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A revised concept for deriving reference values for internal exposures to chemical substances and its application to population-representative biomonitoring data in German children and adolescents 2014–2017 (GerES V)

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A B S T R A C T

HBM reference values, in contrast to toxicologically derived values, are statistically derived values that provide information on the exposure of the population. The exceedance frequency (if applicable for individual population groups) is often a first assessment standard for the local exposure situation for municipalities. More than 25 years have passed since the German Human Biomonitoring Commission (HBMC) formulated the first recommendations for the derivation of population-based reference values (HBM reference values, RV_{95}) for substance concentrations based on HBM studies. A fundamental revision is timely, for several reasons. There have been considerable advances in relevant statistical methods, which meant that previously time-consuming and inaccessible procedures and calculations are now widely available. Furthermore, not all steps for the derivation of HBM reference values were clearly elaborated in the first recommendations. With this revision we intended to achieve a rigorous standardization of the entire process of deriving HBM reference values, also to realise a higher degree of transparency.

In accordance with established international practice, it is recommended to use the 95th percentile of the reference distribution as the HBM reference value. To this end, the empirical 95th percentile of a suitable sample should be rounded, ensuring that the rounded value is within the two-sided 95% confidence interval of the percentile. All estimates should be based on distribution-free methods, and the confidence interval should be estimated using a bootstrap approach, if possible, according to the BCa ("bias-corrected and accelerated bootstrap"). A minimum sample size of 80 observations is considered necessary. The entire procedure ensures that the derived HBM reference value is robust against at least two extreme values and can also be used for underlying mixed distributions.

If it is known in advance that certain subgroups (different age groups, smokers, etc.) show differing internal exposures, it is recommended that group-specific HBM reference values should be derived. Especially when the sample sizes for individual subgroups are too small, individual datasets with potential outliers can be excluded in advance to homogenize the reference value population.

In the second part, new HBM reference values based on data of the German Environmental Survey for Children and Adolescents (GerES V, 2014–2017) were derived in accordance with the revised recommendations. The GerES V is the most recent population-representative monitoring of human exposure to pollutants in Germany on children and adolescents aged 3–17 years (N = 2294). RV_{95} for GerES V are reported for four subgroups (males/females and 3–11/12-17 years) for 108 different substances including phthalates and alternative plasticisers, metals, organochlorine pesticides, polychlorinated biphenyls (PCB), per- and polyfluoroalkyl substances (PFAS), parabens, aprotic solvents, chlorophenols, polycyclic aromatic hydrocarbons (PAH) and UV filter, in total 135 biomarkers. Algorithms implemented in R were used for the statistics and the determination of the HBM reference values. To facilitate a quality control of the study data, the corresponding R source code is given, together with graphical representations of results.

The HBM reference values listed in this article replace earlier RV_{95} values derived by the HBMC for children and adolescents from data of precedent GerES studies (e.g. published in Apel et al., 2017).

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1. Introduction

The Commission "Human Biomonitoring" of the German Environment Agency (HBM Commission) first set out its concept of reference and human biomonitoring values in a policy paper in 1996 (HBM Commission, 1996) and supplemented it by means of an addendum in 2009 (HBM Commission, 2009). The methodology described for deriving HBM reference values (RV_{95}) is based in particular on the work of IFCC (International Federation of Clinical Chemistry; Solberg, 1984) and IUPAC (International Union of Pure and Applied Chemistry; Poulsen et al., 1997). After more than 25 years of successful implementation of the concept, an updated addendum is presented here, which considers new methodical developments. Computers and statistical software have improved continuously since 1996, so that even formerly time-consuming statistical procedures can now be carried out without substantial time expenditure. This allows the reorientation from previously favoured distribution-based methods to distribution-free methods. Thus, from the Commission's point of view, an improved RV_{95} derivation algorithm can be proposed. In particular, it depends less on the statistical distribution of the measured values and significantly less on extreme values.

In the original concept (HBM Commission, 1996), under "Definition of the reference value", it is stated: "The reference value for a chemical substance in a body medium … is a value derived from a series of corresponding measured values of a random sample from a defined population group according to a specified statistical procedure. It is a purely statistically defined value that describes the concentration of a substance in the body medium in question for a population group at the time of the study. Reference values are generally expected to change over time as a result of changing environmental burden." The HBM reference value thus describes, by definition, a merely statistically defined assessment value for the body burden.

- of a defined population group, i.e. the reference population; namely
- at a certain point in time, usually at the time of the study on the basis of which the HBM reference values were derived.

An (upper) HBM reference value indicates an assessment level that is rarely exceeded in the reference population. For the HBM reference values derived by the HBM Commission, no more than about five percent exceedances should be seen. Internationally, other percentiles are also used as an assessment standard for HBM reference value derivation (Vogel et al., 2019); for example, 97.5-percentiles are used for HBM reference value derivation from the comprehensive survey data of the U.S. National Health and Nutrition Examination Survey (NHANES) (Ruckart et al., 2021).

For the derivation of an HBM reference value, however, not only the estimation of the percentile value (technically: point estimator) is decisive, but also its variability and thus its "trustworthiness". The random fluctuations and deviations caused by the selection of the test persons and other random effects have to be considered when deriving an HBM reference value. For this purpose, a confidence interval must be added to the estimate of the 95th percentile value.

In the first concept (HBM Commission, 1996) it is stated that rounding can be performed within the confidence interval in order to be able to specify "as smooth as possible" numerical values as the determined HBM reference values. In deviation to that concept, instead of "rounding up", systematic rounding will be dispensed with in order to better reflect the purely statistical nature of HBM reference values.

Furthermore, there was still a wide scope for the design of the statistical procedure for the quantitative HBM reference value derivation. For example, although the specification of a confidence interval for the 95th percentile has been recommended, no indication was given as to how such a confidence interval should ideally be derived. A precise algorithm for determining the "smooth numerical values" is also missing. Statistical methods, availability of appropriate software, and computational speed have changed over the past 25 years. Taking this into account, the HBM Commission provides in the refined concept concrete proposals for suitable and tested statistical procedures to further standardize the reference value derivation for HBM data.

2. Other concepts of human biomonitoring reference values

The method of selecting reference values from HBM data varies across agencies and research groups around the world, and may be based on varying percentiles with or without a confidence interval (CI). While some studies use or round the percentile value itself, others derive a value in the confidence interval, again others use the exact confidence interval limit, and report several percentiles stressing a percentile value or a value close to it as HBM reference value.

With respect to the percentile of choice often the 95th percentile of the concentration is used to derive HBM reference values, i.e. RV_{95}, for the internal exposure to chemical substances, e.g. Spain, Czech Republic, UK, Canadian CHMS, Democratic Republic of Congo, Brazil (Batáriová et al., 2006; Bevan et al., 2013; Freire et al., 2015; Haines et al., 2017a; Khoury et al., 2018; Kuno et al., 2013; Peña-Fernández et al., 2014; Saravanabhavan et al., 2017; Tuakuila et al., 2015). However, a smaller body of studies took other percentiles as a basis such as the 90th or the 97.5th percentile with or without their CI (Centers for Disease Control and Prevention (CDC), 2012; Den Hond et al., 2015; Hoet et al., 2013; Liu et al., 2012). To conclude, while the majority uses the 95th percentile as a basis for deriving HBM reference values, also other approaches have been defined by others in the worldwide scientific community. However, since the 95th percentile is usually part of the standard descriptive statistics in HBM results, at least and only if no RV_{95} based on the 95th percentile has been derived, the 95th percentile itself can be used to approximate comparisons with RV_{95}.

In Germany, biological reference values for hazardous substances (BAR) are derived for the occupational sector by the Permanent Senate Commission of the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) for the Investigation of Health Hazards of Chemical Compounds in the Work Area (MAK Commission) (Deutsche Forschungsgemeinschaft (DFG), 2022). These BAR values refer to the body burden of a reference population of working-age individuals who are not occupationally exposed to a particular chemical and characterize the existing background exposure to a chemical substance by describing the 95th percentile of the measured values (Göen et al., 2012). Similar assessment values for the evaluation of biomonitoring data from workers have been established e.g. by the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) which derives biological reference values (BRVs) preferably based on data from the general population for biomarkers of *exposure* and on data from workers without exposure (similar target population) for biomarkers of *effects* (ANSES, 2014). Reference values proposed by the European Scientific Committee on Occupational Exposure Limits (SCOEL; European Commission, 2013; version 7), adopted by the European Chemicals Agency (Joint ECHA/RAC-SCOEL Task Force, 2017) are referred to as biological guidance values (BGV), corresponding to the 90 or 95 percentile in a defined reference population, or to the detection limit of the biomonitoring method. In its recently published guidance document on occupational biomonitoring the intergovernmental Organisation for Economic Co-operation and Development (OECD) proposes reference occupational exposure levels (ROBL) (OECD, 2022) based on the 95 percentile of data from large-scale general population biomonitoring studies (excluding individuals with known non-occupational exposure), which could be set at the limit of quantification in case that all or the most measurement values go below it. For an overview see Table 14.

3. Prerequisites

Before starting with the "statistical algorithm" described below, several substantive issues need to be addressed (HBM Commission,

1996, 2009). Only if the uncertainty remaining when answering the questions below can be classified as low, an HBM reference value RV_{95} should be published. Otherwise, only a provisional HBM reference value can be derived for which the uncertainties and limitations of applicability should clearly be stated.

3.1. Reference population

First, it must be determined whether suitable data are available from representative general population studies, such as, e.g., data from the German Environmental Surveys (GerES), the Canadian Health Measure Survey (CHMS) or National Health and Nutrition Examination Survey (NHANES) in the USA.

3.2. Categories for selected variables

Before deriving HBM reference values for a set of data, it should be determined, if separate HBM reference values are intended to be established for specific subgroups of the reference population. In principle, the decision whether to consider categories for certain attributes should be based on content-driven rational reasons and made *a priori*. Typically, sex, age or the region of residence are considered suitable categories. Variables giving information on personal behaviour or consumption, on the other hand, are less suitable for HBM reference value derivations. If systematic group differences are expected or found in the analysis, e.g. cadmium in smokers (tobacco), a differentiating analysis should be initiated.

The question of the exact grouping within a category may not be trivial, especially for continuous attributes. E.g. if stratification by age is to be performed it is still a matter of discretion which age limits should be chosen exactly for the statistical analyses. This applies, e.g. for data from population studies with children and adolescents as the category 'age' as internal exposures during this time often varies substantially.

In case a *a posteriori* approach is considered, various mathematical procedures exist to reveal statistically significant differences between certain subgroups. For the comparison of competing explanatory models or even of different categorizations of a variable, symmetric decision rules, in particular model selection procedures which optimized the model with respect to data fit as well to numbers of used parameters, are suitable. A well-known example of model selection procedures is the variable selection within linear regression, for which different criteria exist, the simplest the adjusted R-squared. For a detailed introduction to model selection procedures see e.g. Linhart and Zucchini (1986).

Nevertheless, they do not answer the question, whether these differences are also biologically meaningful or relevant (relevance vs. significance) (Geffré et al., 2009). Furthermore, if no precise specifications were made in advance, especially for categories with continuous data there are in general too many possibilities to form different subgroups and a homogenous grouping within one category of an HBM study as prerequisite for comparing HBM reference values across further categories (e.g. sex), chemical substances, studies, and time would hardly be possible.

If we look at the population of 3 to 17-years-olds considered in GerES V, any number of theoretical groupings is possible without a biological rationale for constructing age groups. This would be of course in practice not applicable as for reasons of representativeness a minimum sample size, discussed below, should also be available for each subgroup. These prerequisites being fulfilled, any partitioning that is deemed meaningful may be used. We de-cided to use the age groups 3–11 and 12–17 for each sex separately. However, in theory, a large number of potential age groupings is possible, as elaborated in the beginning.

Insofar supporting statistics are used, it should be checked whether the empirical quantiles differ relevantly for potential subgroups (e.g., check via quantile regression procedures). Differences in central estimator location alone should not be decisive for deriving specific HBM reference values.

The HBM Commission holds the view, that in principle, content-driven *a priori* considerations should decide on whether to derive specific HBM reference values for certain categories and subgroups. *A posteriori* analyses based on percentile regression and model selection procedures should be used only as a supporting tool.

If this is possible also depends on the given and necessary sample size as discussed below.

3.3. Sample size

A crucial question is how large the minimum sample size must be in order to derive reliable confidence intervals and, ultimately, reliable HBM reference values. The sample size criterion was chosen combinatory such that up to 2 upper outliers could be present in the data without affecting the derived right confidence interval limit of the reference value with probability near 0.95. This requirement demands a minimum total sample size of $N \geq 80$, below which derivation of the HBM reference value RV_{95} does not appear to be sufficiently certain and therefore not meaningful.

The HBM-K decided to use a nonparametric approach with a conservative method for the selection of the RV95% within the 95% confidence interval. By this, inhomogeneity of internal exposure within the survey population might influence the selection of the RV95%. The claim of e.g. StatCan (Haines et al., 2017b; Saravanabhavan et al., 2017; StatCan, 2011) for low coefficients of variation (CV) with respect to all estimates is an approved way to characterize uncertainty of reference value estimates. In general, the skewness of the HBM-measurement distribution influences the CV of the bootstrap distribution. If known influence factors affect the distribution of HBM measurements, this would give reason for a stratified analysis within the HBM-K approach.

3.4. Data quality

The question of how many data values below the limit of quantification (LOQ) of the chemical substance or its metabolite are acceptable in the process of deriving HBM reference values is frequently discussed. With the chosen distribution free (nonparametric) approaches the relevance of intrinsically non-differentiable values below the limit of quantification has become practically irrelevant - at least for the question of deriving a reference value. So, the requirement that at least 10% of the observed values have been determinable (measured values $\geq$ LOQ) is usually quite sufficient to arrive at a value above the LOQ for the right confidence interval limit of the percentile estimate. For sum parameters the same criteria should apply, i.e. each individual biomarker used for the sum parameter should have at least 10% values $\geq$ LOQ.

HBM reference values can also be derived based on descriptive statistical data from publications without access to the raw data. In this case, the general principles for deriving reference values must be paid attention in equal measure. In particular, it must be checked whether the published study is based on a representative sample of the general reference population or on a sample whose composition is sufficiently representative at least with regard to the main influencing categories. In analogy to the procedure for the HBM reference value derivation from raw data, the sample size should consist of at least $N = 80$ individual samples. At least 10% of the measured values must be quantified, i.e. equal to or above the analytical limit of quantification (10% $\geq$ LOQ). Furthermore, the derivation of the HBM reference value requires the specification of the 95th percentile of the selected, clearly defined population. The statistical procedures used for this purpose should be comprehensibly documented in that publication together with confidence intervals of the estimated parameters. In general, such RV should be labelled as "provisional RV".

An HBM reference value can also be derived by a pooled analysis across studies. In this case, the strengths and weaknesses of the various studies, especially with regard to representativeness and sample size, must be recorded and taken into account in the evaluation by data

weighting.

An HBM reference value should always be derived on the basis of sample surveys that are as up-to-date as possible. If there are indications of a change in the background level, the HBM reference value should be updated. This may also be based on German environmental specimen bank data or other robust time trend studies in the absence of a current representative sample. The shift in the upper range of the distribution (95th percentile) determined from the time series can be applied to a previous representative sample to project a current HBM reference value. Limitations on the representativeness of these data from time series, such as the specific age range of the German environmental specimen bank, must be considered and corrected if necessary. HBM reference values derived according to all the procedures mentioned here can be named "provisional" or "preliminary" HBM reference values, depending on existing uncertainties, to indicate the qualitative deviations.

4. Statistics

4.1. Data distribution

The usual basic requirement of statistics, namely that of "good data", is also required for the derivation of HBM reference values. However, this does not imply that the HBM data must follow a certain statistical distribution - such as the frequently assumed log-normal distribution. In any case, the assumption that mixed distributions are more likely to exist for HBM measurements often seems more realistic than the assumption that an entire population with different risk and exposure profiles could be described as a homogeneous distribution. Since it is often not possible to make a safe distributional assumption, distribution-free methods seem to be more advantageous then distribution-based alternatives.

On the one hand, an advantage of distribution-based versus distribution-free methods is that the distribution-based statistics, e.g. assuming a (log) normal distribution, are usually more efficient: Estimates are more accurate, tests are more discriminating and/or require smaller sample sizes. On the other hand, distribution-based statistics can be virtually useless if the selected distribution assumption does not hold. Thus, even if there is a low degree of uncertainty as to whether a theoretical distribution assumption is true for the complete data set, distribution-free methods should be used, though it has to be mentioned, that the results of this methodological approach are in general less susceptible to extreme individual values.

A further disadvantage of many exact distribution-free methods has been the computational intensity leading to long lasting calculation procedures, and often, therefore, only asymptotic test statistics could be given. Nevertheless, this disadvantage vanished over the last decade thanks to increasing computer capacities making especially bootstrap methods an interesting tool as described below.

4.2. Determination of the 95th percentile (P95)

The starting point in the determination is the empirical 95th percentile of the sampling distribution as the point estimate for the 95th percentile in the reference population. If the 95th percentile lies between two values, the next empirical measurement value below is chosen as the 95th percentile. No additional weighting of data is performed beforehand.

4.3. Estimation of the 95% confidence interval by means of a bootstrap approach

To determine a 95% confidence interval with identical coverage probability at both half intervals, we propose to use a distribution free bootstrap approach: Since the initial publications on bootstrap by Efron (1979), bootstrap methods have been established as resampling methods in many fields over the following 40 years with increasing computer capacities (see, e.g., Carpenter and Bithell, 2000; Davison and Hinkley, 1997; Efron, 1979; Efron and Tibshirani, 1994). This applies in particular to the estimation of confidence intervals for location parameters, i.e. also for the 95% quantile. For each resampling run, a new sample is drawn from the original sample in order to infer the variability of the empirical percentiles determined in each resampling step and to derive confidence intervals directly from this simulated distribution ("bootstrap distribution"). The "bootstrap confidence intervals" determined in this way are far more robust than those of the distribution-based methods and are usually more efficient than customary distribution-free confidence intervals (Linnet, 2000). The intervals are approximately scale invariant, i.e. it is irrelevant whether the concentration values are used in the original scaling or whether transformed.

In the bootstrap procedures for deriving confidence intervals for percentiles there are various alternatives that are comparable in terms of results. To adjust the skewness of the bootstrap distribution, it is recommended - if possible - to use the bias-corrected and accelerated bootstrap (BCa) instead of the intuitively derived bootstrap percentile method. The BCa-based interval corrects for bias and skewness of the bootstrap estimator distribution (Hesterberg, 2015). If the BCa procedure cannot be performed, the simple bootstrap percentile procedure is recommended. In some rare cases the 95% confidence interval cannot be determined as the bootstrap procedure fails in numerically difficult data sets (namely: if the data set of one biomarker consists of only very few unique values, i.e. many measurements with the same value), although the empirical P95 can be determined.

5. Determination of HBM reference values

In the last step exactly one "rounded" value is to be defined within the determined confidence interval of the 95th percentile as the HBM reference value.

For this purpose, the 95th percentile is rounded to two significant digits (i.e. coming from the left, the position of the first non-zero digit as well as the following position). If the value obtained falls outside the confidence interval, it is rounded to three significant digits. The accuracy of the derived HBM reference value should not exceed analytical precision.

Algorithms in R for the statistics and determination of the HBM reference values are given in Appendix B.

6. Documentation

When documenting an HBM reference value derivation, the bootstrap procedure and the evaluation software used should be specified in addition to the number of bootstrap runs. This has the background of partly different calculation procedures for quantiles and deviating methods of the bootstrap estimation. By specifying the mentioned parameters, the calculation becomes comprehensible. Furthermore, data should be documented graphically, which facilitates quality control of study data (Appendix C).

7. Risk communication

To explain the meaning and applicability of the non-toxicologically based HBM reference value is a central role of risk communication (Covello and Sandman, 2001; Covello, 2003; Hoopmann, 2018). The questions "What is an HBM reference value?" and "What does the exceedance of an HBM reference value mean?" are heard again and again, especially in HBM investigations due to specific concern about exposure to environmental pollutants. The appropriate interpretation of individual HBM measurement results is important for the study participants. The exceedance frequency (if applicable for individual population groups) is often a first assessment standard for the local exposure situation for municipalities. The fundamental difference between HBM

Table 1
Chemical substances measured in the German Environmental Survey V (GerES V, 2014–2017). For these chemicals reference values were derived if possible (Tables 2–13).

Chemical substance group	Chemical substance	Abbr.	CAS-Nr	Metabolite	Abbr.	Reference
phthalates						
	dimethyl phthalate	DMP	131-11-3	mono-methyl phthalate	MMP	Schwedler et al. (2020c), Lemke et al. (2021a)
	diethyl phthalate	DEP	84-66-2	mono-ethyl phthalate	MEP	Schwedler et al. (2020c), Lemke et al. (2021a)
	butylbenzyl phthalate	BBzP	85-68-7	mono-benzyl phthalate	MBzP	Schwedler et al. (2020c), Lemke et al. (2021a)
	diisobutyl phthalate	DiBP	84-69-5	mono-iso-butyl phthalate	MiBP	Schwedler et al. (2020c), Lemke et al. (2021a)
				mono-hydroxy-iso-butyl phthalate	OH-MiBP	
	di-n-butyl phthalate	DnBP	84-74-2	mono-n-butyl phthalate	MnBP	Schwedler et al. (2020c), Lemke et al. (2021a)
				mono-hydroxy-n-butyl phthalate	OH–MnBP	
	dicyclohexyl phthalate	DCHP	84-61-7	mono-cyclohexyl phthalate	MCHP	Schwedler et al. (2020c), Lemke et al. (2021a)
	di-n-pentyl phthalate	DnPeP	131-18-0	mono-n-pentyl phthalate	MnPeP	Schwedler et al. (2020c), Lemke et al. (2021a)
	diethylhexyl phthalate	DEHP	117-81-7	mono(2-ethylhexyl) phthalate	MEHP	Schwedler et al. (2020c), Lemke et al. (2021a)
				mono(2-ethyl-5-hydroxyhexyl) phthalate	5OH-MEHP	
				mono(2-ethyl-5-oxohexyl) phthalate	5oxo-MEHP	
				mono(2-ethyl-5-carboxypentyl) phthalate	5cx-MEPP	
				sum 5OH-MEHP and 5oxo-MEHP		
				sum 5OH-MEHP and 5cx-MEPP		
	diisononyl phthalate	DiNP	28553-12-0	mono(4-methyl-7-hydroxyoctyl) phthalate	OH-MiNP	Schwedler et al. (2020c), Lemke et al. (2021a)
				mono(4-methyl-7-oxooctyl) phthalate	oxo-MiNP	
				mono(4-methyl-7-carboxyheptyl) phthalate	cx-MiNP	
				sum OH-MiNP, oxo-MiNP and cx-MiNP		
	diisodecyl phthalate	DiDP	26761-40-0	mono-hydroxy-isodecyl phthalate	OH-MiDP	Schwedler et al. (2020c), Lemke et al. (2021a)
				mono-oxo-iso-decyl phthalate	oxo-MiDP	
				mono(2,7-methyl-7-carboxyheptyl) phthalate	cx-MiDP	
	di-n-octyl phthalate	DnOP	117-84-0	mono-n-octyl phthalate	MnOP	Schwedler et al. (2020c), Lemke et al. (2021a)
	di-2-propylheptyl phthalate	DPHP	53306-54-0	mono (2-propyl-6-hydroxyheptyl) phthalate	OH-MPHP	Schwedler et al. (2020a), Lemke et al. (2021a)
				mono (2-propyl-6-oxo-heptyl) phthalate	oxo-MPHP	
				mono (2-propyl-6-carboxyhexyl) phthalate	cx-MPHxP	
	various phthalates*			sum OH-MPHP and oxo-MPHP Non-specific metabolite mono(3-carboxypropyl) phthalate	MCPP	Schwedler et al. (2020c), Lemke et al. (2021a)
alternative plasticisers						
	diethylhexyl terephthalate	DEHTP	6422-86-2	1-mono-(2-ethyl-5-hydroxyhexyl) benzene-1,4-dicoaboxylate	5OH-MEHTP	Schwedler et al. (2020b), Lemke et al. (2021a)
				1-mono-(2-ethyl-5-oxohexyl) benzene-1,4-dicarboxylate	5oxo-MEHTP	
				1-mono-(2-ethyl-5-carboxylpentyl) benzene-1,4-dicarboxylate	5cx-MEPTP	
				1-mono-(2-carboxylmethylhexyl) benzene-1,4-dicarboxylate	2cx-MMHTP	
	diisononyl 1,2-cyclohexanedicarboxylic acid	DINCH	166412-78-8; 474919-59-0	cyclohexane-1,2-dicarboxylic acid-mono (hydroxyl-iso-nonyl) ester	OH-MINCH	Schwedler et al. (2020a), Lemke et al. (2021a)

(continued on next page)

Table 1 (*continued*)

Chemical substance group	Chemical substance	Abbr.	CAS-Nr	Metabolite	Abbr.	Reference
				cyclohexane-1,2-dicarboxylic acid-mono (oxo-iso-nonyl) ester	oxo-MINCH	
				cyclohexane-1,2-dicarboxylic acid-mono (carboxy-iso-octyl) ester	cx-MINCH	
				sum OH-MINCH and cx-MINCH		
	tris(2-ethylhexyl) trimellitate	TOTM	3319-31-1	1-mono-(2-ethylhexyl) trimellitate	1-MEHTM	Murawski et al. (2021a), Lemke et al. (2021a)
				2-mono-(2-ethylhexyl) trimellitate	2-MEHTM	Murawski et al. (2021a), Lemke et al. (2021a)
				1-mono-(2-ethyl-5-hydroxyhexyl) trimellitate	5OH-1-MEHTM	Murawski et al. (2021a), Lemke et al. (2021a)
				2-mono-(2-ethyl-5-hydroxyhexyl) trimellitate	5OH-2-MEHTM	Murawski et al. (2021a), Lemke et al. (2021a)
				1-mono-(2-ethyl-5-carboxypentyl) trimellitate	5cx-1-MEPTM	Murawski et al. (2021a), Lemke et al. (2021a)
				2-mono-(2-ethyl-5-carboxypentyl) trimellitate	5cx-2-MEPTM	Murawski et al. (2021a), Lemke et al. (2021a)
metals						
	lead	Pb	7439-92-1	–	–	Vogel et al. (2021), Hahn et al. (2022)
	antimony	Sb	7440-36-0	–	–	
	arsenic	As	7440-38-2	–	–	
	arsenobetaine	AsB	64436-13-1	–	–	
	sum of As(III) and As(V)	As_{tox}	–	–	–	
	sum of As(III), As(V), MMA and DMA	As_i	–	–	–	
	cadmium	Cd	7440-43-9	–	–	Vogel et al. (2021)
	chromium	Cr	7440-47-3	–	–	Vogel et al. (2021)
	mercury	Hg	7439-97-6	–	–	Vogel et al. (2021)
	selenium	Se	7782-49-2	–	–	
	selenite	Se(IV)	26970-82-1	–	–	
	selenate	Se(VI)	13410-01-0	–	–	
	trimethylselenium ion	TMSe	25930-79-4	–	–	
	seleno-methylselenocysteine	MeSeC	26046-90-2	–	–	
	seleno-methionine	SeMet	3211-76-5	–	–	
	seleno-ethionine	SeEt	2578-27-0	–	–	
	selenious acid	MeSeA	7783-00-8	–	–	
	seleno-methylselenoglutathione	MeSeG	–	–	–	
	methyl-2-acetamido-2-deoxy-1-seleno-b-d-galactopyranoside	SeSug1	526222-32-2	–	–	
	methyl-2-acetamido-2-deoxy-1-seleno-b-d-glucopyranoside	SeSug2	3946-01-8	–	–	
	methyl-2-amino-2-deoxy-1-seleno-b-d-galactopyranoside	SeSug3	–	–	–	
organochlorine pesticides						
	hexachlorobenzene	HCB	118-74-1	–	–	Bandow et al. (2020)
	α-hexachlorocyclohexane	α-HCH	319-84-6	–	–	Bandow et al. (2020)
	β-hexachlorocyclohexane	β-HCH	319-85-7	–	–	Bandow et al. (2020)
	γ-hexachlorocyclohexane	γ-HCH	58-89-9	–	–	Bandow et al. (2020)
	dichlorodiphenyltrichloroethane	DDT	50-29-3	–	–	Bandow et al. (2020)
	dichlorodiphenyldichloroethylene	DDE	72-55-9	–	–	Bandow et al. (2020)
	dichlorodiphenyltrichloroethane	DDD	72-54-8	–	–	Bandow et al. (2020)
PCB						Bandow et al. (2020)
	2,4,4′-trichlorobiphenyl	PCB 28	7012-37-5	–	–	Bandow et al. (2020)
	2,2′,5,5′-tetrachlorobiphenyl	PCB 52	35693-99-3	–	–	Bandow et al. (2020)
	2,2′,4,5,5′-pentachlorobiphenyl	PCB 101	37680-73-2	–	–	Bandow et al. (2020)
	2,3′,4,4′,5-pentachlorobiphenyl	PCB 118	31508-00-6	–	–	Bandow et al. (2020)
	2,3,4,2′,4′,5′-hexachlorobiphenyl	PCB 138	35065-28-2	–	–	Bandow et al. (2020)
	2,2′,4,4′,5,5′-hexachlorobiphenyl	PCB 153	35065-27-1	–	–	Bandow et al. (2020)
	2,3,4,5,2′,4′,5′-heptachlorobiphenyl	PCB 180	35065-29-3	–	–	Bandow et al. (2020)
	sum of PCB 138, 153, and 180 × 2		–	–	–	Bandow et al. (2020)
PFAS						
	perfluorooctanoic acid	PFOA	335-67-1	–	–	Duffek et al. (2020)
	perfluorooctanesulfonic acid	PFOS	1763-23-1	–	–	Duffek et al. (2020)
	perfluorobutanoic acid	PFBA	375-22-4	–	–	Duffek et al. (2020)
	perfluorobutanesulfonic acid	PFBS	29420-49-3	–	–	Duffek et al. (2020)
	perfluoropentanoic acid	PFPeA	2706-90-3	–	–	Duffek et al. (2020)
	perfluorohexanoic acid	PFHxA	307-24-4	–	–	Duffek et al. (2020)
	perfluorohexanesulfonic acid	PFHxS	355-46-4	–	–	Duffek et al. (2020)
	perfluoroheptanoic acid	PFHpA	375-85-9	–	–	Duffek et al. (2020)
	perfluorononanoic acid	PFNA	375-95-1	–	–	Duffek et al. (2020)
	perfluorodecanoic acid	PFDA	335-76-2	–	–	Duffek et al. (2020)
	perfluoroundecanoic acid	PFUdA	2058-94-8	–	–	Duffek et al. (2020)
	perfluorododecanoic acid	PFDoA	307-55-1	–	–	Duffek et al. (2020)
parabens						

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Table 1 (*continued*)

Chemical substance group	Chemical substance	Abbr.	CAS-Nr	Metabolite	Abbr.	Reference
	methylparaben	MeP	99-76-3	–	–	Murawski et al. (2021b)
	ethylparaben	EtP	120-47-8	–	–	Murawski et al. (2021b)
	iso-propylparaben	*i*-PrP	4191-73-5	–	–	Murawski et al. (2021b)
	n-propylparaben	*n*-PrP	94-13-3	–	–	Murawski et al. (2021b)
	iso-butylparaben	*i*-BuP	4247-02-3	–	–	Murawski et al. (2021b)
	n-butylparaben	*n*-BuP	94-26-8	–	–	Murawski et al. (2021b)
	benzylparaben	BeP	94-18-8	–	–	Murawski et al. (2021b)
	pentylparaben	PeP	6521-29-5	–	–	Murawski et al. (2021b)
	heptylparaben	HeP	1085-12-7	–	–	Murawski et al. (2021b)
bisphenols						
	bisphenol A	BPA	80-05-7	–	–	Tschersich et al. (2021)
aprotic solvents						
	N-methyl-2-pyrrolidone	NMP	872-50-4	5-hydroxy-*N*-methyl-2-pyrrolidone	HNMP	Schmied-Tobies et al. (2021a)
				2-hydroxy-*N*-methylsuccinimide	HMSI	
				sum NMP		
	N-ethyl-2-pyrrolidone	NEP	2687-91-4	5-hydroxy-*N*-ethyl-2-pyrrolidone	HNEP	Schmied-Tobies et al. (2021a)
				2-hydroxy-*N*-ethylsuccinimide	HESI	
				sum NEP		
chlorophenols						
	pentachlorophenol	PCP	87-86-5	–	–	Schmied-Tobies et al. (2021b)
	2-chlorophenol	2-MCP	95-57-8	–	–	Schmied-Tobies et al. (2021b)
	4-chlorophenol	4-MCP	106-48-9	–	–	Schmied-Tobies et al. (2021b)
	2,4-dichlorophenol	2,4-DCP	120-83-2	–	–	Schmied-Tobies et al. (2021b)
	2,5-dichlorophenol	2,5-DCP	583-78-8	–	–	Schmied-Tobies et al. (2021b)
	2,6-dichlorophenol	2,6-DCP	87-65-0	–	–	Schmied-Tobies et al. (2021b)
	2,3,4-trichlorophenol	2,3,4-TriCP	15950-66-0	–	–	Schmied-Tobies et al. (2021b)
	2,4,5-trichlorophenol	2,4,5-TriCP	95-95-4	–	–	Schmied-Tobies et al. (2021b)
	2,4,6-trichlorophenol	2,4,6-TriCP	88-06-2	–	–	Schmied-Tobies et al. (2021b)
	2,3,4,6-tetrachlorophenol	2,3,4,6-TeCP	58-90-2	–	–	Schmied-Tobies et al. (2021b)
PAH						
	fluorene	–	86-73-7	2-hydroxyfluorene	2-OH-flu	Murawski et al. (2020b)
	naphthalene	–	91-20-3	1-hydroxynaphthalene	1-OH-nap	Murawski et al. (2020b)
				2-hydroxynaphthalene	2-OH-nap	
	phenanthrene	–	85-01-8	1-hydroxyphenanthrene	1-OH-phe	Murawski et al. (2020b)
				2-hydroxyphenanthrene	2-OH-phe	
				3-hydroxyphenanthrene	3-OH-phe	
				4-hydroxyphenanthrene	4-OH-phe	
				9-hydroxyphenanthrene	9-OH-phe	
				sum phenanthrene metabolites		
	pyrene	–	129-00-0	1-hydroxypyrene	1-OH-pyr	Murawski et al. (2020b)
UV filter						
	4-methylbenzylidene camphor	4-MBC	36861-47-9	3-(4-carboxybenzylidene) camphor	cx-MBC	Murawski et al. (2021a)
				3-(4-carboxybenzylidene)-6-hydroxycamphor	cx-MBC-OH	
	2-ethylhexyl salicylate	EHS	118-60-5	5-(((2-hydroxybenzoyl)oxy)methyl) heptanoic acid	5cx-EPS	
				2-ethyl-5-hydroxyhexyl 2-hydroxybenzoate	5OH-EHS	
				2-ethyl-5-oxohexyl 2-hydroxybenzoate	5oxo-EHS	
	octocrylene	OC	6197-30-4	2-cyano-3,3-diphenylacrylic acid	CPPA	
				2-(carboxymethyl)butyl 2-cyano-3,3-diphenyl acrylate	DOCCA	
				2-ethyl-5-hydroxyhexyl 2-cyano-3,3-diphenyl acrylate	5OH-OC	
	benzophenone-1	BP-1	131-56-6	–	–	Tschersich et al. (2021)
	benzophenone-3	BP-3	131-57-7	–	–	Tschersich et al. (2021)
	benzophenone-8	BP-8	131-53-3	–	–	Tschersich et al. (2021)
other						
	triclosan	–	3380-34-5	–	–	Tschersich et al. (2021)
	triclocarban	–	101-20-2	–	–	Tschersich et al. (2021)
	2-phenylphenol	–	90-43-7	–	–	Tschersich et al. (2021)
	butylphenyl methylpropional	Lysmeral	80-54-6	tert-butylbenzoic acid	TBBA	Murawski et al. (2020a)

(*continued on next page*)

Table 1 (*continued*)

Chemical substance group	Chemical substance	Abbr.	CAS-Nr	Metabolite	Abbr.	Reference
				lysmerol	–	
				hydroxy-lysmerylic acid	–	
				lysmerylic acid	–	
	methylisothiazolinone, methylchloroisothiazolinone	MI, MCI	2682-20-4; 26172-55-4	*N*-methylmalonamic acid	NMMA	Murawski et al. (2020c)
	butylated hydroxytoluene	BHT	128-37-0	BHT acid		Murawski et al. (2021a)
	2-mercaptobenzothiazole	2-MBT	149-30-4	–	–	Murawski et al. (2020d)
	cotinine	–	486-56-6	–	–	Hahn et al. (2023)
	benzene	–	71-43-2	*N*-Acetyl-*S*-phenyl-cysteine	SPMA	Schwedler et al. (2021)
	acrylamide	–	79-06-1	*N*-acetyl-*S*-(3-amino-3-oxopropyl)-cysteine	AAMA	Schwedler et al. (2021)
				N-acetyl-*S*-(2-carbamoyl-2-hydroxy-ethyl)-cysteine	GAMA	
	glyphosate	–	1071-83-6	-	–	Lemke et al. (2021b)
				aminomethylphosphonic acid	AMPA	Lemke et al. (2021b)
	creatinine	crea	60-27-5	–	–	

[*] di-n-butyl phthalate (DnBP), mono-n-butyl phthalate (MnBP), di-n-octyl phthalate (DnOP), mono-n-octyl phthalate (MnOP), di-isooctyl phthalate (DiOP), di-isononyl phthalate (DiNP), di-isodecyl phthalate (DiDP), di-(2-ethylhexyl) phthalate.

reference values RV_{95} versus HBM-I and HBM-II assessment values is that the HBM reference value provides information on the exposure of the population, whereas the HBM values can be used for a health-related interpretation of HBM results.

Therefore, as central task for a well-managed risk communication, an appropriate processing, representation and explanation of human biomonitoring results is needed, depending on the target group and its scientific background.

8. Application: derivation of HBM reference values for population-representative biomonitoring data of chemicals in children and adolescents in Germany (GerES V, 2014–2017)

8.1. German Environmental Survey V (GerES V)

The German Environmental Survey (GerES) is the only population-representative monitoring of human exposure to pollutants in Germany and, thus, provides suitable data to derive HBM reference values (RV_{95}), which are representative for the German population. For almost 40 years, it has been used to record the exposure to pollutants in the people's bodies and in their living environment. Extensive measurements and surveys are used to determine the level of exposure as well as to identify sources of exposure. In the fifth survey, GerES V (2014–2017), 2.294 children and adolescents aged 3–17 years from 167 municipalities across Germany were examined. GerES V was conducted in close cooperation with the German Health Interview and Examination Survey for Children and Adolescents (KiGGS Wave 2) of the Robert Koch Institute (RKI). Using human biomonitoring (HBM), 109 different substances (phthalates and alternative plasticisers, metals, organochlorine pesticides, polychlorinated biphenyls (PCB), per- and polyfluoroalkyl substances (PFAS), parabens, aprotic solvents, chlorophenols, polycyclic aromatic hydrocarbons (PAH), UV filter; among others) were analysed in whole blood, blood plasma or first-morning void urine of the participants (see Table 1, abbreviations for these substances and their metabolites and further information on CAS numbers and references for GerES results). Additionally, tap water samples, measurements of ultrafine particles, samples of house dust and indoor air are collected to gain insight into the environmental exposure. Furthermore, an interview was conducted on e.g. exposure-relevant behaviour, product use, diet, and information on the living environment (Schulz et al., 2021, https://doi.org/10.1016/j.ijheh.2021.113821). A "scientific use file" of the data is available and can be ordered via the research data centre of the RKI (Robert Koch Institute and the German Environment Agency, 2022).

The prerequisites for determining HBM reference values as described in the concept are fulfilled by the GerES V data as follows: GerES comprises a representative sample of the general population, all groups used to derive a RV_{95} contain at least 83 samples (and are thus $N \geq 80$) and RV_{95} are only given if at least 10% of the measurements were at or above the respective LOQ. The latter results in 37 biomarkers for which no RV_{95} can be derived and further 10 biomarkers where the RV_{95} could not be derived for single subgroups (i.e. specific sex/age groups). All sum parameters contain only biomarkers that were quantified in at least 10% of the samples. Values below the respective LOQ were considered LOQ/2 for calculating sums. Subgroups were selected *a priori* such that the prerequisite of $N \geq 80$ data points was fulfilled, i.e. only two age groups per sex were considered instead of e.g. four age groups. The two age groups used are 3–11 years and 12–17 years to allow for easier comparison with other HBM surveys such as CHMS (Health Canada, 2021), NHANES (CDC, 2023), and Esteban (Fréry et al., 2011) which also frequently split their young population between the ages of 11 and 12 for reporting. In summary, RV_{95} for GerES V are reported for 135 biomarkers and four subgroups (males/females and 3–11/12-17 years) each (Tables 2–13).

Algorithms in R (Appendix B) were used for the statistics and determination of the HBM reference values. An example data file is provided in Appendix A to serve as an illustration of how HBM data of the chemical substances were implemented in R (version 4.2.2.). The 95% confidence intervals were derived by means of the bias-corrected and accelerated bootstrap (BCa) procedure as described above performing 15,039 bootstrap runs. Data is documented graphically as shown in Appendix C.

As smoking is known as a factor leading to higher body loads for several determined chemical substances in the subgroups of adolescents (12–17 years), HBM data from smokers (cotinine >50 µg/L urine (Gruber und Schuurmans, 2018; Sharma et al., 2019)) were excluded for the derivation of reference values for lead, cadmium, PAH, benzene, acrylamide and cotinine (Goniewicz et al., 2018; Rodgman and Cook, 2009; Tranfo et al., 2017; ATSDR, 2007).

For some chemicals noticeable RV_{95} differences of factor two or higher between different subgroups exist. For example, RV_{95} values for some phthalates are higher in younger children either only for females (DPHP, DINCH), or only for males (DEHTP). Vice versa DEP RV_{95} values in females are higher in older age groups (12–17 years). Further differences can be found for RV_{95} values of some parabens: For younger children (2–11 years) higher values have been derived for MeP (males) and 2,4,5-TriCP (females), but lower for MeP, EtP and n-PrP (females). Referring to the sex, females have higher RV_{95} values for MeP, EtP, n-PrP and other chemicals like UV-filters EHS, BP-1 and BP-3, and triclosan, which might indicate a broader application of personal care products in young women. For PCB (sum) HBM reference values are higher in

younger children (females) indicating possibly higher exposure levels due to more recent breastfeeding. For several chlorophenols RV_{95} values are higher in older age groups. These and further differences probably mainly reflect different exposure pattern in childhood and adolescence but age- and gender-dependent metabolic differences might also contribute. Identification of influencing variables should be addressed in further studies. Understanding age- and sex-specific exposure patterns is helpful for the risk assessment of environmental chemicals as well as for prioritizing and choosing adequate risk management options.

HBM reference values listed in Tables 2–13 replace earlier RV_{95} values published by the HBM commission for children and adolescents derived from data of precedent GerES studies (e.g. published in Apel et al., 2017).

8.2. Reference values

8.2.1. Phthalates and alternative plasticisers

Table 2
HBM reference values (RV_{95}) GerES V (2014–2017) for metabolites of phthalates and alternative plasticisers in µg/l. At least 10% of the measured values must be quantified, i.e. equal to or above the analytical limit of quantification (10% ≥ LOQ), for RV_{95} derivation (also for sum RV_{95}). Sum RV_{95} is derived for indicated or all metabolites in case health-based sum HBM (guidance) values (HBM Commission or HBM4EU) exist. Urine = first-morning void urine, f = female, m = male, N = number of individuals, LOQ = Limit of quantification, P95 = 95th percentile, LCI = lower confidence interval, UCI = upper confidence interval, abbr. = abbreviations as explained in Table 1.

Chemical substance (abbr.)	Metabolite (abbr.)	Matrix	LOQ [µg/l]	sex	Age [years]	N	% ≥ LOQ	min	max	P95	LCI 95%	UCI 95%	RV_{95} [µg/l]
DMP	MMP	urine	1	f	3–11	657	98.3	< LOQ	410	46	37	75	46
					12–17	485	97.3	< LOQ	160	28	22	39	28
				m	3–11	668	98.4	< LOQ	880	54	41	68	54
					12–17	447	97.3	< LOQ	620	33	23	45	33
DEP	MEP	urine	0.5	f	3–11	657	100	0.9	3500	130	100	170	130
					12–17	485	100	0.7	2900	360	250	470	360
				m	3–11	668	99.9	< LOQ	2300	150	110	210	150
					12–17	447	100	1.5	2200	220	150	410	220
BBzP	MBzP	urine	0.2	f	3–11	657	99.7	< LOQ	120	19	16	25	19
					12–17	485	100	0.2	66	13	11	14	13
				m	3–11	668	99.7	< LOQ	160	26	19	39	26
					12–17	447	99.3	< LOQ	380	16	11	21	16
DiBP	MiBP	urine	1	f	3–11	657	100	1.9	520	110	94	130	110
					12–17	485	100	1.3	510	110	83	130	110
				m	3–11	668	99.9	< LOQ	540	130	110	150	130
					12–17	447	100	2.6	2300	100	85	130	100
	OH-MiBP	urine	0.25	f	3–11	657	100	0.6	160	38	33	47	38
					12–17	485	100	0.5	230	36	28	44	36
				m	3–11	668	99.9	< LOQ	160	41	35	51	41
					12–17	447	100	0.6	560	31	26	43	31
DnBP	MnBP	urine	1	f	3–11	657	100	1.7	390	74	67	83	74
					12–17	485	100	1.9	590	68	60	76	68
				m	3–11	668	99.7	< LOQ	360	78	66	87	78
					12–17	447	100	1.9	390	66	57	78	66
	OH–MnBP	urine	0.25	f	3–11	657	99.7	< LOQ	45	9.6	8.6	11	9.6
					12–17	485	99.8	< LOQ	88	7.8	6.8	9.1	7.8
				m	3–11	668	99.6	< LOQ	28	11	9.2	12	11
					12–17	447	98.7	< LOQ	26	7.4	5.9	9	7.4
DCHP	MCHP	urine	0.2	f	3–11	657	6.1	< LOQ	2.7	0.3	–	–	–
					12–17	485	3.7	< LOQ	1.4	< LOQ	–	–	–
				m	3–11	668	5.5	< LOQ	3.2	0.3	–	–	–
					12–17	447	6.3	< LOQ	89	0.2	–	–	–
DnPeP	MnPeP	urine	0.2	f	3–11	656	7.2	< LOQ	6	0.3	–	–	–
					12–17	484	4.8	< LOQ	3.4	< LOQ	–	–	–
				m	3–11	668	7.3	< LOQ	13	0.3	–	–	–
					12–17	447	5.4	< LOQ	13	0.2	–	–	–
DEHP	MEHP	urine	0.5	f	3–11	657	85.4	< LOQ	16	5.8	4.7	6.7	5.8
					12–17	485	85.8	< LOQ	26	6.1	5	7.2	6.1
				m	3–11	668	86.2	< LOQ	34	6.5	5.5	7.7	6.5
					12–17	447	86.6	< LOQ	23	6.5	5.3	7.7	6.5
	5OH-MEHP		0.2	f	3–11	657	100	1.5	130	42	38	52	42
					12–17	485	100	0.3	110	30	26	35	30
				m	3–11	668	99.9	< LOQ	190	46	40	56	46
					12–17	447	100	0.6	89	28	25	41	28
	5oxo-MEHP		0.2	f	3–11	655	100	1	100	30	27	37	30
					12–17	484	100	0.2	70	20	18	23	20
				m	3–11	666	99.8	< LOQ	150	34	31	40	34
					12–17	447	100	0.3	63	19	18	29	19
	5cx-MEPP		0.2	f	3–11	657	100	0.7	140	50	43	57	50
					12–17	485	100	0.3	140	34	27	41	34
				m	3–11	668	99.9	< LOQ	240	58	49	67	58
					12–17	447	100	0.6	110	32	28	46	32
	sum 5OH-MEHP and 5oxo-MEHP		–	f	3–11	655	–	2.6	210	73	65	88	73

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Table 2 (*continued*)

Chemical substance (abbr.)	Metabolite (abbr.)	Matrix	LOQ [µg/l]	sex	Age [years]	N	% ≥ LOQ	min	max	P95	LCI 95%	UCI 95%	RV_{95} [µg/l]
					12–17	484	–	0.5	180	49	44	58	49
				m	3–11	666	–	0.2	320	80	70	97	80
					12–17	447	–	0.9	150	48	42	68	48
	sum 5OH-MEHP and 5cx-MEPP		–	f	3–11	657	–	3.2	260	94	77	110	94
					12–17	485	–	0.6	250	61	54	78	61
				m	3–11	668	–	0.2	430	100	89	120	100
					12–17	447	–	1.2	190	60	52	91	60
DiNP	OH-MiNP	urine	0.2	f	3–11	655	100	0.6	270	34	27	41	34
					12–17	484	99.8	< LOQ	1300	20	16	26	20
				m	3–11	665	99.8	< LOQ	87	25	24	31	25
					12–17	447	100	0.3	250	21	16	24	21
	oxo-MiNP		0.2	f	3–11	657	99.8	< LOQ	160	14	12	17	14
					12–17	485	99.6	< LOQ	380	8.7	7.4	13	8.7
				m	3–11	668	99.9	< LOQ	54	12	10	13	12
					12–17	447	99.3	< LOQ	100	9.2	7.4	12	9.2
	cx-MiNP		0.2	f	3–11	656	100	0.5	380	34	28	43	34
					12–17	484	99.8	< LOQ	520	22	16	32	22
				m	3–11	667	99.9	< LOQ	160	24	21	30	24
					12–17	447	100	0.4	380	20	16	23	20
DiDP	OH-MiDP	urine	0.2	f	3–11	657	99.1	< LOQ	56	9.4	7.8	11	9.4
					12–17	485	97.7	< LOQ	68	5.2	3.9	6.6	5.2
				m	3–11	668	99.3	< LOQ	48	6.7	5.8	8.6	6.7
					12–17	447	96.4	< LOQ	44	5.4	4.5	6.4	5.4
	oxo-MiDP		0.2	f	3–11	657	89.5	< LOQ	56	3.8	3.3	5.5	3.8
					12–17	485	85.6	< LOQ	59	2.8	1.9	3.1	2.8
				m	3–11	668	91.3	< LOQ	30	3.1	2.6	4.5	3.1
					12–17	447	84.6	< LOQ	19	2.4	1.9	2.9	2.4
	cx-MiDP		0.2	f	3–11	656	98.6	< LOQ	38	4.8	3.8	5.5	4.8
					12–17	485	96.9	< LOQ	16	2.8	2.2	3.6	2.8
				m	3–11	668	98.7	< LOQ	18	3.8	3.1	4.2	3.8
					12–17	447	95.1	< LOQ	19	2.7	2.2	3.1	2.7
DnOP	MnOP	urine	0.2	f	3–11	657	0.8	< LOQ	2.1	< LOQ	–	–	–
					12–17	485	0.6	< LOQ	0.5	< LOQ	–	–	–
				m	3–11	668	0.4	< LOQ	0.6	< LOQ	–	–	–
					12–17	447	0	< LOQ	< LOQ	< LOQ	–	–	–
DEHTP	5OH-MEHTP	urine	0.3	f	3–11	602	71.9	< LOQ	65	5.3	4.2	8.4	5.3
					12–17	457	56.2	< LOQ	140	3.7	2.2	6.1	3.7
				m	3–11	621	76.7	< LOQ	39	5.9	4.3	7.3	5.9
					12–17	438	55	< LOQ	74	2.6	2.1	2.8	2.6
	5oxo-MEHTP		0.2	f	3–11	602	84.7	< LOQ	41	4.6	4	6.4	4.6
					12–17	457	71.8	< LOQ	170	3.9	2	6.7	3.9
				m	3–11	621	86	< LOQ	22	5.2	3.8	6.5	5.2
					12–17	438	67.6	< LOQ	41	2.2	1.8	2.7	2.2
	5cx-MEPTP		0.2	f	3–11	602	100	0.23	570	73	53	87	73
					12–17	457	99.8	< LOQ	1600	41	29	75	41
				m	3–11	621	100	0.29	700	54	48	68	54
					12–17	438	99.8	< LOQ	210	24	20	37	24
	2cx-MMHTP		0.4	f	3–11	602	26.6	< LOQ	17	1.5	1.2	1.7	1.5
					12–17	457	10.9	< LOQ	33	0.81	0.55	1.5	0.81
				m	3–11	621	27.9	< LOQ	14	1.2	0.97	1.5	1.2
					12–17	438	7.3	< LOQ	4.6	0.53	–	–	–
DINCH	OH-MINCH	urine	0.05	f	3–11	650	99.8	< LOQ	2500	20	14	25	20
					12–17	478	99.8	< LOQ	360	9.9	8.6	14	9.9
				m	3–11	657	99.8	< LOQ	180	19	14	24	19
					12–17	443	99.5	< LOQ	130	11	9.2	16	11
	oxo-MINCH		0.05	f	3–11	650	98.6	< LOQ	590	8.9	7.4	11	8.9
					12–17	477	96.9	< LOQ	160	6.1	4.1	7.4	6.1
				m	3–11	658	98.6	< LOQ	81	9.1	7.1	12	9.1
					12–17	441	94.6	< LOQ	72	7	4.9	9.4	7.0
	cx-MINCH		0.05	f	3–11	649	99.1	< LOQ	890	8.6	7	11	8.6
					12–17	477	99.2	< LOQ	180	4.4	3.7	5.6	4.4
				m	3–11	656	99.5	< LOQ	72	8.9	7.5	11	8.9
					12–17	442	98.4	< LOQ	38	5.2	4.4	7.7	5.2
	sum OH-MINCH and cx-MINCH		–	f	3–11	649	–	0.05	3400	27	21	38	27
					12–17	477	–	0.05	540	16	12	18	16
				m	3–11	656	–	0.26	250	29	21	33	29
					12–17	441	–	0.05	170	16	14	25	16
DPHP	OH-MPHP	urine	0.3	f	3–11	141	49.6	< LOQ	7.5	2.3	1.1	3.2	2.3
					12–17	110	40.9	< LOQ	17	0.93	0.65	2.1	0.93
				m	3–11	170	57.1	< LOQ	18	1.4	1.1	1.6	1.4
					12–17	94	52.1	< LOQ	15	1.9	1.2	6.3	1.9
	oxo-MPHP		0.25	f	3–11	141	63.1	< LOQ	11	3.1	1.1	4.2	3.1

(*continued on next page*)

Table 2 (*continued*)

Chemical substance (abbr.)	Metabolite (abbr.)	Matrix	LOQ [µg/l]	sex	Age [years]	N	% ≥ LOQ	min	max	P95	LCI 95%	UCI 95%	RV_{95} [µg/l]
					12–17	110	57.3	< LOQ	28	1.2	0.99	2.2	1.2
				m	3–11	170	67.1	< LOQ	23	1.8	1.2	2	1.8
					12–17	95	69.5	< LOQ	11	2.1	1.2	11	2.1
	cx-MPHxP		0.15	f	3–11	141	2.1	< LOQ	0.43	< LOQ	–	–	–
					12–17	110	0.9	< LOQ	0.53	< LOQ	–	–	–
				m	3–11	170	0.6	< LOQ	0.83	< LOQ	–	–	–
					12–17	95	1.1	< LOQ	0.25	< LOQ	–	–	–
	sum OH-MPHP and oxo-MPHP		–	f	3–11	141	–	0.28	18	5.4	2.2	7.3	5.4
					12–17	110	–	0.28	44	2.2	1.7	4.4	2.2
				m	3–11	170	–	0.28	40	3.3	2.1	3.8	3.3
					12–17	94	–	0.28	26	4	2.3	17	4.0
various phthalates*	MCPP	urine	0.5	f	3–11	657	95	< LOQ	72	7.9	6.5	8.5	7.9
					12–17	485	87	< LOQ	110	4.3	3.4	5.5	4.3
				m	3–11	668	97	< LOQ	48	5.9	4.9	6.4	5.9
					12–17	447	86.1	< LOQ	56	4.1	3.5	5	4.1
TOTM	1-MEHTM	urine	0.11	f	3–11	107	0	< LOQ	< LOQ	< LOQ	–	–	–
					12–17	96	0	< LOQ	< LOQ	< LOQ	–	–	–
				m	3–11	134	2.2	< LOQ	0.3	< LOQ	–	–	–
					12–17	94	0	< LOQ	< LOQ	< LOQ	–	–	–
	2-MEHTM		0.15	f	3–11	107	3.7	< LOQ	0.34	< LOQ	–	–	–
					12–17	96	1	< LOQ	0.22	< LOQ	–	–	–
				m	3–11	134	4.5	< LOQ	4	< LOQ	–	–	–
					12–17	94	2.1	< LOQ	0.24	< LOQ	–	–	–
	5OH-1-MEHTM		0.26	f	3–11	107	0	< LOQ	< LOQ	< LOQ	–	–	–
					12–17	96	0	< LOQ	< LOQ	< LOQ	–	–	–
				m	3–11	134	1.5	< LOQ	0.4	< LOQ	–	–	–
					12–17	94	0	< LOQ	< LOQ	< LOQ	–	–	–
	5OH-2-MEHTM		0.24	f	3–11	107	0	< LOQ	< LOQ	< LOQ	–	–	–
					12–17	96	0	< LOQ	< LOQ	< LOQ	–	–	–
				m	3–11	134	0.7	< LOQ	2.2	< LOQ	–	–	–
					12–17	94	0	< LOQ	< LOQ	< LOQ	–	–	–
	5cx-1-MEPTM		0.23	f	3–11	107	0	< LOQ	< LOQ	< LOQ	–	–	–
					12–17	96	0	< LOQ	< LOQ	< LOQ	–	–	–
				m	3–11	134	1.5	< LOQ	0.87	< LOQ	–	–	–
					12–17	94	0	< LOQ	< LOQ	< LOQ	–	–	–
	5cx-2-MEPTM		0.09	f	3–11	107	0	< LOQ	< LOQ	< LOQ	–	–	–
					12–17	96	0	< LOQ	< LOQ	< LOQ	–	–	–
				m	3–11	134	0	< LOQ	< LOQ	< LOQ	–	–	–
					12–17	94	0	< LOQ	< LOQ	< LOQ	–	–	–

* di-n-butyl phthalate (DnBP), mono-n-butyl phthalate (MnBP), di-n-octyl phthalate (DnOP), mono-n-octyl phthalate (MnOP), di-isooctyl phthalate (DiOP), di-isononyl phthalate (DiNP), di-isodecyl phthalate (DiDP), di-(2-ethylhexyl) phthalate.

8.2.2. Metals

Table 3
HBM reference values (RV_{95}) GerES V (2014–2017) for metals in µg/l. At least 10% of the measured values must be quantified, i.e. equal to or above the analytical limit of quantification (10% ≥ LOQ), for RV_{95} derivation. Urine = first-morning void urine, blood = whole blood, f = female, m = male, ns = only non-smokers (cotinine <50 µg/L), N = number of individuals, LOQ = Limit of quantification, P95 = 95th percentile, LCI = lower confidence interval, UCI = upper confidence interval, abbr. = abbreviations as explained in Table 1.

Chemical substance (abbr.)	Matrix	LOQ [µg/l]	sex	Age [years]	note	N	% ≥ LOQ	min	max	P95	LCI 95%	UCI 95%	RV_{95} [µg/l]
Pb	blood	2.1	f	3–11	ns	199	100	2.4	48	19	17	22	19
				3–11		200	100	2.4	48	19	17	22	–
				12–17	ns	127	100	2.9	130	16	14	26	16
				12–17		138	100	2.9	130	16	14	26	–
			m	3–11	ns	211	100	2.9	37	22	20	25	22
				3–11		211	100	2.9	37	22	20	25	–
				12–17	ns	157	100	2.2	24	20	17	22	20
				12–17		171	99.4	< LOQ	24	20	17	21	–
Sb	urine	0.04	f	3–11		656	78.5	< LOQ	0.43	0.14	0.12	0.16	0.14
				12–17		484	77.1	< LOQ	0.52	0.15	0.13	0.16	0.15
			m	3–11		666	82.3	< LOQ	0.56	0.14	0.12	0.14	0.14
				12–17		446	77.4	< LOQ	0.66	0.16	0.12	0.17	0.16
As	urine	0.6	f	3–11		656	100	1.3	360	58	49	80	58
				12–17		484	99.8	< LOQ	630	53	37	69	53
			m	3–11		666	100	1	300	55	45	72	55

(*continued on next page*)

Table 3 (*continued*)

Chemical substance (abbr.)	Matrix	LOQ [µg/l]	sex	Age [years]	note	N	% ≥ LOQ	min	max	P95	LCI 95%	UCI 95%	RV_{95} [µg/l]
				12–17		446	100	1.2	180	38	31	58	38
AsB	urine	0.1	f	3–11		656	83.5	< LOQ	200	42	33	52	42
				12–17		484	83.5	< LOQ	520	35	25	49	35
			m	3–11		666	87.2	< LOQ	260	42	34	50	42
				12–17		446	88.3	< LOQ	120	28	20	39	28
As_{tox}	urine	–	f	3–11		656		0.1	3	0.95	0.8	1	0.95
				12–17		484		0.1	4.2	1.1	0.9	1.2	1.1
			m	3–11		666		0.1	5.5	1	0.8	1	1.0
				12–17		446		0.1	6.4	1.2	1	1.4	1.2
As_i	urine	–	f	3–11		656		0.9	170	13	12	15	13
				12–17		484		0.2	84	9.6	8.5	13	9.6
			m	3–11		666		0.5	97	14	12	16	14
				12–17		446		0.7	53	11	9.6	13	11
Cd	urine	0.05	f	3–11	ns	653	69.2	< LOQ	1.9	0.19	0.17	0.19	0.19
				3–11		656	69.4	< LOQ	1.9	0.19	0.16	0.19	–
				12–17	ns	454	83.7	< LOQ	0.72	0.27	0.22	0.31	0.27
				12–17		484	84.1	< LOQ	0.72	0.27	0.23	0.31	–
			m	3–11	ns	666	73.3	< LOQ	1.2	0.22	0.19	0.25	0.22
				3–11		666	73.3	< LOQ	1.2	0.22	0.19	0.25	–
				12–17	ns	408	80.4	< LOQ	0.57	0.25	0.21	0.27	0.25
				12–17		446	81.6	< LOQ	0.57	0.25	0.21	0.27	–
	blood	0.12	f	3–11	ns	199	33.2	< LOQ	0.43	0.2	0.17	0.21	0.20
				3–11		200	33.5	< LOQ	0.43	0.2	0.17	0.21	–
				12–17	ns	127	52.8	< LOQ	0.38	0.23	0.21	0.28	0.23
				12–17		138	56.5	< LOQ	1.9	0.29	0.23	1.5	–
			m	3–11	ns	211	29.4	< LOQ	0.27	0.21	0.16	0.22	0.21
				3–11		211	29.4	< LOQ	0.27	0.21	0.16	0.22	–
				12–17	ns	157	53.5	< LOQ	0.31	0.22	0.19	0.22	0.22
				12–17		171	56.1	< LOQ	2.7	0.31	0.24	0.99	–
Cr	urine	0.2	f	3–11		656	93	< LOQ	2.6	0.79	0.75	0.83	0.79
				12–17		484	88	< LOQ	1.8	0.8	0.72	0.83	0.80
			m	3–11		666	94.7	< LOQ	2.2	0.84	0.79	0.89	0.84
				12–17		446	88.3	< LOQ	1.3	0.8	0.73	0.84	0.80
Hg	urine	0.02	f	3–11		653	94.6	< LOQ	0.93	0.22	0.16	0.23	0.22
				12–17		483	90.9	< LOQ	6.2	0.24	0.2	0.27	0.24
			m	3–11		664	96.1	< LOQ	1.2	0.24	0.18	0.28	0.24
				12–17		446	95.7	< LOQ	4.3	0.3	0.22	0.36	0.30
Se	urine	0.5	f	3–11		621	100	2.9	140	68	64	72	68
				12–17		467	100	2.8	110	69	67	73	69
			m	3–11		641	99.8	< LOQ	150	78	71	82	78
				12–17		440	100	5.2	100	67	63	75	67
Se(IV)	urine	0.11	f	3–11		109	0.9	< LOQ	0.2	< LOQ	–	–	–
				12–17		100	1	< LOQ	0.2	< LOQ	–	–	–
			m	3–11		136	3.7	< LOQ	0.3	< LOQ	–	–	–
				12–17		94	1.1	< LOQ	0.2	< LOQ	–	–	–
Se(VI)	urine	0.1	f	3–11		109	10.1	< LOQ	0.93	0.19	< LOQ	0.33	0.19
				12–17		100	18	< LOQ	1.2	0.19	0.12	0.47	0.19
			m	3–11		136	22.8	< LOQ	1.5	0.35	0.2	0.38	0.35
				12–17		94	19.1	< LOQ	1.1	0.29	0.17	0.73	0.29
TMSe	urine	0.27	f	3–11		109	25.7	< LOQ	6.4	3	1.7	3.4	3.0
				12–17		100	17	< LOQ	4.4	2.6	0.9	3.5	2.6
			m	3–11		136	23.5	< LOQ	5.1	3	1.9	4.2	3.0
				12–17		94	16	< LOQ	14	3	1.9	6.7	3.0
MeSeC	urine	0.19	f	3–11		109	5.5	< LOQ	0.9	0.3	–	–	–
				12–17		100	14	< LOQ	1.4	0.5	0.3	0.8	0.50
			m	3–11		136	11.8	< LOQ	1.7	0.4	< LOQ	0.4	0.40
				12–17		94	19.1	< LOQ	1.8	0.5	0.3	0.8	0.50
SeMet	urine	0.22	f	3–11		109	2.8	< LOQ	0.8	< LOQ	–	–	–
				12–17		100	3	< LOQ	0.6	< LOQ	–	–	–
			m	3–11		136	5.9	< LOQ	1.4	0.3	–	–	–
				12–17		94	7.4	< LOQ	1.7	0.3	–	–	–
SeEt	urine	0.22	f	3–11		109	0	< LOQ	< LOQ	< LOQ	–	–	–
				12–17		100	0	< LOQ	< LOQ	< LOQ	–	–	–
			m	3–11		136	0	< LOQ	< LOQ	< LOQ	–	–	–
				12–17		94	0	< LOQ	< LOQ	< LOQ	–	–	–

(*continued on next page*)

Table 3 (*continued*)

Chemical substance (abbr.)	Matrix	LOQ [µg/l]	sex	Age [years]	note	N	% ≥ LOQ	min	max	P95	LCI 95%	UCI 95%	RV_{95} [µg/l]
MeSeA	urine	0.28	f	3–11		109	92.7	< LOQ	2.7	1.9	1.5	2.5	1.9
				12–17		100	87	< LOQ	3.2	1.7	1.2	2.3	1.7
			m	3–11		136	92.6	< LOQ	6.1	2.1	1.8	2.9	2.1
				12–17		94	91.5	< LOQ	3.1	1.8	1.2	2.1	1.8
MeSeG	urine	0.28	f	3–11		109	11.9	< LOQ	2.4	0.8	0.3	0.9	0.80
				12–17		100	6	< LOQ	0.6	0.4	–	–	–
			m	3–11		136	12.5	< LOQ	1.4	0.8	0.3	0.9	0.80
				12–17		94	6.4	< LOQ	1.1	0.4	–	–	–
SeSug1	urine	0.3	f	3–11		109	99.1	< LOQ	18	11	8	14	11
				12–17		100	98	< LOQ	13	6.6	5.7	10	6.6
			m	3–11		136	100	0.6	26	11	8.7	16	11
				12–17		94	100	0.4	23	9.1	5.8	11	9.1
SeSug2	urine	0.29	f	3–11		109	2.8	< LOQ	0.8	< LOQ	–	–	–
				12–17		100	1	< LOQ	0.5	< LOQ	–	–	–
			m	3–11		136	1.5	< LOQ	0.8	< LOQ	–	–	–
				12–17		94	3.2	< LOQ	0.5	< LOQ	–	–	–
SeSug3	urine	0.29	f	3–11		109	92.7	< LOQ	8.1	6	4.8	6.8	6.0
				12–17		100	89	< LOQ	7.3	4.1	3.5	5	4.1
			m	3–11		136	91.9	< LOQ	9.5	6.3	4.3	8.7	6.3
				12–17		94	88.3	< LOQ	13	6.1	3.9	8	6.1

8.2.3. Organochlorine pesticides

Table 4
HBM reference values (RV_{95}) GerES V (2014–2017) for organochlorine pesticides in µg/l. At least 10% of the measured values must be quantified, i.e. equal to or above the analytical limit of quantification (10% ≥ LOQ), for RV_{95} derivation. Plasma = blood plasma, f = female, m = male, N = number of individuals, LOQ = Limit of quantification, P95 = 95th percentile, LCI = lower confidence interval, UCI = upper confidence interval, abbr. = abbreviations as explained in Table 1.

Chemical substance (abbr.)	Matrix	LOQ [µg/l]	sex	Age [years]	N	% ≥ LOQ	min	max	P95	LCI 95%	UCI 95%	RV_{95} [µg/l]
HCB	plasma	0.07	f	3–11	305	43	< LOQ	0.26	0.15	0.14	0.16	0.15
				12–17	291	16.8	< LOQ	0.18	0.099	0.088	0.11	0.099
			m	3–11	307	45.9	< LOQ	0.26	0.15	0.13	0.16	0.15
				12–17	231	34.2	< LOQ	0.18	0.12	0.1	0.14	0.12
α-HCH	plasma	0.03	f	3–11	305	0	< LOQ	< LOQ	< LOQ	–	–	–
				12–17	291	0	< LOQ	< LOQ	< LOQ	–	–	–
			m	3–11	307	0.3	< LOQ	0.14	< LOQ	–	–	–
				12–17	231	0	< LOQ	< LOQ	< LOQ	–	–	–
β-HCH	plasma	0.02	f	3–11	305	16.4	< LOQ	0.88	0.05	0.031	0.082	0.050
				12–17	291	6.9	< LOQ	0.63	0.029	–	–	–
			m	3–11	307	15.6	< LOQ	0.64	0.038	0.031	0.079	0.038
				12–17	231	7.8	< LOQ	0.3	0.026	–	–	–
γ-HCH	plasma	0.05	f	3–11	280	14.3	< LOQ	0.19	0.079	0.068	0.088	0.079
				12–17	260	15.8	< LOQ	0.38	0.078	0.064	0.093	0.078
			m	3–11	278	15.8	< LOQ	0.28	0.074	0.063	0.097	0.074
				12–17	205	17.1	< LOQ	0.15	0.081	0.063	0.098	0.081
DDT	plasma	0.02	f	3–11	305	18.7	< LOQ	0.67	0.053	0.033	0.064	0.053
				12–17	291	14.4	< LOQ	0.12	0.039	0.028	0.051	0.039
			m	3–11	307	16.3	< LOQ	0.74	0.049	0.036	0.057	0.049
				12–17	231	16.5	< LOQ	0.31	0.042	0.03	0.058	0.042
DDE	plasma	0.02	f	3–11	305	100	0.034	7.6	0.93	0.69	1.5	0.93
				12–17	291	99.7	< LOQ	1.8	0.62	0.5	0.93	0.62
			m	3–11	307	100	0.029	3.3	0.92	0.68	1.2	0.92
				12–17	231	100	0.044	1.4	0.56	0.46	0.68	0.56
DDD	plasma	0.02	f	3–11	305	1	< LOQ	0.033	< LOQ	–	–	–
				12–17	291	0	< LOQ	< LOQ	< LOQ	–	–	–
			m	3–11	307	0	< LOQ	< LOQ	< LOQ	–	–	–
				12–17	231	0.4	< LOQ	0.061	< LOQ	–	–	

8.2.4. PCB

Table 5
HBM reference values (RV_{95}) GerES V (2014–2017) for PCB in µg/l. At least 10% of the measured values must be quantified, i.e. equal to or above the analytical limit of quantification (10% ≥ LOQ), for RV_{95} derivation. Plasma = blood plasma, f = female, m = male, N = number of individuals, LOQ = Limit of quantification, P95 = 95th percentile, LCI = lower confidence interval, UCI = upper confidence interval, abbr. = abbreviations as explained in Table 1.

Chemical substance (abbr.)	Matrix	LOQ [µg/l]	sex	Age [years]	N	% ≥ LOQ	min	max	P95	LCI 95%	UCI 95%	RV_{95} [µg/l]
PCB 28	plasma	0.3	f	3–11	305	2.3	< LOQ	0.65	< LOQ	–	–	–
				12–17	291	3.4	< LOQ	0.45	< LOQ	–	–	–
			m	3–11	307	3.3	< LOQ	0.45	< LOQ	–	–	–
				12–17	231	1.7	< LOQ	0.5	< LOQ	–	–	–
PCB 52	plasma	0.2	f	3–11	305	3.3	< LOQ	0.44	< LOQ	–	–	–
				12–17	291	4.1	< LOQ	0.42	< LOQ	–	–	–
			m	3–11	307	3.3	< LOQ	0.39	< LOQ	–	–	–
				12–17	231	2.2	< LOQ	0.34	< LOQ	–	–	–
PCB 101	plasma	0.03	f	3–11	305	8.2	< LOQ	0.14	0.042	–	–	–
				12–17	291	7.6	< LOQ	0.12	0.043	–	–	–
			m	3–11	307	9.8	< LOQ	0.11	0.044	–	–	–
				12–17	231	7.4	< LOQ	0.1	0.043	–	–	–
PCB 118	plasma	0.02	f	3–11	305	24.9	< LOQ	0.097	0.041	0.036	0.048	0.041
				12–17	291	12	< LOQ	0.065	0.025	0.021	0.027	0.025
			m	3–11	307	25.4	< LOQ	0.062	0.034	0.029	0.038	0.034
				12–17	231	15.6	< LOQ	0.054	0.028	0.024	0.034	0.028
PCB 138	plasma	0.02	f	3–11	305	88.2	< LOQ	0.38	0.2	0.16	0.26	0.20
				12–17	291	89.3	< LOQ	0.27	0.11	0.096	0.11	0.11
			m	3–11	307	90.2	< LOQ	0.48	0.17	0.16	0.2	0.17
				12–17	231	93.5	< LOQ	0.27	0.14	0.12	0.15	0.14
PCB 153	plasma	0.03	f	3–11	305	83.9	< LOQ	0.6	0.31	0.25	0.39	0.31
				12–17	291	83.5	< LOQ	0.3	0.15	0.13	0.16	0.15
			m	3–11	307	84	< LOQ	0.84	0.29	0.26	0.34	0.29
				12–17	231	89.6	< LOQ	0.39	0.22	0.17	0.23	0.22
PCB 180	plasma	0.02	f	3–11	305	70.2	< LOQ	0.33	0.19	0.14	0.23	0.19
				12–17	291	71.1	< LOQ	0.2	0.085	0.076	0.095	0.085
			m	3–11	307	72	< LOQ	0.87	0.17	0.13	0.22	0.17
				12–17	231	82.3	< LOQ	0.25	0.13	0.1	0.15	0.13
sum PCB (138, 153, 180) x 2	plasma	–	f	3–11	305	–	0.07	2.6	1.3	1.2	1.9	1.3
				12–17	291	–	0.07	1.5	0.64	0.57	0.69	0.64
			m	3–11	307	–	0.07	4.4	1.2	1.1	1.5	1.2
				12–17	231	–	0.07	1.8	0.96	0.78	1.1	0.96

8.1.5. PFAS

Table 6
HBM reference values (RV_{95}) GerES V (2014–2017) for PFAS in µg/l. At least 10% of the measured values must be quantified, i.e. equal to or above the analytical limit of quantification (10% ≥ LOQ), for RV_{95} derivation. Plasma = blood plasma, f = female, m = male, N = number of individuals, LOQ = Limit of quantification, P95 = 95th percentile, LCI = lower confidence interval, UCI = upper confidence interval, abbr. = abbreviations as explained in Table 1.

Chemical substance (abbr.)	Matrix	LOQ [µg/l]	sex	Age [years]	N	% ≥ LOQ	min	max	P95	LCI 95%	UCI 95%	RV_{95} [µg/l]
PFOA	plasma	0.5	f	3–11	299	88.6	< LOQ	6.3	3.3	3	3.6	3.3
				12–17	290	82.8	< LOQ	4.3	2.8	2.7	3	2.8
			m	3–11	296	91.9	< LOQ	5.1	3.6	3	4	3.6
				12–17	233	89.3	< LOQ	3.6	3	2.7	3.2	3.0
PFOS	plasma	0.25	f	3–11	299	100	0.61	11	5.6	4.7	7.8	5.6
				12–17	290	99.7	< LOQ	17	5.9	4.9	6.2	5.9
			m	3–11	296	100	0.99	130	6.3	5.3	7.6	6.3
				12–17	233	100	0.62	16	5.7	4.8	6.9	5.7
PFBA	plasma	1	f	3–11	299	0	< LOQ	< LOQ	< LOQ	–	–	–
				12–17	290	0	< LOQ	< LOQ	< LOQ	–	–	–
			m	3–11	296	0	< LOQ	< LOQ	< LOQ	–	–	–
				12–17	233	0	< LOQ	< LOQ	< LOQ	–	–	–
PFBS	plasma	0.25	f	3–11	299	0	< LOQ	< LOQ	< LOQ	–	–	–
				12–17	290	0	< LOQ	< LOQ	< LOQ	–	–	–
			m	3–11	296	0	< LOQ	< LOQ	< LOQ	–	–	–
				12–17	233	0	< LOQ	< LOQ	< LOQ	–	–	–
PFPeA	plasma	0.25	f	3–11	299	0	< LOQ	< LOQ	< LOQ	–	–	–
				12–17	290	0	< LOQ	< LOQ	< LOQ	–	–	–
			m	3–11	296	0	< LOQ	< LOQ	< LOQ	–	–	–
				12–17	233	0	< LOQ	< LOQ	< LOQ	–	–	–
PFHxA	plasma	0.25	f	3–11	299	0.3	< LOQ	0.58	< LOQ	–	–	–
				12–17	290	0	< LOQ	< LOQ	< LOQ	–	–	–
			m	3–11	296	0	< LOQ	< LOQ	< LOQ	–	–	–
				12–17	233	0	< LOQ	< LOQ	< LOQ	–	–	–
PFHxS	plasma	0.25	f	3–11	299	68.2	< LOQ	7.5	1.4	0.97	1.9	1.4
				12–17	290	67.6	< LOQ	20	0.91	0.79	1.3	0.91
			m	3–11	296	80.1	< LOQ	34	1.4	0.95	2.4	1.4
				12–17	233	78.1	< LOQ	2.8	1	0.96	1.4	1.0
PFHpA	plasma	0.25	f	3–11	299	0.3	< LOQ	0.39	< LOQ	–	–	–
				12–17	290	1	< LOQ	0.34	< LOQ	–	–	–
			m	3–11	296	1	< LOQ	0.67	< LOQ	–	–	–
				12–17	233	0	< LOQ	< LOQ	< LOQ	–	–	–
PFNA	plasma	0.5	f	3–11	299	13	< LOQ	3.5	0.82	0.65	0.88	0.82
				12–17	290	7.6	< LOQ	1.3	0.64	–	–	–
			m	3–11	296	10.1	< LOQ	2.3	0.66	0.6	0.75	0.66
				12–17	233	9.9	< LOQ	1.6	0.68	–	–	–
PFDA	plasma	0.25	f	3–11	299	10.4	< LOQ	0.7	0.36	0.31	0.49	0.36
				12–17	290	10	< LOQ	0.91	0.33	0.28	0.41	0.33
			m	3–11	296	12.5	< LOQ	3	0.37	0.32	0.44	0.37
				12–17	233	9	< LOQ	0.76	0.34	–	–	–
PFUdA	plasma	0.25	f	3–11	299	1.3	< LOQ	0.47	< LOQ	–	–	–
				12–17	290	0.7	< LOQ	0.37	< LOQ	–	–	–
			m	3–11	296	1.4	< LOQ	0.78	< LOQ	–	–	–
				12–17	233	0.4	< LOQ	0.28	< LOQ	–	–	–
PFDoA	plasma	0.25	f	3–11	299	0	< LOQ	< LOQ	< LOQ	–	–	–
				12–17	290	0	< LOQ	< LOQ	< LOQ	–	–	–
			m	3–11	296	0.3	< LOQ	0.96	< LOQ	–	–	–
				12–17	233	0	< LOQ	< LOQ	< LOQ	–	–	–

8.2.6. Parabens

Table 7
Reference values (RV_{95}) GerES V (2014–2017) for parabens in µg/l. At least 10% of the measured values must be quantified, i.e. equal to or above the analytical limit of quantification (10% ≥ LOQ), for RV_{95} derivation. Urine = first-morning void urine, f = female, m = male, N = number of individuals, LOQ = Limit of quantification, P95 = 95th percentile, LCI = lower confidence interval, UCI = upper confidence interval, abbr. = abbreviations as explained in Table 1.

Chemical substance (abbr.)	Matrix	LOQ [µg/l]	sex	Age [years]	N	% ≥ LOQ	min	max	P95	LCI 95%	UCI 95%	RV_{95} [µg/l]
MeP	urine	0.5	f	3–11	134	97.8	< LOQ	4900	240	89	730	240
				12–17	104	100	0.82	3100	540	190	1100	540
			m	3–11	154	97.4	< LOQ	2900	860	240	1400	860
				12–17	91	97.8	< LOQ	1500	35	20	240	35
EtP	urine	0.5	f	3–11	141	57.4	< LOQ	100	8.5	3.4	16	8.5
				12–17	109	72.5	< LOQ	690	39	16	130	39
			m	3–11	170	72.4	< LOQ	960	14	4.6	99	14
				12–17	95	71.6	< LOQ	19	8	5.2	12	8.0
i-PrP	urine	0.5	f	3–11	141	0	< LOQ	< LOQ	< LOQ	–	–	–
				12–17	109	2.8	< LOQ	8.1	< LOQ	–	–	–
			m	3–11	170	0	< LOQ	< LOQ	< LOQ	–	–	–
				12–17	95	0	< LOQ	< LOQ	< LOQ	–	–	–
n-PrP	urine	0.5	f	3–11	141	31.2	< LOQ	400	5.2	2.2	36	5.2
				12–17	109	52.3	< LOQ	650	49	13	340	49
			m	3–11	170	27.1	< LOQ	700	11	1.8	32	11
				12–17	95	18.9	< LOQ	110	8.3	0.84	50	8.3
i-BuP	urine	0.5	f	3–11	141	0	< LOQ	< LOQ	< LOQ	–	–	–
				12–17	109	1.8	< LOQ	19	< LOQ	–	–	–
			m	3–11	170	0	< LOQ	< LOQ	< LOQ	–	–	–
				12–17	95	0	< LOQ	< LOQ	< LOQ	–	–	–
n-BuP	urine	0.5	f	3–11	141	2.8	< LOQ	6.3	< LOQ	–	–	–
				12–17	109	5.5	< LOQ	32	0.65	–	–	–
			m	3–11	170	1.8	< LOQ	10	< LOQ	–	–	–
				12–17	95	0	< LOQ	< LOQ	< LOQ	–	–	–
BeP	urine	0.5	f	3–11	141	2.1	< LOQ	2.8	< LOQ	–	–	–
				12–17	109	6.4	< LOQ	5.1	0.93	–	–	–
			m	3–11	170	4.1	< LOQ	1.2	< LOQ	–	–	–
				12–17	95	5.3	< LOQ	2	0.56	–	–	–
PeP	urine	0.5	f	3–11	141	0	< LOQ	< LOQ	< LOQ	–	–	–
				12–17	109	0	< LOQ	< LOQ	< LOQ	–	–	–
			m	3–11	170	0	< LOQ	< LOQ	< LOQ	–	–	–
				12–17	95	0	< LOQ	< LOQ	< LOQ	–	–	–
HeP	urine	0.5	f	3–11	141	0	< LOQ	< LOQ	< LOQ	–	–	–
				12–17	109	0	< LOQ	< LOQ	< LOQ	–	–	–
			m	3–11	170	0	< LOQ	< LOQ	< LOQ	–	–	–
				12–17	95	0	< LOQ	< LOQ	< LOQ	–	–	–

8.2.7. Bisphenols

Table 8
HBM reference values (RV_{95}) GerES V (2014–2017) for bisphenols in µg/l. Urine = first-morning void urine, f = female, m = male, N = number of individuals, LOQ = Limit of quantification, P95 = 95th percentile, LCI = lower confidence interval, UCI = upper confidence interval, abbr. = abbreviations as explained in Table 1.

Chemical substance (abbr.)	Matrix	LOQ [µg/l]	sex	Age [years]	N	% ≥ LOQ	min	max	P95	LCI 95%	UCI 95%	RV_{95} [µg/l]
BPA	urine	0.5	f	3–11	141	95	< LOQ	24	8	5.1	13	8.0
				12–17	108	97.2	< LOQ	400	6.8	5.9	16	6.8
			m	3–11	170	96.5	< LOQ	100	10	6.2	13	10
				12–17	94	96.8	< LOQ	23	9.1	6.7	13	9.1

8.2.8. Aprotic solvents

Table 9
HBM reference values (RV_{95}) GerES V (2014–2017) for aprotic solvents in µg/l. Urine = first-morning void urine, f = female, m = male, N = number of individuals, LOQ = Limit of quantification, P95 = 95th percentile, LCI = lower confidence interval, UCI = upper confidence interval, abbr. = abbreviations as explained in Table 1.

Chemical substance (abbr.)	Metabolite (abbr.)	Matrix	LOQ [µg/l]	sex	Age [years]	N	% ≥ LOQ	min	max	P95	LCI 95%	UCI 95%	RV_{95} [µg/l]
NMP	HNMP	urine	2.5	f	3–11	628	100	7.8	680	180	160	190	180
					12–17	466	100	3.6	1100	170	150	190	170
				m	3–11	643	100	4.2	1100	190	150	210	190
					12–17	439	100	4.1	440	170	150	190	170
	HMSI		2	f	3–11	638	100	10	320	99	91	110	99
					12–17	478	100	6.5	480	110	100	110	110
				m	3–11	655	100	7.6	520	100	95	120	100
					12–17	445	100	5.3	340	100	92	110	100
	sum HNMP and HMSI		–	f	3–11	628	–	19	1000	270	240	300	270
					12–17	466	–	10	1600	280	240	300	280
				m	3–11	643	–	13	1600	260	250	320	260
					12–17	439	–	13	780	270	230	300	270
NEP	HNEP	urine	2.5	f	3–11	636	27.7	< LOQ	770	51	22	90	51
					12–17	474	32.7	< LOQ	1800	130	80	250	130
				m	3–11	654	30	< LOQ	1100	75	40	150	75
					12–17	443	34.3	< LOQ	1900	150	120	220	150
	HESI		2	f	3–11	633	82.1	< LOQ	510	66	41	110	66
					12–17	466	87.8	< LOQ	1300	120	96	190	120
				m	3–11	639	86.9	< LOQ	970	85	59	150	85
					12–17	440	87.5	< LOQ	720	150	110	190	150
	sum HNEP and HESI		–	f	3–11	630	–	2.3	1100	110	71	180	110
					12–17	462	–	2.3	3100	250	160	410	250
				m	3–11	638	–	2.3	1500	230	120	300	230
					12–17	438	–	2.3	2000	300	220	360	300

8.2.9. Chlorophenols

Table 10
HBM reference values (RV_{95}) GerES V (2014–2017) for chlorophenols in µg/l. Urine = first-morning void urine, f = female, m = male, N = number of individuals, LOQ = Limit of quantification, P95 = 95th percentile, LCI = lower confidence interval, UCI = upper confidence interval, abbr. = abbreviations as explained in Table 1.

Chemical substance (abbr.)	Matrix	LOQ [µg/l]	sex	Age [years]	N	% ≥ LOQ	min	max	P95	LCI 95%	UCI 95%	RV_{95} [µg/l]
PCP	urine	0.1	f	3–11	126	86.5	< LOQ	6.7	1	0.6	1.3	1.0
				12–17	110	85.5	< LOQ	1.5	0.6	0.4	0.8	0.60
			m	3–11	149	88.6	< LOQ	1.7	0.7	0.5	0.9	0.70
				12–17	99	81.8	< LOQ	3.5	1.6	0.6	2.1	1.6
2-MCP	urine	0.1	f	3–11	126	98.4	< LOQ	2	1	0.7	1.4	1.0
				12–17	110	95.5	< LOQ	1.5	0.7	0.5	0.8	0.70
			m	3–11	150	98.7	< LOQ	5.2	0.9	0.6	1.5	0.90
				12–17	99	96	< LOQ	2.6	0.8	0.5	0.9	0.80
4-MCP	urine	0.1	f	3–11	126	100	0.1	18	3.6	2.8	5.9	3.6
				12–17	110	99.1	< LOQ	29	7.4	4.6	13	7.4
			m	3–11	150	100	0.2	16	3.3	2.8	4.4	3.3
				12–17	99	100	0.2	6.6	4.4	3.3	5.4	4.4
2,4-DCP	urine	0.1	f	3–11	126	97.6	< LOQ	1.2	0.6	0.5	0.6	0.60
				12–17	110	94.5	< LOQ	5.4	0.7	0.5	1.8	0.70
			m	3–11	150	97.3	< LOQ	5.6	0.8	0.6	0.8	0.80
				12–17	99	93.9	< LOQ	15	0.8	0.5	2.2	0.80
2,5-DCP	urine	0.1	f	3–11	126	96	< LOQ	6.1	1.2	0.8	1.6	1.2
				12–17	110	92.7	< LOQ	3	1.5	0.6	1.9	1.5
			m	3–11	150	96	< LOQ	13	1	0.8	1.6	1.0
				12–17	99	94.9	< LOQ	360	2.8	0.6	7.6	2.8
2,6-DCP	urine	0.1	f	3–11	126	27	< LOQ	0.3	0.1	–	–	0.10
				12–17	110	27.3	< LOQ	0.7	0.2	0.1	0.5	0.20
			m	3–11	150	22	< LOQ	0.3	0.1	–	–	0.10
				12–17	99	22.2	< LOQ	0.4	0.2	0.1	0.2	0.20
2,3,4-TriCP	urine	0.1	f	3–11	126	13.5	< LOQ	0.4	0.1	< LOQ	< LOQ	0.100
				12–17	110	14.5	< LOQ	0.4	0.1	< LOQ	< LOQ	0.100
			m	3–11	150	11.3	< LOQ	0.5	0.1	< LOQ	< LOQ	0.100
				12–17	99	19.2	< LOQ	0.5	0.2	0.1	0.2	0.20
2,4,5-TriCP	urine	0.1	f	3–11	126	28.6	< LOQ	0.5	0.2	0.1	0.2	0.20
				12–17	110	16.4	< LOQ	0.8	0.1	< LOQ	< LOQ	0.100
			m	3–11	150	22	< LOQ	0.2	0.1	–	–	0.10
				12–17	99	24.2	< LOQ	0.9	0.1	–	–	0.10
2,4,6-TriCP	urine	0.1	f	3–11	126	70.6	< LOQ	1.4	0.7	0.4	1	0.70
				12–17	110	71.8	< LOQ	2.8	0.6	0.3	2	0.60
			m	3–11	150	73.3	< LOQ	1.8	0.6	0.4	0.6	0.60
				12–17	99	71.7	< LOQ	1.9	0.5	0.3	0.5	0.50
2,3,4,6-TeCP	urine	0.1	f	3–11	124	53.2	< LOQ	2.2	0.4	0.3	1.3	0.40
		0.1		12–17	109	36.7	< LOQ	0.7	0.3	0.2	0.2	0.300
		0.1	m	3–11	150	41.3	< LOQ	0.4	0.2	–	–	0.20
		0.1		12–17	99	46.5	< LOQ	2	0.4	0.2	0.4	0.40

8.2.10. PAH

Table 11
Reference values (RV_{95}) GerES V (2014–2017) for PAH in µg/l. Sum RV_{95} are derived for metabolites in case health-based sum HBM values (by HBM Commission) exist. Urine = first-morning void urine, f = female, m = male, ns = only non-smokers (cotinine <50 µg/L), N = number of individuals, LOQ = Limit of quantification, P95 = 95th percentile, LCI = lower confidence interval, UCI = upper confidence interval, abbr. = abbreviations as explained in Table 1.

Chemical substance	Metabolite (abbr.)	Matrix	LOQ [µg/l]	sex	Age [years]	note	N	% ≥ LOQ	min	max	P95	LCI 95%	UCI 95%	RV_{95} [µg/l]
fluorene	2-OH-flu	urine	0.05	f	3–11	ns	141	87.9	< LOQ	11	2.2	1.4	4	2.2
					3–11		141	87.9	< LOQ	11	2.2	1.4	4	–
					12–17	ns	101	89.1	< LOQ	50	2.2	1.3	5.6	2.2
					12–17		109	89.9	< LOQ	50	2.2	1.6	5.6	–
				m	3–11	ns	170	91.2	< LOQ	8.1	1.7	1.3	2.7	1.7
					3–11		170	91.2	< LOQ	8.1	1.7	1.3	2.7	–
					12–17	ns	83	88	< LOQ	6.3	1.4	1.1	2.7	1.4
					12–17		95	88.4	< LOQ	6.3	1.8	1.3	2.7	–
naphthalene	1-OH-nap	urine	0.05	f	3–11	ns	141	97.2	< LOQ	9.9	4.7	3.2	7.1	4.7
					3–11		141	97.2	< LOQ	9.9	4.7	3.2	7.1	–
					12–17	ns	101	96	< LOQ	9.7	5.3	2.9	6.9	5.3
					12–17		109	96.3	< LOQ	13	6.9	3.1	9.7	–
				m	3–11	ns	170	96.5	< LOQ	29	5.3	3.9	9.1	5.3
					3–11		170	96.5	< LOQ	29	5.3	3.9	9.1	–
					12–17	ns	83	95.2	< LOQ	9.5	3.7	2.1	5.6	3.7
					12–17		95	95.8	< LOQ	12	9	5.3	11	–
	2-OH-nap		0.05	f	3–11	ns	141	99.3	< LOQ	160	21	10	31	21
					3–11		141	99.3	< LOQ	160	21	10	31	–
					12–17	ns	101	100	0.58	43	22	16	31	22
					12–17		109	100	0.58	43	23	18	31	–
				m	3–11	ns	170	100	0.54	42	20	14	24	20
					3–11		170	100	0.54	42	20	14	24	–
					12–17	ns	83	100	0.57	40	16	10	37	16
					12–17		95	100	0.57	40	24	14	37	–
phenanthrene	1-OH-phe	urine	0.005	f	3–11	ns	141	100	0.022	0.93	0.47	0.32	0.72	0.47
					3–11		141	100	0.022	0.93	0.47	0.32	0.72	–
					12–17	ns	101	100	0.026	0.81	0.45	0.37	0.54	0.45
					12–17		109	100	0.026	0.9	0.51	0.38	0.59	–
				m	3–11	ns	170	100	0.023	1.6	0.57	0.35	0.64	0.57
					3–11		170	100	0.023	1.6	0.57	0.35	0.64	–
					12–17	ns	83	100	0.026	1.2	0.51	0.37	1	0.51
					12–17		95	100	0.022	1.2	0.69	0.41	1	–
	2-OH-phe		0.005	f	3–11	ns	141	99.3	< LOQ	0.88	0.25	0.2	0.4	0.25
					3–11		141	99.3	< LOQ	0.88	0.25	0.2	0.4	–
					12–17	ns	101	99	< LOQ	0.39	0.27	0.22	0.34	0.27
					12–17		109	99.1	< LOQ	0.39	0.32	0.23	0.36	–
				m	3–11	ns	170	98.8	< LOQ	1	0.27	0.23	0.36	0.27
					3–11		170	98.8	< LOQ	1	0.27	0.23	0.36	–
					12–17	ns	83	100	0.013	0.79	0.34	0.25	0.68	0.34
					12–17		95	100	0.013	0.79	0.35	0.29	0.68	–
	3-OH-phe		0.005	f	3–11	ns	141	100	0.028	1.3	0.38	0.3	0.42	0.38
					3–11		141	100	0.028	1.3	0.38	0.3	0.42	–
					12–17	ns	101	100	0.037	0.48	0.37	0.31	0.4	0.37
					12–17		109	100	0.037	1.1	0.39	0.33	0.48	–
				m	3–11	ns	170	100	0.023	1.1	0.49	0.39	0.7	0.49
					3–11		170	100	0.023	1.1	0.49	0.39	0.7	–
					12–17	ns	83	100	0.034	0.99	0.41	0.36	0.59	0.41
					12–17		95	100	0.032	1	0.51	0.41	0.99	–
	4-OH-phe		0.001	f	3–11	ns	141	99.3	< LOQ	1.5	0.29	0.22	0.44	0.29
					3–11		141	99.3	< LOQ	1.5	0.29	0.22	0.44	–
					12–17	ns	101	100	0.004	0.47	0.18	0.14	0.3	0.18
					12–17		109	100	0.004	0.48	0.18	0.14	0.32	–
				m	3–11	ns	170	99.4	< LOQ	2.3	0.42	0.25	0.7	0.42
					3–11		170	99.4	< LOQ	2.3	0.42	0.25	0.7	–
					12–17	ns	83	100	0.008	0.51	0.28	0.16	0.49	0.28
					12–17		95	100	0.008	0.51	0.36	0.17	0.49	–
	9-OH-phe		0.005	f	3–11	ns	141	95.7	< LOQ	1.2	0.3	0.23	0.64	0.30
					3–11		141	95.7	< LOQ	1.2	0.3	0.23	0.64	–
					12–17	ns	101	99	< LOQ	0.59	0.24	0.15	0.4	0.24
					12–17		109	99.1	< LOQ	0.59	0.25	0.19	0.4	–
				m	3–11	ns	170	97.6	< LOQ	1	0.52	0.31	0.63	0.52
					3–11		170	97.6	< LOQ	1	0.52	0.31	0.63	–
					12–17	ns	83	96.4	< LOQ	1.8	0.15	0.12	0.5	0.15
					12–17		95	96.8	< LOQ	1.8	0.24	0.16	0.66	–

(*continued on next page*)

Table 11 (*continued*)

Chemical substance	Metabolite (abbr.)	Matrix	LOQ [µg/l]	sex	Age [years]	note	N	% ≥ LOQ	min	max	P95	LCI 95%	UCI 95%	RV_{95} [µg/l]
pyrene	1-OH-pyr	urine	0.01	f	3–11	ns	141	97.9	< LOQ	0.42	0.29	0.22	0.35	0.29
					3–11		141	97.9	< LOQ	0.42	0.29	0.22	0.35	–
					12–17	ns	101	99	< LOQ	0.5	0.32	0.26	0.4	0.32
					12–17		109	99.1	< LOQ	1.1	0.37	0.28	0.47	–
				m	3–11	ns	170	98.2	< LOQ	1.2	0.43	0.28	0.58	0.43
					3–11		170	98.2	< LOQ	1.2	0.43	0.28	0.58	–
					12–17	ns	83	100	0.013	0.73	0.32	0.26	0.72	0.32
					12–17		95	100	0.013	0.73	0.57	0.32	0.72	–

8.2.11. UV filter

Table 12
HBM reference values (RV_{95}) GerES V (2014–2017) for UV filters in µg/l. At least 10% of the measured values must be quantified, i.e. equal to or above the analytical limit of quantification (10% ≥ LOQ), for RV_{95} derivation. Urine = first-morning void urine, f = female, m = male, N = number of individuals, LOQ = Limit of quantification, P95 = 95th percentile, LCI = lower confidence interval, UCI = upper confidence interval, abbr. = abbreviations as explained in Table 1.

Chemical (abbr.)	Metabolite (abbr.)	Matrix	LOQ [µg/l]	sex	Age [years]	N	% ≥ LOQ	min	max	P95	LCI 95%	UCI 95%	RV_{95} [µg/l]
4-MBC	cx-MBC	urine	0.15	f	3–11	110	1.8	< LOQ	0.6	< LOQ	–	–	–
					12–17	100	2	< LOQ	1.2	< LOQ	–	–	–
				m	3–11	135	2.2	< LOQ	13	< LOQ	–	–	–
					12–17	96	0	< LOQ	< LOQ	< LOQ	–	–	–
	cx-MBC-OH		0.3	f	3–11	110	0	< LOQ	< LOQ	< LOQ	–	–	–
					12–17	100	0	< LOQ	< LOQ	< LOQ	–	–	–
				m	3–11	135	0	< LOQ	< LOQ	< LOQ	–	–	–
					12–17	96	0	< LOQ	< LOQ	< LOQ	–	–	–
EHS	5cx-EPS	urine	0.02	f	3–11	114	98.2	< LOQ	92	8.3	3.2	14	8.3
					12–17	103	97.1	< LOQ	100	3.2	1.6	17	3.2
				m	3–11	140	96.4	< LOQ	37	6.1	2.1	19	6.1
					12–17	97	92.8	< LOQ	8.1	1.3	0.62	3.6	1.3
	5OH-EHS		0.05	f	3–11	114	80.7	< LOQ	52	4.1	1.8	8.2	4.1
					12–17	103	81.6	< LOQ	43	2.5	0.94	6.9	2.5
				m	3–11	140	81.4	< LOQ	27	4.2	1.2	6.1	4.2
					12–17	96	62.5	< LOQ	5	1.1	0.44	3	1.1
	5oxo-EHS		0.15	f	3–11	111	32.4	< LOQ	35	2.3	1.3	4.9	2.3
					12–17	100	25	< LOQ	31	1.5	0.52	3.5	1.5
				m	3–11	138	26.8	< LOQ	11	2.4	0.68	7.3	2.4
					12–17	95	13.7	< LOQ	2	0.56	0.21	1.3	0.56
OC	CPPA	urine	0.5	f	3–11	111	100	1	1300	680	250	1000	680
					12–17	103	98.1	< LOQ	1200	87	46	370	87
				m	3–11	140	100	1	2100	850	210	1300	850
					12–17	95	98.9	< LOQ	360	70	42	260	70
	DOCCA		0.05	f	3–11	114	84.2	< LOQ	26	8.8	3.9	21	8.8
					12–17	103	68	< LOQ	7.5	0.8	0.55	4.9	0.80
				m	3–11	140	90.7	< LOQ	50	9.2	4	17	9.2
					12–17	97	60.8	< LOQ	4	1.2	0.5	1.8	1.2
	5OH-OC		0.015	f	3–11	114	45.6	< LOQ	1.4	0.31	0.15	0.78	0.31
					12–17	103	19.4	< LOQ	0.7	0.063	0.032	0.48	0.063
				m	3–11	140	44.3	< LOQ	2.4	0.39	0.17	0.58	0.39
					12–17	97	15.5	< LOQ	0.14	0.047	0.025	0.063	0.047
BP-1		urine	0.5	f	3–11	141	44	< LOQ	200	4.1	2.1	8.9	4.1
					12–17	109	63,3	< LOQ	89	28	11	43	28
				m	3–11	170	35,9	< LOQ	9.2	2.5	1.7	3.4	2.5
					12–17	95	42,1	< LOQ	110	14	1.9	20	14
BP-3		urine	2	f	3–11	141	35,5	< LOQ	850	13	5.7	21	13
					12–17	109	55	< LOQ	230	99	55	220	99
				m	3–11	170	28,2	< LOQ	28	7.7	6.3	14	7.7
					12–17	95	41,1	< LOQ	170	43	6.8	160	43
BP-8		urine	0.5	f	3–11	141	0	< LOQ	< LOQ	< LOQ	–	–	–
					12–17	109	0	< LOQ	< LOQ	< LOQ	–	–	–
				m	3–11	170	0	< LOQ	< LOQ	< LOQ	–	–	–
					12–17	95	0	< LOQ	< LOQ	< LOQ	–	–	–

8.2.12. Other chemicals

Table 13
Reference values (RV_{95}) GerES V (2014–2017) for other chemicals in µg/l. At least 10% of the measured values must be quantified, i.e. equal to or above the analytical limit of quantification (10% ≥ LOQ), for RV_{95} derivation. Urine = first-morning void urine, f = female, m = male, ns = non-smoker (cotinine <50 µg/L) N = number of individuals, LOQ = Limit of quantification, P95 = 95th percentile, LCI = lower confidence interval, UCI = upper confidence interval, abbr. = abbreviations as explained in Table 1.

Chemical substance (abbr.)	Metabolite (abbr.)	Matrix	LOQ [µg/l]	sex	Age [years]	note	N	% ≥ LOQ	min	max	P95	LCI 95%	UCI 95%	RV_{95} [µg/l]
Triclosan		urine	1	f	3–11		141	8.5	< LOQ	10	2.5	–	–	–
					12–17		109	23.9	< LOQ	800	120	3	370	120
				m	3–11		170	20	< LOQ	660	10	3.3	20	10
					12–17		95	13.7	< LOQ	320	10	1.1	280	10
Triclocarban		urine	1	f	3–11		141	0	< LOQ	< LOQ	< LOQ	–	–	–
					12–17		109	0	< LOQ	< LOQ	< LOQ	–	–	–
				m	3–11		170	0	< LOQ	< LOQ	< LOQ	–	–	–
					12–17		95	1.1	< LOQ	5.5	< LOQ	–	–	–
2-Phenylphenol		urine	2	f	3–11		141	0	< LOQ	< LOQ	< LOQ	–	–	–
					12–17		109	0	< LOQ	< LOQ	< LOQ	–	–	–
				m	3–11		170	1.8	< LOQ	8.3	< LOQ	–	–	–
					12–17		95	1.1	< LOQ	2.2	< LOQ	–	–	–
Lysmeral	TBBA	urine	0.2	f	3–11		609	100	0.48	190	58	46	65	58
					12–17		461	100	0.5	250	54	45	73	54
				m	3–11		627	99.8	< LOQ	320	52	43	59	52
					12–17		438	100	0.4	190	38	33	50	38
	lysmerol		0.2	f	3–11		609	98.5	< LOQ	36	6.3	4.9	7.3	6.3
					12–17		461	99.3	< LOQ	91	8.9	7.1	12	8.9
				m	3–11		627	98.7	< LOQ	30	5.6	4.9	6.9	5.6
					12–17		438	98.9	< LOQ	63	6.5	5.3	7.7	6.5
	hydroxy-lysmerylic acid		0.4	f	3–11		609	19.7	< LOQ	6.3	1.1	0.88	1.4	1.1
					12–17		461	24.1	< LOQ	40	1.6	1.2	2.1	1.6
				m	3–11		627	20.7	< LOQ	33	1.1	0.89	1.3	1.1
					12–17		438	30.6	< LOQ	33	1.6	1.2	2.1	1.6
	lysmerylic acid		0.2	f	3–11		609	34.8	< LOQ	9	0.89	0.72	1.1	0.89
					12–17		461	44	< LOQ	3.8	1.3	0.94	1.8	1.3
				m	3–11		627	36.5	< LOQ	5.5	1	0.82	1.1	1.0
					12–17		438	39.3	< LOQ	3.1	0.86	0.71	1.1	0.86
MI, MCI	NMMA	urine	0.5	f	3–11		583	100	0.94	33	15	14	16	15
					12–17		448	99.8	< LOQ	52	14	13	17	14
				m	3–11		613	100	0.6	37	15	14	16	15
					12–17		435	100	1.2	47	15	14	17	15
BHT	BHT acid	urine	0.2	f	3–11		589	100	0.23	250	14	9.5	20	14
					12–17		453	99.1	< LOQ	73	11	9.4	15	11
				m	3–11		615	99.8	< LOQ	150	15	11	22	15
					12–17		436	99.3	< LOQ	190	8.7	6.4	11	8.7
2-MBT		urine	1	f	3–11		141	42.6	< LOQ	44	4.5	2.8	7.6	4.5
					12–17		109	45	< LOQ	17	3.4	2.7	7.8	3.4
				m	3–11		170	55.9	< LOQ	38	6.6	4.5	8.5	6.6
					12–17		95	51.6	< LOQ	8.5	5.1	3.3	7.4	5.1
cotinine		urine	0.1	f	3–11	ns	654	48.2	< LOQ	47	7.6	6.2	9.8	7.6
					3–11		657	48.4	< LOQ	260	7.9	6.3	11	–
					12–17	ns	454	46.5	< LOQ	45	7.7	3.6	12	7.7
					12–17		485	49.9	< LOQ	1400	110	32	230	–
				m	3–11	ns	669	48	< LOQ	48	6.4	3.7	8.6	6.4
					3–11		669	48	< LOQ	48	6.4	3.7	8.6	–
					12–17	ns	409	47.9	< LOQ	44	9	4.8	13	9.0
					12–17		447	52.3	< LOQ	2000	630	200	890	–
benzene	SPMA	urine	0.02	f	3–11	ns	655	97.6	< LOQ	0.96	0.35	0.3	0.36	0.35
					3–11		658	97.6	< LOQ	2.7	0.36	0.31	0.37	–
					12–17	ns	454	96.3	< LOQ	5.4	0.38	0.32	0.41	0.38
					12–17		485	96.5	< LOQ	5.4	0.75	0.41	1.2	–
				m	3–11	ns	669	98.5	< LOQ	1.4	0.32	0.29	0.38	0.32
					3–11		669	98.5	< LOQ	1.4	0.32	0.28	0.38	–
					12–17	ns	409	96.3	< LOQ	0.75	0.29	0.24	0.29	0.29
					12–17		447	96.6	< LOQ	8.1	0.99	0.58	2.5	–
acrylamide	AAMA	urine	1	f	3–11	ns	655	100	5	590	200	180	260	200
					3–11		658	100	5	590	200	180	270	–
					12–17	ns	454	100	2.8	620	220	200	300	220
					12–17		485	100	2.8	1300	280	220	320	–
				m	3–11	ns	669	100	4.1	840	230	190	260	230
					3–11		669	100	4.1	840	230	190	260	–
					12–17	ns	409	100	5	1500	240	200	280	240
					12–17		447	100	5	1500	290	250	360	–
	GAMA		1	f	3–11	ns	655	100	1.3	99	38	34	41	38

(continued on next page)

Table 13 (*continued*)

Chemical substance (abbr.)	Metabolite (abbr.)	Matrix	LOQ [µg/l]	sex	Age [years]	note	N	% ≥ LOQ	min	max	P95	LCI 95%	UCI 95%	RV_{95} [µg/l]
					3–11		658	100	1.3	99	38	34	41	–
					12–17	ns	454	99.6	< LOQ	72	36	33	39	36
					12–17		485	99.6	< LOQ	170	37	35	43	–
				m	3–11	ns	669	100	1.6	110	41	36	47	41
					3–11		669	100	1.6	110	41	37	47	–
					12–17	ns	409	100	1.3	130	38	35	42	38
					12–17		447	100	1.3	130	43	39	47	–
glyphosate		urine	0.1	f	3–11		614	52	< LOQ	2.1	0.49	0.41	0.57	0.49
					12–17		467	47.8	< LOQ	2.6	0.51	0.4	0.61	0.51
				m	3–11		630	54.6	< LOQ	11	0.51	0.44	0.64	0.51
					12–17		439	53.1	< LOQ	2.6	0.46	0.39	0.52	0.46
	AMPA		0.1	f	3–11		614	45	< LOQ	3.1	0.48	0.42	0.54	0.48
					12–17		467	45.6	< LOQ	13	0.44	0.36	0.54	0.44
				m	3–11		630	47	< LOQ	2.4	0.48	0.41	0.53	0.48
					12–17		439	49.7	< LOQ	2.6	0.49	0.38	0.57	0.49
creatinine		urine	5	f	3–11		658	100	140	4400	2000	1800	2100	2000
					12–17		485	100	110	4200	3000	2800	3100	3000
				m	3–11		669	100	89	3700	2000	1900	2100	2000
					12–17		447	100	200	5000	2900	2800	3200	2900

Table 14
Reference values for internal exposures to chemical substances for the occupational sector, exemplary.

Acronym	Term	Definition	Agency/Country
BAR	Biological reference values for hazardous substances	Values refer to the body burden of a reference population of working-age individuals who are not occupationally exposed to a particular chemical (95th percentile of the measured values)	Permanent Senate Commission of the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG)
BRV	Biological reference values	Based on data from the general population for biomarkers of exposure and on data from workers without exposure (similar target population) for biomarkers of effects	French Agency for Food, Environmental and Occupational Health & Safety (ANSES)
BGV	Biological guidance values	Corresponding to the 90 or 95 percentile in a defined reference population, or to the detection limit of the biomonitoring method	European Scientific Committee on Occupational Exposure Limits (SCOEL; European Commission, 2013; version 7)
ROBL	Reference occupational exposure levels	Based on the 95 percentile of data from large-scale general population biomonitoring studies (excluding individuals with known non-occupational exposure)	Intergovernmental Organisation for Economic Co-operation and Development (OECD)

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Does exposure to nature make children more intelligent? Analysis in Polish children with and without ADHD

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ABSTRACT

Previous studies have shown that exposure to nature and physical activity (PA) may be associated with higher intelligence in children. We examined whether there is an association between lifelong exposure to greenspace and bluespace and intelligence in children aged 10–13 with and without attention deficit hyperactivity disorder (ADHD), and whether PA mediates this association. The sample (N = 714) was collected within the NeuroSmog case-control study, where children with (N = 206) and without ADHD (N = 508) were recruited from 18 towns in Southern Poland. Nature exposure was estimated as the sum of the z-scores of the objective and perceived measures. Objective greenspace exposure was defined as the percentage of grass and tree cover in 500 m and 1 km buffers around lifelong residential addresses, respectively. Objective bluespace exposure was defined as the percentage of water cover in 500 m and 1 km buffers. Perceived greenspace/bluespace was measured as the parent-rated availability, quality, and use of greenspace/bluespace. Intelligence was assessed using the Polish version of the Stanford-Binet Intelligence Scales, 5th edition (SB5). SB5 Full Scale Intelligence Quotient (IQ), Nonverbal IQ, Verbal IQ, five factor and ten subtest scores were analysed as outcomes. The associations between nature and IQ scores were assessed by linear regressions separately for cases and controls, adjusting the models for sex, parental education, and urbanicity. Structural equation modeling was implemented to test whether PA mediated the association between nature and intelligence. None of the greenspace or bluespace measures were consistently associated with intelligence. PA was not found to be a mediator. We did not find evidence that higher lifelong nature exposure is associated with higher intelligence in Polish schoolchildren with or without ADHD. This casts doubts on whether exposure to nature has relevant influence on IQ.

1. Introduction

According to a United Nations report (United Nations Department of Economic and Social Affairs, 2018), 55% of the world's population lives in urban areas. Living in cities is associated with higher risks for both physical and mental health issues (Nieuwenhuijsen et al., 2017).

Air and noise pollution and limited access to natural environments are frequently mentioned factors that contribute to higher health risks for urban populations. Therefore, the beneficial health effects of population exposure to nature (typically referred to as "greenspace" and "bluespace", respectively meaning vegetated and water feature elements of the environment) have become the focus of an emerging field in environmental epidemiology, including research across various disciplines, such as ecology, urban planning, medicine, psychology, and neurosciences. Consequently, there is a growing body of evidence on the positive impact of greenspace and bluespace on human health and well-being (Yang et al., 2021). Previous research also suggests associations between exposure to the natural environment and cognitive functioning in both adults and children (Buczyłowska et al., 2023; de Keijzer et al., 2016; Luque-García et al., 2022), but the number of studies is limited

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and their findings are inconclusive. This topic should be more investigated in children, since crucial stages of cognitive development occur during childhood and adolescence. These developmental stages are also considered critical windows of vulnerability (Davis, 2011; Rice and Barone, 2000), and sensitive periods for exposure to environmental neurotoxicants (Miodovnik, 2011); thus, children and adolescents may also be more strongly affected by greenspace and bluespace exposure than adults.

The beneficial effects of nature on children's cognition might occur through multiple pathways that can be presented in three domains according to their function: reducing harm (e.g., reducing exposure to air pollution, noise, and heat), restoring capacities (e.g., attention restoration and physiological stress recovery), and building capacities (e.g., encouraging physical activity [PA] and facilitating social cohesion) (T. Hartig et al., 2014; Markevych et al., 2017).

The function of restoring capacities is represented by two well-established theories of environmental psychology: attention restoration theory (ART; Kaplan, 1995) and stress reduction theory (SRT; Ulrich et al., 1991), which are further extended by relational restoration theory (RRT) and collective restoration theory (T. Hartig, 2021). All four theories elaborate on the different ways nature enables restoration, thereby improving cognitive functioning. Importantly, the domain of restoring capacity is considered to interact with two other restoration domains (Markevych et al., 2017). The interrelations among different restoration pathways, including PA and social cohesion, should be further examined (Dzhambov et al., 2020; Dzhambov et al., 2018a). In particular, the indirect effect of nature on cognition through the PA pathway requires further investigation, as the effects of PA on cognition in children have frequently been demonstrated (Bidzan-Bluma and Lipowska, 2018; Hillman et al., 2019).

The focus of research devoted to the potential association between exposure to nature and cognition has been on greenspace and/or bluespace, and several different cognitive abilities. In a recently published systematic review on exposure to greenspace and bluespace and cognitive functioning in children (Buczyłowska et al., 2023), the majority of the included studies investigated attentional control and reaction and decision speed (12 studies), and attentional control and processing speed (10 studies). Eleven studies investigated the working memory capacity. Eight studies examined visual processing and psychomotor speed, parent-reported attention, early childhood/cognitive development, and decision-making and self-regulation. Nine of the studies focused on intellectual functioning.

Investigating the association between nature exposure and intellectual functioning appears particularly meaningful, as intelligence is considered one of the best predictors of several crucial life outcomes such as educational attainment, job performance, income, health, and longevity (Deary et al., 2004; Gottfredson and Deary, 2004; Sternberg et al., 2001). Moreover, intelligence test scores usually depict the highest level of cognitive capacity and are correlated with all essential cognitive functions (McDonough et al., 2018).

All nine studies that previously examined the association between exposure to nature and intelligence in children were observational. Only two studies considered bluespace in addition to greenspace exposure. All employed standardised intelligence tests to assess intellectual functioning. The results of these studies are rather inconsistent, which is most likely due to the heterogeneity of the methodological approaches, including both exposure and outcome assessments.

Consequently, the low number of studies, heterogeneity of methodological approaches, and inconsistency in the results across and within the studies do not allow for clear conclusions about the association between nature exposure and intelligence. It is not possible to determine whether some measures of greenspace or bluespace exposure show more consistent associations with cognition than others, and whether associations differ across age groups. Thus, further research using diverse state-of-the-art methodological approaches is required (Buczyłowska et al., 2023).

Nature exposure has also been examined in association with attention deficit hyperactivity disorder (ADHD) (de Keijzer et al., 2016; Luque-García et al., 2022), which is the most common neurodevelopmental disorder in school-aged children with an estimated worldwide prevalence approximately 5–7% (Polanczyk et al., 2007; Thomas et al., 2015). However, studies focusing on nature exposure and intelligence in ADHD are lacking. Based on evidence suggesting that ADHD is associated with deficits across a variety of cognitive domains, including intelligence (Pievsky and Mcgrath, 2018), the potential impact of nature exposure should also be investigated in ADHD.

The current study aimed to examine the associations between lifelong exposure to greenspace and bluespace and intelligence in schoolchildren with and without ADHD. Another objective was to test the indirect effect of nature exposure on intelligence through PA. We hypothesised associations between greenspace and bluespace exposure and intelligence on individual subtest level rather than on the overall intelligence test score level. Further, we expected an indirect effect of greenspace exposure on intelligence through PA. We assumed differences in both the direct and indirect examined effects between children with and without ADHD.

2. Methods

2.1. Sample, recruitment, and testing procedure

The current analysis is a spin-off of the case-control NeuroSmog study, which aims to explore the associations between long-term air pollution and neurodevelopment in children with ADHD (cases) and their ADHD-free counterparts (controls). The data used in the current analysis were collected from October 2020 to September 2022 in 18 towns in southern Poland. Children without ADHD were randomly sampled from randomly selected schools. A team of 25 field clinical psychologists with at least five years of clinical experience was responsible for recruiting potential cases and conducting psychological assessments of all children. Children at risk of ADHD were referred to field psychologists by cooperating schools, psychological-educational counselling centres, and mental health centres, or parents applied for participation on their behalf. A two-step model was used to verify ADHD diagnosis in cases and controls, respectively. Firstly, a comprehensive psychological assessment was conducted and evaluated by field psychologists. Secondly, three consultant clinical psychologists reviewed the assessments to verify the diagnoses according to the International Classification of Diseases 11th Revision (ICD-11) criteria (WHO, 2019).

The study sample consists of children aged 10–13 years with ADHD and without ADHD. Since deficits in executive functions are one of the diagnostic criteria of ADHD (WHO, 2022), we focused on children from the age range of intensive development of executive functions. The included children were fluent Polish speakers, aged between 10 and 13 years (born between 2007 and 2011), with average or above-average intelligence, and attending a school in the selected towns. To be enrolled, children had to be born at $\geq$ 35 weeks of gestation with a birth weight of $\geq$2500 g and Apgar score $\geq$8. Children with severe comorbidities, such as diagnosed intellectual disability, neurological or psychiatric disorders, or other serious medical conditions were excluded. Excluded were also children with contraindications to magnetic resonance imaging (MRI), or those not residing in Poland for at least the previous year.

Clinical psychologists were trained in administering the psychological measures utilised in the study. Psychological assessments, including cognitive and behavioural tasks, were conducted in three meetings with a field psychologist. Each meeting lasted for approximately 2 h. The intelligence assessment was conducted on one occasion during the first meeting. Additionally, children were invited for one session of MRI scanning within three months of the psychological evaluation. For more details on recruitment and testing procedure, please refer to the NeuroSmog study protocol (Markevych et al., 2022).

The sample size was not based on previous power analysis. According to the main goal of the NeuroSmog project, the sample was collected based on the feasibility of MRI scanning. Initially, 756 participants were recruited and tested. Our analytic sample comprised 714 participants: 206 cases and 508 controls with outcome, exposure, main confounder, and mediator data (for details, see Supplement 1 Fig. S1).

The current residential addresses of the study participants superimposed over the study area, as well as the locations of the study area in Poland and Europe, are depicted in Fig. 1.

NeuroSmog was approved by the Ethics Committee of the Institute of Psychology, Jagiellonian University, Krakow, Poland (# KE_24042019A). Written informed consent was collected from the legal guardians of the participants, and written informed assent was collected from the participants themselves. The clinical trial identifier was NCT04574414.

2.2. Intelligence assessment

For intelligence assessment Stanford-Binet Intelligence Scales, Fifth Edition (SB5) – Polish version (Roid et al., 2017a) was used. SB5 is an individually administered intelligence test battery designed to assess cognitive abilities in individuals between 2 and 85 years of age.

The SB5 was revised according to the Cattell-Horn-Carroll (CHC) theory (Carroll, 1993; Cattell, 1943, 1963; Cattell and Horn, 1978), representing three levels of cognitive ability: general ability (stratum III), broad ability (stratum II), and narrow ability (stratum I). Narrow and broad ability scores are obtained from the individual subtests: Fluid Reasoning, Knowledge, Quantitative Reasoning, Visual-Spatial Reasoning, and Working Memory. Each of the subtests is available in both nonverbal and verbal versions; as a result, for each cognitive domain, a composite factor score based on verbal and nonverbal subtests can be calculated. Based on five nonverbal subtests and five verbal subtests, Nonverbal Intelligence Quotient (NVIQ) and Verbal Intelligence Quotient (VIQ) composite scores are calculated. The Full Scale Intelligence Quotient (FSIQ) score representing CHC general ability is derived from the administration of all 10 subtests.

For the Polish version of SB5, the following reliability coefficients were reported: split-half-reliability across age groups for the FSIQ ranged from 0.97 to 0.99, and for the NVIQ and VIQ from 0.93 to 0.98. Internal consistency reliability across age groups for the FSIQ ranged from 0.95 to 0.98, and for the NVIQ and VIQ, it ranged from 0.91 to 0.95 (Roid et al., 2017b). The standardised mean for the SB5 composite scores is 100, and the standard deviation is 15. The standardised mean for the SB5 subtests is 10 and the standard deviation is 3. Within the current analysis, SB5 composite scores (FSIQ, NVIQ, and VIQ, Fluid Reasoning, Knowledge, Quantitative Reasoning, Visual-Spatial Reasoning, and Working Memory), as well as subtest scores, were treated as outcomes.

2.3. Residential greenspace and bluespace exposure assessment

The lifelong addresses of the NeuroSmog participants were collected using a NeuroSmog-specific paper-and-pencil Address Questionnaire that was then digitalised, and validated. Google's Geocoding application programming interface (API) was used to geocode the addresses in the World Geodetic System 84 (WGS 84) coordinate system, as implemented in the R (R Core Team, 2021) ggmap package (function geocode()) (Kahle and Wickham, 2013).

The land cover data set for 2018 (Geoportal, 2022) from the Poland-wide database of topographic objects 10k (BDOT10k) was used to derive information on tree, grass/shrub, and water cover in square meters (Supplement 1, Fig. S2). First, the relevant vector layers were transformed into rasters with a resolution of 20 m × 20 m using the gdal_rasterise function of GDAL version 3.0.4. Second, the focal sum in ArcGIS Pro 2.5.1 (ESRI, Redlands, CA) was used to calculate the areas of each land cover category within a circular 500 m and 1 km buffer for each residential address starting at birth. The assignments were performed in Python version 3.9.6 using the tifffile package version 2022.5.4. Lifelong exposures to greenspace and bluespace were calculated by averaging exposures over all residential addresses, while weighing living duration at each residence. The areas of the land cover categories were then transformed into percentages.

Percentage tree and grass/shrub cover were used as objective greenspace measures, and percentage water cover was used as an objective bluespace measure. In addition, perceived greenspace and bluespace measures were used. Perceived greenspace/bluespace exposure was assessed by parents on a seven-point Likert scale (strongly agree [1] to strongly disagree [7]) with five questions covering the following aspects: (1) greenspace/bluespace usage in the neighbourhood, (2) visible greenspace/bluespace from window view, (3) quality of greenspace/bluespace, (4) greenspace/bluespace neighbourhood use, and (5) use of greenspace/bluespace outside of the neighbourhood. The exact questions can be found in Supplement A of Markevych et al. (2022). The mean score of the responses across the five items for each greenspace and bluespace variable was used as a proxy for the perceived greenspace/bluespace. The internal consistency of the scales was acceptable for both greenspace (Cronbach's alpha = 0.77) and bluespace scores (Cronbach's alpha = 0.70). Lifetime greenspace exposure was calculated as the sum of z-scores derived from percentage tree cover in the 500 m buffer, grass cover in the 500 m buffer, and perceived

Fig. 1. Study area location and current residential addresses of the study participants superimposed over the study area.

greenspace measure. Lifetime bluespace exposure was calculated as the sum of z-scores derived from percentage water cover in a 1 km buffer and perceived bluespace measure. These buffer sizes were selected based on previous research showing that children aged 10–12 years travel no further than 500 m to access greenspace and 1 km to access other common activity spaces (Hand et al., 2018; Loebach and Gilliland, 2014; Villanueva et al., 2012).

2.4. Covariates

We considered several covariates as potential confounders, mediators, and effect modifiers. A directed acyclic graph (DAG), as implemented in dagitty.net (Textor et al., 2016), was used to select a minimum sufficient set of confounders (Greenland et al., 1999) to avoid over-adjustment and to identify potential mediators. Sex, parental education, and urbanicity were identified as confounders, whereas PA was identified as a potential mediator (Supplement 1, Fig. S3).

Sex, minimum education of both parents (low vs. medium vs. high), and PA were reported by parents via a questionnaire. The three levels of parental education were defined as follows: "low" – primary school or/and vocational training, "medium" – high school or/and vocational training after high school, and "high" – bachelor's degree or higher. The level of urbanicity was estimated via proxy which was the percentage of sealed soil in 1000 m buffer around home; these data were derived from the Imperviousness Density for the year 2018 (European Environment Agency, 2020).

To estimate the duration of children's PA per week (None; <1 h; 1–2 h; 3–4 h; 5–6 h; >6 h), the parents were asked the following question: "Approximately how many hours per week does your child usually do strenuous PA outside of school that makes her or him get out of breath or sweat more than usual (e.g., playing team sports, dancing, swimming)?" This question was adopted from the RHINESSA study (Ekström et al., 2022; Lindberg et al., 2020).

As an additional confounder, we considered the presence of learning disabilities such as dyslexia, dyscalculia, and dysorthographia, as they may have an impact on intelligence assessment results. The diagnosis of learning disabilities was verified by clinical psychologists who conducted the psychological assessments.

2.5. Analytic strategy

2.5.1. Main analysis

Data pre-processing and statistical analysis were performed using the statistical software R, version 4.0.4 (R Core Team, 2018). Descriptive characteristics of the entire sample (i.e., children with and without ADHD) are presented as frequencies and percentages for categorical variables, and as means and standard deviations for continuous variables. The distributions of the exposure variables were tested using histograms. One outlier was identified by visual inspection of the scatter plots and was removed.

All regression analyses were conducted separately for the cases and controls. Linear regression was used to examine the associations among residential lifetime greenspace/bluespace exposure, and intelligence. Regressions were performed separately for each exposure-outcome pair. SB5 composite scores (FSIQ, NVIQ, VIQ, Fluid Reasoning, Knowledge, Quantitative Reasoning, Visual-Spatial Reasoning, and Working Memory) were used as outcomes. All models were adjusted for DAG-selected confounders including sex, minimum parental education, and urbanicity. The results are presented as b coefficients and their corresponding 95% confidence intervals (CIs). Residual diagnostics were performed using the DHARMa package (F. Hartig, 2022). Non-linearity of exposure-outcome relationships was checked using generalised additive models (GAMs; Hastie and Tibshirani, 1986).

2.5.2. Sensitivity analyses

To verify the impact of the confounding factors, we conducted unadjusted analyses. In addition, we restricted the main model to children without learning disabilities (cases, N = 204; controls, N = 500). To test whether there were associations between greenspace and bluespace exposure and SB5 performance at the subtest level, we used subtest scores separately from the nonverbal and verbal domains. To test whether there was an association between the single exposure measures and intelligence, we separately regressed each of the residential tree cover, grass/shrub, and water cover in 500 m and 1 km buffers and perceived greenspace and bluespace scores on each of the outcome variables. For this analysis, water cover was dichotomised into the presence or absence of water bodies in line with other analyses in landlocked areas (Dzhambov et al., 2018b) as the data were extremely right-skewed.

2.5.3. Testing effect modification

To check for the presence of effect modification, we stratified our main models by changing place of residence in "movers" (participants who changed their place of residence at least once) vs "non-movers" (participants who never changed their place of residence), minimum parental education in low vs. medium vs. high, and median urbanicity in rural vs. urban place of residence. We concluded an effect modification if 95% CIs of the strata-specific associations did not overlap, disregarding formal statistical significance.

2.5.4. Mediation analyses

To investigate whether PA is involved in the association between greenspace and intelligence, we used structural equation modelling (SEM). We used a robust diagonally weighted least squares (DWLS) estimator (Muthén, 1993) with bootstrapping (1000 draws) to estimate standard errors. SEM modelling was performed using the R lavaan v. 0.6–12 package (Rosseel, 2012). A p-value of <0.05 was selected as a threshold for statistical significance.

3. Results

The main characteristics of the participants are listed in Table 1. The majority of the case subsample were boys (74.27%). In the control subsample, the proportion of both sexes was almost equal (51.48% boys). The cases and controls were similar with respect to age, urbanicity, and presence of learning disabilities. In 75.33% of the cases and 84.81% of the controls, at least one parent had a medium or high level of education. There were also differences in the proportion of low parental education levels between the cases (24.27%) and controls (15.19%). There were no substantial differences in the proportion of medium parental education levels between the cases (40.29%) and controls (43%). The proportion of participants performing PA for more than 6 h per week was higher in cases (21.36%) than in controls (9.86%). Moreover, the proportion of participants who never performed PA was higher in the controls (12.23%) than the cases (4.37%). No substantial differences were observed in the remaining PA duration levels. As presented in Table S1 (Supplement 1), there were differences in SB5 composite scores with controls outperforming the cases. In general, participants with ADHD showed SB5 performance levels closer to the mean of the norming sample (i.e. FSIQ = 100) than did participants without ADHD. As shown in Table S2 (Supplement 1), there were also differences between the two subsamples with respect to exposure to greenspace and bluespace, such that controls were more exposed to greenspace than cases, whereas cases were more exposed to bluespace than controls.

In both cases and controls, no consistent associations were found between residential lifetime exposure to greenspace (Fig. 2) and bluespace (Fig. 3) and SB5 composite scores. Excluding participants with learning disabilities, stratification by changing place of residence (movers vs non-movers), urbanicity (rural vs urban), and parental education level (low vs medium vs high) did not reveal any consistent associations. In controls, several significant negative b coefficients in

Table 1
Descriptive characteristics of the study sample (N = 714) across the analytic samples of children with and without ADHD.

Variable: N (%) or mean ± SD (min. – max.)	Cases (with ADHD, N = 206)	Controls (without ADHD, N = 508)
Sex		
Female	53 (24.73)	246 (48.52)
Male	153 (74.27)	261 (51.48)
Age (years)	11.15 ± 0.9 (9.28–13.32)	11.32 ± 0.75 (9.32–13.23)
Minimum parental education		
Low	50 (24.27)	77 (15.19)
Medium	83 (40.29)	218 (43)
High	73 (35.4)	212 (41.81)
Dyslexia (n = 704)		
No	195 (95.59)	485 (96.6)
Yes	9 (4.41)	17 (3.4)
Dyscalculia (n = 704)		
No	202 (99.02)	483 (96.6)
Yes	2 (0.98)	2 (0.4)
Dysorthographia (n = 704)		
No	201 (98.53)	492 (98.4)
Yes	3 (1.47)	8 (1.6)
Changing place of residence		
No	84 (40.78)	254 (50.1)
Yes	122 (59.22)	253 (49.9)
Urbanicity* (continuous variable, %)	21.53 ± 13.07 (1–59)	19.57 ± 11.3 (1–56)
Urbanicity (categorical variable)		
Urban	102 (49.51)	247 (48.72)
Rural	104 (50.49)	260 (51.28)
Physical activity		
>6 h per week	44 (21.36)	50 (9.86)
5–6 h per week	20 (9.71)	58 (11.44)
3–4 h per week	49 (23.79)	122 (24.06)
1–2 h per week	53 (25.73)	123 (24.26)
<1 h per week	26 (12.62)	69 (13.61)
Never	9 (4.37)	62 (12.23)
Don't know	5 (2.43)	23 (4.54)

Note. SD = standard deviation; * based on percent sealed soil in 1000 m buffer around home.

regression models for bluespace, Quantitative Reasoning, and Visual-Spatial Processing indicated an association in an unexpected direction.

Further, no consistent associations were found when looking at greenspace, bluespace, and performance on SB5 individual subtests from the nonverbal and verbal domains (Supplement 1, Fig. S4). In addition, no associations were detected with SB5 composite scores and greenspace and bluespace single exposures, including those within a 1 km buffer around residence, and perceived greenspace and bluespace (Supplement 1, Fig. S5). All the numerical regression results are presented in Supplement 2.

The mediation analysis did not reveal any significant results for PA as a mediator in the pathway between lifelong exposure to greenspace and FSIQ, NVIQ, and VIQ for cases or controls (Supplement 1, Table S3). Furthermore, there were no direct effects of PA on IQ scores. Nevertheless, the association between greenspace and physical activity was significant.

4. Discussion

Considering the conflicting results derived from previous research, our goal was to analyse the association between nature exposure and intelligence using a state-of-the-art methodology. We conducted a comprehensive analysis of the associations between lifelong residential exposure to greenspace and bluespace and intelligence in schoolchildren. We used a sample composed of randomly selected healthy controls and children with ADHD as cases, whose diagnoses were verified by clinical psychologists. We used land cover data and lifetime addresses to estimate the lifelong exposure to greenspace and bluespace. In addition, perceived greenspace and bluespace exposure measures were applied. In the outcome assessment, we implemented SB5, a comprehensive intelligence test battery used worldwide that allows the assessment of both overall intelligence and domain-specific cognitive abilities. Our analysis showed that in both cases and controls, none of the greenspace and bluespace measures were consistently associated with intelligence, either on the overall performance level or on a domain-specific level. Considering confounders and modifiers did not change the results. PA was not found to be a significant mediator, either.

Comparing the results of our study with those of previous studies is possible only to a limited extent due to differences in the methodology and heterogeneity within previous research. Five previous studies reported a positive association in at least one outcome (Almeida et al., 2022; Asta et al., 2021; Bijnens et al., 2020; Binter et al., 2022; Lee et al., 2021). These studies applied various versions of the Wechsler intelligence test for outcome assessment, and one of them used the British Picture Vocabulary Scale in addition to the Wechsler test (Binter et al., 2022). For exposure assessment, two studies used normalised difference vegetation index (NDVI) (Asta et al., 2021; Lee et al., 2021), two studies implemented both NDVI and nature availability/distance (Almeida et al., 2022; Binter at el., 2022), and one study used land cover data (Bijnens et al., 2020). Four studies reported no association (Flouri et al., 2022; Jimenez et al., 2021; Julvez et al., 2021; Reuben et al., 2019). These studies used the British Ability Scales, Kaufman Brief Intelligence Test, Raven's Coloured Progressive Matrices test, and Wechsler intelligence tests for outcome assessment. For exposure assessment, three studies applied NDVI, whereas one study used land cover data (Flouri et al., 2022).

To the best of our knowledge, this study is the first to use SB5 in relation to nature exposure and intelligence. Although all previous studies have employed standardised intelligence tests, there might be differences in the aspects of intellectual functioning being assessed, as well as in the reliability and validity of these measures. Generally, the application of full versions of intelligence test batteries is recommended (Strauss et al., 2006). Studies that reported positive associations between nature exposure and intelligence used a full version of a Weschler intelligence test; however, in one study (Binter et al., 2022) different cognitive measures in four different cohorts were used and study conclusions were formulated based on the results derived from these different measurements, which is not in line with the standards of cognitive assessment (Lezak et al., 2012). Studies that reported negative or no associations used short versions of intelligence test batteries (Jimenez et al., 2021; Reuben et al., 2019), measures composed only of three subtests (Flouri et al., 2022) or assessing only one facet of intelligence (Julvez et al., 2021).

Furthermore, the heterogeneity of exposure assessment, including the quality of greenspace or bluespace metrics, should be considered. Our greenspace and bluespace measures were derived from land cover data. Additionally, we utilised parent-rated availability, quality, and frequency of greenspace and bluespace use; on the contrary, none of the previous studies implemented perceived exposure measures. Two previous studies used land cover data, five used only NDVI as a greenspace measure, and two used both NDVI and nature availability/distance to nature. However, these metrics do not provide information on the quality of green spaces. Moreover, information on the frequency and duration of visits to green spaces is essential (Dzhambov et al., 2018b) as it would help evaluate potential associations with intelligence.

Three of the five previous studies that reported positive associations between exposure to greenspace and intelligence in children had rather inconsistent results. In a study by Almeida et al. (2022), only residential availability of structured green spaces was positively associated with higher intelligence quotient (IQ) scores, whereas NVDI in different buffers around school was negatively associated with all IQ scores. In a

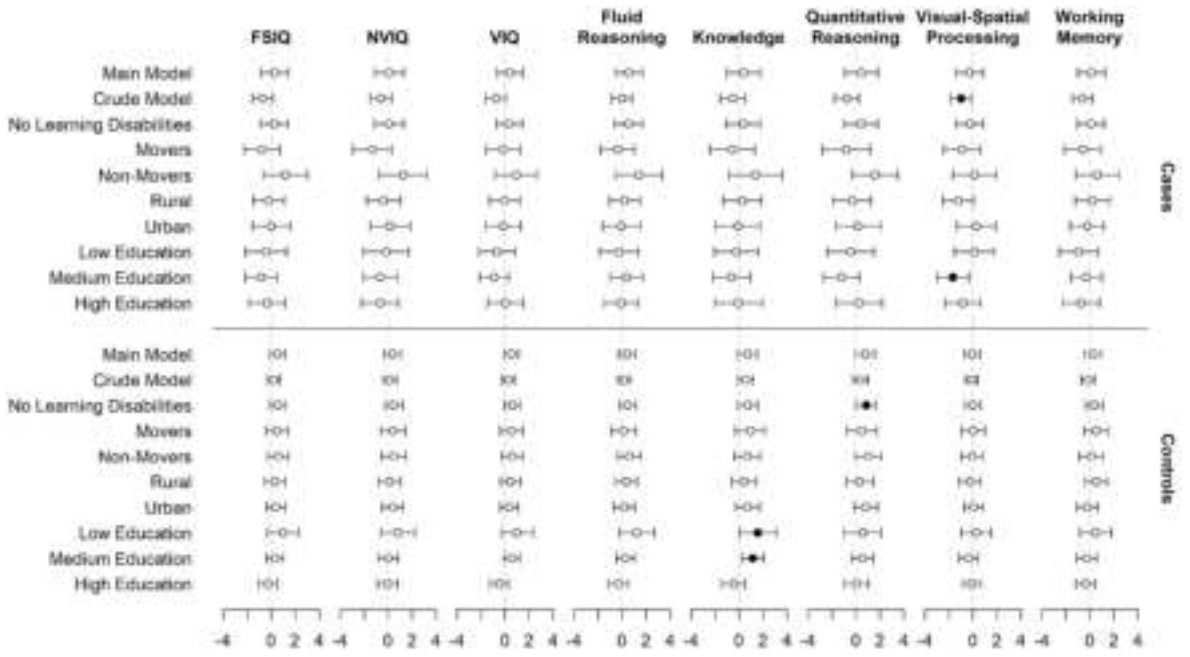

Fig. 2. B coefficients and their corresponding 95% confidence intervals for the associations between lifetime **greenspace** exposure and SB5 IQ and factor scores in cases and controls.
Note. FSIQ = Full Scale Intelligence Quotient, NVIQ = Nonverbal Intelligence Quotient, VIQ = Verbal Intelligence Quotient. Statistically significant results are indicated by filled circles.

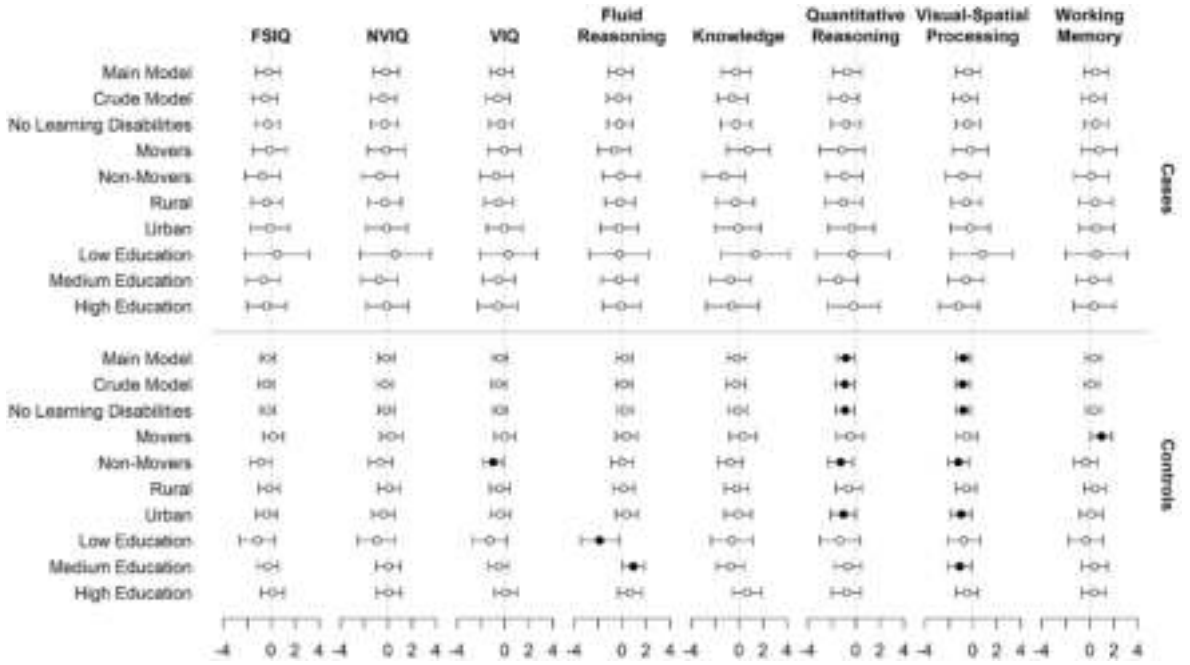

Fig. 3. B coefficients and their corresponding 95% confidence intervals for the associations between lifetime **bluespace** exposure and SB5 IQ and factor scores in cases and controls.
Note. FSIQ = Full Scale Intelligence Quotient, NVIQ = Nonverbal Intelligence Quotient, VIQ = Verbal Intelligence Quotient. Statistically significant results are indicated by filled circles.

study by Asta et al. (2021), NDVI was positively associated with only one subtest score for one buffer size, whereas no associations were detected between NDVI and the remaining test scores. In a study by Lee et al. (2021), the percentage of total, natural, and built prenatal and postnatal greenness was measured in four different buffer sizes around residences; however, the measured IQ scores were inconsistently associated with one, two, or three different buffer sizes of built and total greenness, but not natural greenness. In addition, in two studies, the associations were opposite to the expected direction. In the aforementioned study by Almeida et al. (2022), NDVI in two different buffer sizes around school was negatively associated with IQ scores. Furthermore, in a study by Julvez et al. (2021), a higher NDVI during pregnancy was associated with lower intelligence. In our study, bluespace exposure was negatively associated with two SB5 factors in controls; nevertheless, there seems to

be no plausible explanation for these results. Two previous studies that considered bluespace exposure (Almeida et al., 2022; Binter et al., 2022) reported no association with intelligence. Examining the association between cognition and bluespace exposure seems to be even more challenging than is the case with greenspace as bluespace assessment is less standardized than greenspace. Thus, future research should consider the specific characteristics of bluespace, such as bluespace types, duration of time spent around bluespace, and activities conducted in or around bluespace.

In the current study, we were also interested in whether greenspace would have an indirect effect on intelligence through increased PA. Similar to previous research (Almeida et al., 2022; Jimenez et al., 2021; Lee et al., 2021), the mediation analysis did not reveal any significant results for the three main IQ scores for either cases or controls. PA had no direct effect on IQ scores. These results are not surprising. Although previous research (Bidzan-Bluma and Lipowska, 2018; Hillman et al., 2019) has shown positive effects of PA on cognition, these findings mainly included attention, memory, language, and executive functions. Consequently, PA may be positively associated with cognition. However, IQ scores represent the highest level of cognitive performance and are likely to be affected by several lifelong factors. Thus, lifelong data for variables considered meaningful for the development of intelligence, including PA are required to answer this research question.

5. Limitations

Several limitations of this study should be considered when interpreting its results. Although we calculated lifelong objective greenspace and bluespace, the information was derived from the land cover data referring to a single year (2018), and thus, no land use changes over time were reflected. In addition, we did not have well-resoluted land use data, but only land cover data, thus, we could not differentiate greenspace by its functionality (i.e., natural, urban, agricultural).

Although we used lifelong nature exposure data, intelligence assessments were conducted at only one time point. Using a longitudinal study design with several consecutive outcome assessments at different time points to capture several stages of cognitive development would strengthen future studies. Our intelligence assessments were conducted by qualified clinical psychologists according to the standards of psychological assessment; however, as there were 25 individuals involved in the assessments, individual characteristics of the assessing psychologist might have contributed to the measurement error and decreased the objectivity of assessments.

Furthermore, we used both objective and perceived exposure measures, but unlike objective measures of greenspace and bluespace, our perceived exposures were cross-sectional and parent-reported, and the same concerns PA. In addition, we did not consider specific characteristics of nature exposure, such as the type of greenspace/bluespace or the type of activities conducted therein.

6. Conclusions

We could not find evidence that higher lifelong exposure to greenspace and bluespace is associated with higher intelligence in Polish schoolchildren with or without ADHD. PA did not mediate the association between greenspace and intelligence. The current results raise doubts on whether exposure to nature has relevant influence on IQ.

Declaration of interest statement

Declarations of interest: none.

Authors' contributions

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Availability of data and materials

Data were obtained from the NeuroSmog study, which is bound to the local ethical and legal restrictions with respect to the study data. The informed consent provided by the NeuroSmog study participants did not include data posted in public databases. However, all data used for this publication are available upon request. Contact persons are Dr Dorota Buczyłowska (dorota.buczylowska@uj.edu.pl), and Dr Iana Markevych (iana.markevych@uj.edu.pl).

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Appendix A. Supplementary data

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Exposure assessment for repeated sub-concussive head impacts in soccer: The HEalth and Ageing Data IN the Game of football (HEADING) study

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ABSTRACT

The purpose of this paper was to develop exposure estimates for repetitive sub-concussive head impacts (RSHI) for use in epidemiological analyses. We used a questionnaire to collect lifetime history of heading and other head contacts associated with training and playing football from 159 former footballers all members of the English professional football association. We used linear mixed effect regression with player as the random effect, to model the number of headers, blows to the head and head-to-head impacts as a function of potential exposure affecting factors, which were treated as the fixed effects. Exposure affecting factors included playing position, league, context of play (game vs training) and decade of play. Age at time of response to the questionnaire was also included in the models. In model results, playing position was important, with RSHIs being highest among defenders and lowest among goalkeepers. Players headed the ball more during games than in training, and when playing in amateur or youth leagues compared with semi-professional or professional leagues. The average number of reported head impacts declined linearly throughout the observation period (1949–2015). The derived final model for headers explained 43%, 9% and 36% of the between player, within player and total variance in exposure, respectively with good precision and predictive performance. These findings are generally in agreement with previously published results pointing towards the models forming a valid method for estimating exposure to RSHI among former footballers although some further external validation is still warranted.

1. Introduction

Head impacts such as those from heading in association football (soccer) are often referred to as repetitive sub-concussive head impacts (RSHIs) (Gysland et al., 2012). In contrast to concussion, RSHIs do not generally cause acute neurological symptoms, and therefore their clinical significance is more uncertain. The short and medium-term effects of RSHIs on human neurological health have been investigated (Bahrami et al., 2016), although they remain relatively ill-defined, and the long-term consequences of RSHIs are largely unknown (Pearce, 2016). Nonetheless, it has been suggested that RSHIs may result in persistent cognitive impairments and behavioural changes (McKee and Robinson, 2014) and recently published register-based studies suggested increased risks among former football players compared to the general population (Mackay et al., 2019; Ueda et al., 2023). A recent systematic review of sub-concussive injuries in sport concluded that there was insufficient to weak evidence of an association between RSHIs and neurological health (Mainwaring et al., 2018). To what extent the dynamics, the cumulative effect, and the location of the RSHIs have a role in modulating long term neurological impairment, is unclear from these data. The review also noted that studies that measured impact exposures used various indices, including linear acceleration, rotational acceleration, along with location and frequency of hits, with little consistency between studies.

The appropriateness of exposure indices for assessing the long-term

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risk from RSHIs in soccer players is uncertain. To further examine this, another recent systematic review focused on identifying those factors that determine the magnitude of head impact acceleration (Basinas et al., 2022). Exposure data from 27 observational and 33 experimental studies involving soccer play were included in standard statistical analysis approaches including student t-tests, correlation and simple linear regression analysis. Overall, the data from experimental studies appeared to be a poor proxy for normal play: in observational studies there was a close association between peak linear and angular accelerations, whereas in experimental studies these measures were less well correlated. The method of attaching the measurement accelerometer to the head was also seen to affect the magnitude of the measured acceleration. The linear acceleration experienced by female players and adults was on average higher than for male players and young people, respectively. Available evidence also suggested that the range of head acceleration during soccer playing is generally quite small and does not vary much between playing positions. In line with these findings, the authors suggested that for epidemiological studies of former soccer players the assessment of historical exposures to RSHI should be based on exposure metrics that rely on estimates of the cumulative number of RSHI over a playing career.

The HEalth and Ageing Data IN the Game of football (HEADING) Study explores the long-term cognitive function of around 200 former male professional footballers, aged 50 or over and who were members of the Professional Footballers' Association. In the present analysis, we used questionnaire data collected from 159 participants of the HEADING study to derive empirical exposure models capable of predicting historical cumulative exposures to RSHI. The elaborated models and predicted estimates from them will be used to estimate cumulative exposure to RHSI within the HEADING study.

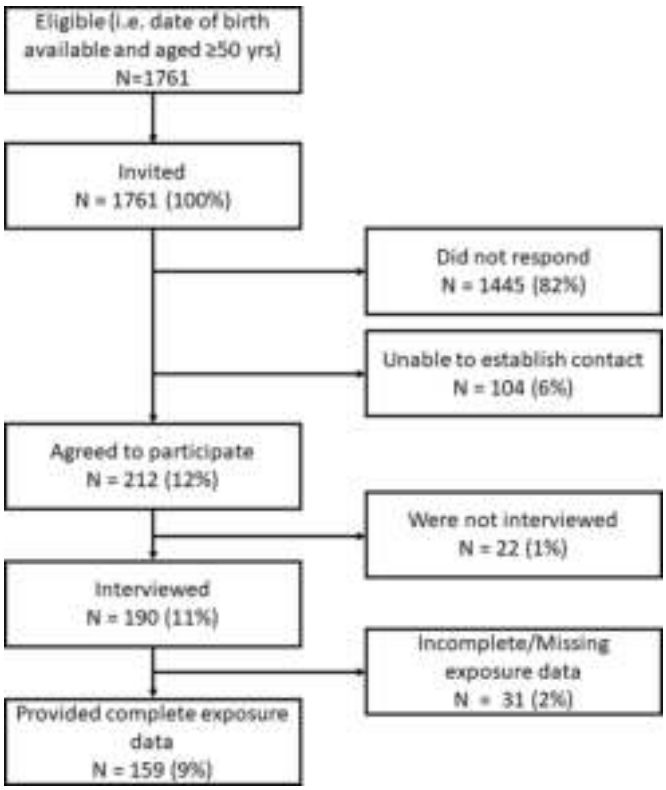

Fig. 1. Flow chart of the enrolment and data collection process.

2. Materials and methods

The HEADING study was designed to investigate the associations between concussion and/or RSHIs from heading the ball in soccer and cognitive function among former male professional soccer players in England. It is a cross-sectional study with a protocol for data collection similar to the BRAIN study of former professional English rugby players (Gallo et al., 2017), but data collection was simplified due to the restrictive measures put in place during the first years of the COVID-19 pandemic, with part of the assessment conducted online only (Seghezzo et al., 2021). A schematic representation of the enrolment and information collection process is provided in Fig. 1. Briefly, recruitment took place between July 2019 and December 2021. Current and former members of the Professional Footballers' Association (PFA – see https://www.thepfa.com/[thepfa.com]) who were known to be aged over 50 were invited by the PFA (for confidentiality reasons) to contact the study team if they were interested in taking part in the study. Some were invited by post and email (n = 1569), and the remainder by email only (n = 192). Of the 1761 invited to participate, 212 agreed to participate, and 190 of these were interviewed. Of the 190 interviewed, 159 provided complete exposure data. Data collection involved a personal interview using a standardised questionnaire and a health examination. At the end of the interview, which covered health, lifestyle and occupational characteristics, participants were asked to provide detailed information about their history of heading, head trauma and concussion while playing and training before, during and after their professional career. The telephone version of the BRAIN-Q test (BRAIN-Qt) already validated in the BRAIN study (Gallo et al., 2022; James et al., 2021), was used to assess exposure to concussion.

2.1. Questionnaire administration

For those participants recruited early in the data collection, the interviews and associated questionnaires were administered in person. However, due to COVID-19 restrictions, the administration of the questionnaires was switched to video communication software such as Zoom or Facetime. This was considered acceptable for the for collecting information on playing and concussion history, but there was some concern over the assessment of cognition using the Pre-Alzheimer Cognitive Composite (PACC) (Donohue et al., 2014). The reliability of the latter assessments was formally assessed in a sub-study, which found the online assessment was just as good as the in-person assessment provided the person facilitating the data collection had been suitably trained (Seghezzo et al., 2021).

2.2. Playing history data

During its development, the exposure assessment section of the HEADING questionnaire (see Online supplement for details) was piloted amongst groups of professional players. This was initially carried out using the coaching staff at Sheffield United football club and subsequently with the study Player Advisory Committee (PAC), which was formed from senior members of the PFA and Trustees of the PFA, as well as nominees from the Football Association. Members of the PAC provided feedback on the questionnaire until it was finalised. For both sessions, members of the HEADING study team administered the questionnaires.

Information on playing careers were collected separately for school, youth, professional/semi-professional, and amateur playing periods. A playing period was defined as a separate playing contract and participants were asked to fill a separate entry for each team for which they played. Players were asked to fill out a timeline of playing history including details of their experience at each club. Participants were also asked to provide information about play, including estimates of the typical number of games and training sessions they participated in each week, both in and out of each playing season. Estimates of the typical number of headers, blows to the head and (separately) head-to-head impacts they experienced during each playing session (i.e. game, training) were also provided. A playing season was assumed to be 38

weeks in length. A record of any prolonged absence from play along with the underlying reason and duration of absence was also provided.

2.3. Data curation and handling

The data on football career, including heading and other head impact information were collected initially in person, and then by Zoom with the onset of the pandemic. Prior to statistical analysis a thorough quality control of the responses of each participant was undertaken. Playing periods ranged in duration from less than a year to 24 yrs. Playing periods exceeding 10 years (n = 25) in duration were split into the decades they were associated with (to facilitate examining the importance of playing decade in any modelling). Decade of play was defined as the decade a playing period started. For any RSHI values reported as ranges the corresponding mid-point was used. In addition, league information was converted to numerical indicator to better define the league levels of play into eight categories (1 = Premier League; 2 = Championship; 3 = League One; 4 = League 2; 5 = Semi-professional; 6 = Amateur; 7 = Reserves/Youth and 8 = Overseas). Historical equivalent leagues were recognised where the name of the division had changed. Where league information was missing or unclear, contextual data (i.e. team played and calendar years) from the playing period was used to determine the league level. Playing level was also categorised into four groups: apprentice; amateur; semi-professional and professional. Where multiple levels were specified for a single period the highest level of play was used i.e. apprentice combined with professional was regarded as professional. In cases where playing level was omitted, historical information on leagues was used to fill any data gaps (see: http://www.englishfootballleaguetables.co.uk/). All playing periods with missing data on playing position (n = 34) or missing information on the frequency of RSHI during play (n = 174) were removed from the final dataset.

To allow the fit and performance of the elaborated models to be evaluated, prior to statistical analysis the dataset was randomised into two sub-sets of size equivalent to 70% and 30% of the total playing periods, respectively. The larger sub-set of the data was used to determine the predictive models, hereafter called the model training dataset, and the smaller sub-set was used to evaluate the model performance, hereafter called the model test dataset. Randomisation was made using SAS software SURVEYSELECT procedure (SAS Institute, 2020).

2.4. Data analysis

The number of headers, blows and head-to-head impacts, as reported on the questionnaire responses, followed a lognormal distribution and so prior to the initiation of the main analysis all data were log-transformed. Geometric means (GM) and geometric standard deviations (GSD) were used to summarise the exposure data presented alongside the relevant arithmetic means (AM).

Linear mixed effect regression (Peretz et al., 2002) was used to model the number of headers and other head impacts as a function of potential exposure affecting factors including decade of play (categorical), playing position (categorical), level of play (categorical), league (categorical) and context of event (games vs training). To account for possible differences in ability to recall, the age of the participant at the time of responding to the questionnaire, was included as a continuous variable in the model. Prior to the statistical modelling, all observations (n = 17, n = 13, and n = 24 for headers, blows and head-to-head impacts, respectively) with a value exceeding the mean of the distribution plus three standard deviations (i.e. 53.9, 11.2 and 7.4 for headers, blows and head-to-head impacts, respectively) were replaced by this value. Models were determined with player identifier as the random effect and potential exposure affecting factors as the fixed effect. Association between fixed effects and RSHI impacts were first examined at a univariable level. Amid small numbers and very similar effects and to preserve degrees of freedom for the multivariate analysis, utility players (i.e. those frequently playing in more than one position) were merged with forwards and league 2 players with league 1 players. Multivariable model build followed a forward selection approach where variables entered the model based on level of significance and improvement of fit as assessed by the Akaike and Bayesian information criteria. Model adequacy was assessed through influence diagnostic and residual plots. In all models, estimation of variance components was based on the restricted maximum likelihood method. The robustness of the derived model coefficients was examined in a series of sensitivity analyses. These included excluding all observations with reported typical number of headers exceeding 29 (the 95th percentile of the distribution of reported headers), removing all observations sourcing from goalkeepers, and excluding all observation sourcing from subjects aged above 74.5 years (the 90th percentile of the age distribution of all participants) or above 61.27 years of age (the median of the age distribution of all participants) to examine for the presence of effects of recall. Pearson and, when appropriate, Spearman correlation coefficients were also used to describe associations between the observed values of the different types of head impacts and potential exposure affecting factors.

The derived model equations were used to predict geometric mean numbers of RSHIs per event by position, playing level and decade of play. Separate estimates for games and training sessions were determined. All model predictions were made assuming the participant's age was equal to the median of all study participants included in the dataset (i.e. 61.27 years).

To evaluate its overall fit, precision and predictive performance, the final model was re-fitted on the test dataset and the results, including predictions, were compared to those from the model training dataset. Comparisons involved estimating the Proportion Change in Variance (PCV) overall, between-players and within players, and after model predictions the Root Mean Square Error (RMSE) and the Mean Absolute Error (MAE). The MAE was calculated as the mean absolute difference between the predicted and absolute values whereas the RMSE was estimated as the square root of average of the square difference between the predicted values and the actual values. The MAE is a measure of the size of the residual in a dataset whereas the RMSE is an indicator of the average distance of observed data points from the fitted model line.

All data were analysed using the SAS statistical software version 9.4 (SAS Institute, 2020).

3. Results

The basic characteristics of the model training and test datasets are shown in Table 1. Overall, 1888 observations representing different periods of play across the 159 participants were available, of which, following randomisation, 1322 were allocated to the model build/training dataset and 566 to the evaluation/test dataset. Slightly more players were present in the model dataset albeit with no further systematic differences between the two datasets across all the exposure affecting factors and/or outcome head impact numbers included. Of all 1888 observations included, 43% were sourced from defenders with the other half almost equally distributed among forwards (25%) and midfielders (28%). The vast majority (83%) of career playing by the study participants occurred between 1970 and 2000 and were mostly at a professional level (67%).

Table 2 summarizes the distribution characteristics of the number of different types of RSHIs across relevant exposure affecting factors. The geometric mean number of reported head impacts was highest among defenders (GM = 13.7; GSD = 2.7) and lowest among goalkeepers (GM = 0.36; GSD = 13.7). Goalkeepers also reported the fewest head-to-head collisions compared to players in other positions. For blows to the head, the fewest occurred among midfielders (Table 2). Both blows to the head and head-to-head collisions were most frequently reported among forward/utility players (AMs of 2.78 and 1.26, respectively). Players reported, on average, that they headed the ball more during games than during training sessions (GM of headers per session: 10.95 vs 6.88, respectively) and when playing in semi-professional or professional

Table 1
Basic characteristics of the model training and test datasets (n = 1888). Data were based on self-reported information from 159 male former professional English football players.

Characteristic	Model training dataset	Model test dataset
n	1322	566
k	157	148
Age of players, mean (SD)	62.6 (8.8)	62.2 (8.0)
Event		
Match	675 (51.1)	293 (51.8)
Training	647 (48.9)	273 (48.2)
Position		
Defender	554 (41.9)	257 (45.4)
Forward	340 (25.7)	139 (24.6)
Goalkeeper	31 (2.4)	13 (2.3)
Midfield	374 (28.3)	149 (26.3)
Utility	23 (1.7)	8 (1.4)
Decade of play		
≤1950	19 (1.4)	5 (0.9)
1960	113 (8.6)	37 (6.5)
1970	296 (22.4)	135 (23.9)
1980	463 (35.0)	204 (36.0)
1990	337 (25.5)	137 (24.2)
≥2000	94 (7.1)	48 (8.5)
League		
Reserves/Youth	61 (4.6)	27 (4.8)
Amateur	93 (7.0)	46 (8.1)
Semi-professional (Leagues < league 2)	280 (21.2)	123 (21.7)
League one or two	367 (27.8)	181 (32.0)
Championship	270 (20.4)	97 (17.1))
Overseas leagues	34 (2.6)	14 (2.5)
Premier league	217 (16.4)	78 (13.8)
# of headers per game, AM (SD)	14.2 (13.3)	13.5 (11.1)
	0–100	0–100
# of blows per game, AM (SD)	1.3 (3.3)	1.2 (2.6)
	0–30	0–30
# of head-to-head contacts per game, AM (SD)	1.1 (2.1)	1.2 (2.2)
	0–15	0–15

n = number of playing periods; k = number of players.

leagues (range of GMs: 8.34–9.53 headers per session) compared to amateur and youth leagues (range of GMs: 7.21–7.28 headers per session). Correlations between the different types of RSHI ranged from low ($r_{spearman}$ of 0.18 between headers and blows) to moderate ($r_{spearman}$ of 0.68 between blows and head-to-head impacts).

Table 3 presents the final multivariable model on the associations between position, league, decade of play, age and playing context and the number of reported headers per training or play event. The corresponding models for blows to the head and head-to-head impacts are presented in the online supplement (Table S1). Overall, the model for headers explained more than 42% of the variation in reported headers between players and 36% of the total variation in reported number of headers. Forward/utility and midfield players reported heading the ball less per session than defenders by a factor 0.83 and 0.67 (Table 3, reported GMR values), respectively, which was statistically significant for the latter. Playing in the English Premier League was associated with statistically significantly greater number of headers compared to semi-professional, amateur, and youth leagues. The number of headers appeared to significantly decrease with an increased decade of play whereas training was associated with a reduced number of headers by a factor 0.66 (Table 3, GMR values). An increased participant age at reporting was associated with a decreased number of reported headers.

The models for blows to the head and head-to-head impacts both explained approximately 13% of the total variability in exposure to head impacts. League and position remained important determinants of the reported frequency of these head impacts albeit with differences in the reported patterns compared to headers. Similarly, the number of reported impacts for blows and head-to-head impacts appeared to increase with an increased decade of play although this trend was not statistically significant.

Sensitivity analysis by excluding all observations with reported number of headers ≥29 (the 95th percentile of the distribution of reported headers) or those from goalkeepers showed no change in direction of effects and very little change in the effect sizes of the model for headers (data not shown). Excluding all observations from participants aged 74.5 and above and 61.27 and above did also not produce systematic differences in the main model results, although in the latter case no effect of participant age at responding was observed (data also not shown).

The results of the comparison of the fit and predictive ability of the model when fitted with the training and test data respectively are shown in Table 4. In general, model performance was relatively similar, with the training dataset explaining slightly more variability in exposure. Small differences were also seen when looking at the estimated RMSE and MAE values, which were generally low, indicating the presence of small model errors in both datasets.

The predictions from the final models for all types of RSHI, across all relevant strata (i.e. position, league, decade, and context) for estimating exposure within the HEADING study may be obtained by request to the authors.

Table 2
Distribution of reported per session average number of headers, blows to the head, and head-to-head collisions by position, league and context of play. Results of the model training dataset are shown that are based on self-reported data from 157 former professional English players.

Category	n	k	# of headers			# of blows to the head			# of head-to-head collisions		
			AM	GM (GSD)	Range	AM	GM (GSD)	Range	AM	GM (GSD)	Range
Position											
Forward/utility	363	50	13.30	9.92 (2.5)	0–100	2.78	0.43 (16.4)	0–30	1.26	0.23 (14.8)	0–9
Goalkeeper	31	6	2.06	0.36 (13.7)	0–10	0.81	0.20 (7.0)	0–4	0.10	0.01 (14.5)	0–0.3
Midfield	374	62	9.02	5.17 (5.1)	0–40	0.60	0.07 (13.3)	0–6	0.90	0.06 (15.8)	0–10
Defender	554	85	18.80	13.65 (2.7)	0–100	0.82	0.18 (9.2)	0–15	1.05	0.17 (11.5)	0–15
League											
Reserves/Youth	61	30	11.70	7.21 (4.3)	0–40	1.02	0.21 (11.9)	0–6	0.58	0.09 (16.5)	0–3
Amateur	93	30	13.25	7.28 (5.9)	0–40	0.48	0.06 (14.3)	0–3	0.50	0.07 (10.9)	0–10
Semi-professional (Leagues < league 2)	280	80	13.32	8.94 (3.8)	0–40	1.09	0.18 (10.3)	0–12	1.01	0.11 (15.6)	0–10
League one or two	367	107	15.53	9.53 (3.6)	0–100	1.29	0.17 (14.3)	0–30	1.14	0.14 (15.9)	0–15
Championship	270	84	13.95	8.74 (3.6)	0–100	1.48	0.23 (12.4)	0–30	1.36	0.19 (13.4)	0–15
Overseas leagues	34	19	16.29	8.38 (3.3)	0–100	2.24	0.22 (24.9)	0–20	1.3	0.17 (25.0)	0–10
Premier league	217	74	13.96	8.34 (4.9)	0–100	1.74	0.21 (13.4)	0–30	0.94	0.14 (12.4)	0–15
Context session											
Match	675	154	15.7	10.95 (2.7)	0–100	1.32	0.18 (13.1)	0.-30	1.06	0.13 (14.7)	0–15
Training	647	148	12.8	6.88 (2.7)	0–100	0	–	0	0	–	0

N = number of observation periods; K = number of players, AM = Arithmetic mean, GM = Geometric mean, GSD = Geometric Standard deviation.

Table 3
Effects of playing characteristics on the average number of reported headers per training or play event.

Parameter	n	*β*	*e*	*GMR*	*p*
Intercept		2.73	0.12	15.38	<.0001
Position					
Forward/utility	363	−0.18	0.13	0.83	0.15
Goalkeeper	31	−3.93	0.38	0.02	<.0001
Midfield	374	−0.40	0.12	0.67	<0.001
Defender	554	Ref			
League					
Reserves/Youth	61	−0.25	0.15	0.78	0.10
Amateur	93	−0.46	0.14	0.63	0.001
Semi-professional (Leagues < league 2)	280	−0.22	0.10	0.81	0.03
League one or two	367	−0.04	0.09	0.96	0.66
Championship	270	0.05	0.10	1.05	0.62
Overseas leagues	34	−0.28	0.18	0.76	0.13
Premier league	217	Ref			
Decade of play (starting)					
≤1950	19	0.27	0.25	1.31	0.29
1960	113	0.30	0.13	1.35	0.02
1970	296	0.10	0.08	1.10	0.20
1990	337	−0.09	0.07	0.92	0.23
≥2000	94	−0.29	0.13	0.75	0.02
1980	463	Ref			
Context					
Training	647	−0.41	0.05	0.66	<.0001
Match	675	Ref			
Age (continuous)		−0.04	0.01	0.96	<.0001
$_{bp}\sigma^2$(naive estimate)		0.67 (1.24)	0.09		
$_{wp}\sigma^2$(naive estimate)		0.76 (0.82)	0.03		
Explained variability					
PCV_{bp}		42.6%			
PCV_{wp}		9.3%			
PCV_{total}		36.0%			

n = number of observations with specific characteristic; β = regression coefficient for log-transformed head impact (header) data; e = standard error; p = p-value; $bp\sigma^2$ = between-player variance; $wp\sigma^2$ = within-player variance; PCV_{wp} = Proportion change in variance within players; PCV_{total} = Proportion change in total variance; GMR = Geometric Mean Ratio. Naïve estimates are derived by a model with only the random effects included.

4. Discussion

We have developed empirical exposure models to predict historical exposures to RSHIs among former male soccer players in England. Our approach is based on our earlier analysis, which indicated that the magnitude of the acceleration experienced when heading a ball was similar regardless of other factors such as playing position (Basinas et al., 2022). Consequently, we tailored our approach to enable estimates of the cumulative number of head impacts for study participants of epidemiological studies. The final models include playing position, league, decade played, context (play versus training) and age, and separately predicts number of RSHIs in each playing or training event from headers, blows to the head and head-to-head contacts. These data can then be combined with data on the players' careers to estimate lifetime cumulative exposures, although we recommend keeping the cumulative number of the three types of RSHIs separate because the magnitude of acceleration in each case is likely different.

The models show that goalkeepers infrequently head the ball during play, and midfield players head the ball less frequently than other outfield players. Defenders were found to head the ball most frequently compared to all other player categories. Recently published observational data collected by the English Football Association (FA) corroborated these findings (FA, 2021). In these data, collected between the period 2013–2021, defenders were observed to head the ball almost twice as much as other players (average 7.5 vs 3.6 to 4.5 per game). Similar findings were also reported among semi-professional French players where average headers per hour were found to be highest among center backs followed by forwards (Cassoudesalle et al., 2020). A study of US college age players that included males in their study population also reported defenders experienced somewhat higher numbers of head impacts compared to players in other positions (Reynolds et al., 2017a). However, in this study any type of head impact, including impacts other than headers, were accounted for in this comparisons. Predicted estimates from our models for Premier league players for the period after 2000 correspond to 7.7, 9.6 and 11.5 headers per game for midfield, forward/utility and defenders, respectively. These results are higher than the corresponding values of 3.7, 4.9 and 7.0 reported for the period 2013–2021 in the FA report (FA, 2021). On the other hand, for the same period our championships predictions (5.4, 6.6, and 8.0 headers per game for midfield, forward/utility and defenders, respectively) seem more comparable to those reported by the FA (4.4, 6.2, 9.3 headers per game for midfield, forward/utility and defenders, respectively). Our sample included only one player that had a career extending beyond 2010 and so any implementation of our models for periods not covered by the data should be made with caution.

The league of play, decade of play and context were, in our study, important exposure predictors for the frequency of headers performed. Previous results from US observational studies suggested somewhat conflicting results in relation to the difference in frequency of head impacts between practice and gaming sessions (Press and Rowson, 2017; Reed et al., 2002; Reynolds et al., 2017a, 2017b; Rich et al., 2019). However, it is important to note that most of those studies collected information on head impacts for the purpose of measuring impact acceleration, where typically all impacts above a certain threshold of acceleration are registered. In addition, several of those studies involved measurements exclusively among female players (Press and Rowson, 2017; Rich et al., 2019), and all involved young non-professional players of college or youth age. Sex is an important difference for RSHI impact and existing data also suggest important differences in head impact patterns between youth and older aged players (Basinas et al., 2022). League and decade of play have seen little study before. In our data set, there were fewer predicted headers for players in the amateur game, compared to fully professional players, with semi-professional and reserve/youth players predicted to have the number of headers between these two. There were also fewer headers predicted for play during later decades of play compared to the 1950s and 60s. In its recent training guidance the English Football Association (FA) suggested that the average number of headers performed per game is higher in leagues lower than the premier league with the highest number observed in players in league 2 (range of 5.1–10.1 vs 3.7–7.0 headers per game in the premier league) (FA, 2021). Our models suggest little differences between Premier league, championship and leagues 1 and 2. However, our grouping of leagues differs slightly from the one used in the FA report. In addition, playing style of English football has evolved through the

Table 4
Comparison of model results using the model training and test datasets.

Dataset	n	AIC*	BIC*	PCV_{bp}	PCV_{wp}	PCV_{total}	RSME	MAE
Training	1322	3739.7 (3832.2)	3745.8 (3838.3)	42.6	9.3	36.0	1.20	0.78
Test	566	1554 (1664.1)	1560 (1670.1)	46.9	9.4	40.7	1.02	0.73

n = number of observations; AIC = Akaike information criterion; BIC = Bayesian information criterion; PCV_{bp} = Proportion change in variance between players; PCV_{wp} = Proportion change in variance within players; PCV_{total} = Proportion change in total variance; RMSE = Root Mean Square Error; MAE = Mean absolute error.

decades (Bush et al., 2015), which could potentially explain differences observed.

Overall, estimated variability in the frequency of head impacts is larger between than within players – irrespective of the type of impact involved (see naïve estimates in Table 3 and Table S1). Differences in exposure to RHSI, as determined by frequency, arise primarily from factors that are different between players but consistent over time. Such factors include playing position and league, for which generally remain stable for players for longer periods of time. The fact that our model explains almost half of the between players variance in header frequency implies that the included fixed factors are good predictors of those differences. On the contrary, changes within the career of individual players (e.g. in playing league) appear to occur less frequently and to have negligible effect on the variability in exposure. The total variance explained by our final model for headers was approximately 36% whereas the corresponding value for blows to the head and head-to-head collisions were approximately 13%. These performances are in line with what we see from similar exercises performed for chemical and physical exposures where proportions of explained variance typically range between 10 and 40% (Basinas et al., 2014; Peters et al., 2011; Stokholm et al., 2020). The lower variance explained for blows to the head and head-to-head impacts suggest that potential important exposure affecting factors for these RSHIs may remain unaccounted for by our final models. Such impacts according to our data occur less frequently compared to headers accounting, according to our data, for less than 10% of the total impacts received by a player during a typical session. Previous experimental results suggested that players experience higher peak linear acceleration (PLA) values during head-to-head collisions compared to ball-to-head impacts (i.e. 300 m/s^2 at 2.5 m/s impact speed and around 700 m/s^2 at 3.5 m/s impact speed for head-to-head collisions vs ~150 m/s^2 at 8 m/s impact speed for ball-to-head impacts, respectively) (Hanlon and Bir, 2010) suggesting that these differences need to be accounted for when calculating the cumulative exposure across a player's career. In observational studies, however, the above patterns are far from clear. Amongst collegiate female players median PLA from head-to-head contacts were reported to be 350 m/s^2 compared to around 200 m/s^2 during headers from passes (Lamond et al., 2018), but other studies, primarily among youth players, suggest PLA associated with non-header-to-ball impacts to be much lower compared to headers events (Nevins et al., 2017; Sandmo et al., 2019; Saunders et al., 2020). At present observational data comparing the acceleration of blows to the head and/or head-to-head impacts with the acceleration of head to ball impacts during actual adult professional play are yet to become available (Basinas et al., 2022). Such information are essential for allowing relative differences in PLA between different types of impacts to be accounted for when calculating the cumulative burden of head impacts across the career of former professional players.

The overall participation rate into our study was 11% which raises a prospect for selection bias by excluding participants with severe cognitive impairment. In addition, the playing histories and reported RSHI frequencies are subject to potential bias originating from the ability of the participants to recall the past. Such information bias can lead to misclassification effects potentially affecting the estimated exposure-response relationships in epidemiological analysis. In principle, imperfect recall that is not subject to disease status (i.e. non-differential misclassification) will bias risk estimates towards the null. Recall that is differential to the status of disease however, can bias risk estimates either towards or away from the null (Pearce et al., 2007). To tackle the potential presence of recall issues we have taken several measures, both at a design (i.e. exposure assessment completed at the end of the interview to keep participants blind to concussion status and level of exposure to RSHI), and modelling/statistical analysis stage including removing outliers when determining prediction models, and adding age at time of data collection as a parameter in the developed models. The effects of the main model remained directionally robust when all data from subjects aged ≥74.5 years and ≥61.27 years were excluded from the modeling process which suggests limited effects in terms of cognition. In our study, it is unlikely that any selection or recall has been differential in nature and thereby any such bias, if present will likely result in attenuating the relationships between the exposure and the outcomes of interest towards the null. By modelling exposure based on similar exposure groups defined by key exposure affecting factors we allow exposure to be assigned at a group level, limiting thereby the presence of bias on the associations between exposure and health outcomes due to the introduction of the so called "Berkson error" (Armstrong, 1998). Nevertheless, the presence of selection and/or recall bias cannot be excluded and in the future, we aim to further validate the predicted exposure estimates and the participants recall by a) using the statistics of historical playing careers to evaluate the participants ability to recall their career and b) by performing direct validation exercises on the accuracy of reported and modeled head impact number estimates using video recordings of historical games. The intent will be to calibrate model results based on those findings.

The developed models can be used to retrospectively assign RSHI exposure estimates to the participants of HEADING and similar studies on the basis of their career characteristics in the prospect of estimating their cumulative whole life burden to RSHI. Although, the models were developed for, and using information from, former male players in England, we see no reason why they should not be used in epidemiological studies of soccer male players in countries other than the United Kingdom. However, given the differences in playing style between England and European or other continent's teams we would not recommend using the models in these situations without first undertaking some additional validation checks. In addition, the frequency and intensity (i.e. Peak Linear Acceleration) of head impacts have been reported to be systematically different between genders (Basinas et al., 2022). In general, males are reported to experience higher frequency of headers per hour than females whereas acceleration from heading is reported to be much higher among females as a result of a lower muscle neck strength. As a result we do not recommend use of our models on studies of female population without a prior extensive validation check against data from such populations.

5. Conclusions

We have developed empirical exposure models that enable us to predict exposure to RSHI on the basis of the context, position, decade, and league involved. The derived effects and impact estimates across positions are in agreement and of the same magnitude of recently published data concerning heading statistics among English football leagues. Exposure estimations therefore using the developed models a valid measure for such exposures, although further work is required on some aspects of their external validity. The models and related predictions will be used to estimate the cumulative lifetime exposure to heading and other head impacts from soccer within the HEADING study, a cross-sectional epidemiological study among former professional English footballers. The authors are happy to make the models and exposure estimates available to other *bone fide* researchers upon request.

Conflicts of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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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.2023.114235.

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Exposure to multiple air pollutant mixtures and the subtypes of hypertensive disorders in pregnancy: A multicenter study

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ABSTRACT

Background: Hypertensive disorders in pregnancy (HDP) have heterogeneous etiologies. Previous studies have linked individual air pollutants to overall HDP with inconsistent results. Moreover, it has not been explored how exposure to a mixture of multiple air pollutants may affect the risks of the subtypes of the disorders.
Objectives: To investigate the associations of exposure to air pollutant mixture in the 1st and 2nd trimesters of pregnancy with the risks of HDP and its subtypes.
Methods: Pregnancy data were obtained from the China Labor and Delivery Survey, a nationwide cross-sectional survey in 2015 and 2016. Levels of air pollutants [including fine particulate matter ($PM_{2.5}$), carbon monoxide (CO), nitrogen dioxide (NO_2), ozone (O_3), and sulfur dioxide (SO_2)] in the 1st and 2nd trimesters were estimated based on the model developed by the Institution of Atmospheric Physics, Chinese Academy of Science. Generalized linear mixed models were built to assess the single-exposure effects of air pollutants in early gestation on HDP. The restricted cubic spline function was further applied to assess the potential non-linearity. The weighted quantile sum (WQS) regression was used to investigate the effects of co-exposure to multiple air pollutants.
Results: A total of 67,512 pregnancies were included, and 2,834 were HDP cases. The single-effect analysis showed that CO, $PM_{2.5}$, and SO_2 exposure in the 2nd trimester was positively associated with the risks of gestational hypertension (GH), with adjusted odds ratios (aORs) and 95% confidence intervals (CI) of 1.16 (1.04, 1.28), 1.19 (1.04, 1.37), and 1.13 (1.04, 1.22), respectively. The first-trimester O_3 exposure was also associated with an increased preeclampsia/eclampsia (PE) risk (aOR = 1.17; 95%CI: 1.02, 1.33). WQS regression confirmed positive associations of air pollutant mixture with HDP subtypes, with $PM_{2.5}$ as the main contributing pollutant to GH, and CO and O_3 as the main pollutants to PE.
Conclusions: Exposure to multiple air pollutant mixtures in early pregnancy was associated with increased risks of hypertensive disorders in pregnancy.

1. Introduction

Hypertensive disorders in pregnancy (HDP), a group of complications encompassing chronic hypertension, gestational hypertension (GH), preeclampsia/eclampsia (PE), and preeclampsia superimposed on chronic hypertension, remain one of the major causes of perinatal morbidity and mortality worldwide (Garovic et al., 2022). Affected women and newborns are also at increased risk of poor long-term health, such as cardiovascular diseases (Garovic et al., 2020), chronic kidney disease (Barrett et al., 2020), stroke (Garovic et al., 2020), mental and behavioral problems (Brand et al., 2021), and even death (Huang et al., 2022). Several maternal characteristics, such as pre-pregnancy obesity, advanced age, and the use of assisted reproductive technologies (ART), have been recognized as potential risk factors for HDP (Bartsch et al.,

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2016; Umesawa and Kobashi, 2017). However, the etiology and pathogenesis of the disorders remain largely unclear.

Exposure to air pollution is one of the leading risk factors for human disease. Higher exposure levels of air pollution have been linked to increased risks of respiratory diseases (Doiron et al., 2019), cardiovascular system impairment (de Bont et al., 2022), adverse pregnancy outcomes (e.g., low birth weight, preterm birth, and stillbirth) (Nyadanu et al., 2022), and millions of premature deaths (Collaborators, 2020). The association between exposure to ambient air pollutants and the risk of HDP has also raised interest in recent years, but previous results remain inconsistent. For example, some epidemiological studies reported a positive association of fine particulate matter ($PM_{2.5}$) levels with HDP (Nobles et al., 2019; Xue et al., 2018), while others observed no or even an inverse association (Assibey-Mensah et al., 2019; Melody et al., 2020). Variations in air pollutant concentrations, study sample size, and population susceptibility may partly explain these contradictory results. Besides, most studies often examined one pollutant at a time, ignoring the health effects of other air pollutants. No study has assessed the mixture effect of multi-pollutants. As humans are exposed to multiple pollutants simultaneously, an analysis of mixture exposure, rather than the traditional "one chemical at a time" approach, should be applied to capture the interplay of different exposures and make clear if an observed association in a single pollutant model reflects the effect of the pollutant of interest or if it acts as a surrogate for another pollutant (Stafoggia et al., 2017).

Furthermore, HDP have heterogeneous etiologies and clinical manifestations (Gyselaers, 2022; Stefańska et al., 2021). Various pollutants may affect different pathways and have different impacts on HDP subtypes. For a better understanding of the underlying mechanisms, it is essential to examine the associations between air pollutant exposure and the risks of HDP subtypes separately. However, only a few studies have been available on this topic so far (Choe et al., 2018; Lee et al., 2013; Michikawa et al., 2015; Nobles et al., 2019).

Using a nationwide survey dataset, the present study aimed to assess the associations of both single and mixture air pollutant exposure with HDP and its subtypes in China.

2. Materials and methods

2.1. Study population

Data of the current study were from the China Labor and Delivery Survey (CLDS), a multicenter study in China (March 1, 2015, to December 31, 2016), which was aimed at describing the epidemiology of pregnancy complications and factors affecting labor and delivery. The study design, organization, and implementation of CLDS have been described in detail in our previous work (Chen et al., 2019). In brief, hospitals with 1,000 or more deliveries per year and expressed an interest in participating in this study were eligible. Six weeks within consecutive 12 months for hospitals with an annual delivery of at least 6,000 births or 10 weeks for hospitals with a yearly delivery of fewer than 6,000 births were randomly selected for data collection. Information on mother-newborn pairs, including demographic characteristics, reproductive and disease histories, pregnancy complications, labor admission assessment, delivery summary, and newborn health, were abstracted from medical records by trained research assistants and underwent logic checks to avoid missingness and typing errors.

A total of 74,192 obstetric records were at first collected from 96 hospitals in 25 provinces (Fig. 1). Subjects with the following conditions were excluded: missing data on HDP, chronic hypertension or hypertension with unknown cause (n = 1,237), multiple pregnancies (n = 1,880), stillbirths (n = 897), gestational age $\leq$ 28 weeks or $>$ 44 weeks (n = 649), pre-pregnancy diabetes (n = 794), pre-pregnancy heart diseases (n = 125), and pre-pregnancy renal diseases (n = 280). We further excluded records with missing data on essential maternal and child characteristics (e.g., maternal age, ethnicity, census, mode of conception, fetus gender, and parity) (n = 1,514). Ultimately, 67,512 pregnancies were included in the analyses (Fig. S1). Approval of the study was obtained from the Ethics Committees of Xinhua Hospital (XHEC-C2015-006). No individual informed consent was required because anonymous information was collected via chart review for research.

2.2. Outcome definitions

The Diagnosis and Treatment Guidelines of Hypertensive Disorders in Pregnancy was followed to diagnose HDP and classify cases into subtypes (Yang and Zhang, 2015), which include (1) gestational hypertension [blood pressure (BP) $\geq$ 140/90 mmHg after 20 gestational

Fig. 1. The geographic location of the study area and the distribution of enrolled hospitals.

weeks with normal BP at 12 weeks postpartum], (2) preeclampsia (BP ≥ 140/90 mmHg combining proteinuria after 20 gestational weeks or abnormal BP plus involving one organ or system with previously normal BP), and (3) eclampsia [new-onset grand mal seizures within pre-eclampsia]. Considering that eclampsia is often a severe status of pre-eclampsia and rare, we combined eclampsia with preeclampsia. Pregnant women without HDP served as the comparison group.

2.3. Estimation of air pollution exposure

Our study included $PM_{2.5}$, carbon monoxide (CO), nitrogen dioxide (NO_2), ozone (O_3), and sulfur dioxide (SO_2) as the main air pollutants. Their exposure levels were estimated using the chemical data assimilation system (ChemDAS) developed by the Institute of Atmospheric Physics, Chinese Academy of Sciences. Details of the system have been described elsewhere (Kong et al., 2021). Briefly, ChemDAS assimilates more than 1000 air quality monitoring stations from China National Environmental Monitoring Centre based on the Nested Air Quality Prediction Modeling System and the ensemble Kalman filter. It provides surface-gridded fields of air pollutants with a spatiotemporal resolution of 15 km and 1 h, respectively. The data accuracy has been proven through cross-validation and independent data validation, with the estimated root of mean square error of 0.38 (0.54) mg/m^3 for CO, 12.7 (16.4) μg/m^3 for NO_2, 17.5 (21.9) μg/m^3 for O_3, 15.2 (21.3) μg/m^3 for $PM_{2.5}$, and 16.9 (24.9) μg/m^3 for SO_2 at assimilation (validation) sites for hourly concentrations during 2013–2018 (Kong et al., 2021).

Daily averages of the five air pollutants were calculated from the ChemDAS data and assigned to pregnant women according to their last menstrual period. The average levels in the 1st trimester (1–13 weeks of gestation), 2nd trimester (14–27 weeks of gestation), and the 1st and 2nd trimesters combined (1–27 weeks of gestation) were then calculated. As CLDS did not collect participants' family addresses, geocoded hospital addresses were used as proxies for home addresses to estimate the exposure levels of air pollutants. In addition, data on daily meteorological factors were downloaded from the China National Weather Data Sharing System (http://data.cma.cn/site/index.html). The average apparent temperature in the gestational windows for each woman was then calculated using the data from the nearest monitoring station to each hospital. Compared to temperature, apparent temperature additionally incorporates wind speed and humidity and thus can reflect the thermal sensations experienced by humans more objectively (Liu et al., 2020).

2.4. Statistical analyses

For descriptive analyses, mean ± standard deviation (SD) or median and inter-quantile range (IQR) were calculated for continuous variables with normal or skewed distributions, respectively. Frequencies and proportions were used for categorical ones. Student t-test and chi-square test were used to assess the differences between HDP cases and normotensive women for continuous and categorical variables, respectively. Spearman rank correlation tests were performed to evaluate the pairwise correlations between air pollutants and apparent temperature.

Generalized linear mixed models with the province as a random effect were first applied to estimate the single effects of individual air pollutants on overall HDP and subtypes during different gestational windows. Covariates adjusted in the models were determined according to a directed acyclic graph (Fig. S2) and available evidence, including maternal age (≤ 35/> 35 years), ethnicity (Han/other), maternal body mass index [BMI, weight (kg)/height(m)^2, < 18.5/18.5–24.0/≥ 24.0] at the first prenatal visit, maternal education levels [middle school or below/high school/college (< 4 years)/college graduate or higher], resident status (registered population/migrants), insurance status (yes/no), parity(nulliparous/multiparous), mode of conception (natural/ART), the season of conception (spring/summer/autumn/winter), fetal gender (male/female), region (categorized variable), average annual Gross Domestic Product per province (GDP, categorized variable) and apparent temperature (continuous variable). The multiple imputation with a fully conditional specification method specifies the multivariate imputation model on a variable-by-variable basis by a set of conditional densities to yield unbiased estimates for missingness in large data sets (Liu and De, 2015). It was used to handle missing values of maternal BMI, education levels, and insurance status. Five datasets with imputations for missing data were created and modeled separately. The results were pooled to obtain the final effect parameters by Rubin's rule (Marshall et al., 2009) and presented as odds ratios (ORs) with 95% confidence intervals (CIs) for an IQR increase in air pollution levels.

Restricted cubic spline (RCS) function with four knots was further applied to visualize the concentration-response relationships between air pollutant exposures and the risk of HDP subtypes and assess the potential non-linearity. Besides, stratified analyses were performed to assess the modification effects by maternal age, fetal gender, maternal BMI, mode of conception, and season of conception [warm (April–September)/cold (October–March)]. An interaction term of modification factor by air pollutants was added to the regression models to detect whether the interaction was statistically significant.

To explore the association of exposure to air pollutant mixture with HDP in the two trimesters separately, a weighted quantile sum (WQS) regression was applied (Carrico et al., 2015). The statistical model constructs a weighted index representing the correlated chemical mixtures based on the quantiles of chemical components, assuming that all features of the index work in the same direction, with individual weights ranging from 0 to 1 and the total sum of 1. By analyzing the association between the outcome and the index, the WQS regression can estimate the overall effects due to the mixture exposure and determine the contributions of each predictor to the overall index effect (Carrico et al., 2015).

We constructed the WQS index based on the quartiles of air pollutant levels. Because no prior assumption was made regarding the direction of the associations between air pollutant levels and HDP, WQS regressions grouping all components of the index to work in the positive or negative directions were performed, adjusting for all the covariates described above. Five hundred bootstrap samples were set to increase the sensitivity in detecting significant predictors and obtain stable weights. The inverse of the number of air pollutants (0.2) was selected as the weight cutoff to determine the main contributors to the mixture effect (Carrico et al., 2015). The log-transformed WQS index was interpreted as the elevated risk of HDP per quartile increase in air pollutant mixture level.

To test the robustness of the results, we repeated the above analyses based on the complete data, which consisted of 47,233 obstetric records. We also adjusted for the 1st-trimester exposure to air pollutants in the 2nd-trimester single-effect analyses. RCS and WQS regressions were performed using R 4.2.0 software, with the packages *rms* and *gWQS*, respectively. All other analyses were conducted in SAS 9.4 (SAS Institute Inc., Cary, NC, USA). The statistical significance level was set at 0.05 (two-tailed).

3. Results

3.1. Descriptive statistics

Table 1 presents the characteristics of the study population. The prevalence of HDP was 4.1% (2,834/6,9026), with 1,307 GH cases and 1,527 PE cases. The means (±SD) of maternal age and BMI at the first prenatal visit in HDP cases were 30.03 ± 5.66 years and 23.23 ± 3.86 kg/m^2, respectively; both were higher than the estimates in normotensive women (28.64 ± 5.10 years and 21.70 ± 3.18 kg/m^2, respectively). Compared to the non-cases, HDP cases also tended to have lower education and lower coverage by private insurance, but higher proportions of migrants and ART pregnancies. No significant difference in fetal gender or parity was observed.

Table 2 shows the average air pollutant levels during different

Table 1
Sociodemographic characteristics of the study population.

	Total (n = 69,026)	Normotensive women (n = 66,192)	HDP cases (n = 2,834)	GH (n = 1,307)	PE (n = 1,527)
Maternal age* (years)	28.70 ± 5.13	28.64 ± 5.10	30.03 ± 5.66	29.80 ± 6.06	30.23 ± 5.29
Maternal BMI* (kg/m^2)	21.76 ± 3.22	21.70 ± 3.18	23.23 ± 3.86	23.58 ± 3.73	23.32 ± 3.94
Maternal ethnicity*					
Han	95.3%	95.4%	93.9%	93.7%	94.1%
Other	4.5%	4.4%	5.8%	6.0%	5.6%
Unknown	0.2%	0.2%	0.3%	0.3%	0.3
Maternal education*					
Middle school or below	22.6%	22.3%	29.0%	28.2%	29.6%
High school	17.8%	17.8%	18.3%	18.0%	18.6%
College (< 4 years)	22.1%	22.1%	20.6%	19.4%	21.6%
College graduate or higher	27.2%	27.4%	22.8%	24.5%	21.4%
Unknown	10.3%	10.3%	9.3%	10.0%	8.8%
Parity					
Nulliparous	56.1%	56.2%	54.9%	54.1%	55.6%
Multiparous	43.7%	43.6%	45.0%	45.9%	44.2%
Unknown	0.2%	0.2%	0.1%	0%	0.2%
Insurance status*					
Yes	58.5%	58.7%	55.2%	53.7%	56.4%
No	38.2%	38.0%	41.4%	43.1%	40.1%
Unknown	3.3%	3.3%	3.4%	3.2%	3.5%
Resident status*					
Registered populations	85.6%	85.7%	84.2%	83.7%	84.5%
Migrants	13.9%	13.8%	15.2%	15.6%	15.0%
Unknown	0.5%	0.5%	0.6%	0.7%	0.5%
Mode of conception*					
Natural conception	94.0%	94.1%	92.4%	91.8%	92.9%
ART	5.6%	5.5%	7.3%	7.9%	6.8%
Unknown	0.4%	0.4%	0.3%	0.3%	0.3%
Fetal gender					
Male	53.0%	53.0%	52.9%	53.6%	52.3%
Female	46.1%	46.1%	46.2%	45.6%	46.8%
Unknown	0.9%	0.9%	0.9%	0.8%	0.9%
Season of conception*					
Spring	21.5%	21.4%	23.6%	26.2%	21.3%
Summer	29.4%	29.4%	30.0%	31.5%	28.6%
Autumn	23.2%	23.2%	21.4%	18.2%	24.3%
Winter	25.9%	26.0%	25.0%	24.0%	25.8%
Region*					
Central	12.2%	12.1%	13.6%	14.6%	12.6%
East	33.7%	33.9%	30.7%	33.9%	28.0%
North	15.9%	15.8%	19.7%	19.9%	19.5%
Northeast	6.0%	6.0%	6.6%	4.0%	8.9%
Northwest	6.9%	6.9%	7.0%	3.0%	10.4%
South	12.2%	12.2%	11.2%	12.6%	10.0%
Southwest	13.1%	13.1%	11.2%	12.0%	10.6%

Note: HDP, hypertensive disorders in pregnancy; GH, gestational hypertension; PE, preeclampsia/eclampsia; BMI, body mass index at the first prenatal visit. ART, assisted reproductive technology. * Statistically significant difference between overall HDP cases and normotensive women examined by *t*-test for continuous variables and chi-square test for categorical variables ($p < 0.05$).

gestation windows. The median (IQR) concentrations of CO, NO_2, O_3, $PM_{2.5}$, and SO_2 during the entire pregnancy were 1.0 (0.4) mg/m^3, 37.6 (18.7) μg/m^3, 49.1 (18.8) μg/m^3, 49.7 (28.0) μg/m^3 and 17.8 (12.8) μg/m^3, respectively. Pairwise correlation analysis showed a positive association between apparent temperature and O_3, but negative associations with other air pollutants, with Spearman correlation coefficients ranging from −0.61 to 0.74 in the 1st trimester and −0.68 to 0.78 in the 2nd trimester (Table S1).

Table 2
Average exposure levels to air pollutants of interest during different gestation windows for all subjects in CLDS.

	Median (IQR)				
	CO (mg/m^3)	NO_2 (μg/m^3)	O_3 (μg/m^3)	$PM_{2.5}$ (μg/m^3)	SO_2 (μg/m^3)
1st trimester	1.0 (0.4)	36.3 (20.1)	50.8 (24.5)	48.3 (29.8)	16.9 (11.9)
2nd trimester	1.0 (0.4)	38.0 (20.2)	48.3 (27.4)	48.3 (31.2)	17.3 (12.4)
1st + 2nd trimester	1.0 (0.4)	37.6 (18.7)	49.1 (18.8)	49.7 (28.0)	17.8 (12.8)

Note: CO, carbon monoxide; NO_2, nitrogen dioxide; O_3, ozone; $PM_{2.5}$, fine particulate matter; SO_2, sulfur dioxide; IQR, interquartile range.

3.2. Associations between single air pollutant exposure and HDP risk

Table 3 summarizes the associations between single air pollutant exposure and HDP risk during different gestation windows. The risk of overall HDP was positively associated with an IQR increase in CO, NO_2, $PM_{2.5}$, and SO_2 in the 2nd trimester, with aORs (95%CIs) of 1.11 (1.04, 1.19), 1.13 (1.01, 1.27), 1.10 (1.01, 1.22) and 1.07 (1.01, 1.13), respectively. These positive associations were statistically significant for GH but not for PE. Interestingly, O_3 exposure showed a positive association with PE in the 1st trimester (aOR = 1.17; 95%CI: 1.02, 1.33) while an inverse association with GH in the 2nd trimester (aOR = 0.78; 95%CI: 0.67, 0.90). Additionally, the association between CO exposure and the overall HDP risk was stronger in the cold season (aOR = 1.38; 95%CI: 1.19, 1.61, $p_{\text{interaction}} = 0.035$). However, no other significant differences were observed when stratified by maternal age, maternal BMI, fetal gender, and mode of conception (all $p > 0.05$) (Table S2).

Fig. S3 shows the concentration-dependent curves of air pollutant exposures with the risks of HDP subtypes using RCS. Non-linear relationships ($p_{\text{non-linear}} < 0.05$) appeared more common in PE, while the associations between air pollutants and GH were mostly linear.

Table 3
Associations between single air pollutant exposure and hypertensive disorders in pregnancy during different gestation windows[a].

Pollutants	aOR (95%CI)		
	Overall HDP	GH	PE
CO			
1st trimester	1.06 (0.98, 1.15)	1.08 (0.96, 1.21)	1.04 (0.94, 1.15)
2nd trimester	1.11 (1.04, 1.19)	1.16 (1.04, 1.28)	1.07 (0.98, 1.18)
1st + 2nd trimester	1.14 (1.04, 1.24)	1.19 (1.05, 1.36)	1.08 (0.96, 1.22)
NO_2			
1st trimester	1.00 (0.89, 1.12)	0.99 (0.84, 1.17)	1.04 (0.89, 1.22)
2nd trimester	1.13 (1.01, 1.27)	1.12 (0.95, 1.32)	1.15 (0.98, 1.34)
1st + 2nd trimester	1.06 (0.94, 1.19)	1.03 (0.88, 1.22)	1.10 (0.94, 1.28)
O_3			
1st trimester	1.02 (0.93, 1.12)	0.91 (0.79, 1.03)	1.17 (1.02, 1.33)
2nd trimester	0.90 (0.81, 1.01)	0.78 (0.67, 0.90)	1.04 (0.90, 1.19)
1st + 2nd trimester	0.97 (0.89, 1.07)	0.84 (0.73, 0.95)	1.14 (1.00, 1.29)
$PM_{2.5}$			
1st trimester	0.95 (0.85, 1.06)	0.99 (0.85, 1.16)	0.94 (0.81, 1.08)
2nd trimester	1.10 (1.01, 1.22)	1.19 (1.04, 1.37)	1.06 (0.93, 1.20)
1st + 2nd trimester	1.04 (0.93, 1.16)	1.13 (0.97, 1.33)	0.99 (0.85, 1.15)
SO_2			
1st trimester	0.99 (0.93, 1.05)	1.05 (0.96, 1.15)	1.08 (0.99, 1.16)
2nd trimester	1.07 (1.01, 1.13)	1.13 (1.04, 1.22)	1.02 (0.95, 1.09)
1st + 2nd trimester	1.05 (0.97, 1.14)	1.17 (1.04, 1.31)	1.05 (0.86, 1.16)

Note: aOR, the adjusted odds ratio of per IQR increase in air pollutants; CI, confidence interval; HDP, hypertensive disorders in pregnancy; GH, gestational hypertension; PE, preeclampsia/eclampsia; CO, carbon monoxide; NO_2, nitrogen dioxide; O_3, ozone; $PM_{2.5}$, fine particulate matter; SO_2, sulfur dioxide; [a] Generalized linear mixed regression models were used, and all models were adjusted for maternal age, ethnicity, BMI, insurance status, resident status, education, parity, mode of conception, the season of conception, fetal gender, region, GDP, apparent temperature, and random contribution of the province.

3.3. Associations between exposure to air pollutant mixture and HDP risk

Table 4 presents the association between exposure to air pollutant mixture and HDP. In the positive direction, the WQS index was significantly associated with overall HDP risk during different gestation windows, with aORs (95%CIs) of 1.14 (1.06, 1.23) in the 1st trimester, 1.13 (1.04, 1.21) in the 2nd trimester, and 1.14 (1.06, 1.23) in the two trimesters combined, respectively. The GH risk was elevated by 11% for a quartile increase in air pollutant mixture during early pregnancy (aOR = 1.11; 95%CI: 1.01, 1.22). Exposure to air pollutant mixture also contributed to 14% increased PE risks in the entire window (aOR = 1.14; 95%CI: 1.05, 1.24), in which the aORs (95%CIs) were 1.15 (1.03, 1.28) in the 1st trimester and 1.25 (1.11, 1.41) in the 2nd trimester, respectively. No significant mixture effect in the negative direction was observed (Table 4). Additionally, the assigned weights indicated that $PM_{2.5}$ was the main contributor to the mixture effect on GH, whereas CO and O_3 were on PE (Table S3).

3.4. Sensitivity analyses

We repeated the above analyses with complete data only. Opposite effects of O_3 exposure on the two HDP subtypes remained, and the 2nd trimester was still a more susceptible gestational window for GH (Table S4). Stratification analyses did not provide additional information (Table S5). The mixture effects of air pollutant exposure decreased slightly but the overall results confirmed the primary findings (Tables S6 and S7). After adjusting for the 1st-trimester exposure to air pollutants, the single-effect estimates in the 2nd-trimester did not change substantially (Table S8).

4. Discussion

The present study assessed the associations of exposure to single and multiple air pollutants during pregnancy with HDP based on a nationwide survey in China. We found that higher ambient levels of CO, $PM_{2.5}$, and SO_2 in the 2nd trimester were significantly associated with increased risks of GH, and $PM_{2.5}$ was the main contributor to the mixture. We also showed that O_3 and CO exposures in the 1st trimester were positively associated with PE risk and were the main contributors in the mixture.

The association between exposure to air pollutants and the risk of HDP has attracted increasing attention in recent years, but few studies have examined the HDP subtypes. It is well known that GH and PE have different etiologies. In PE, insufficient trophoblast invasion and incomplete spiral artery remodeling occur in early pregnancy. The resultant placental ischemia increases the production of angiogenic factors, which further induces vascular endothelial dysfunction, oxidative stress, and microemboli in multiple organ systems, leading to the clinical feature of PE (Ives et al., 2020). As such, PE is increasingly accepted as a placental disease. GH, however, is more related to a susceptible or impaired cardiovascular system (Gyselaers, 2022). On the other hand, different air pollutants may exert their health effects via different mechanisms, pathways, and targets. These differences in etiology and pathogenesis highlight the necessity of assessing the associations between exposure to air pollution and HDP subtypes separately.

Table 4
The effect of exposure to air pollutant mixture on hypertensive disorders in pregnancy estimated by weighted quantile sum regression.

WQS index	Gestation window	aOR (95% CI)		
		Overall HDP	GH	PE
P	1st trimester	1.14 (1.06, 1.23)	1.07 (0.98, 1.17)	1.15 (1.03, 1.28)
	2nd trimester	1.13 (1.04, 1.21)	1.08 (0.98, 1.19)	1.25 (1.11, 1.41)
	1st +2nd trimester	1.14 (1.06, 1.23)	1.11 (1.01, 1.22)	1.14 (1.05, 1.24)
N	1st trimester	1.04 (0.96, 1.12)	1.11 (0.98, 1.26)	0.93 (0.83, 1.05)
	2nd trimester	1.07 (0.96, 1.18)	0.99 (0.87, 1.12)	1.02 (0.92, 1.15)
	1st +2nd trimester	1.02 (0.93, 1.11)	1.05 (0.94, 1.17)	0.94 (0.83, 1.07)

Note: aOR, the adjusted odds ratio; CI, confidence interval; HDP, hypertensive disorders in pregnancy; GH, gestational hypertension subtype; PE, preeclampsia/eclampsia subtype; Estimates were adjusted for maternal age, ethnicity, BMI, insurance status, resident status, education, parity, mode of conception, the season of conception, fetal gender, GDP, region, and apparent temperature. "P" indicates the WQS index was modeled in the positive direction, whereas "N" indicates the WQS index was modeled in the negative direction.

Among various air pollutants, $PM_{2.5}$ was the most studied but with inconsistent results. As $PM_{2.5}$ is a mixture comprising a variety of chemical compositions (Liang et al., 2016), the heterogeneity of $PM_{2.5}$-HDP risk associations across studies has partially been attributed to the variations of critical components and corresponding toxicity with spacetime. However, the most recent meta-analysis by Cao et al. (2021) showed that $PM_{2.5}$ exposure in the 1st and 2nd trimesters was associated with increased risks of HDP, and the associations were significant in GH only, with pooled ORs (95%CIs) of 1.11 (1.01, 1.23) and 1.16 (1.05, 1.29) per 5 $\mu g/m^3$ increase, respectively, which supports our findings. These observations are biologically plausible. Previous experimental and epidemiological studies have shown that chronic exposure to particulate matter can activate local inflammatory responses in the lungs, promote systemic inflammation, and trigger oxidative stress, resulting in endothelial injury and cardiovascular dysfunction (Konduracka and Rostoff, 2022). The affected pregnant women may have a poor ability to balance the cardiac output and vascular resistance because of increasing body water volume from the 2nd trimester and onward, ultimately resulting in GH.

As for O_3 exposure, we found an increased risk of PE while a decreased risk of GH. Michikawa et al. (2015) also observed a positive association of O_3 exposure in the 1st trimester with PE based on the Japan Perinatal Registry Network database. However, Nobel et al. (2019) showed that O_3 exposure did not affect GH but decreased the risk of PE in the U.S. population. Although the biological mechanisms underlying the observed bi-directional association patterns in the current study are far from clear, there is evidence that O_3 exposure could cause lower heart rate, blood pressure, and cardiac output in rodents, suggesting a hypotensive effect of O_3 (Watkinson et al., 2001), thus possibly lowing the risk of GH. In contrast, an experimental study showed that O_3 exposure during the window of embryo implantation had little effect on most of the circulating markers in pregnant rats, whereas the serum from O_3-exposed dams downregulated the invasion of HTR-8/SVneo trophoblasts and increased the release of soluble fms-like receptor 1 (sFlt 1), a critical inhibitor of angiogenesis and invasion, suggesting O_3 exposure during early pregnancy may hinder the crucial process of placentation (Miller et al., 2019), which could lead to PE late in pregnancy (Ives et al., 2020).

Evidence on the associations between other gaseous air pollutants and the risks of overall HDP is rather limited, let alone for HDP subtypes. In the single-effect models, we observed positive associations between CO and SO_2 exposure in the 2nd trimester and the risk of GH. The results were in accordance with the findings from a retrospective cohort study in the U.S., in which 5% and 14% elevated GH risks per IQR increase in CO and SO_2, respectively, were reported (Nobles et al., 2019). In addition, Michikawa et al. (2015) and Choe et al. (2018) assessed the associations between NO_2 and SO_2 exposure and HDP subtypes but failed to show any significant findings.

The inconsistency in the epidemiological findings of the HDP effects of gaseous air pollutants might be attributable to many factors, such as population characteristics, assessment of air pollution exposure levels, sample size, and adjustment for confounding among different studies. Therefore, evidence from large-scale birth cohorts with accurate exposure measurement is needed to better understand the HDP effect of

chronic exposure to gaseous air pollutants.

In recent years, thanks to more advanced statistical methods, quantifying the effects of multi-pollutant exposure on health and diseases has become feasible (Lazarevic et al., 2019; Taylor et al., 2016). Using WQS regression models, our study confirmed that air pollutant mixture was positively associated with the risks of overall HDP and its subtypes. The multi-pollutant models provided further new insights: (1) $PM_{2.5}$ was a main culprit among the air pollutants in the development of GH. (2) CO and O_3 were the main contributors to the development of PE, even though the estimates of CO in the single-exposure models were statistically nonsignificant. (3) The inverse association between O_3 and GH risk was no longer statistically significant in the mixture with other air pollutants.

Currently, there is no direct experimental evidence on the positive association between CO and PE. However, a recent randomized controlled trial may provide a clue, where chronic exposure to CO induced hypoxic effects similar to those of exposure to high altitude (Schmidt et al., 2020). The latter has been observed to be associated with an increased risk of PE partially because of reduced uteroplacental blood flow and placental oxygen availability in the hypoxia environment (Palmer et al., 1999). Additionally, the low weight of O_3 in the positive directional mixture effect indicated the possibility that the "protective effect" of O_3 in single-pollutant models may reflect its inverse correlation with the truly harmful pollutant $PM_{2.5}$ (Carey et al., 2013; Hvidtfeldt et al., 2019). More research is warranted to confirm these findings.

Our study has several advantages. We considered multiple air pollutants as a mixture and evaluated their associations with the risks of HDP and its subtypes using WQS regression models, which allowed us to assess the overall effects and determine the main culprits. In addition, the current study was based on a nationwide survey, which allowed us to explore the effects of interest in a wide range of air pollution concentrations and geographic regions.

Our study also has some limitations. First, the exposure levels of air pollutants were estimated based on hospital addresses rather than home addresses. Nonetheless, our previous sensitivity analyses based on two other cohort populations that could reflect the general state of the CLDS population showed that the median distance between the home addresses and delivery hospitals of pregnant women was less than 8 km. The associations between different air pollutant indexes and disease risk within 5–15 km buffers of the delivery hospitals were quite stable, suggesting that the estimations of air pollutant exposure based on the delivery hospital in the current study were mostly valid (Yu et al., 2020a). However, the method might have poorer performance in the northwest and northeast areas where relatively fewer hospitals were enrolled. Second, we have no data on pregnant women's daily and residential mobility, which may have caused misclassifications. However, previous studies have demonstrated that such misclassifications may have little impact on the effect estimations (Chen et al., 2010; Yu et al., 2020b). Third, we only assessed the mixture effect of five conventional air pollutants and could not consider other co-exposure contaminants. Fourth, we did not adjust for multiple comparisons considering this is an observational study with the goal of hypothesis-generating with relatively limited associations being tested (Savitz, 2013). Last, although a set of essential confounders were adjusted in the regression models, residual or unmeasured confounding is still possible.

5. Conclusions

Our nationwide study showed that exposure to multiple air pollutants mixture was associated with increased risks of HDP subtypes. The mixture effects were mainly driven by $PM_{2.5}$ for GH and by CO and O_3 for PE. Reducing exposure to air pollution and improving protection in gestation may be advised for pregnant women.

Declaration of competing interest

The authors declare no competing financial interests.

Data availability

The exposure and health data used in this study are available on request via email from the corresponding authors.

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Appendix A. Supplementary data

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Occupational exposure to inhalable pathogenic microorganisms in waste sorting

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ABSTRACT

This study assessed microorganisms in personal inhalable work air samples aiming to identify potential human pathogens, and correlate exposure to adverse health outcomes in waste workers. Full-shift personal exposure was measured in six different waste sorting plants. Microbial concentrations in inhalable air samples were analysed using MALDI-TOF MS for cultivable, and next generation sequencing (NGS) for non-cultivable microorganisms. Concentrations of bacterial and fungal CFUs varied substantially within and between waste sorting plants, ranging from no identifiable organisms to a maximum concentration in the order of 10^5 CFU/m^3. *Bacillus* and *Staphylococcus* were among the most abundant bacterial genera, whilst fungal genera were dominated by *Aspergillus* and *Penicillium*. Approximately 15% of all identified species were human pathogens classified in risk group 2, whereas 7% belonged to risk group 1. Furthermore, significant correlations between concentrations of fungi in risk group 1 and self-reported adverse symptoms, such as wheezing were identified in exposed workers. The combination of culture-based methods and NGS facilitated the investigation of infectious microbial species with potential pathophysiological properties as well as non-infectious biological agents in inhalable work air samples and thereby contributed to the risk assessment of occupational exposure in waste sorting.

1. Introduction

Global sustainability goals promote technological progresses in the waste management sector in various way, such as the introduction of new work operations and the implementation of fully automated waste sorting machines that increases the overall waste treatment capacity and reduces the need for manual labour and thus potential exposure moments. However, exposure during cleaning and maintenance of waste sorting machines is an underestimated health challenge. Previous studies have reported differences in exposure levels at automated waste sorting plants and identified cleaning with compressed air as potential high exposure moment (Eriksen et al., 2022, 2023a).

Changes in sorting processes and the introduction of new waste fractions affect pre-sorting routines and waste collection intervals of domestic waste with a general decrease in collection frequencies. Despite dedicated sorting strategies for various waste fractions, about 50% of all food waste is discarded as residual waste (Haslegaard, 2019; ROAF, 2023). Organic material that contaminates residual waste not only reduces the recyclability but also poses potential exposure risk for workers, as prolonged waste collection intervals may promote microbial growth under beneficial conditions (Madsen et al., 2019). Furthermore, effects of climate change need to be considered, as increased temperature and humidity are beneficial for microbial growth in waste bins (Gladding and Gwyther, 2017).

During the handling and sorting of residual waste biological agents, such as microorganisms are aerosolised and dispersed as bioaerosols. Exposure to bacteria and fungi as well as associated toxins such as endotoxins and mycotoxins have repeatedly been linked to occupational diseases of the respiratory tract leading to decline in lung function, the gastrointestinal tract, eyes and skin (Bolund et al., 2017; Hambach et al., 2012; Heldal et al., 2001; Poulsen et al., 1995). As inhalation often is considered the main exposure route in an occupational setting, it is of interest to investigate the biodiversity of inhalable fungi and bacteria to identify potential human pathogens. However, so far there is limited knowledge on the composition of microbial communities in bioaerosols emitted in waste sorting plants.

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Traditionally, workplace assessments investigate the exposure to microorganisms by using culture-based methods, which capture cultivable fungi and bacteria only and potentially underestimate the exposure to non-infectious biological agents with pathophysiological properties. Molecular-biological approaches, such as next generation sequencing (NGS), on the other hand, provide tools for a holistic assessment of microbial work air contaminants (Duquenne, 2018). Degois et al. (2017) conducted a metagenomics analysis in a waste sorting plant and identified *Enterobacteriaceae*, *Staphylococcus*, and *Acinetobacter* to be among the most prevalent bacterial genera, whereas fungal genera were dominated by *Penicillium, Aspergillus* and *Rhizopus*. However, the authors reported their findings on the genus level, and it remains unclear if the samples contained potentially pathogenic species. The present study attempted to combine NGS and culture-based methods to identify non-cultivable potential pathogens on the species level as pathogenicity within a genus can vary substantially. Species in the genus *Aspergillus*, such as *A.fumigatus* have the potential to colonise the airways and cause pulmonary infections. Furthermore, *Aspergillus* spp. have been reported to be problematic in a clinical setting as they develop azole resistance (Chowdhary et al., 2013; Prigitano et al., 2019; Vermeulen et al., 2013). Due to its relevance as human pathogen, *Aspergillus* has been proposed as potential bioindicator for health risks assessment in waste management (Viegas et al., 2015a).

The present study aimed to 1) identify airborne inhalable bacteria and fungi at species level, 2) to identify variability in microbial communities that can be attributed to seasonal variation and different waste sorting plants, 3) to identify human pathogens and potentially harmful non-pathogenic microorganisms in the waste sorting work environment and 4) to evaluate the relationship between concentrations of biosafety level classified microorganisms and the symptoms reported by workers.

2. Material and methods

2.1. Study design

Six Norwegian waste sorting plants that treat residual waste from private homes and small businesses were visited between June 2020 and November 2021 for working environment air sampling. Two waste sorting facilities were in the western part of Norway, and 4 facilities were located in the greater Oslo area. Three of the facilities used fully automated waste sorting lines that sort automatically waste products from private homes, whereas the remaining three facilities use manual labour and excavators for sorting waste from industry and gastronomy (Eriksen et al., 2023a). A total of 59 workers (58 males, 1 female) participated voluntarily and helped recover 114 and 47 samples for NGS and MALDI-TOF MS, respectively (Table 1). Sampling was conducted on two consecutive days (NGS).

Table 1
Overview: Number of participants and collected samples b waste sorting plant.

WSP	season	participants n	NGS n (personal + stationary)		MALDI n (personal + stationary)
			Monday	Tuesday	Tuesday
A	summer	8	8 + 2	8	9[a] + 1
A	autumn	8	8 + 2	8	8 + 1
A	summer	9	9 + 2	9	8 + 1
B	autumn	8	8 + 2	8	5 + 1
B	summer	6	5 + 2	6	2 + 1
C	autumn	5	5 + 2	5	5 + 1
D	autumn	6	6 + 2	6	6 + 1
E	autumn	3	3 + 1	0	0
F	autumn	6	6 + 2	6	4 + 1
Total		**59**	**58 + 17**	**56**	**47 + 8**

[a] One participant was equipped with two samplers that were carried as parallels.

2.2. Sampling methods

Airborne microorganisms were sampled on 37 mm PC membrane filters with a pore size of 1 μm (Frisenette, DK) using personal and stationary conical inhalable air samples (CIS, JS Holdings, Hertfordshire, UK) that were operated at an average airflow of 3.5 L/min. Stationary samples were placed in previously selected spots in the respective waste sorting plant (WSP). Outdoor references were sampled at all sampling sites (approximately 50–100 m from main buildings). For personal samples, sampling devices were placed in the workers' breathing zone and carried for a full shift. Mean sampling time for personal samples was 7.1 h (min: 5.1, max: 8.7). Each participant was equipped with parallel air sampling sets, one dedicated to CFU count and MALDI-TOF MS analyses, the other for DNA extraction and sequencing. At plant E no samples for MALDI-TOF MS analysis were collected. Stationary samples were collected at selected sites of each respective WSP for an average of 27.8 h (min: 6.67, max: 35.3). Filter cassettes were exchanged after approximately 10 h of operation and DNA from filters collected at the same site were pooled during the DNA extraction step. Filter elution and DNA extraction were performed as previously described by Straumfors et al. (2019).

The DNA yield was measured spectrophotometrically on a Qbit 4 Fluorometer (Thermo Scientific, DE, USA) and varied between 0.021 and 15 ng/μL.

2.3. DNA amplification, sequencing, and data analysis

DNA samples were pre-amplified on an Eppendorf Mastercycler X50s (Eppendorf SE, Hamburg, Germany) under the following conditions: Initial denaturation at 90 °C for 30 s, followed by 30 cycles of amplification (98 °C for 10 s, 60 °C for 30 s and 72 °C for 30 s). A final elongation step at 72 °C for 5 min was added. Each reaction contained 5 μL 5x HF PCR buffer, 0.5 μL dNTPs (40 mM), 5 μL of each the forward and reverse primer, 0.25 μL Phusion polymerase (2U/μL), 7.25 μL PCR-grade water and 2 μL template DNA. For bacterial amplification the primer set 515FB/926R that targets the 16S rRNA V4–V5 region was used (Parada et al., 2016; Walters et al., 2016) (Table S1). Fungal amplification was achieved using the fungal specific primer set ITS86(F)/ITS4(R) that targets the ITS2 region as recommended by Op De Beeck et al. (2014). PCR reactions were carried out in duplicates (25 μL each) and pooled after amplification. Subsequently PCR products were purified using a PCR Purification kit (Norgen Biotek Corp., Thorold, Canada) following the manufacturer's recommendations.

Sequencing was performed by the IMR sequencing service (Halifax, Canada) using the same primer sets as had been used in the pre-amplification step. Raw amplicon sequences (fastq) were processed using the DADA2 pipeline (Callahan et al., 2016). Primers were removed prior to downstream analyses using the package's remove primer function allowing for 0 unidentified (N) bases. Further filtering steps were applied allowing a maximum expected error rate of 2 in both forward and reverse bacterial sequences and 2 and 5 errors in fungal sequences, respectively. The minimum sequence truncation length was defined based on the consent quality scores for bacterial amplicons at 230 bases for forward and 180 bases for reverse sequences, respectively. For fungal sequences a minimum sequence length of 50 bases was used due to large variation in intra-specific ITS sequences. Sequences with a quality (phred) score above 30 were included in further analysis. Forward and reverse reads were matched allowing 0 mismatches between sequences and enforcing a 12bp minimum overlap for bacterial sequences and 5bp overlap for fungal sequences. Chimeric sequences were removed based on consensus sequences for each sequence variant. Taxonomy was assigned using the SILVA138.1 database for bacteria (Quast et al., 2012; Yilmaz et al., 2013) and the UNITE database for fungi (Kõljalg et al., 2020), respectively. A mock community with known community composition was included in the analysis (from filter extraction to sequencing) to evaluate the accuracy of the sequences. A

bootstrap cut off at 80 on the species level was used to remove low support amplicon sequence variants (ASV). ASV analysis was conducted using the phyloseq package (McMurdie and Holmes, 2013), and the vegan package (Oksanen et al., 2022). A Permutational multivariate analysis of variance (PERMANOVA) was applied to investigate the differences in clustering of microbial communities between WSP during summer and autumn. The PERMANOVA was based on ordination using principal coordinate analysis (PCoA) and a bray distance matrix.

2.4. Analyses of bacteria and fungi using MALDI-TOF MS

2.4.1. Filter extraction

Filters dedicated to CFU count and MALDI-TOF MS analyses were transferred to sterile tubes and extracted in 5 mL sterile MilliQ +0.85% NaCl and 0.05% Tween 80 by orbital shaking at 500 rpm for 15 min at room temperature. Filter eluates were aliquoted, and glycerol was added. Samples were kept at −80 °C until analyses.

2.4.2. Cultivation

Cultivable bacteria and fungi were grown on nutrient agar (NA; Thermo Fisher Scientific Oxoid, Basingstoke, UK) and Dichloran Glycerol agar (DG18; Dichloran-Glycerol Agar Base; Thermo Fisher Scientific Oxoid, Basingstoke, UK) medium, respectively in a serial dilution as previously described in Madsen et al. (2016). NA plates were incubated at 25 °C ($bacteria_{25}$) and DG18 plates were incubated at 25 °C ($fungi_{25}$) and 37 °C ($fungi_{37}$) for four to seven days, respectively. Colony forming units were counted on day four and seven. 50% of autumn samples collected at WSP A did not contain any microorganisms. Re-plating of the samples at higher dilution produced identical results. It is unsure whether this is due to biases in sampling, handling, or extracting the filters. Samples containing zero CFUs were not included in the summary statistics.

2.4.3. MALDI-TOF MS

DG18 and NA plates with optimal coverage and separation of colony forming units were chosen for analyses. Bacterial colonies were directly transferred to MALDI target plates and analysed on the MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) as previously described by Madsen et al. (2016). Fungal colonies were identified based on the ethanol extraction protocol as described by (Madsen et al., 2015). In short, fungal material was transferred to sterile Eppendorf tubes containing Sabouraud agar (Sabouraud Dextrose Liquid Medium, Oxoid, Basingstoke, United Kingdom, 2% agar) under sterile conditions and cultivated overnight. Subsequently, isolates were extracted in alcohol, formic acid, and acetonitrile before they were transferred to the MALDI target plate.

MALDI-TOF MS analyses were performed on a Microflex LT mass spectrometer (Bruker Daltonics, place). All isolates were analysed in duplicates. Bacterial and fungal identification was based on the BDAL standard library and filamentous library 1.0. included in the Bruker Biotyper 3.1 software. A cut off of 1.70 was used for positive identification of isolates (Stein et al., 2018). Isolates with a score between 1.70 and 1.79 were identified on the genus level, whereas identification on the species level was assigned at a score above 1.80. The GESTIS database (IFA, 2023) was used as reference tool to identify human pathogens in work air samples.

2.5. Questionnaire

Self-reported health data were collected using an adapted version of the questionnaire published by Susitaival and colleagues (Eriksen et al., 2023a; Susitaival et al., 2003) and health outcomes in exposed workers were described in Eriksen et al. (2023b). The present study used the data to investigate the correlation between the prevalence of health symptoms and exposure to viable microorganisms.

2.6. Data analysis

All data analyses were executed in R (version 4.2.2) and RStudio (version 2022.07.2) using the following packages: statix (Kassambara, 2022) for statistical analysis, the ggplot2 for graphics (Wickham, 2016), phyloseq (McMurdie and Holmes, 2013) and vegan (Oksanen et al., 2022) for community analyses and lme4 (Bates et al., 2015) for model fitting. Log transformed exposure levels were reported as time weighted average (TWA) for bacterial and fungal CFU/m^3, respectively. The levels were calculated based on average air follow, sampling time and dilution (filter and inoculation). Results were considered as statistically significant at a p value below 0.05 and borderline significant at a p value between 0.05 and 0.1.

2.6.1. Modelling

A linear mixed effect model stratified by growth medium/temperature was applied to investigate the effect of season (categorical: summer vs autumn) and exposure time (categorical: over vs under 360 min in sorting hall) on personal exposure levels. The model allowed for random intercepts between waste sorting plants,- $\beta_{1\ and}\ \beta_2$ the respective slopes, and Ɛ the residual error.

$$\log(\mathrm{CFU/m^3}) = \beta_1\ \mathrm{season} + \beta_2\ \mathrm{exposure\ time} + (1|\mathrm{WSP}) + Ɛ \quad [1]$$

Furthermore, a logistic regression model was used to investigate the effect of microbial concentrations in risk group 1, 2, and “no risk group”, respectively on the occurrence of health symptoms in exposed workers. The probability of having symptoms is linked to a linear predictor by means of a logistic link function:

$$\mathrm{logit}(\mathrm{Prob}(\mathrm{symptom} = 1)) = \beta\ \mathrm{pathogen\ frequency} \quad [2]$$

where symptom = the symptom prevalence in exposed waste workers (categorical, 0 = no symptoms, 1 = symptoms), β = slope, and pathogen frequency = the concentration of human pathogens in personal air samples (continuous, measured as concentration of pathogens in risk group 1, 2 and “no risk group”).

3. Results

3.1. Exposure to bacteria and fungi

The time-weighted geometric mean (GM) CFU/m^3 levels in personal samples across all samples were 8.1×10^3 CFU/m^3 (range: 1.5×10^1–8.5×10^5) for $fungi_{25}$, 1.8×10^3 CFU/m^3 (range: no CFU – 1.0×10^5) for $fungi_{37}$, and 2.7×10^3 CFU/m^3 (range: no CFU – 3.9×10^5) for $bacteria_{25}$, respectively. Average CFU/m^3 levels in outdoor references were 6.0×10^2 CFU/m^3 (range: 1.8×10^2–6.3×10^3), 4.5×10^1 CFU/m^3, and 7.5×10^1 CFU/m^3 (range: no CFU – 1.3×10^3), for $fungi_{25}$, $fungi_{37}$, and $bacteria_{25}$, respectively (Table S2). Four of the 8 samples collected at WSP A during autumn months did not contain viable microorganisms, these samples were excluded from further analyses.

Significant differences in microbial concentrations were identified between seasons and WSP (Fig. 1, Fig. S1, Table S4). At plant A, levels of $fungi_{25}$ differed significantly between seasons with elevated levels during summer months. In plant B, autumn levels tended to be higher than summer levels. Autumn $fungi_{25}$ CFU/m^3 concentrations were significantly elevated at WSP B (p-value <0.001), and WSP C (p-value = 0.023) compared to plant A. Bacterial CFU levels were significantly different between plant A and B during autumn months with highest levels at the latter. At WSP A average CFU/m^3 concentration were generally lover compared to summer concentrations, whereas autumn concentration in WSP B were on average higher compared to summer CFU/m^3 levels.

The exposure to microorganisms in personal samples was higher than the outdoor background samples during both seasons at each plant, respectively, however only in a few cases the difference was statistically

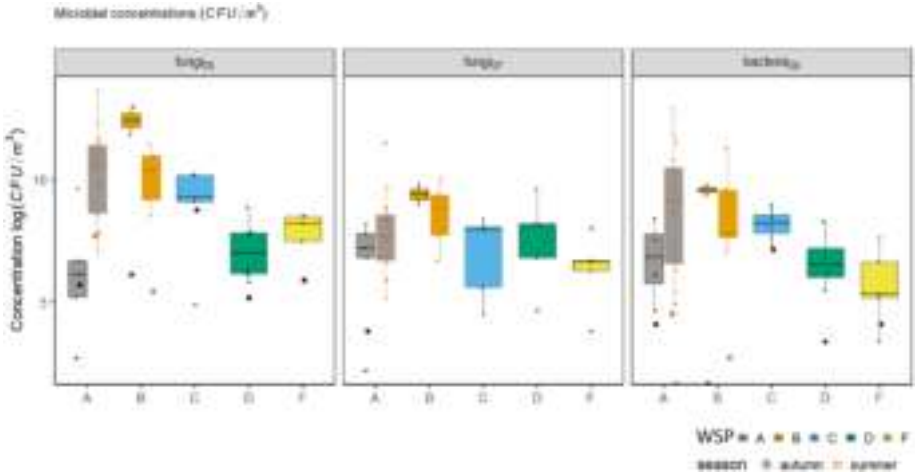

Fig. 1. Microbial concentrations in personal samples (boxplot and dots) and background samples (squares) by WSP (A–F) and season (black: autumn, orange: summer). No samples were collected at plant E. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

significant (WSP A: bacteria$_{25}$ summer, WSP B: fungi$_{25}$ and bacteria$_{25}$, bacteria$_{25}$ autumn, WSP F: fungi$_{25}$). Average autumn exposure levels of fungi$_{25}$ were 5x higher (p-value <0.001) in personal samples compared to outdoor references, whereas fungi$_{37}$ levels were 25x higher (p-value <0.001) and bacteria$_{25}$ levels were 11x higher (p-value <0.001) compared to outdoor references. Summer exposure levels in personal samples were, however, 38x higher for fungi$_{25}$ (p-value <0.001) and as much as 1600x higher (p-value <0.001) for bacteria$_{25}$ (Table S3).

3.2. Modelling determinants of CFU concentrations in personal samples

Average summer CFU levels were statistically higher compared to autumn levels for fungi$_{25}$ as well as bacteria$_{25}$ (Equation (1), Table 2). Time spent in waste sorting did not affect any of the assessed exposure levels. The proportion of the within-WSP variance reported as inter class coefficient (ICC) was relatively low in all cases. Variance within-WSP accounted for 9% for fungi$_{25}$, 4% for fungi$_{37}$ and 1% for bacteria$_{25}$ of the total variance observed in the data set. Thus, the variance in the exposure levels could largely be attributed to different WSP.

3.3. Microbial species in personal air samples identified with MALDI-TOF MS

A total of 42% of bacterial and 78% of fungal CFUs were identified on the species level (MALDI score ≥ 1.8), and 47% of bacterial and 86% of fungal CFUs were identified on the Genus level (MALDI score ≥ 1.7), respectively (Table S5). Thirty three percent of the analysed bacterial CFUs had specific spectra, were however not found in the reference library, whereas 8% of all fungal sequences were unidentifiable. Personal exposure was on average 2.7 × 10^3 CFUs/sample (GSD: 9.4) in bacterial samples, 8.0 × 10^3 (GSD: 10.2) CFUs/sample in fungi$_{25}$ and 1.8 × 10^3 (GSD: 6.0) CFUs/sample for fungi$_{37}$. The average number of identified species in outdoor references was 6.0 × 10^2 (GSD: 3.8) CFUs/sample for fungi$_{25}$ and 7.5 × 10^1 (GSD: 4.6) CFUs/sample for bacteria$_{25}$.

3.3.1. Bacterial diversity

A total of 82 bacterial species were identified in personal air samples belonging to the most abundant genera *Bacillus* (29%), *Staphylococcus* (24%), *Streptomyces* (13%) and *Enterobacter* (8%). Among bacteria, *Bacillus pumilus* (11%), *Streptomyces albidoflavus* (9%), *Staphylococcus equorum* (9%), *Bacillus subtilis* (8%), *Staphylococcus saprophyticus* (7%) and *Enterobacter cloacae* (6%) were among the most prevalent species (Table S6). Bacterial richness was generally higher at automated WSP (A:C) compared to manual WSP (D,F) (Fig. 2). Species in the genus *Bacillus* and *Staphylococcus* were dominant in personal samples collected at automated WSP, whereas *S.albidoflavus*, *Bacillus* spp. and *A.johnsonii* were identified in manual WSP. *E.cloacae* was identified in WSP C only, where it accounted for 32% of the bacteria. The composition of bacterial CFUs in personal samples varied somewhat between seasons with *B. pumilus*, *S.saprophyticus*, *S.albidoflavus* and *S.equorum* dominating

Table 2
Model output of linear mixed effect model accounting for season (autumn as reference) and time spent in waste sorting plant (exposure time <360 min per workday as reference) on fungal and bacterial CFU/m^3 concentrations. The model allows for variation in baseline exposure between plants (WSP included as random effect).

	fungi$_{25}$		fungi$_{37}$		bacteria$_{25}$	
Predictors	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*
Intercept	7.5	**<0.001**	7.2	**<0.001**	6.8	**<0.001**
season (ref 'autumn')	2.5	**<0.001**	0.96	0.12	1.7	**0.02**
exposure time (ref '<360min')	0.51	0.69	−0.15	0.89	0.22	0.87
Random Effects						
σ^2	4.0		3.0		4.5	
τ_{00}	0.38 $_{WSP}$		0.11 $_{WSP}$		0.03 $_{WSP}$	
ICC	0.09		0.04		0.01	
N	5 $_{WSP}$		5 $_{WSP}$		5 $_{WSP}$	
Observations	48		48		48	
Marginal R^2/Conditional R^2[a]	0.26/0.32		0.073/0.11		0.14/0.14	

N = number of grouping variables in random intercept model.
τ_{00} variance of random intercepts.
[a] Marginal R^2 accounts for variance in fixed effects. Conditional R^2 accounts for variance of fixed and random effects.

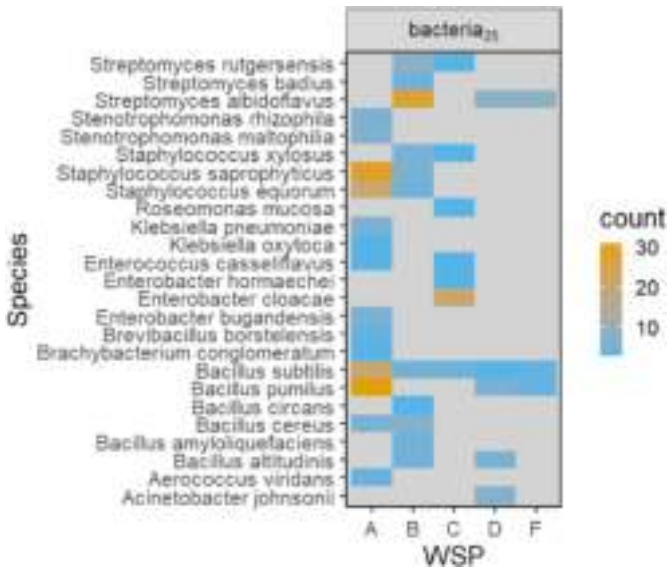

Fig. 2. Most abundant bacterial species in personal air samples stratified by WSP (A:F). Species with abundance greater than 2 CFU/sample are included in the graph.

summer samples (41% of all CFUs in summer samples), whereas *E. cloacae*, *S.xylosus*, *S.equorum* and *S.albidoflavus* dominated autumn samples and accounted for 38% of all CFUs identified in autumn samples (Table S7). Bacterial species profiles were unique for each WSP with highest number of unique species at WSP D (16 species) followed by WSP C (8 species), F (6 species) and B (4 species), whereas only one unique species was identified at WSP A. No bacterial species were common in all WSP (Fig. S4 A). For $fungi_{37}$ and $fungi_{25}$ the species profiles were less distinct and many common species were identified between WSP (Fig. S4 B&C).

3.3.2. Fungal diversity

In total, 36 different fungal species were identified in personal air samples. 60% of the identified fungal species belonged to the genus *Aspergillus*, followed by species in the Genus *Penicillium* that accounted for 33% of the fungal biodiversity. The most abundant species were *A. niger* (26%), *A.fumigatus* (15%), *A.flavus* (8%) and *P.commune* (6%) (Table S7). The species *A.niger* and *P.commune* dominated $fungi_{25}$ in personal samples, accounting for 21% and 12% of all identified fungal CFUs, respectively, were however absent in samples collected at WSP F (Fig. 3). The dominating $fungi_{37}$ in personal samples were *A.niger,* and *A. fumigatus*, and *A.flavus*, accounting for 32%, 27% and 16% of all fungal CFUs, respectively (Table S7).

3.3.3. Seasonal variation in plants A and B

Bacterial diversity in identified isolates was significantly higher during summer months in both WSP with 90% and 74%, respectively (Fig. S2). Only 6% and 13% of all isolates were present during both seasons at plant A and B (Fig. S2). Fungal species richness was generally higher during summer months ranging from 14% to 43% (Fig. S3). Approximately 50% of the fungal species were present during both seasons (Fig. S3).

3.4. Human pathogens in personal and background samples

A total of 14 bacterial species in risk group 2, and 5 species in risk group 1 that have been reported as potential human pathogens in association with occupational exposure were identified in personal air samples (Table 3, Table S6). *B.cereus*, *E.casseliflavus*, and *S.maltophilia* and *A.viridans* were among the most abundant pathogens and were present in between 13% and 23% of personal air samples. Furthermore, 4 fungal species assigned to risk group 2, as well as 3 fungal species assigned to risk group 1 were identified (Table 3, Table S7). Among these, *A.niger* and *A.fumigatus* were among the most prevalent species and were found in 66 and 51% of all personal samples. *A.terreus* and *R. oryzae* were found in a few samples only. Pathogenic species were substantially less present in background samples.

The proportion of fungal species that belong to risk group 2 varied substantially between WSP and seasons, with higher pathogen prevalence at WSP A (72%), B (54%) and C (34%) compared to WSP D (10%) and F (0%), as well as increased levels of risk group 2 species during autumn months (Fig. 4, Fig. S5). The proportion of pathogenic bacterial species in risk group2 in personal air samples was, however, rather similar at all five WSP during both seasons (min: 6%, max:18%).

3.5. Amplicon variants

A total of 1110 bacterial and 1049 fungal ASVs were identified. Principal coordinate analysis (PCoA) of the distance matrix of personal air samples identified clusters of WSP-specific bacterial taxa in autumn samples (Fig. S6). Fungal taxa, however, were largely common between WSP. No distinct clustering was visible for summer samples (Fig. S7, Fig. S8). However, the PERMANOVA analysis indicated that about 40% (p-value <0.001) of the clustering of both fungal and bacterial communities during autumn months was due to differences in WSP.

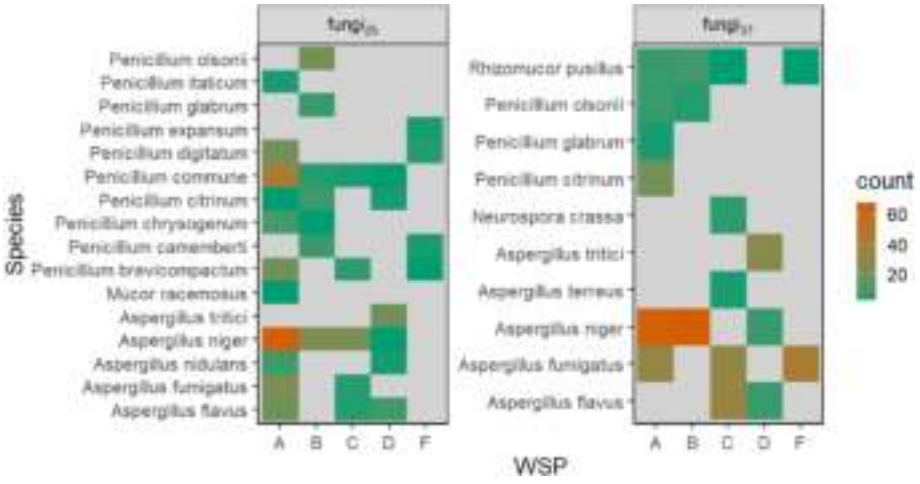

Fig. 3. Most abundant fungal species in personal air samples stratified by WSP (A:F). (Left panel: $fungi_{25}$, right panel: $fungi_{37}$). Species with abundance greater than 2 CFU/sample are included in the graph.

Table 3
Human pathogens identified in personal and background samples. Risk group classification in reference to GESTIS.

	Species	risk group	identified in # personal samples	identified in # stationary samples	Ref
Bacteria	*Bacillus cereus*	2	11	1	
	Enterococcus casseliflavus	2	7	0	
	Aerococcus viridans	2, ht	6	0	
	Staphylococcus saprophyticus	2, ht	6	0	
	Stenotrophomonas maltophilia	2	6	1	
	Enterobacter cloacae	2	3	0	
	Acinetobacter lwoffii	2	2	0	
	Enterobacter bugandensis	2	2	0	
	Enterococcus gallinarum	2, ht	2	0	
	Klebsiella pneumoniae	2	2	0	
	Acinetobacter johnsonii	2	1	0	
	Enterobacter hormaechei	2	1	0	
	Enterobacter ludwigii	2	1	0	
	Enterococcus faecalis	2	1	0	
	Enterococcus faecium	2	1	0	
	Klebsiella oxytoca	2, ht	1	0	
	Kosakonia cowanii	2	1	0	
	Leclercia adecarboxylata	2	1	0	
	Lelliottia amnigena	2	1	0	
	Pantoea eucrina	2	1	0	
	Pantoea septica	2	1	0	
	Pseudomonas putida	2, ht	1	0	
	Roseomonas mucosa	2	1	0	
	Bacillus licheniformis	1	3	0	Haydushka et al., 2012
	Lysinibacillus fusiformis	1	2	0	Wenzler et al., 2015
	Rhodococcus fascians	1	1	0	Austin et al., 2016
	Acinetobacter baylyi	1	1	0	Chen et al., 2008
	Acinetobacter radioresistens	1	1	0	Wang et al., 2019
Fungi	*Aspergillus niger*	2	31	3	
	Aspergillus fumigatus	2	24	0	
	Aspergillus flavus	2	23	1	
	Aspergillus terreus	2	2	0	
	Paecilomyces variotii	2	1	0	
	Rhizopus oryzae	2	1	0	
	Penicillium commune	1	24	4	
	Penicillium brevicompactum	1	22	4	
	Rhizomucor pusillus	1	20	0	St-Germain et al., 1993
	Penicillium digitatum	1	19	5	Oshikata et al., 2013
	Penicillium camemberti	1	17	2	
	Penicillium olsonii	1	17	5	
	Penicillium chrysogenum	1	15	4	
	Penicillium citrinum	1	14	1	Beena et al., 2021
	Penicillium glabrum	1	10	2	
	Neurospora crassa	1	9	0	
	Aspergillus tritici	1	7	0	

ht = Pathogenic for humans and vertebrates, but normally no transmission between the host groups.
Ref = reference articles that have identified the organism as potential human pathogen.
[a] Risk group classification in accordance with GESTIS database (IFA, 2023).

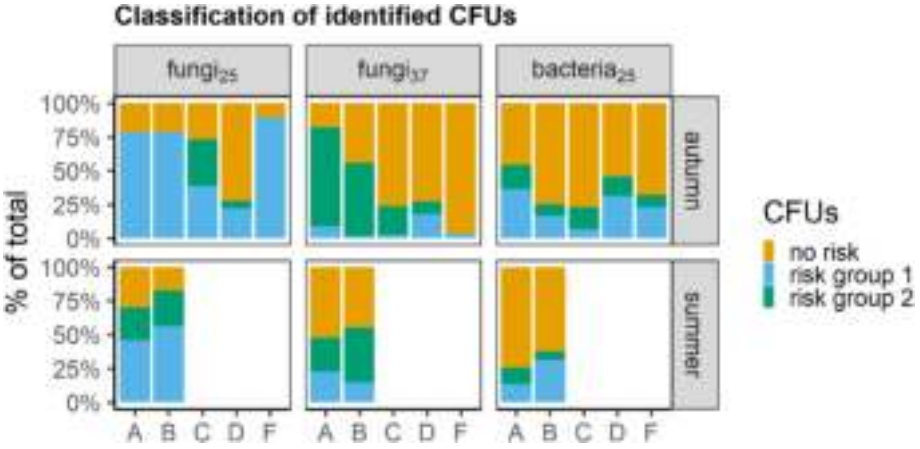

Fig. 4. Classification of identified microbial CFUs in personal air samples by WSP (A:F) and season. No risk group assigned (orange), CFUs in risk group 1 including species that are suspected of being pathogenic for humans and vertebrates in individual cases (blue), CFUs in risk group 2 (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Furthermore, differences in WSP accounted for 12% (p-value <0.001) and 22% (p-value <0.001) of the clustering of bacterial and fungal communities during summer, respectively.

3.5.1. Pathogenic species in NGS samples

A total of 838 taxa (bootstrap of 80% on species level) were identified in NGS samples. Of these, 78 were fungal taxa (13 in risk group 2) and 760 bacterial taxa (136 in risk group 2) (Table S8, Fig. S9). There was substantial variation in the composition of risk assessed microorganisms in personal filter samples between WSP and individuals (Fig. S9). The abundance of risk group 2 classified species was generally low in individual samples. However, the number of bacterial pathogens was somewhat higher at AWSP compared to MWSP, whereas fungal pathogens appeared at similar frequency.

3.5.2. Species present in MALDI and NGS

22 species (10 fungi, 12 bacteria) were identified in both MALDI and NGS samples on the species level. Seven bacterial taxa belonged to risk group 1, and five (*Acinetobacter johnsonii, Acinetobacter lwoffii, Klebsiella pneumoniae, Kosakonia cowanii, and Pantoea septica*) were classified in risk group 2. Seven of the identified fungi were in risk group 1, and three (*Aspergillus flavus, Aspergillus fumigatus,* and *Paecilomyces variotii*) risk group 2.

3.6. Microbial exposure in correlation to symptom frequency

The odds ratio (OR) for experiencing wheezing was positively and significantly correlated to increased levels of risk group 1 fungi, whereas the OR for doctor diagnosed asthma was positively and significantly associated with increased risk group 2 pathogen concentrations in personal samples (Equation (2), Table 4). The OR for exposure to risk assessed bacterial pathogens was borderline significant for coughing (risk group 2, p-value = 0.066) and nausea (risk group 1, p-value = 0.074). No significant impact of bacteria and fungi without risk classification was identified on the symptom prevalence in waste workers.

4. Discussion

This study assessed the microbial burden in personal air samples to identify potentially pathogenic species that may promote adverse health outcomes in susceptible individuals. The results show large seasonal variation in bacterial and fungal CFU concentrations within and between waste sorting plants as well as substantial variation in plant specific microbial species composition (Fig. 1, Tables S2–S4). *Aspergillus* was the most prevalent fungal genus accounting for 58% of all identified fungal CFUs, whereas dominating bacterial species belonged to the genus *Bacillus* and *Staphylococcus* accounting for 29% and 26% of all identified bacterial CFUs, respectively. Furthermore, significant correlation between self-reported symptoms as well as doctor diagnosed asthma and concentrations of risk assessed pathogens was observed in waste workers (Table 4).

4.1. Microbial concentrations in work air

The global shift towards greener societies and the sustainable use of natural resources has caused changes in national waste management regimes. In Norway, this is expressed in the segregation of household waste in different fractions, such as paper and cardboard, glass and metal, organic food waste, plastic and residual waste that are collected with different frequencies with varying time intervals. Hence, residual waste may remain in waste collection containers for up to 4 weeks before it is collected and processed. This prolonged storage contributes, especially during summer months, to accelerated microbial growth and thus to increased exposure levels during collecting and handling when microorganisms are aerosolised (Madsen et al., 2021; Viegas et al., 2016). However, concentrations of air-borne microorganisms have been reported to vary substantially regardless of environmental factors. This may be due to differences in exposure intensities during various non-comparable work tasks but also due to non-standardised sampling and evaluation protocols (Reinthaler et al., 1999).

Fungal and bacterial CFU levels presented in the current study were on average a 10-fold higher (Tables S2–S3, Fig. 1) compared to microbial concentrations reported in a Danish study that investigated occupational exposure to microorganisms in cardboard waste sorting (Madsen et al., 2019), and a Spanish exposure study in household waste sorting conducted by Solans et al. (2007). Results in the present study were comparable to levels reported for Polish waste soring plants (Szulc et al., 2022), whereas Kontro et al. (2022) reported air-borne concentrations of *Aspergillus fumigatus* and *Streptomyces* spp. measured in biowaste processing plants in Finland with concentrations as high as $10^5/m^3$ and $10^6/m^3$, respectively. However, these studies were conducted in manual WSP, comparable to plant D & F assessed in the present study. Microbial concentrations were generally higher in automated WSP (WSP A, B & C) compared to manual WSP (WSP D & F), with significantly higher bacterial CFU levels during autumn (Fig. S1). This supports results presented in a previous study that investigated exposure levels of dust, endotoxins and microbial DNA in the same population (Eriksen et al., 2023a). Seasonal variation in CFU concentrations was observed with contradicting trends at WSP A& B (Fig. 1). Summer levels were generally higher at WSP A, whereas autumn levels were higher at WSP B (Figs. S2 and S4). However, the number of autumn samples was lower in both cases. Furthermore, results from WSP A included two independent samplings during summer months, as well as samples collected during autumn. 50% of the autumn samples did not contain any viable cultivable microorganisms. This is most likely due to biases introduced during the handling of exposed filters, such as filter elution. Variance in CFU levels was generally higher between WSP than within WSP, which can be explained by differences in plant type (automated WSP versus manual WSP) (Fig. S1), observed work operations, as well as geographic location. The large variance in CFUs at plant A and B can largely be explained by the investigated work tasks. Samples with highest concentrations were collected on workers who used compressed air during cleaning operation, which has previously been reported as high exposure moment for various agents (Eriksen et al.,

Table 4
Model output generalised linear model investigating the effect of risk group levels (1, 2, no risk) on odds ratio (OR) of symptom occurrence in exposed waste workers. Only symptoms with significant (<0.05) or borderline significant (0.05–0.1) p values are shown.

	doctor diagnosed asthma		sore eyes		wheezing		coughing		nausea	
Predictor	*OR*	*p*	*OR*	*p*	*OR*	*p*	*OR*	*p*	*OR*	*p*
(Intercept)	0.008	0.002	0.081	0.001	0.022	0.001	0.032	0.001	0.042	<0.001
fungi risk group 1			1.09	0.052	1.1	**0.035**				
fungi risk group 2	1.1	**0.043**								
bacteria risk group 1									1.1	0.074
bacteria risk group 2							1.1	0.066		
Observations	40		41		41		41		41	
R^2	0.19		0.098		0.15		0.099		0.12	

2023a).

Microbial concentrations in personal air samples tended to be higher compared to outdoor samples, however, the differences were only in a few cases statistically significant (Fig. 1). This may be because the background samples were collected too close to the respective WSP in a distance of approximately 50–100 m. However, Cyprowski et al. (2021) reported significant differences in microbial concentrations between work-environmental and background samples that were collected approximately 50 m from the respective waste sorting plants, suggesting that 50 m should be enough distance between the main source of exposure in order to avoid contamination of reference samples. Furthermore, concentrations of airborne microorganisms generally increase in areas where waste with potentially high microbial content is handled (Tables S2–S4). The presence of pathogens, such as *Aspergillus* in background samples may result from transfer from the waste sorting plants to the surrounding environment and indicates that microbial contamination can be of concern for workers and residents in neighbouring areas (Schlosser et al., 2016).

4.2. Community composition and pathogenic potential of microorganisms

The assessment of the microbial community on the species level is crucial to adequately risk assess occupational exposure and potential exposure related health risks, as not all species within a genus may be pathogenic to humans (Duquenne, 2018). A total of 28 cultivable bacterial and 17 cultured fungal species with potentially pathophysiological properties were identified in personal air samples using culture-based methods in combination with MALDI-TOF MS (Table 3), whereas analysis of NGS data identified 136 biosafety level 2 pathogenic bacterial species and 13 fungal species with potentially pathogenic effects (Table S8). However, abundance of risk group 2 classified pathogens varied substantially between WSP and seasons with generally higher pathogen burden at automated WSP (A, B & C) compared to manual WSP (D & F) indicating that each WSP had characteristic microbiomes (Fig. S2:S5). These results were supported by WSP-specific clusters of bacterial communities (Figs. S6–A). Fungal taxa however, appeared to be largely shared between WSP (Figs. S6–B). Species in the genus *Aspergillus* were among the prevalent risk classified fungal pathogens observed in the present study and accounted for approximately 50% of the fungal exposure. Occupational exposure to various species in the genus *Aspergillus* has been associated with adverse health outcomes in susceptible individuals (Bafadhel et al., 2014; Bush et al., 2006; Greenberger, 2002). The presence of *Aspergillus* in the work environment has been proposed as bioindicator for pathogenic fungal exposure with toxicological potential (Sabino et al., 2019). *A.niger, A.fumigatus* and *A. flavus* were among the most abundant *Aspergillus* species, each of which is classified as risk group 2 pathogen that are associated with human respiratory disease. *A.flavus* is one of the main aflatoxin-producing fungi with carcinogenic properties (IARC, 2012) whose prevalence is suspectedly affected by climate change related increase in humidity and temperature (AssunJo et al., 2018; Viegas et al., 2015b). In addition to *Aspergillus*, a large number of species belonging to the genus *Penicillium* and *Rhizomucor* that are classified in risk group 1 was identified. Although classified in risk group 1, such microorganisms may affect the respiratory system and reduce pulmonary function in susceptible individuals (Beena et al., 2021; Oshikata et al., 2013) as well as contribute to antibiotic resistance by transferring antibiotic resistant genes to pathogenic species, as has been reported for bacteria (Jiang et al., 2017).

The most abundant cultured bacterial species belonged to the genus *Bacillus*, *Enterococcus*, *Staphylococcus* and *Streptomyces*, most of which are commonly found in soil, plant material and the natural skin-biota. Some of these were classified in risk group 2 or have been assessed as potential causative agents for human disease or opportunistic pathogens that can elicit an immune response in immunocompromised individuals (Table 3, Table S8). *Bacillus cereus* was the most abundant cultured bacterial species in personal air samples, and appears to be a common contaminant in waste as it was also reported as most prevalent species in cardboard waste sorting in a Danish study (Madsen et al., 2019). *B.cereus* has been discussed as opportunistic human pathogen and causative agent for gastrointestinal symptoms (Messelhäußer and Ehling-Schulz, 2018). The presence of *B.cereus* in combination with other human pathogens in the inhalable fraction indicates that occupational exposure during waste handling and sorting may elicit an immune response in exposed workers with compromised immune systems.

4.3. Fast emerging pathogens

The present study revealed the presence of three fungi with high pathophysiological potential in inhalable work-air samples (Table 3, Table S8). *A.fumigatus* was among the most prevalent fungal species identified in personal air samples. This mould is ubiquitous in the environment and has been reported as causative agent of pulmonary disease such as aspergillosis (Latgé and Chamilos, 2019). Due to its invasiveness and anti-fungal resistance *A. fumigatus* is classified as critical fast emerging pathogen and included the WHO's fungal priority pathogen list (WHO, 2022). Furthermore, we identified eight different species in the genus *Fusarium* that were highly prevalent in personal air samples (Table S8). Some of these filamentous fungi are predominantly found in decomposed organic material are classified as high emerging human pathogens due to their ability to cause serious infections of the respiratory tract especially in immune-compromised individuals (Nucci et al., 2021). Other members of WHO priority list, such as *Candida albicans* and *C. tropicalis* were identified at rather high frequencies in the present study. The opportunistic pathogenic yeast *Pichia kudriavzeveii* was identified in one personal air sample (Table S8). Albeit common in the human microbiome, infections of the skin or mucosa may cause serious health outcomes, such as invasive or oropharyngeal candidiasis (Coronado-Castellote et al., 2013; Pappas et al., 2018). The presence of relatively high levels of fast emerging pathogens in the work air samples implies that the waste sorting industry potentially provides the perfect breeding ground for proliferating microorganisms.

4.4. Exposure-related health outcomes

A great variety of symptoms that are related to exposure to bioaerosols have been reported in waste workers by (Schlosser, 2019; Wikuats et al., 2022). An increased prevalence of respiratory and gastrointestinal symptoms was identified among exposed waste workers in previous studies (Eriksen et al., 2023a, 2023b). The present study revealed correlations between concentrations of cultivable human pathogens in the inhalable work air and the frequency of self-reported health outcomes (Table 4). Significant correlation between symptoms of the respiratory tract, such as wheezing can be explained by high concentrations of infectious biological agents such as species in the genus *Aspergillus* and *Stenotrophomonas* (Chawla et al., 2014; Walsh et al., 2008). Symptoms such as diarrhoea and nausea have been reported in association with exposure to *Bacillus cereus*, a pathogen commonly causing gastrointestinal symptoms (Griffiths and Schraft, 2017). However, despite relatively high concentrations of *B.cereus* measured in the present study, correlations to health effects were non-significant. The prevalence of nausea was, however, borderline significantly correlated to exposure level of bacteria in risk group 1. Even though the frequency of health effects coincided with levels of infectious biological agents in the present study, the effects of non-infectious biological agents with pro-inflammatory and allergenic potential, such as endotoxins and β-glucans, remain unclear (Heldal et al., 2003; Straumfors et al., 2016). The importance of these in regards to occupational exposure in waste sorting cannot be disregarded.

4.5. Limitations of the study

Microorganisms were cultivated on a limited number of culture

media, selected temperature, and oxygen levels. Thus, only fungi and bacteria capable of growing on the provided substrate under the constraining conditions could be cultivated. The use of different culture media and growth conditions may have promoted the growth of other species, such as anaerobic bacteria. Furthermore, only a subfraction of the filter extracts was used for cultivation. Thus, it remains uncertain to what extent rare species were identified.

Identification of viable cultivable fungi at species level using MALDI-TOF MS technology was successful in 78% of all analysed CFUs, however only 42% of the analysed bacterial colonies could be identified on the species level using the reference database provided by the manufacturer. It can be assumed that the unidentified CFUs contained actinobacteria that are difficult to identify. Furthermore, the large differences in the number of unidentified CFUs in MALDI-TOF MS may be due to limitations of the reference database that predominantly included clinically relevant pathogens and may thus have underestimated the presence of potentially harmful species in the analysed work air samples.

In order to risk assess microorganisms that are contained in work air, different tools need to be considered as national and international directives for exposure to microbial agents vary considerably. This study used the GESTIS database (IFA, 2023) for classification of potential human pathogens, which is more detailed than the reference database used in Norway (Arbeidstilsynet, 2020). However, the use of other reference databases might have produced different results. This shows that there is a need for harmonised classification and legislation on microbiological exposure.

5. Conclusions

The present study contributed to increasing the knowledge on occupation exposure to microorganisms and the prevalence of potential exposure related health effects in contemporary waste sorting plants. Microbial biodiversity in personal air samples identified with NGS was higher compared to culture-based methods, indicating that the work environment contained substantial concentrations of not only infectious but also non-infections biological agents. The predominant cultured bacterial species belonged to the genus *Bacillus* and *Staphylococcus*, whereas fungal species were dominated by species in the genus *Aspergillus* and *Penicillium*. However, microbial CFU levels varied substantially between seasons and waste sorting plants. Twenty-three percent of bacterial, as well as 19% of fungal species were classified as human pathogens or biological agents that are suspected of being pathogenic to humans (Arbeidstilsynet, 2020; IFA, 2023). Large variation in the microbial community was identified between WSP as well as between seasons. Furthermore, fast emerging pathogens, such as *A.fumigatus*, were relatively abundant in personal samples. Due to its prevalence and significance as human pulmonary irritant, allergen, and toxin, *Aspergillus* has been proposed as sentinel species for occupational exposure to fungi and *Aspergillus* surveillance in the work air may provide means for occupational risk assessment and health promotion. The presence of risk group 1 assessed fungi was positively and significantly associated to the occurrence of wheezing in exposed workers. This implies that waste workers were potentially exposed to high levels of microbial organisms and metabolites thereof, with pathophysiological potential. However, the discordance between reference databases concerning pathogen classification and national legislations provide challenges in terms of risk assessment of the microbial exposure in waste sorting plants. Nonetheless, the results presented in this study indicate the need for measures to reduce exposure, such as personal protective equipment, to promote workers' health and prevent occupational disease.

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Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Author contributions

Elke Eriksen: Methodology, Investigation, Data curation, Data curation, Visualisation, Writing – original draft, Writing – review & editing.

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Declaration of competing interest

The authors have no competing interests to declare that are relevant to the content of this article.

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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.2023.114240.

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Performance of bacterial and mitochondrial qPCR source tracking methods: A European multi-center study

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ABSTRACT

With the advent of molecular biology diagnostics, different quantitative PCR assays have been developed for use in Source Tracking (ST), with none of them showing 100% specificity and sensitivity. Most studies have been conducted at a regional level and mainly in fecal slurry rather than in animal wastewater. The use of a single molecular assay has most often proven to fall short in discriminating with precision the sources of fecal contamination. This work is a multicenter European ST study to compare bacterial and mitochondrial molecular assays and was set to evaluate the efficiency of nine previously described qPCR assays targeting human-, cow/ruminant-, pig-, and poultry-associated fecal contamination. The study was conducted in five European countries with seven fecal indicators and nine ST assays being evaluated in a total of 77 samples. Animal fecal slurry samples and human and non-human wastewater samples were analyzed. Fecal indicators measured by culture and qPCR were generally ubiquitous in the samples. The ST qPCR markers performed at high levels in terms of quantitative sensitivity and specificity demonstrating large geographical application. Sensitivity varied between 73% (PLBif) and 100% for the majority of the tested markers. On the other hand, specificity ranged from 53% (CWMit) and 97% (BacR). Animal-associated ST qPCR markers were generally detected in concentrations greater than those found for the respective human-associated qPCR markers, with mean concentration for the *Bacteroides* qPCR markers varying between 8.74 and 7.22 $\log_{10}$ GC/10 mL for the pig and human markers, respectively. *Bacteroides* spp. and mitochondrial DNA qPCR markers generally presented higher Spearman's rank coefficient in the pooled fecal samples tested, particularly the human fecal markers with a coefficient of 0.79. The evaluation of the performance of *Bacteroides* spp., mitochondrial DNA and *Bifidobacterium* spp. ST qPCR markers support advanced pollution monitoring of impaired aquatic environments, aiming to elaborate strategies for target-oriented water quality management.

1. Introduction

Conservation of the microbiological quality and safety of water is critical since fecally contaminated water is a major risk to human health (WHO, 2018). Fecal pollution is responsible for remarkable economic losses because of increased hospitalizations and closure of beach and shellfish harvesting areas (Givens et al., 2006; Meschke and Boyle, 2007). Historically, members of the total coliform and fecal streptococci groups have been used as fecal indicator bacteria (FIB) to monitor the levels of fecal contamination of a waterbody (Holcomb and Stewart,

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2020). Enteric bacteriophages (such as somatic coliphages) are viral fecal indicators, which complement FIB, providing more information on the presence of pathogenic viruses (Ballesté et al., 2021, 2022). The discrimination between sources of fecal contamination is paramount for the improvement of remediation measures and identification of potential hazards (Santo Domingo et al., 2007). FIB (*Escherichia coli* (EC) and intestinal enterococci (FE)) and somatic coliphages (SOMCPH) provide valuable information on the existences of fecal contamination. However, these organisms are unable to provide details on the source as they occur in the intestines of several warm-blooded animals (Abbaszadegan et al., 1999). These characteristic limits their use on their own to distinguish from different sources of contamination (Blanch et al., 2006; Newton et al., 2011; Muniesa et al., 2012). Nevertheless, certain genotypes of F-specific coliphages, detected by RT-PCR, and bacteriophages of *Bacteroides* associated with different hosts, as well as the combination of the latter bacteriophages with somatic coliphages have been used to determine the origin of fecal contamination both performed using culture methods (Friedman et al., 2011; Muniesa et al., 2012; Ogorzaly et al., 2009).

The efficiency of fecal indicators as tools for risk assessment would improve by using different methods that are able to characterize the source of such organisms. This knowledge gap led to the development of source tracking (ST) methodologies. The improvement of molecular biology techniques allowed for a shift in the methods used in ST, from library- and culture-dependent to library-, culture-independent methods. Methods targeting eukaryotic-associated mitochondrial DNA (mtDNA) and anaerobic bacteria from the *Bifidobacterium* and *Bacteroides* genus have been developed to discriminate between human and non-human fecal contamination (Gómez-Doñate et al., 2012; Hagedorn et al., 2011; Martellini et al., 2005; Wuertz et al., 2011). Several features make *Bifidobacterium* and *Bacteroides* genus attractive to be used in source tracking studies compared to FIB. Both are able to discriminate between human and non-human fecal contamination due to adaptation to a specific animal host (Carrey et al., 2021; Derx et al., 2021; Diedrich et al., 2023; McLellan and Eren, 2014). They do not replicate in the environment and have short survival times outside the host (Fiskdal et al., 1985). Additionally, they can be found in higher numbers than EC in feces and environmental waters (Allsop and Stickler, 1985; Resnick and Levin, 1981). The methods based on eukaryotic cells, targeting the presence of mitochondrial DNA, have several advantages including being able to identify the animal species directly rather than microbial species it may host, are not affected by climate and diet and therefore are believed to be more geographically stable since host mitochondrial DNA is not affected by diet (Roslev and Bukh, 2011).

However, the application of these host-associated markers, particularly those based on bacterial targets, on a global scale might be biased since little is known on their prevalence, resistance, and abundance in the environment (Baker-Austin et al., 2010; Casanovas-Massana et al., 2015; Mayer et al, 2016, 2018; Monteiro et al., 2021; Reischer et al., 2013). A few studies have covered the prevalence, abundance and resistance of the chosen markers (Ahmed et al., 2020, 2021, 2023; Ballesté et al., 2018, 2020; Demeter et al., 2023; Harwood et al., 2017; Linke et al., 2020; Monteiro et al., 2021; Stange and Tiehm, 2020; Yahya et al., 2017) The HF183 assay was shown to have high levels of specificity and sensitivity in sewage samples, ranging from 85 to 100% in India and the USA. HF183 was also detected in human fecal samples in Ethiopian highlands with lower sensitivity (77%) (Linke et al., 2020). In the study by Yahya et al. (2017), HF183 marker was found in 100% of the target human samples in Spain, Cyprus, and Ireland compared to 57% in Tunisia, with higher concentration determined in samples from Ireland (7.07 log copies/mL) and lower in those from Tunisia (4.71 log copies/mL). This marker also showed low specificity in Spain, Cyprus and Ireland. On the other hand, the HMBif marker was detected in 100% of the human samples from the four countries, with concentrations ranging from 4.71 log copies/mL in Tunisia and 5.79 log copies/mL in Cyprus. HMBif was absent in non-human samples collected in Tunisia and Ireland, and found in 60% of the non-human samples from Cyprus. HF183 was also detected in estuarine sediment samples from Australia (39.1%) at mean concentration of 4.04 log copies/g (Ahmed et al., 2020). HF183 was detected in all wastewater samples from Australia, but was also detected in samples from cat, and dog origin (Ahmed et al., 2023). The BacR and Pig2Bac assays were found in high abundance with high levels of specificity and sensitivity in Ethiopian highlands (8.1 and up to 8.4 log marker equivalents/g of feces, respectively) with BacR being detected in soil samples during the dry season (Linke et al., 2020). BacR was detected in fecal samples from Austria, France, Canada, Israel and the USA, with high sensitivity (ranging from 90% to 100%) and at high concentrations (Mieszkin et al., 2009; Raith et al., 2013; Reischer et al., 2006; Ridley et al., 2014). Mitochondrial DNA have been tested in waters of a karst spring in Germany, with the human-targeted assay found in concentrations has high as 3.69 $\log_{10}$ copies/100 mL and in the River Tagus, at a median concentration of 4.20 $\log_{10}$ copies/100 mL (Monteiro et al., 2021; Stange and Tiehm, 2020). The bovine and poultry markers were found throughout the study conducted in the River Tagus at concentrations of 3.74 and 2.33 log copies/100 mL, respectively. In Germany, the poultry mtDNA marker was detected in higher concentrations but only found during the spring samples, and the bovine mtDNA marker remained undetected throughout the study.

HF183 showed to decay much faster than enteric pathogens, persisting for longer periods at 15 °C compared to 25 °C (Ahmed et al., 2021). A similar result was obtained by Ballesté et al. (2018), with greater decay during summer (absent after 3 days) compared to winter (absent at day 10). The study determined that *Bacteroides* spp. and *Bifidobacterium* spp. markers presented similar environmental persistence. Monteiro et al. (2021), assessed the geographical, temporal, and meteorological fluctuations of four mtDNA (human, bovine, porcine, and poultry) markers. The authors showed that the marker for bovine fecal contamination was impacted by several physico-chemical parameters, including temperature and UV radiation and the poultry-mtDNA marker was influenced by the occurrence of rainfall on the day of sampling (or 24 h prior). A recent review and meta-analysis on genetic methods for fecal pollution analysis of water (Demeter et al., 2023) also comprehensively discusses the status quo and the future research needs on the five basic biological performance characteristics ("the big five") of genetic fecal pollution markers (i.e. fecal specificity, fecal sensitivity, persistence, resistance and mobility).

This European multi-center study compared the existence of host-associated bacteria and mtDNA-based ST qPCR assays. The study was conducted with the analysis of fecal indicator bacteria parallel to the ST markers to extend the setup for the monitoring of water quality. The efficiency of a set of previously described qPCR assays targeting eukaryotic-associated mtDNA and host-associated genetic markers for *Bifidobacterium* and *Bacteroides* was assessed in a set of selected samples. Different geographical areas in Europe (Austria, Finland, Germany, Portugal, and Spain) and five fecal contamination sources (human, cow/ruminant, porcine, poultry, and equine) were covered. It is important to note that the analyzed samples included four of the most important sources of fecal contamination in Europe: human, cow/ruminant, pig, and poultry. molecular markers were (HMBif, HF183, HMMit, BacR, CWMit, Pig2Bac, PGMit, PLBif, and PLMit) (Gómez-Doñate et al., 2012; Green et al., 2014; Mieszkin et al., 2010; Reischer et al., 2006; Schill et al., 2008). The key criteria for the selection of these specific markers were: i) potential to provide quantified results as they are qPCR assays; ii) ST markers with evaluated and reported host-association; iii) previous environmental application; and iv) existence of standard operating procedures (SOP) or clear description of the determination methods.

The aim of this study was to assess the performance of host-associated genetic fecal qPCR ST markers evaluating the following criteria: i) frequency and abundance of each of the specific genetic markers in target and non-target samples (i.e. quantitative sensitivity, specificity, and accuracy); ii) geographical distribution and performance of ST markers; iii) statistical association in and between the occurrence

of the ST markers and fecal indicator bacteria. The study included animal wastewaters and human sewage samples from the different origins and animal fecal slurries (mixed fecal samples from different individuals per slurry), which is in opposition to some practices based on sampling single fecal samples from animal sources (Green et al., 2014; Mayer et al., 2018; Reischer et al., 2013), to obtain a more representative sample.

2. Materials and methods

A total of 77 samples from five relevant fecal sources of discrete origins were collected between November 2013 and September 2014 under the frame of the European Project AQUAVALENS. Samples included human sewage ($n = 23$), and samples of animal origin consisting of animal wastewaters collected from abattoirs or fecal slurries prepared by mixing feces from at least ten individuals in sterile water (cow/ruminant ($n = 17$), pig ($n = 18$), poultry ($n = 15$), and equine ($n = 4$)). Equine samples were used as control of non-targeted sources. Samples were collected in sterile sampling bottles or containers and delivered to the participating laboratories in the liquid form (Table S1 for further details). Samples were collected in three seasons (fall, winter, and spring) in five European countries: Austria, Finland, Germany, Portugal, and Spain. Raw sewage samples were collected from communities with a population between 2.100 and 3.0 million. Animal sewage was sampled from distinct abattoirs and farm animals processing between 400 and 8000 ruminant and porcine animals per day. Samples from poultry abattoirs processed close to 100,000 animals per day. A more detailed information of each sample is provided in Table S1.

Each laboratory performed the analysis for general microbial fecal indicators in their own samples (2.1) and samples to test for specific ST markers were initially sent to a single laboratory for concentration and extraction (2.2) before being distributed to the specific partner responsible for the determination of specific ST markers. Each partner was specialized in analyzing all the samples for a specific molecular marker to avoid variation among the different countries due to technical issues (Table S2). DNA samples were distributed and shipped refrigerated in the dark to the laboratory performing each specific target following standard methods from the International Standardization Organization (ISO) (ISO, 1980, ISO, 1982, ISO, 1983, ISO, 1985, ISO, 1992).

2.1. Determination of general microbial fecal indicators

Three indicators of general fecal contamination (EC, FE, and SOMCPH) were determined using standardized culture methods and all samples were run in duplicate (ISO, 2000a, ISO, 2000b, ISO, 2001). Additionally, the total number of *Bifidobacterium* spp (TBif) was determined using culture techniques (Bonjoch et al., 2005). Universal qPCR markers, AllBac (total *Bacteroides* spp.), TLBif (total *Bifidobacteria*) and FEqPCR (total intestinal enterococci), were tested (Haugland et al., 2012; Layton et al., 2006; Reischer et al., 2006) (Table S2). All the samples were tested in duplicate in qPCR assays including positive and negative controls as described in the respective assays.

2.2. Analysis of host-associated ST markers

A total of nine ST markers were tested by three laboratories (Table S2). The selected ST molecular markers chosen to conduct the study were: i) HMBif, HF183TaqmanII (HF183 hereafter), and HMMit, for human fecal contamination; ii) BacR, and CWMit for cow/ruminant fecal contamination; iii) Pig2Bac, and PGMit for porcine fecal contamination; and iv) PLBif and PLMit for poultry fecal contamination (Gómez-Doñate et al., 2012; Green et al., 2014; Mieszkin et al., 2010; Reischer et al., 2006; Schill et al., 2008). Each laboratory analyzed a set of markers assigned according to its previous expertise: IST (HMMit, CWMit, PGMit, and PLMit), TUWien (HF183, BacR, and Pig2Bac) and UB (HMBif and PLBif). Upon arrival to the laboratory, 10 mL of sample were filtered through a 0.2 μm polycarbonate filter, and the filters were folded, transferred into a 1.5 mL reaction vial and placed immediately at 4 °C (Reischer et al., 2008). Upon filtration of all samples, the filter membranes were stored at −80 °C until further processing.

DNA extraction was performed using bead-beating and phenol/chloroform as described previously (Griffiths et al., 2000; Reischer et al., 2008; Linke et al., 2021). In brief, the filters were transferred into screw vials containing glass beads and placed into a FastPrep 24 benchtop homogenizer (MP Biomedicals Inc., Irvine, CA) for 30 s at a speed of 6.0 m s^{-1} after which they were immediately transferred to ice for 1min. The samples were centrifuged at 13,000×*g* for 5 min at room temperature (rt). The supernatant (approximately 500 μL) was carefully added to 500 μL of chloroform/isoamyl alcohol (24:1) and the mixture was vortexed and centrifuged at 13,000×*g* for 5 min at rt. The aqueous phase was carefully added to 270 μL of isopropanol and the tubes were inverted for mixing. The samples were centrifuged at 13,000×*g* for 15 min at rt after which the supernatant was carefully discarded and 1 mL of ice-cold 70% ethanol was added, and the samples further centrifuged at 13,000×*g* for 5 min at 4 °C. The supernatant was discarded, the pellet dried on a thermomixer (Eppendorf, Germany) at 37 °C for 30 min, finally resuspended in 10 mmol L^{-1} TRIS buffer (pH 8.0). The DNA extracts were kept at −80 °C until qPCR analysis.

Primers and probes employed in this study were according to previously published studies (Table S3 for further information). The cycling conditions for the different qPCR assays are shown in Table S4. Standard curves were generated from genomic bacterial DNA or mitochondrial DNA, as shown in Table S2. To deal with possible inhibitory substances in the samples, 1:4 and 1:16 dilutions were analyzed in parallel with crude samples in the qPCR. Negative and positive controls were added to each qPCR experiment and the samples were analyzed in duplicate.

The samples were concentrated and extracted by the same laboratory before being distributed to the remaining laboratories and the same dilutions were analyzed by PCR to assure that the results were comparable between all markers. Additionally, several controls were conducted to assess for potential cross-contamination during the process. Blank filter controls and extraction controls were performed for each filtration/extraction date and were frozen along with the samples. For the blank filter control, an unused filter was frozen during each sample preparation and then co-extracted along with the samples. In addition, an extraction without filter (reagents only) was performed for each DNA extraction. The blank filter control provided information about potential contamination of the membrane filters, the extraction control about potential contamination of the reagents used. The Quant-it PicoGreen dsDNA Assay kit (Thermo Fischer Scientific, UK) was used for DNA quantification, following the manufacturer's instructions.

The limit of detection for the *Bacteroides* spp. markers, as determined in the present study, was 2.68 $\log_{10}$ GC/10 mL, for the mitochondrial markers was 2.00 $\log_{10}$ GC/10 mL for CWMit and PLMit and 2.20 $\log_{10}$ GC/10 mL for HMMit and PGMit. The limit of detection for PLBif was 2.16 and 2.46 $\log_{10}$ GC/10 mL, depending on the dilution tested (1:4 or 1:16) and for HMBif the limit was 2.51 and 2.81 $\log_{10}$ GC/10 mL, depending also on the dilution tested.

2.3. Data analysis

Data analysis was carried out either with R (version 4.0.5, R Core Team (2021)) or Orange (version 3.31.0, Demsar et al. (2013)). Data were transformed into decimal logarithmic after the addition of 1 to each value prior to $\log_{10}$ conversion. The normality of the data was assessed using the Shapiro-Wilk test. As data was non-normal distributed, non-parametric statistical analysis was used. Correlation coefficients between fecal indicators and ST markers were determined using Spearman's rank correlation.

The library caret (Kuhn, 2021) was used to determine the accuracy, F1 score, sensitivity, and specificity of each ST marker. Sensitivity or recall refers to the ratio of true positives (TP) between the total positives

(TP + false negatives (FN)) in the data, which is calculated as TP/(TP + FN). Specificity refers to the ratio of true negatives (TN) between the total negatives (TN + false positive (FP)) in the data that is calculated as TN/(TN + FP). The accuracy refers to the ratio of the correct predictions between the total predictions, which is calculated as (TP + TN)/(TP + FP + FN + TN). Finally, F1-score or the Sørensen–Dice coefficient is calculated as the harmonic mean of the precision (TP/(TP + FP)) and the recall and is calculated as 2× (recall x precision)/(recall + precision). Noteworthy that while accuracy gives more weight to the true positives and negatives the F1-score gives more weight to the false positives and negatives. The confusion matrix was created using the origin of the sample as the reference variable and the quantitative results of the ST markers were converted into a binary classifier, which was use as the predictive variable.

To determine if ST markers differed by country, the Kruskal-Wallis test was carried out. Additionally, the multivariate visualization approach by a FreeViz projection (Demšar et al., 2007) and the multi-dimensional scaling analysis (MDS) with PCA optimization were carried out using Orange with the same purpose.

3. Results

3.1. Indicators of general fecal contamination

Culture (EC, IE, TBif and SOMCPH) and molecular (AllBac, TLBif and FEqPCR) indicators of fecal contamination were analyzed in all samples (Fig. 1 and Table S5). The indicators of fecal contamination were determined to complement the data obtained with the host-associated

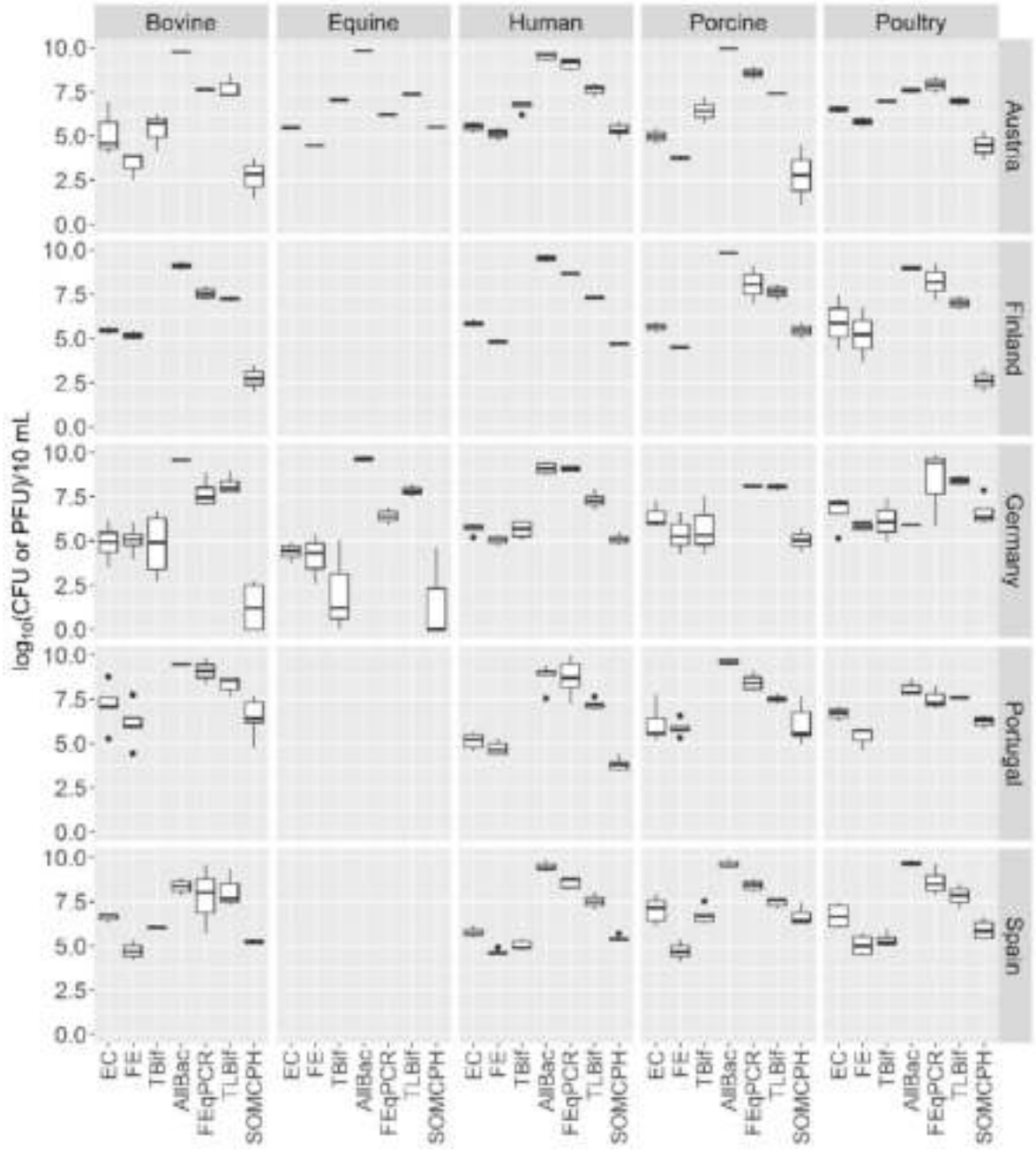

Fig. 1. Boxplot of $\log_{10}$ concentration of the general indicators of fecal contamination in the studied countries. Parameters determined by culture: EC, *E. coli*; FE, intestinal enterococci; TBif, total *Bifidobacterium* spp; SOMCPH, somatic coliphages. Parameters determined by qPCR. AllBac, total *Bacteroides* spp; FEqPCR, intestinal enterococci and TLBif, total bifidobacteria. CFU, colony forming units; PFU, plaque forming units and GC, gene copies.

ST markers, indicating the unequivocal presence of fecal contamination and possible association of certain fecal indicators with a given source of contamination. Data showed the ubiquity of these indicators in all the targeted sources of fecal contamination. EC, FE, AllBac, FEqPCR and TLBif were detected in all samples, SOMCPH and TBif showed a positive detection percentage of 95% and 99%, respectively. Table S5 shows a statistical summary of the analyzed fecal indicators grouped by their contamination origin. Statistically significant differences were observed between human and non-human samples for all fecal indicators except for FE, TBif and SOMCPH when all the samples from the different countries were pooled together (Wilcoxon-test, p-value ≤ 0.05).

3.2. ST markers abundance

All markers were highly abundant in target samples with lower abundance in non-target samples when detected (Fig. 2). The concentration of non-human associated *Bacteroides* spp., *Bifidobacteria* spp., and mtDNA genetic markers was higher in non-human targets than the concentration of human-associated markers in the corresponding human samples.

Animal-associated markers displayed in general higher concentrations than the respective human markers in their target samples. No differences were observed among the different countries in the concentrations of the markers (Kruskal-Wallis test $p > 0.05$).

The *Bacteroides* spp. qPCR markers were found in higher concentrations compared to the remaining markers for each source in target samples (Table S6). The concentration of the ST markers in target and non-target samples is presented in Fig. 2. The mean concentration of human markers was 6.43, 6.61, and 7.22 $\log_{10}$ GC/10 mL for HMMit, HMBif, and HF183, respectively. The qPCR markers found in greater

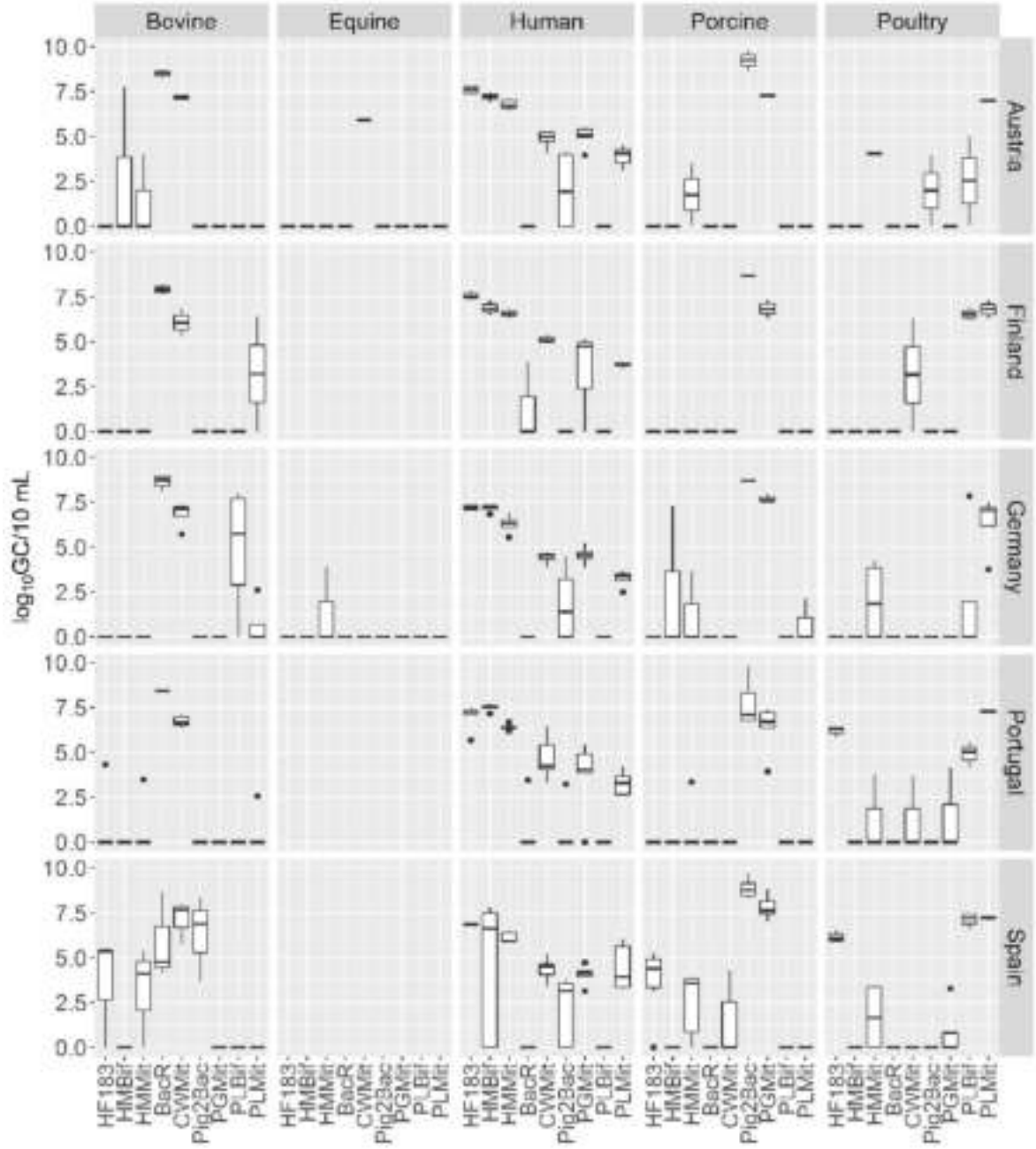

Fig. 2. Boxplot of $\log_{10}$ concentrations of host-associated markers in the different studied countries. HF183, HMBif, and HMMit, human markers; BacR and CWMit, bovine markers; Pig2Bac and PGMit, porcine markers; PLBif and PLMit, poultry markers. GC, gene copies.

abundance were those targeting pig fecal contamination at concentrations of 8.74 $\log_{10}$ GC/10 mL for Pig2Bac and 7.21 $\log_{10}$ GC/10 mL for PGMit. The lowest concentration was determined for PLBif at 4.61 $\log_{10}$ GC/10 mL (Table S6).

3.3. Specificity and sensitivity of host-associated ST markers in target and non-target animal and human sewage samples

All ST markers were practically ubiquitous in target samples, with the lowest sensitivity determined for the bifidobacterial markers with frequencies of detection of 91% and 73% for HMBif and PLBif, respectively (Table 1). Altogether, the specificity displayed a broader distribution among the markers, with mtDNA qPCR markers presenting the highest percentage of false-positive results (specificity ranging between 65% for HMMit to 53% for CWMit), albeit showing high sensitivity. HMBif was the marker with the highest specificity for human source (96%), whereas BacR and PLBif showed a specificity of 97% and 95% for their respective sources. Bifidobacterial markers displayed low levels of false-positive results, but the few positive samples exhibited concentrations similar to those in target samples. In terms of binary data, mitochondrial qPCR markers performed less than desirable with high sensitivity but relatively low specificity. Therefore, in terms of accuracy the markers that performed the best were HMBif, BacR, Pig2Bac and PLBif for the human, bovine, porcine and poultry fecal source detection, respectively.

3.4. Statistical associations of host-associated ST markers to general indicators of fecal pollution and between host-associated ST markers

Correlation data analysis was performed to determine the quantitative relationship between the indicators of fecal contamination (culture and qPCR-based) and the source-associated markers in target samples. Data showed that most source-associated markers had little to no correlation with the fecal indicators measured by either qPCR or the cultivable indicators (Table 2). Among cultivable indicators, correlation was only detected between EC and SOMCPH (rho = 0.69). Correlation between the different markers targeting the same fecal source was also observed for the human HMBif, HF183 and HMMit (rho >0.69), bovine BacR and CWMit (rho = 0.75), and porcine Pig2Bac and PGMit (rho = 0.7). A lower correlation coefficient was found between the poultry PLBif and the PLMit marker (rho = 0.4).

3.5. Geographic distribution of ST markers

As shown in Fig. 3A and Fig. S1, when classes were defined according to their country of origin, FreeViz and MDS analyses provided a misclassification when ST markers were used. This misclassification is displayed in both plots, in which samples from different countries are set near each other whereas some samples of the same country are located further apart. Results were quite different when ST markers were applied in the classification of the source of the contamination (Fig. 3 (B and C) and Fig. S1). When classes were defined according to their source

Table 1
Sensitivity and specificity of MST qPCR assays in the corresponding host-target samples.

MST Marker	Accuracy	F1-Score	Sensitivity	Specificity
HF183	0.805	0.754	1.000	0.722
HMBif	0.948	0.913	0.913	0.963
HMMit	0.753	0.708	1.000	0.648
BacR	0.974	0.944	1.000	0.967
CWMit	0.636	0.548	1.000	0.533
Pig2Bac	0.831	0.735	1.000	0.780
PGMit	0.701	0.610	1.000	0.610
PLBif	0.909	0.759	0.733	0.952
PLMit	0.649	0.526	1.000	0.565

Table 2
Spearman's correlations between studied parameters. Spearman rho coefficients are indicated in bold font (values higher than 0.6 are highlighted) whereas *p*-values are indicated in italics.

	HF183	HMBif	HMMit	BacR	CWMit	Pig2Bac	PGMit	PLBif	PLMit	EC	FE	TBif	AllBac	FEqPCR	TLBif	SOMCPH
HF183		0.70	0.79	−0.30	0.15	−0.10	0.23	−0.07	0.48	−0.04	−0.09	0.06	−0.35	0.36	−0.29	0.09
HMBif	*0.000*		**0.69**	**−0.20**	**0.23**	**−0.13**	**0.24**	**−0.30**	**0.29**	**−0.24**	**−0.08**	**0.05**	**−0.29**	**0.27**	**−0.21**	**−0.24**
HMMit	*0.000*	0.000		**−0.28**	**0.18**	**−0.05**	**0.24**	**−0.32**	**0.35**	**−0.15**	**−0.08**	**0.02**	**−0.32**	**0.34**	**−0.29**	**−0.06**
BacR	*0.009*	0.087	0.014		**0.75**	**−0.27**	**−0.55**	**−0.02**	**−0.38**	**−0.01**	**0.00**	**−0.16**	**0.24**	**−0.03**	**0.24**	**−0.22**
CWMit	*0.179*	0.040	0.109	0.000		**−0.35**	**−0.37**	**−0.14**	**−0.19**	**−0.18**	**−0.14**	**−0.04**	**0.00**	**0.04**	**0.03**	**−0.32**
Pig2Bac	*0.370*	0.260	0.650	0.018	0.002		**0.70**	**−0.32**	**−0.44**	**0.18**	**−0.09**	**0.37**	**0.19**	**0.03**	**−0.04**	**0.25**
PGMit	*0.040*	0.036	0.033	0.000	0.001	0.000		**−0.38**	**−0.22**	**0.09**	**−0.09**	**0.31**	**0.09**	**0.17**	**−0.14**	**0.23**
PLBif	*0.519*	0.008	0.005	0.880	0.209	0.004	0.001		**0.44**	**0.11**	**0.13**	**−0.19**	**−0.04**	**−0.07**	**0.05**	**0.03**
PLMit	*0.000*	0.010	0.002	0.001	0.099	0.000	0.052	0.000		**0.12**	**0.25**	**−0.03**	**−0.36**	**0.22**	**−0.13**	**0.09**
EC	*0.716*	*0.039*	*0.185*	*0.936*	*0.121*	*0.120*	*0.438*	*0.336*	*0.312*		**0.42**	**0.40**	**0.15**	**0.25**	**0.30**	**0.69**
FE	*0.457*	*0.498*	*0.477*	*0.992*	*0.208*	*0.433*	*0.439*	*0.255*	*0.030*	*0.000*		**0.19**	**0.08**	**0.36**	**0.30**	**0.38**
TBif	*0.703*	*0.713*	*0.888*	*0.255*	*0.775*	*0.009*	*0.030*	*0.180*	*0.818*	*0.004*	*0.183*		**−0.09**	**0.13**	**0.02**	**0.27**
AllBac	*0.002*	*0.010*	*0.004*	*0.036*	*0.976*	*0.105*	*0.414*	*0.738*	*0.001*	*0.196*	*0.505*	*0.527*		**0.31**	**0.44**	**0.20**
FEqPCR	*0.001*	*0.018*	*0.003*	*0.809*	*0.755*	*0.767*	*0.150*	*0.526*	*0.054*	*0.029*	*0.002*	*0.352*	*0.006*	*0.547*	**0.07**	**0.36**
TLBif	*0.011*	*0.063*	*0.010*	*0.039*	*0.797*	*0.753*	*0.222*	*0.635*	*0.271*	*0.007*	*0.008*	*0.913*	*0.000*	*0.001*	*0.042*	**0.23**
SOMCPH	*0.457*	*0.037*	*0.590*	*0.050*	*0.005*	*0.026*	*0.041*	*0.826*	*0.461*	*0.000*	*0.001*	*0.056*	*0.089*	*0.001*		0.09

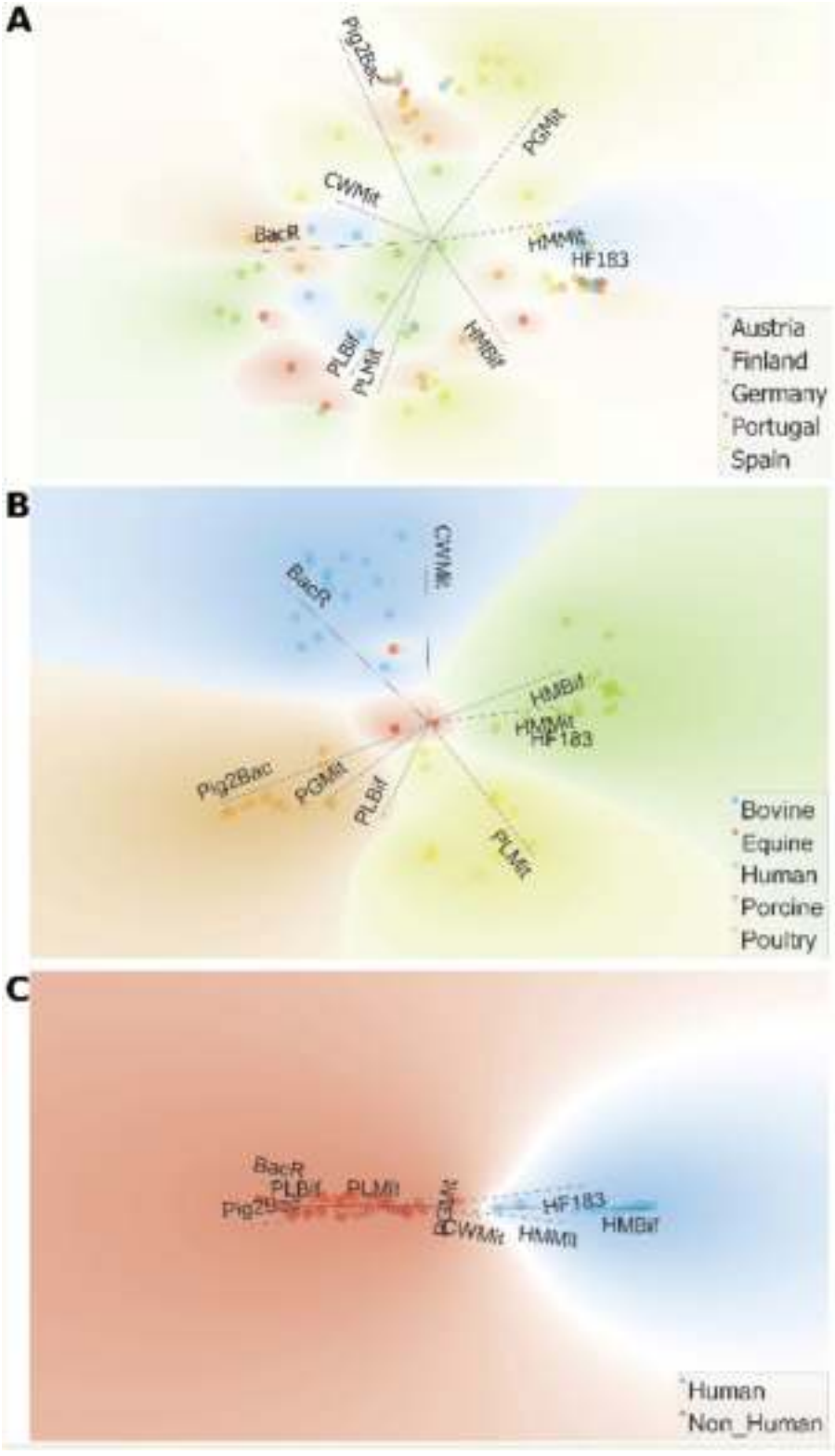

Fig. 3. FreeViz of MST markers. A) represents classifications of samples by country, B) represents the classification of samples by origin of the fecal contamination and in C) is represented the classification of the samples by human or no-human. Individual samples are represented as points and classified according to their geographical origin (countries in plot A) or their origin of contamination (plots B and C). Vectors represent the ST markers, and the colored areas of the graph show the level of influence of the classes. For example, in Fig. 3A, samples from different countries appear mixed; in contrast, when samples were categorized according to the origin of contamination, samples were clearly separated (Fig. 3B and C). In addition, the colored areas of the graph are well defined, and boundaries between classes are well defined. Note that in Fig. 3B, although no horse-specific (equine) marker was used, samples with this fecal origin were separated from the others.

of pollution samples were correctly classified. Similarly, for human *vs* non-human a correct classification was observed, Noteworthy, all equine samples were almost correctly classified in both analyses although no ST markers were specific for this origin, which indicates that the other origins were correctly classified.

4. Discussion

4.1. Indicators of general fecal contamination

A wide variety of fecal indicators were analyzed in this study. Fecal indicators/markers are important in routine monitoring as they provide information on the existence and loads of fecal contamination in a

waterbody, with water quality monitoring being mainly based on the detection of EC and IE. Although they provide important knowledge, they have several drawbacks including the fact that they are capable of replicating in environmental reservoirs (Byappanahalli et al., 2012; Desmarais et al., 2002) and do not provide reliable information on the source of fecal contamination since they are part of the natural flora of warm-blooded animals (Field and Samadpour, 2007). Somatic coliphages, which have been included in latest regulations, do not replicate in the environment but as with bacterial indicators they are not able to distinguish between different sources of fecal pollution on their own (Jofre, 2009). In our study, indicators of general fecal contamination (culture and PCR-based) were detected in all samples, regardless of the source.

4.2. Overall performance of ST markers in animal integrated fecal samples or wastewater samples and human sewage samples

The performance of ST markers is based on different parameters: i) binary specificity; ii) binary sensitivity; iii) accuracy; and iv) quantitative sensitivity.

This work demonstrated that the ST markers were ubiquitous throughout the different regions of Europe. The *Bacteroides*-specific assays provided high levels of sensitivity and, generally, high levels of specificity. The HF183 marker showed the lowest binary specificity whereas the BacR assay had the highest specificity. Nshimyiamana et al. (2017) determined for the HF183 marker a specificity of 88% whereas Ahmed et al. (2019) described a specificity of 96%. Boehm et al. (2013) reported specificity levels of more than 85%. In these studies, incorrect source identification occurred mainly in chicken fecal samples. Green et al. (2014) found false positives among fecal samples of chicken, turkey and dog origin. In our study, false positive results for the HF183 marker occurred for all non-human fecal sources tested (bovine/ruminant, pig, and poultry). However, it is noteworthy that false positives were detected only in samples from Southern European countries. Conversely, the binary sensitivity for HF183 in our study was higher that the reported in the above-mentioned studies. The specificity for BacR assay was similar or even higher to that previously reported likely due to the usage of single fecal samples in these studies (Haramoto and Osada, 2018; Reischer et al., 2013). Pig2Bac showed similar binary results to those reported in Japan (Haramoto and Osada, 2018), but still lower than that reported in the original publication (Mieszkin et al., 2010). A performance trial on different ST markers showed that Pig2Bac had high sensitivity (100%) but low specificity (ranging between 40% and 100%) with three laboratories reporting specificities below 80% (Boehm et al., 2013).

The tested *Bifidobacterium* markers showed high levels of specificity. Furthermore, HMBif had high sensitivity levels. The sensitivity and specificity of mitochondrial DNA markers differed depending on the source of fecal pollution. All mtDNA markers showed high levels of sensitivity. However, they showed less than desirable binary specificity. Schill and Mathes (2008) reported 100% of specificity for the mitochondrial markers but lower sensitivity for the cow and chicken markers (50%). In our study, mitochondrial markers were capable of correctly assigning the origin of fecal contamination in target samples, but showed high levels of interspecies cross-reactivity. Mitochondrial markers are known to be present in non-target samples, mainly because of interactions between humans and animals, either through consumption of animal meat or through farming or between the animals themselves. It has been previously demonstrated that dietary carryover is frequent in human samples, albeit at lower abundance indicating that mitochondrial markers can still be useful in discriminating between sources of fecal contamination (Caldwell et al., 2007; U.S. Geological Survey, 2007). Additionally, samples may become cross-contaminated with mitochondrial DNA from other sources through various pathways. For instance, Portuguese chicken sewage samples were collected from an abattoir that manually stuffed several of the birds with bacon to provide a better taste. Thereafter, the detection of pig and human contamination in this type of samples or for instance in the Spanish pig samples that were collected from the washes of the trucks, which carried pigs, may have had implication on the results, with possible cross-contamination with different mitochondrial DNA sources.

Specificity may introduce a bias in the decision of appropriate ST qPCR markers since it is highly dependent upon several factors including the limit of detection of the assays, the dataset itself and the quantitative difference between the concentrations of the ST markers in target and non-target samples (Boehm et al., 2013; Reischer et al., 2013). To overcome the potential bias that binary sensitivity and specificity introduce in the use of ST markers, this study was careful to choose only ST assays that were able to provide quantification.

The distances found for the distribution of true- and false-positives for the *Bacteroides* spp. markers were much larger than those previously determined (Reischer et al., 2013). This may have resulted mainly from the type of sample analyzed in this study compared to Reischer et al. (2013). In the latter study, only fresh, individual fecal samples were tested whereas in our study, sewage and fecal slurries from different origins were analyzed. In such type of samples, the dilution and aging of the markers are extremely important factors that must be considered, and which may have influenced the results obtained in our study. Concomitantly, the distances in the distribution of true- and false-positives for the mitochondrial markers was also found to be large, although less than the distances found for the *Bacteroides* spp. markers. On the other hand, the *Bifidobacterium* spp. markers although displaying high levels of specificity (96% for HMBif and 95% for PLBif) where positive results were found in non-target samples, the concentrations were on pair with the concentration in target samples.

All the markers/assays chosen in our study were already tested in different countries showing variable results. For example, in China similar values of HF183 in sewage samples were observed (He et al., 2016). Lower values were detected in Australia, Tunisia or Singapore (around 6 $\log_{10}$ GC/10 ml) (Ahmed et al., 2015; Yahya et al., 2017), whereas higher values (around 8 $\log_{10}$ GC/10 ml) were detected in Ireland (Yahya et al., 2017). Differences were also found in the ratios between HF183 and HMBif markers compared to other countries. In our study, HF183 displayed a slightly higher concentration than HMBif, whereas in another study the concentration of HF183 surpassed in more than 1-log the concentration of HMBif in European countries (Yahya et al., 2017). In our study, the concentration of all *Bacteroides* marker was mostly 1–2 $\log_{10}$ GC/10 mL above the concentration of the respective mtDNA markers, with similar concentration to the human-bifidobacterial marker. Similar results were obtained by He et al. (2016).

4.3. Statistical associations of host-associated ST markers

The ST assays demonstrated low correlations to the indicators of fecal contamination, regardless of the detection method used. A positive correlation was determined for the pair HF183 and HMMit (rho = 0.79). Additionally, more associations were found when considering the entire dataset indicating that these markers are quite similar in their behavior. The findings from this collaborative study are noteworthy in that they indicate that the *Bacteroides* population and mitochondrial DNA sequences detected are stable regardless of the regions where samples were collected.

The definition of host-associated microorganisms is extremely difficult since the normal animal gut microbiome is a concoction of microorganisms estimated at 10^{10}-10^{12} microbial cells/g feces and 300–500 bacterial species (Caldwell et al., 2011; Guarner and Malagelada, 2003). While *Bacteroidetes* performs consistently well as an animal-associated marker, very few host-associated bacterial genetic markers have been identified (Caldwell et al., 2011). The addition of new and distinct markers such as mitochondrial DNA and *Bifidobacteria*, displaying a good degree of specificity and sensitivity would increase the efficiency

of determining the sources of fecal pollution.

4.4. Geographical distribution of the ST qPCR assays

The qPCR markers chosen for this study showed no particular geographical patterns in the target samples. Although the number of studies conducted in a broad geographical area are scarce, data obtained for BacR assay are in accordance with those previously published (Reischer et al., 2013) with the remaining *Bacteroides* markers having been tested separately in other parts of the globe (Green et al., 2014; Mieszkin et al., 2010; Haramoto and Osada, 2018; Venegas et al., 2015; Gourmelon et al., 2010; Boehm et al., 2013; Malla et al., 2018; Ahmed et al., 2019). Studies have already demonstrated that the prevalence, abundance, and constitution of the bacterial population are deeply impacted by the distinct dietary regime, the host age and by different climates (Reischer et al., 2013; Benni et al., 1989; Moore and Moore, 1995; Puig et al., 1999; Shanks et al., 2011; Arboleya et al., 2016; Nagpal et al., 2017). The behavioral difference among the qPCR assays underlines the necessity to analyze carefully and with awareness the quantitative patterns of the markers when trying to apply and confirm the performance of such ST assays. It should be mentioned once again that this study was focused on the detection of ST qPCR assays in sewage samples from five different origins and that they were treated as discrete sources not contemplating the possibility of external sources of fecal contamination. Nonetheless, the choice of ST markers should be based on the specific situation and the conditions present in a particular area impairing the creation of a threshold level for the general performance of a marker in ST studies (Harwood and Stoeckel, 2011; Belanche and Blanch, 2011). Specific considerations or even modeling efforts including certain characteristics such as fecal specificity, fecal sensitivity, target abundance, aging and persistence of the markers in the environment or geographical stability is crucial in determining if a definite source identification can be achieved for certain fecal pollution problems (Belanche and Blanch, 2011; Reischer et al., 2011; Derx et al., 2016; Ballesté et al., 2020; Derx et al., 2021).

5. Conclusions

This collaborative study led to encouraging findings since they indicate that the ST markers evaluated are suitable for a reliable quantitative source tracking at the European level. The markers usually performed at a high level with HMBif showing the highest accuracy for human markers, Pig2Bac for porcine, BacR for bovine markers and PLBif for poultry markers. The remaining markers also proved to be ubiquitous in the investigated European region. Several characteristics such as environmental persistence and resistance of the markers, the interactions with the remaining communities and the resistance of the markers populations during sewage treatment should be accounted for in the study design (Reischer et al., 2008, 2011; Ballesté et al., 2018).

Improvements in qPCR methodologies have generated a set of new ST markers. In this study, most of the selected bacterial ST molecular markers for human (HMBif), ruminant and porcine fecal contamination showed extremely high levels of specificity and sensitivity. PLBif marker and the mitochondrial markers may still require further improvements given the high variation observed. What is more important is that the use of a specific set of ST markers to a new area should be validated against fecal samples before being used in field studies. Altogether, data from this study gives specific indications that the markers tested may be used beyond regional level and that a toolbox approach (considering the usage of more than a single ST assay for a specific source) may be useful when multiple sources of fecal contamination are expected in the catchment area.

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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.2023.114241.

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Relationships between growth indicators, liver and kidney function markers, and blood concentrations of essential and potentially toxic elements in environmentally exposed young children

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ABSTRACT

Environmental exposure to multiple metals and metalloids is widespread, leading to a global concern relating to the adverse health effects of mixed-metals exposure, especially in young children living around industrial areas. This study aimed to quantify the concentrations of essential and potentially toxic elements in blood and to examine the potential associations between multiple elements exposures, growth determinants, and liver and kidney function biomarkers in children living in several industrial areas in Dhaka, Bangladesh. The blood distribution of 20 trace elements As, Ag, Bi, Br, Cd, Co, Cr, Cu, I, Mn, Hg, Mo, Ni, Pb, Se, Sb, Tl, V, U, and Zn, growth determinants such as body mass index and body fats, blood pressure, liver and kidney injury biomarkers including serum alanine aminotransferase and alkaline phosphatase activities, serum calcium, and creatinine levels, blood urea nitrogen, and hemoglobin concentrations, and glomerular filtration rate were measured in 141 children, aged six to 16 years. Among these elements, blood concentrations of Ag, U, V, Cr, Cd, Sb, and Bi were measured below LOQs and excluded from subsequent statistical analysis. This comprehensive study revealed that blood concentrations of these elements in children, living in industrial areas, exceeded critical reference values to varying extents; elevated exposure to As, Pb, Br, Cu, and Se was found in children living in multiple industrial areas. A significant positive association between elevated blood Tl concentration and obesity ($\beta = 0.300$, $p = 0.007$) and an inverse relationship between lower As concentration and underweight ($\beta = -0.351$, $p < 0.001$) compared to healthy weight children indicate that chronic exposure to Tl and As may influence the metabolic burden and physical growth in children. Concentration-dependent positive associations were observed between the blood concentrations of Cu, Se, and Br and hepatic- and renal dysfunction biomarkers, an inverse association with blood Mo and I level, however, indicates an increased risk of Cu, Se, and Br-induced liver and kidney toxicity. Further in-depth studies are warranted to elucidate the underlying mechanisms of the observed associations. Regular biomonitoring of elemental exposures is also indispensable to regulate pollution in consideration of the long-term health effects of mixed-elements exposure in children.

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1. Introduction

Exposure to metals and metalloids (referred to hereafter as "metals") from natural and anthropogenic sources is widespread, because of their ubiquitous occurrence in various environmental media such as air, dust, soil, water, and diets (Zeng et al., 2015; Briffa et al., 2020). Mining, smelting, foundry work, the combustion of fossil fuels, and the extensive application of metals in a range of industries have increased the release of metals into the environment, leading to global concerns about potential health risks owing to metal pollution (Dos Santos et al., 2018; Briffa et al., 2020).

The liver and kidneys are the primary targets of metal toxicity, owing to their key roles in xenobiotic metabolism, the excretion of metal ions, and the homeostatic regulation of metal concentrations (Jaishankar et al., 2014; Lentini et al., 2017). Non-essential metals, such as arsenic (As), cadmium (Cd), lead (Pb), and mercury (Hg) are well-known environmental toxicants in man. Acute or chronic exposure to these metals may disrupt cellular events, cell growth and differentiation, and can be detrimental to normal liver and kidney function (Lentini et al., 2017; Orr and Bridges, 2017; Balali-Mood et al., 2021). Epidemiological studies suggest an association of Pb and Cd exposure with abnormal liver functions, and renal dysfunction in the adult population (Chen et al., 2019a, 2019b). Exposures to Pb and Cd, and in combination with Hg and Cu, have been reported to increase the serum alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activities, reduce haemoglobin concentrations, and total serum protein levels in animals (Al-Attar, 2011; Tomaszewska et al., 2015). Nephrotoxicity due to exposure to these metals along with As, chromium (Cr), and uranium (U) have been characterised by reduced glomerular filtration rate (GFR), and elevated serum urea and creatinine levels (Soderland et al., 2010; Pollack et al., 2015; Orr and Bridges, 2017). Exposure to metals in early life, even at a low environmental concentration, is a specific concern since children are known to be more susceptible to metal toxicity than adults, are particularly sensitive owing to developing organs and biologic systems, and such toxicity can be associated with life-long disease and disability (de Burbure et al., 2006; Landrigan et al., 2018).

Some trace elements, including zinc (Zn), copper (Cu), cobalt (Co), Cr, iodine (I), manganese (Mn), molybdenum (Mo), and selenium (Se), are essential for the growth and development and to maintain normal physiological processes (Organization, 1996; Tanvir et al., 2020). In children, deficiency or excessive exposure to these minerals may result in adverse developmental outcomes, such as reduced somatic growth, poor neurobehavioral performance, impaired immune functions, endocrine dysfunctions, and/or altered kidney functions (Oulhote et al., 2014; Vidal et al., 2014; Nascimento et al., 2016; Signes-Pastor et al., 2021; Skogheim et al., 2021). The toxicity mechanisms include the inhibition of enzyme activity, protein synthesis, genomic instability, and/or altered cell membrane permeability *via* oxidative stress (Vidal et al., 2014; Briffa et al., 2020; Balali-Mood et al., 2021).

Although many studies have investigated the effects of metal exposure in man, the majority of the research, however, has been conducted in the occupationally exposed adult population. Moreover, the studies have primarily examined the effects of single, toxic element exposure (such as As, Pb, Hg, or Cd). Information on the effects of multiple metals exposure, however, in combination with other elements [such as Mn, Co, Ni, Cu, Zn, Se, bromine (Br), Mo, I, or thallium (Tl)] in young children, are lacking. This is of key concern for those children and adolescents who may work at and also live in close proximity to industrial sites. The urinary elemental concentrations in children have been used as biomarkers in some epidemiological studies (Roca et al., 2016; Dos Santos et al., 2018; Sanchez et al., 2018). The distribution of some elements such as Pb, Hg, Cu, Zn, or Se, in body fluids, may, however, limit the optimal utility of their urinary measurements, reflecting recent exposure.

The present study, therefore, aimed to measure 20 elements, including essential and non-essential elements in blood samples from children living in industrialised areas, and to examine the effects of multiple metals exposure on hepatic and renal function in this cohort. The study also evaluated the contributions of some demographic and morphometric characteristics including muscular growth, blood pressure, and food-related determinants on blood elemental distribution in children. In addition to the non- and essential elements, to the best of our knowledge, this study is the first comprehensive research to measure the blood Br concentration in young children and to assess its utility as a biomarker for effects on hepatic and renal dysfunctions.

2. Materials and methods

2.1. Study area

A cross-sectional study was carried out in three municipalities (Gazipur, Hazaribagh and Palash) located in Dhaka, Bangladesh between November 2019 and March 2020. The sampling areas were purposely selected considering different industrial and economic activities described previously (Tanvir et al., 2021b) as follows. The Gazipur district (23°59′20″N 90°22′30″E) is dominated by the textile industries, and is identified as a hub of the garments industry of Bangladesh. The Hazaribagh Thana (23°44.1′N 90°22.2′E) is an old tannery industrial area in Dhaka city, located near Buriganga River. It is also a place of tourism, with traditional buildings and cultural sites, and is also one of the most densely populated places on earth. With access to Shitalakshya River, the Palash Upazila (23°57′N 90°37.5′E) is also a popular tourism venue and is another well-established industrial area of Bangladesh (Banglapedia, 2015). Prior to sampling, an environmental pollution awareness program was carried out by the health professionals and educators of elementary schools in each study area. The program described the source of the toxic metals and their adverse health effects and the importance of biomonitoring of metals and metalloids in local children to ensure appropriate growth and development. This educational program was aimed at engaging the parents/carers of the children who had been living in the area for more than three years.

2.2. Study participants

A total of 153 children, aged between six and 16 years were enrolled from the elementary schools at each study area. The participant's recruitment was restricted to healthy children with no pre-existing medical conditions and those who had been living in the area for more than three years. The children were not users of vitamin or mineral supplements and/or other medications during the preceding month of study. A printed consent form describing the study's aims and protocols and a questionnaire related to socio-demographic characteristics and food frequency were provided to the children's parents or carers. Data related to age, sex, education level, geographical location, occupation, known history of liver, kidney, or heart disease or endocrine disorders, frequencies of meals (regular and irregular), and frequencies of consumption of food items such as rice, cereals, vegetables, fruits, egg, poultry meat, and red meat products, fish, and fish products (regular and irregular) were collected. The weight of the children was measured using an electronic balance and the height, waist, and hip circumference were recorded. The standard age- and sex-specific body mass index (z-BMI – kg/m^2) of the children was calculated using BMI cut-points based guidelines from CDC, body weight in kg and divided by the (square of height in metre). Blood pressure (BP) for each participant was determined using a calibrated digital sphygmomanometer. Before measurement, the children were seated calmly and relaxed with their right arm resting at the heart level. The width of the cuff covered at least two-thirds of the upper right arm. The measurement of BP was repeated at least twice to avoid any abnormal high readings recorded. The study and questionnaire were ethically approved by the Biosafety, Biosecurity and Ethical Committee of Jahangirnagar University (Reference: BBEC, JU/M 2019 (12) 5). The study also received approval from the Ethical

Committee of Queensland Health Forensic and Scientific Services, Queensland, Australia.

2.3. Blood collection and serum preparation

Blood samples of 5.0 mL were collected in the morning from each participant from the cephalic or median cubital veins by trained medical staff. A Vacutainer plain tube (Becton-Dickinson, USA) containing sodium heparin as anticoagulant was used to collect whole blood for the metals and haemoglobin estimation. The samples were stored at 4 °C and transported to the Biochemistry Department, Gono University, Dhaka for immediate processing. Whole blood samples were stored at −20 °C initially and the frozen samples were shipped in dry ice to Australia and stored at −80 °C at the University of Queensland for elemental analysis using ICP-MS (see below). To assess liver and kidney function, serum samples were obtained from whole blood samples collected in Vacutainer tubes containing clot activator (Becton-Dickinson, USA), without anticoagulant, and following centrifugation at 3000 rpm for 10 min. The serum samples were stored at −20 °C until analysis.

2.4. Biochemical evaluation

The collected serum was used to estimate hepatic marker enzymes including alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activities, total albumin, total calcium (Ca) levels, and the kidney markers, creatinine, and blood urea nitrogen (BUN) levels, using standard assay kits (HUMAN GmbH, Germany) on a calibrated Humalyzer 300 auto analyser, (HUMAN GmbH, Germany). The total haemoglobin level in whole blood was estimated using standard assay kits (HUMAN GmbH, Germany). The estimated glomerular filtration rate (eGFR) was calculated to assess kidney function using CKD-EPI creatinine equation, based on the Schwartz formula (Schwartz and Work, 2009): eGFR = 0.413 × (height/serum creatinine), where height was expressed in centimetres and serum creatinine level was expressed in mg/dL.

Table 1
Characteristics of the study participants (n = 141).

(a) Demographic, anthropometric and health parameters	Mean ± SD or N (%)
Females	87 (61.7)
Age (y)	10.10 ± 2.29
6–12 y	120 (85.1)
13–16 y	21 (14.9)
z-BMI (kg/m^2)	18.0 ± 6.2
Healthy weight	77 (54.6)
Underweight	29 (20.6)
Overweight	13 (9.2)
Obese	22 (15.6)
Waist circumference (cm)	56.57 ± 8.92
Hip Circumference (cm)	67.52 ± 9.72
WHR	0.84 ± 0.04
SBP (mm Hg)	89 ± 10
DBP (mm Hg)	57 ± 8
Normal BP	114 (80.9)
Low BP	21 (14.9)
High BP	6 (4.3)
(b) Food consumption (regular)	**N (%)**
Meal frequency	131 (92.9)
Egg	104 (73.8)
Poultry meat	133 (94.3)
Red meat	102 (72.3)
Rice, fish, and vegetables	141 (100)
(c) Kidney and liver function parameters	**Mean ± SD (% above or below reference range)**
S.ALT (U/L)	20.0 ± 7.5 (0.7)[a]
S.ALP (U/L)	381 ± 256 (97.2)[a]
S.Albumin (g/dL)	3.60 ± 0.55 (39.0)[b]
S.Calcium (total) (mg/dL)	6.9 ± 0.8 (98.6)[b]
S.Creatinine (mg/dL)	0.73 ± 0.13 (48.2)[a]
BUN (mg/dL)	7.90 ± 1.45 (2.8)[b]
Haemoglobin (g/dL)	8.60 ± 0.73 (95.0)[b]
eGFR (mL/min/1.73 m^2)	78 ± 18 (69.5)[b]

Abbreviations: SD: standard deviation, BMI: body mass index, WHR: Waist-to-hip ratio, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, BUN: Blood urea nitrogen, S.: serum, ALT: alanine aminotransferase, ALP: alkaline phosphatase, eGFR: estimated glomerular filtration rate, a indicates value above reference range and b indicates a value below the reference range. Reference ranges are provided in Table S3.

2.5. Trace elements analysis

Blood concentrations of 20 trace elements: antimony (Sb), As, bismuth (Bi), Br, Cd, Cr, Co, Cu, I, Pb, Mn, Hg, Mo, Ni, Se, silver (Ag), Tl, U, vanadium (V), and Zn were determined using an Agilent 7700x ICP-MS (Inductively coupled plasma-mass spectrometry, Agilent Technologies Ltd, USA). The details of the analytical method and quality control (QC) processes are described in our previous study (Tanvir et al., 2021a). Briefly, 0.25 mL of blood sample was transferred into a polypropylene tube containing an alkaline solution (0.5 mL). The sample solution was vortex mixed for 5 s and then made up to 5.0 mL using deionised water (18.2 MΩ cm) and submitted for ICP-MS analysis. The sample analysis was quality assured by measuring elemental concentrations in certified reference materials, CRM (Seronorm Trace Elements Whole Blood L-1 and L-2), and spiked human blood samples (including those from children). The blood sample collection tubes and pre-acid-washed polypropylene tubes were checked for elemental contamination in each batch of sample analysis; their contributions to elemental concentrations of interest were negligible. The mean recovery values measured for the metals under review were in the acceptable ranges of CRM values (Table S1). The limit of detection (LOD) and limit of quantification (LOQ) were calculated as three times and nine times the standard deviation of seven sets of blanks, respectively, and were corrected by the dilution factor (DF: 20). The LOQ of the metals of interest ranged from 0.07 μg/L (for Tl) to 37.55 μg/L (for Br) (Table S1). The relative standard deviations (RSDs) were calculated by measuring elemental concentrations in CRMs and human blood samples to evaluate intra- and inter-day precision. The RSDs values for the metals under review were in acceptable ranges (≤10.0%) (Table S2).

2.6. Statistical analysis

The descriptive statistics of the children's demographic and morphometric characteristics, biochemical markers for hepatic and renal functions, and blood elemental concentrations were calculated using Statistical Package for the Social Sciences (SPSS), version 26.0 (IBM Corp., USA). Blood elemental concentrations below LOQ were substituted by LOQ/2 and those with a quantification frequency ≤40% were not considered for subsequent statistical analysis. The normality of each analyte was tested by using the non-parametric, Kolmogorov-Smirnov test and the parametric, Shapiro-Wilk test. Blood elemental concentrations and serum levels of biochemical parameters were not normally distributed, except blood Cu, Zn, and Se concentrations and serum creatinine level.

Significant differences for each of the continuous variables between children grouped by categorial variables were determined using appropriate parametric, one-way analysis of variance (ANOVA) with Tukey HSD test and non-parametric, Kruskal-Wallis multiple comparison Dunn test with Bonferroni methods. Spearman's correlation coefficients (r) were calculated to investigate the associations between demographic, and morphometric characteristics, and serum levels of biochemical parameters, and also between blood elemental concentrations and morphometrics and serum biochemical parameters.

Multiple general linear regression analyses were performed to evaluate the independent contributions of the industry types of the living areas, development stage grouped by age (school-age: 6–12 years and

Table 2
Blood concentrations of trace elements in children living in industrial areas in Dhaka, Bangladesh (n = 141).

Elements	DF% ≥ LOQ	DF% ≤ LOD	GM (CI)	Percentile					Maximum	Reference value (RV)	Above RV (%)
				5th	25th	50th	75th	95th			
V (μg/L)	26	60	<0.26 (<0.26)	<0.26	<0.26	<0.26	<0.26	0.73	1.41	0.35a	20
Cr (μg/L)	15	59	<1.44 (<1.44)	<1.44	<1.44	<1.44	<1.44	1.59	16.2	1.48a	7
Mn (μg/L)	100	0	12.00 (11.30–12.70)	6.96	9.43	11.70	14.80	23.00	37.70	14.10a, 16.40 b, 16.0c	12
Co (μg/L)	67	2	0.24 (0.21–0.27)	<0.22	<0.22	0.24	0.37	0.64	6.07	0.38c	21
Ni (μg/L)	90	0	1.31 (1.07–1.61)	<0.52	0.61	0.84	2.27	14.80	76.30	2.44a, 1.10c	29
Cu (μg/L)	100	0	944 (915–974)	653	849	952	1064	1215	1661	1300c	2
Zn (mg/L)	100	0	4.99 (4.86–5.13)	3.73	4.57	4.96	5.61	6.38	7.21	5.8a, 6.8c	1
As (μg/L)	100	0	2.00 (1.88–2.14)	1.05	1.58	1.95	2.56	4.09	5.84	1.40[c]	79
Se (μg/L)	100	0	116 (113–119)	87	107	118	130	145	161	99a, 212 b, 220c	0
Br (mg/L)	100	0	4.05 (3.71–4.42)	1.77	2.57	4.23	5.95	9.33	21.3	–	–
Mo (μg/L)	100	0	1.45 (1.34–1.57)	0.65	1.00	1.38	2.00	3.29	5.8	1.16a, 1.7c	34
Ag (μg/L)	7	44	<0.24 (<0.24)	<0.24	<0.24	<0.24	<0.24	0.25	0.58	<0.27a	4
Cd (μg/L)	28	30	<1.08 (<1.08)	<1.08	<1.08	<1.08	1.00	1.78	2.81	0.41a, 0.27c	100*
Sb (μg/L)	25	44	<0.15 (<0.15)	<0.15	<0.15	<0.15	0.15	0.37	1.02	–	–
I (μg/L)	100	0	47.0 (44.7–49.3)	29.3	39.8	46.2	53.2	78.6	199.0	–	–
Hg (μg/L)	100	0	1.21 (1.12–1.31)	0.64	0.87	1.19	1.54	2.94	6.26	1.33 b, 1.2c	44
Tl (μg/L)	87	1	0.082 (0.076–0.087)	<0.07	0.070	0.083	0.102	0.150	0.300	0.04a	100*
Pb (μg/dL)	100	0	8.9 (8.5–9.4)	5.4	7.1	9.0	11.1	14.7	30.0	3.5d	100
Bi (μg/L)	21	76	<0.27 (<0.27)	<0.27	<0.27	<0.27	<0.27	0.61	1.18	0.05a	100*
U (μg/L)	1	97	<0.16 (<0.16)	<0.16	<0.16	<0.16	<0.16	<0.16	0.24	0.01a	100*

DF: Detection frequency, RV: Reference value, CI: Confidence interval, a: Goullé et al. (2015), b: CDC (2021), c: Saravanabhavan et al. (2017), and d: (Ruckart et al., 2021). * Indicates calculation based on the concentration above LOQ.

adolescent: 13–16 years), age- and sex-specific standardized z-BMI based body weight (healthy weight, underweight, overweight, and obese), body fats (waist-to-hip ratio), meal frequency (regular and irregular), frequency of consumption of egg, poultry meat, red meat, and fish products (regular and irregular) on blood metals concentrations in children. To satisfy the key assumptions of the linear regression models, a natural log transformation was applied for blood concentrations of Mn, Co, Ni, As, Br, Mo, I, Hg, Tl, and Pb. The key assumptions of the linear regression model and the influence of potential outliers were verified using residual plots for each analyte. Any potential outlier was capped by the respective mean or median value as appropriate.

General linear regression models (GLMs) were also performed to examine the associations of multiple metals exposure on hepatic and renal functions. In the GLM, hepatic and kidney biomarkers were evaluated on a continuous scale and blood elemental concentrations were categorised according to quartiles of distribution in the entire study children and entered the models as ordinal categorical variables using integer values (0,1,2 and 3) to calculate the beta-coefficient and 95% confidence interval (CI). The models were then adjusted by the potential confounders including age, sex, BMI, waist-to-hip ratio waist and hip circumferences, frequency of meals, and dietary items based on their biological and statistical consideration. Linear regression models were performed with backward elimination procedure, setting alpha at 0.10 to identify variables to be considered in the final models. BMI and hip circumferences were finally included in the models. A trend test was performed for the quartiles of the elemental distribution entered in the models. The results with the p-value <0.05 were considered significant.

3. Results

3.1. Characteristics of the participants

The study excluded four children who provided no blood samples, and eight with an incomplete questionnaire, from initial 153 participants. Table 1 summarises the demographics, morphometrics, blood pressure, food frequencies, and liver and kidney functions status of 141 children aged six to 16 years living in three industrialised areas in Dhaka, Bangladesh. The participants consisted of more females (61.7%) than males (38.3%) because of higher attendees. The average age of the children was 10 years (SD: 2.29). Most of the participants originated from the multiple industrial areas (n = 73, 52%); the children participating from the textile and tannery industrial areas comprised 29% (n = 41) and 19% (n = 27) of the cohort, respectively. The age distribution revealed that 120 children (85.1%) were in the primary school-aged group (range: 6–12 years) and 21 children (14.9%) were in the adolescent group (range:13–16 years). The mean of standardized z-BMI of the participants was 18.0 kg/m^2 (SD: 6.2) and no significant differences were observed between the three sampling sites (Table S3). According to the standard age- and sex-specific BMI cut-points based guidelines from CDC (z-BMI), 55% of the children were of normal weight, 21% were underweight, 9% were overweight and 15% participants were obese. The waist-to-hip ratio (WHR) determined for the children was 0.84 ± 0.04, where 91.4% of the children had lower values than the reference ranges specified for age-specific Dutch boys (0.93–1.02) and girls (0.89–0.99) (Fredriks et al., 2005) and similar to the mean values (0.86 ± 0.06) reported for healthy adolescent children (aged 12–16 years) in Sylhet metropolitan city of Bangladesh (Akther et al., 2020); a significant difference was found between the textile (0.85 ± 0.05) and tannery (0.82 ± 0.03) groups. The mean systolic- and diastolic-blood pressure (BP) of the children was 89/57 mm Hg and a significant difference was observed between sampling sites (Table S3). Following the recommendations of the American Academy of Paediatrics (AAP), and using the age-, sex-, and height-specific cut-points based systolic and diastolic blood pressure (BP) guidelines, around 81% of children had normal BP while 15% had low BP and 4% had high BP.

The food frequency data demonstrates that about 93% of participants had regular meals consisting of eggs (74%), poultry meat (94%), and red meat, mainly beef (72%). All children had a regular consumption of rice, fish, vegetables, and/or fruits (Table 1).

The liver and renal biomarkers presented in Table 1 differed significantly between participants from textile, tannery, and multi-industrial areas (see Table S3). Relative to specific reference values (RVs) for the children (presented in Table S3), the serum ALT and ALP activities and creatinine levels were above the RVs in 0.7%, 97.2%, and 48.2% of the children, respectively. Conversely, serum levels of albumin were lower in 39.0%, total calcium in 98.6%, BUN in 2.8%, and blood haemoglobin in 95.0% of the participants. The eGFR calculated for the children was 78 ± 18 mL/min/1.73 m^2, where 69.5% children had lower than the RVs (85–150 mL/min/1.73 m^2) (Fadrowski and Furth, 2011). The Spearman's correlation data presented in Table S4 revealed significant associations between demographic and anthropometric characteristics, blood pressure and serum liver and kidney biomarkers.

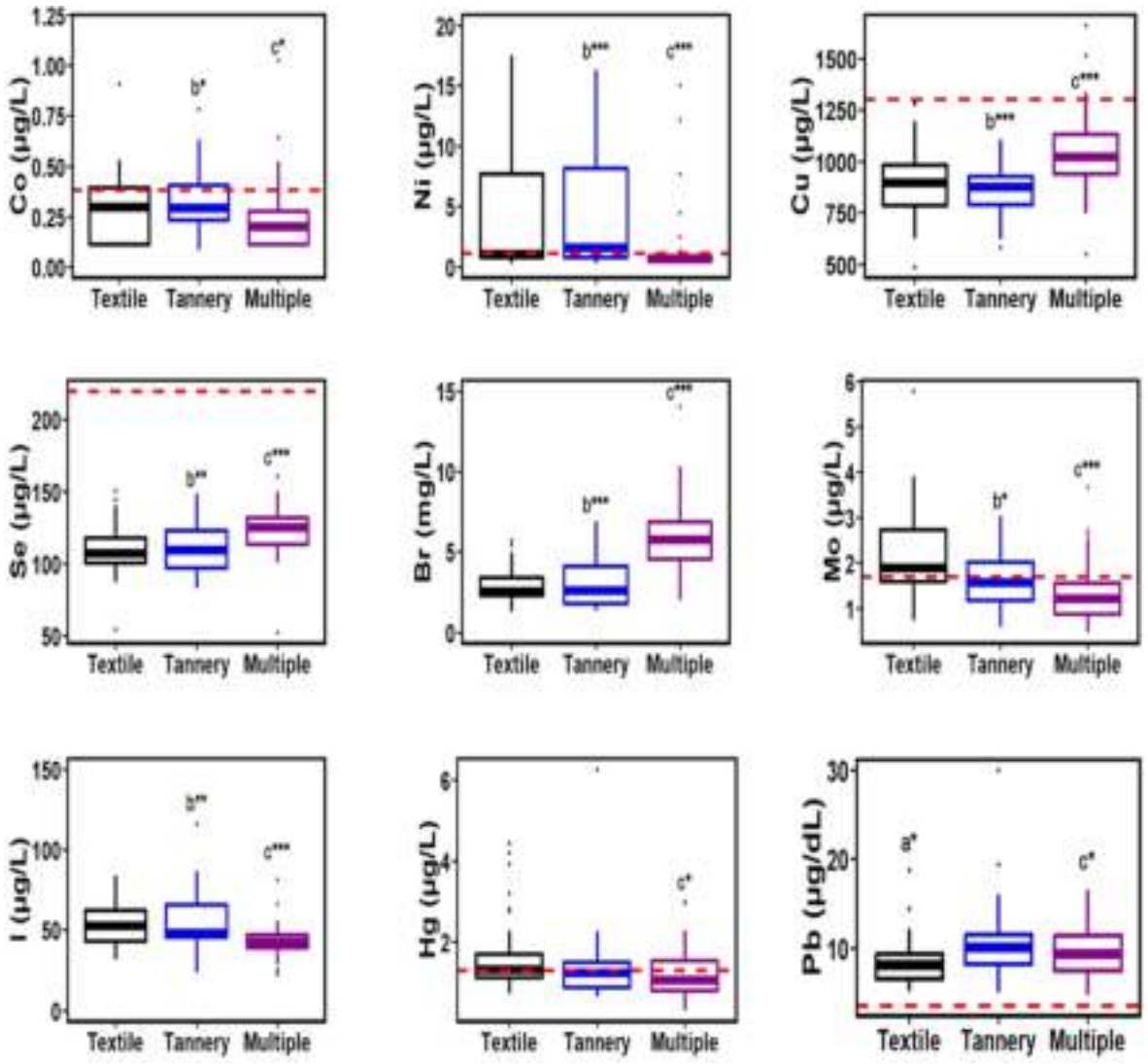

Fig. 1. The significant differences of nine measured blood elemental concentrations according to sampling locations in children.
In each dataset, each letter indicates a significant difference between two groups, determined by Kruskal-Wallis multiple comparison Dunn test with Bonferroni method for Co, Ni, Br, Mo, I Hg and Pb, and determined by ANOVA with Tukey HSD test for Cu and Se. No significant difference was found for Mn, Zn, As, and Tl. a: a significant difference between textile and tannery groups, b: a significant difference between tannery and multiple industry groups, c: a significant difference between textile and multiple industry groups, respectively. * Indicates adjusted p-value at < 0.05, ** indicates adjusted p-value at < 0.01 and *** indicates adjusted p-value at < 0.0001. Red dotted lines indicate reference values according to previous reports (CDC, 2012; Goullé et al., 2015; Saravanabhavan et al., 2017; CDC, 2021; Ruckart et al., 2021). The reference values for Br and I in blood for children were not available.

3.2. Distribution of blood elemental concentrations in children

The blood concentrations and distribution histograms of 20 essential and nonessential elements in children are presented in Table 2 and Fig. S1, respectively. The concentrations of Ag and U were observed to be below LOQ in most of the participants (93% and 99%, respectively); the detection frequency for V, Cr, Cd, Sb, and Bi was also not higher than their respective LOQs in 40% of the cohort. These elements were, therefore, excluded from statistical analysis. The concentrations of the essential elements Cu, Zn, and Se were within the range of RVs recommended by the CDC (2021), and/or other reports Goullé et al. (2015) and/or Saravanabhavan et al. (2017) for the majority of the children. The I and Br concentrations in blood were quantified in all participants, with a geometric mean (GM) value of 47.0 µg/L (CI: 44.7–49.3 µg/L) and 4.05 mg/L (CI: 3.71–4.42 mg/L), respectively. Relative to the RVs, blood Mn, Co, Ni, and Mo levels were exceeded in some children, ranging from 12% of the children exceeding the RV for Mn to 34% for Mo (see Table 2).

The distribution of the toxic elements As, Hg, Tl, and Pb examined here exceeded the RVs in most children. Approximately 79% of the children had higher As levels (GM: 2.0 µg/L) and 44% had higher Hg levels (GM: 1.21 µg/L) than the recommended values of 1.40 µg/L (Saravanabhavan et al., 2017) and 1.33 µg/L (CDC, 2021), respectively. Unexpectedly, 100% of the children had higher blood Pb concentrations than the recommended level (3.5 µg/dL) (Ruckart et al., 2021), with a maximum observed concentration of 30.0 µg/dL.

Multiple comparisons revealed that the distribution of nine elements varied significantly between the three sampling areas (Fig. 1). The study measured significantly higher concentrations of Co and Ni in the blood samples from children in the tannery group compared to the multi-industry group. Similarly, the concentrations of Cu, Se, Br, and Pb were also significantly higher. Mo, I, and Hg were, however, significantly lower in the multi-industry group blood samples compared to the textile group. In respect of the demographic characteristics, no significant disparities were observed for elemental concentrations between males and females, and between school-age and adolescent children.

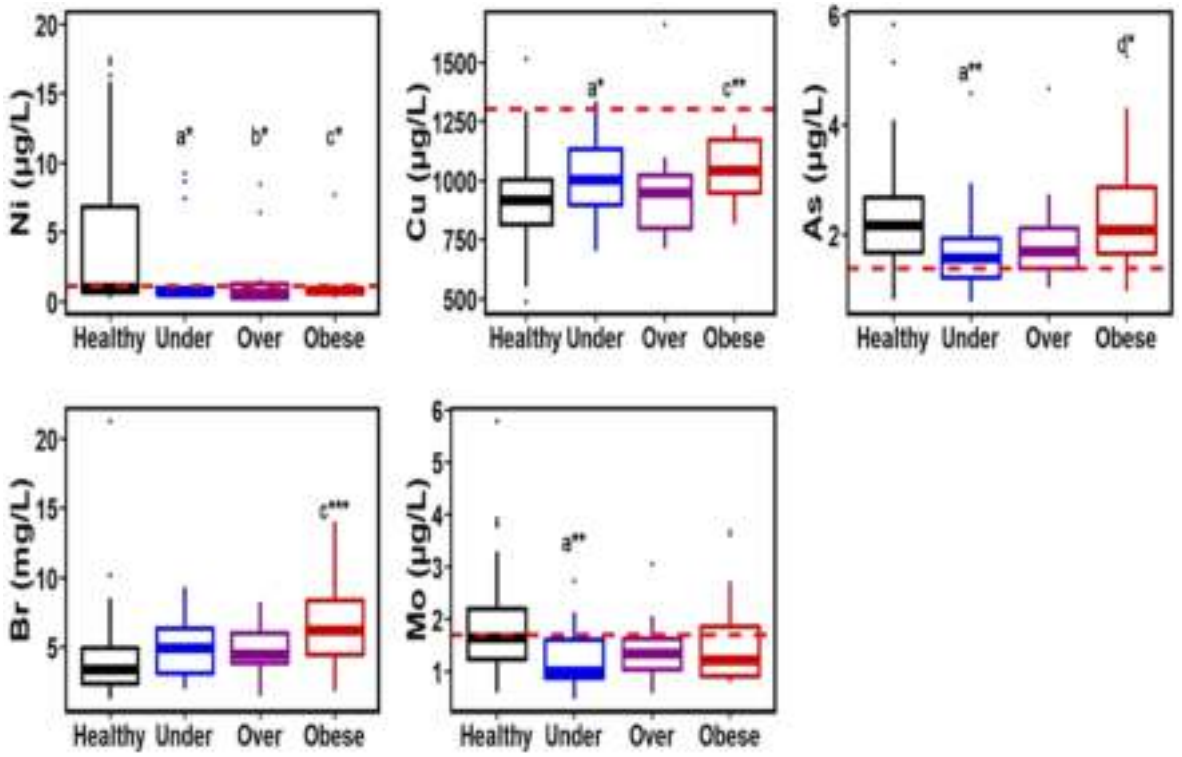

Fig. 2. The significant differences of measured blood elemental concentrations in relation to standard age- and sex-specific z-BMI status in children (n = 141). In each dataset, each letter indicates a significant difference between two groups, determined by Kruskal-Wallis multiple comparison Dunn test with Bonferroni method for Ni, As, Br, and Mo and determined by ANOVA with Tukey HSD test for Cu. No significant differences were found for Mn, Co, Zn, Se, I, Hg, Tl and Pb. a: a significant difference between healthy and underweight children, b: a significant difference between healthy and overweight children, c: a significant difference between healthy and obese children, d: a significant difference between under and obese children, respectively. * Indicates adjusted p-value at < 0.05, ** indicates adjusted p-value at < 0.01 and *** indicates adjusted p-value at < 0.0001. Red dotted lines indicate reference values according to previous reports (CDC, 2012; Goullé et al., 2015; Saravanabhavan et al., 2017; CDC, 2021). The reference values for Br and I in blood for children were not available.

Following an analysis of the BMI data, significant variations of five elements concentrations - Ni, Cu, As, Br, and Mo were observed (Fig. 2). Relative to healthy weight children, the concentrations of Cu and Br were significantly higher in obese children; Ni, Mo, and As were significantly lower in underweight children. The Cu concentrations were, however, significantly greater in underweight children.

3.3. Association of blood elemental concentrations with demographic and anthropometric parameters

Multiple general linear regression analyses were performed to assess the respective independent contributions of age, sex, body height, and weight, waist and hip circumference, industrial activities of the living areas, frequency and consumption of eggs, poultry meat, red meat, fish and their products, cereals, fruits, and vegetables on the blood elemental concentrations. All children reported regular consumption of fish, rice, and vegetables or fruits, and these were, therefore, not considered in the regression models. Table 3 presents the influential factors on blood elemental concentrations. The children's living area with multiple industrial activities was the greatest contributing factor in respect of the higher blood concentrations of Cu, As, Se, Br, and Pb, and lower levels of Ni, Mo, I, and Hg when compared to the textile industry group. Similarly, the tannery industries contributed to higher Pb concentrations, but lower Mo concentrations when compared to the textile area. The consumption of egg, meat, or their products showed significant associations with the blood concentrations of Zn, Se, Br, Tl, and Pb. Regular consumption of eggs showed an inverse association with Zn and Se but a direct positive association with Br, Tl, and Pb concentrations. On the other hand, regular consumption of red meat and meat products had a negative association with Br, Tl, and Pb concentrations, however, had a positive association with Se concentration. BMI was significantly associated with Cu, As, Mo, and Tl distribution in the study population. Relative to healthy body weight children, As and Mo concentrations were lower in underweight children ($\beta = -0.351$, $p < 0.001$, $\beta = -0.216$, $p = 0.047$, respectively), but had higher Cu concentrations ($\beta = 89.3$, $p = 0.015$) while higher Tl concentrations showed a positive association with obesity ($\beta = 0.300$, $p = 0.007$).

3.4. Association between blood elemental concentrations and liver and kidney function biomarkers

Tables 4 and 5 present the concentration-response associations between blood elemental concentrations and the liver and kidney injury biomarkers, respectively, adjusted by the potential confounders using the GLM. Several elements including Co, Ni, Cu, Se, Br, Mo, and I were associated with serum ALT and ALP activities, serum albumin, creatinine, BUN concentrations, and eGFR. A significant trend was observed for the positive association between serum ALT activities and the elemental distribution at higher quartiles of Cu (P-trend = 0.007), Se (P-trend = 0.005), and Br (P-trend <0.001). Inverse associations for the higher quartiles of Co (P-trend = 0.032), Ni (P-trend <0.001), Mo (P-trend = 0.006) and I (P-trend = 0.046) were, however, noted. Similarly, a trend was found for the positive association between the higher quartile concentrations of Mo and Br with higher serum ALP activity and serum albumin level, respectively (Table 4).

The kidney injury marker, BUN, was observed to be directly associated with the higher quartile elemental concentrations of Br (P-trend <0.001). There was, however, an inverse association with the higher quartiles of Mo (P-trend = 0.014). Similarly, serum creatinine concentrations were positively associated with the higher quartile

Table 3
Multiple general linear regression models with the influential factors (demographic characteristics, anthropometric characteristics, and food consumption frequency) on blood elemental concentrations (n = 141).

Elements	Factors	Standardized beta coefficients (95% CI)	p-value	R^2 (Adjusted R^2) of the models
Ni	Multi-industry area	−0.915 (−1.367, −0.463)	<0.001	0.261 (0.191)
	Tannery area	0.250 (−0.306, 0.807)	0.375	
Cu	Multi-industry area	90.294 (29.586, 151.002)	0.004	0.257 (0.188)
	Tannery area	−22.869 (−97.623, 51.886)	0.546	
	Obese	77.615 (−0.866, 156.096)	0.053	
	Overweight	−34.081 (−121.758, 53.597)	0.443	
	Underweight	89.317 (17.398, 161.237)	0.015	
Zn	Egg	−0.715 (−1.347, −0.083)	0.027	0.09 (0.005)
As	Multi-industry area	0.197 (0.038, 0.356)	0.016	0.157 (0.078)
	Tannery area	0.103 (−0.094, 0.299)	0.303	
	Obese	−0.074 (−0.28, 0.132)	0.478	
	Overweight	−0.177 (−0.407, 0.054)	0.132	
	Underweight	−0.351 (−0.539, −0.162)	<0.001	
Se	Multi-industry area	13.85 (7.565, 20.135)	<0.001	0.223 (0.15)
	Tannery area	1.643 (−6.096, 9.383)	0.675	
	Egg	−13.497 (−25.348, −1.647)	0.026	
	Red meat	12.502 (2.052, 22.952)	0.019	
Br	Multi-industry area	0.679 (0.519, 0.84)	<0.001	0.52 (0.475)
	Tannery area	−0.057 (−0.254, 0.141)	0.572	
	Egg	0.392 (0.089, 0.695)	0.012	
	Red meat	−0.433 (−0.7, −0.166)	0.002	
Mo	Multi-industry area	−0.507 (−0.686, −0.327)	<0.001	0.278 (0.211)
	Tannery area	−0.341 (−0.563, −0.12)	0.003	
	Obese	0.041 (−0.191, 0.273)	0.728	
	Overweight	−0.101 (−0.361, 0.158)	0.442	
	Underweight	−0.216 (−0.429, −0.003)	0.047	
I	Multi-industry area	−0.202 (−0.299, −0.106)	<0.001	0.216 (0.142)
	Tannery area	−0.029 (−0.148, 0.091)	0.637	
Hg	Multi-industry area	−0.222 (−0.41, −0.035)	0.020	0.102 (0.018)
	Tannery area	−0.186 (−0.417, 0.045)	0.113	
Tl	Obese	0.300 (0.084, 0.517)	0.007	0.113 (0.03)
	Overweight	0.072 (−0.17, 0.314)	0.559	
	Underweight	0.102 (−0.097, 0.3)	0.312	
	Egg	0.324 (0.008, 0.64)	0.045	
	Red meat	−0.348 (−0.626, −0.069)	0.015	
Pb	Multi-industry area	0.15 (0.025, 0.276)	0.019	0.164 (0.085)
	Tannery area	0.212 (0.057, 0.366)	0.008	
	Egg	0.294 (0.058, 0.531)	0.015	
	Red meat	−0.251 (−0.46, −0.042)	0.019	

Blood concentrations of Mn, Co, Ni, As, Br, Mo, I, Hg, Tl, and Pb were natural log-transformed. Co and Ni were not considered in the models because of the violation of assumptions.
Variable considered: location (tannery and multi-industry areas; textile area represented the reference category), gender (males vs. females; males represented the reference category), age ((school-age: 6-12 years vs. adolescent: 13-16 years; school-age represented the reference category), body mass index (underweight, overweight and obese; healthy weight represented the reference category), weight-to-hip ratio (continuous measures), meal frequency (regular vs. irregular, regular represented the reference category), egg product consumption (regular vs. irregular, irregular represented the reference category), poultry meat product consumption (regular vs. irregular, regular represented the reference category), red meat product consumption (regular vs. irregular, irregular represented the reference category).

concentrations of Br, and the higher Br concentrations showed an inverse association with eGFR alone (Table 5). Both adjusted and unadjusted GLMs (Tables S6 and S7) noted significant trends for the concentration-dependent associations for the higher quartile concentrations of Ni, Cu, Se, Br, Mo, I, Tl, and Pb in the children's blood samples and liver and kidney injury biomarkers; the coefficient values of Spearman's correlations were similar to those of the GLMs (Table S8).

4. Discussion

The quantification of blood concentrations of nutritionally essential elements (Cr, Mn, Co, Cu, Zn, Se, Mo, and I), probably essential (V, Ni, and Br) and potentially toxic elements (As, Cd, Sb, Hg, Tl, Pb, Bi, and U) revealed that children living in the textile, tannery and multi-industrial areas in Dhaka are exposed, to elevated concentrations, the toxic metals As, Cd, Hg, Tl, Pb, or Bi. These elemental concentrations exceeded the critically acceptable/reference values reported for US children aged 6–11 years, from the National Health and Nutrition Examination Survey (NHANES) (CDC, 2021); the Canadian population aged 6–19 years, estimated through the Canadian Health Measure Survey (CHMS) (Saravanabhavan et al., 2017); the RVs reported for a young French population aged <18 years (Goullé et al., 2015). Exposure to the essential elements V, Cr, Mn, Co, Ni, Cu, and Mo also exceeded, by variable proportions of the study children, the guideline values, ranging from a 2% of the participants exceeded for Cu to a 34% for Mo (Table 2). The findings of the current study of elevated exposure to Pb (GM: 8.9 μg/dL) and Se (116.0 μg/L), are similar to the higher blood levels of Pb and Se in urban children in Dhaka aged 7–16 years reported in a previous study in 2008, with mean concentrations of 12.0 μg/dL and 120.0 μg/L, respectively (Linderholm et al., 2011).

The study used multiple comparisons of blood elemental concentration distributions and GLMs to identify the elemental exposure in children and was driven to a great extent by their living areas; the multiple industrial areas were found as a potential contributor to greater exposures to As, Pb, Br, Cu, and Se when compared to the textile area. Similarly, the tannery and textile areas were also potential contributors of Pb exposure, whereas Mo exposure was significantly higher in children living in the textile area, as compared to other industrial sites of the study. The informal recycling of lead-acid batteries, effluents from the textile, dyeing, tannery, and auxiliary industries established near the sampling areas of this study and the urban sewage system in Dhaka have been found previously as the major sources of metal pollution that

Table 4
Multivariate adjusted general linear regression models (GLM) of concentration-dependent associations between blood elemental concentrations and liver function biomarkers.

Elements	Quartiles	ALT	P-trend	ALP	P-trend	Albumin	P-trend	Calcium	P-trend
		B (95% CI)		B (95% CI)		B (95% CI)		B (95% CI)	
Mn (μg/L)	Q1 (≤9.43)	0 (Reference)	0.54	0 (Reference)	0.629	0 (Reference)	0.739	0 (Reference)	0.951
	Q2 (>9.43–11.7)	−2.51 (−5.727, 0.707)		73.406 (−47.154, 193.965)		−0.269 (−0.52, −0.019)		−0.248 (−0.615, 0.119)	
	Q3 (>11.7–14.8)	−2.579 (−5.817, 0.659)		92.318 (−29.041, 213.676)		−0.283 (−0.536, −0.03)		−0.273 (−0.642, 0.097)	
	Q4 (>14.8)	−1.038 (−4.278, 2.202)		25.031 (−96.385, 146.447)		−0.04 (−0.293, 0.212)		−0.004 (−0.374, 0.366)	
Co (μg/L)	Q1 (≤0.22)	0 (Reference)	**0.032**	0 (Reference)	0.734	0 (Reference)	0.503	0 (Reference)	0.077
	Q2 (>0.22–0.24)	−1.351 (−6.007, 3.304)		−21.635 (−198.699, 155.429)		−0.397 (−0.768, −0.026)		−0.592 (−1.122, −0.062)	
	Q3 (>0.24–0.37)	−2.738 (−5.568, 0.092)		−43.539 (−151.177, 64.099)		−0.072 (−0.298, 0.153)		0.13 (−0.192, 0.453)	
	Q4 (>0.37)	−3.141 (−6.014, −0.268)		28.844 (−80.426, 138.113)		−0.019 (−0.248, 0.21)		0.097 (−0.23, 0.424)	
Ni (μg/L)	Q1 (≤0.61)	0 (Reference)	**< 0.001**	0 (Reference)	0.383	0 (Reference)	0.238	0 (Reference)	0.443
	Q2 (>0.61–0.84)	−1.455 (−4.591, 1.681)		−32.551 (−154.914, 89.811)		−0.341 (−0.594, −0.087)		−0.087 (−0.461, 0.288)	
	Q3 (>0.84–2.27)	−4.328 (−7.432, −1.224)		21.016 (−100.097, 142.129)		−0.104 (−0.355, 0.147)		0.112 (−0.259, 0.482)	
	Q4 (>2.27)	−5.173 (−8.388, −1.957)		40.531 (−84.939, 166.001)		−0.243 (−0.502, 0.017)		0.091 (−0.293, 0.474)	
Cu (μg/L)	Q1 (≤849)	0 (Reference)	**0.007**	0 (Reference)	0.746	0 (Reference)	0.082	0 (Reference)	0.225
	Q2 (>849–952)	0.029 (−3.158, 3.216)		133.267 (13.303, 253.231)		−0.24 (−0.491, 0.012)		−0.463 (−0.83, −0.096)	
	Q3 (>952–1064)	1.72 (−1.456, 4.896)		−1.142 (−120.687, 118.403)		0.075 (−0.176, 0.326)		−0.23 (−0.595, 0.136)	
	Q4 (>1064)	4.212 (0.954, 7.47)		66.004 (−56.62, 188.628)		0.135 (−0.123, 0.392)		−0.321 (−0.696, 0.054)	
Zn (mg/L)	Q1 (≤4.57)	0 (Reference)	0.136	0 (Reference)	0.744	0 (Reference)	0.503	0 (Reference)	0.067
	Q2 (>4.57–4.96)	1.004 (−2.245, 4.253)		−25.251 (−147.627, 97.125)		0.128 (−0.131, 0.387)		−0.23 (−0.599, 0.14)	
	Q3 (>4.96–5.61)	1.96 (−1.292, 5.212)		−3.667 (−126.135, 118.801)		0.063 (−0.196, 0.322)		−0.246 (−0.616, 0.124)	
	Q4 (>5.61)	2.281 (−0.971, 5.532)		14.174 (−108.289, 136.637)		0.114 (−0.145, 0.373)		−0.36 (−0.729, 0.01)	
As (μg/L)	Q1 (≤1.58)	0 (Reference)	0.085	0 (Reference)	0.935	0 (Reference)	0.529	0 (Reference)	0.316
	Q2 (>1.58–1.95)	1.141 (−2.054, 4.336)		66.421 (−54.018, 186.859)		−0.043 (−0.299, 0.213)		−0.18 (−0.545, 0.184)	
	Q3 (>1.95–2.56)	1.072 (−2.187, 4.33)		34.562 (−88.288, 157.412)		−0.122 (−0.383, 0.139)		−0.374 (−0.746, −0.002)	
	Q4 (>2.56)	3.022 (−0.215, 6.26)		15.943 (−106.115, 138)		−0.061 (−0.32, 0.198)		−0.134 (−0.503, 0.236)	
Se (μg/L)	Q1 (≤107)	0 (Reference)	**0.005**	0 (Reference)	0.461	0 (Reference)	0.317	0 (Reference)	0.439
	Q2 (>107–118)	4.206 (1.081, 7.331)		−95.56 (−216.004, 24.883)		0.253 (−0.003, 0.51)		−0.02 (−0.394, 0.355)	
	Q3 (>118–130)	5.766 (2.542, 8.99)		−136.861 (−261.121, −12.601)		0.124 (−0.141, 0.389)		0.001 (−0.385, 0.387)	
	Q4 (>130)	4.207 (1.132, 7.283)		−33.157 (−151.702, 85.388)		0.179 (−0.074, 0.432)		−0.16 (−0.529, 0.208)	
Br (mg/L)	Q1 (≤2.57)	0 (Reference)	**< 0.001**	0 (Reference)	0.192	0 (Reference)	**0.001**	0 (Reference)	0.27
	Q2 (>2.57–4.23)	1.22 (−1.464, 3.904)		3.437 (−118.167, 125.041)		0.156 (−0.092, 0.404)		0.091 (−0.281, 0.464)	
	Q3 (>4.23–5.95)	6.687 (4.004, 9.369)		−76.178 (−197.715, 45.36)		0.394 (0.147, 0.642)		−0.065 (−0.437, 0.308)	
	Q4 (>5.95)	10.345 (7.497, 13.193)		−63.911 (−192.949, 65.128)		0.414 (0.151, 0.677)		−0.182 (−0.577, 0.214)	
Mo (μg/L)	Q1 (≤1)	0 (Reference)	**0.006**	0 (Reference)	**0.035**	0 (Reference)	0.067	0 (Reference)	0.701
	Q2 (>1–1.38)	−1.947 (−5.175, 1.281)		44.58 (−77.378, 166.539)		−0.116 (−0.376, 0.144)		0.112 (−0.267, 0.49)	
	Q3 (>1.38–2)	−3.785 (−7.049, −0.521)		50.055 (−73.259, 173.369)		−0.243 (−0.506, 0.02)		0.213 (−0.169, 0.596)	
	Q4 (>2)	−4.342 (−7.648, −1.037)		140.508 (15.636, 265.381)		−0.22 (−0.486, 0.046)		0.046 (−0.342, 0.433)	
I (μg/L)	Q1 (≤39.8)	0 (Reference)	**0.046**	0 (Reference)	0.382	0 (Reference)	0.141	0 (Reference)	0.307
	Q2 (>39.8–46.2)	−1.755 (−5.038, 1.528)		102.987 (−20.675, 226.648)		−0.06 (−0.323, 0.203)		0.107 (−0.274, 0.488)	
	Q3 (>46.2–53.2)	−1.153 (−4.384, 2.078)		38.782 (−82.946, 160.51)		−0.088 (−0.347, 0.171)		0.112 (−0.263, 0.487)	
	Q4 (>53.2)	−3.68 (−6.919, −0.442)		78.598 (−43.407, 200.603)		−0.196 (−0.456, 0.063)		0.204 (−0.172, 0.58)	

(*continued on next page*)

Table 4 (*continued*)

Elements	Quartiles	ALT	P-trend	ALP	P-trend	Albumin	P-trend	Calcium	P-trend
		B (95% CI)		B (95% CI)		B (95% CI)		B (95% CI)	
Hg (μg/L)	Q1 (≤0.87)	0 (Reference)	0.396	0 (Reference)	0.639	0 (Reference)	0.534	0 (Reference)	0.229
	Q2 (>0.87–1.19)	0.115 (−3.093, 3.324)		−47.548 (−168.363, 73.266)		0.222 (−0.034, 0.478)		0.403 (0.044, 0.763)	
	Q3 (>1.19–1.54)	−2.937 (−6.215, 0.341)		−52.906 (−176.316, 70.505)		0.131 (−0.13, 0.393)		0.166 (−0.201, 0.533)	
	Q4 (>1.54)	−0.443 (−3.658, 2.771)		32.171 (−88.848, 153.189)		0.116 (−0.141, 0.372)		−0.153 (−0.513, 0.207)	
Tl (μg/L)	Q1 (≤0.07)	0 (Reference)	0.872	0 (Reference)	0.249	0 (Reference)	0.608	0 (Reference)	0.639
	Q2 (>0.07–0.083)	−4.042 (−7.294, −0.79)		−49.323 (−171.392, 72.746)		−0.035 (−0.299, 0.229)		0.227 (−0.154, 0.607)	
	Q3 (>0.083–0.102)	−2.123 (−5.255, 1.01)		41.715 (−75.871, 159.302)		−0.053 (−0.307, 0.201)		0.13 (−0.236, 0.496)	
	Q4 (>0.102)	−0.361 (−3.612, 2.891)		−105.544 (−227.592, 16.504)		0.078 (−0.186, 0.342)		0.127 (−0.253, 0.508)	
Pb (μg/dL)	Q1 (≤7.1)	0 (Reference)	0.252	0 (Reference)	0.543	0 (Reference)	0.102	0 (Reference)	0.13
	Q2 (>7.1–9)	2.67 (−0.521, 5.86)		−16.102 (−139.566, 107.363)		0.133 (−0.127, 0.392)		−0.163 (−0.539, 0.213)	
	Q3 (>9–11.1)	−1.247 (−4.392, 1.897)		−22.205 (−143.88, 99.471)		0.083 (−0.173, 0.338)		−0.154 (−0.524, 0.217)	
	Q4 (>11.1)	3.292 (0.046, 6.538)		42.837 (−82.768, 168.441)		0.248 (−0.016, 0.512)		−0.313 (−0.695, 0.069)	

GLM was adjusted with variable children's body mass index (underweight, overweight and obese; healthy weight represented the reference category) and hip circumference (continuous measures). Bold indicates significant values.

exceeded the permissible limits for Pb, As, Cu and Cr, contaminating the surrounding water bodies, agricultural fields, soil, rice, and vegetables (Bhuiyan et al., 2011; Nahar et al., 2018). Elevated levels of Ni, Pb, Cd, As, Cu, Cr, and Zn in the street dust in Dhaka city (Rahman et al., 2019), may be the potential source of exposure to higher concentrations of these metals in the blood of the studied children, where the hot-spot areas were associated with industrial activities and heavy traffic density.

Diet could be a major source of elemental exposure, mainly through consumption of cereals (mostly rice and pulse), vegetables, fish, meat, hen eggs, and drinking water. These have been identified as potential contributors of Pb, Cd, Cu, As, and/or Hg exposure in children (Roca et al., 2016; Zhang et al., 2019). The present study found a significant positive association for higher Tl, Pb, and Br levels with regular consumption of eggs; an inverse relationship with the consumption of red meat products was, however, seen. A higher prevalence of Pb in the edible portion of eggs from backyard chickens has been reported in the USA (Bautista et al., 2014) and New Zealand (Cowie and Gartrell, 2019), where Pb was detected in the yolk, eggshell, blood, liver, kidney, and bone of all birds analysed. The consumption of one Pb-contaminated average-size egg daily by a young child was reported as being sufficient to warrant health concerns (Bautista et al., 2014).

Early-in-life exposure to toxic metals can be associated with adverse growth and developmental outcomes. Further, excessive or inadequate exposure to essential elements during this vulnerable period can affect muscular growth, bone health and may increase the risk of hypertension (Bastola et al., 2020; Signes-Pastor et al., 2021). In the present study, significant variations of elemental concentrations of Ni, Cu, As, Br, and Mo were observed between groups of underweight, healthy weight, overweight, and obese children (Fig. 2), as assessed from their BMI. Using GLM, however, the study observed an association of lower blood As and Mo concentrations in underweight children, while a higher Tl level was associated with obesity (Table 3). Our findings are concordant with an observation from the US population, where a direct association between Tl and barium (not tested here) with obesity has been reported in NHANES participants 1999–2002 (Padilla et al., 2010). On the other hand, the decreased body weight observed at the lower As concentrations observed in the present cohort may be due to an adverse effect of chronic exposure to As at sub-micromolar concentrations (Ambrosio et al., 2014; Liu et al., 2015). An adverse health effect of elevated Pb exposure to current study children was the observed association with lower waist- and hip circumference (Table S5), which is similar to the recent observation reported on US children aged 6–11 years by Signes-Pastor et al. (2021), where the children participated in the NHANES program 2013–2016. The present findings of a significant positive association for higher Mo blood concentrations but an inverse relationship for elevated Pb concentrations, with systolic and diastolic BP in children (Table S5) were consistent with previous observations (Shih et al., 2021). These authors reported a similar direct association for Mo and Se and a negative association between Hg concentrations, with systolic and diastolic BP among Bangladeshi rural children aged 5–7 years.

The toxic effects of environmental pollutants are mainly concentration-dependent, and a common mechanism of metal-induced toxicity involves oxidative stress owing to increased production of reactive oxygen and nitrogen species from complex redox reactions, induce cell necrosis and organ dysfunction (Valko et al., 2005; Tanvir et al., 2019; Briffa et al., 2020). Cu, Se, Mo, Co, and I are essential elements in man and are an integral part of various enzymes and hormones, involved in vital biological processes (Briffa et al., 2020). Chronic environmental exposure to excessive Cu or dietary deficiency of Cu, has, however, been linked to oxidative stress, associated with abnormal Cu metabolism and organ dysfunction (Gaetke and Chow, 2003). The concentration-dependent positive association between blood Cu concentration and serum ALT activity in the present study, indicates that the young children living in the industrial areas are at higher risk of Cu-induced liver toxicity. The association of elevated exogenous Cu exposure, derived from milk boiled in Cu vessels or the use of Cu utensils, with childhood liver cirrhosis and hepatic toxicosis, has been previously reported in India and western Austria (Müller et al., 1996; Council, 2000; Taylor et al., 2020).

Adverse health effects of long-term exposures to environmental Se have been reviewed recently (Vinceti et al., 2017; Gebreeyessus and Zewge, 2019). These authors described the epidemiological observations of hepatic and neurological toxicity, dermatitis, hair and nail loss, gastrointestinal abnormalities owing to chronic exposure to elevated levels of environmental Se. The positive trend of association between environmental Se exposure with liver injury biomarker observed in the present study agree with those previous observations and indicate the potential risk of liver damage arising from long-term exposure to elevated environmental Se levels in a young population. The different Se species in the environment and oxidative effects of selenate and selenite may be the most significant contributor in living organisms, accounting for up to 95% of Se toxicity (Lenz and Lens, 2009; Gebreeyessus and

Table 5
Multivariate adjusted general linear regression models (GLM) of concentration-dependent associations between blood elemental concentrations and kidney function biomarkers.

Elements	Quartiles	Creatinine	P-trend	BUN	P-trend	eGFR	P-trend
		B (95% CI)		B (95% CI)		B (95% CI)	
Mn (μg/L)	Q1 (≤9.43)	0 (Reference)	0.740	0 (Reference)	0.846	0 (Reference)	0.802
	Q2 (>9.43–11.7)	−0.035 (−0.098, 0.027)		−0.788 (−1.45, −0.126)		0.673 (−7.378, 8.725)	
	Q3 (>11.7–14.8)	−0.034 (−0.097, 0.029)		−0.312 (−0.978, 0.354)		3.667 (−4.437, 11.772)	
	Q4 (>14.8)	0.011 (−0.052, 0.073)		−0.09 (−0.756, 0.577)		−2.085 (−10.194, 6.023)	
Co (μg/L)	Q1 (≤0.22)	0 (Reference)	0.751	0 (Reference)	0.704	0 (Reference)	0.602
	Q2 (>0.22–0.24)	−0.055 (−0.147, 0.037)		0.221 (−0.769, 1.21)		9.996 (−1.707, 21.7)	
	Q3 (>0.24–0.37)	−0.009 (−0.065, 0.047)		0 (−0.602, 0.601)		3.131 (−3.984, 10.245)	
	Q4 (>0.37)	−0.005 (−0.061, 0.052)		−0.061 (−0.672, 0.549)		0.097 (−7.126, 7.319)	
Ni (μg/L)	Q1 (≤0.61)	0 (Reference)	0.123	0 (Reference)	0.172	0 (Reference)	0.34
	Q2 (>0.61–0.84)	−0.048 (−0.111, 0.015)		0.218 (−0.455, 0.892)		5.19 (−2.939, 13.32)	
	Q3 (>0.84–2.27)	−0.05 (−0.112, 0.013)		−0.425 (−1.092, 0.242)		4.487 (−3.56, 12.534)	
	Q4 (>2.27)	−0.053 (−0.117, 0.012)		−0.29 (−0.98, 0.401)		4.477 (−3.859, 12.814)	
Cu (μg/L)	Q1 (≤849)	0 (Reference)	0.098	0 (Reference)	0.067	0 (Reference)	0.091
	Q2 (>849–952)	−0.024 (−0.087, 0.038)		0.321 (−0.348, 0.989)		3.998 (−4.023, 12.018)	
	Q3 (>952–1064)	0.029 (−0.033, 0.092)		0.028 (−0.638, 0.694)		−2.954 (−10.946, 5.039)	
	Q4 (>1064)	0.039 (−0.025, 0.103)		0.767 (0.084, 1.451)		−5.114 (−13.313, 3.084)	
Zn (mg/L)	Q1 (≤4.57)	0 (Reference)	0.85	0 (Reference)	0.688	0 (Reference)	0.759
	Q2 (>4.57–4.96)	0.051 (−0.011, 0.114)		0.241 (−0.439, 0.921)		−2.561 (−10.707, 5.585)	
	Q3 (>4.96–5.61)	0.024 (−0.039, 0.087)		0.095 (−0.586, 0.776)		−1.082 (−9.234, 7.07)	
	Q4 (>5.61)	0.015 (−0.047, 0.078)		0.195 (−0.486, 0.876)		−1.833 (−9.984, 6.319)	
As (μg/L)	Q1 (≤1.58)	0 (Reference)	0.721	0 (Reference)	0.512	0 (Reference)	0.391
	Q2 (>1.58–1.95)	−0.014 (−0.077, 0.048)		−0.72 (−1.377, −0.064)		−1.448 (−9.433, 6.537)	
	Q3 (>1.95–2.56)	−0.021 (−0.085, 0.043)		−0.205 (−0.875, 0.465)		1.427 (−6.718, 9.572)	
	Q4 (>2.56)	0.014 (−0.049, 0.078)		0.062 (−0.604, 0.727)		−4.672 (−12.765, 3.42)	
Se (μg/L)	Q1 (≤107)	0 (Reference)	0.666	0 (Reference)	0.084	0 (Reference)	0.69
	Q2 (>107–118)	0.019 (−0.045, 0.082)		0.735 (0.066, 1.404)		−3.664 (−11.82, 4.492)	
	Q3 (>118–130)	0.024 (−0.041, 0.09)		0.608 (−0.083, 1.298)		−3.076 (−11.49, 5.338)	
	Q4 (>130)	0.013 (−0.05, 0.075)		0.656 (−0.002, 1.315)		−1.917 (−9.944, 6.11)	
Br (mg/L)	Q1 (≤2.57)	0 (Reference)	**0.027**	0 (Reference)	**< 0.001**	0 (Reference)	**0.001**
	Q2 (>2.57–4.23)	0.016 (−0.047, 0.078)		0.248 (−0.388, 0.884)		−2.836 (−10.692, 5.02)	
	Q3 (>4.23–5.95)	0.054 (−0.009, 0.116)		1.002 (0.366, 1.638)		−8.459 (−16.311, −0.607)	
	Q4 (>5.95)	0.067 (0, 0.133)		1.353 (0.678, 2.028)		−12.869 (−21.205, −4.532)	
Mo (μg/L)	Q1 (≤1)	0 (Reference)	0.523	0 (Reference)	**0.014**	0 (Reference)	0.32
	Q2 (>1–1.38)	−0.021 (−0.085, 0.043)		−0.074 (−0.738, 0.589)		2.265 (−5.98, 10.509)	
	Q3 (>1.38–2)	−0.027 (−0.092, 0.038)		0.03 (−0.641, 0.701)		2.622 (−5.714, 10.958)	
	Q4 (>2)	−0.021 (−0.086, 0.045)		−0.941 (−1.621, −0.262)		4.387 (−4.055, 12.829)	
I (μg/L)	Q1 (≤39.8)	0 (Reference)	0.338	0 (Reference)	0.086	0 (Reference)	0.758
	Q2 (>39.8–46.2)	−0.049 (−0.113, 0.015)		−0.394 (−1.08, 0.291)		5.083 (−3.2, 13.367)	
	Q3 (>46.2–53.2)	−0.029 (−0.092, 0.035)		−0.233 (−0.908, 0.442)		1.84 (−6.314, 9.994)	
	Q4 (>53.2)	−0.039 (−0.103, 0.024)		−0.678 (−1.355, −0.002)		2.43 (−5.743, 10.602)	
Hg (μg/L)	Q1 (≤0.87)	0 (Reference)	0.731	0 (Reference)	0.471	0 (Reference)	0.8
	Q2 (>0.87–1.19)	−0.001 (−0.064, 0.063)		−0.189 (−0.865, 0.487)		−4.444 (−12.519, 3.63)	
	Q3 (>1.19–1.54)	−0.003 (−0.068, 0.061)		−0.362 (−1.053, 0.328)		−3.755 (−12.004, 4.493)	
	Q4 (>1.54)	0.012 (−0.051, 0.076)		−0.203 (−0.88, 0.474)		−1.325 (−9.413, 6.764)	
Tl (μg/L)	Q1 (≤0.07)	0 (Reference)	0.298	0 (Reference)	0.072	0 (Reference)	0.131
	Q2 (>0.07–0.083)	0.002 (−0.063, 0.066)		0.083 (−0.603, 0.769)		−2.677 (−10.904, 5.551)	
	Q3 (>0.083–0.102)	0.003 (−0.059, 0.065)		0.219 (−0.442, 0.88)		−1.617 (−9.542, 6.308)	
	Q4 (>0.102)	0.035 (−0.029, 0.1)		0.618 (−0.068, 1.304)		−7.009 (−15.235, 1.216)	
Pb (μg/dL)	Q1 (≤7.1)	0 (Reference)	0.477	0 (Reference)	0.565	0 (Reference)	0.627
	Q2 (>7.1–9)	0.064 (0.001, 0.127)		−0.195 (−0.881, 0.491)		−3.615 (−11.834, 4.604)	
	Q3 (>9–11.1)	0.007 (−0.055, 0.069)		−0.184 (−0.86, 0.492)		−0.165 (−8.265, 7.934)	
	Q4 (>11.1)	0.043 (−0.021, 0.108)		0.21 (−0.487, 0.908)		−3.318 (−11.679, 5.043)	

GLM was adjusted with variable children's body mass index (underweight, overweight and obese; healthy weight represented the reference category) and hip circumference (continuous measures). Bold indicates significant values.

Zewge, 2019).

Neurotoxic Br and brominated compounds are emerging environmental contaminants, and pose the greatest risk of exposure to workers in the textile and dye industries, gold mining, and the organic and photographic chemicals industries (Brown, 2014). Young children are at higher risk of exposure to brominated compounds, brominated flame retardants (BFRs) owing to hand-to-mouth activities and like to play on the ground (Tilley and Fry, 2015). In the present study, the children living in multiple industrial areas were exposed to higher levels of Br (Fig. 1 and Table 3). The observed blood Br concentration in obese children (Fig. 2) and positive association with egg consumption may, in addition to their lipophilicity suggesting, BFRs maybe a key Br source for exposure to children in the present study. The concentration-dependent positive relationship between Br exposure and serum ALT, albumin, BUN and creatinine levels and an inverse association with eGFR indicates that chronic environmental exposure to Br and/or brominated compounds may impair both liver and kidney functions in young children. Epidemiological studies of the health consequences of BFRs exposure suggested possible associations with diabetes, altered thyroid function, cancer, neurobehavioural and developmental disorders (Kim et al., 2014), which may be directly or indirectly linked to liver and kidney functions.

In contrast, blood iodine concentration measured in the participants reflected the bioavailable supply of iodide to the thyroid gland and assessing the thyroid functions, a biomonitoring approach providing more direct information, compared to the ordinary urinary iodine concentrations (Yu et al., 2018; Mills et al., 2020). The negative trend of association between blood I concentrations and serum ALT levels

observed in the study may be explained as a positive influence of blood iodine on liver functions. Further studies are, however, warranted to investigate the underlying mechanisms of Br toxicity and effects of blood I concentration in young children.

The present study has some limitations. The present cross-sectional study involved children who were living in urban industrial areas and lacks an exposure reference group. As such, the blood elemental distributions observed do not necessarily represent those of rural and nonindustrial urban children. The relationships between blood concentrations of Cd and Sb with BMI, WHR, BP and with liver and kidney functions were not elucidated here due to analytical limitations and higher quantification limits.

The collection of more accurate and comprehensive dietary information would also be appropriate in the identification of the dietary sources of elemental exposures. Further in-depth studies with large sample size are, therefore, important in elucidating the principal foods, amount of food consumption per day and their association with detailed sociodemographic information, such as common mealtime and place, duration of indoor and outdoor activities, parental education, occupation, smoking habits, house distance from industries and highways, source of drinking and cooking water and others. The blood samples were also limited by the absence of a more detailed haematological investigation including but not limited to the assessment of lipid profiles, information on thyroid function as well as oxidative stress biomarkers. This study, therefore, does not describe the metabolic status of the children and was limited in explaining the underlying mechanisms of the observed health effects with multiple elemental exposure. Moreover, lack of information on emerging molecular biomarkers such as Beta-2 microglobulin, kidney injury molecule-1, neutrophil gelatinase-associated lipocalin and/or N-acetyl-glucosaminidase limited in explaining the performance of specific renal glomerular and tubular functions of the participants and associated risk of acute and chronic kidney failure. This study was also limited in explaining the effects of mixed elevated exposures to Pb, Hg, and Mn on neurobehavioural development. The GLMs used here are unable to minimise the influence of some confounders on associations between blood elemental concentrations and some demographic, morphometric, and biochemical determinants, and are also unable to examine potential interactions of multiple mixed metals. Moreover, the observed associations between elemental exposure and health parameters discussed independently here, in fact, are the consequences of the interactions of multiple essential and potentially toxic elements.

5. Conclusion

Despite the above limitations, the results of the present study have uncovered a scenario of multiple elemental exposure in young children living in urban industrial areas in a populated city in a developing country. The blood concentrations of essential (Cr, Mn, Co, Cu, Zn, Se, Mo, and I), probably essential (V, Ni, and Br), and potentially toxic elements (As, Cd, Sb, Hg, Tl, Pb, Bi, and U) have exceeded the critical RVs reported by the CDC and/or CHMS in variable proportions of the children. This comprehensive study permitted the assessment of the associations between multiple elemental exposure and growth determinants and liver and kidney injury biomarkers, also indicating a possible association between elevated exposure to Tl and childhood obesity, while chronic exposure to low levels of As may decrease the body weight of young children. The results also revealed that chronic environmental exposure to excessive Br may alter the specific biochemical parameters, increase the risk of liver and kidney damage. More in-depth studies are warranted to confirm these findings and regular biomonitoring of metal exposure is essential in regulating elemental pollution and the consequential long-term health effects in the population including children. In addition, the government should respond appropriately to encourage industry entrepreneurs to improve existing industrial quality and emission processes.

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Declaration of competing interest

The authors have no conflict of interest.

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Appendix A. Supplementary data

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Special issue editorial: Key results of the european human biomonitoring initiative - HBM4EU

Human Biomonitoring (HBM) has increasingly become an established tool for the risk assessment of chemicals, chemical's safety, and for the steering of chemical policies in Europe, both at national level and worldwide for regulatory purposes. By monitoring internal human exposure, HBM is required to control the effectiveness of regulatory measures and to inform the general public as well as policy makers about the impacts of environmental pollutants and the use of synthetic chemicals on humans and the environment. HBM is important for achieving the goal of a sustainable and non-toxic environment for all by identifying areas with high risk of chemical exposure and by initiating, through science-to-policy approaches, targeted measures to reduce this exposure.

European citizens of all ages, as well as people worldwide, are exposed to a wide range of chemicals through their diet, their environment, the use of consumer products and at the workplace. Exposure to chemicals takes place through a variety of pathways and exposure routes, notably via dermal and oral uptake and by inhalation, with the combined exposure via all routes being the aggregate exposure. Knowledge about levels, co-exposures and extent of exposure are essential to inform effective policy-making to protect the European population from the impacts of exposure to hazardous chemicals on health.

Prior to the implementation of the European Human Biomonitoring Initiative (HBM4EU), a European Joint Programme, there was a clear lack of data on aggregate exposure to single substances and to combinations of chemical substances in the European population (highly exposed and vulnerable sub-groups) and on the actual distributions of exposure in different geographical European regions. Additionally, evidence-based knowledge on the link between external exposure via different routes, internal levels and human health was insufficient despite the productive long-standing national HBM programmes and the large number of Research and Development projects at national and European Union (EU) level. Furthermore, there have been significant limitations to the use of existing data as a basis for policy decisions at the EU level as they were not easily available and not collected according to harmonised protocols and might therefore not be comparable. The existing data were not representative of the European population and differences in the metadata characterising the datasets impede cross-dataset analyses.

At the beginning of this century, the European Commission which is responsible for chemicals policy, started a series of initiatives aiming at the development of a systematic, EU-wide HBM programme with harmonised methods, standards and study materials. First, in support of the Action 3 of the European Environment and Health Action Plan 2004–2010, an HBM Implementation Group was established to bring forward the development of a coherent approach to HBM in Europe in close cooperation with the Member States (Commission of the European Communities, 2003). In a second step, this approach was concretized in the first EU-co-funded project, Expert team to Support human BIOmonitoring on a European scale (ESBIO, 2009). Subsequently, the feasibility was piloted and further tested with the twin projects COPHES (Consortium to Perform Human Biomonitoring on a European Scale) and DEMOCOPHES (DEMOnstration of a study to Coordinate and Perform Human biomonitoring on a European Scale). While COPHES was working on the development of harmonised standards and setting up a framework for a coherent HBM in Europe (Joas et al., 2012; Schindler et al., 2014), DEMOCOPHES consisted in a feasibility study and generated the first HBM data for a limited number of substances in 1,844 children (5–11 years of age) and their mothers from 17 European countries (Den Hond et al., 2015). These results provided strong evidence for ubiquitous exposure of the European populations that were studied but also showed large differences between samples from different countries, thereby underlining the critical need for an EU-wide HBM study. These efforts ultimately led to the implementation of the large European Joint Programme, HBM4EU.

HBM4EU ran from 2017 to 2022 as a joint effort of 30 countries and 116 partner institutions from Europe and Israel with the aim to support and improve the EU's policies for chemicals, the environment and health. It was established as an innovative and unique type of large-scale, multi-national research programme operating at the science-to-policy interface. Importantly, national and EU level policy makers were included already early on in the planning phase of this demanding research programme. Policy needs, open policy and research relevant questions for altogether 18 prioritised substances and substance groups informed the setting up of this ambitious initiative. Important goals of HBM4EU were, besides the research output itself, a broad sharing of collected and produced data in accordance with the EU General Data Protection Regulation (2016/679) on the protection of natural persons with regard to the processing of personal data and a fast and easy access for policy makers and the scientific community to these results including the aggregated data sets, major conclusions, background information, and policy recommendations. A continuous and transparent dialogue between researchers and key actors in chemical regulation and policy-making at the EU level was maintained through an EU policy board. This board included representatives of different EU Departments and agencies such as the European Food Safety Authority (EFSA), the European Chemicals Agency (ECHA) and was led by the European Environmental Agency (EEA) which was also a partner of HBM4EU. In this way, HBM4EU ensured that policy makers gave input to the work plan development, kept track of the progress and were prepared and

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mobilised for optimal use of the research results to shape their policies and decisions.

The overarching objectives of HBM4EU were to:

i) harmonise procedures and tools for HBM at EU level,
ii) provide and, where missing, generate internal exposure data and link these data to aggregate external exposure and the relevant exposure pathways and sources,
iii) develop novel methods to identify human internal exposure to environmental and occupational chemicals and provide evidence for causal links with human health effects,
iv) provide policy-makers and the general public with science-based knowledge on the health risks associated with chemicals exposure, and
v) improve chemical risk assessment and policies in the EU through the effective use of HBM data.

To reach these objectives, the research activities were organized into clusters under three main pillars: 1) Science to Policy, which ensured the translation of results into policy, 2) European HBM Platform, which covered all components of HBM studies, and 3) Exposure and Health, which dealt with the research of innovative methods to determine the relation between exposure and health. By setting such a broad scope that emphasized involving and linking together different communities in the research process, HBM4EU ensured that the results of the initiative were relevant and useful to those most affected by exposures to hazardous chemicals.

This special issue contains the first comprehensive set of results from central activities of HBM4EU and reflects the complementarity of the HBM4EU components. However, it can only provide a glimpse of the extensive and intense work done collaboratively to further developing national HBM programmes in Europe and establishing HBM at a European level. Among the major outcomes of the project, we would like to highlight the importance of harmonisation of knowledge and protocols as a cornerstone for generating reliable scientific information on human exposure to hazardous chemicals in Europe to support the European environmental and health policy and the establishment of highly capable and EU-wide excellence networks.

The presented papers related to Pillar 1 “Science to policy” work expand on how HBM4EU results support current EU strategies, like the Chemicals Strategy for Sustainability and the Zero Pollution Action Plan (Lobo Vicente et al., 2023), and stress the importance of national awareness of HBM results for policy making (Ubong et al., 2023). They further elaborate on tools used to communicate with policy makers: indicators related to phthalates and DINCH exposure are presented as an example (Gerofke et al., 2023) and HBM guidance values for priority substances under the HBM4EU Initiative demonstrate how internal exposure levels can be interpreted concerning their potential health impact (Apel et al., 2023a,b). In addition, the use of HBM in chemical risk assessment is highlighted and methodological aspects, recommendations and lessons learnt are derived from HBM4EU work (Santonen et al., 2023b).

As ethics and ethical approvals are indispensable prerequisites of many studies and especially investigations in which humans are involved, HBM4EU has emphasized the EU-wide implementation and coordination of an ethics framework – and shares experiences and reflections in one of the papers included in this issue (Knudsen et al., 2023).

Harmonisation of materials and methods and operating procedures are essential for the preparation and generation of EU-wide comparable studies and study results. A tailor-made Quality Assurance (QA) program for analytical laboratories was designed and ran to this end. Lessons learned are derived for many aspects of the initiative, but this special issue dedicates three articles specifically to this topic: While one describes the tools and materials developed for a harmonised HBM study design and draws lessons from the development process, including the success in the QA program (Pack et al., 2023), the second one focusses on the coordination of the analytical phase (Vorkamp et al., 2023) and the third one discusses major aspects of administrative and scientific governance and steering of this multi-scale endeavour at the science-policy interface (Kolossa-Gehring et al., 2023).

The results of HBM4EU significantly contribute to understanding the exposure of European populations to a wide range of hazardous chemicals and how these exposures may impact human health. An overview paper showcases the EU-wide exposure data of 11 chemical substance groups in children, teenagers and adults from the HBM4EU Aligned Studies (2014–2021) (Govarts et al., 2023). A series of papers presents additional in-depth analyses of the internal exposure of Europeans.

HBM4EU work was organised around the 18 priority substances and substance groups. The comprehensive research on the substances is presented for two exemplary priority substance groups, the phthalates and substitute plasticisers as well as per- and polyfluoroalkyl substances (PFAS).

The phthalates case study perfectly illustrates the complex exposure situation and its numerous impacts on health and suggests some directions for a chemical policy aiming at a Zero Pollution ambition. Indeed, the study covers: current exposure to phthalates and DINCH in European children and adolescents (Vogel et al., 2023b); time trends following up the success of EU regulation and identifying needs for further action (Vogel et al., 2023a); the assessment of cumulative risks from simultaneous phthalate exposure (Lange et al., 2022); health-based guidance values and indicators as tools for risk description and transfer of results to policy conclusions (Apel et al., 2023a,b).

For the second substance group of high societal, scientific and health concern, PFAS, the study gives a comprehensive overview of European citizens exposure and evidence for the necessity to strengthen the protection of human health. It covers the exposure levels and determinants of variability in exposure in European teenagers (Richterová et al., 2023) a mixture risk assessment (following different methodological approaches) (Bil et al., 2023) and an overview of the PFAS results generated by all Work Packages (Uhl et al., 2023).

Further HBM results from the EU-wide HBM4EU Aligned Studies on the priority substances arsenic (Buekers et al., 2023), cadmium (Snoj Tratnik et al., 2022), and flame retardants in the general population and exposure determinants complement the picture of human internal exposure (van der Schyff et al., 2023).

The importance of HBM as a tool for the investigation of exposure at the workplace in a multi country setting was demonstrated by HBM4EU. Even though some companies may use HBM routinely, data are scarcely accessible. The HBM4EU chromates study underlines the need and use of an extension of occupational HBM studies to improve the protection of workers in the occupational setting in Europe and for collecting harmonised and comparable data (Santonen et al., 2023a).

In order to expand the HBM toolbox with new methods and findings concerning the relation of exposure and health, HBM4EU sought to explore different paths. One of them was the identification of relevant effect biomarkers, which is why a systematic approach to their implementation in HBM studies is explored in a dedicated article (Rodríguez-Carrillo et al., 2023). This Special Issue also elaborates on options to include more extensive health information in HBM studies (Tolonen et al., 2022).

New analytical and interpretation methods address the real-life simultaneous co-exposures to numerous chemicals which do not physiologically belong to the human body and how to deal with these very complex datasets. This special issue looks into the exposure and effects to mixtures of phthalates, PFAS, and pesticides as well as science-based recommendations for mixture risk assessment (Luijten et al., 2023; Ottenbros et al., 2023).

HBM4EU’s key strengths include its interdisciplinary nature, which allows for a more comprehensive understanding of the complex interactions between exposures to hazardous chemicals and human health, and its multi-scale approach to exposure science, which provides

a more complete picture of how environmental exposures impact human health.

Even with all the success in HBM4EU and its legacy, the urgent need in our fast-paced and everchanging world for evaluation and regulation of newly emerging products and chemical use applications, need to be further developed and adapted. HBM4EU, with its systematic establishment of interlinked science-and-policy networks in the field of environmental health and chemical policies spanning national to European to international levels, can now serve as a beacon for a new generation of EU co-funded research partnerships.

HBM4EU's work is being continued in the new Partnership for the Assessment of Risks from Chemicals (PARC), as the next European project operating at the science-policy interface, building on the structures, networks and experiences of HBM4EU. PARC has a larger scope since it also covers exposure and effects of chemicals in the environment and puts an extensive focus on further development of hazard and risk assessment (Marx-Stoelting et al., 2023). Thus, PARC will be the next step towards a sustainable EU-wide HBM, an across-silo risk assessment of chemicals, a support for the European Green Deal and consequently for the protection of humans and the environment in Europe. It is highly expected by the scientific community and by citizens that a long-term sustainable HBM framework will be established in Europe in support of policy making and of environment and health research.

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