought to harmonize across Europe. The number of substances for which little knowledge is available is so substantial that only a sustained effort over time will allow us to face such a challenge.

One of the major issues addressed in the Strategy is how to identify the most relevant mixtures of chemicals and how to address their impacts. HBM4EU has been carrying out relevant research and contributing to working groups on risk assessment and management of chemical mixtures (Bopp et al., 2018; HBM4EU, 2022d; Socianu et al., 2022). This matter is rather intricate, and long-term research is needed. Yet, there is an urgency to act and, based on the available knowledge, decision makers can already draw some conclusions and take action.

Similarly to the objectives of chemical policy, HBM4EU's objectives cannot be achieved by a single discipline. There is a need for epidemiologists, exposure experts, public health specialists, toxicologists, computational scientists, analytical chemists, social scientists, policy advisers and policy makers working side by side. This multi-disciplinary collaboration happened under HBM4EU, but it is also happening in other EU projects in this field. To further expand this multi-disciplinary collaboration towards the social sciences, attention should be given to other specializations within the social sciences and humanities, such as risk governance, risk perception, communication, policy sciences and evaluation and socio-economic inequality.

Crucial to HBM4EU's success was also the number of countries that were part of this programme and contributed to a functional HBM network. HBM4EU's legacy will be built upon in the next partnership, PARC, with an enlarged scope. It will bring together European risk assessment and regulatory agencies, as well as policy makers, academia, and stakeholders to set a joint research and innovation agenda. This agenda will support EU and national chemical risk assessment and risk management bodies with new data, knowledge, methods, networks, and skills to address current, emerging and novel chemical safety challenges. It will facilitate the transition to next generation risk assessment to better protect human health and the environment, in line with the zero-pollution ambition, and will be an enabler for the EU Chemicals Strategy for sustainability.

[4] IPCHEM is the European Commission's reference access point for searching, accessing and retrieving chemical occurrence data collected and managed in Europe. The platform has been developed to fill the knowledge gap on chemical exposure and its burden on health and the environment.

The Strategy represents the first step towards a zero-pollution ambition for a toxic-free environment. The zero-pollution agenda should start from an understanding of how European residents are exposed to synthetic chemicals and how these accumulated in the body and make the reduction of the chemical body burden and associated health impacts a key priority.

In practice, this can only be delivered through a surveillance system for measuring the exposure burden of environmental pollutants in the European population that is embedded in European Union legislation.

4. Conclusion

HBM4EU results demonstrate that exposure of European residents is too high for some chemicals, with a fraction of the population exceeding health based guidance values. If exposure continues, adverse health effects cannot be excluded anymore. This underlines the need to further develop chemicals regulation and management in the EU as well as the research on risks on humans and the environment. The EU strategies aim at filling this gap by increased emphasis on lowering the impact of environmental pollution on health, in line with the European Green Deal's objectives. This rather ambitious legislative packaged, has a set of goals which include a zero-pollution ambition for air, water and soil thus protecting the health and well-being of Europeans as well as reducing environmental and climate pressures.

Protecting the health of European residents is a priority of the European Union and residents are eager to learn about their chemical body burden. In this context, the science-policy interface of HBM4EU is particularly important, ensuring up-to-date and coordinated science-based information for policy makers responsible for managing risks to human health from chemical exposure. Furthermore, informing the public will give additional support for the policy measures. Analysis of exposure determinants reveals how the internal dose may be attributed to multiple upstream sources, emphasising the need to consistently regulate substances across policy domains.

By assessing the internal dose of chemicals, HBM integrates the intake of chemicals from different sources and from different routes (ingestion, inhalation, dermal). In the exposure science field, this is now referred to as the "Aggregated Exposure Pathways" or AEP. HBM takes into consideration the absorption, distribution metabolism and excretion (ADME), that lead to internal dose. Together with computational tools such as PBPK, HBM studies provide critical information both on the actual level of contamination that can initiate or contribute to adverse health effects, and on the contribution of the different exposure sources and pathways.

HBM data complements exposure modelling which is increasingly complex due to the variety of sources and exposure pathways by which the same chemical can enter the human body. A sector-based decision making is not protective enough as sustainable use of chemicals will result in more complex chemical life cycles. By focusing on a substance approach, HBM is an essential step for the implementation of the "one substance one assessment" promoted by the Chemical Strategy for Sustainability. A straightforward implication of this conclusion is that data on chemicals should be presented both from the perspective of the current legislation/sectors, but also from the perspective of the substances themselves.

One of the overarching goals of HBM4EU was to actively engage with policy makers to translate scientific results into effective policies and make a step forward in protecting residents' health across Europe. As the premier European programme in the field of exposure to chemicals and health, HBM4EU looked at the Chemicals' Strategy for Sustainability as a major opportunity to move forward with the protection of European residents and human biomonitoring is mentioned in the CSS to assess the growing number of different hazardous chemicals in the human body. To attain the zero-pollution objective, it is critical to apply tools to monitor the chemical body burden of European residents and assess whether this would be related with associated health impacts. To ensure

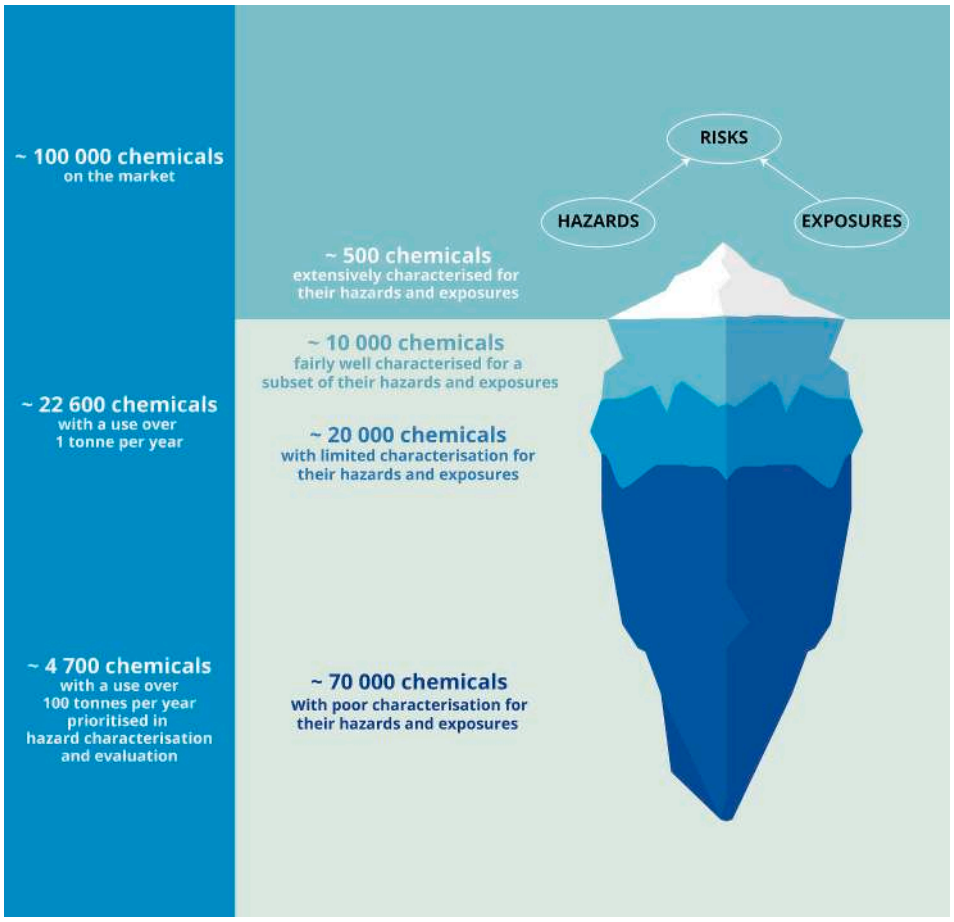

Fig. 9. The unknown territory of chemicals. Note: The numbers in the figure do not include impurities, transformation products or structural variants (isomers) of chemicals placed on the market. ~ 500 chemicals: Chemicals which are considered sufficiently regulated (ECHA, 2019b), typically legacy and well-known chemicals characterised for most known hazards, which have limit values and are regularly monitored by quantitative methods in most media. ~10,000 chemicals: Chemicals on EU or national legislation lists which are characterised for some but not for all known hazards, which have specific limit values, and are monitored quantitatively, but irregularly across time, media, or space. ~20,000 chemicals: Chemicals with hazards characterised mainly by modelling, or where exposure data are based on qualitative screenings done occasionally and in few media. ~70,000 chemicals: typically, low volume chemicals for which usually no or very few hazards characteristics are available and information on uses and exposure is scarce, not characterised or measured in very few media. Source: © EEA, 2020, The European Environment – State and outlook report.

that, HBM4EU established a trust-based cooperation and data-sharing process between all parties to enable the consortium to react on short notice to knowledge needs; partners were actively identifying windows of opportunities in regulatory processes on chemicals where they might feed in evidence; accomplished the vision of a human biomonitoring programme in Europe to support the delivery of chemical safety for Europe's population.

Looking forward, HBM4EU results will be used as baseline against which the success of the EU Chemicals Strategy for Sustainability and Zero Pollution Action Plan supported by the development of indicators. It is therefore crucial that the PARC, the follow-up partnership to HBM4EU, ensures a constant flow of data on priority chemicals to allow for the mapping of trends in population exposure over time at European level and tease out the effects of policy interventions on exposure. Data generated under PARC will also allow to assess progress against objectives in the Zero Pollution Action Plan and the Chemical Strategy for Sustainability and provide comparable data and analysis to assess the impact of chemicals pollution on human health. This will also contribute to estimate the burden of disease from chemical exposure in Europe. Another key element is the development of approaches that are safe and sustainable by design, with the power to fundamentally transform how chemicals are used.

To achieve such an ambitious goal, a sustainable surveillance system is needed, a system embedded in legislation that can be used to measure the chemical burden through human biomonitoring not only to help inform policy actions and environmental health interventions but also to evaluate the efficacy of such actions. This will support sustainable risk assessment, chemical management and legislation in Europe to the benefit of current and future generations.

Another one of HBM4EU's biggest achievements and main drivers was its inclusiveness across the different domains of research and policymaking, as well as capacity building. While this demands time and investment in the short-term, it is certainly more productive in the long-term with the added value of having an open channel between scientists, policy advisers and policy makers acting as a catalyst, and in line with the European spirit.

HBM4EU has contributed to shaping the next research agenda for chemicals at European level, and its legacy with carry on with PARC, as mentioned in the introduction. PARC is an EU-wide research and innovation programme, involving 28 partner countries and three EU agencies, the European Environment Agency (EEA), the European Chemicals Agency (ECHA), and the European Food Safety Authority (EFSA), to cement the link to implementation of the Chemical Strategy for Sustainability. It started in May 2022 and will last for 7 years. It will support EU and national chemical risk assessment and risk management bodies with new data, knowledge, methods, networks and skills to address current, emerging and novel chemical safety challenges. It will facilitate the transition to next generation risk assessment to better protect human health and the environment, in line with the Green Deal's zero-pollution ambition for a toxic free environment and will be an enabler for the EU Chemicals Strategy for sustainability.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114111.

Abbreviations

CLH	Harmonized Classification and Labelling
CLP	Classification, Labelling & Packaging
CSS	Chemicals' Strategy for Sustainability
ECHA	European Chemicals Agency
EEA	European Environment Agency
EFSA	European Food Safety Authority
HBM-GV	Human Biomonitoring guidance value
COPHES/DEMOCOPHES	DEMOnstration of a study to COordinate and Perform Human biomonitoring on a European Scale
HBM	Human Biomonitoring
BPA	Bisphenol A
BPS	Bisphenol S
bw	Bodyweight
Cd	NGOs Cadmium Non-Governmental Organisations
PARC	Partnership for the Assessment of Risks from Chemicals
PBT	Persistent Bioaccumulative and Toxic
PFHxS	Perfluorohexane sulfonic acid
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonic acid (sum of all isomers)
PIC	Prior Informed Consent
SVHC	Substance of Very High Concern

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Heat waves and mortality in the Brazilian Amazon: Effect modification by heat wave characteristics, population subgroup, and cause of death

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ABSTRACT

Background: The Brazilian Amazon faces overlapping socio-environmental, sanitary, and climate challenges, and is a hotspot of concern due to projected increases in temperature and in the frequency of heat waves. Understanding the effects of extreme events on health is a central issue for developing climate policies focused on the population's health.

Objectives: We investigated the effects of heat waves on mortality in the Brazilian Amazon, examining effect modification according to various heat wave definitions, population subgroups, and causes of death.

Methods: We included all 32 Amazonian municipalities with more than 100,000 inhabitants. The study period was from 2000 to 2018. We obtained mortality data from the Information Technology Department of the Brazilian Public Healthcare System, and meteorological data were derived from the ERA5-Land reanalysis dataset. Heat waves were defined according to their intensity (90th; 92.5th; 95th; 97.5th and 99th temperature percentiles) and duration ($\geq$2, $\geq$3, and $\geq$4 days). In each city, we used a time-stratified case-crossover study to estimate the effects of each heat wave definition on mortality, according to population subgroup and cause of death. The lagged effects of heat waves were estimated using conditional Poisson regression combined with distributed lag non-linear models. Models were adjusted for specific humidity and public holidays. Risk ratios were pooled for the Brazilian Amazon using a univariate random-effects meta-analysis.

Results: The pooled relative risks (RR) for mortality from total non-external causes varied between 1.03 (95% CI: 1.01–1.06), for the less stringent heat wave definition, and 1.18 (95% CI: 1.04–1.33) for the more stringent definition. The mortality risk rose as the heat wave intensity increased, although the increase from 2 to 3, and 3–4 days was small. Although not statistically different, our results suggest a higher mortality risk for the elderly, this was also higher for women than men, and for cardiovascular causes than for non-external or respiratory ones.

Conclusions: Heat waves were associated with a higher risk of mortality from non-external causes and cardiovascular diseases. Heat wave intensity played a more important role than duration in determining this risk. Suggestive evidence indicated that the elderly and women were more vulnerable to the effects of heat waves on mortality.

1. Introduction

Heat waves are meteorological events characterized by high temperatures that last for several days, are among the most dangerous of natural hazards and can have significant health impacts (WMO and WHO, 2015). They are associated with increased morbidity, including

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hospitalizations, ambulance call-outs and emergency department visits, and with general mortality from non-external and specific causes (Campbell et al., 2018; Li et al., 2015; Son et al., 2019; Xu et al., 2016; Zuo et al., 2015), principally cardiorespiratory outcomes (Cheng et al., 2019).

The effects of heat waves on the population's health are not homogeneous, and may vary by location and study population. The groups most vulnerable to the effects of heat waves include the elderly, people of low socioeconomic status, heavy-duty workers, individuals with a pre-existing condition, and residents from deprived areas with poor housing conditions, including a lack of air conditioning or ventilation mechanisms (Li et al., 2015; Son et al., 2019; Song et al., 2017). Exposure of the vulnerable (such as children and the elderly) to heat waves has increased in recent years, and there is an increasing trend in heat-related mortality for populations over 65 years (Romanello et al., 2021; Watts et al., 2021).

According to the Intergovernmental Panel on Climate Change (IPCC, 2022), the global surface temperature has increased over recent decades, and extreme events, such as heat waves, are becoming more frequent and intense. Projections based on climate change scenarios indicate that temperatures will continue to rise, and heat waves will become even more severe (IPCC, 2022). Accordingly, an increase in heat- and heat wave-related mortality is expected (Deng et al., 2018; Guo et al., 2018; Sanderson et al., 2017).

The climate in the Amazon region is predominantly hot and humid, with average temperatures over 25 °C, although some locations experience periods of drought. The air temperature over the region has been increasing (Marengo et al., 2018), and projections based on climate models consistently show increases in temperature variability and in the frequency of heat waves in the region (Bathiany et al., 2018; Feron et al., 2019). Another cause for concern is the recent and growing trend in forest fires and deforestation (Hope, 2019; Qin et al., 2021). In addition, the region still faces old socio-environmental and sanitary challenges, which may be exacerbated by the climate crisis, especially in a context of population growth predominantly concentrated in urban centers (Garnelo, 2019; Viana et al., 2016).

The Brazilian Amazon consists of 772 municipalities and 9 states, covers 58.9% of Brazil's territory, and has an estimated population of 28,113,186 inhabitants - 13.3% of the country's population (IBGE, n.d.). It has complex socio-environmental characteristics, with urban centers coexisting with relatively small and isolated towns and villages, and traditional populations (indigenous, quilombolas and riverine peoples) of different ethnicities dispersed across remote rural areas (Garnelo, 2019). The highly predatory development model implemented in the Amazon has led to significant environmental changes, without improving its social or health characteristics. The region has the worst socio-environmental indicators in the country, with high levels of extreme poverty, the lowest human development index, and sanitation coverage well below the national average. Its health indicators, including infant mortality and incidence of infectious diseases, such as dengue, leishmaniasis and leprosy, are also worse than the national average (Viana et al., 2016).

Action and climate policies focusing on the population's health and well-being are urgently required (Yglesias-González et al., 2022). Understanding the effects of climate and climate change on health, highlighting the most vulnerable populations, is a central feature of such action, especially in a region that experiences overlapping socio-environmental, health, and climate challenges. There is a lack of scientific evidence about the effects of heat waves in the Amazon, or in other tropical regions in low- and middle-income countries (Campbell et al., 2018; Son et al., 2019; Xu et al., 2016). Most of the studies on the mortality effects of heat waves in Brazil were restricted to São Paulo (Diniz et al., 2020; Moraes et al., 2022; Son et al., 2016), or focused on heat wave effects at the national level (Guo et al., 2017). In this study, we investigate the effects of heat waves on mortality in the Brazilian Amazon region. Furthermore, we examined how these vary according to different definitions of heat waves, and across different population subgroups and causes of death.

2. Methods

2.1. Study location

We included municipalities with more than 100,000 inhabitants, according to the 2010 Census, giving a total of 32 municipalities: Rio Branco in Acre State; Manaus and Parintins in Amazonas; Macapá and Santana in Amapá; Belém, Altamira, Ananindeua, Santarém, Marabá, Castanhal, Parauapebas, Abaetetuba, Cametá, Bragança and Marituba in Pará; Porto Velho and Ji-Paraná in Rondônia; Boa Vista in Roraima; Palmas and Araguaína in Tocantis; São Luís, Imperatriz, São José de Ribamar, Codó, Paço do Lumiar, Açailândia and Bacabal in Maranhã; Cuiabá, Várzea Grande, Rondonópolis and Sinop in Mato Grosso (Fig. 1). Municipal socio-demographic and geographic information is presented in Supplementary Table S1.

2.2. Data

2.2.1. Mortality and individual-level data

We calculated daily deaths based on mortality data from non-external causes (codes A00 to R99 of the 10th International Classification of Diseases), obtained from the Mortality Information System (SIM) of the Information Technology Department of the Public Healthcare System (DATASUS). The study covered the period from 2000 to 2019, and included information on age, gender and cause of death.

2.2.2. Meteorological data

We calculated the daily weather variables – mean temperature, maximum temperature and specific humidity – based on the ERA5-Land reanalysis dataset from the European Center for Medium-Range Weather Forecasts (ECMWF) for the study period. The ERA5-Land provides hourly data with a grid resolution of 9 km. Daily variables were calculated from hourly data. Gridded data was attributed to each municipality by calculating the weighted average of the grids covered by a 20 km circular buffer centered on the municipality's main offices. We compared the temperature from the ERA5-Land and ground-level

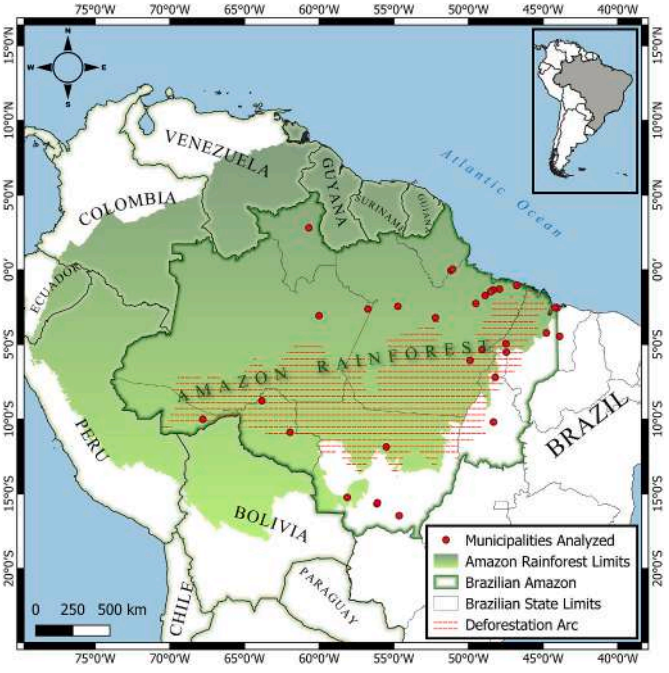

Fig. 1. Study area.

weather stations, examining the Pearson correlation for these series in 17 cities where data were available. Additional details about these data are presented in Supplementary Table S2.2.3 Heat wave definitions.

While there is no unique definition for heat waves, it is understood that they refer to prolonged periods of unusually hot temperatures (WMO, 2015). Generally, definitions differ in relation to their temperature metric, duration (days, weeks), and intensity (temperature threshold), which can be absolute or relative, based on temperature distribution percentiles over a certain period (Xu et al., 2016; Zuo et al., 2015). The best criteria may vary between different regions, and what is considered a climatic extreme in one place may not be considered so in another (Zuo et al., 2015). In order to better understand the effect of heat waves on mortality, we used fifteen definitions, which varied according to heat wave intensity (90th; 92.5th; 95th; 97.5th and 99th percentiles of the daily mean temperature over the study period) and duration ($\geq$2, $\geq$3, and $\geq$4 days). We used the mean temperature as it is more likely to represent the heat level in 24 h (Xu et al., 2016). We did not restrict our analysis to the hottest season as temperatures in the region are generally high throughout the year.

2.3. Study design and analysis

In the first stage of analysis, we used a case-crossover study for each city to estimate the effects of each heat wave definition on mortality, and across different population subgroups (people aged 0–64, people aged 65 or above, men and women) and causes of death (cardiovascular and respiratory diseases). In case-crossover studies, the individuals who experienced the event of interest serve as their own controls, and exposure on the day of the event (case period) is compared with exposure at different times (control periods) (Maclure, 1991). We used the time-stratified control selection strategy, whereby controls are selected on the same day of the week, month, and year as the cases (Janes et al., 2005).

We used a directed acyclic graphic (DAG) to explain the causal hypotheses underlying our study and to identify a sufficient set of adjustment variables to estimate the total effect of heat waves on mortality outcomes (Supplementary Fig. S1). The DAG was created in DAGitty (Textor et al., 2011). Because participants are compared with themselves at different points in time, time-invariant confounders, such as individual characteristics, are adjusted by design (Maclure, 1991). Furthermore, since the cases were matched to controls on the same day of the week, month and year, the potential confounding effects of day of the week, long-term trend, and seasonality were also avoided (Janes et al., 2005). The sufficient set of variables for adjustment to estimate the total effect of heat waves on mortality in our regression model therefore included specific humidity and public holidays. Since relative humidity is a function of temperature, we adjusted for specific humidity rather than relative humidity, potentially reducing over-fitting (Davis et al., 2016). Air pollution was considered a potential mediator and thus was not included in the models.

We ran conditional Poisson regression models (Armstrong et al., 2014) to estimate the effect of heat waves on mortality. Heat waves were represented by a binary variable (0 for non-heat wave days, 1 for heat wave days) while distributed lag non-linear models (DLNM) (Gasparrini et al., 2010) were used to account for their delayed effects. We used a linear function to represent the effect of exposure, and a natural cubic spline, with four degrees of freedom, over the 5-day lag period. The choice of model parameters, spline function and number of knots, was based on a minimization of the sum of the Akaike Information Criteria for quasi-Poisson models. According to our analyses, 5 days is sufficient to account for the delayed effect and any potential harvesting effect (Supplementary Fig. S2). Specific humidity was controlled using a natural cubic spline with 3 degrees of freedom, while public holidays were controlled using a binary variable. The formula representing the conditional Poisson regression model used to estimate the city-specific heat wave-mortality association is in the supplementary material.

In the second stage, we used univariate random-effects meta-analyses (Gasparrini and Armstrong, 2013) to pool the city-specific results and obtain a summary measure for the entire Brazilian Amazon. The regression coefficients of the first stage models were reduced, cumulating the lag-specific contribution, and then used as outcomes for the meta-analyses with the intercept only. The pooled mortality risks were estimated for all definitions of heat waves and each subgroup.

Since the locations studied have different climatic and socioeconomic characteristics, we tested the heterogeneity between the city-specific heat wave-mortality association using the multivariate Cochran Q test and I^2 statistic. We fitted a meta-regression model to assess whether the exposure-outcome association changes according to certain meteorological, socioeconomic and geographical characteristics at city level. We tested the meta-predictors: average mean, minimum and maximum temperatures, mean annual temperature range, the Köppen climate classification, and latitude. The associations between the meta predictors and the exposure-response reduced coefficients were tested using the Wald test. We used the meta-analytical model with average maximum temperature at city level to derive the best linear unbiased prediction (BLUP) (Gasparrini et al., 2012) of heat wave-mortality in each city. Associations predicted with BLUP correspond to a trade-off between the city-specific and pooled associations, allowing locations with a low number of daily deaths to borrow information from locations which have similar characteristics but a higher number of deaths (Gasparrini et al., 2012, 2015).

2.3.1. Sensitivity analyses

We performed certain sensitivity analyses to verify the robustness of our results. For the lagged effects, we used a quadratic b-spline, natural cubic splines with 3 and 5 degrees of freedom and different lag periods (10 and 21 days). Different adjustments were explored, including a model without humidity and a model adjusted for temperature to investigate whether there was an additional effect of heat waves on mortality above the effect of high temperature on the first heat wave day, in line with Guo et al. (2017). We used a natural cubic spline with 4 degrees of freedom, with equally spaced knots, for both temperature and 5-day lag. Finally, the maximum temperature was used to define heat waves. Sensitivity analyses were pooled for the entire Brazilian Amazon, and the results were reported for heat wave definitions with a duration of at least 2 days.

All procedures and statistical analyses were conducted in R software (version 4.1.1), with the microdatasus package to download health data, ecmwfr to download weather data, gnm and dlnm for the city-specific analyses and mvmeta for the meta-analyses.

3. Results

Between 2000 and 2019, there were 831,084 deaths from all non-external causes, 199,330 (24%) from cardiovascular diseases and 85,598 (10.3%) from respiratory ones. The majority occurred among men (58.2%) and people aged up to 64 (52.2%). Supplementary Table S3 presents the descriptive statistics of the outcomes and meteorological variables for each of the 32 cities included in the study, while Table 1 presents a summary of the averages for these cities.

Table 2 shows the average, minimum and maximum number of heat wave days in the cities studied, according to each definition criteria. The number of heat wave days in the 32 municipalities in this study, according to the fifteen heat wave definitions applied, are presented in Supplementary Table S4. As the heat wave definition became more stringent, with increasing duration and intensity, the average, minimum, and maximum numbers of heat wave days fell.

The results of the I^2 statistic and the p-value of the Cochran Q test for the meta-regression with intercept only, equaling 25.9% and 0.093 respectively, suggest that the residual heterogeneity of the association between cities is not high, and not significant at the 5% confidence level (Supplementary Table S5). However, the residual heterogeneity was

Table 1
Summary statistics of the daily mean deaths and meteorological variables in the 32 cities in the Brazilian Amazon.

	Total (and %)	Mean	SD	Min	Median	Max
Total non-external mortality	831,084	3.5	5.3	0.3	1.6	22.2
Male	483,863 (58.2%)	2.1	3	0.2	0.9	12.9
Female	346,561 (41.7%)	1.5	2.3	0.1	0.6	9.3
0–64 years old	433,833 (52.2%)	1.9	2.8	0.1	0.8	12.5
65 years old or above	397,121 (47.8%)	1.7	2.5	0.2	0.8	10.7
Cardiovascular mortality	199,330 (24.0%)	0.9	1.2	0.1	0.4	5.1
Respiratory mortality	85,598 (10.3%)	0.4	0.6	0	0.2	2.8
Average temperature (°C)	–	26.4	0.5	25.4	26.5	27.4
Average specific humidity (kg/kg)	–	0.014	0.001	0.013	0.014	0.015

Table 2
Average, minimum, and maximum number of heat wave days for fifteen heat wave definitions, across 32 cities in the Brazilian Amazon (2000–2019).

Abbreviation	Definition (days, percentile threshold)	mean	minimum	maximum
hw2_90	2 days, 90th	599.5	511.0	671.0
hw2_925	2 days, 92.5th	429.7	363.0	523.0
hw2_95	2 days, 95th	277.8	216.0	330.0
hw2_975	2 days, 97.5th	124.5	90.0	156.0
hw2_99	2 days, 99th	49.25	35.0	62.0
hw3_90	3 days, 90th	468.8	367.0	553.0
hw3_925	3 days, 92.5th	321.7	268.0	420.0
hw3_95	3 days, 95th	198.6	137.0	248.0
hw3_975	3 days, 97.5th	83.28	53.0	119.0
hw3_99	3 days, 99th	29.53	18.0	48.0
hw4_90	4 days, 90th	379.6	270.0	475.0
hw4_925	4 days, 92.5th	250.8	181.0	358.0
hw4_95	4 days, 95th	148.4	93.0	201.0
hw4_975	4 days, 97.5th	57.22	30.0	92.0
hw4_99	4 days, 99th	18.12	7.0	40.0

even lower in the models that contained (separately) the variables: temperature range, and maximum and minimum temperature, which, according to the Wald test, modified the heat wave-mortality association. When we tested the multivariate meta-regression model with these three meta variables, only the maximum temperature remained significantly associated at the 5% level. Given this, and observing the goodness of fit criteria (AIC and BIC), we chose to use the model with the maximum temperature only to derive the BLUP for the city-specific heat wave-mortality association. For this paper we have chosen to present the combined results for the entire region, while the city-specific results are reported in the supplementary material.

Fig. 2 shows the pooled relative risks (RR) and their 95% confidence intervals for the effect of heat waves on mortality, according to the different definitions analyzed, over a lag period of 0–5 days. Although not statistically different, we found higher mortality risk as the heat wave intensity increases (higher thresholds), while there is a small increase as the duration changes from 2 to 3 days (for 95th percentile thresholds or higher), but no difference for a duration of 4 days. The pooled RR for mortality from total non-external causes, comparing heat wave days to non-heat wave days, varied between 1.03 (95% CI: 1.01–1.06), for the less stringent heat wave definition, and 1.18 (1.04–1.33) for the more stringent definition. Most city-specific results (Fig. S3) had a pattern similar to the pooled association, with risks increasing with heat wave intensity, albeit with a higher occurrence of

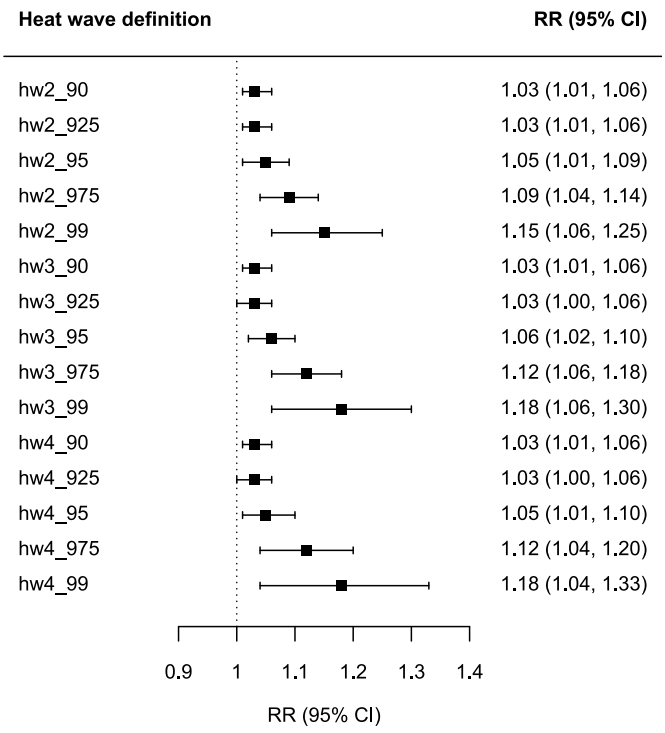

Fig. 2. Pooled effect of heat waves on mortality in the Brazilian Amazon over a lag period of 0–5 days, with heat waves of varying duration and intensity.

non-significant results at the 5% level.

Table 3 presents the relative risks of mortality due to heat waves by subgroup. We have reported the results for heat waves lasting at least 2 days, because this is a less rigorous criterion with a greater number of heat wave days, for the lowest (90th percentile), intermediate (95th), and highest heat wave intensity (99th). Results stratified by age, sex and cause of death were not statistically different, but suggest that the effect was greater for the elderly (≥65 years), and close to the null value for the younger subgroup (0–64 years). Both men and women experienced an increased risk of mortality on heat wave days, but the central RR estimates were greater for women. Although not statistically different, the results also suggest a greater effect for cardiovascular mortality than for non-external and respiratory causes. Supplementary Table S6 shows the stratified results for all the heat wave definitions.

Sensitivity analyses (Supplementary Table S7) show that these results remain robust when the model specifications change. The pooled results for the Brazilian Amazon were similar for a quadratic b-spline function and a natural cubic spline with 3 and 5 degrees of freedom for the lag-response relationship, and when specific humidity was removed from the model. For the longer lag period, we observed a reduction in

Table 3
Overall effect of heat waves on mortality in the Brazilian Amazon, over a lag period of 0–5 days, stratified by age, gender and cause of death. Heat waves of two or more days and varying intensity.

Subgroup	Risk ratio (95% confidence interval)		
	Lowest intensity (90th)	Intermediate intensity (95th)	Highest intensity (99th)
All causes	1.03 (1.01–1.06)	1.05 (1.01–1.09)	1.15 (1.06–1.25)
0–64 years old	1.00 (0.97–1.03)	0.99 (0.96–1.03)	1.03 (0.95–1.12)
65 years or above	1.07 (1.03–1.12)	1.12 (1.05–1.19)	1.34 (1.15–1.57)
Male	1.02 (0.99–1.05)	1.05 (1.01–1.08)	1.11 (1.01–1.22)
Female	1.04 (0.99–1.09)	1.07 (1.00–1.14)	1.24 (1.07–1.43)
Cardiovascular	1.06 (1.02–1.1)	1.07 (1.01–1.13)	1.27 (1.13–1.42)
Respiratory	1.09 (0.98–1.22)	1.07 (0.96–1.21)	1.05 (0.73–1.52)

the relative risks, but the reductions were smaller during the more intense heat waves. When we used the maximum temperature to define heat waves, the effects were slightly greater. When we controlled for temperature, the effect became null, except during the highest intensity heat wave, suggesting that there is an added effect from high intensity heat waves beyond the temperature effect.

4. Discussion

We carried out a time-stratified case-crossover study and a univariate random-effects meta-analysis to estimate the effect of heat waves on mortality in the Brazilian Amazon, according to heat wave definitions, population subgroups and causes of death. Our findings show that heat waves increased the risk of mortality in the Brazilian Amazon, and this risk increased with more intense heat waves. The evidence suggests that the elderly (≥65 years) were more vulnerable to heat wave effects than the younger population, women were more vulnerable than men, and the risk was higher for cardiovascular mortality than for non-external mortality and respiratory causes.

The effect of heat waves on mortality that we observed is consistent with the literature (Campbell et al., 2018; Xu et al., 2016; Zuo et al., 2015), although few studies have analyzed the effect of heat waves on health in Brazilian locations. Findings for Brazil in a multi-country study evidenced both the main effect, due to high temperatures, and the additional effect, due to heat wave duration, on all-cause mortality (Guo et al., 2017). Studies conducted in the city of São Paulo identified an increase in the risk of mortality from all-cause mortality and cardiorespiratory outcomes (Diniz et al., 2020; Moraes et al., 2022; Son et al., 2016).

Most heat-related health outcomes have cardiorespiratory causes (Cheng et al., 2019). The physiological mechanisms underlying these effects are usually explained by cardiovascular changes induced by thermoregulatory responses (Cheng et al., 2019; Kenney et al., 2014; Song et al., 2017). Thermoregulatory responses to high temperature exposure cause an increase in blood flow to the skin and sweating to dissipate heat, Consequently, there is an increase in both respiratory rate and heart rate, causing additional stress, particularly to the heart and lungs, increased dehydration, blood viscosity, and blood pressure (Deng et al., 2018; Hanna and Tait, 2015; Kenney et al., 2014; Liu et al., 2015).

We assessed the effects of fifteen heat wave definitions based on intensity (90th, 92.5th, 95th, 97.5th and 99th daily mean temperature percentiles) and duration (≥2, ≥3, e ≥ 4 days). The effects were greater as the intensity of the heat waves increased, but little change was seen with increased duration. Similar results have been found in other studies (Diniz et al., 2020; Guo et al., 2017; Moraes et al., 2022), and these results corroborate the conclusion of a systematic review which concluded that heat wave intensity plays a more important role than duration in determining the effects on mortality (Xu et al., 2016). The more intense or longer-lasting the heat wave, the greater the load on the cardiovascular system to perform thermoregulation (Chen et al., 2020).

In relation to cause of death, the effects of heat waves on mortality were higher for cardiovascular causes than non-external ones. The effects on respiratory mortality were not consistent. A meta-analysis study reported that heat waves were associated with an increased risk of mortality from cardiovascular and respiratory diseases (Cheng et al., 2019). In addition, the effects of heat waves on mortality from respiratory diseases and chronic obstructive pulmonary disease were significant among the elderly. A study conducted in São Paulo observed a higher risk of death from total cardiovascular and respiratory outcomes among women; for men, the risk of mortality from cerebrovascular disease and ischemic stroke was higher (Moraes et al., 2022). In our study, the inconsistency of the effect on respiratory mortality may be associated with our inclusion of all deaths classified by the ICD-10 chapter J, and the fact that we studied the entire population.

Our stratified analyses evidenced the heterogeneity of heat wave effects according to demographic characteristics. In the age-stratified analyses, heat wave effects were only observed among the elderly (≥65 years), which is similar to other studies (Chen et al., 2020; Green et al., 2019; Van den Wyngaert et al., 2021; Yang et al., 2019; Yin et al., 2018). Even in healthy individuals the aging process is accompanied by the degeneration of the physiological functions involved in thermoregulation and limitations in thermal perception, and may be associated with the presence of chronic diseases (Kenney et al., 2014; Zhou et al., 2014).

Both genders had an increased risk of mortality on heat wave days, although women were more vulnerable than men, in line with findings from other studies (Cheng et al., 2018; Yang et al., 2019). A study carried out on the elderly in São Paulo, found that women had a higher risk of mortality from heart and respiratory diseases (Diniz et al., 2020). In a systematic review, Son et al. (2019) found strong evidence that the risk of heat-related mortality is higher for women than men, although some studies have failed to identify a difference or found a higher risk for men. The greater vulnerability of women may be associated with physiological differences, and occupational and exposure patterns between genders (Son et al., 2019). Furthermore, differences in age structure between genders may explain some of this heterogeneity (Benmarhnia et al., 2015).

To the best of our knowledge, this is one of the largest studies carried out on the effects of heat waves on mortality in Brazil and specifically in the Brazilian Amazon. The study accounted for the lagged effects of heat waves, examining the modification of these effects according to different heat wave definitions, causes of deaths and population subgroups.

This evidence has significant public health implications. In the Brazilian Amazon, heat waves can increase the risk of mortality from all non-external causes and from cardiovascular diseases. In addition, older people and women are more vulnerable to the effects of heat waves on mortality. These results highlight the urgent need to develop and implement heat-health action plans for the region, with a specific focus on the most sensitive sectors of society and including the implementation of warning systems and response strategies to reduce the effects of heat waves on health.

This study has certain limitations. Similar to our previous studies on the effect of temperature on mortality in Brazil, exposure classification errors may have occurred (Silveira et al., 2019, 2020). Due to the lack of high-spatial resolution meteorological data, or geocoded health data, heat wave identification was based on a single temperature series for each city. In addition, we used an environmental measure as a proxy for individual exposure and were unable to consider variations in individual exposure related to daily mobility. However, exposure misclassification tends to be random, which could bias the results towards the null (Guo et al., 2017; Lee et al., 2016). In addition, since we used publicly available mortality data, it was not possible to distinguish between the rural and urban populations, which may have revealed important differences related to exposure and vulnerability to heat waves.

Another possible study limitation refers to the use of climate reanalysis, rather than station-based temperature data. According to Mistry et al. (2022), climate reanalysis represents an alternative to a lack of monitoring station data for use in time series analyses of heat-health effects, although, compared to measured temperature, they observed a lower performance in risk estimates in tropical regions, including the Brazilian Amazon. However, the differences in exposure-response associations based on the two temperature sources can be explained by certain factors, including proximity to the coast or hills, the quality and maintenance of the weather monitoring network, the population density in the cities analyzed, etc. We examined Pearson's correlation between the ERA5-Land and station-based temperature for some of the locations in our study area, and found a mean correlation of 0.78, with two locations below 0.70 (Supplementary Table S2). This low correlation may be explained by the sparse monitoring network in the region and less reliable data, with many missing data in the time series. Ground-level measurements are important inputs for reanalysis datasets.

Future studies should examine the role of the socio-environmental

and infrastructural determinants of the effect of heat waves on health and the vulnerability of specific subgroups in the Amazonian population. According to the 2010 Census, the region contains almost half of the Brazilian indigenous population, and a high percentage of the quilombola and riverine populations. These people are not historically assisted by public policies, face a series of socio-environmental conflicts, aggravated in recent years by pressures from the agricultural sector, deforestation and illegal mining in the region, and may be more vulnerable to the impacts of climate change. We also need to better understand the role of socioeconomic situation on these findings. Some of these data are not available on death certificates, and the completion of the race/color field is still very limited, since it depends on identification by the responsible physician, and there remains a high proportion of missing data. In addition, studies are required to investigate the modification effect of humidity, environmental pollution, mainly resulting from forest fires, the use of air conditioning, and coverage of public policies, such as cash transfer programs, primary healthcare, and hospital care policies. We could also investigate the use of different thermal indices, that consider, for example, humidity conditions, to classify heat wave events.

5. Conclusion

Our study showed that heat waves in the Brazilian Amazon were associated with a higher risk of mortality from non-external causes and cardiovascular diseases. The mortality risk was higher as the intensity of heat waves increased, which played a more important role in determining risk than duration. In addition, suggestive evidence indicated that older people and women were more vulnerable to the effects of heat waves on mortality.

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Declaration of competing interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.114109.

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Human biomonitoring guidance values (HBM-GVs) for priority substances under the HBM4EU initiative – New values derivation for deltamethrin and cyfluthrin and overall results

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ABSTRACT

The European Initiative HBM4EU aimed to further establish human biomonitoring across Europe as an important tool for determining population exposure to chemicals and as part of health-related risk assessments, thus making it applicable for policy advice. Not only should analytical methods and survey design be harmonized and quality assured, but also the evaluation of human biomonitoring data. For the health-related interpretation of the data within HBM4EU, a strategy for deriving health-based human biomonitoring guidance values (HBM-GVs) for both the general population and workers was agreed on. On this basis, HBM-GVs for exposure biomarkers of 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH), phthalates (diethyl hexyl phthalate (DEHP), di-n-butyl phthalate (DnBP), diisobutyl phthalate (DiBP), butyl benzyl phthalate (BBzP), and bis-(2-propylheptyl) phthalate (DPHP)), bisphenols A and S, pyrethroids (deltamethrin and cyfluthrin), solvents (1-methyl-2-pyrrolidone (NMP), 1-ethylpyrrolidin-2-one (NEP), N-dimethylformamide (DMF), N,N-dimethylacetamide (DMAC)), the heavy metal cadmium and the mycotoxin deoxynivalenol (DON) were developed and assigned a level of confidence. The approach to HBM-GV derivations, results, and limitations in data interpretation with special focus on the pyrethroids are presented in this paper.

1. Introduction

Human biomonitoring (HBM) is the determination of human exposure to contaminants that are detectable in the body and is worldwide increasingly used in population studies to provide an overview of the body burden in the population in general or in specific population groups. HBM is a valuable control instrument with regard to regulatory success and an important tool for further policy advice (Ganzleben et al., 2017; Louro et al., 2019; Ubong et al., this issue). In addition, HBM can be used to meet the information needs of citizens on issues related to environmental health protection (Uhl et al., 2021). HBM also plays an important role in occupational settings by complementing air measurements with individual measurements in various human matrices (ANSES, 2014).

In 2017, the European Human Biomonitoring Initiative HBM4EU was launched with the aim of establishing a European network of research institutions involved in the standardized collection and analysis of HBM data, to improve the state of knowledge for environmental and chemicals policy in the European Union and to enable comparability of HBM results across Europe (Gilles et al., 2021; Esteban López et al., 2021).

After five and a half years, the HBM4EU initiative marked a major step forward in the EU-wide establishment, harmonization, and quality assurance of human biomonitoring. This includes not only common agreements on study design, sampling and sample storage, methods to identify and quantify chemicals and/or their metabolites in human biological material, but also the data analysis and interpretation of the measurements results (Govarts et al., this issue).

With regard to the health-related evaluation of HBM results, a joint procedure has been agreed upon for deriving human biomonitoring

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guidance values (HBM-GVs). These values represent benchmark levels relating to the internal concentration of chemicals in e.g. blood or urine and are a key element for health-based risk assessment (Apel et al., 2020b) as well as an easy-to-use tool for comparison with HBM results. The HBM-GVs for the general population (HBM-GV_{GenPop}) indicate the concentration of a compound or its metabolite(s) in a biological matrix (e.g. blood, urine) at and below which a health risk is not anticipated, according to current knowledge (Apel et al., 2020b). The interpretation of these guidance values corresponds to that of the HBM-I values of the German HBM Commission (Apel et al., 2017; HBM Commission of the German Environment Agency, 2007, 2014) and is similar to that of the biomonitoring equivalents (BE values) insofar as these refer to substances with an effect threshold. BE values refer to chemicals' concentrations in biological material consistent with existing external exposure guidance values and were introduced by the US-based consulting firm Summit Toxicology (Hays et al., 2007, 2008).

HBM-GVs were also derived for occupationally exposed adults (HBM-GV_{Worker}). These values each represent the concentration of a substance or its specific and sensitive metabolite(s) in human biological media which should protect workers who are regularly exposed to the substance in question for 8 h per day and 5 days per week during their working lives from the harmful effects of medium- and long-term exposure. The HBM-GV_{Worker} are similar to the biological limit values (BLV) derived by the French Agency for Food, Environmental and Occupational Health and Safety (ANSES) as well as by the former Scientific Committee on Occupational Exposure Limits (SCOEL) or also to the biological tolerance values (BAT) set by the Working Group on "Assessment Values in Biological Material" of the German Research Foundation (DFG) (ANSES, 2014; SCOEL, 2013; DFG, 2021; Apel et al., 2020b). However, by definition, HBM-GVs can only be derived for substances for which a concentration without effect (threshold concentration) can be specified.

The joint procedure to derive HBM-GVs is published in a strategy paper (Apel et al., 2020b) that underwent - as well as each HBM-GV derivation - a consultation process within the HBM4EU consortium to include the expertise of scientists from HBM4EU partner countries.

Eighteen substances and substance groups were identified by the HBM4EU partner countries and EU institutions as of high priority because open policy relevant questions still had to be answered by targeted research (Ougier et al., 2021b). Corresponding biomarkers of exposure, the biological material to be analysed and analytical methods for the detection of the biomarkers of exposure for substances of the 1st and 2nd prioritisation round were compiled (Vorkamp et al., 2021; Esteban López et al., 2021) and formed the basis for further research activities. HBM-GVs were derived for some of the priority substances and used for subsequent health-related assessments of HBM studies. For some substances, only an HBM-GV for the general population or only for workers was proposed. This is partly due to the fact that, for example, exposure scenarios and results do not allow the derivation of HBM-GVs for workers (BPA) or no significant exposure is expected for the respective population (N,N-dimethylacetamide (DMAC) exposure of the general population). For a general overview, all derived values are presented in summary form, while the derivation of HBM-GVs for the pyrethroids is presented in more detail and for the first time.

2. Methods

The HBM-GVs were derived according to a systematic approach commonly agreed on within HBM4EU (Apel et al., 2020b). In short, a stepwise procedure is foreseen in which the existing literature on recent toxicological and epidemiological data on selected substances is reviewed and assessed for scope, quality, and relevance for the derivation of HBM-GVs. The first step consists in the selection of the most relevant biomarker(s). A biomarker is defined as any substance, structure or process that can be measured in the body or its degradation product(s) which influence(s) or predict(s) the incidence of outcome or disease. Biomarkers can be classified into biomarkers of exposure (BME), biomarkers of effects or biomarkers of susceptibility (Apel et al., 2020b). They are selected for the derivation of HBM-GVs according to defined criteria: specificity, sensitivity, half-life, availability of a suitable analytical method, invasiveness of biological sampling, and background level. The derivation of HBM-GVs for the selected biomarkers can then be conducted according to three possible options (Fig. 1). The most robust derivation and thus the 1st option is based on a relationship between human internal biomarker concentrations and adverse health effects. If those human data are unavailable, insufficient, or not reliable, it can be tried to proceed according to the 2nd option by converting existing external toxicity reference values (TRV), such as a Tolerable Daily Intake (TDI), or Occupational Exposure Limits (OEL), set by EU or relevant non-EU bodies, into internal values using toxicokinetic data or correlation equations based on a relationship between exposure and internal concentration. As a 3rd option, if the TRV is considered inappropriate or no TRV is available, an HBM-GV can be derived based on a point of departure (POD) identified in an experimental (animal) study. Uncertainty factors, also called assessment factors, are used depending on the respective data situation. A level of confidence (low, medium or high) is attributed to each calculated HBM-GV. The level of confidence should reflect the uncertainties identified during the derivation of the value and can be a good incentive to later revise values with estimated 'lower' level of confidence. In the following the derived values are shown depending on the derivation path. For the workplace, it is also indicated when it is best to sample so that a meaningful comparison can be made with the HBM-GV_{Worker}.

3. Results

3.1. First option: HBM-GVs based on a relationship between human internal biomarker concentrations and adverse health effects

As part of the HBM4EU work, HBM-GVs were derived in accordance with option 1 for two substances: Cadmium and its compounds and N-Dimethylformamide (DMF).

3.1.1. Cadmium and its compounds

The heavy metal Cadmium (Cd) is an environmental pollutant that occurs naturally and is also released from industrial and agricultural activities. Foodstuffs are the main source of cadmium exposure for the non-smoking general population. Urinary Cd (U–Cd) is a well-known and reliable indicator of long-term exposure to Cd for the general population as well as for occupationally exposed adults. Blood Cd (B–Cd) is a useful indicator of Cd-exposure during the last months (3–6 months). Therefore, B–Cd is a useful biomarker to monitor Cd occupational exposure in addition to U–Cd, especially for new employees at the workplace. There is ample evidence of nephrotoxicity as critical effect in the case of exposure to cadmium and quantitative relationships between the concentration of Cd both in urine (U–Cd) and in blood (B–Cd) and renal dysfunction in workers and in occupationally unexposed persons are available (EFSA, 2009; ANSES, 2018). Tubular and glomerular renal effects have been observed with the former being better characterized. A comprehensive literature review by Lamkarkach et al. (2021) on effects suspected to be related to Cd exposure showed also bone and cardiovascular effects for low cumulated exposure as indicated by U–Cd and/or B–Cd. However, for the time being, these effects were not selected as critical effects for the derivation of HBM-GVs, as the weight of evidence was not considered sufficient within the HBM4EU project (Lamkarkach et al., 2021). Based on a robust meta-analysis conducted by EFSA (2009) on cadmium-related elevated levels of beta-2-microglobulin (β2M) in urine as a marker of low molecular weight proteinuria, an HBM-GV_{GenPop} for U–Cd of 1 μg/g creatinine (creat) is recommended for adults older than 50 years. Taking into account the accumulation of Cd in the human body throughout life, threshold or 'alert' values according to age were estimated for U–Cd

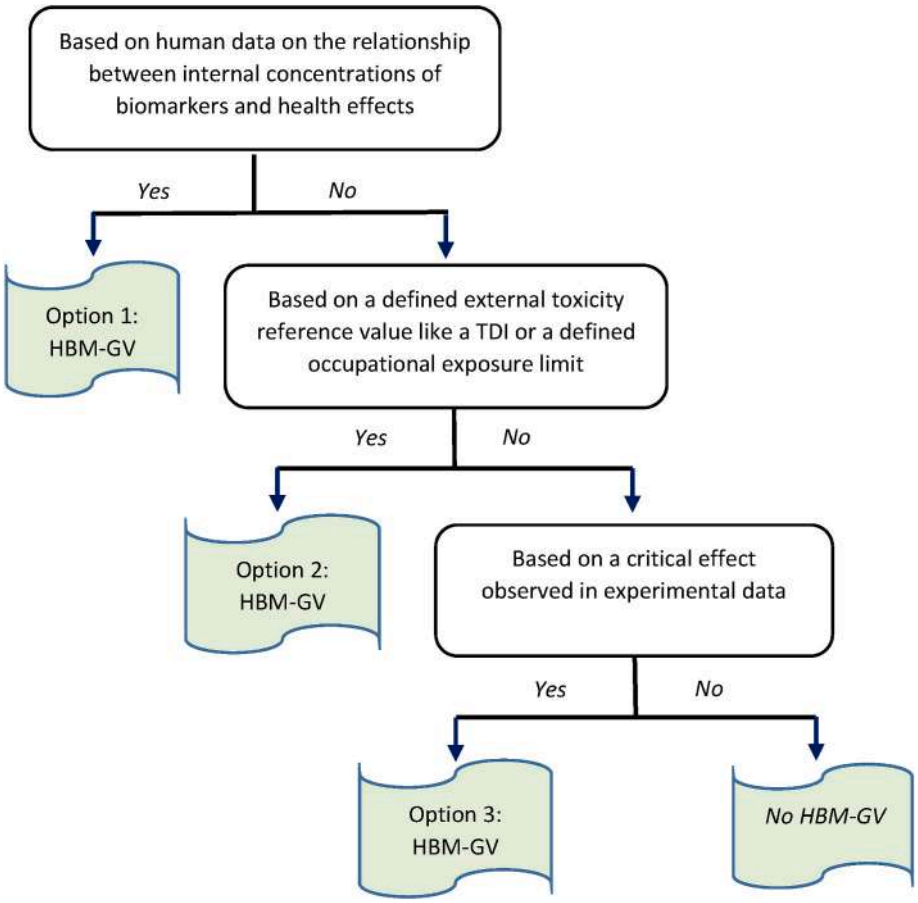

Fig. 1. Decision tree for determining HBM-GVs (taken from Lamkarkach et al., 2022, slightly modified).

according to the physiologically based pharmacokinetic (PBPK) model by ANSES (2018). These values referring to age groups represent a further specification and improvement of the HBM-I values earlier proposed by the German Human Biomonitoring Commission (HBM Commission of the German Environment Agency, 2011) (Lamkarkach et al., 2021). For the workplace, an HBM-GV_{Worker} of 2 µg/g creat was derived from the study of Chaumont et al. (2011) for U–Cd, and additionally an HBM-GV_{Worker} for B–Cd of 5 µg/L was proposed based on the recommendation of the American Conference of Governmental Industrial Hygienists (ACGIH, 2016). Both HBM-GV_{Worker} refer to renal effects. The HBM-GV_{Worker} for U–Cd is in line with the biological limit value (BLV) set by the amendment of the European Carcinogens and Mutagens Directive (EU) 2019/983 of the European Parliament and of the Council in June 2019 (2 µg/g creat for U–Cd) (Lamkarkach et al., 2021). This BLV is based on renal effects and the European Scientific Committee on Occupational Exposure Limits (SCOEL) recommendation.

Table 1
Derivation of HBM-GVs according to option 1, based on a relationship between human internal biomarker concentrations and adverse health effects.

Substance/ Exposure biomarker	Critical effect	Key studies and Point of departure (POD)	Assessment factor	HBM-GV_{GenPop}	HBM-GV_{Worker}	Level of confidence (LoC)
Cadmium/U–Cd	Nephrotoxicity	BMD_5L_{95}: 4 µg/g creat from meta-analysis of 35 studies (EFSA, 2009)	Intra-species variation (Chemical-specific "adjustment factor") = 3.9	In µg/g creat >50 years (y): 1 41–50 y: 0.8 31–40 y: 0.5 21–30 y: 0.3 11–20 y: 0.2 ≤ 10 y: 0.1 (PBPK model by ANSES, 2018)		High
		Chaumont et al. (2011)	Intraspecies variation = 3 (average age 45 y)		2 µg/g creat	High-medium
Cadmium/B–Cd	Nephrotoxicity	BEI: 5 µg/L (ACGIH, 2016)		n.d.	5 µg/L	High-medium
DMF/tNMF	Hepatotoxicity	Several studies carried out at workplace		n.d.	10 mg/L (or 10 mg/g creat)	High
DMF/AMCC	Hepatotoxicity	Several studies carried out at workplace		1 mg/g creat[a]	10 mg/g creat	Medium-low

U–Cd: Urinary Cadmium, B–Cd: Blood Cadmium, tNMF: total N-methylformamide, AMCC: N-acetyl-S-(N-methylcarbamoyl)cysteine.
[a] Provisional value without consultation of HBM4EU partners.

Cadmium is considered a genotoxic carcinogen, causing, among other effects, oxidative DNA damage, inhibition of DNA repair, and deregulation of cell proliferation, with a threshold level for these effects below which no effect is expected (ECHA, 2020c). But it is not possible to establish exposure risks on the basis of Cd's carcinogenicity due to a lack of a consistent relationship in epidemiological studies and the complex interactions with smoking and other co-carcinogenic factors.

The level of confidence attributed to the HBM-GV for the general population is 'high'. For the working population, the levels of confidence attributed to the U–Cd and B–Cd HBM-GVs are 'high-medium' (Lamkarkach et al., 2021).

The essential information for the derivation of, and the numerical values for the HBM-GVs for the general population and workers are summarized in Table 1.

3.1.2. N-dimethylformamide (DMF)

Release to the environment of the solvent N-dimethylformamide (DMF) can occur from industrial use e.g. in processing aids and from application of products like machine wash liquids/detergents, automotive care products, paints and coatings or adhesives, fragrances and air fresheners (ECHA substance infocard). Exposure to DMF in the workplace can occur through both inhalation and dermal contact. Results on DMF biomonitoring show sufficient and robust relationships between health effects (i.e. hepatic effects, antabuse effects, or effects on the gastrointestinal tract) and levels of biomarkers of exposure, mainly total N-methylformamide (tNMF), which is the sum of N-hydroxymethyl-N-methylformamide and N-methylformamide, and N-acetyl-S-(N-methylcarbamoyl)cysteine (AMCC) in urine. These BME were identified as the most appropriate and rather complementary BME for the derivation of HBM-GV_{Worker} (Lamkarkach et al., 2022). Liver damage which is reflected by an elevation of serum hepatic enzymes activity proved to be the critical effect for the derivation of the HBM-GV_{Worker}. Of the studies conducted in the workplace, four were assessed as suitable for deriving an HBM-GV_{Worker} (Sakai et al., 1995; He et al., 2010; Kilo et al., 2016; Wu et al., 2017) and based on these, an HBM-GV_{Worker} of 10 mg tNMF/L (or 10 mg/g creat) was agreed on (Lamkarkach et al., 2022). This value is slightly lower than the values previously proposed by SCOEL (2006), ACGIH (2017) or DFG (2019) (15 mg/L to 30 mg/L). The reasons for this are, on the one hand, the approach used, SCOEL and DFG established the values for tNMF in urine based on the occupational exposure limit (OEL) for DMF and correlations between external exposure and the concentration of biomarker in urine. This is not the best choice as DMF is readily absorbed through the skin. On the other hand, ACGIH has retained option 1, which builds on relationships between internal biomarker concentration and health effects, but has not taken into account the most recent publications by Kilo et al. (2016) and Wu et al. (2017), which were identified by Lamkarkach et al., (2022) as important for deriving the HBM-GV_{Worker}. Considering tNMF half-life time, end-of-shift urine sampling at any day of the workweek can be performed for this biomarker. Complementing the value for tNMF as most reliable biomarker of exposure, an HBM-GV_{Worker} for AMCC was derived by Lamkarkach et al. (2022), which is an indicator of cumulative exposure after several days. The basis was the same four studies which were used for the elaboration of an HBM-GV_{Worker} for tNMF. Due to the wide range of NOAEL values and the large margin between NOAEL and LOAEL values, a conservative limit value of 10 mg/g creat AMCC was agreed on and is provisionally recommended. As DMF LOAEL and NOAEL for developmental effects in the most sensitive species are higher than the corresponding values for hepatotoxic effects, the HBM-GV_{Worker} for tNMF and AMCC which were established to protect against hepatotoxic effects are expected to also protect from developmental toxicity. Further, as DMF carcinogenic effects in animals follow hepatic damage, as there is no sufficient evidence of DMF genotoxicity, and the three reported clusters of testis cancer do not constitute a sufficient evidence of DMF carcinogenicity in humans, the HBM-GV_{Worker} for tNMF and AMCC are expected to also protect from carcinogenic effects (Lamkarkach et al., 2022). Alcohol intolerance can be observed at lower exposure levels in some individuals, therefore workers exposed to DMF should be informed about the risk of alcohol consumption. On the other hand, acute and chronic alcohol consumption interferes with DMF metabolism and tNMF and AMCC elimination kinetics; thus, information on alcohol habits and alcohol consumption should be collected to allow interpretation of the measurement results (Lamkarkach et al., 2022). The levels of confidence attributed by Lamkarkach et al. (2022) to the HBM-GV_{Worker} are set to 'high' for tNMF and 'medium-low' for AMCC. For the purpose of risk assessment, the HBM-GV_{Worker} for AMCC of 10 mg/g creat was used by Mahiout et al. (2022) and divided by another assessment factor of 10 to consider more sensitive subgroups of the population, resulting in a provisional HBM-GV_{GenPop} of 1 mg/g creat for the DMF metabolite AMCC (Mahiout and Santonen, 2022). The essential information for the derivation of HBM-GVs, and the numerical values for the HBM-GVs for workers and the general population are summarized in Table 1.

3.2. Second option: HBM-GVs based on toxicity reference values (TRV) like TDI

As part of the HBM4EU work, HBM-GVs were derived in accordance with option 2 for nine substances: deltamethrin, cyfluthrin, diethyl hexyl phthalate (DEHP), di-n-butyl phthalate (DnBP), diisobutyl phthalate (DiBP), bis-(2-propylheptyl) phthalate (DPHP), 1,2-Cyclohexane dicarboxylic acid diisononyl ester (DINCH), bisphenol A (BPA), and deoxynivalenol (DON). The HBM-GVs derived are for the general population, information on their derivation and the numerical values for the HBM-GVs are summarised in Table 2. The derivation of the HBM-GVs for deltamethrin and cyfluthrin is described in more detail in the present article.

3.2.1. Pyrethroids

3.2.1.1. Deltamethrin. Deltamethrin ((S)-α-cyano-3-phenoxybenzyl (1R, 3R)- 3 -(2,2-dibromovinyl)-2,2-dimethylcyclopropane carboxylate) is a synthetic pyrethroid that is currently registered in the European Union as a biocide (product group 18: insecticides, acaricides, and products to control other arthropods) and as an active substance in plant protection products (ECHA, 2011a; EU pesticides-database). The name refers to cis-deltamethrin as the active isomer; other isomers are considered to be impurities (EC, 2018b). Exposure of the general population to deltamethrin occurs primarily through residues in food. From animal experiments and human examinations, it is known that following oral intake, deltamethrin is rapidly absorbed and a significant proportion is already metabolized in the gastrointestinal tract and thus no longer bioavailable. Via hydrolysis of the ester bond, which results in substantial detoxification, the metabolism leads to the formation of DBCA (3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanoic acid) and 3-PBA (3-phenoxybenzoic acid) (EC, 2018b). While 3-PBA is also formed by the metabolism of pyrethroids other than deltamethrin, DBCA represents a deltamethrin-specific metabolite (Schettgen et al., 2002).

The excretion of deltamethrin was studied by Sams and Jones (2012) in a small group of five volunteers (3 males + 2 females, age 23–55 years old, weight 77–107 kg) who received a single oral dose of 0.01 mg/kg bw/d dissolved in ethanol and diluted in a soft drink. Total urine was collected within 0–60 h after intake at time intervals of initially 2 h and after 8 h at longer intervals. The volume of each sample was recorded and creatinine was determined to adjust for dilution. Urine samples were heated for 2 h after addition of sulphuric acid to hydrolyse any conjugates, were then extracted with chlorobutane and analysed by gas chromatography-mass spectrometry (GC-MS) for the two metabolites, DBCA and 3-PBA. The limit of detection (LoD) for both metabolites was 1 μg/L. Elimination of both metabolites was rapid with mean half-lives of 3.6 h (DBCA) and 7.1 h (3-PBA). The mean recovery of DBCA in urine

Table 2
Derivation of HBM-GVs according to option 2, based on an existing TRV.

Substance/Exposure biomarker/Biological material	Critical effect	Toxicity Reference Value (TRV) [mg/kg bw/d]	Toxico-kinetics	$HBM\text{-}GV_{GenPop}$ [mg/L urine]	Level of confidence
Deltamethrin/DBCA/ urine	Lowered body weight, behavioural changes, and clinical signs of neurotoxiciy	ADI: 0.01 Studies: 90 d, rats; 90 d dogs; 1-year dogs (ECHA, 2011a; EC, 2018b)	Mass balance approach, Fue: 0.45 (Sams and Jones, 2012)	Children: 0.09 Adults: 0.13	Medium
Cyfluthrin/FPBA/urine	Acute neurotoxicity	ADI: 0.01 4-week rat study with β-cyfluthrin (EC, 2017a; EC, 2017b; EC, 2020; EFSA, 2020)	Mass balance approach, Fue: 0.47 (Leng et al., 1997a; Hays et al., 2009)	Children: 0.08 Adults: 0.13	Medium
DEHP/5-oxo-MEHP + 5-OH-MEHP/urine DEHP/5-cx-MEPP + 5-OH-MEHP/urine	Impairment of testicular development	TDI: 0.05 (EFSA, 2005a)	Mass balance approach, 5-OH-MEHP, $Fue_{(48h/24h)}$: 0.156/0.149; 5-oxo-MEHP, $Fue_{(48h/24h)}$: 0.113/0.109; 5-cx-MEPP, $Fue_{(48h/24h)}$: 0.139/0.132 (Anderson et al., 2011)	Children: 0.34 Adults: 0.5 Children: 0.38 Adults: 0.57	Medium
DnBP/MnBP/urine	Delayed germ cell development and male mammary gland changes after exposure from GD 15 to PND 21	DNEL: 0.0067 (ECHA, 2016)	Mass balance approach, $Fue_{(24h)}$: 0.69 Anderson et al. (2001)	Children: 0.12 Adults: 0.19	Medium
DiBP/MiBP/urine	Delayed germ cell development and male mammary gland changes after exposure from GD 15 to PND 21	DNEL: 0.0083 (ECHA, 2016) Read across with DnBP considering 25% potency difference (Saillenfait et al., 2008)	Mass balance approach, $Fue_{(24h)}$: 0.7 Koch et al. (2012)	Children: 0.16 Adults: 0.23	Low
DPHP/oxo-MPHP + OH-MPHP/urine DPHP/oxo-MPHP/urine DPHP/OH-MPHP/urine	Thyroid follicular hypertrophy/ hyperplasia observed in F1 adult rats	RfD: 0.1 (Bhat et al., 2014)	Mass balance approach,oxo-MPHP, $Fue_{(\sim 24h)}$: 0.0795; OH-MPHP, $Fue_{(\sim 24h)}$: 0.0597 (averaged values: Leng et al., 2014 & Klein et al., 2018)	Children: 0.33 Adults: 0.5 Children: 0.19 Adults: 0.29 Children: 0.14 Adults: 0.22	Low
Hexamoll® DINCH/OH-MINCH + cx-MINCH/urine	Nephrotoxicity	TDI: 1 (EFSA, 2006)	Mass balance approach, OH-MINCH, $Fue_{(48h/24h)}$: 0.1073/0.0955; cx-MINCH, $Fue_{(48h/24h)}$: 0.0203/0.0167 (Schütze et al., 2017)	Children: 3 Adults 4.5	Medium
BPA/total BPA/urine	Increased relative mean kidney weight	t-TDI: 0.004 (EFSA, 2015)	TK model by Karrer et al. (2018)	Children: 0.135 Adults: 0.230	Medium
DON/total DON/urine	Reduced body weight gain	group-TDI for DON, 3-Ac-DON, 15-Ac-DON and DON-3-glucoside: 0.001 (EFSA, 2017)	TK model by Mengelers et al. (2019)	0.023 (CI: 0.005–0.033) Based on a 24-h urine sample	Medium

$HBM\text{-}GV_{GenPop}$: Human biomonitoring guidance value for the general population; DBCA: 3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanoic acid; FPBA: 4 fluoro-3-phenoxybenzoic acid; DEHP: diethyl hexyl phthalate; 5-oxo-MEHP: mono(2-ethyl-5-oxohexyl)phthalate; 5-OH-MEHP: mono(2-ethyl-5-hydroxyhexyl) phthalate; 5-cx-MEPP: mono (5-carboxy-2-ethylpentyl) phthalate; DINCH: 1,2-Cyclohexane dicarboxylic acid diisononyl ester; OH-MINCH: cyclohexane-1,2-dicarboxylic acid-mono(hydroxyl-iso-nonyl) ester; cx-MINCH: cyclohexane-1,2-dicarboxylic acid-mono-(carboxy-iso-octyl) ester; DnBP: di-n-butyl phthalate; MnBP: monobutyl phthalate; DiBP: diisobutyl phthalate; MiBP: monoisobutyl phthalate; DPHP: bis-(2-propylheptyl) phthalate; oxo-MPHP: mono(propyl-6-oxo-heptyl) phthalate; OH-MPHP: hydroxy-mono-propylheptyl phthalate; BPA: bisphenol A; DON: deoxynivalenol; F_{ue}: fractional urinary excretion coefficient or urinary excretion factor; TRV: toxicity reference value; bw: body weight; TDI: tolerable daily intake; t-TDI: temporary tolerable daily intake; ADI: acceptable daily intake; RfD: reference dose; DNEL: derived no effect level; TK: toxicokinetic; CI: confidence interval.

as a proportion of the administered dose over the initial 24 h post-dose was 45% (range 18–64%). This value was practically unchanged when taking into account a 48 h collection period (46%, range 19–66%). DBCA was excreted almost twice as rapid as 3-PBA. Also, the level of DBCA in urine was within the first 24 h about five times higher than that of 3-PBA: The mean level of DBCA in 24-h total urine reached 58.3 (range: 42.2–86.7) μmol/mol creat, while that of 3-PBA only reached 11.9 (range 8.0–18.5) μmol/mol creat. Adjusting for a 70 kg bw individual, these values result in 42.8 μmol DBCA/mol creat and 8.7 μmol 3-PBA/mol creat, respectively. The fact that both metabolites were not excreted in equimolar amounts in urine as expected from the molecular structure may be explained by further formation of 4-hydroxy-3-PBA (Sams and Jones, 2012). It is clear from the above that the urinary excretion rate of the specific metabolite DBCA within 24 h is high

enough to use DBCA as a biomarker for deltamethrin exposure, and hereby also to calculate an HBM-GV. Besides the method published by Sams and Jones (2012), there are even more sensitive methods for the determination of DBCA, for example the highly sensitive and specific gas chromatography-tandem mass spectrometry (GC/MS/MS) -method to simultaneously quantify the metabolites of the most common synthetic pyrethroids in urine, described by Schettgen et al. (2016) with a limit of quantification for DBCA of 0.01 µg/L urine.

A Tier II Epidemiology Report focused on non-carcinogenic and carcinogenic health effects in humans for the chemical class of pyrethroids, as well as selected individual pyrethroids including deltamethrin was compiled by the U.S. EPA (2019a). A total of 62 published epidemiological studies was reviewed. Studies regarding non-carcinogenic effects included neurodevelopmental, neurobehavioral and neurocognitive effects, birth defects, male reproductive effects with respect to semen quality, male reproductive hormone levels, sperm damage/genetic abnormalities and other effects like coronary heart disease in adults, respiratory effects, autism spectrum disorders (ASD) in children, thyroid hormone levels in neonates. Based on this review, there is - according to U.S. EPA (2019a) - no or insufficient evidence to suggest a clear associative or causal relationship between exposure to pyrethroid pesticides and the non-carcinogenic health outcomes examined. A quality-based review of pyrethroid epidemiology further concluded that pyrethroids as a group are unlikely to adversely affect human fertility or reproductive capability (Burns and Pastoor, 2018). Regarding carcinogenic effects, the U.S. EPA concluded that "for childhood brain tumors and childhood leukemia, there is insufficient evidence at this time to conclude that there is a clear associative or causal relationship with pyrethroid exposures." Furthermore, "there is no evidence at this time to conclude that there is a clear associative or causal relationship between pyrethroid exposure and breast cancer" (US EPA, 2019a). Many of the published studies were found to be of low quality because they were designed as cross-sectional studies, rather than cohort or case-control studies in which temporal aspects are taken into account.

An acceptable daily intake (ADI) value for deltamethrin of 0.01 mg/kg bw/d was derived in 2000 by the Joint Meeting of the Food and Agriculture Organization (FAO) Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group (JMPR, 2000), based on a NOAEL of 1 mg/kg bw/d in one-year and two-year oral studies with dogs and a two-year oral study with rats using an overall assessment factor of 100. This default uncertainty factor for inter- and intra-species extrapolations in pyrethroids risk assessment is also still recommended by the U.S. EPA (2019b). ECHA and EFSA assessments came, based on results of two dog studies (90 days and one-year) and one rat study (90 days) to the same numerical value for the ADI as JMPR (ECHA, 2011a; EC, 2018b). The value is current and the literature search did not reveal any new findings on the effects of deltamethrin to question this value. The data base is broad: repeated dose toxicity studies, mostly with oral administration of deltamethrin, were conducted with rats, mice, and dogs. A NOAEL of 1 mg/kg bw/d was obtained in a subchronic feeding study with rats, based on non-specific effects (lowered body weight) at the next higher dose. An identical NOAEL of 1 mg/kg bw/d and a NOAEL of 1.1 mg/kg bw/d were also obtained in chronic toxicity studies with rats, based on lower body weight gain and hepatic changes, respectively. Studies with oral exposure of dogs to deltamethrin over 90 days or 1 year also delivered a NOAEL of 1 mg/kg bw/d, based on behavioural changes and clinical signs of neurotoxiciy at higher doses. In vitro and in vivo studies provided no evidence of genotoxicity of deltamethrin, and chronic toxicity/carcinogenicity studies with rats and mice provided no evidence of carcinogenicity. Also, no effects on fertility or development were observed or only observed at doses which already led to maternal toxicity. Furthermore, deltamethrin does not meet the World Health Organization (WHO) definition of an endocrine disruptor, that means it does not alter any function(s) of the endocrine system and does not cause any adverse health effects in this respect in an intact organism, its progeny, or (sub)- populations (WHO, 2012). A number of neurotoxicity studies with single or repeated exposure of adult animals to deltamethrin or with exposure during gestation and postnatal development are available. Studies with adult animals provided acute NOAEL or benchmark doses similar to those NOAEL obtained in studies with repeated exposure, e.g., a "threshold dose" of 0.99 mg/kg bw/d was derived for acute effects of deltamethrin on motor function in rats. Furthermore, the U.S. EPA concluded that the developmental neurotoxicity studies do not provide evidence for a higher sensitivity during development and that an additional assessment factor is not necessary (US EPA, 2019b). Thus, the ADI and, based on this, the HBM-GVs to be calculated in the following are also protective against acute neurotoxic effects (EC, 2018c).

The ADI (EC, 2018a, b; ECHA, 2011a; JMPR, 2000) as external toxicity reference value can be translated into a corresponding $HBM\text{-}GV_{GenPop}$ by using the following mass balance equation, assuming steady-state conditions:

$$HBM\text{-}GV_{GenPop} = \frac{\mathbf{ADI} \bullet \left[\frac{\mathbf{MW(DBCA)} \bullet \mathbf{Fue(DBCA)}}{\mathbf{MW(Deltamethrin)}}\right]}{\mathbf{Daily\ urinary\ flow\ rate\ adjusted\ to\ the\ bw}}$$

Here is MW the molecular weight (DBCA: 298 g/mol, deltamethrin: 505.2 g/mol, ratio: 0.59) and Fue the molar excretion factor of 0.45 (Sams and Jones, 2012). Relating, in a simplified approach, the calculated excretion to the weight proportional amount of urine per day (0.03 L/kg bw/d for children and 0.02 L/kg bw/d for adults (Apel et al., 2020b), the ADI for deltamethrin corresponds to the following concentration of DBCA in urine:

Children: 0.01•0.59•0.45 mg/kg bw/d: 0.03 L/kg bw/d = 0.0885 mg DBCA/L urine

Adults: 0.01•0.59•0.45 mg/kg bw/d: 0.02 L/kg bw/d = 0.133 mg DBCA/L urine

On this basis, $HBM\text{-}GV_{GenPop}$ of 90.0 µg DBCA/L urine for children and 130 µg DBCA/L urine for adults were derived for the interpretation of biomonitoring results on deltamethrin in the general population. The level of confidence is rated medium.

For information and comparison, Aylward et al. have already derived urinary biomonitoring equivalents (BEs) in 2011 (Aylward et al., 2011). At the time this calculation was performed, no human data were available regarding the quantitative excretion of the deltamethrin-specific metabolite DBCA in urine. Instead, Aylward et al. (2011) used data from human toxicokinetic studies with cypermethrin, which differs from deltamethrin only in the halogen groups at the vinyl moiety (chlorine instead of bromine). Based on this read across, BE values of 50 µg DBCA/L (adults) and 7 µg/L (children) were derived consistent with the U.S. EPA reference dose (RfD). With reference to the ADI of 0.01 mg/kg bw/d, urinary BE values of 60 µg DBCA/L were derived for both children and adults (Aylward et al., 2011).

With the human toxicokinetic data of Sams and Jones (2012) it was now possible to propose updated HBM guidance values for the general population of 90 µg DBCA/L for children and 130 µg DBCA/L for adults.

3.2.1.2. Cyfluthrin. Cyfluthrin (IUPAC name, unspecified stereochemistry: (RS)-α-cyano-4-fluoro-3-phenoxybenzyl (1RS,3RS; 1RS,3RS)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate) is a synthetic pyrethroid. The cyfluthrin molecule contains three chiral centers, giving rise to four enantiomeric pairs denoted as diastereomer I to IV. Technical cyfluthrin (thereafter referred to as "cyfluthrin") is a mixture of all four diastereomeric enantiomer pairs (i.e. eight isomers), which are present in the mixture in approximately equal proportions (about 20–35% of each). β-cyfluthrin is the common name for the mixture of enantiomer pairs II and IV in a ratio of about 1:2; the sum of the proportions of the other two pairs is less than 5%. The two diastereomeric

pairs II and IV of β-cyfluthrin represent the biologically active isomers of cyfluthrin. They are present in cyfluthrin at a concentration of about 40%. Cyfluthrin is currently approved in the European Union (and Switzerland) as an active substance for use in biocidal products of product-type 18 (insecticides, acaricides and products to control other arthropods) until 28/02/2028 (EC, 2016; ECHA Dissemination, 2021). For plant protection purposes in the EU, cyfluthrin and also β-cyfluthrin are no longer approved. The reasons put forward by EFSA for the non-renewal of approval for β-cyfluthrin as active substance in plant protection products were specifically because of unacceptable risks for workers loading and sowing beet seeds treated and for workers and operators using the respective plant protection products on tomatoes in greenhouses. Furthermore, in cases of β-cyfluthrin application to potato and wheat fields, high risks to residents have been identified. In addition, based on the available data, the consumer risk could not be conclusively assessed. Despite the non-approval of cyfluthrin and β-cyfluthrin as active substances in plant protection products in the EU, residues in imported food and feed cannot be excluded and this, like the use of cyfluthrin biocides in the EU, can be an explanation for a possible detection of residues in the urine of EU citizens.

The toxicokinetics of cyfluthrin and β-cyfluthrin are similar, and data on cyfluthrin are considered representative for β-cyfluthrin and vice versa. In both, rodents and humans, cyfluthrin is almost completely and rapidly absorbed after ingestion (≥90%). Absorption is also rapid after inhalation. The volume of distribution, as determined in rodent studies, is about 17% of the body volume, indicating that the distribution is primarily in the extracellular fluid compartment (Hays et al., 2009). Hydrolysis of the ester bond and further oxidative metabolism leads to a number of metabolites, and it appears that cleavage of the ester bond results in substantial detoxification. In studies with rats, the main urinary metabolites were identified as cis/trans 3-(2,2-Dichlorovinyl)-2, 2-dimethylcyclopropanoic acid (DCCA) as well as its glucuronide, and as 4 fluoro-3-phenoxybenzoic acid (FPBA) as well as conjugated and free 4′-hydroxy-FPBA. Studies in humans also show the excretion of DCCA and FPBA in urine after cyfluthrin exposure (ATSDR, 2003; Hays et al., 2009; US EPA, 2019a). DCCA is not specific for cyfluthrin and also FPBA is not only known as a metabolite of cyfluthrin, but also as a metabolite of flumethrin, which is used in veterinary medicine. However, human exposure to flumethrin is thought to be very limited and measured urinary FPBA concentrations are likely to reflect recent exposure to cyfluthrin/β-cyfluthrin (Leng et al., 1997a). Quantitative data on the excretion of metabolites is available from a study with controlled oral exposure to cyfluthrin (Leng et al., 1997a). In this study a single male volunteer received a single oral dose of 0.03 mg/kg bw/d (total dose: 2.66 mg). Urine samples were collected at 12-h intervals for 2 days, starting 6 h after exposure. For the quantification of metabolites, the urine samples were acid-treated for the cleavage of conjugates, extracted and subjected to methylation of the free acid metabolites. Separation and detection were performed by GC-MS with a limit of detection of 0.5 μg/L for all metabolites (Leng et al., 1997a). The appearance of metabolites in urine could be described by first-order kinetics with a half-life of elimination of 6.44 h (cis-DCCA: 6.7 h, trans-DCCA: 6.5 h, FPBA: 6.1 h), similar to the results obtained in an inhalation exposure study by Leng et al., 1997b. The authors further found that "in comparison with the intake of 2.6 mg, a total of 1 mg cyfluthrin equivalent was recovered in the urine." Furthermore, of this 1 mg cyfluthrin equivalent, "the total amount of FPBA was twice as much as the total amount of cis/trans-DCCA" (Leng et al., 1997a). This translates into 0.66 mg FPBA excreted in urine following an oral dose of 2.6 mg cyfluthrin, corresponding to a mass fraction of 0.25 (0.66/2.6) FPBA excreted in urine. On a molar basis, this equates to a fraction of 0.47 (Hays et al., 2009). The fraction of FPBA not recovered in urine is likely to be eliminated via faecal excretion or as unmonitored other metabolites in urine (Hays et al., 2009; Leng et al., 1997a).

Epidemiological studies on cyfluthrin did not allow for establishing a relationship between the internal biomarker concentration and critical health effects. In line with the methodology for deriving HBM-GVs, the second option was explored: whether a TRV is available and adequate to derive an HBM-GV. There are 6 evaluations by recognized bodies on cyfluthrin and β-cyfluthrin with derivations of TRVs. Four of them are current, from the years 2018–2020 (EC, 2020/EFSA, 2020 – in the frame of the plant protection products regulation; EC, 2018d – biocidal products regulation; US EPA, 2020; Health Canada, 2018). According to these assessments, the critical effect of cyfluthrin/β-cyfluthrin is neurotoxicity, and quantitatively, beta-cyfluthrin, being the biologically active component of cyfluthrin, is more potent than cyfluthrin. However, biomonitoring via the nearly specific metabolite FPBA no longer allows a distinction to be made here. In studies with several animal species, acute neurotoxic effects were observed at doses lower than those leading to other adverse effects or histological changes in organs. Based on the available findings, there is no evidence of genotoxic or carcinogenic effects of cyfluthrin/β-cyfluthrin. Effects on fertility or development were not or only observed at higher doses, and β-cyfluthrin was concluded unlikely to have endocrine-disrupting properties.

An ADI value of 0.01 mg/kg bw/d was derived from a 4-week rat study with β-cyfluthrin under the EU plant protection products framework (EC, 2017a; EC, 2017b; EC, 2020; EFSA, 2020). This ADI was based on a NOAEL of 1 mg/kg bw/d for acute neurotoxicity, using a standard assessment factor of 100. The rationale for selecting this study as a key study was as follows: The acute toxicity/neurotoxicity of β-cyfluthrin/cyfluthrin is the critical endpoint. This applies also to repeated-dose studies. Due to intensive metabolism and rapid excretion of β-cyfluthrin/cyfluthrin, daily administrations of β-cyfluthrin/cyfluthrin are considered to represent a sequence of acute intoxications. With respect to the occurrence of clinical symptoms, the lowest NOAEL from the repeated-dose study is 1 mg/kg bw/d. Clinical symptoms were evident at the next higher dose of 4 mg/kg bw/d. For cyfluthrin, an ADI of 0.02 mg/kg bw/d was derived under the EU biocidal products regulation based on the NOAEL of 2.0 mg/kg bw/d from an acute neurotoxicity study with rats as well as from a 90 days neurotoxicity study with rats (EC, 2018d). An acute RfD for β-cyfluthrin was derived by the U.S. EPA based on the acute neurotoxicity study in rats by Wolansky et al. (2006) in which 2 h after dosing the decrease in total motor activity was assessed (ED30). EPA performed a benchmark calculation to derive a $BMDL_{1SD}$ of 1.17 mg/kg bw/d. Factors of 10 each for inter- and intraspecies extrapolation were used to derive the acute dietary RfD of 0.0117 mg/kg bw/d for all age groups (US EPA, 2020). The ADI of 0.005 mg/kg bw/d for β-cyfluthrin derived by Health Canada (2017; 2018) is based on a $BMDL_{20}$ of 1.4 mg/kg bw/d for decreased motor activity also from the Wolansky et al. (2006) study. If it is taken into account that Health Canada applies an additional "pesticide factor", which the European bodies do not use, the values in the overall view are very similar despite different approaches and suggest to take the ADI of 0.01 mg/kg bw/d for β-cyfluthrin/cyfluthrin to further derive HBM-GVs.

The ADI as external TRV can be translated into a corresponding $HBM\text{-}GV_{GenPop}$ by using the following mass balance equation, assuming steady-state conditions:

$$HBM\text{-}GV_{GenPop} = \frac{\mathbf{ADI} \bullet \left[\frac{\mathbf{MW(FPBA)} \bullet \mathbf{Fue(FPBA)}}{\mathbf{MW(Cyfluthrin)}}\right]}{\mathbf{Daily\ urinary\ flow\ rate\ adjusted\ to\ the\ bw}}$$

Here is MW the molecular weight (FPBA: 232.2 g/mol, cyfluthrin: 434.3 g/mol, ratio: 0.535) and Fue the molar excretion factor 0.47 for FPBA over two days (Leng et al., 1997a). Relating, in a simplified approach, the calculated excretion to the weight proportional amount of urine per day (0.03 L/kg bw/d for children and 0.02 L/kg bw/d for adults (Apel et al., 2020b), the ADI for β-cyfluthrin/cyfluthrin corresponds to the following concentration of FPBA in urine:

Children: 0.01 mg/kg bw/d•0.535•0.47:0.03 L/kg bw/d = 0.0838 mg/L

Adults: 0.01 mg/kg bw/d•0.535•0.47:0.02 L/kg bw/d = 0.1256 mg/L

On this basis, HBM-GV_{GenPop} of 80.0 μg FPBA/L urine for children and 130 μg FPBA/L urine for adults were derived for the interpretation of biomonitoring results on cyfluthin exposure in the general population.

The proportion of cyfluthrin excreted as FPBA in human urine after oral ingestion is subject to great uncertainty, the reported value of 25% expressed as a mass fraction (47% on a molar basis) of an oral dose is based on a single study with a single individual. Other human inhalation exposure studies to cyfluthrin suggest a factor of about three in the interindividual variability of the amount of FPBA excreted in urine. Therefore, the level of confidence related to this aspect is rated as low and additional toxicokinetic studies or the development of appropriate models are recommended to improve the level of confidence in this respect. The overall level of confidence in the HBM-GV is rated as "medium" due to the large database on toxicity.

For information and comparison, the HBM-GVs derived here are lower by a factor of 2–5 compared to the BE values derived by Hays et al. in 2009 (200–400 μg/L total FPBA in urine). This is partly due to the fact that here the ADI for beta-cyfluthrin was used, but also because the health-based exposure guidance values for cyfluthrin from various agencies have been lowered over time. In addition, it should be noted that Hays et al. used slightly different anthropometric and physiological parameters for calculating the BE: average bodyweights from US EPA (2008), average 24-h urine volumes for children from Remer et al. (2006), average 24-h urine volumes for adults from Perucca et al. (2007). Hays et al. (2009) note that the appearance of FPBA in urine has a half-life of about 6 h so that one day corresponds to four half-lives, resulting in approximately 94% of total excretion of FPBA in the first day following an acute exposure. Therefore, a BE for acute exposure will almost be identical to a BE for a chronic RfD or ADI assuming steady-state (100% of a daily dose excreted per day).

The limits of quantification and detection (LoQs/LoDs) of analytical methods applied are at least about two or three orders of magnitude lower than the HBM-GV proposed. For example, a sensitive method was described by Schettgen et al. (2016) for the simultaneous quantification of eight metabolites of synthetic pyrethroids, including FPBA, in urine of the general population. The method comprises acid hydrolysis of the conjugates, followed by a pH-controlled extraction into n-hexane, derivatization with n-tert-butyldimethylsilyl-n-methyltrifluoroacetamid (MTBSTFA) and quantification by GC/MS/MS using deuterium- and 13C-labelled standards. This method has a limit of quantification of 0.01 μg/L. The 5th report on human biomonitoring of environmental chemicals in Canada describes a method using hydrolysis with glucuronidase followed by extraction, derivatization with hexafluoro-2-propanol and diisopropylcarbodiimide and GC/MS detection using 13C-FPBA as an internal standard. For this method, a LoD of 0.006 μg/L is reported (Health Canada, 2019).

3.2.2. Phthalates and DINCH

Phthalates have long been used in the manufacture of flexible PVC and are found in many consumer-related products. Some are considered as substances of very high concern (SVHC) and are meanwhile regulated, so the effectiveness of restrictions as well as the change of use patterns of substitutes should be controlled by HBM. For selected substances of this substance group and DINCH as a substitute, HBM-GVs for the general population were derived (Lange et al., 2021). In 5 out of 6 cases, the basis of derivation was a TRV, specifically for DEHP (TDI), DnBP (DNEL), DiBP (DNEL), DPHP (RfD) and DINCH (TDI). HBM-GVs derived for adults and children, refer with regard to the phthalates DEHP, DnBP, and DiBP to anti-androgenicity, in view of DPHP to effects on the thyroid and in view of DINCH to nephrotoxicity. Table 2 summarizes the essential information on critical impact endpoints, TRVs, toxicokinetic aspects, relevant biomarkers of exposure and numerical values for the HBM-GV_{GenPop} together with their level of confidence. With regard to BBzP, Lange et al. (2021) decided not to use the TDI value of 0.5 mg/kg bw/d (EFSA, 2005b) for deriving HBM-GVs for the general population. This TDI is based on reduced anogenital distance in F1 and F2 males at 250 mg/kg bw/d, however Howdeshell et al. (2008) showed that BBzP has similar potency to suppress foetal testosterone as DEHP, DiBP and DnBP. Therefore, it cannot be ruled out that effects could be observed at lower doses than the 50 mg/kg bw/d dose tested in the key study used by EFSA. Thus, Lange et al. (2021) decided to use other toxicologically relevant values derived from experimental studies with animals (see Table 3).

3.2.3. Bisphenols – bisphenol A (BPA)

Human exposure to bisphenol A (BPA) is widespread and of particular concern because of its known endocrine-disrupting properties. BPA is considered to be more toxicologically active than the conjugated forms (e.g. BPA-glucuronide (BPA-G) and BPA-sulfate (BPA-S)), and its measurement in blood would be a better surrogate of the biologically effective dose. However, considering the difficulty of implementing blood sampling in large HBM cohorts, as well as the current analytical capacities, total BPA in urine (i.e. the sum of free and conjugated forms of BPA measured after hydrolysis of phase II metabolites) was retained as the relevant exposure biomarker for BPA. The derivation of the HBM-GVs for total BPA by Ougier et al. (2021a) was made based on the concentrations of urinary total BPA equivalent to the steady-state exposure to the temporary Tolerable Daily Intake (t-TDI) of 4 μg/kg bw/day set in 2015 by the European Food Safety Authority (EFSA). HBM-GV_{GenPop} for total BPA in urine of 230 μg/L and 135 μg/L for adults and children, respectively, were developed on the basis of toxicological data and the human physiologically-based pharmacokinetic (PBPK) model developed by Karrer et al. (2018), assuming an oral exposure to BPA at the t-TDI level averaged over 24 h (Ougier et al., 2021a).

For workers, dermal uptake of BPA is suspected to contribute substantially to the total BPA body burden, which, in comparison with the oral route, is generating a higher ratio of free BPA to total BPA in blood. Therefore, the steady-state concentration of urinary total BPA was estimated after a dermal uptake of BPA that would generate the same concentration of free BPA in plasma as would a 24h-averaged intake according to the European Chemicals Agency's oral DNEL of 8 μg BPA/kg bw/day set for workers (ECHA, 2015). The predicted concentration of urinary total BPA at steady-state is equivalent to, or exceeds the 95th percentile of total BPA in urine measured in different European HBM studies conducted in the general population. Thus, no HBM-GV_{Worker} was proposed by Ougier et al. (2021a), as the high background level of BPA coming from environmental exposure - mostly through food intake - is making the discrimination with the occupational exposure to BPA difficult.

The EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP) recently released for consultation a re-evaluation of their t-TDI set in 2015 (EFSA, 2021). According to this proposal, the new TDI would be established at 0.04 ng/kg bw/d of total BPA. This value is 10^5 times lower than the current t-TDI. In fact, when recalculating the HBM-GVs for total BPA in urine of the general population, on the basis of this new TDI proposal, and by the means of the PBPK model by Karrer (2018), the HBM-GV would have to be divided by 10^5 (2.3 ng/L for adults and 1.4 ng/L for children). Thus, the risk for consumers related to BPA exposure would be of concern with regard to this new value proposed, and protective measures would need to be taken (Meslin et al., 2022).

3.2.4. Mycotoxins – deoxynivalenol (DON)

Deoxynivalenol (DON) is a naturally occurring mycotoxin produced mainly by Fusarium graminearum and F. culmorum. The general population is exposed to DON through the consumption of contaminated foodstuff, especially grain-based products. Maximum levels for DON in foodstuffs for human consumption have been established in Regulation (EC) No 1881/2006 (EC, 2006). In the absence of human data to derive an HBM-GV_{GenPop} for DON, and in the absence of new toxicological studies showing chronic effects other than reduction in body weight gain

Table 3
Derivation of HBM-GVs according to option 3, based on a POD.

Substance/ Exposure biomarker/ Biological material	Critical effect	POD [mg/kg bw/d]/key study/Assessment factors (AFs)	Toxicokinetics	HBM-GV_{GenPop} [mg/L]	HBM-GV_{Worker} [mg/L]	Level of confidence (LoC)
DEHP/5-cx-MEPP/urine	Aspermatogenesis and reprotoxicity	NOAEL = 5.8 (David et al., 2000) Total AF: 50	Mass balance approach, 5-cx-MEPP, $Fue_{(48h/24h)}$: 0.139/0.132 (Anderson et al., 2011)		0.62	Medium
DnBP/MnBP/ urine	After *in utero* exposure (GD 12 – GD 19): Reduction of foetal testosterone and reduction in expression of key genes encoding proteins involved in cholesterol transport and steroidogenesis	NOEL = 10 (Lehmann et al., 2004) Total AF: 100	Mass balance approach, $Fue_{(24h)}$: 0.69 Anderson et al. (2001)		3	Medium
DiBP/MiBP/urine	After *in utero* exposure (GD 12 – GD 19): Reduction of foetal testosterone and reduction in expression of key genes encoding proteins involved in cholesterol transport and steroidogenesis	POD = 12.5 Read across with DnBP considering 25% potency difference (Lehmann et al., 2004) Total AF: 100	Mass balance approach, Fue $_{(24h)}$: 0.7 (Koch et al., 2012)		3.5	Low
BBzP/MBzP/ urine	After *in utero* exposure: Suppression of foetal testicular testosterone production; reduced serum testosterone and reduced epididymal sperm count and motility in F1 adult rats	LO(A)ELs: 100 (Furr et al., 2014/Ahmad et al., 2014) Total AF: 900	Mass balance approach, $Fue_{(24h)}$: 0.73 Anderson et al. (2001)	Children: 2 Adults: 3	3	Medium
DPHP/oxo-MPHP & OH-MPHP/ urine	Follicular hypertrophy of the thyroid gland in F0 rats	Human equivalent $BMD_{10}L_{95}$: 6.1 (BASF, 1995; Bhat et al., 2014) Total AF: 45	Mass balance approach, oxo-MPHP, $Fue_{(24h)}$: 0.0795;		0.7	Low
DPHP/oxo-MPHP/urine			OH-MPHP, $Fue_{(24h)}$: 0.0597		0.4	
DPHP/OH-MPHP/urine			(averaged values, Leng et al., 2014 & Klein et al., 2018)		0.3	
BPS/Total BPS/ urine	Mammary gland and neuro-behavioural toxicity	LOAEL: 0.002 (Kolla et al., 2018, 2019; Catanese and Vandenberg, 2017) Total AF: 75	PBPK model by Karrer et al. (2018), 24h constant (worker discon-tinuous) exposure to 0.0266 µg/kg bw/day	0.001	0.003	Medium-low
NMP/5-HNMP & 2-HMSI/urine	Maternal and developmental toxic effects seen in a study on developmental toxicity	NOAEL: 125 (Saillenfait et al., 2002) Total AF: 300	Mass balance approach, 5-HNMP, $Fue_{(9d)}$: 0.44; 2-HMSI, $Fue_{(9d)}$: 0.20 Åkesson & Jönsson (1997)	Children: 10 Adolescents/ adults: 15		Medium
NEP/5-HNEP & 2-HESI/urine	Foetus weight, variations of the skeleton seen in a study on developmental toxicity	NOAEL: 50 LOAEL: 250 (Saillenfait et al., 2007) Total AF: 100	Mass balance approach, 5-HNEP, $Fue_{(96h)}$: 0.289; 2-HESI, Fue $_{(96h)}$: 0.216 Koch et al. (2014)	Children: 10 Adolescents/ adults: 15		Medium-low
DMAC/tNMAC/ urine	Liver toxicity	NOAEC = 25 ppm (Malley et al., 1995) Total AF: 12.5	Correlation atmospheric DMAC and urinary tNMAC: LnCNMAC (mg/g cr) = 0.894 × lnCDMAC(ppm) + 2.47 R2 = 0.54 (Spies et al., 1995a) Log(CNMAC mg/g cr) = 0.685 + 0.455 log (CDMAC mg/m^3) R2 = 0.497 and $p < 0.001$ (Qian et al., 2012)		12 mg/g creat for total NMAC	Medium-low

HBM-GV_{GenPop} = Human biomonitoring guidance value for the general population; DEHP = diethyl hexyl phthalate; 5-oxo-MEHP = mono(2-ethyl-5-oxohexyl) phthalate; 5-OH-MEHP = mono(2-ethyl-5-hydroxyhexyl) phthalate; 5-cx-MEPP = mono (5-carboxy-2-ethylpentyl) phthalate; DnBP = di-n-butyl phthalate; MnBP = monobutyl phthalate; DiBP = diisobutyl phthalate; MiBP = monoisobutyl phthalate; BBzP = butyl benzyl phthalate; MBzP = monobenzyl phthalate; DPHP = bis-(2-propylheptyl) phthalate; oxo-MPHP = mono(propyl-6-oxo-heptyl) phthalate; OH-MPHP = hydroxy-mono-propylheptyl phthalate; BPS: bisphenol S; NEP: N-methyl-2-pyrrolidone; 5-HNMP: 5-hydroxy-N-methyl-2-pyrrolidone; 2-HMSI: 2-hydroxy-N-methylsuccinimide; NEP: N-Ethyl-2-pyrrolidone; 5-HNEP: 5-hydroxy-N-ethyl-2-pyrrolidone; 2-HESI: 2-hydroxy-N-ethylsuccinimide; DMAC: N,N-dimethylacetamide; tNMAC: total N-methylacetamide; Fue = fractional urinary excretion coefficient or urinary excretion factor; AF = assessment factor; POD = point of departure; bw = body weight; NO(A)EL = no observed (adverse) effect level; LOAEL = lowest observed (adverse) effect level; NOAEC = no observed adverse effect concentration; BMDL = benchmark dose lower confidence limit.

as critical, the group TDI for the sum of DON and its acetylated and modified forms set by EFSA in 2017 (EFSA, 2017) was used by Van den Brand et al. (2021) as a starting point for deriving an HBM-GV_{GenPop}. EFSA's Panel on Contamination in the Food Chain (CONTAM) decided to characterize the hazard for the sum of DON, its acetylated forms (3-Ac-DON, 15-Ac-DON) and DON-3-glucoside together, both for chronic and for acute adverse health effects in humans, assuming that i) 3-Ac-DON, 15-Ac-DON and DON-3-glucoside are all metabolized to DON and absorbed to the same extent as DON, ii) the acetylated forms of DON induce the same acute and chronic adverse health effects as DON and iii) similar acute and chronic adverse health effects of DON-3-glucoside as of DON cannot be excluded. The key study (Iverson et al., 1995) selected by EFSA (2017) is a two-year long-term study on mice. Data for both sexes were combined and a lower 95% confidence limit for a benchmark response of 5% additional risk ($BMDL_{05}$) of 0.11 mg/kg bw/d for reduced body weight gain was calculated by EFSA (2017). A group TDI of 1 μg DON/kg bw/d was then set for the sum of DON, 3-Ac-DON, 15-Ac-DON and DON-3-glucoside by using the default assessment factor of 100 for inter- and intraspecies variability. Since the BMDL values calculated for developmental and reproductive toxicity were all larger than the $BMDL_{05}$ of 0.11 mg DON/kg bw/d, the CONTAM Panel concluded that the group TDI was also protective for developmental and reproductive toxicity (EFSA, 2017).

Regarding toxicokinetic data in humans, there is one comprehensive quantitative study on DON urinary excretion profile and metabolism after oral exposure (Vidal et al., 2018; Mengelers et al., 2019). This intervention study involved 20 adult volunteers, including 11 women (55%) and 9 men (45%) at a mean age of 32 years, range 18–61 years. The volunteers followed a diet free of cereals and cereal products for two days before and two days after the administration date. 16 volunteers were administered a single oral dose of DON at the level of the group TDI (1 μg/kg bw), while 4 volunteers served as controls. The individual urine was collected during 24 h after administration and samples were analysed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). DON was rapidly excreted within 24 h, the total DON recovered (free DON + DON glucuronides) was 64.0 ± 22.8% of the administered DON on a molar basis. DON-15-glucuronide was the most prominent urinary biomarker followed by free DON and DON-3-glucuronide. In a further approach, Mengelers et al. (2019) determined the so-called 'reverse dosimetry factor' (RDF). The RDF is the ratio of the intake of a substance to the calculated cumulative excreted amount and is the inverse of the urinary excretion factor Fue. Because the distribution of the ratios was skewed, a logistic transformation was applied to calculate the confidence bounds. According to this, the RDF for total DON is 1.45 (CI 1.03–7.10) and the Fue for total DON is 0.69 with a confidence interval of 0.14–0.97. The derivation of the HBM-GV by Van den Brand et al. (2021) is based on this urinary excretion factor (Fue) of total DON calculated by Mengelers et al. (2019) at 0.69.

Due to the fact that a single dose of DON in humans is almost completely excreted within 12 h of the last exposure to DON and even in case of three exposure time points per day (breakfast, lunch and evening meal) a steady-state will not be reached because the elimination half-lives of all biomarkers are shorter than the dosing interval, a large variation is expected in the excreted amount of DON in a spot urine sample. Thus, it was decided to relate the HBM-GV for DON in urine to a 24-h urine sample, with an average 24h urinary flow rate of 29.4 mL/kg bw/d, as determined by Mengelers et al. (2019):

$$\mathrm{HBM-GV_{GenPop}} = \frac{\textbf{Group TDI} \bullet \textbf{Fue (total DON in 24h)}}{\textbf{Daily urinary flow rate adjusted to the bw}}$$

On this basis the calculated HBM-GV for the general population is (1 μg/kg bw/d•0.69): 29.4 mL/kg bw/d = 0.023 mg/L or 23 μg/L. A differentiation according to age was not made.

The level of confidence in this value is regarded as "medium". Using the confidence interval around the Fue, the following confidence interval around the HBM-GV for total DON was calculated: 0.005–0.033 μg/mL. Due to the short elimination half-life of total DON (<3h) the HBM-GV for DON (23 μg/L) cannot be used for a spot urine sample because the variation in the total DON concentration in such a sample will be much larger than the confidence interval calculated for a 24h urine sample (Van den Brand et al., 2021).

3.3. Third option: HBM-GVs based on a point of departure from an experimental (animal) study

According to option 3, the derivation of HBM-GVs from experimental (here animal) test results, values for nine substances were derived within the framework of HBM4EU: for the phthalates DEHP, DnBP, DiBP, and DPHP for workers; for the phthalate BBzP as well as for bisphenol S (BPS) for both, the general population and workers; for the solvents N-methyl-2-pyrrolidone and N-ethyl-2-pyrrolidone for the general population, and for the solvent N,N-dimethylacetamide for workers. The information on their derivation and the respective numerical values are summarized in Table 3.

3.3.1. Phthalates

HBM-GVs for selected phthalates to apply to workers have all been derived from PODs of animal studies, in particular for DEHP, DnBP, DiBP, BBzP, and DPHP. In addition, the HBM-GV for BBzP to apply to the general population, was also derived on the basis of two PODs from animal studies (Lange et al., 2021). For DEHP, DnBP, DiBP and DPHP, the critical study chosen for deriving the corresponding occupational HBM-GV differs from that chosen for deriving the HBM-GV_{GenPop}, as such studies were chosen that most closely reflect the exposure scenario at the workplace. That is, experimental designs with exposure of adult animals, including exposure in the first trimester of pregnancy, are considered more transferable to the working population. On the other hand, women should no longer be occupationally exposed to classified reproductive toxins from the time they report their pregnancy to the employer (usually from the second trimester of pregnancy) and during breastfeeding, so that this exposure period is considered rather inappropriate.

HBM-GVs for the phthalates DEHP, DnBP, DiBP and BBzP refer to anti-androgenic effect endpoints, for DPHP to effects on the thyroid. Regarding the HBM-GV_{Worker} for DnBP, DiBP and BBzP, the selected critical endpoints are related to the effects on the offspring after *in utero* exposure. Since in this case no differences in the sensitivity of fetuses carried by women from the general population and fetuses carried by working women can be assumed, a default assessment factor (AF) of 10 was applied to the respective PODs for within-species differences instead of the default value of 5 normally applied for systemic effects on the working population as a more homogeneous population. As the critical endpoint selected for deriving the DEHP HBM-GV_{Worker} is bilateral aspermatogenesis observed in adult rats, an AF of 5 was retained in that case. Table 3 provides an overview of the most important parameters to derive the HBM-GVs as well as the relevant biomarkers of exposure and the numerical values themselves.

3.3.2. Bisphenols – bisphenol S (BPS)

BPS is used for epoxy resins, in coatings for cans, in the production of plastics, especially in polycarbonate plastics, as additive in dyes and tanning agents (Chen et al., 2016), and in thermal paper as a substitute for BPA (ECHA, 2020d; Björnsdotter et al., 2017). Absorption of BPS by humans occurs predominantly orally while dermal absorption of BPS by humans proved to be limited (Khmiri et al., 2020). This section summarizes the derivation of HBM-GVs for BPS previously published by Meslin et al. (2022). According to the authors, available human data on BPS is insufficient for the derivation of HBM-GVs, and no TRVs have yet been published by the EU institutions or other recognized international organizations. However, numerous results from animal experiments with BPS show convincingly adverse effects on female reproduction,

neurodevelopment and mammary gland (Beausoleil et al., 2022). The sensitivity to BPS is clearly dependent on the timing of exposure, with specific periods of development being critical. Disruption of estrogenic signaling is likely central in the mediation of these effects although other modes of action may be involved (Almeida et al., 2019; Le Magueresse-Battistoni et al., 2018; Mhaouty-Kodja et al., 2018). The LOAELs of 2 μg/kg bw/d for mammary gland and neurobehavioral toxicity determined in oral studies with perinatal exposure of mice (Kolla et al., 2018, 2019; Catanese and Vandenberg, 2017) were selected by Meslin et al. (2022) as POD for the derivation of HBM-GVs. Appreciating that the clearance of BPS is mainly driven by its glucuronidation and that major differences exist between BPS and BPA toxicokinetics with a lesser capacity for elimination and a 100-fold higher oral bioavailability of BPS compared to BPA, Meslin et al. (2022) translated the POD into the corresponding human urine level of total BPS by using the PBPK model by Karrer et al. (2018). This was based on a 100% oral exposure scenario for the general population as well as for workers. While continuous exposure was assumed for the general population, the workers exposure was adjusted to occupational conditions (discontinuous, 5 days per week, 8 h per working day followed by a non-exposure period of 16 h). Once the human equivalent dose was derived, different assessment factors were applied to estimate the HBM-GVs. A factor of 3 was applied for extrapolating the LOAEL to the NOAEL, an assessment factor of 2.5 for remaining interspecies differences (mostly for toxicodynamic differences) and a factor of 10 accounting for intra-species differences. Generally, this factor is set to 5 when workers are the targeted population for which the HBM-GV is derived (ECHA, 2012). However, as the most sensitive endpoint(s) to be protected from are the effects on the unborn child, no differences can be assumed between the foetuses of the general population and of the workers. The HBM-GV_{GenPop} was rounded to 1.0 μg/L and should be applied to the whole general population. The HBM-GV_{Worker} was rounded to 3.0 μg/L. The level of confidence attributed to the HBM-GVs for the general and working population was set to 'medium-low' (Meslin et al., 2022).

3.3.3. Solvents

3.3.3.1. Pyrrolidones. N-methyl-2-pyrrolidone (NMP) is an aprotic and medium polar organic solvent which is completely miscible with water. It is used e.g. for extraction purposes in the petrochemical industry, in the production of polymers (membranes), in stripping and cleaning applications in the microelectronics industry, in coating products, in waterborne paints and in functional fluids like coolants, insulators, refrigerants, hydraulic fluids. Different consumer products may include NMP, such as (printer) inks, toners, coatings, cleaners (RIVM, 2013). N-Ethyl-2-pyrrolidone (NEP), also a polar aprotic solvent, is used in many applications as substitute for the structural analogue NMP.

Consumers are expected to be exposed to NEP by using anti-freezing products, coating products, lubricants and greases, adhesives and sealants, air care products, non-metal-surface treatment products, inks and toners, leather treatment products, polishes, waxes and cleaning products (ECHA, 2011b). Exposure to both substances is mainly via the skin and the respiratory tract.

Specific biomarkers of exposure in urine are 5-HNMP (5-hydroxy-N-methyl-2-pyrrolidone) and 2-HMSI (2-hydroxy-N-methylsuccinimide) for NMP and 5-HNEP (5-hydroxy-N-ethyl-2-pyrrolidone) and 2-HESI (2-hydroxy-N-ethylsuccinimide) for NEP (Åkesson and Jönsson, 1997; Koch et al., 2014; RIVM, 2013). Half-lives of the urine metabolites are 4 h for 5-HNMP, 17 h for 2-HMSI, 7 h for 5-HNEP and 27 h for 2-HESI. David et al. derived in 2021 HBM-GV_{GenPop} for the sum of the respective specific metabolites of NMP and NEP.

Based on the knowledge that reproductive toxicity is the most critical effect of NMP (ECHA, 2020b), one key oral developmental study from Saillenfait et al. (2002) on rats and one supporting oral reproductive toxicity study from Sitarek et al. (2012) on rats were selected for the determination of a POD. Although the oral pathway is secondary due to the indications for use of NMP, oral studies were used for the derivation of HBM-GVs, as a conceivable derivation of HBM-GVs based on animal studies with dermal administration was considered too uncertain due to differences in dermal absorption in rodents and humans. With regard to the question of whether it is possible to extrapolate to the inhalation route, it was investigated and confirmed that the NOAEC of the main inhalation study by Saillenfait et al. (2003), which was used by RIVM (2013) to derive the $DNEL_{inhalation}$ for workers (including pregnant women), would result in a body burden of a similar magnitude.

The study of Saillenfait et al. (2002) resulted in a NOAEL of 125 mg/kg bw/d for maternal and developmental toxic effects, that was only slightly below the 150 mg/kg bw/d that showed already maternal and developmental toxic effects in the study of Sitarek et al. (2012). Since no NOAEL could be determined in the Sitarek et al. study (2012), and since the distance between NOAEL and LOAEL of the two studies is small, an additional assessment factor of 3 was applied to the NOAEL of 125 mg/kg bw/d from the Saillenfait et al. (2002) study to consider the uncertainties in the underlying database. In order to account for inter- and intraspecies differences further assessment factors of 10 each were applied resulting in a TRV-like value of 0.42 mg/kg bw/d. Finally, an HBM-GV_{GenPop} of 15 mg/L for adolescents/adults and 10 mg/L for children was determined for the sum of the selected urine exposure biomarkers 5-HNMP und 2-HMSI with excretion fractions of 44% for 5-HNMP (Fue: 0.44) and 20% for 2-HMSI (Fue: 0.20) (Åkesson and Jönsson, 1997).

Also for NEP, reproductive toxicity is the most critical effect endpoint (ECHA, 2020a) and David et al. (2021) derived the HBM-GV_{GenPop} in this case on the basis of a developmental toxicity study in rats as key study with a LOAEL of 250 mg/kg bw/d and a NOAEL of 50 mg/kg bw/d (Saillenfait et al., 2007), supported by a developmental toxicity study in rabbits with a LOAEL of 200 mg/kg bw/d and a NOAEL of 60 mg/kg bw/d (BASF, 2007). Assessment factors for inter- and intraspecies variability of 10 each resulted in a TRV-like value of 0.5 mg/kg bw/d. Finally, an HBM-GV_{GenPop} of 15 mg/L for adolescents/adults and 10 mg/L for children was calculated for the sum of the selected urine exposure biomarkers 5-HNEP and 2-HESI based on excretion fractions of 28.9% for 5-HNEP ($Fue_{(96\ h)}$: 0.289) and 21.6% for 2- HESI ($Fue_{(96\ h)}$: 0.216) (Koch et al., 2014).

The overall level of confidence (LoC) considering uncertainties in e. g. reliability of the key study used to derive the TRV-like value, uncertainties related to the extrapolations leading to the TRV-like value, to toxicokinetic data on the substance of interest, and to the calculation of the final HBM-GV was set by David et al. (2021) to 'medium' for NMP and 'medium-low' for NEP.

3.3.3.2. N,N-dimethylacetamide (DMAC). In the EU, DMAC is predominantly used in the production of agrochemicals, pharmaceuticals and fine chemicals, accounting for 65–70% of tonnage. About 20–25% of EU tonnage is used in the production of man-made fibres which are mainly used for the production of clothing. DMAC is also used as a solvent in coatings for industrial use (approximately 3–5% of EU tonnage). Non-occupational exposure to DMAC is uncommon. At workplace, DMAC exposure occurs by inhalation and dermal routes. The literature provides occupational studies with relationships between exposure and health effects, and between exposure and biomarkers concentrations. There is no data linking biomarker levels and health effects.

Although the metabolite S-acetamidomethyl-mercapturic acid (AMMA) in urine may be a relevant biomarker of DMAC, sufficient data to allow the derivation of a biological value are according to research by Meslin et al. (2021) only available for total N-methylacetamide (tNMAC), which is the sum of N-hydroxymethyl-N-methylacetamide and NMAC.

After it was determined by Meslin et al. (2021) that a derivation of an HBM-GV according to the second option is not possible, because on the

one hand SCOEL recommendations are outdated, on the other hand assessment factors were applied for the derivation of MAK or ACGIH values, which are neither consistent with ECHA nor HBM4EU methodology, they proposed initially as part of the third option a TRV-like value of 2 ppm. This TRV-like value is based on the NOAEC of 25 ppm for the most sensitive effect endpoint "hepatotoxicity" resulting from the study by Malley et al. (1995), and on the application of assessment factors of 2.5 for the extrapolation to humans and 5 to take into account interindividual variabilities in the working world (note: the Malley et al. (1995) study was also key study for MAK and ACGIH values derivation.).

In a further step, the most relevant studies providing correlations between DMAC concentration in air and tNMAC in urine were selected (Spies et al., 1995; Qian et al., 2012), allowing the calculation of an HBM-GV_{Worker} of 12 mg/g creat for total NMAC based on the TRV-like value. The sampling time recommended for comparison of analytical results with this HBM-GV is at the end of the shift after at least two days of exposure.

The derivation of the HBM-GV_{Worker} presents according to Meslin et al. (2021) many uncertainties: lack of data on relationship between health effects and biomarker levels, calculation of a TRV-like value and the use of a correlation between airborne DMAC levels and biomarkers, despite of a potentially significant dermal contribution on intake. The level of confidence to this value is therefore given "medium-low".

4. Discussion - obstacles in derivation and limitations in interpretation of HBM-GVs

The synopsis of the results shows that the approach of deriving HBM-GVs based on a relationship between human internal biomarker concentrations and adverse health effects, which is considered to be the most suitable, could only be applied for few substances within the framework of the HBM4EU activities. In addition to cadmium and DMF, this also applies to mercury, although further consultation is still required, so that these values have been classified as provisional for the time being. Although numerous results from human studies are available for some substances, including phthalates and pyrethroids, several limitations do not allow to use these data. This is due to the following reasons, among others: Although longitudinal studies are most suitable for linking exposures to health outcomes, a cross-sectional design was often chosen by research groups instead of a longitudinal design, and the results of individual samples do not in themselves allow conclusions to be drawn about earlier events leading to the observed health effects. Furthermore, the health questionnaires that accompany human biomonitoring are not always targeted enough to conclude causality. Many of the studies cannot exclude effects from co-exposures and often the long latency period between exposure and effect makes interpretation difficult. The possibility of exposure misclassification was also considered a limitation, as exposure determination was mostly based on a single sample. In some cases, concentrations of exposure biomarkers were below the LoD of the method, which meant that suboptimal statistical evaluations and/or default assumptions had to be made. Also, sometimes the results of the studies were seen as contradictory or inconsistent and did not necessarily lead to a unified conclusion.

TRV values such as TDI, ADI, or OEL from recognized national and international bodies, on the other hand, represent - as long as they are still current and a minimum of TK data and/or correlations between exposure and exposure biomarker levels are available - a valuable basis for a less complex derivation of HBM-GVs. One drawback here is the update of guidance values which is lagging far behind the generation of new toxicological data and knowledge – this process has to speed up. The working group which derived the HBM-GVs under HBM4EU has made use of this approach in nine cases and it seems appropriate to establish a mutual information mechanism for further activities, so that e.g. upcoming amendments to a TDI, such as for BPA, can be directly included in the derivation process and conversely, research results of large EU projects can be used by European bodies in a timely manner (e.g. elaborations on BPS).

Deriving HBM-GVs based on a POD of an animal experiment can sometimes be more difficult because comparatively few studies are available for some substances. For example, chronic studies may be missing, the number of animal species studied may be small, or the exposure pathway may not be the most relevant for humans. Also, the dosing regimen may not be sufficient to derive meaningful dose-effect relationships or to determine a NOAEL. In these cases, extrapolation is necessary so that the level of confidence for values derived under such conditions is in general lower. In this way, according to the third option, HBM-GVs were derived for nine substances, whose level of confidence lies between low and medium.

When deriving HBM-GVs for the biological medium urine using the simple urine mass balance approach, the proportion of the absorbed substance that is excreted in the form of the respective biomarker over a certain time (Fue) plays an essential role. Unfortunately, this Fue is often calculated on the basis of data from a few volunteers which do not fully cover inter-individual variability, e.g. according to age or gender. Also, usually only a few dose levels are examined. This uncertainty in the calculation of an HBM-GV must be taken into account when interpreting the measured data, as must the body weight-dependant urinary flow rate, which is subject to variation within- and between individuals, but is likely to have increased on average over time. So, the German HBM Commission assumes an excretion rate of 20 mL/kg bw/d for adults. Aylward et al. (2015) came, after a literature review, to a similar statement that the urinary flow rates in adults were consistent across the range of ages from 15 to 80, averaging approximately 20 mL/kg bw/d with no consistent differences between males and females, but a high coefficient of variation (in the order of 100% based on spot samples). Lermen et al. (2019) showed then in 2019 that geometric mean values for daily urinary flow rates of young adults (20–30 years) increased from 1997 to 2016 with a similar rate in both sexes (in males by 32%, from 1532 mL/24 h in 1997 to 2039 mL/24 h in 2016; in females by 36% from 1459 mL/24 h in 1997 to 1987 mL/24 h in 2016) meaning 33.12 mL/kg bw/d for females and 25,5 mL/kg bw/d for males which is confirmed by Mengelers et al. (2019) with a 24-h urinary flow rate of 29.4 $\pm$ 8.5 mL/kg bw/d determined on 20 volunteers. Thus, a certain degree of uncertainty must be assumed, which becomes particularly relevant when measured concentrations are close to the HBM-GV and thus the margin of safety is low. To reduce this uncertainty in general, further data collection, especially on urinary flow rate for children and pregnant women is recommended. It must also be decided whether the median or rather the 95th percentile should be the appropriate reference parameter.

For some substances, PBPK models are available which can predict the absorption, distribution, metabolism and excretion (ADME) of chemicals in humans or animals. These models facilitate the transfer of findings or assumptions from one species to another or the extrapolation from one exposure pathway to another (e.g. inhalation to oral). For BPA, a model is available with which the oral and dermal uptake, the subsequent distribution in blood, and the excretion in urine can be calculated, so that more precise statements on kinetics and a more precise HBM-GV derivation are possible. In order to improve risk assessments, such models need to be developed all the more urgently for substances whose measured biomarker concentration is close to an HBM-GV calculated with simpler one-compartment models. Thus, a tiered approach is suggested, based on the amount and quality of data available: 1st tier one-compartment modelling based derivation of HBM-GV, 2nd tier in cases e.g. where the risk characterization ratio is close to 1, refinement by PBPK modelling, and 3rd tier, most robust, supplementary determination and use of correlations between external exposure to specific chemicals and the concentration of the chemical itself and/or its metabolite(s) in the human body.

Although HBM - compared to external exposure assessment – provides a better estimate of actual exposure by direct measurement of the chemicals or their metabolites in the human body, thus reducing

uncertainties in health-based risk assessment, uncertainties remain in discontinuous exposure to substances with short biological half-lives of the biomarkers of exposure. This is why for the analysis of individual samples, 24 h urine collections instead of spot urine samples are recommended. However, on a population basis, this aspect is not relevant: spot and 24 h urine samples produce comparable results provided that the population is large enough (Christensen et al., 2012) and sampling is at random in relation to meal ingestion and bladder-emptying times. However, the analysis also suggests that caution should be exercised when interpreting the high end of spot sample data sets. For workers, HBM-GVs are recommended associated with a sampling time, which is mainly based on half-lives.

So far, HBM-GVs have only been derived for single substance risk assessment. But different substances with similar mechanisms of action occur simultaneously in the environment. For the assessment of such real mixtures, the Hazard Index Approach can be applied (Apel et al., 2020a; Lange et al., this issue) or a MAF (mixture assessment factor) can be introduced. Other working groups under HBM4EU have explored this topic in more depth and developed initial proposals (Kortenkamp, 2020; Socianu et al., 2022).

5. Conclusions and future challenges

The derivation and application of health-based guidance values in the context of HBM4EU has shown that HBM-GVs are an effective tool for an easy and comprehensible evaluation of HBM results. Used at the population level, these values can not only help to refine public health risk assessment, but also indicate potential regulatory priorities and the need for (additional) measures to reduce exposure or for continuous monitoring. At the individual level a careful interpretation is necessary recognising the limitations of these values and taking into account individual and environmental factors as well as personal behaviours. It must also be kept in mind that HBM data are not necessarily obtained in the most sensitive exposure window, they only show the internal exposure at a certain point in time, which may not be the critical age or life circumstance (for example pregnancy).

The establishment of a commonly agreed upon procedure for deriving HBM-GVs (Apel et al., 2020b), as well as the consultation of experts from all partner countries prior to finalizing the values, has led to this type of assessment tool becoming better known, more widely used and broadly quality assured. Furthermore, even in other working contexts corresponding values have been derived based on the concept paper (Tarazona et al., 2022). These values have not yet gone through a consultation process and are therefore referred to as “provisional” HBM-GVs. Within the framework of the recently launched project PARC (The European Partnership for the Assessment of Risks from Chemicals) this agreement on content is to be made up for.

Since HBM-GVs describe a threshold concentration at and below which no health effect is to be expected, no HBM-GVs can usually be derived for genotoxic carcinogens. Nevertheless, an analogous calculation method can be used to derive risk-based values for internal exposure from external doses that relate to specific additional lifetime cancer risks. This was done for chromium VI by deriving an HBM Exposure Equivalent for Cancer Risk (HBM-EECR) in the amount of 8 μg/g creatinine for workers of the chrome-plating sector (corresponding to an excess lifetime risk of 20 cases of lung cancer per 1000 workers) (Sissoko et al., 2022).

The work on the derivation of HBM-GVs is to be continued under PARC, whereby PBPK modelling and the evaluation of HBM results on substance mixtures will play a greater role. It is also planned to intensify the mutual transfer of information at international level (i-HBM Working Group; OECD Occupational Biomonitoring, see references) in order to avoid duplication of work and to achieve mutual recognition of interpretation standards.

New scientific findings may require a re-evaluation of substances. HBM-GVs must therefore be checked from time to time with regard to new scientific findings and updated if necessary.

Declarations of interest

None.

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Human urinary arsenic species, associated exposure determinants and potential health risks assessed in the HBM4EU Aligned Studies

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ABSTRACT

The European Joint Programme HBM4EU coordinated and advanced human biomonitoring (HBM) in Europe in order to provide science-based evidence for chemical policy development and improve chemical management. Arsenic (As) was selected as a priority substance under the HBM4EU initiative for which open, policy relevant questions like the status of exposure had to be answered. Internal exposure to inorganic arsenic (iAs), measured as Toxic Relevant Arsenic (TRA) (the sum of As(III), As(V), MMA, DMA) in urine samples of teenagers differed among the sampling sites (BEA (Spain) > Riksmaten adolescents (Sweden), ESTEBAN (France) > FLEHS IV (Belgium), SLO CRP (Slovenia)) with geometric means between 3.84 and 8.47 μg/L. The ratio TRA to TRA + arsenobetaine or the ratio TRA to total arsenic varied between 0.22 and 0.49. Main exposure determinants for TRA were the consumption of rice and seafood. When all studies were combined, Pearson correlation analysis showed significant associations between all considered As species. Higher concentrations of DMA, quantitatively a major constituent of TRA, were found with increasing arsenobetaine concentrations, a marker for organic As intake, e.g. through seafood, indicating that other sources of DMA than metabolism of inorganic As exist, e.g. direct intake of DMA or via the intake of arsenosugars or -lipids. Given the lower toxicity of DMA(V) versus iAs, estimating the amount of DMA not originating from iAs, or normalizing TRA for arsenobetaine intake could be useful for estimating iAs exposure and risk. Comparing urinary TRA concentrations with formerly derived biomonitoring equivalent (BE) for non-carcinogenic effects (6.4 μg/L) clearly shows that all 95th percentile exposure values in the different studies exceeded this BE. This together with the fact that cancer risk may not be excluded even at lower iAs levels, suggests a possible health concern for the general population of Europe.

1. Introduction

Arsenic (As) is omnipresent in the environment. In the past it was used in e.g. pesticides and wood treatment products which led to a wide disperse environmental contamination. Local contamination can be found near metal producing/processing industries. Soil arsenic concentrations originating from natural sources are generally lower in Northern European countries compared to Southern (Tarvainen et al., 2013). Local or regional natural hotspots from geological origin exists as

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Abbreviations

As(III)	arsenite
As(V)	arsenate
MMA	monomethylarsonic acid
DMA	dimithylarsinic acid
AB	arsenobetaine
As_{tot}	total arsenic
TRA	toxic relevant arsenic

well (e.g. Cornwall, N-Italy, Hungary), which influence groundwater As concentrations. Untreated well water is sometimes used as drinking water or for e.g. showering, watering of vegetables. Thus, direct intake of well water, which can be relatively rich in As concentrations, or intake of vegetables irrigated with such water, can also lead to substantial human As exposures. Overall, concentrations in drinking water distributed in the EU are well below the WHO provisional guideline value of 10 μg/L (WHO, 2011) which is based on technical limitations rather than evidence from toxicological & epidemiological studies (WHO, 2018). Exceedances of the WHO guideline value are registered in several EU countries, which is mainly due to regional natural hotspots (Van Halem et al., 2009).

Arsenic concentrations in food differ across the EU and are related to concentrations in agricultural soils. Some items that need attention are e.g. cereals (rice), grains and mushrooms because they may contain relatively high As concentrations at weight basis (USFDA, 2022). But also seafood (fish, mollusks, crustaceans, seaweed) and food supplements based on seafood can contain relatively large As concentrations (Gao et al., 2018). However, arsenic in the environment is present as numerous chemical species, differing greatly in properties and toxicity (Francesconi et al., 2002; Hughes, 2002). As species observed in the environment are a. o. inorganic iAs (arsenite As(III), arsenate As(V)), arsenobetaine (AB), methylated arsenic species and organoarsenicals (sugars, lipids). Main sources of iAs human exposure already identified are the use of As containing well water, the consumption of rice and grains (EFSA, 2021). Arsenobetaine, DMA and organoarsenicals were already identified to be present in seafood (Luvonga et al., 2020) with AB the predominant species in finfish and shellfish and arsenosugars and arsenolipids the predominant species in algae (Krishnakumar et al., 2016). Mushrooms contain DMA, AB and some iAs as well (Braeuer and Goessler, 2019).

Overall, iAs is considered as the most toxic arsenic species. In the human body iAs is metabolized to MMA (monomethylarsonic acid) and DMA (dimithylarsenic acid) (Drobna et al., 2009). Opposite, after ingestion of arsenobetaine (AB) rapid excretion without interaction and toxicity in the body is noted (Lai et al., 2004). Further, there is a variation in toxicity of methylated arsenic species, naturally present in food or metabolized after ingestion of inorganic arsenic, arsenosugars and -lipids. As(III) and As(V) are categorized as nonthreshold Class I carcinogens (IARC, 2012), with acute toxicity of LD50 (lethal dose, 50%) = 15–42 mg/kg body mass, while simple methylated arsenicals are deemed to pose intermediary toxicity (LD50 = 890–10600 mg/kg body weight), and the tetraalkylated compound AB, present in fish and the principal dietary source of arsenic exposure for humans, is considered nontoxic with LD50 = >10000 mg/kg body weight and is primarily eliminated intact by humans in urine (Luvonga et al., 2020). After intake of arsenosugars, DMA(V) is the main species after metabolization (Francesconi et al., 2002; Feldmann and Krupp, 2011; Chen et al., 2019). Arsenosugars show less cytotoxicity than MMA or DMA. Structural characteristics (presence of sugar groups) are important and complexes with enzyme sulfhydryls cannot easily be formed (Andrewes et al., 2004). After intake of arsenolipids, biotransformation to possibly toxic species may take place as for arsenosugars (Taylor et al., 2017). Toxicity data on arsenolipids are limited. Arsenohydrocarbons, a subcategory of arsenolipids, show cytotoxicity (Meyer et al., 2014) and have neurotoxic properties (Witt et al., 2017).

When focusing on urinary arsenic, the sum of urinary inorganic arsenic (iAs = As(III)+As(V)) and metabolites MMA (monomethylarsonic acid) and DMA (dimithylarsenic acid) is often used as an indicator for exposure to toxic inorganic arsenic. However, there is increasing evidence that DMA in human urine is not only originating from the metabolization of inorganic arsenic but also results from direct intake or from the metabolization of organic arsenic compounds (Francesconi et al., 2002; Schmeisser et al., 2006; Yusà et al., 2018). According to Luvonga et al. (2020) also arsenosugars and arsenolipids, which are present at higher levels in e.g. seaweeds can be of importance from a toxicological point of view. Recent toxicokinetic studies show that some organoarsenicals are bio-accessible and cytotoxic (Luvonga et al., 2020).

There is a need for harmonization of analytical methods, statistical processing and reporting of HBM-data to enable a sound comparison of exposure-levels, to obtain information on exposure determinants and e.g. time-trends. Therefore, in this manuscript we investigate data of ongoing and newly conducted human biomonitoring (HBM) studies, aggregated and harmonized by HBM4EU for differences and similarities between the different study countries, for common exposure determinants and to compare biomarker levels with existing risk-based guidance values. This study was carried out under HBM4EU, co-financed under Horizon 2020. The main goal of HBM4EU was to coordinate and advance HBM in Europe in order to provide science based evidence for chemical policy development and improve chemical management (Ganzleben et al., 2017). More information is available on the HBM4EU website (www.HBM4EU.eu). The HBM4EU Aligned Studies are a survey aimed at collecting HBM samples and data as harmonized as possible from existing (national) studies or newly conducted studies to derive current internal exposure data for the European population/citizens across a geographic spread (Gilles et al., 2021). Within the HBM4EU Aligned Studies, arsenic was one of the prioritized chemicals. As metabolites were measured in urine samples of teenagers (12-18y). Samples were sometimes analysed in different laboratories but data were quality assured/controlled (QA/QC) following a scheme designed and implemented in HBM4EU (Esteban López et al., 2021).

2. Methods

2.1. Data

2.1.1. Population

Information about the involved HBM studies and the study design can be found in (Gilles et al., 2021). Arsenic data were obtained from Germany (GerES V-sub; 2015–2017), Sweden (Riksmaten adolescents; 2016–2017), Slovenia (SLO CRP; 2018), Spain (BEA; 2017–2018), France (ESTEBAN; 2014–2016) and Belgium (FLEHS IV; 2017–2018). Characteristics of the study participants and ethics can be found in Gilles et al. (2022). Briefly, the age of participants ranged between 12 and 18 years. A 50:50 ratio of male and female participants, with individuals living in rural areas, in towns/suburbs and in cities, and with at least 10% of individuals from low, medium and high educational level (of the household) was a recommendation for the studies. No samples were taken from persons living in known natural or industrial hot spots.

2.1.2. Urinary arsenic concentrations

Urinary measurements of the following arsenic species were collected: As(III): arsenite, As(V): arsenate, MMA: monomethylarsonic acid, DMA: dimithylarsenic acid, AB: arsenobetaine, As_{tot}: total arsenic, TRA: toxic relevant arsenic. In most studies, TRA was calculated as a sum parameter (see further) except in the ESTEBAN study where it was determined by HPLC-ICPMS as a measured sum of species (As(III), As(V), MMA, DMA). Data were normalized for specific gravity (SG) except

for ESTEBAN where data on SG were not available. Biomarker data were normalized as follows:

$$C_{SG} = \frac{C_{bio}\ (1.024 - 1)}{SG - 1}$$

With C_{SG} the biomarker concentration in urine normalized for specific gravity, C_{bio} the wet weight concentration of the biomarker in urine and SG Specific Gravity. The value 1.024 was taken as average urinary specific gravity (Kuiper et al., 2021).

Methodologies for handling data below limits of detection (LOD) or quantification (LOQ) have been a long-recognized issue. Biomarker data are typically left-censored data; data below a LOD/LOQ for which the true value is unknown are often referred to as "left-censored". Depending on the laboratory, the LOD or LOQ was used as lower limit to report measured values. For measurements indicated as below these limits, possible values were randomly imputed by using a truncated lognormal distribution (R statistical analysis software package; R Core Team, 2018). This imputation was only performed if at least 30% of the concentrations had a value above the LOD or LOQ for a specific biomarker per study. Geometric means (GM) were only calculated if at least a quantification rate of 60% was obtained.

Sum parameters (e.g. TRA or toxic relevant arsenic: As(III)+As(V)+ MMA + DMA; mass based) were only calculated if all of the compounds constituting the sum were analysed in a study and if at least one of the compounds was detected for at least 60%. For the construction of sum parameters, values below LOD/LOQ were substituted by LOD/LOQ divided by 2 (as the randomly imputed biomarker data are not always available if detection frequency <30% as described above). LOD and LOQ values and detection frequencies per study are reported in Table A1 (see supplementary material). For the study of ESTEBAN, information on concentrations of different As species was not available. TRA was reported by ESTEBAN as a measured sum of species without giving information on the contribution of the individual As species.

2.1.3. QA/QC

Within the framework of the HBM4EU project, a complete QA/QC programme offering 95 biomarkers was conducted (Esteban López et al., 2021). The programme included an interlaboratory comparison (ICI) for the analysis of arsenic in urine, covering total arsenic, As(III), As(V), MMA, DMA and arsenobetaine, to assess the comparability and reliability of analytical methods across the participating expert laboratories. Three rounds of ICI were performed from February to March 2020. For each round, two different test samples consisting of 5 mL urine spiked with arsenic at two different concentrations were prepared and sent to the participating expert laboratories for single analysis. Homogeneity and stability assessment of the control materials confirmed that the materials were adequately homogeneous and stable. Consensus values were calculated by averaging the values obtained by the expert labs when the relative uncertainty of the mean was within 17.5%. In order to express the proficiency of the laboratories in a numerical way, Z-scores were calculated using the consensus value and a fixed fit-for-purpose relative target standard deviation (FFP-RSDR) of 25%. Three expert laboratories from different countries participated in the ICI.

2.1.4. Questionnaires

Questionnaires used within the new and ongoing HBM campaigns under HBM4EU were harmonized. Questions about socio-demographic characteristics were included in all studies; the majority of studies also captured information on dietary habits and health status of the participant, lifestyle, and residential environment. For some ongoing campaigns questionnaire data needed to be harmonized *post factum*. Therefore, some information may be available in some studies while not in others. For upcoming HBM studies, base and substance specific harmonized questionnaires are described by (González-Alzaga et al., 2022). Specific questionnaires for teenagers have been developed for arsenic and are available in the HBM4EU library for upcoming studies (https://www.hbm4eu.eu/online-library/).

2.2. Statistical analysis

Statistical analyses were performed in SPSS Statistics 28. Descriptive statistics were calculated. Determinants of variability in As concentration were analysed (ANOVA). Variables considered were among others personal factors such as sex and BMI, questions from the questionnaires related to the living environment as exposure to groundwater, questions related to food consumption, information on socio-economic status (SES; categories for equivalent household income and highest education in household), information on degree of urbanization at the home address (see Table 1). The chosen variables were in the first instance based on associations already observed in literature and science based hypotheses about potentially influencing factors. Studies were analysed separately as based on the questionnaires, not all questions were answered in all studies (see Table 1). Arsenic concentrations were normalized for SG and ln transformed. In a first analysis, linear regression was used to identify single variables which might explain differences in As concentrations with matrix (spot urine or first morning urine) and SG forced into the model. Secondly, a multiple linear regression analysis model was built for each ln-transformed biomarker "per study" starting from variables with $p < 0.2$ in the former analysis and backward selection was applied. Only those variables having a significance level of $p < 0.05$ were kept in the final multiple linear regression model. Variables reflecting socio-economic differences can be proxies for other, underlying, determinants of exposure and were added to the models as final steps. For the multiple linear regression model, only the following sum parameter was considered: sum of As(III), As(V), MMA, DMA. A model with and without arsenobetaine as a covariate was built. Arsenobetaine was taken up in the model to correct for seafood intake. A pooled regression analysis with all studies was not performed seeing that not all information from the questionnaires was available in each study. Additionally, a correlation analysis between different As species was performed on ln-transformed non-imputed values for all studies combined.

3. Results and discussion

3.1. Exposure in Europe and associations between as species

Although many efforts were put to obtain quality and comparable results for arsenic and species there were some limitations due to the approach of the aligned studies. All new arsenic analysis under HBM4EU were done in expert laboratories that participated successfully in the QA/QC HBM4EU programme and all of them analysed all the biomarkers under study: total arsenic, As(III), As(V), MMA, DMA and arsenobetaine. However, some studies provided arsenic results that were generated before HBM4EU. For that reason, the results did not cover all the biomarkers in the different studies and the results cannot be considered at the same level of comparability. In those cases, the HBM4EU Quality Assurance Unit requested information related to the analytical method applied, quality control measures, LOQs, etc. in order to assess the quality and comparability of the results. However, since this retrospective assessment cannot ensure the comparability, different labels were assigned to the data: a) biomarker data quality assured by HBM4EU QA/QC program; b) biomarker data generated before HBM4EU QA/QC program but deemed comparable; c) biomarker data generated before HBM4EU QA/QC program but not deemed comparable.

In Table 2 urinary concentrations of different As species are shown. What is noticed immediately, is that results of recent As analysis are still heterogenous. As explained above not all studies have information on all As species. HBM4EU took a step forward in aligning the design and

Table 1
Overview of considered exposure determinants based on individual characteristics, geographical information and data retrieved from questionnaires per study.

Variable	Study					
	BEA (Spain)	Riksmaten adolescents (Sweden)	GerES V-sub (Germany)	SLO CRP (Slovenia)	FLEHS IV (Belgium)	ESTEBAN (France)
Sex (F/M)	x	x	x	x	x	x
BMI	x	x	x	x	x	x
NUTS region	x	x	x		x	x
Degree of urbanization	x	x	x	x	x	x
Sampling month	x	x	x	x	x	x
Sampling season	x	x	x	All same category	x	x
Frequency consumption of seafood	x	x	x	x	x	x
Frequency consumption of fish food	x	x	x	x	x	x
Frequency consumption of shellfish		x	x	x	x	x
Frequency consumption of meat	x		x	x	x	x
Frequency consumption of organ meat			x	x	x	x
Frequency consumption of poultry			x	x		x
Frequency consumption of milk	x		x	x	x	x
Frequency consumption of eggs			x	x	x	x
Vegetarian (no/yes)		x	x	All same category (no vegetarians)		x
Frequency consumption of vegetables		x	x	x	x	x
Frequency consumption of fruit		x	x	x	x	x
Frequency consumption of bread		x	x	x	x	x
Frequency consumption of cereals			x	x	x	x
Frequency consumption of rice			x	x		x
Recent consumption of rice (3 days before sampling)		x[a]		x	x	
Recent consumption of seafood (3 days before sampling)		x[a]	x	x	x	x
Frequency consumption of juices/sugar drinks			x	x		x
Frequency consumption of local food				x	x	
Frequency consumption of tea/coffee			x	x		x
Using vitamin B supplements (no/yes)				x		
Using folic acid supplements (no/yes)						
Using sea food supplements (no/yes)						
Use of pesticides indoor (no/yes)			x	x	x	x
Type of drinking water (bottled, tap, ground, other)	x			x		x
Tap water source at home (public, private, both public & private)	x	x	All same category	x	x	
Living close to waste incinerator (no/yes/do not know)	x			x		x
Smoking (no/yes)	x	x	x	All same category	x	x
Passive smoking (no/yes)	x		x	x	x	x
Use of alcohol (no/yes)	x	x	x	x	x	
ISCED	x	x	x	x	x	x
Income	x			x	x	x

An x means that data were available.
Data were not always uniformly distributed over different categories of the variables. For some categories the number of participants was small. Therefore in the statistical analysis categories were sometimes combined.
NUTS: https://ec.europa.eu/eurostat/web/nuts/background.
Degree of urbanization: DEGURBA: cities/towns and suburbs/rural areas (Dijkstra, 2014).
ISCED: International Standard Classification of Education, highest education in the household.
Income: equivalent household income.
The food frequency questions had six options according to individual frequency consumption of each item in the prior four weeks: Never, Rarely: <1 time/month, Sometimes: ≤ 1 time/week but ≥ 1 time/month, Often: 2–3 times/week, Very Often: 4–6 times/week, Everyday: ≥ 7 times/week.
[a] Based on registered consumption the day before urine sampling (and not on 3 days).

conduct of national and regional HBM surveys, but more effort is needed to further improve harmonization of As analysis across European studies. The Horizon Europe PARC project (PARC: Partnership on the Assessment of Risks of Chemicals) aims to increase upfront harmonization of HBM study designs (including exposure biomarkers), protocols and questionnaires.

For total arsenic, it is clear that the studies of Riksmaten adolescents (Sweden) and BEA (Spain) have a significantly higher geometric mean As_{tot} concentration (or As(III)+As(V)+MMA + DMA + AB) compared to the studies of FLEHSIV (Belgium) and CRP (Slovenia). This is indicated in Table 2 by the different letters between brackets, showing the result of a Tukey analysis. Total arsenic measured in ESTEBAN was not compared in this analysis as results were generated before HBM4EU and found to be not deemed comparable. For Germany no values were available for total arsenic in HBM4EU. An earlier study described in literature reported for Germany a P50 of 4.5 μg/L and a P95 of 14 μg/L for total arsenic in children of GerES IV (Schulz et al., 2009). This is at the lower part of current findings in other EU countries in HBM4EU, however, as the study was not part of HBM4EU, an exact comparison can't be made.

When focusing on toxic relevant arsenic, absolute GM values of TRA in HBM4EU varied between 3.84 and 8.47 μg/L (normalized for SG; see Table 2). Previous studies in literature reported for TRA a GM of 4.8 μg/L in adolescents of FLEHSII (Schoeters et al., 2012), a median of 4.54 μg/L in children and adolescents of GerES IV (Becker et al., 2008), a GM of 4.26 μg/L in children of Italy (Bocca et al., 2020) and a GM of 2.17 μg/L in children of the CROME-LIFE + study, Slovenia (Stajnko et al.,

Table 2
Speciated HBM urinary As concentrations (μg/L normalized for SG, except for ESTEBAN) in HBM4EU studies.

Study		As (III)	As(V)	MMA	DMA	AB	As_{tot}	As(III)+As (V)+MMA + DMA +AB	As(III)+ As(V) +MMA	TRA = As(III)+ As(V)+MMA + DMA	TRA/(As(III)+As (V)+MMA + DMA + AB)[a]	TRA/ As_{tot}[a]
FLEHS IV (Belgium; n=148)	GM	0.19 (A)[d]	NC	0.39 (A)[d]	3.46 (BC)[d]	1.25 (A)[d]	NA	9.24(A)	0.91(A)	4.52(A)	0.49	
	GSD	4.48		3.90	1.77	20.60		2.71	2.06	1.71		
GerES V-sub (Germany; n=300)	GM	0.28 (B)[d]	NC	NA	2.98 (AB)[d]	0.78 (A)[d]	NA	NA	NA	NA	NA	
	GSD	1.98			1.95	9.02						
Riksmaten adolescents (Sweden; n=300)	GM	0.31 (B)[c]	0.24 (A)[c]	1.48 (C)[c]	3.76 (C)[c]	9.59 (B)[c]	27.78 (B)[c]	22.29(B)	2.15(C)	6.20(B)	0.28	0.22
	GSD	1.74	1.95	1.60	1.93	7.28	3.06	3.14	1.43	1.60		
SLO CRP (Slovenia; n=97)	GM	0.32 (B)[d]	NC[e]	0.78 (B)[d]	2.48 (A)[d]	NA	7.94 (A)[d]	NA	1.22(B)	3.84(A)		0.48
	GSD	2.01		1.78	1.99		2.34		1.65	1.78		
BEA (Spain; n=300)	GM	0.45 (C)[c]	0.34 (B)[c]	1.31 (C)[c]	5.70 (D)[c]	10.73 (B)[c]	32.06 (B)[c]	25.42(B)	2.34(C)	8.47(C)	0.33	0.26
	GSD	2.04	2.50	1.82	2.08	6.49	2.97	3.08	1.62	1.79		
ESTEBAN[b] (France; n=300)	GM	NA	NA	NA	NA	NA	17.34[e]	NA	NA	5.48(B)		0.32
	GSD						2.51			1.80		

AB: arsenobetaine; MMA: methylarsonate acid; DMA: dimethylarsinate; As_{tot}: total arsenic; TRA: toxic relevant arsenic, i.e. As(III)+As(V)+MMA + DMA.
All data of different quality assured/quality controlled categories were included for this table and further analysis (Gilles et al., 2021).
GerES V-sub is an unweighted subset of the nationally representative GerES V.
GM: geometric mean; GSD: geometric Standard Deviation.
All concentrations were normalized for specific gravity (SG) except for ESTEBAN for which SG was not available.
NA: Not Available.
NC: Not Calculated. For AsV in FLEHS IV, CRP and GerES V-sub no GM was calculated as the detection frequency was less dan 60%. LOD and LOQ values are given in the supplementary material (Table A1).
Different letters between brackets point to significant differences between studies by a Tukey test (p at 0.05). No adjustments for influencing factors as age, sex, sampling year were done.
[a] Based on geomean values.
[b] TRA is available as a measured sum of species in ESTEBAN. No data on individual species was made available. For all other studies analysed species were summed to calculate TRA.
[c] Biomarker data quality assured by HBM4EU QA/QC program (Esteban López et al., 2021).
[d] Biomarker data generated before HBM4EU QA/QC program but deemed comparable.
[e] Biomarker data generated before HBM4EU QA/QC program but not deemed comparable.

2019). These values are all in the same order of magnitude as those observed in the HBM4EU Aligned Studies but comparability is hampered as these studies did not participate in the HBM4EU QA/QC.

The total urinary As concentration may be higher in some countries compared to others but this does not mean that the share of toxic arsenic is higher. When the contribution of TRA to As_{tot} or to the sum of As(III), As(V), MMA, DMA and AB is studied, it is shown that two HBM4EU studies reported a relatively larger contribution of TRA. Based on the geomeans following ratios were calculated: 0.22 to 0.33 for Riksmaten adolescents, BEA and ESTEBAN while for FLEHSIV and CRP this ratio varied between 0.48 and 0.49 (see Table 2). This means relatively to the total measured arsenic, more urinary toxic relevant arsenic is observed in Belgium and Slovenia.

Next, correlations between different As species were studied. Table 3 shows Pearson correlation coefficients between the As species based on non-imputed ln-transformed data for all studies combined. In human urine, all measured As species are significantly correlated, with the weakest significant correlation for As(V) and MMA (Pearson coefficient 0.103; p-value = 0.014). Associations between different As species are graphically displayed in the supplementary material in Figure A1. The highest Pearson correlation coefficient observed with TRA was for DMA (coefficient equal to 0.943; p-value = 0.001). DMA is quantitatively the major constituent of TRA as can be seen from Table 2. DMA is not a perfect marker for the intake of toxic inorganic arsenic. It originates from the intake of iAs but there are indications for direct intake and intake through arsenosugars and -lipids. It has already been shown that arsenobetaine is a main marker for the intake of seafood and that DMA co-occurs with arsenobetaine (Navas-Acien et al., 2011). HBM4EU data show that larger DMA concentrations are found with larger arsenobetaine concentrations (Table 3). Also Navarro Serrano et al. (2016) reported in their analysis a connection between urinary DMA and urinary arsenobetaine, indicating that the estimated risk when using As (III)+As(V)+MMA + DMA as a marker for exposure to iAs could be misleading (Navarro Serrano et al., 2016). The association clearly shows that a fraction of DMA is probably linked to similar sources as arsenobetaine. Therefore the suggestion is made to also measure As species as DMA in food items to get a clearer view of how much DMA originates from the intake of iAs and how much comes from other As sources (Carlin et al., 2016). It could be useful to calculate the contribution of directly ingested DMA because this could be used to subtract such DMA from urinary DMA levels for estimation of exposure to inorganic arsenic, as DMA(V) is less toxic than iAs (Hughes, 2002). The contribution of arsenosugars and -lipids should be explored as well. Another option could be to normalize TRA for the intake of arsenobetaine when a comparison would be made with a guidance value for toxicity for iAs. In doing so, one could take into account DMA associated with the intake of arsenobetaine as a marker for the intake of organic arsenic. However this would only solve partly the problem because there is also DMA coming from rice for example, not associated with AB (Guillod-Magnin et al., 2018) (Šlejkovec et al., 2021) (EFSA, 2021).

3.2. Exposure determinants

In the current study, we used questionnaire information filled out by the teenagers to associate information on food intake with their individual HBM data and this for a large dataset (see Table A2 in the

Table 3
Pearson correlation analysis between different As species (sg normalized and ln transformed).

		As(V)	MMA	DMA	AB	As_{tot}	As(III)+As(V)+MMA + DMA + AB	As(III)+As(V)+ MMA	TRA = As(III)+As(V)+ MMA + DMA
As(III)	N	677	764	1037	881	666	681	768	768
	Pearson coefficient	0.356	0.157	0.462	0.168	0.219	0.222	0.510	0.525
	p-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
As(V)	N		572	711	683	531	572	572	572
	Pearson coefficient		0.103	0.391	0.194	0.213	0.231	0.547	0.498
	p-value		0.014	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
MMA	N			812	691	691	721	812	812
	Pearson coefficient			0.220	0.118	0.195	0.226	0.854	0.426
	p-value			<0.001	0.002	<0.001	<0.001	<0.001	<0.001
DMA	N				957	696	748	844	844
	Pearson coefficient				0.447	0.619	0.601	0.418	0.943
	p-value				<0.001	<0.001	<0.001	<0.001	0.001
AB	N					592	713	713	713
	Pearson coefficient					0.925	0.931	0.154	0.411
	p-value					<0.001	0.001	<0.001	<0.001
Astot	N						600	696	696
	Pearson coefficient						0.997	0.355	0.635
	p-value						<0.001	<0.001	<0.001
As(III)+As(V)+MMA + DMA + AB	N							748	748
	Pearson coefficient							0.329	0.623
	p-value							<0.001	<0.001
As(III)+As(V)+MMA	N								844
	Pearson coefficient								0.656
	p-value								<0.001

Not imputed values were used; Data below LOQ not included.
Data of ESTEBAN were not included as no sg normalized values were available.

supplementary material for detailed information on questionnaire answers). Multiple linear regression analysis showed that the sum of As (III), As(V), MMA, and DMA was significantly associated with (recent) seafood and/or rice consumption in all cohorts ($p < 0.05$) and not significantly with other food types (see Table 4). It is shown in Table 1 which food types were questioned in which study. It also can be seen from Table 1 that there are many gaps in the questionnaire data so more harmonization is needed in advance. Regarding As from drinking water, in the HBM4EU Aligned Studies, the distribution of participants across different categories of use of drinking water was not uniform and often there was a limited amount of participants in some categories (e.g. use of groundwater as drinking water). Studies in HBM4EU were not performed in areas with highly contaminated soil and groundwater. For smoking and alcohol, no significant association was detected in the multiple regression analysis of the current HBM4EU studies, however few adolescents reported smoking.

No significant associations were found with studied food types excluding rice and seafood. However literature data on As levels in food show that the main sources of exposure to iAs in European adolescents and adults are rice, grains and grain-based products, drinking water, and to a lesser extent vegetables, fruits, and seafood (Cubadda et al., 2016; Mania et al., 2017; González et al., 2019; Menon et al., 2020; EFSA, 2021). Some seaweed and mushroom types, dietary supplements based on seaweed and algae, and green teas can have a high iAs content, but since consumption is relatively limited in the average population, their contribution to total iAs exposure is generally small (EFSA, 2014; Mania et al., 2014; Braeuer and Goessler, 2019; EFSA, 2021). Nevertheless, a Danish study concluded that dietary supplements based on herbs, other botanicals, and algae may contribute to iAs exposure considerably (Hedegaard et al., 2013). Also, Cheyns et al. (2021) reported that food supplements based on algae or cyanobacteria may pose a health risk due to elevated concentrations of arsenic species.

Literature data on HBM show that associations of the sum As(III), As (V), MMA, and DMA measured in urine with consumption of seafood were also reported in adult populations in France, Italy and Slovenia (Fillol et al., 2010) (Saoudi et al., 2012) (Vimercati et al., 2017) (Minichilli et al., 2018) (Snoj Tratnik et al., 2019). Associations were also found with wine and beer consumption in France and Italy, hypothesized to be related to pesticide use (Fillol et al., 2010) (Saoudi et al., 2012) (Minichilli et al., 2018). In children living near agricultural sites in Spain, increased arsenic concentrations were found in hair (Molina-Villalba et al., 2015). Vimercati et al. (2017) did however not find an association between the sum of urinary As(III), As(V), MMA and DMA and the use of pesticides. Nowadays, the application of arsenic containing pesticides is not allowed in the EU. In areas affected by natural or anthropogenic arsenic contamination in Italy, Hungary, Slovakia, Romania, and the UK, correlations of the sum of As(III), As(V), MMA and DMA with tap, spring or well water consumption were found (Lindberg et al., 2006) (Middleton et al., 2016) (Vimercati et al., 2017) (Minichilli et al., 2018). A number of studies reported (weak) associations of urinary sum TRA with smoking (Lindberg et al., 2006) (Vimercati et al., 2017) (Minichilli et al., 2018). It should be noted that of all studies cited above, only one (Fillol et al., 2010) included teenagers (48 participants between 7 and 18 years of age); an association of As(III)+As(V)+MMA + DMA levels with age was however in general not identified. Saoudi et al. (2012) did find a positive association with age until the age of 55. Associations with sex are not that clear. Some studies show lower concentrations in women while in others it's the other way around (Fillol et al., 2010) (Vimercati et al., 2017). In some studies findings with sex may also be related to BMI, in case there is no correction for BMI. There may be an association between BMI and As with As being a possible causal contributor to obesity (Eick and Steinmaus, 2020). Also women in

Table 4
Final backward multiple linear regression analysis for ln TRA.

Study	Variable	Overall p value	Categories	N	Beta	95%CI		Pairwise p value
						LL	UL	
Riksmaten adolescents	Intercept	0.039			9.08	0.46	17.70	
	SG	0.108			−6.91	−15.35	1.53	
	Recent rice consumption	**0.006**	No	206	−0.16	−0.27	−0.04	0.006
			Yes	94	Reference = 0			
	Recent seafood consumption	**0.006**	No	203	−0.16	−0.27	−0.05	0.006
			Yes	97	Reference = 0			
	Highest ISCED household	**0.013**	ISCED 0-2	23	0.10	0.09	0.49	0.004
			ISCED 3-4	103	0.06	−0.12	0.10	0.870
			ISCED ≥5	174	Reference = 0			
	Model	**<0.001**						
FLEHS IV	Intercept	0.477			−5.80	−21.90	10.30	
	SG	0.322			7.85	−7.79	23.50	
	BMI	**0.008**			−0.04	−0.07	−0.01	
	Sex	**0.052**	Female	60	0.20	−0.01	0.41	0.052
			Male	51	Reference = 0			
	Recent rice consumption	**0.014**	No	76	−0.28	−0.50	−0.06	0.014
			Yes	35	Reference = 0			
	NUTS2 regions	**0.018**						
	Model	**<0.001**						
SLO CRP	Intercept	0.895			1.72	−14.12	17.57	
	SG	0.955			0.44	−15.07	15.95	
	Matrix (sampling method)	0.996	Morning urine	81	0.01	−0.28	0.28	0.996
			Spot urine	15	Reference = 0			
	Sampling month	**0.005**	January	40	−0.32	−0.56	−0.06	0.013
			February	27	−0.44	−0.72	−0.16	0.002
			March	29	Reference = 0			
	Seafood consumption	**0.002**	Never	43	−0.43	−0.67	−0.18	<0.001
			Rarely (<1 time/month)	22	−0.14	−0.43	0.14	0.327
			Sometimes (≤ 1 time/week but ≥ 1 time/month)	31	Reference = 0			
	Recent consumption seafood	**<0.001**	No	74	−0.45	−0.70	−0.19	<0.001
			Yes	22	Reference = 0			
	Model	**<0.001**						
BEA	Intercept	0.293			6.39	−4.99	17.78	
	SG	0.483			−3.97	−15.10	7.16	
	Matrix (sampling method)	0.719	Morning urine	197	−0.03	−0.19	0.13	0.719
			Spot urine	72	Reference = 0			
	Seafood consumption	**0.022**	Never	11	−0.46	−0.92	−0.01	0.049
			Rarely (<1 time/month)	8	−0.79	−1.30	−0.28	0.003
			Sometimes (≤ 1 time/week but ≥ 1 time/month)	39	−0.20	−0.56	0.14	0.252
			Often (2–3 times/week)	99	−0.18	−0.51	0.14	0.262
			Very Often (4–6 times/week)	97	−0.12	−0.44	0.20	0.460
			Everyday (≥ 7 times/week)	15	Reference = 0			
	Model	**<0.001**						
ESTEBAN	Intercept	<0.001						
	Recent consumption of seafood	**<0.001**	No	157	−0.46	−0.67	−0.24	<0.001
			Yes	37	Reference = 0			
	NUTS3 regions	**0.005**						
	Model	**<0.001**						

ISCED: International Standard Classification of Education: Low education (ISCED 0–2), Medium education (ISCED 3–4), High education (ISCED ≥ 5).
NUTS: Nomenclature Of Territorial Units for Statistics.
CI: Confidence Interval; LL: lower limit; UL: upper limit.
SG and matrix (first morning urine; spot urine) forced into the model except for ESTEBAN.
All biomarker data (μg/L) were normalized for specific gravity (SG) except for ESTEBAN for which SG was not available.
Only those variables with $p < 0.05$ were kept in the multiple regression analysis.
Beta is the value by which ln TRA changes. The actual change of TRA equals exp (beta).
For GerES V-sub no data on TRA were available.

childbearing age may have a higher As methylation efficiency (Lindberg et al., 2008).

Table 5 presents the multiple linear regression analysis for the sum of As(III), As(V), MMA and DMA with arsenobetaine taken up in the model as a co-variate. In the study of Riksmaten adolescents the consumption of seafood, dropped both out of the model as significant exposure determinant ($p > 0.05$) (Table 5). Considering food, only recent rice consumption remained in the model. For the FLEHS IV study, also only recent rice consumption remained in the model possibly overshadowing other determinants. In the BEA study no significant variables associated with food consumption were observed. This could be related by a lack of information on drinking water concentrations and on consumption of specific food types high in iAs (dietary supplements, seaweed, mushrooms). This information is not available (see Table 1). In the Riksmaten adolescents cohort, a significant effect of sampling season was found, with lower observed concentration of TRA in summer.

Another reason why few significant association with food items were observed in the HBM4EU studies can be that besides consumption of certain food types associated with higher iAs exposure, food types decreasing As exposure takes place as well. In the US, higher consumption of milk and milk products, vegetables, organ and other meats, and nutritional drinks was found to be associated with lower concentrations of organic as well as inorganic arsenic. It will be of interest to investigate possible interactions/mechanisms responsible for lower excretion of As when certain foods like milk and milk products, vegetables, and organ meats are consumed (Jain, 2021). Associations may reflect nutrients found in certain foods associated with As metabolism such as Zn and Fe, Se, vit B12, etc. (Steinmaus et al., 2005; Krohn et al., 2016; Howe et al., 2017).

3.3. Toxicity and comparison with HBM guidance values (non-) carcinogenic effects

A causal association between human arsenic exposure and lung, skin,

Table 5
Final backward multiple linear regression analysis for ln TRA with arsenobetaine in model.

Study	Variable	Overall p value	Categories	N	Beta	95%CI		Pairwise p value
						LL	UL	
Riksmaten adolescents	Intercept	0.071			7.79	−0.63	16.21	
	Arsenobetaine	<0.001			0.09	0.06	0.11	
	SG	0.172			−5.71	−13.93	2.51	
	Sampling season	**0.051**	Spring	70	−0.05	−0.19	0.09	0.538
			Summer	25	−0.25	−0.44	−0.05	0.013
			Autumn	100	0.01	−0.12	0.14	0.828
			Winter	74	Reference = 0			
	Recent rice consumption	**0.020**	No	188	−0.13	−0.25	−0.02	0.020
			Yes	81	Reference = 0			
	Highest ISCED household	**0.044**	ISCED 0-2	22	0.25	0.05	0.44	0.013
			ISCED 3-4	93	0.03	−0.07	0.15	0.545
			ISCED ≥5	154	Reference			
	Model	**<0.001**						
FLEHS IV	Intercept	0.825			−1.55	−13.55	10.45	
	Arsenobetaine	**0.002**			0.04	0.02	0.07	
	SG	0.541			3.63	−8.08	15.34	
	BMI	**0.013**			−0.03	−0.05	−0.01	
	Recent rice consumption	**<0.001**	No	97	−0.31	−0.48	−0.14	<0.001
			Yes	51	Reference = 0			
	NUTS2 regions	**<0.001**						
	Model	**<0.001**						
BEA	Intercept	0.110			7.92	−1.51	17.36	
	Arsenobetaine	**<0.001**			0.15	0.11	0.18	
	SG	0.217			−5.81	−15.06	3.43	
	Matrix (sampling method)	0.865	Morning urine	197	−0.01	−0.14	0.12	0.865
			Spot urine	74	Reference = 0			
	NUTS1 regions	**0.015**						
	Model	**<0.001**						

NUTS: Nomenclature Of Territorial Units for Statistics.
CI: Confidence Interval; LL: lower limit; UL: upper limit.
SG and matrix (first morning urine; spot urine) forced into the model.
All datasets were normalized for specific gravity (SG).
Beta is the value by which ln (As(III)+As(V)+MMA + DMA) changes. The actual change of As(III)+As(V)+MMA + DMA equals exp (beta).
Only those variables with $p < 0.05$ were kept in the multiple regression analysis.
For SLO CRP and ESTEBAN no data on arsenobetaine was available and for GerES V-sub no data on TRA.

and bladder cancer has been recognized at high exposure (NRC, 2013; USEPA, 2019). The strength of evidence of causal associations with ischemic heart disease and cardiovascular disease, hypertension, stroke, diabetes, skin lesions, and effects on pregnancy outcomes (fetal and infant morbidity, fetal loss, stillbirth, and neonatal mortality) is also considered to be relatively robust. Causal associations with liver and kidney cancer, non-malignant respiratory disease, neurodevelopment, and effects on the immune system are less certain according to US EPA (NRC, 2013) (USEPA, 2019). US EPA is currently performing an in depth investigation of the shape of the dose-response curves in the low dose region.

In 2010 Hays et al. derived guidance values for TRA in urine (Hays et al., 2010). For non-cancer effects the BE (Biomonitoring Equivalent) value was equal to 6.4 μg/L or 8.3 μg/g creatinine (persons >6y). The point of departure (POD) is 0.8 μg/kg BW/day (USEPA: https://iris.epa.gov/static/pdfs/0278_summary.pdf) which originates from a human study (hyperpigmentation and vascular complications). Assuming steady state conditions an internal urinary concentration of 19.3 μg/L was estimated. By applying an assessment factor of 3 for intrahuman differences a value of 6.4 μg/L was obtained. This corresponds US EPA's Reference Dose and the Agency for Toxic Substances and Disease Registry's Minimal Risk Level of 0.3 μg/kg bw/day. Above this value, health effects cannot be excluded. This value needs an update as new information on toxicity of arsenic is available and epigenetic changes are reported (Kenyon, 2021; Chakraborty et al., 2022). In addition, as pointed out by Hays et al. (2010), this BE value does not consider carcinogenicity of arsenic. For the Canadian Health Measures Survey (CHMS) St-Amand et al. (2014) set the BE value for the sum of MMA and DMA at 5.8 μg/L. This was done because of detection problems of arsenite and arsenate (Aylward et al., 2013; St-Amand et al., 2014). In France, INRS stated that the reference value in the general population of As(III)+As(V)+MMA + DMA should be lower than 10 μg/L or 10 μg/g creatinine and As(III)+As(V) should be lower than 2.2 μg/L (Fillol et al., 2010). So a dual guidance value was set.

Studies included here (Fig. 1) show that P50 value was lower than the BE-value of 6.4 μg/L except in the dataset of Spain (BEA). P95 values all exceeded the BE value. In the BEA study the P95 value equaled 21.5 μg/L. The percentage of teenagers exceeding the BE-value varied between 16 and 52%: SLO CRP 16%, FLEHS IV 22%, Riksmaten adolescents 38%, ESTEBAN 39% and BEA 52%. No data on MMA were available for GerES V-sub which means that TRA could not be calculated. Values higher than the BE value are an indication for policymakers and risk managers and pinpoint the need to have a closer look at the risk assessment. It is an indication for a reason of concern, certainly for highly exposed individuals or vulnerable populations. To reduce exposure in children, it is for example recommended that products based on rice (rice waffles) are not consumed by children on a regular basis (BFR, 2014). This is supported by the Health Council in Belgium (Hoge Gezondheidsraad, 2018).

Inorganic arsenic is a carcinogen. There is uncertainty related to the dose-response relation of arsenic-induced cancers at low exposure levels; although there is mechanistic evidence suggesting a potential threshold for its carcinogenicity (Snow et al., 2005; Yager et al., 2013), the data have been usually considered insufficient for the identification of such thresholds and a linear approach for the cancer risk assessment has been used as a default. Risk specific doses (RSD) have been calculated for a range of risk levels of interest from $1x10^{-4}$ to $1x10^{-6}$ (i.e. BE_{RSD} or biomonitoring equivalents at the RSD). They provide an estimate of the steady-state concentrations that would result from chronic exposure, over a lifetime at the RSD (Aylward et al., 2013). The BE_{RSD} value corresponding to an extra cancer risk of $1x10^{-6}$ equals 0.0065 μg TRA/L (Sean M. Hays et al., 2010), based on a cancer slope derived by USEPA for a study on lung and bladder cancer among a Taiwanese population exposed via drinking water (Morales et al., 2000). Cancer risks for the HBM4EU Aligned Studies corresponding to the 50th exposure percentile were estimated assuming linear extrapolation. Extra lifetime cancer risks equaled $5.7x10^{-4}$ to $1.0x10^{-3}$ depending on the study. However, discussions on the existence of a threshold for lung- and bladder cancers continue (Tsuji et al., 2021). Other assumptions used in the assessment include linearity of the dose-response relation, lifetime exposure, constant bodyweight, satisfactory nutritional status and no changes in metabolism. The calculated values must be interpreted with caution, particularly as the development of an updated assessment of toxicity of iAs, including the shape of dose-response curves in the low dose region, is ongoing by USEPA (NASEM, 2019).

The study addresses exposure, exposure determinants and risk of arsenic in adolescents of the general population. Strengths are the homogenization of protocols under HBM4EU and the QA/QC so that

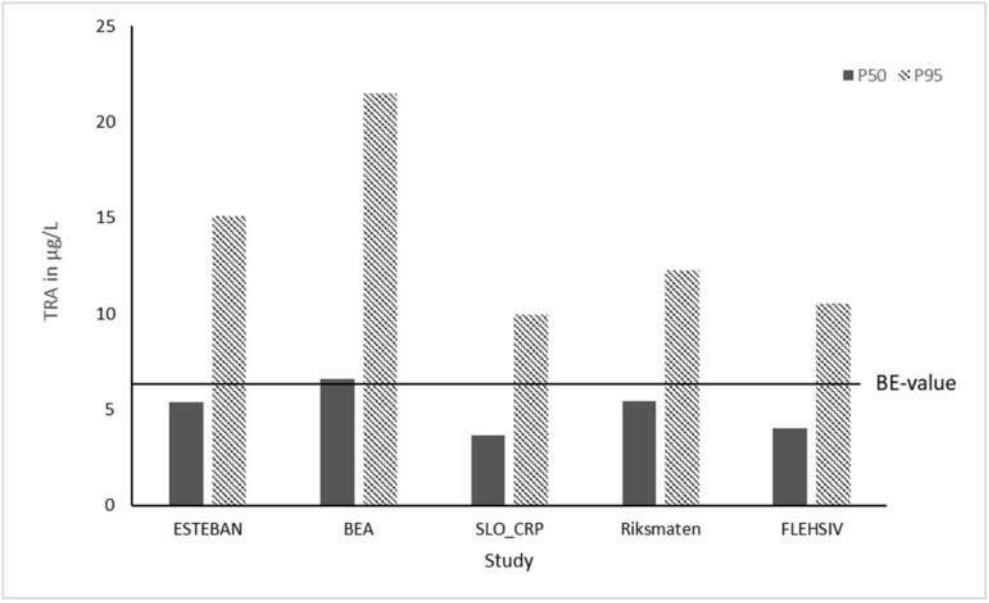

Fig. 1. Comparison of 50th percentile (P50) and 95th percentile (P95) arsenic concentrations (TRA) with HBM guidance value by Hays et al., (2010) (BE-value: 6.4 μg/L based on non-carcinogenic effects). Values in this figure were not normalized by SG. Riksmaten = Riksmaten adolescents. GerES V-sub is not presented as TRA was not determined.

harmonized results for exposure and questionnaire data can be obtained if taken into account *a priori*. The study covered the different EU regions (North, South, East, West) and by selection adolescents it is focusing on a vulnerable population for which the possibility exists that later in life resampling may take place. Some limitations are that not all As species were measured and that also in the questionnaires some information was missing. Therefore questionnaire were developed within HBM4EU to use in future studies.

4. Conclusion

HBM4EU made a big step in collecting comparable HBM data creating the largest dataset representing As exposure of adolescents living in Europe. Nevertheless, analysis of different As species needs more harmonization in the EU in future campaigns as results of recent analysis are still too heterogeneous. Also future questionnaire data with specific questions on arsenic exposure determinants (e.g. consumption of food supplements, seaweed, selenium status, smoking status) need more harmonization in advance. This will allow a combination of datasets which will increase statistical power. Besides, when addressing certain research questions on the source or determinant of exposure, more attention can go to selection of participants as not each study is *a priori* designed to answer all the questions for a certain chemical. Toxicologically relevant arsenic concentrations (TRA) varied a factor 2 between studies. The P95 exceeded the current biomonitoring equivalent (BE) of 6.4 μg/L for non-cancer effects in each study, and cancer risks related to arsenic exposure cannot be neglected although there is a lot of uncertainty at low dose exposure. Exposure determinants pointed in the direction of rice and seafood as main exposure determinants for TRA which overshadow the role of others. After adjusting for seafood intake, only rice intake remained significant. More HBM data (additional age groups, wider geographical spread) are needed related to arsenic exposure through groundwater consumption/use, co-exposure with other pollutants, and for vulnerable populations including information on ethnicity, genetic polymorphism and epigenetics. By combining HBM4EU studies, the statistical power increased and significant associations between all studied arsenic species appeared with some strong and some weak associations. A strong association was found between DMA and arsenobetaine. DMA may originate from inorganic arsenic but also from the direct intake of food or from the intake of organoarsenicals. Therefore, to properly identify human iAs exposure and to reduce iAs exposure efficiently in vulnerable populations, more information on the contribution of direct intake of DMA from food could be obtained which can be subtracted from urinary DMA concentrations. The role of arsenosugars and arsenolipids as sources of urinary DMA should be explored as well.

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Appendix A. Supplementary data

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Implementation and coordination of an ethics framework in HBM4EU – Experiences and reflections

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ABSTRACT

Human biomonitoring involves the use of human samples and data to investigate exposure to environmental chemicals and their impact on human health. HBM4EU developed a coordinated and harmonized approach involving 29 countries in Europe plus Israel. Addressing ethical issues has been an indispensable prerequisite, from the application phase, grant agreement, project performance to the closing of the project. HBM4EU has established a better understanding of the ethics in such projects and the need for a standardised way of reporting and handling of ethics and data exchange, securing compliance with ethics standards, transparency, transferability and sustainability. The main reflections were:

Knowledge: Ethics awareness, norms and practices are dynamic and increased throughout the project, much learning and experience is achieved by practice and dialogue.

Attitude: Rules and standards were very diversely known and needed to adhere to local practices.

Assistance: Good results achieved from webinars, training, help desk, and individual consultations.

Standardisation: Was achieved by templates and naming convention across documents.

Management: The establishment of the SharePoint directory with uploading of all requested documents assisted collaboration and exchange. Also, a designated task for ethics within the management/coordination work package and the enthusiasm of the task leader were essential.

Compliance: Some, but not all partners were very good at complying with deadlines and standards.

Transferability and sustainability: All documents are archived in the SharePoint directory while a system assuring updating is recommended.

Transparency: Assured by public access to annual ethics reports. The ethics reports bridged to the annual work plans (AWPs).

Evaluation: The Ethics Check by the Commission was successful.

1. Introduction

The European Human Biomonitoring Initiative (HBM4EU) is a pan-European consortium from 2017 to 2022 and follows in succession to the Expert Team to Support Biomonitoring in Europe (ESBIO) 2005–2008, Consortium to Perform Human Biomonitoring on a European Scale (COPHES) and its demonstration sister project DEMO-COPHES 2009–2012 (Joas et al., 2012, 2015; Becker et al., 2014; Den Hond et al., 2015). These projects established guidance and practices of

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Abbreviations

AWP	Annual Work Plan
COPHES	Consortium to Perform Human Biomonitoring on a European Scale
DEMOCOPHES	Demonstration project for Consortium to Perform Human Biomonitoring on a European Scale
EB	Ethics Board
EC	European Commission
ESBIO	Expert Team to Support Biomonitoring in Europe
GDPR	General Data Protection Regulation
HBM4EU	The European Human Biomonitoring Initiative
IPCHEM	the Information Platform for Chemical Monitoring
MDTA	Material and Associated Data Transfer Agreement
MoM study	Methylmercury-contrOl in expectant Mothers
SPECIMEn	Survey on PEstiCIde Mixtures in Europe
SOP	Standard Operating Procedure
WP	Work Package

ethics in the field of human biomonitoring as part of being pilot projects (Casteleyn et al., 2015).

Compared to the previous projects, HBM4EU aimed to include more countries, and regulatory and research institutions in the efforts to develop a sustainable human biomonitoring programme for Europe. The project should support chemical regulation, in particular the risk assessment of chemicals, and enhance knowledge of the relationship between environmental exposure to chemicals and human health. An evaluation of existing measures was foreseen in order to improve the health and well-being of the population.

Specifically, key objectives of HBM4EU included:

- Harmonizing procedures for human biomonitoring across 29 European countries and Israel, providing policy makers with reliable data on human internal exposure to chemicals at the level of the European Union (EU);
- Linking data on internal exposure to chemicals to aggregate external exposure and identifying exposure pathways and upstream sources;
- Generating scientific evidence on the causal links between human exposure to chemicals and health outcomes; and
- Adapting chemical risk assessment methodologies to use human biomonitoring data and account for the contribution of multiple external exposure pathways to the total chemical body burden.

HBM4EU was structured into 16 work packages (WPs). Special attention was given to national efforts and all participating countries have referred to existing HBM structures or set up National Hubs with relevant institutions from research, government bodies, Non-Governmental Organizations and other stakeholders. The management WP included a specific task on ethics and a number of other WPs addressing topics such as training, science to policy, study protocols, health studies, field studies and methods used for analysis of exposures and early effects cover ethics as part of their tasks. The project structure is detailed in Ganzleben et al. (2017).

An Ethics Board (EB) was established within the first five months of the project. The EB members were selected according to the following criteria: Actively engaged in HBM4EU; documented experiences with HBM studies and/or surveillance studies and biobanks; documented experience with ethics and data protection related to sensitive personal information; and experience with EBs. The EB consisted of five members: the ethics coordinator, representatives of the project coordination team, the health studies WP, the National Hub coordinator, the data management team, and a specialist in social sciences. The EB met annually and was consulted in all important ethics issues of HBM4EU. All EB members are co-authors of this paper.

The project used both existing data which needed to be harmonized - this was termed 'post-harmonized' - and new data. Furthermore, in the project both biobanked samples from completed or ongoing national or regional HBM programs were used, as well as newly collected human samples from EU co-funded projects under HBM4EU.

Biobanked samples and previously published data from some of the 17 countries participating in the previous European DEMOCOPHES project were re-used. The DEMOCOPHES samples were originally collected in 2011–2012 using common protocols including an obligation to store samples for 10 years from 2011 on (Fiddicke et al., 2015; Schwedler et al., 2017; Casteleyn et al., 2015). New analysis of the biobanked samples required renewed ethical approval in some countries. In addition, the HBM4EU Aligned Studies built on existing HBM capacity in Europe by aligning national or regional HBM studies. The aim of the HBM4EU Aligned Studies was to generate comparable HBM data with European wide coverage to derive current internal exposure data representative for the European population (Gilles et al., 2021a). For the HBM4EU Aligned Studies a combination of renewed ethical approval and new ethical approvals were required. Finally, some new studies were initiated under HBM4EU such as the HBM4EU Survey on PEstiCIde Mixtures in Europe (SPECIMEn study, Huber et al., 2022) performing suspect screening of pesticide exposures, studies on occupational health of exposures to chromate (Santonen et al., 2022), e-waste (Scheepers et al., 2021) and diisocyanates and the HBM4EU MoM study (Methylmercury-contrOl in expectant Mothers through suitable dietary advice for pregnancy).

The different types of research objects are shown in Table 1 which also shows the ethics requirements from the initial review in HBM4EU.

Given this combination of re-use of existing samples/data and newly performed studies, ethics approaches included several elements (Fig. 1). Renewed ethics approvals with amendments to dossiers were required in cases of previous studies. Thus, some existing approvals had to be extended to new parameters, however not requiring new informed consents as the studies were already within the environmental health application domain. New studies required full ethical approval. Furthermore, questionnaires and surveys to the public and stakeholders for dissemination also required ethics approvals in some countries and anyway transparency on the quality assurance for ethical aspects and data protection.

Handling human samples and data requires full compliance with national ethics requirements as well as ethics principles and European and international legislation including the EU's Charter of Fundamental Rights and the European Convention on Human Rights and its Supplementary protocols. In addition, data protection has been considered together with questions on ethics. Since the General Data Protection Regulation (GDPR) entered into force very early in the HBM4EU project (May 2018), many questions arose as to use of directly identified (never the case), pseudonymised or fully anonymized data and samples. The diverse nature of the studies and data included in HBM studies and the wide scope of requirements by national ethics committees represented a major challenge.

In this paper we describe the organization of ethics within HBM4EU and challenges met as well as lessons learned. These include aspects of standardization, questions of responsibilities, measures of transparency, while meeting data protection standards, and possibilities of transfer to new projects, covering the HBM4EU Aligned Studies, new studies as well as social science studies. As HBM4EU is funded by Horizon 2020) the compliance with the ethics requirements set up in the application phase as well as the reporting phase will be included.

This description feeds a retrospective attitude in the ethics governance of a research organisation, and more in particular its compliance with ethics regulatory standard. Such an approach is appraised by authors in the field of responsible research as complementary to the more prospective, rules-based compliance with ethics requirement and any on-going ethical monitoring in the context. According to Dawson et al.

Table 1
Addressing the ethics requirements from the ethics review initially in HBM4EU for studies with different research objects.

Objects	Concerns	Comments
1 Human Embryos/ Foetuses	Clarify whether the work will involve human embryos/ foetuses and if it relates to results from other studies or work done in this initiative. Copies of relevant approvals from competent ethics/legal bodies	Samples from pregnant women and umbilical cord blood etc from newborns are not foetal as the child is born and thus a human. The pseudonymised placental tissue which is of foetal origin is included with proper informed consent and ethics approvals and is to be considered human cells
2 Humans	Clarify whether children or adults unable to give consent will be involved and if yes justify participation. Explain how consent/assent will be ensured for children and adults unable to give consent.	HBM4EU will make use of available and new samples from children and adults from existing biobanks and new collections. HBM4EU will ensure compliance with national regulations by appropriate consent from both parents and assent from participating children and appropriate consent from relatives with persons unable to consent. HBM4EU will provide the relevant documents to the Commission upon request and store them in a database
2 Humans	Measures taken to prevent enhancing vulnerability/ stigmatization of vulnerable individuals/groups	A policy on providing the study persons with individual and personal results will be developed. The policy will take measures to avoid stigmatization of particular social groups, political or financial retaliation and malevolent use.
2 Humans	Measures taken to prevent potential detrimental socio-economic disadvantages of individuals who have indicated they want to receive their individual results	
3 Human cells	Details on the types of human cells/tissues obtained within the project and ethics approval	Addressed in specific WP7 that develops templates for recruitment, information and informed consent
3 Human cells	Details on the types of human cells/tissues obtained within another project and authorisation by primary owner (incl. reference to ethics approval)	Biobanked samples will be analysed and only after ethics approvals as well as transfer documents are provided ensuring legal handling
3 Human cells	Details on the types of human cells/tissues obtained from a biobank and details on the biobank and access to it	
4 Data	Measures taken to prevent the re-identification of individual through the merger of identifiers	The data protection issues complying with GDPR are described in the Data management plan (DMP) also referring to the Legal and Ethics Policy document IPCheM Data Policy Document will describe legal transfer of data from HBM4EU to IpChem
4 Data	Document from the responsible data management structure/individual stating compliance with national and EU legislation, incl. reference to the legislation	The documents will be collected and stored in the database and made available to the Commision upon request. Description of existing data sets in IPCheM are publicly available.
4 Data	Copies of the notifications/ approvals/opinions/ authorisations from the relevant data protection authorities at National/ regional level for the proposed data collection and processing as well as reuse	
4 Data	Information on procedures for data collection, storage, protection, retention and destruction and confirmation that they reply with national and EU standards.	Information to be provided for the dataset kept at the EU level, see DMP as well as information to be made available for how the data flow is at the data provider.
4 Data	Information on informed consent procedures for collection, storage and protection of personal data. Templates of informed consent forms and information sheets	Procedures were set up for collecting and storing these documents centrally in the database
8 Other	Provide copies of all Material Transfer Agreements	Material transfer agreements for all transfers have been requested and archived
9 General	Copies of all partners ethics approvals relevant to the project	These documents have been requested and archived in the database

n/a = not applicable.

(2019) for instance, a retrospective review has "the potential to promote critical reflection on ethics and help to develop ethical sensitivity and integrity within the research team". Based on experiences with the development and dynamic update of the ethics framework in HBM4EU, and "lessons learned", solutions for future human biomonitoring initiatives will be described.

2. Organization of ethics in HBM4EU

2.1. Project development

Application for funding from the Horizon 2020 Programme required attention to ethics from the very beginning on when identifying studies and programs to be included as well as new studies to be set up. The ethics self-assessment templates issued by the European Commission (EC) were followed identifying issues of collection and exchange of foetal tissue (placental tissue), humans (new studies with adults and children), human tissues (from existing biobanks or cell collections) and data (personal data from new studies as well as existing data collections), use of animals as well as environmental aspects. Thus, an extensive annex to the application, covering the ethics, was submitted with the proposal, addressing each of the relevant items and including description of ethics considerations for each of the included countries.

An ethics review was performed by the EC after the positive scientific evaluation and prior to the contract negotiations for HBM4EU. An ethics review (assessment) report was issued including an ethics analysis, and nine ethics requirements. Animal studies were not included in HBM4EU and thus not relevant in this respect (see Table 1). The requirements were answered and most of them were transferred into deliverables to be provided as reports during the project.

Addressing ethics is a contractual obligation with any EC funded project with a robust review and analysis process. Separate obligations of forming an EB as well as delivering an Ethics Policy Paper were included as deliverables. Annual reporting of ethics was requested as deliverables and organized to follow the progress of including studies and humans with ethics compliance. Annual reporting was organised to comply with the requirements set in the initial ethics analysis as seen in Table 1.

2.2. Annual reporting and establishment of an ethics board

The first tasks were to organise the collection of ethics approvals as well as drafting the Legal and Ethics Policy Paper. Since GDPR was such an important issue much attention was devoted to interpretation and understanding the demands from GDPR related to HBM4EU. Special attention had to be given to the secondary use of data and samples to

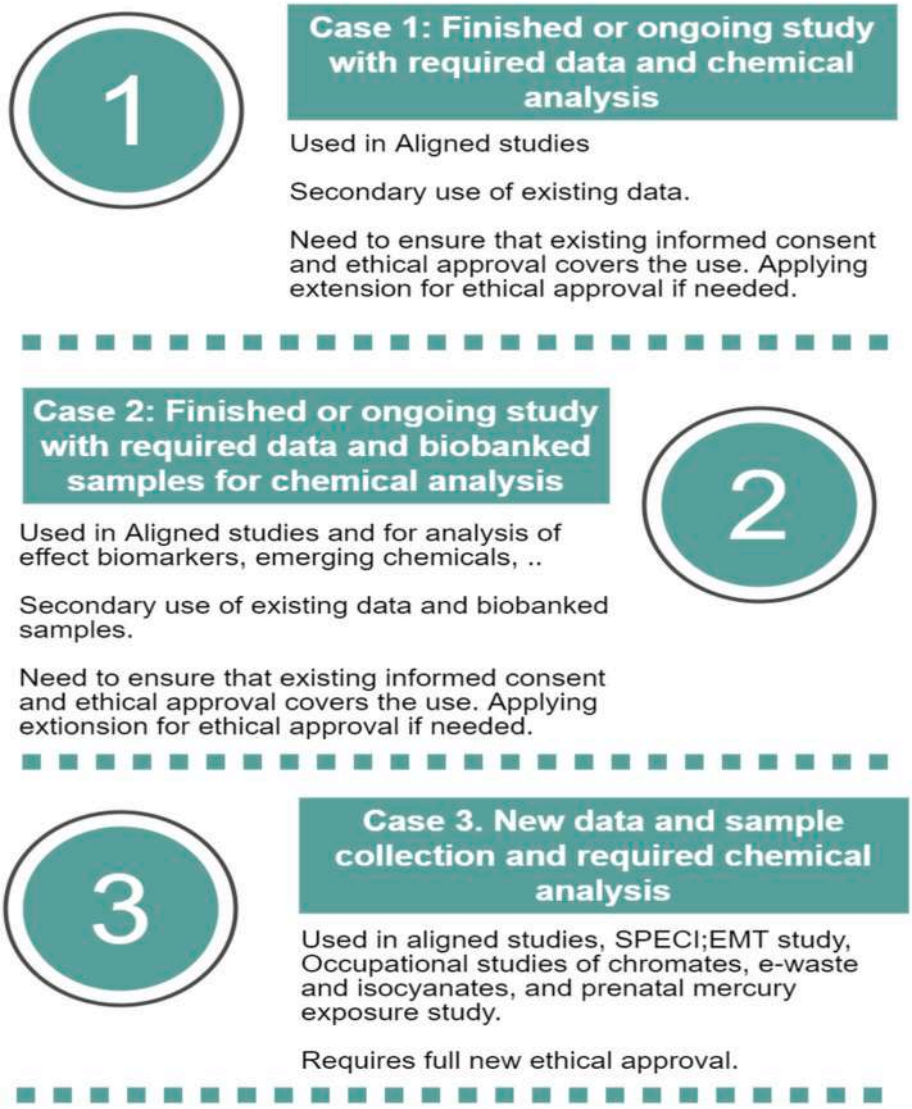

Fig. 1. Overview of which initiatives to be taken for finished, ongoing and new studies within the HBM4EU.

ensure initial or renewed consent from the study persons, proper local/national ethics and data protection approvals as well as legal data sharing.

The annual ethics report included updates of received ethics approvals and planned approvals which requested a close dialogue with the WP and task leaders, supported by the HBM4EU management board.

2.3. Documentation of the ethics compliance

The Ethics Policy Paper developed within the HBM4EU project described ethics issues related to each of the WPs as shown in Table 2. The Ethics Policy Paper served as a reference for ethics related questions and was a corner stone in the training activities (HBM4EU. Legal and Ethics Policy Paper. Available online: https://www.hbm4eu.eu/work--packages/deliverable-1-5-legal-and-ethics-policy-paper-september-2018/).

Recruitment of study participants in HBM studies requires protocols, communication materials and templates for informed consent forms approved by local ethics and data committees. When study programs provided data and samples for HBM4EU these documents were requested by the HBM4EU ethics coordinator, to be submitted with copies of all approvals and an Excel file explaining the documents as the original documents were in national languages. Fig. 2 shows this template. The validity of the documents was checked as to name of approving committee, project title, expiry dates and responsible scientist.

However, the data and sample owner were responsible for accuracy of the content. Fig. 3 shows the process. Details regarding processes in the individual countries linked to the renewed or first-time approval were not monitored by the HBM4EU ethics coordinator and therefore not included in this article.

Secondary use of samples and data sharing also required transfer agreements between providers and recipients of samples and data for which templates were developed in HBM4EU. These transfer agreements were organised by the providers of data and samples and collected uniformly in a SharePoint system organised by the HBM4EU data management team. A standardised format for the naming of the documents was important for later traceability of specific documents and requested:

The file name should be descriptive of the contents and should include as prefix: “InstituteAcronym_StudyAcronym_”, using standardized and unambiguous acronyms throughout HBM4EU. For the different document types a different suffix was used, such as “InformedConsent”,”InformationLeaflet”,”EthicalApproval”, … or corresponding descriptions (e.g. UBA_ESB_InformedConsent). The document names were in English and not in in national languages, in order to avoid any confusion.

Table 2
Overview of ethics issues identified in HBM4EU.

WP	Ethics	Comment
WP1: Project coordination and management	Agreement in Consortium on how to handle timely provision of ethics documents to ensure full compliance by issue of a Policy Paper	Deliverables of annual ethics reports, and contributions to annual work plans complied with the agreed procedure holding the WP leader and partners responsible for delivering, and Task 1.5 of keeping track.
WP2: Knowledge Hub	Training	Webinars and courses, all including ethics – basic and advanced.
WP3: Internal calls	–	Ethics and consent procedures and national ethics approvals need to be in place before HBM4EU financing.
WP4: Prioritisation and development of scoping documents	Focus interviews with citizens will be performed	Guidelines for focus interviews and how to protect participants was developed within the WP.
WP5: Translation of results into policy	Primary data to be stored legally in IPCHEM	Compliance with the Data Management Plan (DMP) and alignment with principles for lawful processing of data according to GDPR is requested.
	Workshops and surveys in the science-policy uptake	The approach was developed within the WP and described in each report for the bilateral interviews, the workshop participation and the science-policy questionnaires with the management board, policy board and stakeholder forum.
WP6: Sustainability and capacity building	Questionnaire and focus interview	Guidelines for focus interviews and questionnaires to individuals - how to protect participants were developed within the WP.
WP7: Survey design and fieldwork preparation	Material for communication to participants, including informed consent was developed	Guidelines for focus interviews and how to ensure the rights of participants as research participants and data subjects to were developed within the WP. Specific focus on ethics: Children, and other vulnerable groups Information and informed consent (D 7.4)
WP8: Targeted field work surveys and alignment at EU level	Aligned and new studies. Secondary use of samples, data, and health information	Compliance with HBM4EU procedures was set up to ensure all documents were in place before starting. Children and vulnerable groups?
WP9: Laboratory analysis and quality assurance	Secondary use of samples, data and issues of transfer Animal data new or existing	Compliance with HBM4EU procedures ensuring informed consent and Material Transfer Agreements collected
WP10: Data management and analysis	Sharing of data via IPCHEM, Article 6 of the IPCHEM Data Policy[a] Developing HBM4EU database and ethics in a share point database	The protection of personal data, licensing conditions, commercial interests and intellectual property rights, and contractual obligations restricting access to data to be ensured – and the alignment with principles of lawful processing according to GDPR
WP11: Linking HBM, health studies and registries	Data protection issues related to linking of HBM to health information and to administrative registers	Compliance with data protection according to GDPR and according to other EU data protection regulations regulating the processing of data by EU institutions Children and vulnerable groups?
WP12: From HBM to exposure	Secondary use of data	Compliance with the DMP and the HBM4EU procedures ensured by Material Transfer Agreements collected
WP13: Establishing exposure-health relationships	Secondary use of data and samples, transfer New human studies initiated	
WP14: Effect biomarkers	Secondary use of data and samples, transfer	Special attention to Children and vulnerable groups
WP15: Mixtures, HBM and human health risk	New human studies initiated	
WP16: Emerging chemicals		

[a] IPCheM Data Policy (http://publications.jrc.ec.europa.eu/repository/bitstream/JRC95307/lb-na-27163-en-n%20.pdf).

It was important that all other documentation regarding data collection used the same acronym for institute and study, such as the IPCHEM metadata fiche, data transfer agreement, material transfer agreement, and others.

Material and associated data transfer agreement (MDTA) documents should include the source and receiving institute – who both signed a media transferred and date (HBM4EU. Material and Associated Data Transfer Agreement. Available online: https://www.hbm4eu.eu/mdocs-posts/material-and-associated-data-transfer-agreement/):

MDTA_[StudyAcronym]_from_[Institute acronym]_to[Institute acronym] _[medium]_[date] e.g. MDTA_Specimen_from_ISCIII_toFISABIO_blood_01042020.

Special attention was given to new population based studies within HBM4EU for which templates for protocols, recruitment and information material as well as informed consent templates were prepared for submission to ethics committees in participating countries (Pack et al., 2022). The templates were also used for occupational studies of chromates (Santonen et al., 2022) and e-waste (Scheepers et al., 2021). The templates were adjusted for prenatal and maternal studies of mercury exposures initiated in 2021. A more dynamic consent procedure which could ease communication with study persons for participation in follow-up studies was not installed as not yet practiced in the EU, however discussed in relation to bridging of HBM and health information (Tolonen et al., 2022).

Standard Operating Procedures (SOPs) including information material and consent templates were developed and applied in all new HBM4EU studies (Santonen et al., 2019; Scheepers et al., 2021; Uhl et al., 2021, Matisāne et al., 2022). This included the social science studies with focus groups and questionnaire surveys completed online or in presence with external parties or citizens.

Finally, the use of human material for quality assurance of chemical and biological assays required informed consent from donors and an ethics approval in one validation study using material from employed personnel at the institution providing the requested informed consents.

Ethics check was requested as part of the HBM4EU contract and took place in continuation of the scientific midterm review with all ethics documents made available to the ethics check panel. The ethics check was organized by the EC and performed by independent reviewers with access to all documents and files collected at the time of assessment. This independent evaluation went satisfactorily from presentation of the status by the scientific officer. The scientific officer - the civil servant who is responsible for the follow-up of the project on behalf of the EU-commission -, had collected all relevant documents in advance and forwarded to the ethics check panel and the presence of project partners was not considered necessary.

3. Results

In the initial phases of HBM4EU ethics guidelines were produced and shown at presentations and webinars. In these meetings attendants were alerted to adherence to ethics (e.g., Helsinki Declaration) as presented in general by the Commission guidelines (see appendix). These guidelines were detailed in requirements transformed into deliverables in the contract. Documents for new studies were developed including the full

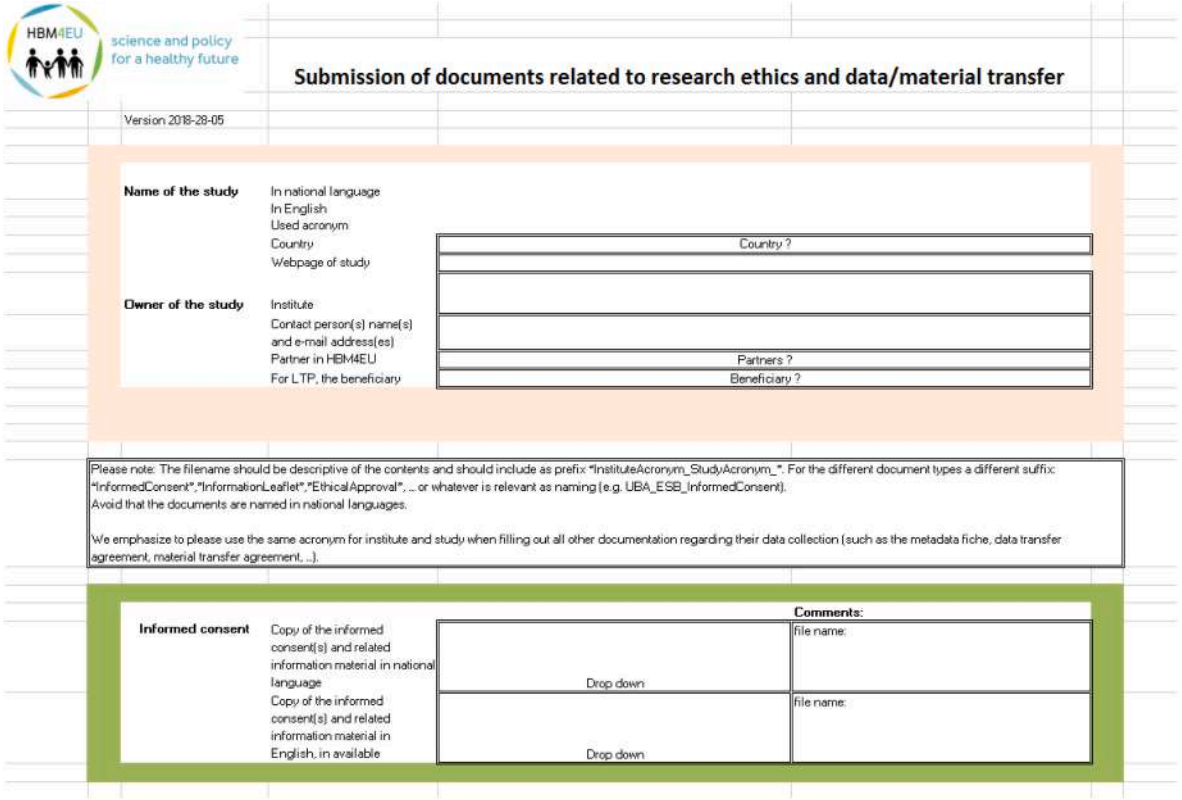

Fig. 2. A template for declaration of ethics within the HBM4EU project was developed.

process from announcement, recruitment, performance and reporting (Fiddicke et al., 2021). The instructions uploaded in the HBM4EU website were welcomed and refined during the first year where the SharePoint directory provided the tool for keeping an overview. For data sharing, the HBM4EU collaboration with IPCHEM - the Information Platform for Chemical Monitoring finally enabled public access to metadata from 143 HBM4EU research activities of in total 179 activities at the IPCheM Portal (europa.eu).

Ethics approvals from the 25 studies from 21 countries, included in the HBM4EU Aligned Studies with supplementary measurements on existing samples were collected (Gilles et al., 2022), accompanied with 64 MDTAs (Table 3). The supplementary measurements in DEMOCOPHES samples were performed on samples from 10 countries providing the relevant documents and accompanied with 21 MDTAs. The SPECIMEn study included samples from 6 countries with ethics declared and 6 MDTAs.

Occupational studies of exposures to chromates (Galea et al., 2021; Santonen et al., 2022) were performed in 9 countries with ethics declared and sample exchange covered by 21 MDTAs. Occupational studies of exposures to e-waste (Scheepers et al., 2021) were performed in 8 countries with ethics declared and sample exchange covered by 33 MDTAs. Occupational studies of exposures to diisocyanates were performed in 5 countries with ethics declared and sample exchange covered by 11 MDTAs. MoM study was performed in 5 countries with ethics declared and 5 MDTAs.

4. Discussion

All studies of the HBM4EU Aligned Studies followed national and European ethics regulation. They all acquired approval from their country's ethics committees. In all studies participation was on a voluntary basis. Written informed consent was obtained from all participants and withdrawal from the study was possible at any time. Each study also confirmed that informed consent and approval were in place for secondary use of the collected data. New biomarker analysis in the frame of HBM4EU was covered by renewed ethics approvals in each country if the original approval did not cover surplus analyses. The project developed an inventory of all ethics and data protection approvals to ensure full compliance with EU requirements and also installed an EB. The data provider ensured legal and GDPR compliant use of data in pseudonymised format. Below of the main points are discussed further.

5. Challenges

All studies in HBM4EU including their preparations and reporting have proceeded within a comprehensive ethics framework. Given the complexity of HBM4EU, this required the generation of a variety of clearly defined and communicated procedures that all partners had to comply with. Specifically, partners providing samples for chemical or biological analyses also needed to provide the corresponding ethical approval for the planned study. In addition, MDTAs had to be established to allow for the pan-European approach to analysis and data evaluations.

6. Lessons learned

Knowledge – Partners with hands on human research experience were well acquainted with national and EU ethics compliance while training of less updated partners on newest national and EU standards and formats of requests was necessary. Including data and material from ongoing studies required attention to validity of ethics approvals issued at the time of sampling. It was especially important to ensure the coverage of the consent given by participants for use in other projects such as the EU-wide and trans-national HBM4EU studies. Such studies were in many instances designed for a specific purpose not taking future use of data and samples into account when applying for approvals and obtaining consent from the participants. The informed consent provided

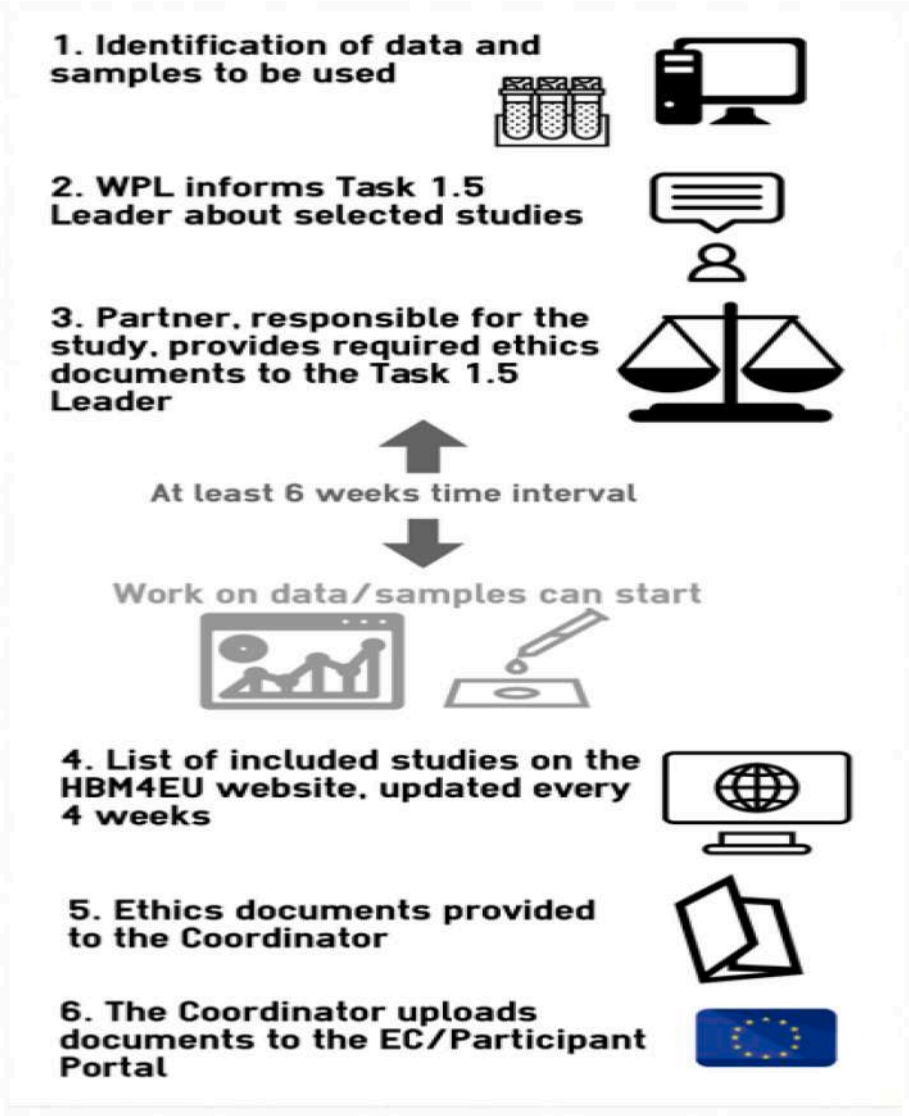

Fig. 3. Process for handling ethics approval in HBM4EU.

Table 3
Numbers of ethics approvals for studies in HBM4EU.

Study in HBM4EU	Number of countries with ethics declared	Number of MDTAs[a]
HBM4EU Aligned Studies	21	64
Supplement to DEMOCOPHES	10	21
SPECIMEn study	6	6
MoM study	5	5
Occupational studies – chromate	9	21
Occupational studies – e-waste	8	33
Occupational studies - diisocyanate	5	11

[a] Material and Data Transfer Agreements.

from each study participant varied from very broad consent allowing future analyses to very specific study questions. In many cases the material from those studies was only accessible by HBM4EU after renewed approval of an ethics committee, however depending on the scope of the initial studies. All DEMOCOPHES studies had followed the common protocol with the broad scope of exposures to environmental toxicants with common consent forms stating expiry after 10 years – i.e. in 2021 (Casteleyn et al., 2015). The extent of the approval varied as some countries asked approval for renewed protocols (e.g., Cyprus and Denmark) while other countries were allowing use based on the initial approvals from 2011.

In conclusion, the ethics compliance is dynamic in the sense that new standards are set continuously, as with GDPR setting new rules for data handling. Knowledge of ethics in a project like HBM4EU was increasing in large part from learning by doing and also due to instructions provided in training courses and seminars.

Practice – Well-described guidelines for handling of ethics documents submitted to the management were assured by setting up templates to be filled out and well-defined procedures to be followed (Figs. 2 and 3). The HBM4EU task covering the ethics also regularly responded to issues that occurred. In countries with persons specifically allocated to the handling of ethics documents their experiences assured uniform reply to requests and proper responses. This was the case especially for the occupational studies where the task lead organized submissions of documents and thus speeded up the process enabling initiation of studies in different countries at the same time of the year. The Ethics Policy Paper with reference to relevant documents also eased the process.

In conclusion, the practice had to be established by partners with very different backgrounds, some of them with good knowledge while

others had less experience. Well-described procedures as well as personal assistance through a helpdesk and individual consultation assured compliance.

Assistance – Webinars and presentations on ethics and data management were part of all initial activities alerting the partners to these issues (HBM4EU online library) (HBM4EU Online Library Guidelines, protocols and questionnaires/Materials for the occupational studies under HBM4EU. Available online: https://www.hbm4eu.eu/online-library/ (accessed October 28th 2022)). Some partners questioned the necessity of the requested compliance which paved the way to more detailed discussions. All providers delivered the required documents. Individual assistance was provided in many instances to ensure completeness with all documents provided and correct naming of the documents.

In conclusion, the organization of webinars on ethics and data management within the project assisted in common practice. Training in the training courses educated partners participating with their obligations to disseminate, the helpdesk was well appreciated and, in many cases, individual consultations assisted the progress.

Standardisation by templates and terminology for naming of files enables sustainability of the project and easy updating and transferability to future use in other programs. However, continuous updating is needed if the documents present valid status.

For the occupational studies and the MoM-study protocols were adapted from the templates developed in HBM4EU and full comparability assured within the countries by submitting a common protocol translated into national languages to the national ethics committees. The templates covered the full process from recruitment to enrolment. In many instances requests were issued personally as responses were not sufficient following the generic (non-personal) requests.

For the management– The scientific officer initially stressed the importance of collecting relevant ethics documents and these requests were met by the management by including the topic in all meetings in the management board in the first months of the project and allocating resources for alertness to the issue. The establishment of a platform for upload of documents provided overview and transparency. The ethics task was part of the management and not independently represented in the management board as seen in other Horizon 2020 programs. Communication was assured by frequent invitation of the task lead to the management board meetings and extensive collaboration with the WP lead of the health studies who had major insight into ethics.

In conclusion, the establishment of an interactive database (such as SharePoint) with all documents uploaded and restricted access served as a major source of documentation and transparency.

Compliance-was assured by allowing initiation of activities such as sampling and analysis only when ethics was recorded in the database. Ethics reports were delivered each year. The reports included an overview of past activities and future plans. The bridging to the Annual Work Plans ensured insight from the WP leaders who all had to ensure consistency by checking the text written about their WP.

Transferability – was assured by the unanimous naming of all documents in a common SharePoint while updates are needed for sustainability of HBM4EU.

Transparency – was assured by announcements uploaded to the HBM4EU website with proper references to relevant ethics approvals.

7. Conclusion

Comprehensive and up-to-date ethics procedures are a pre-requisite in human biomonitoring activities. It was a general feature in HBM4EU that partners presented a diversity of backgrounds and levels of experience. While some partners had long-term experience with human biomonitoring programs, others may have conducted specific studies and were now faced with the challenge of renewing or extending ethical approval. It has thus been a major achievement of HBM4EU that the central role of ethics in this project has been recognized and supported by all partners and that study designs, ethics documentation and chemical analyses were harmonized.

Furthermore, ethics procedures have been developed and implemented for a multi-national, multi-partner project, covering different types of human biomonitoring applications with potentially different ethics requirement. This experience will be beneficial in follow-up or continued human biomonitoring activities.

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Appendix 1. Additional instruments of relevance in the context of HBM4EU

- European Convention for the Protection of Human Rights and Fundamental Freedoms (1950)[1];
- Charter of Fundamental Rights of the European Union, OJC 326, 26 October2, 012[2]
- Council of Europe Convention for the Protection of Human Rights and Dignity of the Human Being with regard to the Application of Biology and Medicine: Convention on Human Rights and Biomedicine (Oviedo Convention), 4 April1, 997[3]; as well as relevant additional protocols such as Additional Protocol on the Prohibition of Cloning Human Beings, 12 January1, 998[4];
- Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004 on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells[5];
- Directive 2006/17/EC implementing Directive 2004/23/EC as regards certain technical requirements for the donation, procurement and testing of human tissues and cells[6];
- Directive 2006/86/EC implementing Directive 2004/23/EC as regards traceability requirements, notification of serious adverse reactions and events and certain technical requirements for the coding, processing, preservation, storage and distribution of human tissues and cells[7];
- Directive 98/44/EC of the European Parliament and of the Council of 6 July 1998 on the legal protection of biotechnological inventions[8];
- REGULATION (EU) 2016/679 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 27 April 2016 on the protection of natural persons with regard to the processing of personal data and on the free movement of such data, and repealing Directive 95/46/EC (General Data Protection Regulation)[9]
- WMA Declaration of Helsinki, Brazil, 2013; The World Medical Association (WMA) has developed the Declaration of Helsinki as a

[1] https://www.echr.coe.int/Documents/Convention_ENG.pdf.
[2] https://eur-lex.europa.eu/legal-content/En/TXT/HTML/?uri=OJ:C:2012:326:FULL.
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[5] http://eur-lex.europa.eu/Lex-UriServ/LexUriServ.do?uri=OJ:L:2004:102:0048:0058:en:PDF.
[6] http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv:OJ.L_.2006.038.01.0040.01.ENG&toc=OJ:L:2006:038:TOC.
[7] http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32006L0086.
[8] http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A31998L0044.
[9] http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32016R0679&from=EN.

statement of ethical principles for medical research involving human subjects, including research on identifiable human material and data[10]

- o OECD Guidelines for Human Biobanks and Genetic Research Databases (HBGRDs),2009[11];
- o Council of Europe Rec(2004)10 concerning the Protection of the Human Rights and Dignity of Persons with Mental Disorder[12];
- o Recommendation CM/Rec(2016)6 of the Committee of Ministers to member States on research on biological materials of human origin[13];
- o ISBER Best practices for repositories: collection, storage, retrieval, and distribution of biological materials for research, third edition,2012[14];
- o EGE, European Group on Ethics in Science and New Technologies relevant Opinions[15];
- o Article 29 Data Protection Working Party opinions and recommendations[16];
- o OECD Principles and Guidelines for Access to Research Data from Public Funding (2007)[17];
- o The Oviedo Convention on Human Rights and Biomedicine[18]
- o Council of International Organizations of Medical Sciences and WHO in 2002: International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS and WHO, 2002).[19]
- o The Belmont report "Ethical Principles and Guidelines for the Protection of Human Subjects of Research" (NIH, 1979)[20];

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Inflammatory profiles, gut microbiome, and kidney function are impacted after high-fidelity firefighter training

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ABSTRACT

Background: Firefighters are frequently exposed to high temperatures, environmental toxicants, and strenuous physical demands. The health impacts of these occupational exposures on processes including inflammation and kidney function as well as on the gut microbiota are poorly understood. A firefighter training course may provide a controlled environment to assess these health risks.

Methods: Basic health measures, stool, and blood samples were obtained from 24 firefighters participating in a one-week, heat-intensive training course. Indicators of inflammation, gut permeability, kidney health, and stool microbiota composition were measured before and after the training course in 18 participants. Urine specific gravity was measured before and after a heat-intensive training day to evaluate dehydration.

Results: The majority of firefighters in this cohort were categorized as hypertensive and experienced multiple heat-related illness symptoms during the training week and dehydration after the heat-intensive training day. While plasma IL-1β, CXCL8, and NGAL decreased over the training week, other indicators of inflammation and acute kidney injury increased, and estimated kidney function declined. Microbiota composition shifted over the course of the training week, with changes in *Peptostreptococcus anaerobius* and *Streptococcus*.

Conclusions: This pilot study conducted in a controlled field setting suggests that the occupational environment of firefighters may increase their risk for systemic inflammation and kidney disease.

1. Introduction

Occupational heat stress represents one of the many hazards experienced by firefighters. Both environmental and physiological heat stress contribute to increased core body temperatures for firefighters (Games et al., 2020; McEntire et al., 2013) and can lead to dehydration and multiple heat-related-illness (HRI) symptoms (Nerbass et al., 2017). Firefighters are also exposed to numerous toxicants such as wood smoke (Fabian et al., 2011), polycyclic aromatic hydrocarbons (Stec et al., 2018), and heavy metals (Al-Malki, 2009). This unique combination of occupational heat and toxicant exposure has been associated with changes in the body's inflammatory profile, such as increased interleukin (IL)-6, IL-1β, immunoglobulin G, and C-reactive protein (CRP) (Watkins et al., 2021).

There is also a growing body of literature that connects occupational heat exposure with kidney dysfunction, and this phenomenon has been documented among agricultural workers (Smith et al., 2021), kitchen workers (Singh et al., 2016), and also firefighters (Schlader et al., 2017). The mechanisms linking heat and kidney injury are not well understood, though dehydration is likely involved (Houser et al., 2021;

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Roncal-Jimenez et al., 2015), and inflammation-related mechanisms have been proposed (Sato and Yanagita, 2018). The role of the microbiome in inducing these inflammatory changes and their sequelae also warrants investigation. Heat stress has been associated with changes in the microbiome in animal models such as cattle (Chen et al., 2018), ducks (He et al., 2019), and pigs (Pearce et al., 2013). It has also been reported that heat stress decreases the integrity of intestinal tight junctions, allowing translocation of inflammatory bacterial products such as lipopolysaccharide to the blood stream (Dokladny et al., 2006).

Workplace hazards, including heat stress, can be challenging to examine in firefighter groups due to the unpredictable frequency of fire calls and the need for rapid response which often prevents the collection of biological samples in temporal proximity to an acute exposure. This pilot study utilized a repeated measures design to assess the effects of a physically demanding, heat-intensive training course designed to mimic real-life firefighting scenarios on heat illness, inflammation, kidney health, and the gut microbiome of firefighters. This design enabled us to detect changes in bacterial populations, immune mediators, and indicators of kidney damage following exposure to high heat and hazardous conditions.

2. Materials and methods

2.1. Study design and population

Study participants were attendees or instructors of the Georgia Smoke Diver Association's (GSD) week-long training program in January–February 2017. The class hones the skills of firefighters through drills designed to mimic real-life situations, with the goal of improving safety during critical incidents. Drills, called evolutions, are taught incrementally, beginning with the basics early in the week and becoming more complex as the week progresses until they are completed in a simulated fireground environment with smoke, heat, fire, zero visibility, running water, and loud noises. Because evolutions are often designed based on past incidents where there may have been a line-of-duty death, there is also an emotional component in the drill which contributes to the stress level during evolutions, yielding a high-fidelity training environment. Each day of class begins with high intensity physical training (PT) in full gear. PT is followed by a strenuous strength-based obstacle course and a three-mile run. The purpose of this regimen is to tire the student in an effort to compromise decision-making ability, adding another level of stress (Glick-Smith, 2016). All participants were provided the same meals for lunch and dinner during the training days; however, breakfast and evening snacks may have differed among participants.

Forty students and 6 instructors were approached during in-processing for the training course, and 18 students and 6 instructors were enrolled. Participants provided informed consent at the on-site study office and were compensated with $100 gift cards if completing baseline and follow-up data collection. All procedures were carried out in accordance with the Declaration of Helsinki and approved by the Emory University Institutional Review Board (IRB00093822).

2.2. Data collection

Immediately before the training week began, during orientation, medical screenings, and in-processing, participants completed a questionnaire covering demographics and health-related practices, and waist circumference, body mass index (BMI), and blood pressure (bp) were obtained, along with blood glucose, triglycerides, and cholesterol through a fingerstick blood sample. A combination of these measures was used to represent pseudo-metabolic syndrome (pseudo-due to the non-fasting status of participants). We considered participants with at least three of the following to meet criteria for pseudo-metabolic syndrome: central abdominal obesity (waist circumference $\geq$ 102 cm), stage 2 hypertension (systolic bp $\geq$ 140 OR diastolic bp $\geq$ 90), elevated blood glucose ($\geq$ 140), elevated triglycerides ($\geq$ 200), or low high-density lipoprotein (HDL) (<40).

Additional baseline measures included cytokines, chemokines, and other indicators of inflammation, gut permeability, and kidney health which were measured in plasma and in stool collected via Catch-All rectal swabs (Epicentre). These inflammation measures were repeated immediately after the conclusion of the training week (day 5), and participants provided information about HRI symptoms experienced during the week. Analytes were measured by the Emory Multiplexed Immunoassay Core on the QuickPlex instrument (Meso Scale Diagnostics) using V-PLEX Proinflammatory Panel 1 (human), Kidney Injury Panel 5 (human), Human LBP, and V-PLEX Human CRP kits (Meso Scale Discovery). Glomerular filtration rate was estimated (eGFR) from plasma cystatin C levels using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) cystatin C equation (Inker et al., 2012) and evaluated using Kidney Disease Improving Global Outcomes threshold for GFR indicative of kidney disease (KDIGO, 2013).

During the training, a single day was denoted as especially heat-intensive, in which firefighters were exposed to increased environmental heat compared to the other days (burn building temperature 150°F-500 °F). On this day, urine specific gravity (USG) was measured to assess dehydration before and after the training. For USG readings which indicated dehydration (USG $\geq$ 1.020) (Sawka et al., 2007), we informed the participant and instructed them to follow up with the onsite medical team who advised them on rehydration. For other out-of-range study-related lab values or anthropometric readings, participants were notified of the result and given a copy to share with their healthcare provider at their next routine visit.

16 S sequencing was performed by the Emory Integrated Genomics Core. DNA was isolated from stool samples collected before and after the training week and from clean swabs as negative controls using the MoBio PowerSoil DNA Isolation Kit per the manufacturer's protocol. The 16 S rRNA gene V3 and V4 regions were amplified and tailed with Illumina sequencing adapters and barcodes per the Illumina 16 S Metagenomic Sequencing Library Preparation guide (version 15044223-b). Quantified libraries were pooled and sequenced at 10pM loading density on the Illumina MiSeq using the 600cycle MiSeq Reagent Kit v3 and 20% Phix Control Kit v3 spike-in.

2.3. Data analysis

Analyses were performed in R (R Core Team, 2021) and RStudio (RStudio Team, 2020). Data were formatted and summarized using packages "tidyverse" (v1.3.0) (Wickham et al., 2019), "arsenal" (v3.6.2) (Heinzen et al., 2021), and "ggpubr" (v0.4.0) (Kassambara, 2020). Data were summarized as mean and standard deviation (SD) and percentages of total. Wilcoxon signed-rank tests were used to compare measures pre- and post-training week and pre- and post-heat-intensive day for each participant.

For microbiome analysis, raw 16 S sequences were trimmed, dereplicated, and processed including chimera removal, paired reads merging, and taxonomic assignment using the "dada2" package (v1.16.0) (Callahan et al., 2016). Taxonomy was assigned by alignment with Silva database (v132) sequences (Quast et al., 2013). The "decontam" package (v1.6.0) (Davis et al., 2018) was used to identify and remove sequences associated with contaminants (50) based on both presence in negative control samples and frequency in different sizes of sequence libraries. One pre-training sample had relatively few reads that met quality thresholds, so it and its post-training paired sample were excluded from analysis. Alpha diversity (Shannon and inverse Simpson indices) and beta diversity (non-metric multidimensional scaling [NMDS] method, Bray-Curtis dissimilarity) were evaluated using the "phyloseq" package (v1.30.0) (McMurdie and Holmes, 2013). Differences in bacterial composition pre- and post-training week were assessed by PERMANOVA (adonis2, 999 permutations). After removing taxa with counts of three or fewer in at least 20% of the samples, taxa

which differed significantly in abundance before and after the training week were identified using packages "DESeq2" (v1.26.0) (Love et al., 2014) and "apeglm" (v1.8.0) (Zhu et al., 2019).

For all tests, $p < 0.05$ was considered statistically significant. Analysis code and output are provided in **Supplementary File 1**.

3. Results

Eighteen participants completed the study protocol with attrition (n = 6) due only to training course failure, meaning participants did not complete the week-long program and thus did not complete the study. Participants were males aged 23–46 with 1–19 years in fire service, and the majority were non-smokers (Table 1).

3.1. Pseudo-metabolic syndrome rare among participants

The majority of participants were categorized as either overweight or obese by BMI, though only one student and two instructors met the criterion for central abdominal obesity by waist circumference. The majority of students had HDL in the acceptable or desirable range and low-density lipoprotein (LDL) and total cholesterol in the desirable range, but half of instructors were classified as having high total cholesterol. Most participants had normal triglyceride and blood glucose levels, but none had normal blood pressure according to the 2017 American College of Cardiology/American Heart Association Task Force guidelines (Whelton et al., 2018); in fact, over half of participants were classified as having stage II hypertension. Nonetheless, only one student and one instructor met criteria for pseudo-metabolic syndrome based on the combination of these variables (Supplementary Table 1). GSD is an elite firefighter training program requiring applicants to complete physical fitness tests and receive recommendations from the leadership of their home stations in order to qualify for the course. It is not unexpected, therefore, that these participants are, on average, younger and have higher cardiometabolic fitness relative to the broader career firefighter population in the U.S. (Moffatt et al., 2021).

3.2. Dehydration prevalent after heat-intensive training

Before the heat-intensive training day, half of instructors and two-thirds of students were euhydrated; at the end of the day, only a third of instructors and students were adequately hydrated, and 25% of students were severely dehydrated (Table 2). The difference in USG bordered on statistical significance ($p = 0.052$) (**Supplementary File 1**).

Table 1
Participant demographics and health-related practices.

	Instructor (N = 6)	Student (N = 18)	Total (N = 24)
Age (years)			
- Mean (SD)	32.7 (5.4)	30.1 (6.3)	30.8 (6.0)
- Min - Max	24.0–38.0	23.0–46.0	23.0–46.0
Years in fire service			
- Mean (SD)	9.5 (4.9)	7.6 (5.2)	8.0 (5.1)
- Min - Max	3.0–15.0	1.0–19.0	1.0–19.0
Smoked more than 100 cigarettes in lifetime			
- No	4 (80.0%)	13 (72.2%)	17 (73.9%)
- Yes	1 (20.0%)	5 (27.8%)	6 (26.1%)
- Missing	1	0	1
Current smoking frequency			
- Not at all	5 (83.3%)	17 (94.4%)	22 (91.7%)
- Some days	1 (16.7%)	1 (5.6%)	2 (8.3%)
- Every day	0 (0.0%)	0 (0.0%)	0 (0.0%)
Current use of chewing tobacco			
- Not at all	4 (80.0%)	11 (64.7%)	15 (68.2%)
- Some days	1 (20.0%)	2 (11.8%)	3 (13.6%)
- Every day	0 (0.0%)	4 (23.5%)	4 (18.2%)
- Missing	1	1	2

Table 2
Participant dehydration status pre- and post-heat-intensive day.

	Instructor (N = 6)	Student (N = 12[a])	Total (N = 18)
Urine specific gravity pre-heat day			
- Mean (SD)	1.018 (0.010)	1.018 (0.009)	1.018 (0.009)
- Min - Max	1.005–1.030	1.007–1.033	1.005–1.033
Pre-heat day dehydration status			
- Euhydrated (<1.020)	3 (50.0%)	8 (66.7%)	11 (61.1%)
- Dehydrated (1.020–1.030)	1 (16.7%)	2 (16.7%)	3 (16.7%)
- Severely dehydrated (>1.030)	2 (33.3%)	2 (16.7%)	4 (22.2%)
Urine specific gravity post-heat day			
- Mean (SD)	1.023 (0.010)	1.022 (0.009)	1.023 (0.009)
- Min - Max	1.008–1.036	1.004–1.034	1.004–1.036
Post-heat day dehydration status			
- Euhydrated (<1.020)	2 (33.3%)	4 (33.3%)	6 (33.3%)
- Dehydrated (1.020–1.030)	3 (50.0%)	5 (41.7%)	8 (44.4%)
- Severely dehydrated (>1.030)	1 (16.7%)	3 (25.0%)	4 (22.2%)
Percent change in urine specific gravity pre- to post-heat day			
- Mean (SD)	0.496 (0.998)	0.404 (0.724)	0.434 (0.797)
- Min - Max	−0.485 - 2.069	−0.883 - 1.390	−0.883 - 2.069

[a] Data are missing from 6 students who provided baseline data.

3.3. HRI symptoms common during firefighter training

Two-thirds of participants reported experiencing at least two symptoms consistent with HRI during the training week (Table 3). The most common were heavy sweating (reported by all participants), cramps, and headache. One student and one instructor reported experiencing dizziness, and one student reported experiencing both confusion and gastrointestinal symptoms (nausea and vomiting).

Table 3
Heat-related illness (HRI) symptoms experienced by participants during training week.

	Instructor (N = 6)	Student (N = 12[a])	Total (N = 18)
Cramps			
- No	5 (83.3%)	6 (50.0%)	11 (61.1%)
- Yes	1 (16.7%)	6 (50.0%)	7 (38.9%)
Nausea and vomiting			
- No	6 (100.0%)	11 (91.7%)	17 (94.4%)
- Yes	0 (0.0%)	1 (8.3%)	1 (5.6%)
Heavy sweating			
- No	0 (0.0%)	0 (0.0%)	0 (0.0%)
- Yes	6 (100.0%)	12 (100.0%)	18 (100.0%)
Confusion			
- No	6 (100.0%)	11 (91.7%)	17 (94.4%)
- Yes	0 (0.0%)	1 (8.3%)	1 (5.6%)
Dizziness			
- No	5 (83.3%)	11 (91.7%)	16 (88.9%)
- Yes	1 (16.7%)	1 (8.3%)	2 (11.1%)
Fainting			
- No	6 (100.0%)	12 (100.0%)	18 (100.0%)
- Yes	0 (0.0%)	0 (0.0%)	0 (0.0%)
Headache			
- No	4 (66.7%)	9 (75.0%)	13 (72.2%)
- Yes	2 (33.3%)	3 (25.0%)	5 (27.8%)
Number of HRI symptoms			
- Mean (SD)	1.7 (0.5)	2.0 (1.0)	1.9 (0.8)
- Min - Max	1.0–2.0	1.0–4.0	1.0–4.0
Two or more HRI symptoms			
- No	2 (33.3%)	4 (33.3%)	6 (33.3%)
- Yes	4 (66.7%)	8 (66.7%)	12 (66.7%)
HRI with CNS involvement			
- No	3 (50.0%)	8 (66.7%)	11 (61.1%)
- Yes	3 (50.0%)	4 (33.3%)	7 (38.9%)

[a] Data are missing from 6 students who provided baseline data.

3.4. Changes in indicators of inflammation and kidney injury after firefighter training

After the training week, plasma levels of IL-1β, CXCL8, and neutrophil gelatinase-associate lipocalin (NGAL) had decreased (Fig. 1A). Levels of plasma lipopolysaccharide-binding protein (LBP), an indicator of the levels of bacterial lipopolysaccharide in circulation, rose, as did levels of CRP in plasma and stool (Fig. 1B). Kidney injury markers cystatin C, epidermal growth factor (EGF), and osteopontin (OPN) in plasma had increased (Fig. 1C). Cystatin C levels in particular can serve as an indicator of kidney function and can be used to estimate glomerular filtration rate (Inker et al., 2012) (Supplementary Table 2). Immediately prior to the training week, one third of participants (3 of 6 instructors and 3 of 12 students) had cystatin C levels that exceeded normal reference ranges (Erlandsen and Randers, 2018). After the training week, 89% of participants – all but one instructor and one student – had plasma cystatin C levels that exceeded the reference range.

The average eGFR of the study cohort prior to training was 90.29 mL/min/1.73 m^2 of body surface area, in the range considered normal according to KDIGO criteria (KDIGO, 2013) (Supplementary Table 2). None of the students had eGFR less than 60 mL/min/1.73 m^2, the commonly utilized threshold for impaired kidney function, before the training week, although two instructors did. Participants' eGFR declined significantly from the beginning to the end of the training week (Fig. 1C), with the average dropping to 66.86 mL/min/1.73 m^2 and 50% of both students and instructors having eGFR less than 60 mL/min/1.73 m^2.

3.5. Changes in stool microbiota composition after firefighter training

While we did not observe statistically significant differences in alpha diversity from the beginning of the training week to the end, there was a

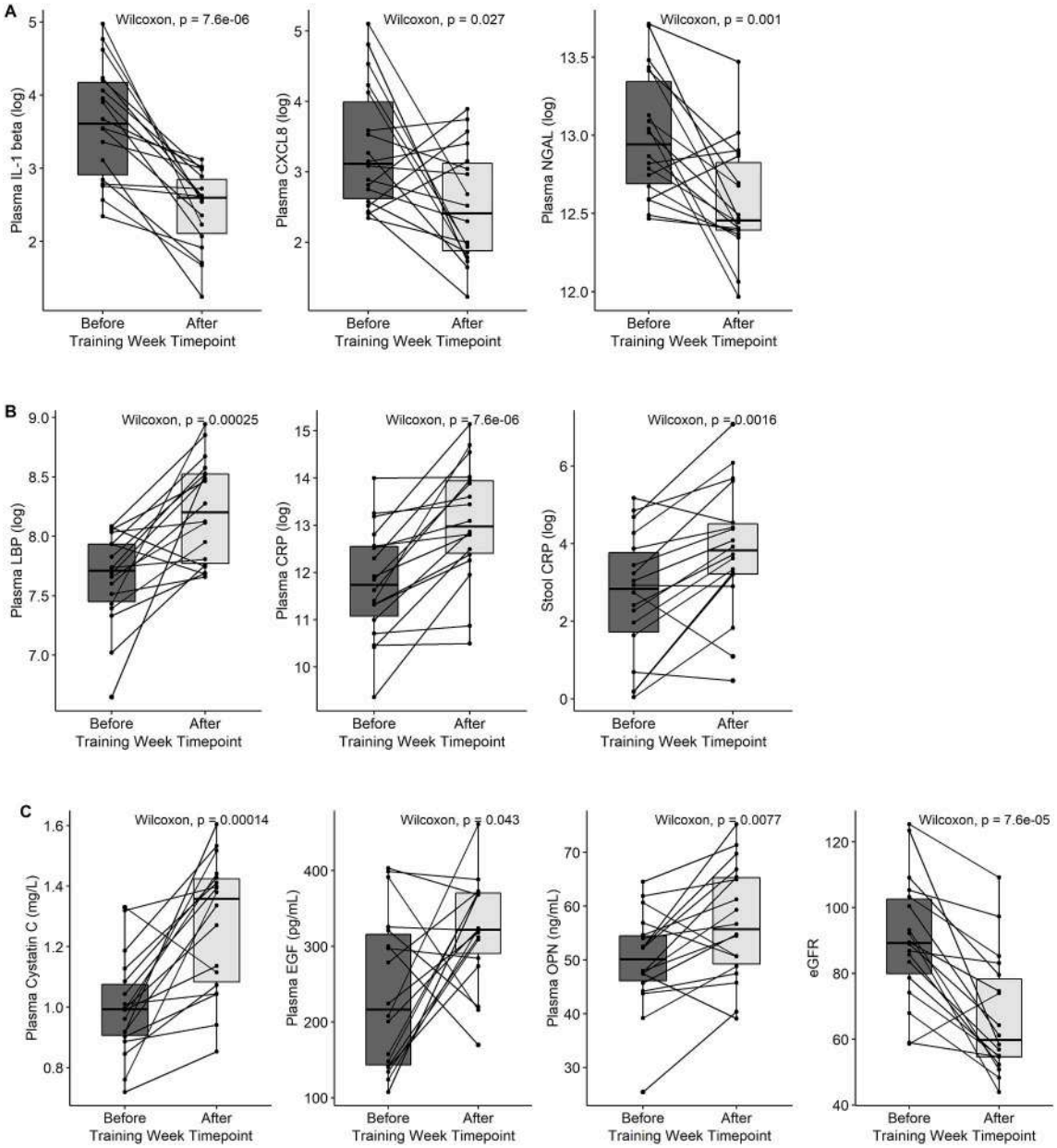

Fig. 1. Levels of Inflammatory Factors and Kidney Function Indicators Change During Training Week
Measurements obtained before and after the training week of **A)** plasma cytokines, **B)** lipopolysaccharide-binding protein (LBP) in plasma and C-reactive protein (CRP) in plasma and stool, and **C)** kidney injury markers in plasma and eGFR calculated from cystatin C. Wilcoxon signed-rank test, n = 18.

trend for a reduction in the inverse Simpson index ($p = 0.057$) (Fig. 2A). Microbial composition did shift over the course of the week (Fig. 2B), and PERMANOVA indicated a significant effect of timepoint ($p = 0.012$). Specifically, the abundance of the bacterium *Peptostreptococcus anaerobius* increased over the training week while the abundance of *Streptococcus* decreased to undetectable levels in most individuals but increased for two participants (Fig. 2C).

4. Discussion

Firefighters' occupation entails regular physical activity and exertion as well as frequent exposure to extreme heat and environmental toxicants. The week-long GSD training course presented a unique opportunity to study the potential impact of these circumstances on firefighters' health in a controlled environment in which participants' activities, exposures, and even diet were consistent. This pilot study also highlighted the value of field training programs for the reliable collection of biospecimens and assessment of firefighters' physiological state in close temporal proximity to acute exposure to occupational hazards. Along with other recent studies examining physiologic effects during multiple bouts of firefighting activity (Smith et al., 2022; Watkins et al., 2021), this pilot demonstrates the importance of monitoring the potential impacts of their occupational environment on firefighters' health. While the small sample size of this pilot study necessitates the validation of its findings in larger cohorts, we anticipate that the results will guide the design and implementation of future studies. As the number of participants in a firefighter training course is usually limited, efforts should be made to increase study enrollment by enhancing the appeal of research participation to training course students, and data could be collected from multiple cohorts of training course participants to increase the sample size for future studies and ensure the consistency of research findings.

Despite the diverse occupational hazards that firefighters face, cardiovascular disease remains a leading cause of on-duty fatalities (Kahn et al., 2019; Kales et al., 2007). The prevalence of hypertensive blood pressure readings, overweight and obesity, and other cardiometabolic risk factors in this study cohort highlight the importance of continued efforts to promote cardiovascular health among firefighters. This is especially true since our study participants represent a select group that is generally younger and in better health than the average career firefighter (Moffatt et al., 2021), and as such, adverse health outcomes observed in this study likely represent an underestimate of those in the full firefighter population. Moreover, our findings regarding gastrointestinal and renal impacts add to the growing evidence of

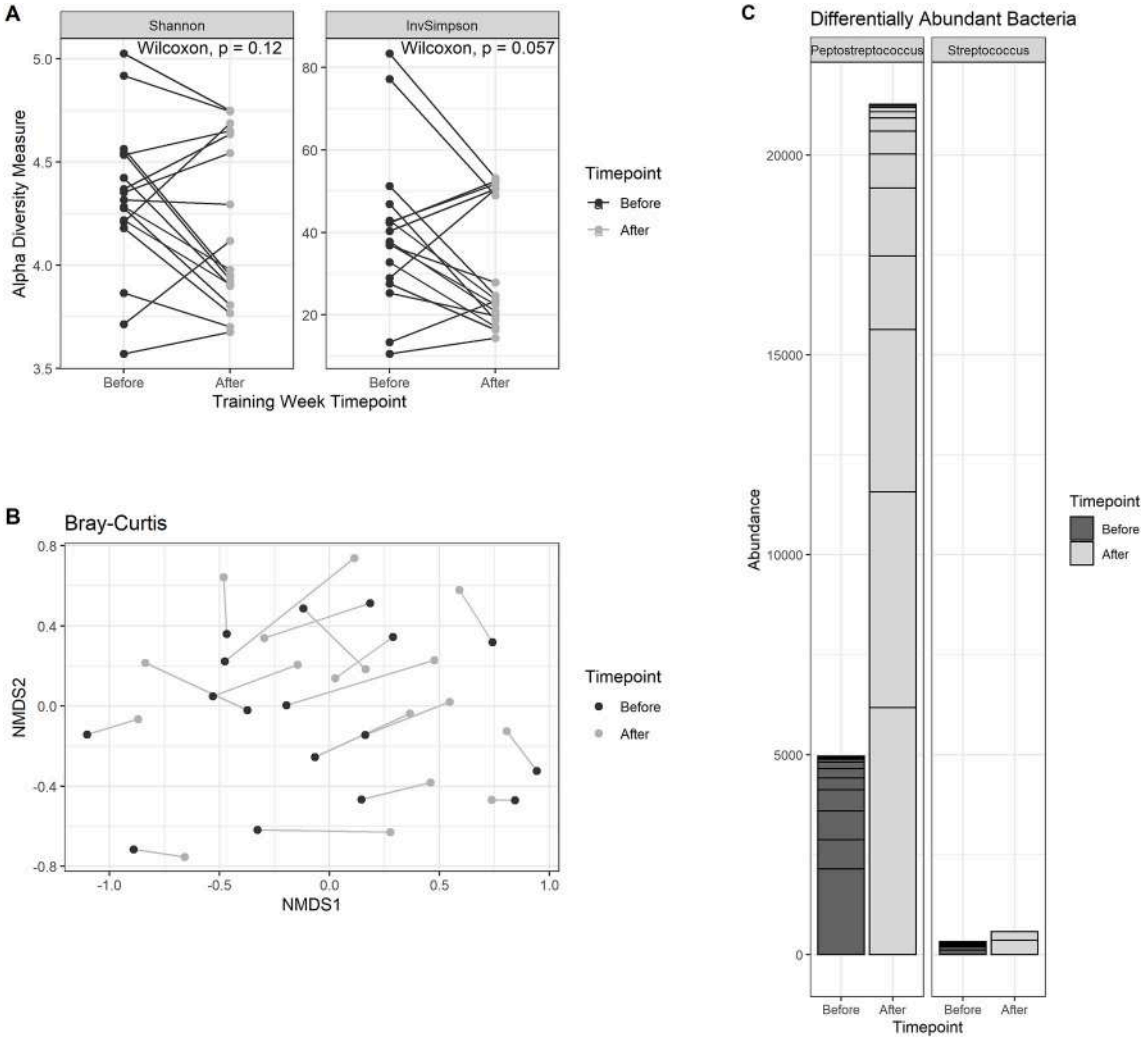

Fig. 2. Subtle Microbiota Composition Changes Detected Following Training Week
Sequencing data from before and after the training week were used to calculate **A)** Shannon and inverse Simpson indices representing alpha diversity of bacteria in stool (Wilcoxon signed-rank test, $n = 17$), **B)** non-metric multidimensional scaling (NMDS) using Bray-Curtis dissimilarity representing beta diversity of stool bacteria ($n = 17$), and **C)** abundance of bacteria belonging to two taxa, *Peptostreptococcus anaerobius* and *Streptococcus*, which differed significantly between the two timepoints. Each subdivision of a bar represents relative abundance in a different participant's stool ($n = 17$).

pathophysiologic changes to multiple body systems [*e.g.* cardiovascular (Kales and Smith, 2017)] and hematologic (Smith et al., 2022; Watkins et al., 2021) that can occur in fireground environments.

Over the course of the training week, plasma LBP rose significantly, in keeping with what is known regarding increases in gut permeability following heat exposure and strenuous exercise (Armstrong et al., 2018). CRP is commonly used as an indicator of inflammation, and higher levels of CRP were found in plasma and stool after the training week. This suggests that the training activities may have induced gut inflammation and gut leakiness which likely contributed to systemic inflammation. However, levels of certain inflammation-related molecules – IL-1β, CXCL8, and NGAL – were significantly reduced in plasma after the training week. This may reflect the anti-inflammatory impact of regular physical activity. While plasma NGAL levels typically rise immediately after exercise, there is some evidence suggesting that this may not be true following repetitive bouts of exercise, such as the daily runs and strength challenges during the training course, or in highly athletic individuals (Andreazzoli et al., 2017; Moghadasi and Mohammadi Domieh, 2014). IL-1β is often produced in the early stages of inflammatory immune responses, but its production is reportedly suppressed in response to lipopolysaccharide in the context of exercise (Nielsen et al., 2016).

Despite these adaptive anti-inflammatory changes, levels of kidney injury markers cystatin C, EGF, and OPN in the plasma had increased after the training week, and eGFR calculated using cystatin C values had decreased, with half of participants having eGFR values indicative of impaired kidney function at the end of the week. While eGFR derived from cystatin C is an imperfect measure of kidney function, the clear change in it and other renal markers raises concern about acute kidney injury and long-term kidney health among firefighters. This concern is amplified because the majority of firefighters in this cohort met the criteria for hypertension and overweight or obesity, both factors that are known to increase the risk for chronic kidney disease (Herrington et al., 2017). This study also demonstrated that after a day involving intense heat exposure, the majority of participants were dehydrated. Dehydration also increases the risk for kidney injury (Chapman et al., 2020; Houser et al., 2021; Roncal-Jimenez et al., 2015), and it impairs adaptive heat stress responses (Hillman et al., 2011), increasing the likelihood of HRI. Two-thirds of participants in this pilot study experienced multiple symptoms consistent with HRI during the training week, and more than a third had symptoms indicating central nervous system involvement. While it is expected that heat exposure contributed markedly to these symptoms and to dehydration, and while the training environment did seek to limit exposure to some hazards which would be encountered in a real fireground (*e.g.* using only untreated wood for burning), this study could not distinguish between the effects of heat and of other exposures. Further studies will be needed to delineate the contributions of exertion and hazards such as noise, products of combustion in smoke particles, residual chemical compounds on hoods and turnout gear, and fire itself to the physiological responses observed.

A novel component of this research was the evaluation of the gut microbiome in participants before and after the training course. Even in this small cohort, we observed a trend for a reduction in the inverse Simpson index, which reflects the richness and evenness of bacterial taxa in an environment, and changes in the composition of the gut microbiota over the week. We observed an increase in the relative abundance of *Peptostreptococcus anaerobius* and changes in *Streptococcus* levels. Though *Peptostreptococcus anaerobius* is a common component of the human gut microbiota, it is also considered an opportunistic pathogen, and it has been implicated in development of colorectal cancer (Long et al., 2019). The genus *Streptococcus* includes species with diverse functions. They can be associated with opportunistic infections and are also over-represented in colorectal cancer patients (Wang et al., 2012), but some species are used as probiotics and may have particular benefits in instances of physical exertion (Pane et al., 2018) and in individuals with chronic kidney disease (Vitetta et al., 2019). Interestingly, bacteria of the family Streptococcaceae reportedly increased in abundance after heat stress in chickens (Suzuki et al., 1983). It will be important to confirm and expand upon these findings in a larger cohort and to examine the relationships between gut microbiota composition and HRI and kidney function in firefighters.

5. Conclusions

This pilot study demonstrates the importance of examining associations between exposure to occupational hazards and firefighters' inflammatory profiles, kidney health, and gut microbiota composition, and it highlights factors that may contribute to long-term health concerns such as chronic kidney disease. Our findings emphasize the need to ensure adequate hydration during trainings, which will take place repeatedly during a firefighter's career, as well as during any exposure to high heat conditions on the job. They also highlight the need for monitoring and further studies of systemic inflammation and metabolic health among firefighters to determine their potential contributions to chronic kidney damage in this population.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.114107.

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Internal radiation exposure from TENORM for workers conducting cleaning activities on equipment used at geothermal energy plant

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ABSTRACT

Geothermal energy is predicted to be one of the most important renewable energy sources in the near future. In geothermal energy plants, the secondary products such as the scale containing naturally occurring radioactive material (NORM) and adhering to the surface of equipment produce radiation fields. The workers who maintain and clean such equipment are at a risk to be exposed by the technically enhanced NORM (TENORM). To estimate the risks of radiation exposure to the workers, we assessed internal doses resulting from the cleaning activities on 150 heat exchanging boards used at a geothermal energy plant, focusing on ^{222}Rn, ^{226}Ra, ^{210}Pb, ^{228}Ra and ^{228}Th. The experiment was performed with the subjects of workers and office workers as control, supplying prepared foods and drinks. Using the analytical results of ^{210}Pb, ^{226}Ra, ^{228}Ra, and ^{228}Th in the excretions of subjects, committed effect doses were determined. The annual internal dose for the workers with protective clothing due to the cleaning activities on removing scale, assuming the cleaning activities requires 170 h (standard monthly working time) a year, was obtained as 26 μSv/y and the total dose including ^{222}Rn inhalation dose was calculated as 323 μSv/y. The additional dose for the cleaning workers was less than the dose limit of 20000 μSv/y for radiation workers, even less than for general population (1000 μSv/y) recommended by International Commission on Radiological Protection (ICRP). However, the elevated inhalation dose for workers conducting cleaning activities may present a health hazard to workers if they deal with excessive materials containing TENORM, work for excessive time or are under inappropriate safety measures.

1. Introduction

Energy supply by nuclear power generation contributed about 11% of total energy supply in Germany in 1990 and about 6 % in 2019. The German Government planned to have nuclear free energy by 2022. The renewable sources of energy, such as wind, sunlight and geothermal, are predicted to replace the part of nuclear power supply. Geothermal energy generating from the original formation of the planet (20 %) and from radioactive decay of nuclides (80 %) (Turcotte and Schubert, 2002) is expected to grow rapidly over the next decades as the resource is generally abundant, ubiquitous, versatile, low carbon generation, and non-intermittent. Presently the United States has the world largest installed geothermal capacity with approximately 3.7 GWe (IEA Geothermal, 2021). During the last 30 years, the share of renewables in power generation increased from 10 % in 1990 to about 37 % in 2020 in the European Union (IEA International Energy Agency, 2021). Geothermal energy production is forecasted still a high potential for development. It was estimated that the geothermal energy plants could contribute approximately 4–7 % to European electricity generation by the middle of the century (Dalla Longa et al., 2020) and more than 8 % in the U.S. (IEA International Energy Agency, 2021). However, geothermal power production entails associated environmental impacts which should be taken into consideration (Bayer et al., 2013; Sayed et al., 2021). Regarding the human health, in addition to the hazardous elements such as arsenic (Morales-Simfors and Bundschuh, 2021), technically enhanced naturally occurring radioactive materials (TENORM) are mobilized and deposited as waste or scale in equipment like pipes and heat exchanging boards. Such TENORM increase radiation exposure of the workers on site.

In the deep saline fluids of geothermal energy plants, high concentration of radionuclides, e.g., ^{226}Ra, ^{210}Pb, ^{228}Ra, and ^{228}Th are found. Ra isotopes and ^{210}Pb are accumulated in $BaSO_4$/$SrSO_4$ and PbS-bearing

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precipitants, respectively, leading to the occurrence of scale with enhanced radioactivity levels (> 100 Bq/g) (Degering et al., 2011). Such TENORM wastes associated with geothermal energy production are like those associated with oil and gas production: temperature changes lead to precipitation of solids from hot formation waters in piping, equipment, and retention ponds at the surface. Annually generated masses of scale and scale-like materials are in the order of several 100 kg per geothermal facility. The maximum activity concentrations in residues from geothermal energy production were reported in Bq/g as ^{226}Ra: 1347, ^{210}Pb: 1100, ^{228}Ra: 442, ^{224}Ra: 384, ^{228}Th: 459 (Cuenot et al., 2011) and ^{226}Ra: 270, ^{210}Pb: 300, ^{228}Ra: 210, ^{228}Th: 190 (Degering and Köhler, 2015) at Upper Rhine Valley and Neustadt-Glewe in Germany, respectively, while the activity concentration of ^{226}Ra in general soil and granite rocks are usually less than 0.1 Bq/g. The elevated activities produce a radiation field with equivalent dose rate up to about 10 μSv/h of external dose at Neustad-Glewe (Degering and Köhler, 2015). The value may be significant considering the criterion of the effective annual dose, which is maximally 1 mSv/y for the general population in the EU regions (Official Journal of the European Union, 2014). For health care of the workers, it is important to understand the radiation dose from TENORM at geothermal energy plants.

In this study we assessed internal dose from TENORM for workers conducting cleaning activities on heat exchanging boards, which were in contact with hot fluids, using a novel experimental approach. We organized the model experiment with “cleaning workers”, who were the staff of our institute and performed cleaning activities, and “office workers” for comparison. The heat exchanging boards used at a geothermal energy plant in Germany were transported to a radiation controlled area for cleaning. The experiment was continued for 7 days including 3-day cleaning activities, providing the workers and controls, who were non-smokers, with known foods and drinks to reduce the influence of variety in the subjects’ diets on the results.

Radon was considered as an influencing radionuclide to calculate the final internal dose for workers due to cleaning activities in this study. The longest-lived radon, ^{222}Rn, is the decay product of ^{226}Ra, one of the main radioactive nuclides in scale adhering to equipment at geothermal facilities. ^{222}Rn concentrations in air surrounding the subjects were continuously measured the whole day during the experiment period at working place and home.

The 24-h feces and urine from the workers and controls were collected separately every day in the morning. The activity concentration of ^{226}Ra, ^{210}Pb, ^{210}Po, ^{228}Ra and ^{228}Th in each excretion was determined and used to calculate the committed effective doses for the subjects.

Finally we determined annual internal dose from TENORM for workers conducting cleaning activities on scale adhering to heat exchanging boards used at a geothermal energy plant.

The experimental approach and the data obtained concerning such risks at geothermal energy plants shown in this study will be important and useful from the viewpoint of radiation protection for workers worldwide.

2. Methods

2.1. Outline of study

The experiment was carried out for continuous 7 days including 3-day cleaning activities on the heat exchanging boards, which were used at a geothermal energy plant in Germany (Figs. 1 and 2). The foods and drinks provided by us were supplied to the subjects from the 1st day through the 6th day during the experiment. 24-hours (24-h) urine and feces were collected separately by the subjects every day in the morning from the 2nd day through the 7th day. The 24-h feces sample represents the excretion of the foods consumed the day before since a mean time of gastrointestinal transit is considered as 24 h (Degen and Phillips, 1996). Accordingly the collection of excretions was started on the second day

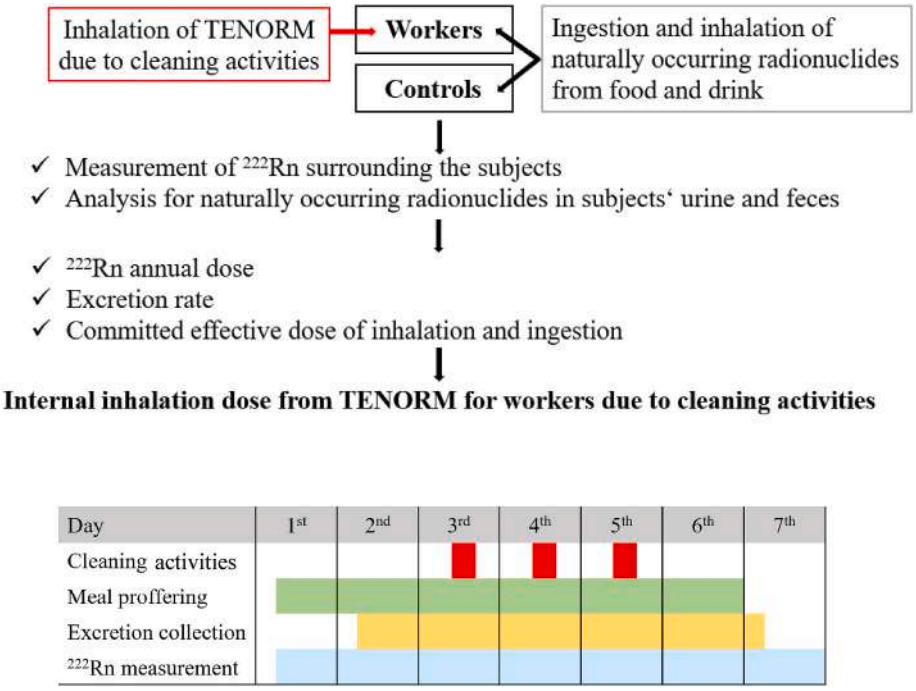

Fig. 1. Outline of study contents and diagram depicting the program. The experiments were carried out continuously for 7 days and the workers were assigned cleaning activities on the 3rd, 4th and 5th days.

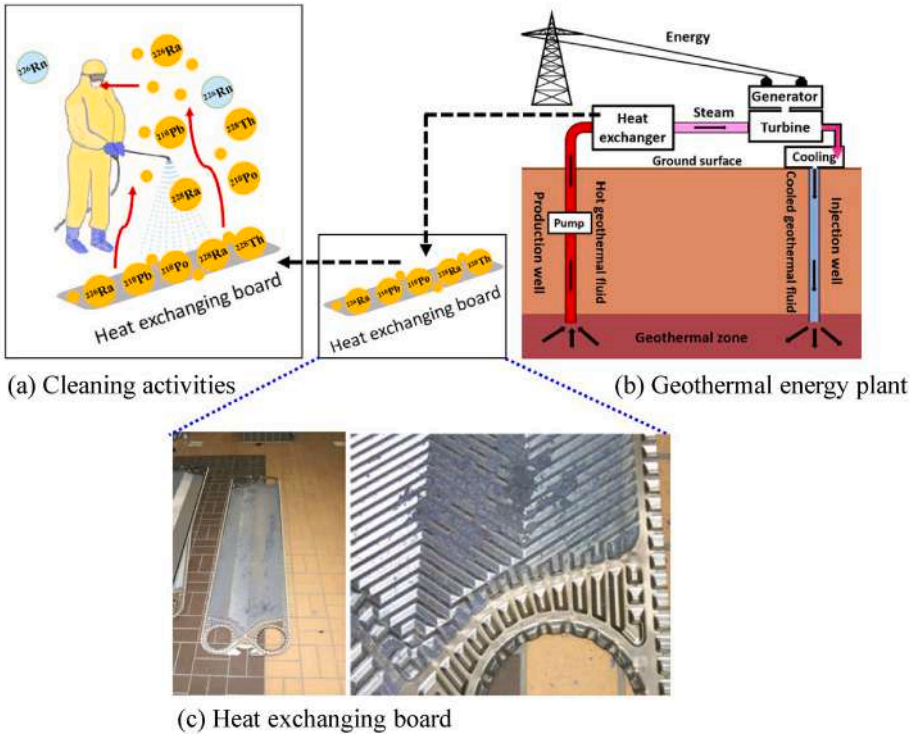

Fig. 2. Example depicting (a) cleaning activities, (b) geothermal energy plant and (c) heat exchanging board.

after taking the prepared foods on the first day. Activity concentrations of ^{210}Pb, ^{210}Po, ^{226}Ra, ^{228}Ra and ^{228}Th in urine and feces were determined by alpha-particle spectrometry, gamma-ray spectrometry and gas-flow proportional counting method, after chemical separation except ^{226}Ra and ^{228}Ra in feces. ^{222}Rn concentration in air surrounding the subjects was measured continuously using a portable electronic radon exposure meter, which the subjects wore throughout 7 days. The subjects recorded the timetable of basic daily life actions such as eating, working, resting and sleeping they executed. The obtained results in the experiments were used to determine the committed effective doses for the subjects and the internal inhalation dose for workers due to cleaning activities.

2.2. Subjects

The subjects in the experiments were all adult males and classified into two groups: the first group of three workers, W1, W2 and W3 (W1 – W3), engaged in cleaning activities on the heat exchanging boards and located in Dresden, Germany; the second group of 6 office workers, C1, C2, C3, C4, C5 and C6 (C1 – C6) located in Munich, Germany, as control for a comparison of the results. They were limited to non-smokers to avoid taking a high amount of naturally occurring radionuclides from the tobacco leaves (Papastefanou, 2009) and influencing the dose assessments. W2 performed main part of cleaning activities and W1 and W3 assisted W2. The doses for the workers WN1, WN2 and WN3 (WN1 – WN3), the same persons as W1, W2 and W3, respectively, but neither performing cleaning activities nor undergoing dieting control, were additionally studied sometime later for comparison.

2.3. Dieting program

The subjects chose for lunch and dinner from eight cooked-frozen set meals, which were prepared by a catering service. The commercial foods and drinks were supplied for breakfast as well as for additional meals. The prepared foods and drinks were as followings.

- Set meal: (1) beef goulash + spaetzle, (2) braised beef + beetroot + potato wedge, (3) cheese noodle + no side dish, (4) chili con carne + rice, (5) coalfish filet + mashed potato + carrot, (6) poulard + potato, (7) pork cutlet + mashed potato + sauerkraut, (8) sausage + noodle.
- Commercial food: bread, butter, camembert cheese, cereal, chocolate, edam cheese, honey, jam, Lyonnaise sausage (pressed meat), roll bread, salami (processed meat).
- Commercial drink: apple juice, milk, mineral water, orange juice.

Due to significantly high concentration of naturally occurring radionuclides in unfiltered drinks (Wang and Chen, 2006), e.g., brewed beer from wheat were forbidden to drink. The filtered drinks like wine, champagne and hard liquor were accepted to drink. Coffee and tea were prepared using mineral water provided.

2.4. Cleaning activities on heat exchanging boards

The heat exchanging boards cleaned in this experiment were practically used at the facility “Heat exchanger” of a geothermal plant in Germany (Fig. 2). The cleaning activities by the workers were performed on the 3rd, 4th and 5th days for each 4 h during the experiment as shown in Fig. 1. 150 pieces of heat exchanging boards made of titanium were cleaned at one of the radiation controlled areas of our institutes. The workers carried out unpacking, cleaning activities and re-packing the boards. The radioactivity on surface board before and after cleaning was measured under inherent protection combined with supplied air in a hermetically sealed room, where air exchange rate was about 14/h. The workers wore the protective clothing required for the controlled area and additionally face mask during the cleaning activities (Fig. 2). The gray-black scale adhering to the heat exchanging boards, which were in contact with hot fluid, were mechanically removed using wire brushes and washed away using water. The concentration of ^{210}Pb (+^{210}Po), ^{226}Ra, ^{228}Ra and ^{228}Th on the surface of each board was measured using a surface contamination monitor (α, β detector) before and after cleaning. The cleaning was finished when the surface activity concentration

of radionuclides reached to the criteria defined by the regulation in Germany (BGB1 Federal Law Gazette, 2018), i.e., ^{210}Pb ($+^{210}$Po): 1 Bq/cm^2, ^{226}Ra: 1 Bq/cm^2, ^{228}Ra: 1 Bq/cm^2 and ^{228}Th: 0.1 Bq/cm^2.

The total mass of scale removed from the surface of 150 heat exchanging boards was 14 kg. The activity amount of ^{210}Pb ($+^{210}$Po), ^{226}Ra, ^{228}Ra and ^{228}Th in the scale determined by gamma-ray spectrometry was obtained as ^{210}Pb ($+^{210}$Po): 1.2×10^4 Bq, ^{226}Ra: 7.6×10^4 Bq, ^{228}Ra: 6.2×10^4 Bq and ^{228}Th: 4.3×10^4 Bq.

The work described has been carried out in accordance with The Code of Ethics of the World Medical Association and precaution was taken to protect the privacy of research subjects and confidentiality of their personal information.

2.5. Measurement of ^{222}Rn in air surrounding subjects

The subjects, both workers and controls, wore individual electronic radon exposure meters (Karinda et al., 2008) continuously throughout the experiment. The portable meter recorded the radon activity concentration in air surrounding the subjects during the 7 days. During cleaning activities, the workers wore the portable meter inside the protective clothing. The portable meter consists of a passive diffusion chamber with a filtering medium and pin-photodiodes for alpha-particle detection, allowing continuous concentration records. Radon gas diffuses through the inlet filter into the detection chamber. The filter prevents the entry of radon decay products into the chamber and makes the device independent of aerosol concentrations and humidity. The alpha radiation emitted from ^{222}Rn and its decay products ^{218}Po and ^{214}Po are recorded using the alpha detectors inside the chamber. The induced voltage drop caused by ionization currents is amplified, shaped, counted, and stored on a microprocessor. The count rate was proportional to the ^{222}Rn concentration (Karinda et al., 2008; Irlinger et al., 2016). Calibration of the radon exposure meters was performed in a high radon concentration environment (up to 2300 Bq/m^3) within a closed vessel, using a stable radon source. A calibrated radon exposure meter, AlphaGuard (Bertin Instruments, Germany), was applied for the secondary standard. The detection limit and uncertainty for a 1 h measurement period were determined as 44.0 Bq/m^3 and ± 5.6 %, respectively (Irlinger, 2015). Thoron (^{220}Rn) was not assessed in this study.

2.6. Dose conversion factor for ^{222}Rn inhalation

The effective dose from inhalation of ^{222}Rn and its short-lived decay products is usually expressed in terms of a dose conversion factor using the following formula (Brudecki et al., 2014), which is given as effective dose per unit potential alpha exposure in Sv per J h/m^3.

$$E = \sum_{i=1}^{4} \sum_{j=1}^{4} C_{j,i}\ B\ t\ f_{pj}\ E_{j,i}$$

where i corresponds to inhaled ^{222}Rn (i = 1, 2, 3 and 4, for ^{218}Po, ^{214}Pb, ^{214}Bi and ^{214}Po, respectively); j corresponds to the aerosol size distribution (j = 1, 2, 3 and 4 for the unattached, nucleation, accumulation and coarse modes, respectively); Cj,i is the activity concentration of decay product i with activity size distribution j corresponding to a ^{222}Rn decay products mixture of 3.54×10^{-3} h/m^3 (Table 1); B is the mean breathing rate for each age group; t is taken as the exposure period of 170 h (standard monthly working time); f_{pj} is the fraction of Potential Alpha Energy Concentration (PAEC) associated with mode j; and Ej,i is the effective dose coefficient for decay product i with an aerosol size distribution for mode j.

Table 1
Activity concentrations, $C_{j,i}$ of a mixture of short-lived ^{222}Rn decay products corresponding to the concentration of 3.54 x 10^{-3} h/m^3 for either the unattached or the attached decay products.

j	Diameter	f_{pj}	$C_{j,i}$ (Bq/m^3)			
			^{218}Po (i=1)	^{214}Pb (i=2)	^{214}Bi (i=3)	^{214}Po (i=4)
1	1 nm	0.1	24100	2410	0	0
2	50 nm	0.135				
3	230 nm	0.747	5210	3910	3130	3130
4	2500 nm	0.018				

The ratio of the activity concentrations in air is assumed as following: ^{218}Po : ^{214}Pb : ^{214}Bi : ^{214}Po = 1 : 0.1 : 0 : 0 (unattached); 1 : 0.75 : 0.6 : 0.6 (attached).

In monitoring ^{222}Rn and its decay products, it is useful to know the dose contributions of each progeny to the total effective dose of ^{222}Rn decay products. The contributions were calculated using the following formula (Brudecki et al., 2014), assuming the ratio of activity concentrations of the decay product and aerosol sizes (with ^{214}Po being in equilibrium with ^{214}Bi) as shown in Table 1.

$$\frac{C_{j,i}\ \ f_{pj}\ \ E_{j,i}}{\sum_{i=1}^{4} \sum_{j=1}^{4} C_{j,i}\ \ f_{pj}\ \ E_{j,i}}$$

The parameters of equations and the values are taken from Table 1.

The ^{222}Rn annual doses for the workers were calculated, employing the mean ^{222}Rn concentrations measured during this experiment and the dose conversion factor of 13 nSv/(Bq·h/m^3), which is recommended by the International Commission on Radiological Protection (ICRP) (ICRP International Commission on Radiological Protection, 2017). The radon equilibrium factor of 0.4 indoor and occupancy factor of 0.8 were used. Air exchange rate at the working place was about 14/h, which is a typical value for laboratories. We were unable to determine the true F value as we did not measure the fractions of PAEC nor the activity concentrations of each progeny, consequently, we employed the recommended mean indoor value of radon equilibrium factor: F = 0.4 (ICRP International Commission on Radiological Protection, 2017) for the calculation. The additional exposure to ^{222}Rn during the cleaning activities was calculated, assuming that the workers work for 170 h (standard monthly working time) a year and using the mean ^{222}Rn concentrations measured. The uncertainties of radon annual doses to the subjects were calculated using the variation of radon concentration in the measurements.

2.7. Analytical methods for ^{210}Pb, ^{210}Po, ^{226}Ra, ^{228}Ra and ^{228}Th in excretions

The 24-h urine samples were acidified using HNO_3 and HCl solutions and stored at 4 °C. ^{209}Po (~15 mBq) and ^{229}Th (~15 mBq)/^{225}Ra: decay product from ^{229}Th for ^{226}Ra analysis, from certified standard solutions were added as spikes to an aliquot of urine sample for isotope dilution method analysis. The urine samples with the spikes were evaporated to dryness and the residue was fumed using 65 % HNO_3 and 30 % H_2O_2 for several times. The feces samples were frozen at −18 °C soon after they arrived at the institute to limit the growth of microorganism. ^{209}Po (~15 mBq) and ^{229}Th (~15 mBq) from certified standard solutions were added as spikes to an aliquot of feces sample for isotope dilution method analysis. The feces samples with the spikes were ashed at 450 °C and the ash was put in a mixture of 65 % HNO_3 and 75 % $HClO_4$ and heated to dryness. All chemicals used throughout the chemical separation procedure were from analytical grade.

A sequential method for chemical separation was developed to isolate each radionuclide from excretions and the method was applied for the sample analysis. The dry residues were dissolved using 6 M HCl and after addition of ascorbic acid, Po and Bi were autodeposited onto a nickel plate in 0.5 M HCl, with Pb, Ra and Th remaining in the solution. The nickel plate was rinsed using deionized water and ethanol, dried in air and wrapped in aluminum foil.

The supernatant solution left from the autodeposition was used for

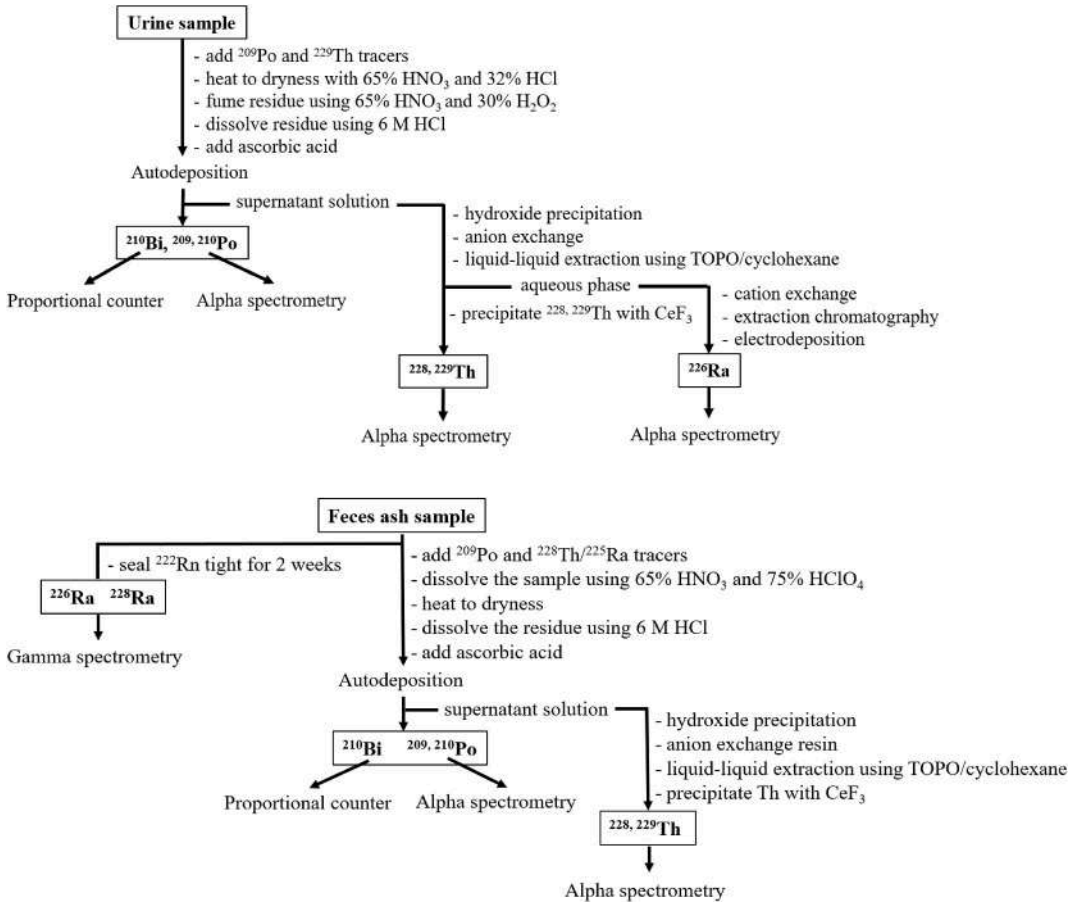

Fig. 3. Schematic explanation of chemical separation procedures for urine and feces samples.

further chemical separation for Ra and Th. After adjusting the oxidation state of various elements in matrix using H_2O_2, Th was extracted twice using a 5 % trioctyl phosphine oxide (TOPO) in cyclohexane, recording the date and time of the first organic extraction. The organic phase was washed using 0.5 M HNO_3. From the organic phase, Th was re-extracted using 0.3 M H_2SO_4. The re-extract was washed using cyclohexane and collected in a plastic tube. Th was coprecipitated with Ce(III)fluoride adding Ce (50–100 μg) and 20 % hydrofluoric acid and the precipitate was gathered on a membrane filter of 0.1 μm pore size and glued on a stainless steel plate. The liquid phases before re-extraction were collected for the analysis of ^{226}Ra in urine samples. Ra was separated from the matrix using cation exchange resin: Dowex 50Wx8 (Merck, KGaA) and further using extraction chromatographic resin: Sr-Resin (Triskem International). ^{226}Ra was electrically deposited on a stainless steel plate. ^{228}Ra in urine samples could not be directly quantified as the analytical method had at that stage not been established. The result of ratio ^{228}Ra/^{226}Ra in feces was used for calculation.

^{210}Pb was measured via its daughter nuclide ^{210}Bi, which was autodeposited together with ^{210}Po on nickel plate wrapped in aluminum foil, using a proportional counter (PC). After removing the aluminum foil from the nickel plate, ^{210}Po and ^{209}Po were measured using alpha spectrometers. ^{229}Th and ^{228}Th as well as ^{226}Ra were measured using alpha spectrometers.

The chemical recovery of ^{209}Po ranged from 35 to 95 %. The reason for the non-volatilization of Po can be assumed as due to embedding of Po in the matrix such as sodium, potassium, ammonium chloride, nitrate and others, which can retain Po. The chemical recovery of ^{210}Pb was determined using a certified standard solution of ^{210}Pb in equilibrium with ^{210}Bi and ^{210}Po, from which defined activity aliquots were autodeposited and measured via proportional counter. The chemical recovery of ^{210}Bi ranged from 92 to 98 %. The chemical recovery of ^{226}Ra was determined via ^{225}Ac, ^{221}Fr or ^{217}At, the daughter nuclides of ^{225}Ra, which is the decay product from ^{229}Th in the tracer added to the samples and already unsupported by ^{229}Th, using alpha spectrometry. The chemical recovery for ^{226}Ra and ^{229}Th ranged from 35 to 95 % and from 35 to 85 %, respectively.

The activity concentration of ^{226}Ra and ^{228}Ra in the feces ash samples were determined using non-destructive method and gamma-ray spectrometer equipped with high purity germanium (HPGe) detector with a relative efficiency of 30 % and 90 %, respectively, at the underground laboratory (Niese et al., 1998; Köhler et al., 2009). 1–5 g feces ash was filled into a cylindrical container, sealed radon tight and stored for 2–3 weeks for equilibration between mother and daughter nuclides. ^{226}Ra was determined using its daughter nuclides ^{214}Pb and ^{214}Bi, and ^{228}Ra was determined using its daughter nuclide ^{228}Ac.

The schematic explanation of chemical separation procedures is shown in Fig. 3.

2.8. Dose assessment for workers resulting from cleaning activities

The lifetime cumulative dose based on the effective dose is called committed effective dose (Sv), where the lifetime is set as 50 years for adults. To estimate the committed effective dose for the workers resulting from cleaning activities, we follow the standard dose assessment method employing monitoring exposure data in three steps: the individual workplace monitoring measurements, such as urinary and fecal excretions; the assessment of intake from the measurements and the calculation of dose from the assessed intake (EC European Commission, 2018). In the working place monitoring in this study, a single excretion rate R_{exc} was used, the intake I can be estimated comparing the

Table 2
Mean activity concentration of ^{222}Rn (Bq/m^3) obtained from each subject each day[a] and ^{222}Rn annual doses (μSv/y) estimated in this study using the mean activity concentration.

	Mean activity concentration of ^{222}Rn (Bq/m^3)								
Day	W1	W2	W3	C1	C2	C3	C4	C5	C6
1	25 ± 21	24 ± 26	31 ± 24	70 ± 26	-	-	286 ± 35	103 ± 25	60 ± 28
2	27 ± 21	19 ± 26	35 ± 24	176 ± 37	-	-	68 ± 23	314 ± 40	79 ± 28
3	27 ± 21	156 ± 32	28 ± 24	81 ± 26	36 ± 19	-	45 ± 22	129 ± 29	116 ± 30
4	36 ± 21	51 ± 27	35 ± 25	96 ± 31	40 ± 10	-	111 ± 28	97 ± 26	84 ± 30
5	66 ± 23	99 ± 29	32 ± 25	86 ± 28	71 ± 12	-	69 ± 23	121 ± 27	59 ± 28
6	56 ± 23	107 ± 30	16 ± 24	97 ± 27	36 ± 9	-	143 ± 30	116 ± 29	242 ± 37
7	n.m.	n.m.	n.m.	24 ± 25	40 ± 11	-	67 ± 23	85 ± 24	16 ± 26
^{222}Rn Annual dose (μSv/y)	1440 ± 320	2770 ± 420	1010 ± 360	3280 ± 400	1620 ± 210	-	4100 ± 370	5020 ± 400	3410 ± 410

[a] Dieter, 2015. W: worker (located in Dresden, Germany), C: control (located in Munich, Germany). ±: combined uncertainty. n.m.: not measured. -: no data due to breakdown of exposure meter.

result of the measurement with the appropriate value predicted by the corresponding bioassay function $E_f(t)$ at time t after intake: $I = R_{exc}/E_f(t)$. For routine monitoring, the assumed time of a suspected acute intake corresponds to the mid-point of the monitoring interval. In our monitoring, the incorporation of the activity within the collection period of six days is assumed, accordingly the fecal excretion function rate at the middle of the interval, i. e. the third day after intakes were applied. Using an estimated value of a radionuclide intake I, the committed effective dose E_{50} can be evaluated by multiplying the intake by the appropriate dose coefficient: $E_{50} = I \cdot e_{50}$. Accordingly the committed effective doses for an exclusive inhalation and ingestion by the workers were calculated using the following formula.

$$E_{50} = \frac{e_{50} \cdot Rexc}{E_f(t)}$$

where E_{50} is committed effective dose for 50 y, e_{50} is dose coefficient (Sv/Bq) for E_{50}, R_{exc} is excretion rate (Bq/d) and $E_f(t)$ is standardized excretion rate (Bq/d) on the day t after a single intake of 1 Bq through inhalation or ingestion.

3. Results

3.1. ^{222}Rn concentration in air surrounding subjects and annual doses

The daily mean ^{222}Rn exposures to each subject (Dieter, 2015) and ^{222}Rn annual doses (μSv/y) calculated in this study using the daily mean values are shown in Table 2. The ^{222}Rn measurement was not performed on the 7th day for workers as no activity was allowed at their usual working place due to limitation of radiation exposure. Additional effective doses for the workers due to cleaning activities for 170 h (standard monthly working time) a year were estimated employing the radon concentration at workplace measured in this study. The results are shown in Table 3.

3.2. Determination of excretion rates of ^{210}Pb, ^{210}Po, ^{226}Ra, ^{228}Ra and ^{228}Th

The amount of 24-h feces samples ranged from 12 to 541 g per day with the mean value of 157 ± 96 g. The ratio of ash mass to fresh mass of the feces was 0.032. The volume of 24-h urine samples of the subjects ranged from 1.4 to 4.3 L per day with a mean value of 2.3 ± 0.7 L. The mean excretion rates of ^{210}Pb, ^{210}Po, ^{226}Ra, ^{228}Ra and ^{228}Th from the subjects were calculated (Table 4). Because no analysis for ^{228}Ra in urine sample was possible during the execution of studies, ^{228}Ra activity concentration in urine was calculated using the ^{228}Ra/^{226}Ra ratio of feces obtained using gamma-ray spectrometry, multiplying the ^{226}Ra activity concentration of urine. The results of ^{228}Ra in urine are, however, possibly lower than the indicated values due to the short physical half-life of ^{228}Ra (5.75 y) compared to the long biological half-life of Ra (50 y). The exchange of ^{228}Ra among tissues may be slower than its physical decay, also the transfer pattern of ^{228}Ra into urine could be different from that into feces. The most of results for ^{210}Po were lower than the detection limit (Table 4). The daily records of excretion rates from each subject are shown in Appendix A, Table S1.

3.3. Inhalation and ingestion committed effective doses

Intake of radionuclides occurs through inhalation and ingestion. As the workers performed cleaning activities on heat exchanging boards in a radiation controlled area wearing protective clothing, ingestion of radionuclides presumably does not occur. However, to compare the potential dose ranges for the two actions of inhalation and ingestion, the committed effective doses were calculated, applying the standardized excretion rates (ICRP International Commission on Radiological Protection, 2017, 2019) due to insufficient data for statistical evaluation in this study (Table 5).

E_f: standardized excretion rate after one-time incorporation of 1 Bq, e: dose coefficient. E_f (inh); E_f (ing): the fecal excretion function at the third day after intake (ICRP International Commission on Radiological

Table 3
Mean additional ^{222}Rn exposure (Bq/m^3) for workers (W1, W3 and W3) conducting cleaning activities on heat exchanging boards and additional effective dose (μSv/y) for 170 h cleaning activities a year.

Day	W1		W2		W3	
	(Bq/m^3)	(μSv/y)	(Bq/m^3)	(μSv/y)	(Bq/m^3)	(μSv/y)
3rd	311 ± 78		1272 ± 172		39 ± 62	
4th	224 ± 70		195 ± 77		128 ± 68	
5th	484 ± 94		344 ± 89		39 ± 67	
170 h working time a year		300 ± 83		530 ± 12		61 ± 67

±: combined uncertainty.

Table 4
Mean of excretion rate (mBq/d) of ^{210}Pb, ^{210}Po, ^{226}Ra, ^{228}Ra and ^{228}Th from subjects.

Isotope	Excretion	MDC (mBq/d)	W1-W3			WN1-WN3			C1-C6		
			n	Mean (mBq/d)	StdDev	n	Mean (mBq/d)	StdDev	n	Mean (mBq/d)	StdDev
^{210}Pb	Feces	20	15	149	129	9	79	42	29	85	70
^{210}Po		2	2	62	31	9	< 86		32	< 227	
^{226}Ra		4.6	15	153	99	8	81	33	32	90	43
^{228}Ra		11	12	230	117	7	115	40	21	148	71
^{228}Th		0.5	15	62	48	9	27	16	31	39	24
^{210}Pb	Urine	9	12	18	3	5	14	3	18	25	13
^{210}Po		0.9	18	< 41		9	< 23		36	< 472	
^{226}Ra		0.35	13	1.6	0.6	9	1.3	0.5	21	0.9	0.5
^{228}Ra		—		2.4			1.9			1.5	
^{228}Th		0.23	9	0.70	0.30	4	0.55	0.11	12	0.76	0.21

MDC: minimum detectable concentration. n: number of analyses, mean: mean value, StdDev: standard deviation. —: not analyzed (see the text).

Table 5
Committed effective dose (μSv) of inhalation and ingestion for subjects.

Nuclide	Inhalation					Ingestion				
	E_f (inh) (Bq/d)/Bq	e (inh) (μSv/Bq)	E_{50} (inh), μSv			E_f (ing) (Bq/d)/Bq	e (ing) (μSv/Bq)	E_{50} (ing), μSv		
			W1-W3	WN1-WN3	C1-C6			W1-W3	WN1-WN3	C1-C6
^{210}Pb	0.17	0.62	0.54	0.29	0.31	0.18	0.32	0.26	0.14	0.15
^{226}Ra	0.18	1.4	1.19	0.63	0.70	0.21	0.13	0.09	0.05	0.06
^{228}Ra	0.18	1.2	1.53	0.77	0.99	0.21	0.34	0.37	0.19	0.24
^{228}Th	0.17	9	3.28	1.43	2.06	0.23	0.031	0.01	0.004	0.01
Total			6.55	3.11	4.06			0.74	0.38	0.45

E_f: standardized excretion rate after one-time incorporation of 1 Bq, e: dose coefficient. E_f (inh); E_f (ing): the fecal excretion function at the third day after intake (ICRP, 2019). e (inh); e (ing): the inhalation and ingestion dose coefficients (ICRP, 2017).

Protection, 2019). e (inh); e (ing): the inhalation and ingestion dose coefficients (ICRP International Commission on Radiological Protection, 2017).

3.4. Total annual effective inhalation dose for workers

The mean of annual internal dose from TENORM for W1 – W3 was calculated assuming 170 h working time which is equivalent to 4 times-cleaning week (Table 6).

4. Discussion

4.1. Elevated ^{222}Rn exposure during cleaning activities

Elevated inhalations of ^{222}Rn for W2, who performed the main cleaning activities during the 3rd through 5th day, were observed (Table 2). An evaluated inhalation for W1 was also observed on the 5th day when W1 assisted the activities of W2. Referring that the ^{222}Rn concentration in dwellings in Germany reported as annual mean 50 Bq/m^3 (BfS Federal Office for Radiation Protection, 2019), the additional dose for W2 resulting from the cleaning activities is significant. The large variations in ^{222}Rn concentration for the controls were observed during the experiment, although clarification of cause could not be identified in this study.

Table 6
Annual internal dose (μSv/y) for workers (W1 – W3) from TENORM during cleaning activities on scale adhering to heat exchanging boards used at a geothermal energy plant, assuming 170 h cleaning activities a year.

TENORM	W1 – W3 (μSv/y)	u_c (μSv/y)
^{210}Pb,^{226}Ra,^{228}Ra,^{228}Th	26	11
^{222}Rn	297	36
Total	323	38

u_c: combined uncertainty.

The ^{222}Rn annual doses estimated for the workers ranged from 1000 to 2800 μSv/y. The obtained values are higher than those of the mean annual indoor radon dose (900 μSv/y) in Germany (BMUV Federal Ministry for the Environment, Nature Conservation, Nuclear Safety and Consumer Protection, 2014). On the other hand, the higher ^{222}Rn annual doses for the controls than the workers were found to be 1600–5100 μSv/y, presumably due to higher daily exposure in an indoor environment or the different geological environment of the area the workers were located in.

Additional effective doses for the workers due to cleaning activities for 170 h (standard monthly working time) a year were estimated employing the radon concentration at workplace measured in this study. The results show that the additional effective doses due to cleaning activities range from 61 to 530 μSv/y (Table 3).

4.2. Differences of excretion rates of ^{210}Pb, ^{210}Po, ^{226}Ra, ^{228}Ra and ^{228}Th among subjects

The excretion rates of all isotopes in the feces show higher values than those in the urine. The excretion rates of ^{210}Pb, ^{226}Ra, ^{228}Ra and ^{228}Th in feces of W1 – W3 are about factor 1.5–1.7 of WN1 – WN3 as well as of C1 – C6. The higher rates of W1 – W3 and comparable results between WN1 – WN3 and C1 – C6 imply that the higher uptake of radionuclides is a result from the inhaled ^{222}Rn and ^{210}Pb, ^{210}Po, ^{226}Ra, ^{228}Ra or ^{228}Th during cleaning activities. The excretion mean rates (mBq/d) of ^{226}Ra, ^{210}Pb and ^{210}Po in feces; urine were reported in previous studies as.

- ^{210}Pb: 1–395 (Schäfer and Seitz, 2005; Santos et al., 1994); 1–187 (Schäfer and Seitz, 2005; Naumann et al., 1998; Santos et al., 1994),
- ^{226}Ra: 52–65 (ICRP International Commission on Radiological Protection, 1975; UNSCEAR United Nations Scientific Committee on the Effects of Atomic Radiation, 1993; Naumann et al., 1998); <2 (Schäfer and Seitz, 2005) and

- ^{210}Po: 13–141 (Hölgye, 2013); <1–248 (Schäfer and Seitz, 2005; Naumann et al., 1998; Santos et al., 1994; Hölgye, 2013).

4.3. Inhalation and ingestion committed effective doses for workers

One-time incorporation of activities during the 6 days is assumed and the standardized excretion rate E_S of the corresponding radionuclide for feces during the cleaning activities was used for the calculation. The E_f (inh) for ^{226}Ra was substituted for ^{228}Ra, assuming that their biokinetic behavior is the same. E_{50} (Sv) was calculated without consideration of the contribution from ^{222}Rn inhalation nor ingestion through diets. The annual inhalation committed effective dose for workers conducting cleaning activities calculated using the mean excretion rates of feces from W1 – W3 was 6.55 μSv. The value indicates about 10 times higher than the ingestion committed effective dose for workers. Comparison among the three subjects groups indicates that the inhalation and ingestion doses for workers W1 – W3 are higher than those for the other two control groups, (WN1 – WN3) and (C1 – C6), confirming that there were additional doses during cleaning activities.

4.4. Total annual effective inhalation dose for workers

The monitoring of ^{222}Rn exposure for workers W1 – W3 showed increased ^{222}Rn concentrations during cleaning activities (Table 2). This may be due to that the inhalation of ^{222}Rn gas cannot be avoided by usual protective measures and this additional inhalation should be considered in dose assessment of TENORM. The mean of annual internal dose from ^{210}Pb, ^{210}Po, ^{226}Ra, ^{228}Ra and ^{228}Th and ^{222}Rn for W1 – W3 was calculated as 26 μSv/y and 297 μSv/y, respectively, and 323 μSv/y for total (Table 6), using the results of committed effective dose (Table 5) and assuming 170 h working time which is equivalent to 4 times cleaning week. The dose caused by ^{222}Rn exposure is about 10 times higher than the inhalation dose caused by the other nuclides analyzed in this study as shown in Table 6. The results of inhalation dose for the workers are in good agreement with the data from the literature (UNSCEAR United Nations Scientific Committee on the Effects of Atomic Radiation, 2000; BMUV Federal Ministry for the Environment, Nature Conservation, Nuclear Safety and Consumer Protection, 2013, 2014).

Considering the uncertainties of ^{222}Rn exposure for subjects each day (Table 2), exposure from other nuclides and dose limit for workers, the additional exposure 323 μSv/y is tolerable. However, the elevated inhalation dose for workers conducting cleaning activities leads to endanger workers if they deal with more scale/waste, work for excessive time or are under inappropriate protective wearing. The risk of exposure to TENORM could be decreased by reducing working time, before the exposure exceeds regulations, an appropriate ventilation during the cleaning activities and wearing appropriate protective clothes. Conclusively it is important to perform the dose estimation regularly for the health care of workers.

4.5. Comparison of internal exposure among general public and workers at geothermal energy plant and oil and gas industries

The internal exposure from ^{222}Rn for general public varies depending on location. For example, annual effective doses due to ^{222}Rn inhalation for staff or students at school buildings in various locations have been assessed in the past. The ranges of values were reported in μSv/y as Greece: 30–390 (Papaefthymiou and Georgiou, 2007), Italy: 150–1800 (Gaidolfi et al., 1998; Malanca et al., 1998; Malisan and Padovani, 1994), Slovenia: 3700–6700 (Vaupotič et al., 2001), Kuwait: 200–650 (Maged, 2006), Pakistan: 160–1740 (Kahn et al., 2005; Rafique et al., 2010; Rahmann et al., 2010), and Nigeria: 130–450 (Obed et al., 2011). The quantity of exposure shows a greater variability depending on location and probably the environment of school buildings. The ^{222}Rn exposure for the controls located in Munich, Germany observed in this study (1600–5100 μSv/y, Table 2) is also one of the examples of variation depending on area. The additional dose from ^{222}Rn during the cleaning activities for 1 month observed in this study (297 μSv/y) is in the range of those annual doses in the school buildings, excepting the situation in Slovenia with one order of magnitude higher values.

The radiation exposure at the oil and gas industry has been investigated in various countries and the problem with occupational exposure has been noted (Kolb and Wojcik, 1985). The studies were summarized in the recommendations of the International Atomic Energy Agency (IAEA International Atomic Energy Agency, 2003). The internal annual doses at oil industries were reported in μSv/y as Saudi Arabia: 13–118 (Alshahri and El-Taher, 2018), Egypt: 306000–335000 (Abo-Elmagd et al., 2010), Algeria: 10–600 (Ali et al., 2019), Argentina: 10–1600 (Canoba et al., 2008), Ghana: 10010–128920 (Darko et al., 2012), Hungary: 50–700 (Jonas et al., 2018), Albania: 40–70 (Xhixha et al., 2015). The values are in the same order of magnitude as the results obtained in this study, except for the case of Egypt and Ghana. The exposure of workers to ^{222}Rn was also studied in gas and oil industries, investigating ^{222}Rn from gas and oil materials (Gesell, 1975; Al-Masri and Shwiekani, 2008) as well as from wastes/scale (Ali et al., 2019; Nowak et al., 2020). Most of those exposures were indicated below 10000 μSv/y. However, an exposure level higher than 10000 μSv/y was found in Egypt and Ghana, indicating regional variabilities and necessity of additional studies for various regional geological environments for comprehensive assessments.

5. Conclusions

In this study we demonstrated the experimental approach for internal exposure assessment resulting from TENORM for workers conducting cleaning activities on equipment used at geothermal energy plants. The additional exposure due to the cleaning activities on the scale adhering to equipment indicated 323 μSv/y. The value was less than the dose limit of 20000 μSv/y for radiation workers, even less than for general population (1000 μSv/y) recommended by ICRP (ICRP International Commission on Radiological Protection, 2007). However, the increased radiation exposure for workers implies that it may exceed the limit depending on the working time, dealing materials, on-site situation or inappropriate safety measures, requiring periodic inspections of doses for the workers. According to the results on TENORM exposure for workers obtained in this study, the ^{222}Rn contribution is much greater than the other nuclides. For the safety management of workers, the ^{222}Rn exposure can be assessed as the first step, whereby the time and cost could be appropriately reduced. A recent study reported that masks are effective in filtering radon progeny and thus are capable of reducing the total effective dose to the lungs, investigating with the filtration properties of FFP2 masks and surgical masks (II R) (Hinrichs et al., 2022). The doses determined in this study may also have been influenced by the kinds of face masks which the workers wore and the total uncertainty of the results may be larger. The influence of masks on inhalation should be important to study as the next stage for reducing doses.

The methods of experiments and data evaluations shown in this study will be increasingly important in various types of works to protect and manage the health of workers, consistent with the increasing demands of geothermal energy in the future. In reference to the numerous databases on TENORM exposure in oil and gas industries, greater variability of data can be expected, dependent on location of geothermal energy plants. Additional studies for various regional geological environments are required to provide comprehensive assessments.

Author contribution

TS designed and implemented the study; evaluated data; wrote the original manuscript; edited and finalized the manuscript. DW designed and implemented the study; performed excretions analysis; evaluated data; reviewed and edited the manuscript. WBL evaluated data;

calculated doses; reviewed and edited the manuscript. JT launched the project and gained fundings; calculated doses; reviewed and edited the manuscript.

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Declaration of competing interest

We declare that we have no competing interests.

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Appendix A. Supplementary data

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Abbreviation

BfS: Federal Office for Radiation Protection (Bundesamt für Strahlenschutz), Germany
BGB1: Federal Law Gazette (Bundesgesetzblatt), Germany
BMBF: Federal Ministry of Education and Research (Bundesministerium für Bildung und Forschung), Germany
BMUV: Federal Ministry for the Environment, Nature Conservation, Nuclear Safety and Consumer Protection (Bundesministerium für Umwelt, Naturschutz, nukleare Sicherheit und Verbraucherschutz), Germany
IAEA: International Atomic Energy Agency
ICP-MS: Inductively coupled plasma mass spectrometry
ICRP: International Commission on Radiological Protection
IEA: International Energy Agency
EC: European Commission
NORM: Naturally occurring radioactive material
PAEC: Potential alpha energy concentration
PC: Proportional counter
StdDev: Standard deviation
TENORM: Technically enhanced naturally occurring radioactive material
TOPO: Trioctyl phosphine oxide
UNSCEAR: United Nations Scientific Committee on the Effects of Atomic Radiation