TARGETED ANALYSIS OF ORGANIC CONTAMINANTS, EXPOSURE ASSESSMENT, AND VULNERABILITY OF POPULATIONS TO HAZARDOUS COMPOUNDS

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Doctoral Dissertation
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Doctoral Dissertation

TARČNA ANALIZA ORGANSKIH KONTAMINANTOV, OCENA IZPOSTAVLJENOSTI IN RANLJIVOSTI POPULACIJ ZA NEVARNE SPOJINE

Doktorska disertacija

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“A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales.”

— Marie Curie

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Abstract

From the moment of conception until death, humans are exposed to chemicals at varying intensities and frequencies. While an individual's level of resilience is commonly high enough to tolerate these kinds of stressors, specific exposures or genetic predispositions can lower it. Therefore, the assessment of chemical exposure, the development of analytical methods, the evaluation of the consequences for the population, and the assessment of individual susceptibilities are key aspects in exposome studies.

Within this dissertation, exposure to persistent organic pollutants (POPs), and non-persistent compounds, such as phthalates (PHs) and their alternatives, bisphenols (BPs), parabens (PBs), and triclosan (TCS) was assessed in two populations of 1) men and primiparous lactating women from Slovenia (first national human biomonitoring (HBM) project in Slovenia) and of 2) Slovenian men, women, and children (DEMOCOPHES). Potential exposure sources and differences in exposure were determined using questionnaire data. The results revealed that the Slovenian population is widely exposed to PHs and di(isononyl)cyclohexane-1,2-dicarboxylate, Hexamoll®DINCH (DINCH) and common BPs and PBs, whereas the levels of less-common BPs, PBs, and TCS were low. Likewise, exposure to POPs is low, especially if compared to populations from the Northern side of the Alps, whereas in Slovenia, POP levels were the highest in Bela krajina and Ljubljana. Associations with questionnaire data revealed that the most common sources of exposure for POPs, high molecular weight PHs, DINCH, and BPs were dietary, whereas exposure to PBs and low molecular weight PHs is mostly related to personal care products. We observed differences in exposure based on sociodemographic characteristics and the residential environment.

In the form of a review study, we summarized and evaluated analytical methods for the determination of contaminants of emerging concern (CECs) and concluded that the existing methods are suitable for application in HBM studies and do not require out-of-the-norm modifications in the laboratory.

To assess the possibility of harmful health effects resulting from exposure to PHs, DINCH, BPs, PBs, and TCS, we calculated the hazard quotient that is below the threshold of 1 for the evaluated population and contaminants. We suggest to repeat this assessment on other populations and to include a wider range of analytes.

As genetic predisposition can influence a person's detoxification ability, we investigated for the first time the influence of single nucleotide polymorphisms (SNPs) in genes of cytochrome P450 enzymes (CYPs) and UDP-glucuronyl transferases (UGTs) that can influence the biotransformation of PHs and DINCH using HBM data. The results indicate that SNPs in genes of *CYP2C9, CYP2C19, CYP2D6, UGT2B15* and *UGT1A7* can influence the biotransformation of PHs and DINCH and are, therefore, suitable biomarkers of susceptibility.

From this dissertation we can conclude that the available analytical methods for determining CECs are appropriate in the implementation of HBM. The Slovenian population is exposed to selected chemicals through food, personal care products, and the living environment. Exposure levels generally do not exceed critical thresholds, but

cumulative exposure to multiple chemicals at the same time and genetic susceptibility should be considered in the risk assessment. An important result of this work is also the identification of biomarkers of susceptibility to PH and DINCH exposure.

Povzetek

Od trenutka spočetja do smrti so ljudje izpostavljeni kemikalijam različnih intenzivnosti in frekvenc. Medtem ko je posameznikova raven odpornosti običajno dovolj visoka, da prenaša tovrstne stresorje, jo lahko posebne izpostavljenosti ali genetske predispozicije znižajo. Zato so ocena izpostavljenosti kemikalijam, razvoj analiznih metod, vrednotenje posledic za populacijo in ocena individualne občutljivosti ključni vidiki pri tovrstnih raziskavah.

V okviru te disertacije so bile prvič izvedene raziskave nacionalnega humanega biomonitoringa (HBM), s katerim smo želeli opredeliti izpostavljenost slovenske populacije obstojnim organskim onesnažilom (POPs) in neobstojnim spojinam, kot so ftalati (PH) in njihove alternativne spojine, bisfenoli (BP), parabeni (PB) in triklosan (TCS). V raziskavo so bile vključene naslednje skupine: 1) moški in nosečnice prvorodke na območju celotne Slovenije ter 2) moški, ženske in otroci v mestnem in podeželskem okolju (študija DEMOCOPHES). Potencialni viri izpostavljenosti in razlike v izpostavljenosti so bili določeni s podatki iz vprašalnikov. Rezultati so pokazali povišano izpostavljenost PH in di(izononil)cikloheksan-1,2-dikarboksilatu, Hexamoll®DINCH (DINCH) ter pogosto prisotnih BP in PB, medtem ko je bila raven izpostavljenosti manj pogostih BP, PB in TCS mnogo nižja. Prav tako je izpostavljenost POPs nizka, še posebej v primerjavi s prebivalci s severne strani Alp. S Sloveniji so bile ravni POPs najvišje v Beli krajini in Ljubljani. Povezave s podatki iz vprašalnika so pokazale, da so bili najpogostejši viri izpostavljenosti POPs, PH z visoko molekulsko maso, DINCH in BP s prehrano, medtem ko je izpostavljenost PB in nizkomolekularnim PH večinoma povezana z izdelki za osebno nego. Opazili smo razlike v izpostavljenosti glede na sociodemografske značilnosti in bivalno okolje.

V obliki pregledne študije smo povzeli in ovrednotili analizne metode za določanje skrb vzbujajočih kemikalij (CECs) in ugotovili, da so obstoječe metode primerne za uporabo v študijah HBM in ne zahtevajo sprememb za laboratorije.

Za oceno možnosti škodljivih učinkov na zdravje, ki so posledica izpostavljenosti PH, DINCH, BP, PB in TCS, smo izračunali količnik nevarnosti, ki je pod pragom 1 za ocenjeno populacijo in onesnaževala. Predlagamo, da to oceno ponovimo na drugih populacijah in vključimo širši nabor analitov.

Ker lahko genetska predispozicija vpliva na sposobnost razstrupljanja osebe, smo prvič raziskali vpliv polimorfizmov posameznih nukleotidov (SNP) v genih encimov citokroma P450 (CYP) in UDP-glukuronil transferaz (UGT), ki lahko vplivajo na biotransformacijo PH in DINCH z uporabo podatkov HBM. Rezultati kažejo, da lahko SNP v genih CYP2C9, CYP2C19, CYP2D6, UGT2B15 in UGT1A7 vplivajo na biotransformacijo PH in DINCH in so zato primerni biomarkerji občutljivosti.

Iz dela te disertacije lahko sklepamo, da so razpoložljive analitične metode za določanje CECs primerne pri izvedbi HBM. Slovenska populacija je izbranim kemikalijam izpostavljena preko hrane, izdelkov za osebno nego in bivalnega okolja. Raven izpostavljenosti v splošnem ne presega kritičnih pragov, vendar je treba pri oceni tveganja upoštevati kumulativno izpostavljenost več kemikalijam hkrati ter genetsko občutljivost.

Pomemben rezultat tega dela je tudi opredelitev biomarkerjev občutljivosti pri izpostavljenosti PHom in DINCHu.

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Abbreviations

5cx-MEPP	...	mono(2-ethyl-5-carboxypentyl)phthalate
5OH-MEHP	...	mono(2-ethyl-5-hydroxyhexyl)phthalate
5oxo-MEHP	...	mono(2-ethyl-5-oxohexyl) phthalate
ACN	...	acetonitrile
ADI	...	acceptable daily intake
ANOVA	...	analysis of variance
BBzP	...	benzylbutyl phthalate
BE	...	biomonitoring equivalent
BK	...	Bela krajina
BMI	...	Body-mass index
BPA	...	bisphenol A
BPF	...	bisphenol F
BPS	...	bisphenol S
BPs	...	bisphenols
BuP	...	butyl paraben
BzP	...	benzyl paraben
CE	...	Celje
CECs	...	contaminants of emerging concern
CRT	...	creatinine
cx-MINCH	...	cyclohexane-1,2-dicarboxylate-mono-(7-carboxylate-4-methyl)heptylester
CYPs	...	cytochrome P450 enzymes
DDT	...	dichlorodiphenyltrichloroethane
DEHP	...	di-(2-ethylhexyl)phthalate
DEP	...	di-ethyl phthalate
DiBP	...	di-*iso*-butyl phthalate
DINCH	...	di(isononyl)cyclohexane-1,2-dicarboxylate, Hexamoll® DINCH
dl-PCB	...	dioxin-like polychlorinated biphenyls
DnBP	...	di-n-butyl phthalate
ECD	...	electron capture detector
EPA	...	Environmental Protection Agency
EtP	...	ethyl paraben
EWAS	...	environment-wide association studies
GC	...	gas chromatography
GCMS	...	gas chromatography mass spectrometry
GerES	...	German Environmental Survey
GO	...	Posočje and Idrija
HBM	...	human biomonitoring
HBM I	...	first national HBM project

HBM4EU	...	human biomonitoring for Europe
HCE	...	heptachlor epoxide
HCH	...	hexachlorocyclohexane
HCB	...	hexachlorobenzene
HMW	...	high molecular weight
HQ	...	hazard quotient
HRMS	...	high-resolution mass spectrometer
*i*BuP	...	*iso*-butyl paraben
*i*PrP	...	*iso*-propyl paraben
JSI	...	Jožef Stefan Institute
KO	...	Kočevje and Cerknica
KP	...	Koper
KR	...	Jesenice
LC	...	liquid chromatography
LCMS	...	liquid chromatography mass spectrometry
LJ	...	Ljubljana
LLE	...	liquid liquid extraction
LMW	...	low molecular weight
LOQ	...	limit of quantification
MAFs	...	minor allele frequencies
MB	...	Maribor
MBzP	...	monobenzyl phthalate
MCHP	...	monocyclohexyl phthalate
MCI	...	methylchloroisothiazolinone
MEHP	...	mono(2-ethylhexyl)phthalate
MEP	...	monoethyl phthalate
MI	...	methylisothiazolinone
MiBP	...	mono-*iso*-butyl phthalate
MINCH	...	mono-isononyl-cyclohexane-1,2-dicarboxylate
MMP	...	monomethyl phthalate
MnBP	...	mono-n-butyl phthalate
MnOP	...	mono-n-octyl phthalate
MP	...	methyl paraben
MRM	...	multiple reaction monitoring
MS	...	Pomurje
MS/MS	...	tandem mass spectrometry
ndl-PCB	...	non-dioxin-like polychlorinated biphenyls
NHANES	...	National Health and Nutrition Examination Survey
NOAEL	...	no-observed-adverse-effect level
OCPs	...	organochlorine pesticides
OH-MIDP	...	mono(6-hydroxy-2-propylheptyl) phthalate
OH-MINCH	...	cyclohexane-1,2-dicarboxylate-mono-(7-hydroxy-4-methyl)octyl ester
OH-MINP	...	mono(4-methyl-7-hydroxyoctyl) phthalate
oxo-MIDP	...	mono(6-oxo-2-propylheptyl) phthalate
oxo-MINCH	...	cyclohexane-1,2-dicarboxylate-mono(oxo-isononyl) ester
oxo-MINP	...	mono(4-methyl-7-oxooctyl) phthalate
p,p'-DDE	...	dichlorodiphenyldichloroethylene
PAHs	...	polycyclic aromatic hydrocarbons
PBs	...	parabens

PBPK	...	Physiologically based pharmacokinetics
PCA	...	principal component analysis
PCBs	...	polychlorinated biphenyls
PCDD/Fs	...	polychlorinated dibenzodioxins and dibenzofurans
PCPs	...	personal care products
PH	...	phthalate
POPs	...	persistent organic pollutants
PrP	...	propyl paraben
PVC	...	polyvinyl chloride
RA	...	Mežica valley
RCR	...	risk characterization ratio
SG	...	specific gravity
SNP	...	single nucleotide polymorphism
SP	...	Savinjsko-Posavska
SPE	...	solid phase extraction
TCS	...	triclosan
TEQ	...	toxic equivalent
UGTs	...	UDP-glucuronyl transferases
UHPLC-MS/MS	...	Ultra-high-performance liquid chromatography tandem-mass spectrometry
UV	...	ultra-violet
ZA	...	Zasavje

Chapter 1

Introduction

1.1 The Exposome

From the perspective of disease development, the need for a research area complementing the genome became inevitable once it was apparent that the genetic predisposition contributes to merely 10% of disease risk (Rappaport, 2011). This redirected the attention of researchers to external influences and their respective impact on the individual. As such, the term "exposome" was introduced for the first time in 2005 by Dr. Christopher Wild (Wild, 2005) with the purpose to capture the lifelong exposure history from the time of conception until death. Wild, (2012) divides exposure into three main categories: internal, specific external, and general external exposure. The internal exposure covers the endogenous processes within the body, such as metabolism, oxidative stress, hormones, or gut microflora, to name a few. The specific external exposure captures distinct environmental factors (e.g. radiation, chemical contaminants), whereas the general external exposure focuses on the overall environment including social, economic, and psychological influences (education, financial status, stress, climate, etc.). These categories should not be regarded separately, but rather in context of one another with some factors falling into several classes.

Unlike the genome that shows minimal variation during the lifetime of an individual, the exposome is prone to changes and dynamic in nature. Origin, characteristics, and intensities of life-long exposure are constantly fluctuating making its assessment a challenge. However, some studies hypothesize that spot exposure assessments can serve as an approximation of continuous exposure especially, but not exclusively, during vulnerable developmental stages (Mimoto et al., 2017). As such, exposure assessment studies like the one presented in this dissertation have become an indispensable asset to the exposome.

This PhD is part of the ITN project NEUROSOME that aims at developing an integrative framework to evaluate the potential relationships between genetic susceptibility, exposure to chemicals and chemical mixtures, and the development of neurological disorders. It, thereby, relies on human biomonitoring data and combines exposure monitoring, toxicological analyses, and advanced modeling approaches in order to follow the exposure-to-health effect continuum within the exposome paradigm.

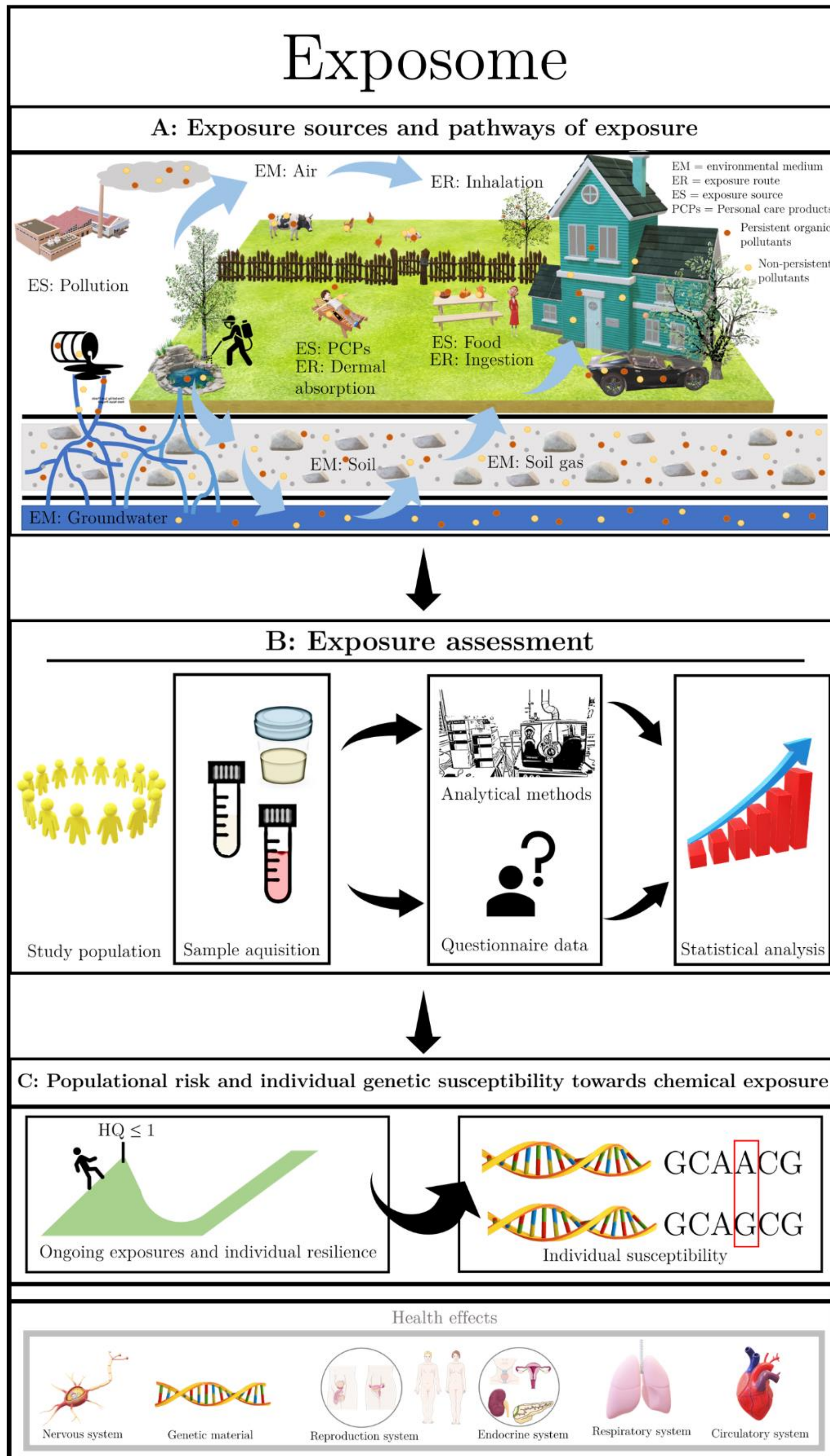

Figure 1: Illustration of the three main compartments of this doctoral dissertation. A: An illustration of exposure sources and pathways of exposure, B: exposure assessment of populations using HBM data, method development, and statistical analysis, C: the assessment of populational risks and individual susceptibilities within the concept of resilience. Adverse health effects are not a part of this PhD.

1.1.1 Exposure sources and pathways of exposure

Chemical exposure can be a one-time event or ongoing, high-dose or low-dose, and via different pathways, as illustrated in Figure 1A. The pathway of exposure can be of high importance, as it determines the site of metabolism and the biologically active dose. Ingestion may result in higher concentrations of the chemical in the liver, while dermal absorption and inhalation are followed by circular distribution throughout the body (Bekö et al., 2013; Jo et al., 1990). Thus, understanding the influence of the pathway on the total exposure is a crucial part in exposome-based studies. Decisive hereby are the contaminant's physiochemical characteristics. In the case of phthalates (PHs), high molecular weight (HMW; 7-13 C atoms in the side chain) PHs enter the human body mainly via ingestion of contaminated food or dust, whereas for low molecular weight (LMW; 3-6 C atoms in the side chain), dermal absorption and inhalation are more frequent pathways. LMW PHs occur to a large fraction in the gas phase (Bekö et al., 2013) and some of them can penetrate the skin barrier (Pan et al., 2014). Dermal absorption delivers the chemical directly into the blood stream via which it distributes throughout the body. Ingested and inhaled compounds first enter the gastrointestinal tract and lungs, respectively, before they reach the blood (Bekö et al., 2013). These 3 pathways – ingestion, inhalation, and dermal absorption – represent the major exposure routes to environmental contaminants.

Environmental media such as air, water, and soil as well as dietary and consumer products are sources of exposure (Figure 1A), but intensity and frequency of such can vary among compounds. Organic and inorganic contaminants are often regarded as two separate groups, however, this concept neglects associations between inorganic elements and organic molecules (Gochfeld, 2003). More appropriate in the context of this doctoral dissertation is the differentiation between compounds with the ability to bioaccumulate in the environment and in the human body (persistent pollutants) and those that can be rapidly degraded via (a)biotic degradation processes (non-persistent pollutants).

1.1.2 Persistent organic pollutants

Persistent organic pollutants (POPs) can be characterized by their stability and ability to bioaccumulate and biomagnify in the food chain. Thus, the main pathway of human exposure is via dietary products, especially if derived from animals with a high trophy status. POPs can be either of natural origin, such as polychlorinated dibenzo-dioxins and polychlorinated dibenzofurans (PCDD/Fs) or man-made, such as polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs). Industrial POPs find a wide range of applications ranging from pesticides over insulators to flame retardants. Due to the often high degree of halogenation (Porta et al., 2007), POPs pose a threat to human health as well as to the environment and wildlife. POPs can be linked to neurological diseases such as Parkinson's or Alzheimer's disease. Adverse health effects in humans include neurotoxicity, developmental effects, chronic illnesses, death, increased cancer risk, endocrine disruption within the reproductive system, the central nervous system, and the immune system. Especially vulnerable are humans during critical developmental periods inside the womb or during early childhood. The effects resulting from exposure to POPs during these critical stages can last throughout a person's lifespan (Damstra, 2002). Furthermore, they are the cause of multiple human and environmental disasters, such as the decline of insect, bird, and aquatic animal populations in the United States vividly described by Rachel Carson in her book Silent Spring (Carson, 1962) or the 1968 mass poisoning of Japanese women, men, and children with PCBs and PCDD/Fs (Kuratsune et

al., 1996). In Slovenia, especially the monitoring of PCB concentrations in the environment and in humans is of interest, ever since the contamination of the Krupa River in Bela krajina between 1962 and 1983 has become public knowledge. In response to the pollution, PCB concentrations in river sediment reached 55 µg/g, whereas 117 µg/g was the concentration determined in local fish. In 1984, as study by (Jan & Tratnik, 1988) investigated the PCB blood concentrations of residence of the area and determined mean values of 155 ng/g. With increasing distance from the location (20 km), PCB blood levels dropped to 5 ng/g (Jan & Tratnik, 1988). The toxicity of POPs is widely recognized and restrictions of these compounds have been in place since the 1970s. In 2001, the Stockholm Convention on Persistent Organic Pollutants officially initiated the ban and continuous monitoring of POPs in the environment and in humans that came into force in 2004. The human internal exposure is, however, only slowly decreasing as POPs can have a half-life of several decades and do not undergo vast biotransformation in the body. Environmental concentrations have since been decreasing but remain widespread and detectable in the environment (Figure 1A) in both populated and isolated regions due to their persistence and capacity of long-range transport via oceanic and atmospheric currents (UNEP, 2017).

1.1.3 Non-persistent organic pollutants

Non-persistent contaminants commonly have short half-lives in the human body and in the environment, which limits their toxic potential in the case of one-time exposures. Adverse health effects related to the exposure to these chemicals can, however, often be attributed to the compound-specific metabolites that can be the toxic agent instead of the parent compound. For many non-persistent pollutants, their mode of action is understood – the estrogenic capacity of bisphenol A (BPA) has already been suspected in the 1930s (Vandenberg et al., 2009), whereas for others it is merely suspected. Neurological disorders are a particularly ambitious endpoint as the development of the brain is a dynamic process with somewhat increased intensity during childhood and adolescence that is abating later on in life. Recently, certain non-persistent chemicals such as PHs and BPA have been drawn into the center of attention as they are increasingly suspected to alter brain development in children (Rochester et al., 2018). Endocrine disruptors, such as PHs and BPA affect the human body mainly through their anti-androgenic effects and via disruption of thyroid's hormonal functions (Jeddi et al., 2016). As they share certain structural similarities with hormones such as estrogen or testosterone, they are able to bind to their specific receptors mimicking the activity of the actual hormone. Other health endpoints that have been observed with regard to endocrine disruptors involve: different kinds of cancer (Bonde et al., 2016; Cashman & Warshaw, 2005; Hsieh et al., 2011; Soto & Sonnenschein, 2010), respiratory diseases (North et al., 2014), impaired human fertility (Chen et al., 2017), obesity (Goodman et al., 2014), diabetes (Shapiro et al., 2015). As an additional reason for concern, many members of the group of non-persistent pollutants, such as PHs, bisphenols (BPs), parabens (PBs), and triclosan (TCS) have reached the status of pseudo-persistency as they can be detected in human samples at high concentrations and frequencies due to widespread industrial applications. For instance, HMW PHs and BPs are utilized in plastics, whereas LMW PHs, PBs, and TCS are additives in many personal care products (PCPs) to enhance their properties and longevity. Therefore, humans can be exposed to non-persistent contaminants via ingestion, inhalation, and dermal absorption. As none of the mentioned compounds are chemically bound to the applied matrix, they tend to migrate into the environment and into products via evaporation, leaching, and abrasion (Figure 1A). Rudel et al., (2003) detected between 6

and 42 different compounds in household dust, of which PHs, PBs, antimicrobials, alkylphenols, synthetic musks, and cyclosiloxanes have endocrine disruptive potential with different severity and pathways. Calafat et al., (2018) detected 24 individual biomarkers of pesticides, phenols, PBs, PHs, and polycyclic aromatic hydrocarbons (PAHs) in urine and blood of 122 children from the USA.

Human exposure to chemicals is an important and crucial part of the study design step within HBM surveys that mainly targets three key questions

1) Who is exposed?
2) To which compounds are humans exposed?
3) Via which pathways are humans exposed?

Exposure assessment studies aim to fill the existing knowledge gaps in each of these questions.

1.2 Exposure Assessment

As indicated in the previous section, the direct link between exposures and health effects is nearly impossible to detect. One option is, however, to target specific aspects of exposure (e.g. selected compounds, a source or a specific pathway) one at a time (Figure 1B). Several bottom-up approaches for estimating exposure exist that can be based on the direct and targeted measurement of contaminants or their metabolites in human matrices, non-targeted and suspect screening for chemical profiling of human samples, or computational exposure science that is based on large databases and computational modeling in order to - for instance - predict concentrations, exposure pathways, but also to identify so far unrevealed associations among variables (Bonnell, Zidek, Griffiths, & Gutzman, 2018; Isaacs et al., 2020; Ring et al., 2019). With the help of pharmacokinetic (PBPK) modeling, important exposure routes can be identified and the factors that underlie variability in the determined concentrations among individuals can be revealed (Isaacs et al., 2020). However, also modeling approaches rely on exposure data obtained through the measurement of contaminants in human samples.

Wild, (2012) hypothesizes that exposures leave measurable traces that can be followed towards the corresponding effect. The remaining unsolved variable in this attempt of source apportionment lies in the chemical or structural identification of those traces that are hereby referred to as biomarkers (Ladeira & Viegas, 2016; Vineis et al., 2020). They represent the measurable evidence that exposure occurred (**biomarkers of exposure**), caused alterations in the body immediately or long after the event (**biomarkers of effect**), and can serve as indicators of individual susceptibility or resilience towards exposure (**biomarkers of susceptibility**). Vineis et al., (2020) point out that the current challenges in exposome research lie in the identification of new biomarkers and the development of analytical methods for their determination (Figure 1B).

The half-life of POPs can range over several decades and only small amounts are excreted from the body at a time. Therefore, blood is a suitable matrix for compounds with accumulative and lipophilic tendencies. Blood sampling, however, is an invasive procedure that requires technical skills and yields limited sample volume, which needs to be considered in the planning phase of exposure assessment studies. Additionally, during data interpretation it needs to be considered that bioaccumulation and lipophilicity often go hand in hand. Additionally, maternal milk, due to its high lipid content, is an often chosen

matrix. Therefore, it is wise to adjust the measured concentrations in blood or maternal milk to the lipid content if a correlation is detected.

Non-persistent contaminants pose a challenge in the laboratory due to contamination and during data interpretation due to their fast excretion from the human body. Non-persistent contaminants are often biotransformed to more hydrophilic metabolites that can be more easily excreted in urine compared to the parent compound. Well investigated is the example of di-(2-ethylhexyl)phthalate (DEHP) that undergoes phase I and phase II metabolism in humans. Upon entering the body, the di-ester is rapidly hydrolyzed to mono(2-ethylhexyl)phthalate (MEHP) via esterases and lipases (Phase I biotransformation) that can be further modified via cytochrome P450 enzymes (CYPs) to form hydroxy, oxo, and carboxy secondary metabolites (Phase I). Primary as well as secondary metabolites can undergo conjugation mainly via UDP-glucuronyl transferases (UGTs) (Phase II biotransformation), which facilitates their excretion in either urine or faeces depending on their lipophilicity. Among the compounds included in this dissertation, only PHs and their alternative di(isononyl)cyclohexane-1,2-dicarboxylate, Hexamoll® DINCH (DINCH) undergo phase I biotransformation. Some LMW PHs, BPs, PBs, and TCS go through phase II biotransformation and are, thus, excreted from the body in mostly conjugated form. The half-life of non-persistent compounds in the human body is less than 24h, which makes reliable source apportionment and exposure assessment challenging and highlights the need for identifying reliable biomarkers of exposure. Such biomarkers can additionally serve as a proxy for the exposure timing if the compound of interest undergoes the complete phase I biotransformation. It has been hypothesized that the ratio between a carboxy (mono(2-ethyl-5-carboxypentyl)phthalate; 5cx-MEPP) and a hydroxy (mono(2-ethyl-5-hydroxyhexyl)phthalate; 5OH-MEHP) metabolite of DEHP can serve to estimate the time of exposure based on differences in elimination half-life between the two compounds (Lorber et al., 2011; Meeker et al., 2012).

To reliably assess exposure and related health effects, such biomarkers have to be identified, their mode of action understood, and excretion pathways and urinary excretion factors established (Koch et al., 2017). Excretion factors are valuable information in exposure science as they allow an estimation of how much of a chemical is excreted in urine as a certain biotransformation product. Accordingly, they allow the back-calculation of the original intake dose of the parent.

Challenges in data interpretation arise from the uncertainty of exposure timing. With half-lives often as short as several hours, the time-period between exposure and sample acquisition determines the concentrations detected in urine. 24h urine is widely accepted as the most reliable source to estimate exposure, but population-based studies rarely allow for such extensive sampling campaigns. Spot urine samples are a commonly used method in studies with a large sample size, despite the related uncertainty. Studies suggest, though, that despite large intra-day variations in urinary metabolite concentrations, the median concentrations in a month are comparable to those obtained in spot urine samples (Aylward et al., 2014; Koch et al., 2013, 2017). Hauser & Calafat, (2005) observed that individuals are continuously exposed to phthalates, with relatively constant exposure sources over time. Thus, even though phthalates have a half-life of less than 24h in the human body, the continuous exposure underlies little variation and the concentrations detected in one first morning urine sample are representative for approximately 3 months.

In an attempt to increase comparability among studies, first morning urine samples are commonly preferred in human biomonitoring (HBM) studies as they somewhat unify the time period between the exposure and sampling (Frederiksen et al., 2013).

1.2.1 Human biomonitoring as a tool in exposure assessment

As illustrated in Figure 1B, HBM is an established tool to assess the chemical burden of either targeted sub-populations, or the representative general population. It is the "repeated, controlled measurement of a chemical or chemical marker in fluids, tissues, or other accessible samples from subjects exposed to chemical, physical, or biological risk factors" (Ladeira & Viegas, 2016). Furthermore, it aims at the determination and validation of biomarkers as tools in exposure assessment and reflects the total body burden, also known as internal exposure (Koch & Calafat, 2009; World Health Organization, 2015). Information on the levels of contaminants in human matrices can then be associated with questionnaire data on dietary habits and individual lifestyles reported by the participants obtained within HBM studies in the frame of exposure association studies.

Exposure reconstruction makes an important part in exposure assessment studies. The most simple approach that is, however, only applicable to non-persistent contaminants, involves the intake dose calculation via excretion factors, metabolite concentrations, and the urinary output (Runkel et al., 2020). However, more complex PBPK modeling can be combined in a reverse dosimetry approach to associate the concentrations of contaminants in a chosen matrix with a distribution of exposures (Martínez et al., 2021). Reverse dosimetry is a sophisticated tool for the reconstruction of the original exposure dose by estimating the distribution of exposure in the environment (Clewell et al., 2008).

Results obtained from those studies can be further utilized in medical studies, various modelling approaches, and risk assessment. As already implied in the introduction to the exposome concept, HBM aims at assessing the internal exposure of the population in an attempt to link it to the general and specific external exposure. This includes

- the determination of contaminants in human matrices (Manuscript 1, 2, 4)
- identifying determinants of exposure via questionnaire data (Manuscript 1,2)
- reconstructing exposure via the calculation of intake doses (Manuscript 1)
- addressing individual susceptibility (Manuscript 5)
- evaluation of the risk of the population in response to exposure (Manuscript 4)
- the evaluation of analytical methods for the determination of contaminants (Manuscript 3), and
- linking or modeling potential resulting health impacts on the human body, which is beyond the scope of this doctoral dissertation.

Within the frame of this dissertation, two populations were selected. The first consists of 77 men, 155 women, and 155 children from two regions of Slovenia, that were recruited in 2011 within the European DEMOCOPHES project. The second study population consists of 536 primiparous lactating women and 548 men that were recruited from 12 regions in Slovenia within the first national HBM project (HBM I) during two sampling campaigns (2009-2009; 2011-2014).

Exposure assessment is in constant need of updated exposure data and in an ever need of sensitive and robust analytical methods for the determination of contaminants in human matrices.

1.2.2 Analytical methods for the determination of compounds of interest

As illustrated in Figure 1B, the development of analytical methods and the quality assurance of measurements are key stones in exposure assessment. The methodology in chemical analysis can roughly be divided into three large chapters: sample pretreatment, sample preparation, and analyte separation and detection.

Compounds that undergo phase II metabolism additionally require a deconjugation step, where the analyte is enzymatically cleaved from the respective conjugate. Sample preparation usually is the most time-consuming fraction during analytical procedures. Clean-up, extraction, and pre-concentration can take an average of 60 % of the total time (Andrade-Eiroa et al., 2016). These steps are critical for the overall performance of the analysis, as contamination and analyte loss can occur at many stages during sample preparation. Therefore, the lower the concentration of the target analyte in a sample, the higher the risk. To remove impurities from the complex biological matrix, different extraction methods can be applied that isolate the analyte from the mixture of compounds present in the matrix (Azzouz et al., 2016; Rocha et al., 2018). For a number of different matrices (urine, blood, water, soil, tissues, etc.), solid phase extraction (SPE) is the most commonly applied method for clean-up, extraction, and pre-concentration of environmental pollutants (Andrade-Eiroa et al., 2016). The extraction involves four critical steps: conditioning of the sorbent, loading of the sample, washing, and eluting of the analyte. The basic principle relies on the solution containing the dissolved or suspended analyte being loaded onto a solid phase where the target analyte is retained and the unwanted leftover matrix is being discarded. At last, another solvent is added that elutes the analyte into a collection tube. Nowadays, the solid phase is usually packed in a cartridge (Andrade-Eiroa et al., 2016). Conditioning of the cartridge is commonly carried out by wetting the surface with elution solvents and equilibration by a matrix comparable to that of the sample. The sample afterwards interacts with the sorbent and is hereby retained. Hence, when choosing the sorbent, the polarity of the analyte plays a critical role. In the washing step, a milder elution solvent (which elutes the matrix interferences, but not the analytes, depending on analyte polarity and pKa) is flushed through the solvent to remove further impurities. At last, a polarity-matched solvent is used to elute the analyte from the cartridge and collected. The method is, among others, very suitable for the measurement of phthalate metabolites and BPA in urine and blood (Azzouz et al., 2016).

Especially, but not exclusively, analyses with gas chromatography (GC) often require an additional derivatization step during sample preparation. Hereby, the physical and chemical properties of the analyte are being modified to meet the analytical requirements. We applied this method to PBs, BPS, and TCS in order to increase their volatility, chromatographic properties, and thermal stability (Manuscript 4).

The most commonly used instruments for trace organic analysis are gas chromatography coupled to mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS). The basic principle of chromatography lies in the separation of compounds based on their properties. In the first step, the sample enters the system in a mobile phase that is then forced through a stationary phase. While the mobile phase moves along the stationary phase, the analyte(s) interact with the latter mainly depending on their polarity. The main difference between GC-MS and LC-MS is the analyte carrying mobile phase that is a gas in the former and a liquid in the latter (Richter & Pirola, 2017). The separation efficiency during analysis largely relies of the column of choice. The appropriate column is the one that balances sample capacity, resolution, analysis time, retention time, stationary phase breakdown, and is compatible with the polarity of the

analyte. Post column, the eluted compounds enter the mass spectrometer. In the ionization source of the mass spectrometer, the molecules ionize, in the simplest case with electron impact ionization (GC-MS) or electrospray ionization (LC-MS). Then the ions undergo further separation according to the mass-to-charge ratio of parent to daughter ion transitions, which is commonly referred to as multiple reaction monitoring (MRM). The latter is the most common acquisition mode in tandem mass spectrometry (MS/MS), which is particularly important when determining trace-level analytes. An example is the analytical method for the determination of selected BPs, PBs, and TCS which includes an offline-SPE sample preparation procedure followed by analysis by GC-MS/MS (Manuscript 4).

HBM relies on the analysis of biomarkers of exposure and is, thus, dependent on improvements in the field of analytical chemistry (Aylward et al., 2014). Therefore, one manuscript included in the present dissertation (Manuscript 3) is dedicated to the review of available analytical methods for biomarkers of exposure for contaminants of emerging concern (CECs). These are of interest, as humans can be widely exposed to these compounds while they are to date not part of regular HBM studies and information on analytical procedures is scarce. Included in the manuscript are isocyanates, benzotriazoles, pyrroliodones, ultra-violet (UV) filters, the antimicrobials methylchloroisothiazolinone and methylisothiazolinone (MCI, MI), fragrances, and selected non-phthalate plasticizers.

1.2.3 Exposure association studies

Exposure association studies are a common tool in HBM with the aim to link the determined compound concentrations to exposure sources and to reveal associations among and between contaminants and influencing factors (Figure 1B). Such factors include physiology or the socio-economic background of a person. The information necessary for such associations can be obtained via questionnaire data. However, there are some general uncertainties associated with this approach that include a lack of standardization among studies, the false interpretation of the questionnaire by the participant, incomplete recall of, for instance, consumption frequencies, or intentional false reporting in the case of delicate questions (e.g. alcohol consumption, mental health) (Coggon, 1995; Galbete et al., 2018). Especially for the assessment of exposure to non-persistent contaminants, detailed information on the last 48h prior to sampling is essential. Additionally, the targeted sample size is a matter of discussion as the analytical measurements and data interpretation have to be achievable while obtaining a sufficient sample size to guarantee statistical power. In order to reliably assess exposure in a population, a minimum sample size of 120 per population group is recommended by the World Health Organization (World Health Organization, 2015).

In the general population, additional challenges arise during source apportionment, as information on exposure time, location, and magnitude is generally scarce. Limited data and the extrapolation from a smaller subset to country scale populations require necessary simplifications and assumptions that largely increase the number of uncertainties for this kind of exposure assessment (McKone & Daniels, 1991).

In order to reliably assess exposure and to identify the sources, different statistical approaches are currently utilized, among them correlation and regression analyses. Commonly used are multiple linear regression modeling and generalized linear modeling to identify associations among variables. However, less common methods, such as ordinal logistic regression can be applied if the circumstances allow it. An example of such is demonstrated in Manuscript 4, where the lack of necessary confounders increases the uncertainty of the results and has a negative effect on the model performance. To overcome

this issue, the analyte concentrations are grouped into categories of increasing value, which shifts the focus from the actual determined value towards the relative ordering between values. However, to assess associations between exposure and health effects, environment wide association studies (EWAS) are a powerful tool for the interpretation of epidemiological data (Patel et al., 2010).

In the present dissertation, we conducted two kinds of association studies. Firstly, we investigated associations among compounds that can indicate a common source of exposure. This can be expected as chemicals are often added to products as mixtures. This is for instance the case for methyl paraben (MP) and propyl paraben (PrP) that result in a higher preservative effect in PCPs and food when applied together (Manuscript 1, 2, 4). Secondly, we identified potential sources of exposure as well as lifestyle factors that increase or decrease exposure to phthalates and POPs (Manuscript 1 and 2).

Subsequent to exposure association studies between determined contaminant concentrations and questionnaire data, the last chapter in exposure assessment focusses on the resulting risk of the population as well as the vulnerability of individuals (Figure 1C).

1.3 Risk and Vulnerability Assessment Towards Chemical Exposure

The matter of on-going and past exposures has been elaborated in the previous chapters, however, the picture is incomplete without the complex interrelationship among exposure, hazard, risk, and susceptibility. In the frame of this, the general concept of resilience needs to be highlighted that can be specifically applied to exposure studies. Resilience can be defined by the ability of an individual or an (a)biotic system to recover from a disruptive event and to maintain functioning with no external assistance (Garcia-Dia et al., 2013). As such, the level of resilience – or the amount of disturbance an organism can withstand without losing the capacity to recover (Figure 1C) – within an individual is a key factor in maintaining health (Manyena, 2006). Within exposome studies, biomarkers of susceptibility are a measure of estimating an individual's resilience towards exposure to chemicals. The concept is commonly applied to (epi-)genetic factors, but the physiological status including factors like age, body-mass index (BMI), or life-style can as well be considered markers of susceptibility. Over the years, study approaches including gene-environment interaction studies or environment-wide association studies (EWAS) have emerged to address the matter and to re-evaluate the equation where hazard × vulnerability equals risk (Dennis et al., 2016; Manyena, 2006). The understanding of gene-environment interactions is a key stone in the estimation of risk resulting from exposure to environmental pollutants (Zeiger, 1994).

1.3.1 Populations at risk

Within chemical risk assessment, two terms must be differentiated, namely hazard and risk. Where the hazard mainly describes the potential of a chemical to cause adverse health effects (its toxicity), the risk establishes a probability relationship depending on the hazard, the occurrence of this chemical in the environment, and the actual exposure. In order to perform risk assessment, three steps generally need to be followed (WHO/IPCS, 2009):

1) hazard characterization, which determines the biological response to a chemical at different doses,

2) exposure assessment, which consists of either the measurement of the chemical in a human matrix or exposure estimation,
3) risk characterization, which combines the hazard – determined in step 1 – and the actual exposure as described in step 2.

Additionally, when dealing with real-life scenarios, the effects of cumulative exposures have to be considered. For many chemicals, the dose-response relationship is not linear and similarly can the effects of substances in a mixture with others be additive, synergistic or antagonistic (Carpenter et al., 2002). To date, risk assessment for chemical mixtures is an underdeveloped area that suffers from many generalizations in response to a severe lack of data (Louro et al., 2019). Dose additivity estimates the effect of the mixture via the sum of the doses assuming a common mode of action. This approach, however, can easily mislead as it is the case for cyanide and hydrogen sulfide that share their mode of action - respiratory inhibition - but act antagonistic towards each other (Borgert et al., 2004). Unfortunately, with the current state-of-the-art it is rarely possible to test the infinite range of interactions of chemicals in mixtures due to a lack of data, guidance, and experience. Attempts to overcome these limitations exist in the form of adverse outcome pathways, *in vitro* testing, omics techniques, the use of threshold values, and pharmacokinetic models and the Horizon 2020 project European Test and Risk Assessment Strategies for Mixtures is developing strategies for the understanding of chemical mixtures and the resulting risk (Pletz et al., 2020; Rotter et al., 2018). For the moment, risk assessment of chemical mixtures is still at a preliminary stage and dose addition remains the most commonly applied approach (Simon, 2020).

For the compounds included in this doctoral dissertation, the hazard has been widely characterized as either the biomonitoring equivalent (BE), the acceptable daily intake (ADI) or the no-observed-adverse-effect level (NOAEL). The BE value describes the concentration of a chemical (or its breakdown products) in a biological matrix that is consistent with given health-related guidance values, such as the ADI among others (Hays et al., 2008). The ADI describes the maximum amount of a chemical that can be ingested daily without resulting in any adverse health effects (Lu, 1988). The NOAEL describes the highest concentration of a chemical at which no adverse health effects have been observed (Louro et al., 2019). The risk can, therefore, be characterized utilizing the ratio between the measured concentrations of the chemical of interest and the given BE, ADI or NOAEL values (EFSA, 2013). This ratio is commonly referred to as the hazard quotient (HQ) or the risk characterization ratio (RCR). The value obtained based on the given approach cannot be regarded as the probability for the development of adverse health outcomes, but rather as an indicator if health effects are likely to occur or not. As such, if the ratio exceeds the threshold of 1, it implies that the determined concentration of the chemical exceeds the reference value at which no adverse health effects were observed and adverse health outcomes cannot be excluded. This concept can be applied to individuals as well as to populations (Sanchis et al., 2020) and has been done so within the frame of this doctoral dissertation as presented in Manuscript 4. However, this concept generalizes the biological response of an organism to chemical stressors and assumes equal levels of resilience for all individuals. Per contra, individual susceptibilities can underlie often large variations that need to be considered.

1.3.2 Individual susceptibility

How an organism responds to chemical stressors is largely determined by its intrinsic characteristics (Figure 1C). Underlying diseases or normal physiological characteristics,

such as age, can, for instance, influence the absorption rate of xenobiotics and the body's metabolic rate (Zeiger, 1994; Zeise et al., 2013). In this aspect, the focus of this doctoral dissertation lies on genetic biomarkers of susceptibility (Ladeira & Viegas, 2016). Genetic predisposition can influence enzymes involved in the detoxification of certain compounds or metabolites, the capacity of cells to repair DNA damage, or the general sensitivity towards chemical exposure resulting from inherited genetic defects (Ladeira & Viegas, 2016; Zeiger, 1994). In the case of the former, genes that are coding for enzymes involved in the biotransformation of xenobiotics are especially of interest. The respective sequence coding for these genes can vary among individuals and such variations can accumulate in a population in response to selective pressures. If the frequency of variation exceeds the 1% threshold, it is referred to as a genetic polymorphism rather than a mutation (Kelada et al., 2003). So-called "silent mutations" have no effect on the phenotype, whereas functional polymorphisms can alter gene expression and consequently enzyme functioning and/or stability. The majority of these genetic variations (~90%) are single nucleotide polymorphisms (SNPs), where single base pairs are altered. The average gene is assumed to contain 3 – 4 SNPs of which less than half is functional. The influence of such SNPs on the biotransformation of xenobiotics can be rather significant, considering that the metabolic fate of some compounds is dependent on the activity of a single enzyme (Ladeira & Viegas, 2016; Zeise et al., 2013).

The role of SNPs has been profoundly studied in relation to trace elements and to specific exposure – disease scenarios; an example of the latter being the smoking – lung cancer causation. However, little has been done on organic contaminants and among these even less on non-persistent contaminants. Within the frame of this doctoral dissertation, we studied the influence of SNPs in genes coding for enzymes (CYPs and UGTs) involved in the biotransformation of PH and DINCH using HBM data, which to date has never been done before (Manuscript 5).

Revealing direct links between biomarkers of susceptibility and disease development is to date the exception rather than the norm, especially if the disease is more triggered by exposure than by genetic predispositions (Ladeira & Viegas, 2016). Additionally, large knowledge gaps exist in understanding the interrelation between the genetic make-up, environmental influences, and the development of disease. This is partially due to the complexity of genetic predisposition and the involvement of multiple genes rather than single ones in the development of health effects. An example of such complex interactions being the dose-response curve between known endocrine disruptors and health outcomes that follows a U-shape rather than a linear correlation (Rochester, 2013).

Using individualized estimates on susceptibility to specific chemical exposures, public health protection can be improved and, thus, the risk assessment process can be largely refined (Kelada et al., 2003). However, both exposure-health effect studies as well as detailed risk assessment processes go beyond the scope of this doctoral dissertation and will not be elaborated further.

Chapter 2

Aims and Hypotheses

Within the frame of this doctoral dissertation, two populations have been selected consisting of the general population of Slovenian women, men, and children (DEMOCOPHES) and a population of Slovenian lactating primiparous women and their male partners (HBM I). Assessed will be the exposure of named populations to non-persistent contaminants (PHs, DINCH, BPs, PBs, and TCS) and persistent organic pollutants (POPs) (PCDD/Fs, PBDEs, PCBs, and organochlorine pesticides (OCPs), namely dichlorodiphenyltrichloroethane (DDT), hexachlorobenzene (HCB), aldrin, endrin, dieldrin, hexachlorocyclohexane (HCH), heptachlor, chlordane, heptachlor epoxide (HCE), isodrin, and endosulfan) via existing databases and GC-MS/MS based methods. In order to identify so far unrecognized knowledge gaps in the field of analytical methods, available analytical methods for the determination of isocyanates, benzotriazoles, pyrroliodones, UV-filters, methylchloroisothiazolinone and methylisothiazolinone (MCI, MI), fragrances, and selected non-phthalate plasticizers will be reviewed. The acquired exposure data will be utilized in the frame of association studies, where sources and pathways of exposure will be determined using questionnaire data and biostatistical analyses. Subsequent to the assessment of exposure, the resulting risk of the population will be evaluated applying an established chemical risk assessment approach. As this approach, however, simplifies individual biological responses to chemical exposure, the influence of SNPs in *CYP2C9, CYP2C19, CYP2D6, UGT2B15* and *UGT1A7* genes on the biotransformation of PHs and DINCH will be investigated using HBM data.

Hypotheses:

1. Despite their fast degradation and partial restriction, selected non-persistent chemicals are ubiquitously present in the environment and can be quantified in human samples with high detection frequencies and in often high concentrations. Exposure of individuals can be strongly associated with personal dietary habits and lifestyle.
2. Maternal transfer of persistent organic pollutants through *in utero* exposure and lactation is an important part of the individual exposome and the inclusion of such populations is valuable in HBM studies. POPs remain detectable in the environment 50 years after the first restrictions have come into action. The Slovenian population is mainly exposed via diet and differences in exposure occur based on local sources, contamination history, geography, and individual lifestyles.
3. Despite advances in the field of analytical chemistry, there is a lack of methods for the determination of contaminants that are so far escaping the attention of HBM projects.

4. As exposure to non-persistent contaminants is frequent and can range from low doses to high doses, the population is approaching an exposure load where adverse health outcomes can no longer be excluded.
5. Conventional approaches in chemical risk assessment are neglecting the influence of individual susceptibility towards chemicals. Selected SNPs in genes of *CYP2C9, CYP2C19, CYP2D6, UGT2B15* and *UGT1A7* can influence the biotransformation of PHs and DINCH and are, therefore, suitable biomarkers of susceptibility.

Chapter 3

Scientific Publications

The present dissertation consists of five manuscripts of which four are published and one is in preparation. Manuscripts 1 (3.1) and 2 (3.2) cover the first and second of the listed hypotheses, namely the exposure of two populations to PHs and POPs. Following the assessment of exposure, manuscript 3 covers the methodological approach in exposure assessment studies, thereby describing existing methods for chemicals of emerging concern (3.3). Manuscript 4 (3.4) leads further to the question if current exposure levels pose a health risk for the population, which corresponds with the fourth hypothesis. This widely applied approach in chemical risk assessment neglects the influence of individual susceptibility on the organism's biological response. Therefore, the last manuscript (3.5) covers the hypothesis that selected SNPs in *CYP2C9, CYP2C19, CYP2D6, UGT2B15* and *UGT1A7* genes can influence the biotransformation of PHs and DINCH, which makes them suitable biomarkers of susceptibility.

To address these aspects, two study populations were selected. The first consists of family units sampled in 2011-2012 as part of the European project DEMOCOPHES and consists of 155 women (age 30 – 52), 155 children (age 6 – 11), and 77 men (age 30 – 53) from Ljubljana and Šmarje pri Jelšah. First morning urine samples were obtained from each participant and a detailed questionnaire on their residential environment, diet, life style, occupation, and socio-demography was filled out. The second study population consists of 536 primiparous lactating women and 548 men (age 18 – 49) that were recruited from 12 regions in Slovenia within the HBM I project during two sampling campaigns (2008–2009; 2011–2014). The regions were selected based on their specific characteristics, such as a high degree of urbanization (Ljubljana, LJ; Maribor, MB; Koper, KP), potential pollution or industrial activity (Mežica valley, RA; Posočje and Idrija, GO; Jesenice, KR; Zasavje, ZA; Celje, CE; Bela krajina, BK), and the absence of known contamination sources (Kočevje and Cerknica, KO; Pomurje, MS; Savinjsko-Posavska, SP). The obtained samples were spot urine, maternal milk, plasma (men only) and serum (men only). The population of lactating women was selected to monitor vulnerable sub-populations.

Thereby, the present dissertation covers the various aspects of exposure assessment, in detail the evaluation of analytical methods, the determination of environmental pollutants in human matrices, the exposure source and pathway assessment via questionnaire data, the assessment of potential health risks resulting from such exposures, and the evaluation of individual susceptibilities.

3.1 Manuscript 1: Urinary Phthalate Concentrations in the Slovenian Population: An Attempt to Exposure Assessment of Family Units

Published: Runkel AA., Snoj Tratnik J., Mazej D., Horvat M., (2020). Environmental Research, 186, 109548.

PHs are industrial organic chemicals that are synthesized via the reaction between phthalic acid and an alcohol. The wide range of physicochemical properties can be derived by the alcohol of choice (Koch & Calafat, 2009). PHs generally fall into either of two categories depending on the number of carbon atoms in their side chain. HMW PHs (6 – 13 C atoms) find numerous applications as plasticizers in end products such as polyvinyl chloride (PVC) and plastic packaging materials. LMW PHs (3 – 6 C atoms), on the other hand, are more volatile and more hydrophilic compared to HMW PHs. Thus, they find more applications as additives in PCPs, fragrances, solvents, paints, and lubricants (Berger et al., 2019; Koch & Calafat, 2009; Lorber et al., 2017; Saravanabhavan et al., 2014).

Human exposure to PHs is widespread and their biotransformation products can be detected in mostly urine at high detection frequencies and concentrations. In recent years, concerns have been rising over the known or assumed health effects of PHs and their metabolites, mainly due to their endocrine disruptive properties (Koch et al., 2007, 2017; Koch & Calafat, 2009; World Health Organization, 2015).

In the presented manuscript (Runkel et al., 2020), we determined the levels of seven metabolites of five PHs (DEHP; benzylbutyl phthalate, BBzP; di-*iso*-butyl phthalate, DiBP; di-n-butyl phthalate, DnBP; and di-ethyl phthalate, DEP) in first morning urine samples of Slovenian men, women, and children in the frame of the European project DEMOCOPHES. Potential sources and pathways of exposure were identified using detailed questionnaire data provided by the participants and the intake dose of the PH parent compounds has been calculated using available excretion factors. Additionally, we evaluated two common methods for the adjustment to urine dilution, namely creatinine (CRT) and specific gravity (SG).

In the absence of an in-house analytical method for the determination of PH metabolites in urine at the time, samples were sent to the VITO NV laboratory in Belgium for analysis according to the COPHES SOP protocol (Schindler et al., 2014). Associations with questionnaire data were performed by the PhD candidate using analysis of variance (ANOVA), spearman's rank correlation, and multiple linear regression analysis.

Firstly, as the results indicated that CRT adjustment leads to a significant underestimation of exposure, SG adjustment seems to be the more reliable approach. The results indicate a generally higher exposure of children compared to women and men. Among all analytes, only MEHP could not be detected above the limit of quantification (LOQ) in 4 samples, which translates to an overall detection rate of 97% for this compound. All others could be detected at a frequency of 100%. Several lifestyle characteristics, such as the living environment (rural or urban), the level of education or the occupation were associated with PH exposure. Furthermore, dietary sources and packaging materials, such as canned food, frozen food, and food and drinks from plastic packaging were positively associated with levels of HMW PH metabolites. Especially LMW PHs were associated with several personal care products, such as makeup, lotions, and fragrances. The associations obtained in this study are based on the metabolite concentrations detected in urine. In the

case of complex exposure reconstruction using PBPK modeling approaches, it may be feasible to associate the questionnaire data with the calculated intake dose of the parent phthalate. However, in the present manuscript the intake dose was calculated using generalized values for daily urinary output and excretion factors. Such generalizations would increase the uncertainty of the results. Therefore, we chose the metabolites as the better proxy for exposure.

In addition to associations with each of the population subgroups, we performed an analysis to evaluate the exposure of the whole family unit. Especially the available living space, the presence of PVC in the house, and home-grown food (potentially due to freezing in plastic bags) were associated with increased exposure.

In general, both the observed urinary metabolite concentrations as well as the calculated intake doses are comparable to the results of other studies within the DEMOCOPHES project.

As such we conclude that exposure to PHs in the Slovenian population is widespread and associated with industrial and dietary products. Furthermore, certain lifestyles seem to increase exposure to these compounds. Children are generally more exposed than adults, which is of concern as endocrine disruptors have a larger effect on the developing organism.

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Urinary phthalate concentrations in the slovenian population: An attempt to exposure assessment of family units

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ABSTRACT

Phthalates are widespread contaminants with differing chemical characteristics, which largely determine their product applications, and they can leach into the environment. Due to their endocrine disruptive properties at long-term low-level exposure, they propose a health threat to people that has been associated with several adverse health effects such as: decreased male fertility and impacts on neurological development. People are exposed to different phthalates on a daily basis. Accordingly, this study aims to determine urinary concentrations of seven phthalate metabolites in Slovenian mothers (n = 155), fathers (n = 77), and children (n = 155) within the European project DEMOCOPHES and to identify potential sources of exposure using questionnaire data on sociodemographic characteristics. Furthermore, the appropriateness of two adjustment methods (creatinine and specific gravity) has been evaluated. First morning urine samples were obtained from one urban and one rural location in 2011. Samples were analysed with Ultra Performance Liquid Chromatography Tandem Mass Spectrometry according to the COPHES SOP protocol by VITO NV laboratory in Belgium. All investigated metabolites were detected in all populations. Children's urinary concentrations exceeded those of adults for most metabolites. We observed variations in concentrations depending on sociodemographic and geographic characteristics, such as food and product sources (e.g. plastic packaging, tins, personal care products, PVC) as well as lifestyle and habits (e.g. living space, time spent outside). We observed geographic and sociodemographic differences in our populations that could be confirmed for the three populations separately and for family units. Concentrations are significantly higher at the rural sampling location as well as in households with a lower level of education. We found both the urinary concentrations and the intake doses to be within the European range as presented in the literature. Between creatinine and specific gravity, we found specific gravity the more appropriate option for phthalates. To our knowledge, this is the first study investigating exposure to phthalates in the Slovenian population while considering the common exposure of family units.

1. Introduction

Esters of phthalic acid are synthesised via the reaction between phthalic acid and alcohol, forming a group of chemicals commonly referred to as phthalates. Their industrial applications vary depending on the properties of the phthalate in use. Long-chain phthalates (LCPs) can be characterised based on the length of their alkyl chain (7–13 carbon atoms). They are mainly used in plastics, such as PVC flooring, wallpaper, cables, clothing, packaging materials, and toys, to increase their flexibility, longevity, and durability (Koch and Calafat, 2009). Short-chain phthalates (SCPs) (3–6 carbon atoms) are most commonly used as industrial solvents, in pesticide formulations, lubricants, paints,

Abbreviations: 5-OH-MEHP, Mono(2-ethyl-5-hydroxyhexyl) phthalate; 5-oxo-MEHP, Mono(2-ethyl-5-oxohexyl) phthalate; As, Arsenic; BBzP, Benzylbutyl phthalate; BMI, body mass index; BPA, Bisphenol A; CE, creatinine excretion; Co, Cobald; Cr, Chromium; CRT, creatinine; Cu, Copper; d, diester; DBP, dibutyl phthalate; DEHP, Diethylhexyl phthalate; DEP, diethyl phthalate; DI, Daily intake; DiBP, Diisobutyl phthalate; DnBP, Di-n-butyl phthalate; FMU, First morning urine; FSH, follicle-stimulating hormone; F_{ue}, urinary excretion factor; GM, Geometric mean; hAR, human androgen receptor; HBM, Human Biomonitoring; ID, intake dose; LCP, Long Chain Phthalates; LOQ, Limit of quantification; m, monoester; MBzP, Monobenzyl phthalate; MEHP, Mono(2-ethylhexyl) phthalate; MEP, Monoethyl phthalate; MiBP, Monoisobutyl phthalate; Mn, Manganese; MnBP, Monobutyl phthalate; MW, molecular weight; NA, not available; Ni, Nickel; NMEC, National Medical Ethics Committee; P95, 95th percentile; Pb, Lead; PCA, principle component analysis; PCPs, Personal care products; SCP, Short Chain Phthalates; SD, standard deviation; Se, Selenium; SG, specific gravity; UE, urinary excretion; Zn, Zink

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and personal care products (PCPs) (Berger et al., 2019; Koch and Calafat, 2009; Lorber et al., 2017; Saravanabhavan et al., 2014). People are exposed mainly via ingestion of LCPs (Koch et al., 2003a) and via dermal adsorption and inhalation of SCP (Bekö et al., 2013).

The length of the chemical backbone has further influence on the metabolic fate of the compound. Once they have entered the human body, all phthalates rapidly undergo hydrolysation to form their hydrolytic monoesters (primary metabolites). Additionally, LCPs can undergo further oxidation and form oxidised monoesters (secondary metabolites) (Koch et al., 2017; Koch and Calafat, 2009). For some phthalates, urinary excretion factors have been established that estimate the amount of the parent compound that is excreted as the respective metabolite, allowing the back calculation of the parent compound intake dose. Thus, the analysed metabolites in urine are suitable biomarkers of phthalate exposure.

Long-term exposure to low doses of phthalates has been associated with a number of health impacts due to the endocrine-disrupting properties of certain phthalates. Health effects that are suspected to be related to phthalate exposure include a decrease in male fertility (phthalate syndrome), adverse child neurodevelopment, cancer, feminisation in men, decreased testicular levels of testosterone, and increased levels of follicle-stimulating hormone (FSH) (Koch et al., 2017, 2007; Koch and Calafat, 2009; World Health Organization, 2015). Overall, they seem to contribute to the global decline in human fecundity (Skakkebæk et al., 2006). Humans at a young age are especially susceptible to the effects of endocrine disruptors, and children have been found to be exposed to higher levels of phthalates due to child-typical behaviour (hand-to-mouth, proximity to the ground)(Koch and Calafat, 2009; World Health Organization, 2015).

Due to the application of phthalates in PCPs, personal lifestyle factors are important variables that can influence urinary concentrations. Studies have associated the use of certain products, such as deodorant, hair products, make-up, nail polish, and sunscreen, with specific SCPs, such as diethyl phthalate (DEP) with increasing concentrations found as the number of products increased (Braun et al., 2014; Buckley et al., 2012; Parlett et al., 2013). Through their lifetime, people are exposed to a variety of contaminants from often shared sources, such as food packaging or household dust. (Koch and Calafat, 2009; von Goetz et al., 2010). Bisphenols are one example of chemicals that might share sources with phthalates. Even though, chemical mixtures are not the focus of this study, we hypothesize that correlations between contaminants could indicate co-exposure, which could be important for future studies.

Human biomonitoring (HBM) is frequently used to assess the chemical burden of the general population. In the framework of EU-funded projects, COPHES (Becker et al., 2014a)—a harmonised HBM methodology—was developed and then implemented in the pilot study Life + DEMOCOPHES (Den Hond, 2015b; Schindler et al., 2014). The aim of these studies was to demonstrate a well-designed HBM programme with selected chemicals (mercury, cotinine, cadmium, phthalates, and bisphenol A) (Sarigiannis et al., 2019; Tratnik, 2019) and to evaluate the exposure of the general population to these chemicals. The study population included 120 mother–child pairs in 17 European countries. The study population in Slovenia also included fathers in order to allow exposure assessment at the family level. Urine samples have been analysed for seven phthalate metabolites (Mono(2-ethyl-5-hydroxyhexyl) phthalate (5OH-MEHP), Mono(2-ethyl-5-oxohexyl) phthalate (5oxo-MEHP), Monoethyl phthalate (MEP), Monobenzyl phthalate (MBzP), Monoisobutyl phthalate (MiBP), Monobutyl phthalate (MnBP)) as listed in Table A1 in the supplements. Questionnaire data were included in the statistical analysis to identify potential sources and lifestyle factors that can be associated with higher urinary concentrations of phthalate metabolites in the studied population. To our knowledge, this is one of the rare studies in which phthalates have been measured in family members with the aim to identify common sources of exposure in individual homes and family lifestyle factors.

2. Material and methods

2.1. Study population

In this study, families consisting of mother, child, and father/partner (male) were recruited in equal parts in two locations in Slovenia, representing one urban and one rural site with general exposure levels. Out of 990 invited families, 313 could be reached, and of these 155 decided to participate in the study. A total of 155 children (age 6–11), 155 mothers (age 30–52), and 77 fathers (age 30–53) were included in the study. The capital of Slovenia, Ljubljana, was chosen as the urban location, as it has the highest population density and the largest community size. An area free of industry with high air quality in the Eastern part of the country between the towns of Bizeljsko and Šmarje pri Jelšah was chosen as the rural location. It had the lowest population density in the country with enough children available for the study. Recruitment and sampling were organised via participating primary schools according to the project protocol (Becker et al., 2014b). First-morning urine samples were collected by the participants in urine collection vessels distributed prior to the sampling date, and 5 mL aliquots were stored at −20 °C at the Jožef Stefan Institute, Ljubljana, Slovenia. Participants were interviewed during school visits with the aim to collect data on: i) their residential environment, ii) diet, iii) smoking habits, iv) occupation, v) socio-demography, and vi) exposure-related lifestyle factors. The latter included questions about the usage of PCPs, and the presence of PVC in the indoor environment.

The National Medical Ethics Committee (NMEC) of the Republic of Slovenia granted approval of the study (number of accordance 64/06/11), and all participants gave written consent of their voluntary participation. Participants could withdraw from the study at any time.

2.2. Laboratory analysis

Urine samples for mothers and children were sent to the VITO NV laboratory in Belgium, while those of fathers were sent to the Institut national de sante publique Quebec in Canada. A total of seven phthalate metabolites (MEHP, 5OH-MEHP, 5oxo-MEHP, MEP, MBzP, MiBP, MnBP) were analysed in both laboratories according to the COPHES SOP protocol (Schindler et al., 2014) using ^{13}C-labelled standards instead of D-labelled standards. Reported limits of detection and quantification are presented in Table A1 in the supplements. Average recovery based on 20 μg/L additions on real urine samples was 101%, 96%, 99%, 105%, 106%, 104%, and 103% for MEHP, 5OH-MEHP, 5oxo-MEHP, MnBP, MBzP, MiBP, and MEP, respectively. Quality controls have been included in the analysis every 20 samples and included spiked water samples (20 μg/L), spiked urine samples (20 μg/L), as well as EQUAS samples. Blanks were included every 20 samples and all analytes were below 1 μg/L. Variations in QC samples did not exceed ± 20%. Coefficients of variation were (8%, 11%, 10%, 11%, 13%, 10%, and 9% for MEHP, 5OH-MEHP, 5oxo-MEHP, MnBP, MBzP, MiBP, and MEP, respectively. The laboratories were approved for the study after a succession of interlaboratory comparisons.

Enzyme buffer beta-glucuronidase *E. coli* type K12 was added to 1 mL of sample together with 13C-MEP, 13C–MnBP, 13C-MBzP, 13C-MEHP, 13C–OH-MEHP, and 13C-oxo-MEHP as internal standards. After 90 min of incubation at 37 °C, 10 μL of solution were injected and analysed with Ultra Performance Liquid Chromatography Tandem Mass Spectrometry (UPLC-MS/MS). Quantification was achieved via internal standards and a calibration range from 0.1 to 250 μg/L in acetonitrile:water (1:9).

The analytical method for analysis of bisphenol A (BPA) has been published elsewhere (Tratnik, 2019).

Creatinine excretion was measured at the subcontracting laboratory Synevo Polska Sp. in Poland using the Jaffe method with a limit of detection of 0.042 g/L. Quality was assured using two-point calibration and through internal quality controls and external quality control

programmes (COBJwDL Lodz, Poland, Labquality Helsinki, Finland). SG was measured using a PAL-10 S refractometer. Calculation of SG-adjusted urinary concentrations of phthalate metabolites was based on the method described by Suwazono et al. (2005).

2.3. Statistical analysis

Participants outside the range of 300–3000 mg/L creatinine and outside the range of 1.000–1.035 SG were excluded from the statistical analysis, as suggested by the World Health Organization and the United Nations Environment Programme (WHO-UNEP (2018)). All statistical analyses were carried out using the RStudio programme version 3.5.1 (R Core Team, 2018a). The data were not normally distributed, and an analysis of variance (ANOVA, package: stats) was used to assess differences in log-transformed biomarker concentrations between mothers, fathers, and children. Correlations between continuous variables were assessed individually using Spearman's correlation coefficient from the stats package (R Core Team, 2018b) and in a correlation matrix with Benjamini–Hochberg method post-hoc test using the ggstatsplot package (Patil, 2018). For analysis of two groups of non-normally distributed data, the Wilcoxon test was used (package:stats). (Multiple) linear regression modelling was included into the statistical analysis of continuous variables using the lm function from the stats package. The level of significance was set to (p-value) < 0.05 in all tests. Values below the respective LOD were assigned the value of LOD/2. For intra-household correlations questionnaire data applicable to all household members were correlated with the family mean concentration of the respective metabolite. Cluster analysis was carried out on scaled data after determining the optimal amount of clusters using the Elbow method (fviz_nbclust() function, factoextra package). K-means clustering was carried out using the kmeans () function from the package “stats”. Distance was measured using the dist() function from the factoextra package, method “euclidean”. Principle component analysis (PCA) was done using the ggplot2 package.

3. Results and discussion

3.1. Descriptive statistics and distribution of phthalate metabolite concentrations among children and adults

All investigated phthalate metabolites could be detected in all three populations. The descriptive statistics are summarized in Table 1. Among mothers and children, four and five samples, respectively, contained MEHP concentrations below the limit of quantification (LOQ), which translates into detection rates of 97% in both cases. The detection rates for all other analytes was 100% for women and children. For the men, all metabolites could be measured above LOQ in all samples (detection rate 100%). In the adult population, the highest concentrations could be observed for MEP, while children had the highest concentrations of MiBP. DEP and DiBP are common additives in PCPs. In the past, DiBP has been associated with a number of health outcomes and has, thus, been restricted in toys and childcare articles, while DEP is currently not regulated. DEP is widely present in fragrances, coatings, insecticides, and a number of PCPs (Wormuth et al., 2006), leading to extensive exposure.

We observed that the investigated phthalate metabolites within and between populations were correlated with each other and that the level of correlation varied depending on the population and the adjustment method used (creatinine or SG). Most correlations were observed for unadjusted data and the least after creatinine adjustment. Phthalate metabolite concentrations correlated the most in children's urine and the least in men's urine. The colored fields in Fig. 1 indicate significant correlations after Benjamini and Hochberg adjustment, with red implying positive correlations and blue negative correlations. The strongest correlations were observed among the DEHP metabolites and between DEHP metabolites and MBzP, MiBP, and MnBP, respectively. MnBP and MiBP were strongly correlated in all populations as well, while the weakest correlations were found for MEP. DEP differs in its industrial applications from the other compounds, as it is widespread in a number of PCPs (Berger et al., 2019; Wormuth et al., 2006). The correlations among the DEHP metabolites confirm the shared metabolic pathway that follows DEHP exposure (Liu et al., 2012). Correlations among other metabolites might indicate cumulative exposure resulting from contact with sources that contain more than one of the respective parent compounds. These findings suggest a need to further investigate the effects of cumulative exposure on the human body.

3.2. Correlation between SG, creatinine, and unadjusted phthalate concentrations

We compared differences in metabolite concentrations after adjustment to SG and creatinine and without adjustment. The results indicate very little change after adjustment to SG compared to unadjusted data. The results were significantly ($p < 0.004$) lower after adjustment to creatinine, except for MnBP, where the same (non-significant) trend was observed. Creatinine adjustment is a subject of debate in the literature, and its appropriateness for certain compounds has been thoroughly questioned in recent years (Johns et al., 2016). However, it is still the most widespread way to adjust for the dilution factor of urine, as it is thought to be a relatively stable indicator of renal functioning. This assumed stability is questionable, though, at it has been shown to be influenced by many factors, such as muscle mass, age, gender, physical activity, and diet. Additionally, it has been reported that creatinine should only be used as an adjustment method when the analyte of interest does not undergo active tubular secretion in the kidney (Boeniger et al., 1993). As phthalates undergo both active and inactive secretion (Johns et al., 2016), it can be assumed that the metabolite concentrations adjusted to creatinine will have larger uncertainties compared to SG - adjusted levels. SG, on the other hand, can be taken as an indicator of urinary osmolarity (Johns et al., 2016). Even though it is usually correlated with creatinine and thus may share some of the influencing factors, it is less prone to the effect of influencing factors. Therefore, it is still recommended to use SG over creatinine. As we observed significantly deviating results after adjustment to creatinine that confirm the doubts regarding the appropriateness of creatinine adjustment for phthalates, the results presented in this paper will be based on SG-adjusted concentrations unless otherwise indicated. Results for creatinine-adjusted data and unadjusted data are included in the supplements to allow comparison with other studies. Future work should assess the suitability of other measured biomarkers of kidney function for urine adjustment.

3.3. Correlation between phthalate metabolites and BPA

Many chemicals present in the environment can cause similar adverse health effects in humans. Endocrine disruptors are a prominent example of that. Studies have shown that certain phthalate parent compounds as well as BPA can affect the human androgen receptor (hAR) antagonistically and thus share a potential pathway of effect within the body (Christen et al., 2012). When assessing the risk of exposure to endocrine disruptors it is therefore essential to consider the cumulative effect of chemicals with similar potential health effects. As BPA was one of the chemicals included in the DEMOCOPHES study, we included this data into the analysis in order to investigate if there is any correlation between phthalate and BPA concentrations in urine that might indicate common sources of exposure. We observed significant (p-values between 4.5E-05 and 0.005) positive correlations for DEHP metabolites with total BPA in mothers in unadjusted data (Fig. 1) using spearman's rank correlation test. As described by Tratnik, 2019, the intake of contraceptive pills can influence the total BPA concentration in urine through upregulation of uridine diphosphate-glucuronosyl transferase (UGT). Thus, the positive correlation between BPA and

Table 1
Descriptive statistics for urinary phthalate metabolite concentrations in the Slovenian population.

Adjustment	metabolite	Mothers						Children						Fathers					
		Median	GM	CI	Max.	SD	95%	Median	GM	CI	Max.	SD	95%	Median	GM	CI	Max.	SD	95%
Unadjusted (μg/L)	MEHP	3.75	3.52	4.63–6.98	52.0	7.34	19.7	2.50	2.56	3.14–4.54	33.0	4.38	10.4	5.00	4.03	4.41–7.35	40.0	6.06	14.3
	HMEHP	19.0	17.9	21.8–31.0	216	28.8	71.8	29.0	29.2	32.1–44.9	383	40.1	78.4	25.5	22.9	23.2–37.6	210	29.7	70.1
	OMEHP	9.40	9.47	11.5–16.1	103	14.4	37.6	18.0	18.3	20.1–28.5	204	26.1	49.8	13.0	12.8	13.0–19.7	91.0	13.9	40.7
	MEP	48.0	53.3	81.8–133	1050	163	380	38.0	43.8	44.2–144	3810	312	257	40.0	51.1	57.3–206	2100	308	494
	MBZP	5.10	5.15	6.21–8.45	48.0	7.01	21.4	8.10	8.77	11.4–17.4	136	18.9	37.2	5.55	5.47	5.70–10.5	71.0	9.90	18.7
	MIBP	40.0	39.9	45.1–58.3	289	41.3	140	58.0	61.7	69.1–90.1	550	66.0	200	47.0	47.5	46.3–70.3	390	49.6	107
	MNBP	25.0	26.5	19.6–92.6	2800	228	115.8	38.0	40.1	45.6–59.9	290	44.8	134	28.0	27.6	29.2–44.2	170	31.1	88.8
CRT (μg/g_{CRT})	MEHP	2.95	2.73	3.22–4.62	42.6	4.38	10	2.11	2.14	2.46–3.49	28.	3.24	6.67	2.92	2.50	2.61–4.16	19.1	3.19	9.64
	HMEHP	13.5	14.0	15.1–20.8	177	17.7	42.5	24.6	24.4	25.6–34.7	264	28.4	62.0	15.5	14.2	14.0–21.0	100	14.4	36.1
	OMEHP	7.05	7.41	8.07–10.9	84.4	8.70	22.2	14.5	15.3	16.0–22.1	156	18.9	39.7	7.95	7.92	7.81–11.0	43.5	6.66	20.3
	MEP	38.4	41.7	60.1–102	1242	132	291	32.3	36.7	41.9–92.5	1877	159	198	28.0	31.7	35.2–104	981	143	268
	MBZP	4.11	4.03	4.54–6.00	30.2	4.53	16.2	6.54	7.33	8.80–13.3	115	14.2	37.3	34.5	3.39	3.48–5.84	340	4.88	12.2
	MIBP	29.4	31.2	32.7–40.0	128	22.5	84.3	49.5	51.6	55.7–69.2	286	42.2	138	28.0	29.4	27.6–43.2	258	32.2	66.9
	MNBP	19.1	20.8	13.0–66.2	2059	166	83.0	32.1	33.6	36.6–47.3	224	33.5	104	18.4	17.1	17.0–28.2	175	23.2	53.2
SG (μg/L)	MEHP	3.60	3.67	4.29–6.12	52.0	5.74	15.1	2.66	2.66	3.04–4.54	48.4	4.70	9.30	4.64	4.27	4.48–7.19	32.9	5.60	14.64
	HMEHP	18.2	18.7	20.4–27.8	216	23.1	59.0	29.6	30.5	32.0–43.6	291	36.2	85.3	26.4	24.3	24.2–36.3	173	24.9	65.45
	OMEHP	9.95	9.90	10.8–14.4	103	11.2	29.6	18.4	19.1	19.8–28.5	270	27.0	47.8	13.4	13.5	13.5–19.1	74.8	11.8	39.88
	MEP	50.2	55.7	82.3–137	1442	171	364	39.0	45.8	50.3–135	3175	20.7	257	39.7	54.1	61.8–196	1858	278	556
	MBZP	5.50	5.38	6.13–8.09	43.4	6.13	20.4	8.64	9.16	11.0–17.7	199	263	48.9	5.96	5.80	6.00–10.1	58.3	8.43	18.8
	MIBP	38.0	41.7	44.5–55.3	198	33.7	110.2	61.4	64.4	69.9–88.0	403	56.7	195	47.3	50.3	47.0–74.8	472	57.5	109
	MNBP	25.3	27.7	17.1–93.7	2956	239	105.0	36.7	41.9	46.0–59.3	242	41.7	140	29.4	29.2	28.9–49.2	326	41.9	77.5

SD = standard deviation.
CI = Confidence Interval.
GM = geometric mean.

DEHP metabolites in mothers might be explained by such intake. We tested this hypothesis using multiple regression modelling and obtained a significant positive relationship (p value 0.03) between BPA and MEHP (r^2 = 0.08). 5OH-MEHP and 5oxo-MEHP were associated only marginally significantly (p value 0.09, r^2 = 0.9 and p value 0.07, r^2 = 0.07, respectively). Potential reasons for the low correlation factors in this case might be the small number of women using contraceptive pills (25 out of 155). PCA results placed BPA in a group separated from the phthalate metabolites, though, indicating differences between the groups that could reject the hypothesis. Despite the inconsistent results, this hypothesis finds further support by the fact that this correlation was not observed when testing girls only. Interestingly, the opposite trend, a negative correlation between BPA and DEHP and BPA and DiBP metabolites was found to be significant (p = 0.05 and 0.03, respectively). No significant trend could be observed for boys or fathers. No relationship could be observed, however, when correlating the family's exposure to BPA with the exposure to phthalates. As such, a common household source of BPA and phthalates can be excluded, but the data do not allow any further interpretation regarding why we observed significant correlations for mothers and girls. A detailed survey of the overall exposure of the Slovenian population to BPA has been published elsewhere (Tratnik, 2019). As both BPA and certain phthalates are known to migrate from matrices into edibles (Cutanda et al., 2015b; Koch et al., 2013), cumulative exposure via those pathways is theoretically possible and must be considered in exposure-based studies. Therefore, we conducted k-means cluster analysis as well as PCA in order to reveal any patterns within the dataset. Both methods group the DEHP metabolites separately from the short chain phthalates. Furthermore, BPA was grouped together with the SCP in the cluster analysis, while PCA revealed differences between phthalates and BPA, plotting them on different sites of the graph. Thus, results from these analyses are inconclusive and can only hint towards a possible shared source between SCP and BPA. The cumulative effects of chemical mixtures on the human body are not well understood (Christen et al., 2012). Studies investigating the matter have observed either antagonistic or synergistic relationships between compounds, with additional variations depending on the magnitude of exposure (Christen et al., 2012). The respective effects of cumulative exposure to BPA and phthalates are known to be concentration dependent, with antagonistic activity being reported for low concentration mixtures and synergistic activity being described for high concentrations (Christen et al., 2012). In the present study, we observed large ranges of urinary BPA and phthalate metabolite concentrations. Our results allow us to exclude a common-source exposure scenario for our population, but exposure to different compounds from different sources must still be considered.

3.4. Sociodemographic differences in phthalate exposure

Significant differences ($p < 0.05$) were observed between the populations, especially between children and adults (significant for all metabolites except MEP). No significant difference could be observed between girls and boys while the difference between mothers and fathers was significant only for 5OH-MEHP and 5oxo-MEHP and marginally significant ($p < 0.1$) for MiBP. Children's urinary phthalate concentrations exceeded those of adults in all cases except MEHP and MEP. The different patterns for these two metabolites can be explained by the toxicokinetic pathway of DEHP as well as by the industrial applications of DEP. MEHP represents a primary metabolite of DEHP that is further oxidised to a number of secondary metabolites. Thus, comparing the urinary concentrations of the primary metabolites of long chain phthalates is not recommended and the secondary metabolites (5oxo-MEHP, 5OH-MEHP) should be used instead (Koch et al., 2017). DEP, on the other hand, is used in a variety of personal care products such as makeup, lotions, and fragrances. We assume that these products are less commonly used by children and more often by women than by men, which is an observable pattern in our results.

Furthermore, we observed differences among the age groups in all three populations. Children were grouped into different age groups, and the results were compared. **In the first test**, 6–7-year-olds formed one group (n = 44) while 8–12-year-olds formed a second group (n = 109). The younger children had significantly higher concentrations of 5OH-MEHP and 5oxo-MEHP in their urine compared to the older children, while no significant difference could be observed for the other metabolites. **In a second test**, 6–8-year-olds (n = 63) were compared to 9–12-year-olds (n = 90). No significant differences could be observed. **In a third test**, three groups were formed: 6–8-year-olds (n = 63),

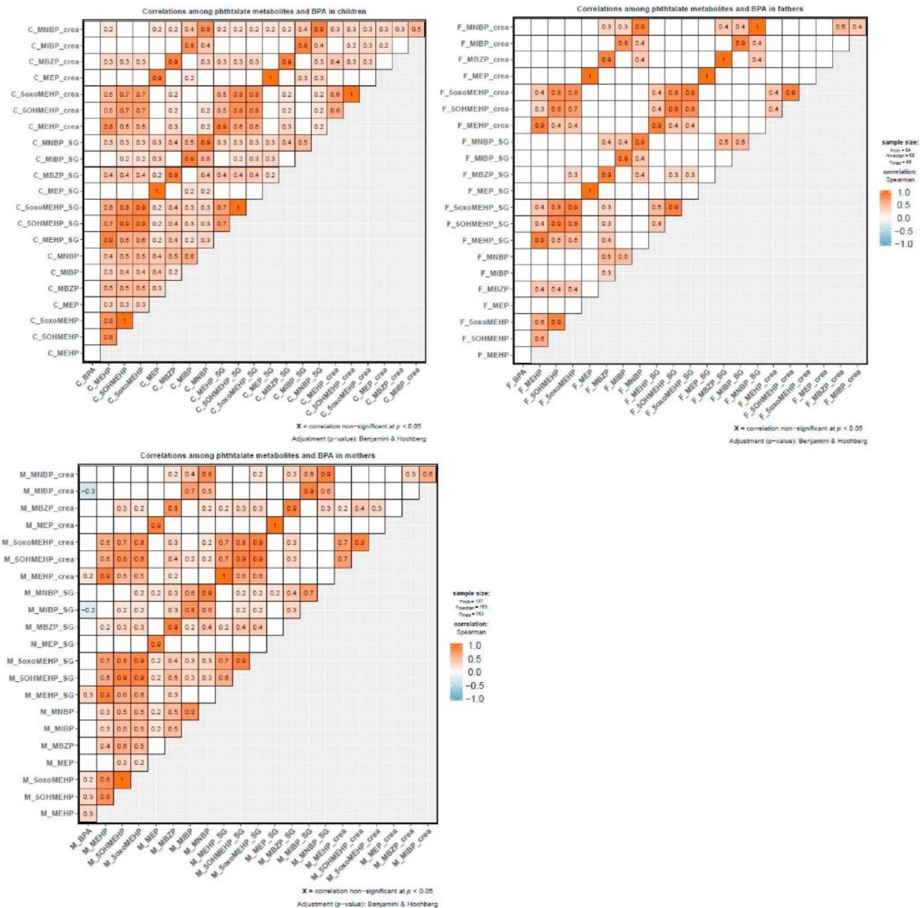

Fig. 1. Correlations between BPA, phthalate metabolites and adjustment methods. Non-significant correlations are left blank. Red colors are indicating significant positive correlations with the number in the cells representing Spearman's Rho. SG = specific gravity, crea = creatinine. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

9–10-year-olds (n = 57), and 11–12-year-olds (n = 33). MEHP metabolite levels were significantly higher in the oldest children compared to the youngest. However, this trend could not be observed for the secondary metabolites. This result, therefore, should be viewed with caution, as MEHP metabolised further to 5OH-MEHP and 5oxo-MEHP. As the participants collected the urine samples themselves, differences in sampling time and temporal distance to exposure might influence the measured concentration of MEHP in urine. To assess exposure to DEHP, studies widely agree to place the focus on secondary metabolites for a more reliable comparison (Koch et al., 2017). No significant differences could be observed for any other metabolites. **In a fourth test**, 6–9-year-olds (n = 87) were compared to 10–12-year-olds (n = 66). The difference in MEHP concentrations was significant in this test. The urinary levels of older children were higher than those in the younger age group. The difference in the secondary metabolites of DEHP was not significant, however, and must be regarded with the same caution that should be applied to the result of the third test. Other metabolites (MEP, MnBP, MiBP, MBzP) did not differ significantly between the age groups. These results are contradictory to the literature, which suggests that younger children have higher concentrations compared to older children due to differences in physiology, increased hand-to-mouth behaviour, and more time spent on the ground (Koch et al., 2003b). The suggested reasons for this age-related difference imply that the age span in this study might have been too narrow to lead to any observable differences, or the sample size per age group might have been too small. The significant results for the secondary metabolites of DEHP that were obtained **in the first test** (6–7-year-olds vs. 8–12-year-olds) are in line with those in the literature. It has been hypothesised that younger children are exposed to more sources of phthalates and, additionally, increase their uptake of the chemicals through child-typical behaviour (Sathyanarayana et al., 2008; Wormuth et al., 2006). Our results can be explained by this hypothesis. However, when 6–7-year-olds were compared to 11–12-year-olds, this trend vanished. Linear regression modelling was included in the statistical analysis, but as the analytical plots suggest very bad model performance, group comparisons between age groups in children provide more reliable results.

Interestingly, an age-dependent trend could be observed for the mothers as well, where the younger age group (age 30–37, n = 106)

had significantly higher urinary phthalate concentrations of DEHP metabolites as well of MBzP compared to the older women in the study (age 38–52, n = 49). This result could be confirmed with linear regression. No significant difference could be observed for other metabolites. Younger men (age 30–41, n = 39) had significantly higher concentrations of MEHP in their urine, while concentrations of MiBP and MnBP were significantly higher in the older participants (age 42–53, n = 31). Linear regression confirms the results for MEHP but does not reveal any correlation between age and other metabolites. The literature widely agrees that age-based differences can be observed between children and adolescents (Koch et al., 2005), whereas the observed age trends among adults are inconsistent and, to date, lack an explanation (Brock et al., 2002; CDC, 2009; Koch et al., 2017). Some studies report increasing urinary concentrations of MEP with age in the general population (Saravanabhavan et al., 2013), while others do not find any significant gender-related differences (Fromme et al., 2007). As phthalate exposure in the general population is largely dependent on lifestyle, the differences observed between age groups may be a result of changing lifestyles and not physiologically induced. Thus, we compared occurrences of positively associated phthalate sources between the age groups, but no age-related differences could be revealed. Younger participants were found to be mainly living at the rural sampling location, though, that has been associated with higher urinary phthalate metabolite concentrations (see section 3.4 Living area).

3.4. Living area

Phthalate exposure results mainly from dietary sources, PVC, or PCPs (Wormuth et al., 2006). In Slovenia, where people in different regions have equal access to commercial products, we observed significant differences in urinary phthalate concentrations between individuals living in urban (n = 75) and rural areas (n = 80). Children's concentrations of MEHP, 5OH-MEHP, 5oxo-MEHP, MEP, and MBzP were significantly higher in rural areas, while the differences in MiBP and MnBP levels were non-significant. Mothers as well had significantly higher levels of DEHP metabolites and MEP in rural areas, while the differences in MBzP and MiBP levels were non-significant. MnBP was present in (significantly) higher concentrations in samples taken in urban areas. For the men, concentrations of MEHP were only marginally significantly higher ($p < 0.1$) in rural areas, but the trend was significant for the other DEHP metabolites. In urban locations, MiBP and MnBP were present in urine in significantly higher concentrations, while the difference for MEP and MBzP was not significant. Those results are presented as graphs in the supplements (Figure A1.1, A1.2 & A1.3). The results did not show any adjustment-dependent variations for women or men but differed largely between the creatinine-adjusted and SG-adjusted concentrations and unadjusted data for children. After creatinine normalisation, the significance for all DEHP metabolites and MEP disappeared, while the trends of higher concentrations of MiBP and MnBP in urban areas became significant. Additionally, we tested if the results held on a family level. MEHP, 5OH-MEHP, 5oxo-MEHP, and MEP were significantly higher in families living in rural areas, while in urban areas MiBP and MnBP levels were significantly higher. The results for MBzP were only marginally significant (p value < 0.1) indicating higher concentrations in rural areas. All results are based on specific gravity adjusted data. In order to identify the drivers underlying these trends, we compared the occurrence of known sources of phthalates as well as factors that were associated with urinary phthalate concentrations to assess the spatial distribution of the sources in our study population. To begin with, older participants with a higher educational level and higher income were mainly recruited from the urban sampling location, which might bias the results (further discussed in section 3.7). Additionally, we detected an uneven distribution of known sources for phthalates between urban and rural areas (Table 2). Some sources of phthalates, such as new cars, consumption of food from plastic packaging, application of PCPs, and PVC, have been described in the literature (Bornehag et al., 2005; Geiss et al., 2009; Wormuth et al., 2006) and repeatedly associated with phthalate exposure in humans. We observed a number of such sources occurring at higher levels in the rural sampling location, which might explain the observed regional differences. This approach was not able to explain the higher concentrations of MiBP and MnBP in urban areas, however. One possible explanation is the higher abundance of external sources potentially leading to higher concentrations of phthalates in urban air compared to rural air as reported in the literature (Rakkestad et al., 2007; Rudel and Perovich, 2009), but this hypothesis requires further investigation of urban outdoor air concentrations of phthalates. Naturally, these listed sources and processes influence indoor air concentrations, as indoor and outdoor living environments are relatively open systems.

Table 2
Observed geographical distribution of phthalate sources. + indicating higher values than -.

Sources of phthalates	Urban region	Rural region
Education	+	–
Income	+	-
Age (adults)	+	-
Living space	–	+
Age of car	+	–
Consumption of frozen food from plastic packaging	–	+
Application of (eye) make up, fragrances, hair styling products	–	+
Working environment	+ Offices, schools	+ Factories[a], shops
PVC flooring/wallpaper	–	+
BMI	–	+
Age of building	–	+

[a] Further discussed in section 3.8.

3.5. Correlations between phthalate metabolite levels and dietary sources

The literature widely agrees that dietary sources of phthalates are abundant in the environment and that ingestion is the main pathway of exposure to long chain phthalates. Thus, we combined the data on urinary phthalate concentrations with the obtained questionnaire data to confirm known or to identify potential sources. The results varied strongly depending on the adjustment method. As previously discussed, the reliability of data adjusted to creatinine is questionable (Rudel et al., 2011), therefore, the significant ($p < 0.05$) results obtained for data normalised to SG will be discussed here, while those obtained for unadjusted and creatinine-adjusted results can be found in the supplements (Table A3 and A4). In Table 3 the significant results from this correlation study are presented. Urinary phthalate metabolite concentrations in mothers were positively correlated with the consumption of the following: cider, canned food, frozen food packed in paper, hazelnut spread, non-frozen food packed in plastic, food reheated in plastic, and drinks from sports bottles. Negative correlations were observed for the consumption of the following: frozen food packed in plastic, ice cream, canteen food, food from plastic containers, and drinks from older sport bottles compared to younger ones. For children, we observed negative correlations with ice cream consumption, food stored in plastic containers, refilling of non-reusable plastic bottles, and frozen food packed in plastic, while frozen food packed in paper, use of sports bottles, and canned food were positively associated. We were not able to explain the negative correlations observed, but uncertainties within the questionnaire might have influenced the results. For future studies, a detailed revision of the questionnaire would be advisable to ensure identical data sets for children, mothers, and fathers that cover the potential sources of phthalates that a person could come in contact with 48 h prior to sampling. In many ways, the prepared questionnaire

Table 3
Significant correlations between questionnaire data and urinary phthalate metabolites adjusted to specific gravity for women (W), children (C), and men (M).

Variable	MEHP			5OH-MEHP			5oxo-MEHP			MEP			MBzP			MiBP			MnBP		
	W	C	M	W	C	M	W	C	M	W	C	M	W	C	M	W	C	M	W	C	M
Cider										+						+			+		
Hazelnut spread																(+)					
Fast food																			+		
Ice cream		-			-			-		(−)				-							
Canteen										-											
Frozen food packed in paper		(+)			(+)			+								+					
Frozen food packed in plastic	-			-			-										-				
Canned food (tins)		+								+			+								
Reheating in plastic													+								
Time between reheating in plastic and sampling			-			-			(−)												
Sports bottles	+	+		+	+		+	+										+			
Age of sports bottle	-			-			-											+			
Food/drinks from plastic	+			+			+														
Plastic containers										-						-					
Canned food/drinks		+								+			+								
Refilling of non-reusable plastic bottles																	-			-	
Eye makeup	-									+											
Hair styling																-			-		
Lotions											(+)										
Fragrances										+	+										
Deodorant		(+)																			
Nail polish		+																			
PVC			(+)		(+)								+	+	+						
Lubricants		(+)																			
Oil contact					+									+					-		
Paint contact	(+)	(+)																			
Solvent contact																				(+)	
Plasticizer contact		(−)																			
Pharmaceuticals												(+)			+						
Sunscreen	+			+			+														
Mouth wash		+			+			+						+							
Disinfectant											+										
Smoking										+									+		
Education																					
School	+			+			+						(+)								
University																+		+	+		(+)
Area																					
Rural	+	+		+	+	+	+	+	+		+			+							
Urban																+		+	+	+	+
Year of building construction						+			+		(+)	(+)					-	(−)	-	-	
Living space	+					+			+					-				-		-	
Age of car		+			+			+						+							
Working environment																					
Offices, schools, libraries																					
Factories, warehouses	+			+			+														
Shops, canteens																					
Hospitals, laboratories																					
Traffic											+										
Agriculture																					
BMI																-			-		
Playing outside		-			-			-						-							

+/- significant positive/negative associations.
() marginally significant (p value < 0.1).

was too general, which could influence the results and might be the reason for some of the unexplainable associations that were found. For instance, questions targeted general habits instead of actual source contact within the relevant time frame. Thus, a person who usually applies make-up daily but did not do so within the 48 h prior to sampling would still mark "daily" for the respective question, which would not reflect the actual exposure. The measured concentrations would, thus, be lower than expected and bias the results of the statistical analysis. We observed higher concentrations of phthalate metabolites in men who consumed food reheated in plastic shortly before sampling. Additionally, the use of sports bottles and their respective age were significantly correlated with elevated phthalate levels. Heat-induced leaching is an obvious source of elevated phthalate concentrations in food, which explains some of the correlations that we found. Further, it is known that chemicals leach easier from older materials, which supports the positive correlations that we found between elevated urinary concentrations and the frequency of sport bottle use as well as the bottle's age. Not all our findings have obvious explanations, however. We observed negative associations between metabolites and known sources of phthalates, such as ice cream, canteen food, and frozen food from plastic packaging (Wormuth et al., 2006), while other expected sources (e.g. fast food) did not yield any significant results. One possible reason for this is the relatively small number of participants. The generally high educational level of the study participants as well as their willingness to participate indicate an awareness of and concern about chemical exposure. Additionally, in the present study, the number of participants consuming fresh food was very high compared to the number of people who come in contact with potentially contaminated

food more frequently. Uneven distributions of data can bias statistical testing and can only be solved by either increasing the sample size or assuring an even distribution over all categories. Nevertheless, we obtained associations between a number of products that have been previously reported as sources of different phthalates and increased urinary concentrations of the respective metabolites. Such sources include alcoholic beverages (Karačonji et al., 2017), laminated paperboard and canned food (Fasano et al., 2012), hazelnut spreads (Wormuth et al., 2006), as well as old plastic or plastics exposed to higher temperatures (Cao, 2010). The literature widely agrees that migration is enhanced from plastic materials into fatty foods, as they facilitate the migration of lipophilic chemicals such as phthalates.

3.6. Correlations between phthalate metabolite levels and product sources

As some of the SCPs are regularly added into personal care products, they have been previously detected in a number of products. We observed (marginally) significant positive correlations (Table 3) between phthalate metabolite levels and make-up, lotions, styling products, fragrances, deodorant, nail polish, sunscreen, mouthwash, and disinfectants. Interestingly, associations were not only observed for the SCPs but also between DEHP metabolites and these products, even though only some of the results were significant ($p < 0.05$). Strong associations have been observed between PVC presence in the house and metabolite concentrations, which is in line with the literature that implicates PVC as one of the strongest sources of DEHP since it may contain up to 40% of the chemical (Wittassek et al., 2011). In this study, negative correlations have been observed between eye make-up and MEHP concentrations. This result cannot be explained by our data, but DEHP is not commonly added to eye make-up products. Thus, this result is likely a coincidence. The negative correlations between MiBP and PCPs require further investigation as well, but, interestingly, the same association has been reported in a study by Berger et al. (2019), although the result was not discussed.

These results explain the elevated concentrations of MEP in women's urine, as their use of higher amounts and a larger variety of PCPs compared to men and children exposes them to more sources of SCPs, leading to higher concentrations in their urine (Berger et al., 2019; Wormuth et al., 2006). The results for unadjusted and creatinine-adjusted data are presented in the supplement tables A3 and A4.

3.7. Associations between phthalate metabolite levels and lifestyle

In addition to product sources, we investigated lifestyle data provided by the participants through a questionnaire in order to identify potential associations between urinary phthalate metabolite levels and personal habits and lifestyle (Table 3). Higher levels of DEHP and BBzP metabolites were measured in mothers with lower education but not in fathers with lower education, while MiBP and MnBP concentrations were higher in samples from participants with a university degree. We observed the same trends for the area of residence, which itself is correlated with the level of education (higher in urban areas). Thus, the same drivers underlying the observed differences between urban and rural residence areas might apply to the level of education. We obtained higher metabolite concentrations of DEHP metabolites and MEP in fathers and (partially) children living in younger buildings, while concentrations of MiBP and MnBP in fathers and children were (marginally) significantly lower in younger buildings. The reasons for the observed trends are difficult to assess because, on one hand, aging increases phthalate migration into the environment, but on the other hand, renovation and construction have the same effect. Both, larger places and older cars were associated with elevated levels of DEHP metabolites. Meanwhile, concentrations of SCPs were lower in larger places. This might be due to the volatility of the compounds, which might allow dispersion through ventilation systems. DEHP is more likely to settle into dust, which might explain our results. MBzP has been found in higher concentrations in samples taken from people driving in older cars. Interestingly, we observed significantly higher concentrations of the metabolites of the plasticizing phthalate DEHP in women that reported working in factories (Figure A2 in the supplements). Even though the results were significant, they should be viewed with caution since only eight people reported working in factories, compared to 87 working in offices, 15 in shops, 26 in hospitals, and one in the traffic sector. Naturally, the significant results obtained for the latter are highly questionable. Of the people working in factories, one participant working with industrial electronics (here labelled as participant nr. 7) had 3–4 times higher concentrations of 5OH-MEHP and 5oxo-MEHP compared to other people working in factories, while the MEP concentrations were up to 20 times higher (Table A2 in the supplements). Children who reportedly spent more time outside had significantly lower concentrations of DEHP metabolites in their urine. Despite food being the dominant source of DEHP, household dust is also considered a major contributor (Wormuth et al., 2006). Thus, spending less time inside, where concentrations are generally higher than outside (Rudel and Perovich, 2009), would decrease the exposure time of the child. The influence of Body Mass Index (BMI) has been discussed in the literature in terms of whether or not it might influence urinary phthalate concentrations. The results have often been contradictory, mainly due to the number of influencing factors (Chiang et al., 2016; Goodman et al., 2014). In our study, we observed negative correlations between MiBP and MnBP in women with elevated BMIs that cannot be explained by our data, as phthalates, due to their lipophilic properties, could be expected to accumulate in the short term in fatty tissues, or they might act as obesogens, as some studies suggest (Goodman et al., 2014).

3.8. Associations between phthalate metabolite concentrations on a family level and potential sources

In order to determine domestic sources of phthalates, we investigated potential associations between urinary phthalate concentrations within families and questionnaire data. We observed significant negative correlations between MiBP and MnBP concentrations in urine and the age of the building the family was living in ($p = 0.02$ and 0.0006, respectively). This result is in contradiction with the literature (Bornehag et al., 2005), as age-related differences are generally due to higher phthalate contents in older construction and flooring materials. Thus, the association between younger buildings and higher concentrations of MiBP and MnBP cannot be explained by this dataset. Families living in smaller places had significantly higher concentrations of MnBP in their urine compared to families living in larger places ($p = 0.01$). It is known that phthalates with higher molecular weights tend to accumulate in house dust (Bornehag et al., 2005), and one of the major determinants of household dust accumulation is a lack of airflow that would remove the particles. Thus, in smaller spaces with less direct air flow compared to larger spaces, dust can be expected to accumulate more, leading to higher exposure of people living there (Jones, 1999). Furthermore, if the house/flat in which the family was living at the time of the sampling had been either renovated or redecorated, it could be associated with higher concentrations of secondary phthalate metabolites (5OH-MEHP, 5oxo-MEHP) in the urine of all family members. Studies have shown that air concentrations of a number of chemicals, among them phthalates, are elevated after renovations due to higher dust concentrations during renovation, the presence of fresh paint, and/or constituents of construction materials (Dong and Qian, 2014). Elevated concentrations of both DEHP and BBzP metabolites were significantly associated with the presence of PVC flooring or wallpaper in the house/flat (5OH-MEHP: $p = 0.03$, 5oxo-MEHP: $p = 0.05$, MBzP: $p = 2.47e\text{-}05$). PVC is a known source of LCPs, and the high concentrations of the compounds used in these materials have long been a concern (Afshari et al., 2004).

For the correlations with dietary sources, the mother's questionnaire was taken as a basis, assuming that, even if their eating habits might

Table 4
Geometric means (GM) and 95 percentiles (P95) of urinary phthalate metabolites throughout the DEMOCOPHES states.

Reference	country	year of sampling	gender	n (age)	sample	unit	DEHP						DEP		BBzP		DiBP		DnBP	
							MEHP		5OH-MEHP		5oxo-MEHP		MEP		MBzP		MiBP		MnBP	
							P95	GM	P95	GM	P95	GM	P95	GM	P95	GM	P95	GM	P95	GM
current study	**Slovenia**	**2011**	**female**	**155 (30–52)**	**FMU**	**μg/g creatinine**	**10.0**	**2.70**	**42.5**	**14.0**	**22.2**	**7.40**	**291**	**41.7**	**16.2**	**4.00**	**84.3**	**31.2**	**83.0**	**20.8**
			male	**71 (30–70)**			**9.64**	**2.50**	**36.1**	**14.2**	**20.3**	**7.92**	**268**	**31.7**	**12.2**	**3.39**	**66.9**	**29.4**	**53.2**	**17.1**
			female and male	**172 (6–12)**			**6.67**	**2.14**	**62.0**	**24.4**	**39.7**	**15.3**	**198**	**36.7**	**37.3**	**7.33**	**138**	**51.6**	**104**	**33.6**
current study	**Slovenia**	**2011**	**female**	**155 (30–52)**	**FMU**	**μg/L**	**19.7**	**3.50**	**71.8**	**17.9**	**37.6**	**9.50**	**380**	**53.3**	**21.4**	**5.20**	**140**	**39.9**	**116**	**26.5**
			male	**71 (30–70)**			**14.3**	**4.03**	**70.1**	**22.9**	**40.7**	**12.8**	**494**	**51.1**	**18.7**	**5.47**	**107**	**47.5**	**88.8**	**27.6**
			female and male	**172 (6–12)**			**10.40**	**2.56**	**78.4**	**29.2**	**49.8**	**18.3**	**257**	**43.8**	**37.2**	**8.77**	**200**	**61.7**	**134**	**40.1**
Esteban et al. (2015)	United Kingdom	2012	female and male	(6–12)	FMU	μg/g creatinine	9.96	1.58	39.3	20.4	25.5	12.7	89.3	15.9	21.6	3.84	75.7	28.7	66.4	25.2
			female	(≤50)			4.08	1.11	20.5	8.21	12.4	4.85	120	25.6	4.82	1.56	70.7	16.3	32.4	12.9
Cullen et al. (2017)	Ireland	2011/2012	female and male	120 (6–11)	FMU	μg/g creatinine		3.50		32.8		17.7		38.7		5.40		41.4		26.1
			female	120 (≤45)				2.80		17.0		8.80		50.2		3.10		23.8		18.5
Cutanda et al. (2015a)	Spain	2011/2012	female and male	119 (5–11)	FMU	μg/g creatinine	16.35	6.85	98.1	38.4	58.3	24.3	1705	198	50.1	13.9	237	61.4	214	51.0
			female	118			21.22	6.65	50.7	20.1	34.8	13.0	949	150	29.8	7.99	77.0	35.0	18.8	31.0
Černá et al. (2015a)	Hungary	2009–2012	female and male	117 (6–11)	FMU	μg/L		3.65		33.7		22.9		47.0		7.57				
			female	115 (≤45)				3.83		19.4		12.9		55.0		4.76				
Černá et al. (2015b)	Slovakia	2010–2012	female and male	127 (6–11)	FMU	μg/L		3.95		49.3		33.3		39.6		8.35				
			female	125 (≤45)				3.96		22.4		14.2		54.8		4.70				
Černá et al. (2015b)	Czech Republic	2011–2012	female and male	120 (6–11)	FMU	μg/L		3.8		37.0		24.8		31.6		8.32				
			female	117 (≤45)				3.33		19.6		12.4		56.7		4.62				
Schwedler et al. (2017)	Germany	2011	female and male	120 (6–11)	FMU	μg/g creatinine		35.6[a]						20.6		5.88		37.2		41.9
			female	116 (28–45)				19.1[a]						35.0		4.03		22.2		27.9
Larsson et al. (2014)	Sweden		female and male	97	FMU	μg/g creatinine	11.47	3.08	93.5	27.8	60.5	17.7	179	32.6	96.6	22.49			237	86.8
			female	95			9.11	2.22	39.0	14.1	28.5	8.18	320	40.9	74.2	12.07			161	59.4
(Den Hond, 2015a)	Belgium	2011–2012	female and male	120 (5–11)	FMU	μg/g creatinine	37.3[a]							26.7		9.00		59.2		39.4
			female				21.7[a]							31.7		6.50		38.6		30.5
(Den Hond, 2015a)	Poland	2012–2012	female and male	120 (5–11)	FMU	μg/g creatinine	76.4[a]							46.9		9.30		108		90.4
			female				43.9[a]							42.5		4.50		53.6		48.2
(Den Hond, 2015a; Mørck et al., 2015)	Denmark	2011	female and male	145 (6–11)	FMU	μg/g creatinine	40.9[a]							22.1		8.00		33.6		62.2
			female	145 (31–52)			24.0[a]							37.3		4.50		41.6		21.6
(Den Hond, 2015a)	Switzerland	2011–2012	female and male	120 (5–11)	FMU	μg/g creatinine	28.1[a]							19.7		5.10		20.5		20.1
			female				20.4[a]							31.2		3.90		14.4		13.9
(Den Hond, 2015a)	Cyprus	2012–2012	female and male	121 (5–11)	FMU	μg/g creatinine	25.0[a]							41.2		3.70		51.8		20.6
			female				16.8[a]							87.7		2.40		16.1		43.7
(Den Hond, 2015a)	Luxembourg	2013–2012	female and male	122 (5–11)	FMU	μg/g creatinine	25.8[a]							26.8		5.20		36.9		28.2
			female				15.9[a]							36.4		3.60		21.1		18.3
(Den Hond, 2015a)	Portugal	2014–2012	female and male	123 (5–11)	FMU	μg/g creatinine	48.2 [a]							50.2		8.10		40.3		33.3
			female				37.2[a]							55.9		5.60		28.4		22.3
(Den Hond, 2015a)	Romania	2015–2012	female and male	124 (5–11)	FMU	μg/g creatinine	74.0[a]							34.8		4.10		51.1		43.2
			female				51.5[a]							44.2		2.50		34.7		27.1

FMU = First Morning Urine.
[a] Data presented for the sum of DEHP metabolites.

Table 5
Estimated intake doses in μg/kgbw/day.

Reference	Country	year of sampling	population	N (age)	DEHP			DEP			BBzP			DiBP			DnBP		
					Median	P95	Max	Median	P95	Max	Median	P95	Max	Median	P95	Max	Median	P95	Max
current study	**Slovenia**	**2011**	**female adult**	**155 (30–52)**	**3.84**[a]	**12.1**	**48.7**	**2.01**	**15.3**	**65.2**	**0.15**	**0.61**	**1.14**	**1.33**	**3.81**	**5.78**	**0.88**	**3.80**	**94.4**
			male adult	**71 (30–70)**	**5.60**	**13.6**	**34.1**	**0.17**	**0.58**	**65.8**	**0.17**	**0.58**	**1.63**	**1.62**	**3.87**	**14.9**	**1.08**	**3.12**	**10.3**
			boys	**72 (6–12)**	**5.92**	**15.9**	**32.3**	**1.44**	**9.08**	**83.7**	**0.24**	**1.23**	**2.46**	**1.97**	**5.55**	**8.03**	**1.25**	**4.31**	**8.74**
			girls	**81 (6–12)**	**6.09**	**16.0**	**62.9**	**1.46**	**7.33**	**14.0**	**0.19**	**0.87**	**3.44**	**1.77**	**4.70**	**10.3**	**1.19**	**3.72**	**5.60**
Marsee et al. (2006)	USA	1999–2002	pregnant women	214 (NA)	1.32	9.32	41.1	6.64	112	1260	0.50	2.47	15.5	0.12	0.41	2.90	0.84	2.33	5.86
Kohn et al. (2000)	USA	1988–1994	NHANES III	289 (20–60)	0.71	3.6	46.0	12.0	110	320	0.88	4.00	29.0				1.50	7.20	110[b]
Koch et al. (2003a)	Germany	2002	general population	85 (7–63)	13.8	52.1		2.32	22.1		0.60	2.50					5.22	16.2	
Wittassek et al. (2007a)	Germany	2001–2002	GerES IV	239 (2–14)	4.30	15.2	140												
Koch et al. (2017)	Germany	2002–2002	GerES IV	240 (2–14)							0.42	2.57	13.9				4.07	14.9	76.4
Wittassek et al. (2007b)	Germany	1988–2003	ESBHum	632 (20–29)	3.50	10.1	39.8				0.26	1.6	27.3	1.4	5.7	29	4.1	19.1	116
Frederiksen et al. (2011)	Denmark	2007	boys	(6–10)	5.67	44.2	53.0	0.92	4.63	4.77	0.96	6.28	6.93						
			boys	(11–16)	3.90	11.2	12.6	0.98	3.32	3.57	0.66	2.96	3.60						
			boys	(17–21)	2.96		10.3	1.56		12.8	0.40		9.96						
			girls	(6–10)	5.37	10.8	11.0	1.13	8.24	8.77	0.97	3.57	3.95						
			girls	(11–16)	3.58	10.5	10.9	1.13	15.7	18.2	0.53	4.38	5.81						
			girls	(17–21)	1.74		3.79	1.50		13.4	0.32		0.97						
Dewalque et al. (2014)	Belgium	2013	children	52 (1–12)	3.37	10.63	21.12	1.47	5.81	7.24	0.42	1.73	2.07	2.29	8.04	26.11			
			adults	209 (13–85)	1.43	6.77	17.51	1.44	14.76	59.65	0.20	1.12	4.50	0.88	4.53	26.18			
Saravanabhavan et al. (2014)	Canada	2007–2009	boys	521 (6–11)				0.78											
			girls	511 (6–11)				0.95											
			males	504 (12–19)				1.36											
			females	484 (12–19)				1.50											
			male adult	608 (20–49)				1.76											
			female adult	596 (20–49)				1.27											

NA = not available.
[a] Average value intake estimations based on 5OH-MEHP and 5oxo-MEHP.
[b] Presented as the sum of DnBP and DiBP.

differ, the members of the family share the same sources of food. The consumption of hazelnut spread was positively associated with higher urinary concentrations of MiBP, while fast food was associated with higher concentrations of MnBP. Interestingly, home-grown food was associated with elevated levels of 5OH-MEHP and 5oxo-MEHP, which might be a consequence of the plastic bags or containers used to store the food after harvesting.

The level of education was observed to have an influence on family exposure, as families with higher educational background (university degree) had significantly lower concentrations of DEHP ($p = 0.001–0.003$) and MBzP metabolites ($p = 0.003$), while levels of MnBP and MiBP were higher ($p = 0.01$ and 0.03, respectively). MEP concentrations were comparable in both groups. These results confirm the hypothesis that education has an influence on a family's lifestyle, which is reflected in urinary phthalate metabolite concentrations.

3.9. Distribution of urinary phthalate metabolite concentrations within the DEMOCOPHES countries and intake dose estimations

A total of 17 countries (Slovenia, UK, Ireland, Spain, Hungary, Slovakia, Czech Republic, Germany, Sweden, Belgium, Poland, Denmark, Switzerland, Cyprus, Luxembourg, Portugal, and Romania) participated in the European human biomonitoring study (Schindler et al., 2014). The geometric means (GM) and 95th percentiles (P95) are presented in Table 4. Overall, Slovenia falls into the European range, with most values slightly below the DEMOCOPHES average. As most of the participating countries are members of the European Union and thus fall under common legislation with regard to phthalate restriction, this result is expected.

Urinary excretion factors were used to calculate the intake dose (ID) of the phthalate parent compound from the urinary metabolite concentrations following the instructions available in the literature (David, 2000; Heudorf et al., 2007; Koch et al., 2007, 2003a).

$$ID(\mu g/kg_{body\ weight}/day) = \frac{UE((\mu g/g)) \times CE(mg/kg_{body\ weight}/day)}{F_{ue} \times 1000\,(mg/g)} \times \frac{MW_d}{MW_m}$$

UE = urinary excretion of phthalate metabolite in μg/g creatinine.
CE = creatinine excretion rate normalised by body weight
F_{ue} = metabolite excretion factor.
MW = molecular weight
d = diester
m = monoester.

Based on existing publications, the 24 h excretion rate of creatinine was set to 18 mg/kg/day for women and 23 mg/kg/day for men (Kohn et al., 2000) and to 15.3 mg/kg/day for boys and 14.3 mg/kg/day for girls (Wang et al., 2018). For girls and boys together the average excretion rate of 14.8 mg/kg/da was used. The excretion factors (F_{ue}) for the MEHP, 5OH-MEHP, 5oxo-MEHP, MEP, MiBP, MnBP, and MBzP were 0.024, 0.074, 0.55 (Schmid and Schlatter, 1985), 0.69, 0.71 (Koch et al., 2003a), 0.69, and 0.73 (Anderson et al., 2001), respectively, as obtained from the literature.

Table 5 presents the calculated intake dose estimations for the Slovenian DEMOCOPHES population as well as the results from other studies for comparison. The estimated intake dose calculations in μg/Kg_{bw}/day seem to fall in the range that can be obtained from other studies. Only studies on samples collected before 2015 were included in order to capture the chemical burden of the general population prior to the establishment of restrictions on selected phthalates by the European Union. It should be noted, however, that new regulations were frequently introduced and altered, limiting the comparability of results obtained from samples taken during different years. Contradictory to the comparison with the participating DEMOCOPHES countries, it should furthermore be noticed that differences in the industrial and economic environments of countries outside the European Union could influence the exposure of the general population. The observed differences may therefore be the result of differing and changing markets. Additionally, most studies do not report results on men; therefore, there is very little data available in the literature to compare the results for male adults.

4. Conclusions

This study confirms the common exposure of general populations to phthalates. All seven investigated phthalate metabolites were detected in women, men, and children from Slovenia in a wide range of concentrations, mainly due to personal habits and lifestyle. Children had the highest urinary concentrations of all metabolites, except for MEHP and MEP, as well as the highest daily IDs.

We could confirm the widespread argument that creatinine adjustment is not suitable for all compounds, making SG the more desirable adjustment option.

We confirmed some of the known sources for phthalates, including dietary sources (e.g. packed food, and fatty food), as well as product and lifestyle sources (e.g. such as microwave heating in plastic containers, lotions, eye make-up, PVC, solvents, etc.) Interestingly, urinary phthalate metabolite concentrations exhibited significant geographic differences, with higher concentrations being measured in individuals from the rural sampling location, where most previously positively associated sources were present.

To our knowledge, this is the first investigation of the exposure of the whole family unit to phthalates from common sources in Slovenia. The results indicate that, due to an accumulation of associated sources, the exposure of families is higher in rural areas. Household sources of exposure include for instance younger buildings. The educational level of the parents was connected to exposure, as families with more highly educated mothers and fathers had significantly lower concentrations of DEHP and BBzP metabolites and significantly higher levels of MnBP and MiBP. MEP concentrations were (not significantly) higher among lower educated participants.

Overall, the measured urinary concentrations as well as the calculated daily intakes fall into the range reported in other studies. The DEMOCOPHES project has enabled us to assess the overall exposure to phthalates of Slovenian women, men, children, and family units as well as to evaluate the contribution of potential sources and exposure pathways. However, more detailed environmental data, such as atmospheric concentrations of phthalates, are needed to close some of the persisting knowledge gaps.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2020.109548.

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Credit author statement

1. Agneta Annika Runkel: conceptualization, formal analysis and model creation, investigation, results interpretation, writing – original draft, writing – review & editing, visualization.
2. Janja Snoj Tratnik: project administration, organization, and sampling.
3. Darja Mazaj: project administration, organization, and sampling.
4. Milena Horvat: Corresponding author, supervision, validation, resources, funding acquisition.

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3.2 Manuscript 2: Organohalogens: A Persisting Burden in Slovenia?

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POPs are a group of contaminants that share a wide range of characteristics; persistency, toxicity, the ability to bioaccumulate and biomagnify, and the capacity for long-range transport (Angerer et al., 2007; Curtean-Bănăduc et al., 2020; Govaerts et al., 2018; Islam et al., 2018; Nawab et al., 2020; Rawn et al., 2012; Warenik-Bany et al., 2019). POPs are of concern to the environment and human health and have, hence, been restricted since 2004. Since then, their levels in the environment have been decreasing, however, they can still be determined at often high concentrations due to their long half-life and few active sources (Fång et al., 2015; Schwarzenbach et al., 2010). Active sources can be industrial activities or traffic, whereas legacy pollution is associated with today's background contamination of, for instance, animal products. Therefore, diet is widely regarded as the main pathway of exposure for humans (Bjerregaard-Olesen et al., 2017; Porta et al., 2008).

In Slovenia, in response to modern technologies and pollution prevention only a few active sources still remain. In BK, however, following the pollution of Krupa River with PCBs between 1962 and 1983, the levels of PCBs remain elevated until today (Pezdirc et al., 2011).

In the present manuscript (Runkel et al., 2021), we assessed the levels of selected POPs (PCDD/Fs, PCBs, PBDEs, and OCPs) in pooled and individual samples of maternal milk and men's serum and pooled plasma. The samples of 536 primiparous lactating women and 548 associated men from 12 regions in Slovenia were obtained between 2008 and 2014 during two sampling campaigns.

In pooled samples of maternal milk and plasma, the levels of PCDD/Fs, dioxin-like PCBs (dl-PCBs), and PBDEs were determined, whereas, non-dioxin-like PCBs (ndl-PCBs) and OCPs were measured in individual samples of milk and men's serum. All analyses were carried out at the National Laboratory of Health, Environment, and Food, located in Maribor, following United States Environmental Protection Agency (EPA) methods (EPA 1613, 1668A, and 1614, respectively) using high-resolution mass spectrometry (HRMS) for the determination in pooled samples. An in-house method using electron capture detection (ECD) was applied for the measurement of ndl-PCBs and OCPs at the same institution. All biostatistical analyses including ANOVA, spearman's rank correlation, multiple linear regression analysis, cluster analysis, and principal component analysis (PCA) were performed by the candidate.

The aim of the study was to assess exposure to POPs in men and lactating women, to identify sources and pathways of exposure, and to establish reference values for both populations. The following results were obtained:

1) The determined concentrations and detection frequencies were in general low, but higher in maternal milk compared to men's blood samples.
2) The calculated toxic equivalent (TEQ) values for PCDD/Fs + dl-PCBs were 3.04 pg/gTEQ_{2005} and 0.09 pg/gTEQ_{2005} for pooled milk and plasma samples, respectively. ΣPBDE could be determined at concentrations of 1076 pg/g and 920 pg/g in pooled milk and men's plasma, respectively.
3) In individual samples of maternal milk, only HCB, dichlorodiphenyldichloroethylene (p,p'-DDE), ΣDDT and the ndl-PCB congeners 138, 153, and 180 (concentration range 5-60 ng/g) could be determined, whereas in

men's serum, only p,p'-DDE and Σndl-PCB could be detected (0.25 ng/g and 0.3 ng/g, respectively).

4) We observed strong within-group correlations for PCBs, PCDD/Fs, PBDEs, DDT breakdown products and weaker correlations among compounds from different groups.
5) The levels of OCPs and ndl-PCBs were significantly correlated with age, whereas smoking was inversely correlated. Significant associations were found with dietary products, such as seafood, meat, and alcohol. Other sources determined included old buildings and private water wells.
6) The concentrations observed in this study are very low compared to studies from other countries, especially in comparison with countries on the Northern side of the Alps. However, among the regions, BK has the highest TEQ values, followed by LJ.

We conclude that exposure to POPs in Slovenia is low by comparison to other countries and we hypothesize that one of the underlying reasons might be Slovenia's geographical location. The alpine barrier effect might shelter Slovenia from the influence of the Westerly winds that pose a mechanism of transport for POPs over Northern and Western Europe. Despite this, the effects of the pollution of Krupa River are still visible in a national comparison. We can confirm some known dietary and industrial sources of POP exposure that were associated with POPs' concentrations even at low levels.

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Organohalogens: A persisting burden in Slovenia?

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A R T I C L E I N F O

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A B S T R A C T

Persistent organic pollutants (POPs) represent a concern for the environment and human health due to their persistence and toxicity. Exposure in Slovenia is geographically differentiated because the country, as part of former Yugoslavia, has a history of industry and regional contamination and is – at the same time – known for its clean nature. The PCB pollution of the Krupa River drew the public's attention to the chemical burden of Slovenians, and the demand for studies has been rising since. We assessed the exposure of men (n = 548) and primiparous women (n = 536) to POPs in 12 regions of Slovenia as well as exposure pathways via questionnaires. Most PCDD/Fs, PCBs, and PBDEs could be determined in pooled samples of maternal milk at low concentrations (1.57 pg/gTEQ, 1.47 pg/gTEQ, and 1076 pg/g fat, respectively), but a much lower number of compounds could be measured above the LOQ in pooled men's plasma samples (PCDD/Fs 0.08 pg/gTEQ, PCBs 0.007 pg/gTEQ, ΣPBDE 920 pg/g), and only HCB, p,p'-DDE, ΣDDT, and the non-dioxin-like PCB congeners 138, 153, and 180 could be determined in individual samples of milk (concentration range 5–60 ng/g fat). In individual samples of men's serum, only p,p'-DDE and ΣPCB were detected at concentrations of 0.25 ng/g and 0.3 ng/g, respectively. Nonetheless, we were able to differentiate between polluted and unpolluted areas on a national level, with higher exposure levels in the PCB polluted region of Bela Krajina, the industrial region Zasavje, and the capital, Ljubljana. Despite low concentrations, determinants of exposure, such as age, proximity to roads, old building materials, private water supplies, and consumption of alcohol, fish, meat, and eggs that have previously been observed only at higher levels could still be identified. Furthermore, levels of PCBs and PBDEs were highly correlated suggesting common exposure sources and pathways, whereas PCDD/Fs were correlated to a lesser extent. The calculated ratio between DDT and DDE in maternal milk samples was decreasing with the year of sampling, suggesting no ongoing exposure to DDT. The study findings suggest low exposure of men and lactating women to legacy pollutants in Slovenia, which gave rise to the hypothesis that Slovenia's geographical location might provide shelter from the long-range transport of POPs via Westerly winds. This hypothesis remains to be confirmed within future studies.

1. Introduction

Persistent organic pollutants (POPs) can be characterised by their resistance to environmental degradation, their ability to bioaccumulate, and their potential threat to the environment, wildlife, and human health (Angerer et al., 2007; Curtean-Bănăduc et al., 2020; Govaerts et al., 2018; Islam et al., 2018; Nawab et al., 2020; Rawn et al., 2012; Warenik-Bany et al., 2019). Subjected to restriction and monitoring since 2001, POPs include organochlorine pesticides (OCPs), industrial chemicals, and unintentionally produced by-products. Even though some POPs have been prohibited since the 1970s, they are still present in the environment and detectable in different matrices, including human samples (Fång et al., 2015; Schwarzenbach et al., 2010).

In Slovenia, assessment of exposure to POPs is of interest due to the country's legacy of industrial activity and local PCB pollution. Between 1962 and 1983, a capacitor manufacturer disposed of PCB contaminated oil in the karstic region of Bela krajina (Pezdirc et al., 2011), where it

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Abbreviations

BAT	best available techniques
BDE	brominated diphenyl ether
DDT	dichlorodiphenyltrichloroethane
dl – PCB	dioxin-like PCB
H6CDD/F	hexachlorodibenzo-p-dioxin/furan
H7CDD/F	heptachlorodibenzo-p-dioxin/furan
HCB	hexachlorobenzene
HCE	heptachlor epoxide
MM	maternal milk
ndl–PCB	non-dioxin-like PCB
OCDD/F	octachlorodibenzo-p-dioxin/furan
OCP	organochlorine pesticide
PCDD/Fs	polychlorinated dibenzop-dioxins and polychlorinated dibenzofurans
p,p'-DDE	dichlorodiphenyldichloroethylene
PBDE	polybrominated diphenyl ether
PCB	polychlorinated biphenyl
PeCDD/F	pentachlorodibenzo-p-dioxin/furan
PMM	pooled maternal milk
POPs	persistent organic pollutants
PP	pooled men's plasma
S	men's serum blood samples
SD	standard deviation
TCDD/F	tetrachlorodibenzo-p-dioxin/furan

contaminated the spring of the Krupa River (Fig. 1). Monitoring has been carried out since the pollution became known, and reported values from the 1980s are as follows: 55 ng/g in sediment, 0.3 μg/g in water, 117 μg/g in fish, and 155 ng/g in human blood of the local population (Jan and Tratnik, 1988). The literature is in general agreement on the most important exposure sources for humans. As POPs biomagnify up the food chain they accumulate at the highest trophy levels such as predator species in aquatic and terrestrial environments. Thus, diet, especially meat and fish consumption, is the main contributor to exposure of humans to legacy pollutants and studies report positive correlations among POPs with similar half-lives in the human body (Bjerregaard-Olesen et al., 2017; Porta et al., 2008). Lifestyle factors influencing the metabolism of pollutants, such as alcohol intake and smoking, have been associated with POP concentrations as well (Arrebola et al., 2010; Porta et al., 2008).

The most crucial windows of vulnerability to the effects of POPs are prenatal and neonatal developmental stages. Because maternal history is known to alter POP concentrations in the mother's body (Miyashita et al., 2015), primiparous women were chosen as one study population. The temporary physiological state of these women is of further interest for the interpretation of the study results, as pregnancy induced changes in metabolism include rapid elimination of drugs and pollutants from the body. As highlighted in a review study by Porta et al. (2008), further studies on POP exposure of populations are needed. Men of the same age group were additionally included in this study, which was part of the first national human biomonitoring survey in Slovenia.

Human biomonitoring is an effective means of directly assessing the chemical burden of a population instead of estimating it; it allows comparisons among countries and the identification of time trends of exposure, as done by Fång et al. (2015) who evaluated exposure to POPs globally. As such, it successfully supplements ambient environmental monitoring (Angerer et al., 2007). However, POP concentrations measured below the level of quantification in individual blood samples have been repeatedly reported. Thus, sample pooling is a common strategy for overcoming these limitations (Rawn et al., 2012).

Among the restricted global persistent contaminants, PCDD/Fs, PCBs, PBDEs, and OCPs were included in the national human biomonitoring project. The aim of the study was to collect data on exposure of the general population (men) and primiparous women to POPs and to determine geographical differences among 12 regions classified into rural, urban, and potentially polluted/industrial areas. Additionally, we searched for correlations between questionnaire data and POP concentrations in individual samples of maternal milk (MM) and individual samples of men's serum (S, male participants). Pooled men's plasma (PP, male participants) and pooled maternal milk (PMM) were included for better detection and comparison between regions. We hypothesised that elevated POP concentrations are concentrated in industrial regions rather than in rural environments in Slovenia and that the legacy of the

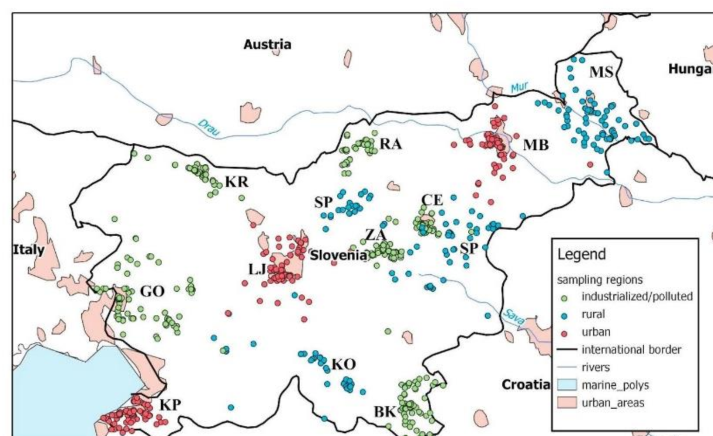

Fig. 1. Sampling regions (Natural Earth quick start for QGIS). Dots represent approximate sampling locations for data protection. Study areas: BK = Bela krajina, LJ = Ljubljana, KO = Kočevje and Cerknica, CE = Celje, GO = Posočje and Idrija, KP = Koper, KR = Jesenice, MB = Maribor, MS = Pomurje, Ra = Mežica valley, SP = Savinjsko-Posavska, ZA = Zasavje.

PCB pollution of the Krupa River in Bela krajina is still measurable.

2. Material and methods

2.1. Study population and design

A detailed description of the study population, design, and questionnaire data has been published elsewhere (Snoj Tratnik et al., 2019). Briefly, 536 primiparous women and 548 men (18–49 years of age) were recruited from 12 regions classified as rural (KO = Kočevje and Cerknica, MS = Pomurje, SP = Savinjsko-Posavska), urban (LJ = Ljubljana, MB = Maribor, KP = Koper), and potentially polluted/industrial areas due to past/present industrial activities, and/or geological presence of metals (RA = Mežica valley, GO = Posočje and Idrija, KR = Jesenice, ZA = Zasavje, CE = Celje, BK = Bela krajina) (Fig. 1) during two sampling campaigns. The pilot phase (2008–2009) included three regions (Ljubljana, Kočevje and Cerknica, and Bela krajina). Other regions (Celje, Posočje and Idrija, Koper, Jesenice, Maribor, Pomurje, Mežica Valley, Savinjsko-Posavska, Zasavje) were included in the follow-up study (2011–2014). Recruitment of women was done in the third trimester of pregnancy via maternity hospitals and schools as well as gynaecologists and their respective partners were invited to participate in the study at any point. Important criteria for inclusion were continuous residency in the area for at least 5 years with no active exposure sources nearby, no occupational exposure, good health of all participants including the child, an unproblematic pregnancy, breastfeeding, and availability for sampling during 8 weeks post-partum. The study design is in line with the WHO protocol on human biomonitoring (WHO, 2007), following the recommended procedures on recruitment, questionnaires, interviews, sampling, chemical analyses, compound selection, and ethics and as such ensures reliability and comparability with other studies. The protocol recommends the use of pooled samples of maternal milk to assess exposure levels of priority POPs equally defined in the document. Additionally, it recommends the exclusion of participants with occupational exposure of active exposure sources in close proximity of the residence. A detailed questionnaire was included in the sampling campaign; it covered information on lifestyle, employment, health, residency, and diet as suggested by the WHO protocol on HBM (WHO, 2007). The population characteristics are summarised in supplementary Table A.1. The National Medical Ethics Committee of the Republic of Slovenia granted approval of the pilot study (number of accordance 42/12/07) and the follow-up study (number of accordance 53/07/09).

2.2. Pooled samples

Samples of maternal milk and men's plasma were obtained from 12 sampling regions (Fig. 1) and pooled into 50 PMM and 33 PP samples according to the respective sampling location. Within each sampling region, sample pools were formed based on the residence of participants, considering industrial activity, emission sources, and geography (relief, wind direction). The final number of samples per pool was largely restricted by project funding and by variations in participation per location (supplementary Table A.2). Pooled samples were prepared and analysed for PCDD/Fs, dl-PCBs, and PBDE as described below.

2.3. Laboratory analysis

Table 1 provides an overview of all the analytes and matrices included. Samples were analysed at the National Laboratory of Health, Environment, and Food, located in Maribor. A detailed version of the analytical methods is provided in the supplementary material.

Table 1
Overview of analytes measured in different matrices.

Pooled maternal milk (PMM)	Pooled men's plasma (PP)	Individual samples of maternal milk (MM)	Individual samples of men's serum (S)
PCDD/Fs	PCDD/Fs		
		PCBs: 28, 52, 101, 138, 180, 153, ΣPCB	PCBs: 28, 52, 101, 138, 180, 153, ΣPCB
PCBs: 105, 114, 123, 126, 156, 157, 167, 169, 189, 77, 81, 118	PCBs: 105, 114, 123, 126, 156, 157, 167, 169, 189, 77, 81, 118		
PBDEs	PBDEs		
		OCs[a]	OCs[a]
			isodrin
		δ - HCH	
		α -, β – endosulfan (sulfat)	

[a] ΣDDT, p,p'-DDT, o,p'-DDT, p,p'-DDE, o,p'-DDE, p,p'-DDD, o,p'-DDD, HCB, aldrin, endrin, dieldrin, α -, β -, γ – HCH, heptachlor, chlordane, cis-HCE, trans-HCE.

2.3.1. Fat extraction

Fat was extracted from human milk (PMM, MM) using Sodium Oxalate ($Na_2C_2O_4$) and acetone for protein participation, and hexane for extraction.

2.3.2. Sample preparation and analysis of PCDD/Fs, dl-PCBs, and PBDEs (maternal milk and men's plasma)

The methodologies applied for the analysis of PCDD/Fs, dl- PCBs, and PBDEs followed the United States Environmental Protection Agency (EPA) methods (EPA 1613, 1668A, and 1614, respectively) that have been presented in detail elsewhere (Horvat et al, 2010, 2015), and a detailed description is provided in the supplementary material.

To summarise briefly, isotopically labelled internal standard was added to all samples. The extracts were cleaned up using a multiple-step protocol, micro-concentrated and analysed using a high-resolution gas chromatograph coupled with high-resolution mass spectrometer (HRGC/HRMS) and isotope dilution quantification. LOQs were 0.05 pg/g for tetra to hepta PCDD/F and 0.1 pg/g for octa PCDD/F, dl-PCBs, and PBDE expressed as whole weight for men's plasma samples and on the basis of fat basis for PMM samples.

2.3.3. Quality control for PCDD/Fs, dl-PCBs, and PBDEs (maternal milk, men's plasma)

Laboratory method blanks were used in every batch and subtracted from the results. To all samples isotopically labelled ^{13}C standards were added at the earliest point of sample processing. Target analyte quantification was performed via isotope dilution. Injection standards were used in every sample extract prior to instrumental analysis, and the concentrations of the surrogates were determined as percent recoveries, which were compliant with standard prerequisites.

2.3.4. Sample preparation of men's serum and maternal milk for analysis of OCPs and ndl-PCBs

For MM samples, fat extracts were used as described in Section 2.3.1. Men's S samples were mixed with 5% formic acid in acetonitrile and centrifuged. The centrifugate was added to a dispersive solid phase reagent. The resulting suspension was centrifuged again and extracted twice with hexane. MM fat extracts and concentrated dried men's S extracts were loaded to the conditioned SPE cartridge, eluted with hexane:dichloromethane and hexane:dichloromethane, and reduced for gas chromatographic analysis.

An Agilent Technologies 6890N GC equipped with two 60 m × 0.25 mm x 0,25 μm capillary columns with slightly different polarities and two ^{63}Ni electron-capture detectors (ECD-ECD) was used. The operating conditions are described in detail in the supplementary material.

For MM samples, limits of quantification (LOQs) were 0.01 mg/kg

for α-HCH, heptachlor epoxide, chlordane, and HCB, 0.015 mg/kg for endosulfan, heptachlor, aldrin, dieldrin, DDE, and β,γ,δ-HCH, and 0.02 mg/kg for endrin, endosulfan sulphate, DDT, DDD, and ndl-PCBs expressed per gram of fat. For men's S samples LOQs were 0.015 μg/kg for HCB, 0.2 μg/kg for α,β,γ-HCH, heptachlor, heptachlor epoxide, aldrin, dieldrin, isodrin, chlordane, and DDE, and 0.3 μg/kg for endrin, DDT, DDD, and ndl-PCBs.

2.3.5. Quality control for OCPs and ndl-PCBs (maternal milk, men's serum)

Real matrix blanks were used in every batch, where all analytes investigated were below the limits of detection (LOD). For quality assurance, internal standard PCB 209 was added to every sample. In every batch, a quality control (QC) sample (fortified blank sample) was analysed. The recoveries for every analyte were calculated. Fortifications used were interchanging from batch to batch throughout the entire range of the calibration curve – from low to high range.

Recoveries ranged from 52 to 114% (MM) and from 43 to 102% (men's S) with variations in QC samples not exceeding 25%. Median recoveries were 82% (MM) and 72% (men's S).

2.4. Emission inventory for Slovenia (2008–2014) and POP sources to air (PAHs, PCDD/Fs, HCB, PCB)

The Slovenian Environmental Agency is responsible for reporting air emissions for Slovenia. These data are gathered each year in Informative Inventory Reports for Slovenia (submission under the UNECE Convention on Long-Range Transboundary Air Pollution). Emissions to air have been reported for the following POPs: PAHs, PCDD/Fs, HCB, and PCBs. The emissions in g (E) are calculated as follows (Allende et al., 2016; Logar et al., 2016; Rode et al., 2010).

$$E = AD \times EF$$

E = emission (g).
AD = activity data (quantity of fuel combusted (t))
EF = emission factor per quality of fuel (g/t).

2.5. Statistical analysis

Questionnaire data were used to determine associations between analytical concentrations of POPs in individual samples with potential exposure sources. All statistical analyses were carried out in R Studio version 3.6.3. Correlations among analytes were evaluated via a correlation matrix using Spearman's correlation coefficient and Benjamini&Hochberg adjustment (Patil, 2018). Other correlations were tested separately using Spearman's correlation test (stats package) and confirmed using multiple linear regression modelling (confounders listed in Table A.7). Differences among groups were obtained via analyses of variance and covariance (ANOVA and ANCOVA) and the Wilcoxon test. Cluster analysis and principal component analysis (PCA) were carried out for pattern recognition of sample concentrations.

Values below LOQ were treated as LOQ/2. 2005 WHO toxicity equivalency factors (TEFs) (Van den Berg et al., 2006) were used to convert analytical concentrations of PCDD/Fs and dl-PCBs into toxic equivalents (TEQs). Analytes with 70% of the samples < LOQ were excluded from the statistical analysis and from the presentation of descriptive statistics. Concentrations were not normally distributed and log-transformed data were thus used to achieve normality. For PP samples, no information on lipid content was available, which made fat normalisation impossible. For S samples, no normalisation was performed because analyte concentrations did not correlate with lipid content (Hebert and Keenleyside, 1995).

2.6. Calculation of proposed reference values

Reference values (RVs) are statistical estimates of the upper margin of background exposure of populations. They were introduced as a tool in science-policy translations and communication to policy makers as they allow an understanding of the exposure burden of population and mirror successes and failures of regulatory actions (Buekers et al., 2018; Schulz et al., 2011). Preliminary RVs for PCDD/Fs, dl-PCBs, and PBDE were established for men and primiparous women (20–40 years of age) following the guidelines suggested by the international project on HBM in Europe (HBM4EU) and the German HBM commission (Govarts, 2018; Schulz et al., 2011). Values were calculated by rounding off the 95th percentile (P95) of measured concentrations within the 95th confidence interval of that value as suggested in the literature (Ewers et al., 1999; Schulz et al., 2011; Solberg, 1987a, 1987b). RVs for PCDD/Fs and dl-PCBs were based on calculated TEQs of the measured concentration. The data for calculation is presented in the supplementary Table A.5.

3. Results

Descriptive statistics of analytes and proposed RVs by sample matrix are presented in Table 2.

3.1. Polychlorinated dibenzodioxins/furans (PCDD/Fs) and dioxin-like-polychlorinated biphenyls (dl-PCBs)

Median concentrations for PCDD/F and dl-PCBs ranged from 0.05 pg/g fat to 24.5 pg/g fat and from 0.42 pg/g to 2700 pg/g fat in PMM samples, respectively. In PP samples, only 1,2,3,4,6,7,8 – H7CDD and 1,2,3,4,6,7,8,9 – OCDD could be detected in more than 30% of the samples with median concentrations of 0.05 pg/g and 0.1 pg/g, respectively. Dl-PCBs were detected in a median range between 0.15 pg/g and 9.8 pg/g (PP), respectively. In PMM and PP samples, 1,2,3,4,6,7,8,9 – OCDD was the largest contributor to ΣPCDD/Fs in PMM samples, followed by 1,2,3,4,6,7,8 – H7CDD and 2,3,4,7,8 – PeCDF (71%, 11%, 6%). In PP samples, among the two detected congeners, 1,2,3,4,6,7,8,9 – OCDD contributed more (67%). The largest contributions to ΣPCB had PCB 118 (PMM: 50% and PP: 55%), followed by PCB 156 in PMM (22%) and PCB 105 in PP (19%) and PCB 105 in PMM (11%) and PCB 156 in PP (14%).

The TEQ values are presented in Table 3 (and in more detail in supplementary Table A.4). The highest contribution to ΣPCDD/Fs TEQ came from 2,3,4,7,8-PeCDF (21%) in PMM samples and from 1,2,3,7,8-PeCDD (29%) in PP samples. Among PCB congeners, PCB 126 made the largest contribution to Σdl-PCBTEQs (36% PMM and 6% PP). ΣPCDD/F TEQs was 1.6 pg/gTEQ_{2005} and 0.08 pg/gTEQ_{2005} for PMM and PP, respectively, and Σdl-PCBTEQs was 1.5 pg/gTEQ_{2005} (PMM) and 0.007 pg/gTEQ_{2005} (PP). The calculated ΣPCDD/Fs + dl-PCBs TEQ was 3.04 pg/gTEQ_{2005} in PMM samples and 0.09 pg/gTEQ_{2005} in PP samples, where PCDD/Fs contributed 52% to ΣTEQ PCDD/Fs + PCBs in PMM and 92% in PP.

3.2. Polybrominated diphenyl ethers (PBDEs)

Median analytical concentrations of PBDEs in pooled samples ranged from 12 pg/g to 505 pg/g fat in PMM and from 0.25 pg/g to 6 pg/g in PP samples. Among PBDEs, BDE 47 contributed the most to ΣPBDE (46% in PMM, 70% in PP).

3.3. Organochlorine pesticides (OCPs)

Organochlorine pesticides were measured only in individual samples. Only HCB, p,p'–DDE, and ΣDDT metabolites could be detected in MM at median concentrations of 5 ng/g fat, 50 ng/g fat, and 60 ng/g fat, respectively. In S samples, only p,p'–DDE could be detected (median = 0.25 ng/g). Using LOQ/2 as a representative value for p,p'-DDT the mean p,p'-DDT:p,p'-DDE ratio was 0.1 in MM samples.

Table 2
Descriptive statistics of pooled samples of maternal milk (n = 50) and men's plasma (n = 33) and individual samples of maternal milk (n = 448) and men's serum (n = 520). CI = confidence interval. Reference values (RVs) for PCDD/Fs and dl-PCBs are presented in pg/g TEQ. Median TEQ values are presented in the supplementary Table A4.

	Pooled samples maternal milk (pg/g fat)									Pooled samples men's plasma (pg/g)								
	P25	Median	A. mean	P75	P95	range	SD	< LOD %	RV	P25	Median	A. mean	P75	P95	Range	SD	< LOD %	RV
1,2,3,4,6,7,8 - H7CDD	2.48	3.70	3.62	4.98	6.72	0.02–7.10	1.89	6	0.07[a]	0.02	0.05	0.06	0.05	0.08	0.02–0.70	0.12	73	0.001[a]
1,2,3,4,6,7,8 - H7CDF	0.17	0.45	0.60	0.99	1.57	0.02–2.10	0.54	22	0.02[a]								100	
1,2,3,4,6,7,8,9 - OCDD	17.0	24.5	25.9	32.8	51.6	0.10–68.0	13.3	2	0.02[a]	0.05	0.10	0.18	0.18	0.43	0.05–1.40	0.24	55	0.0001[a]
1,2,3,4,6,7,8,9 - OCDF								88									97	
1,2,3,4,7,8,9 - H7CDF								88									97	
1,2,3,4,7,8 - H6CDD	0.02	0.26	0.57	0.56	3.17	0.02–4.40	0.96	48	0.30[a]								97	
1,2,3,4,7,8 - H6CDF	0.05	0.73	0.72	1.10	1.86	0.02–2.50	0.63	30	0.20[a]								97	
1,2,3,6,7,8 - H6CDD	1.03	1.45	1.59	2.18	3.21	0.02–4.40	0.96	8	0.30[a]								88	
1,2,3,6,7,8 - H6CDF	0.27	0.70	0.78	1.10	1.71	0.02–3.50	0.64	22	0.18[a]								97	
1,2,3,7,8 - PeCDD	0.03	0.42	0.51	0.85	1.36	0.02–2.10	0.50	32	1.40[a]								88	
1,2,3,7,8 - PeCDF	0.05	0.11	0.15	0.23	0.43	0.02–0.53	0.14	42	0.01[a]								100	
1,2,3,7,8,9 - H6CDD								72									97	
1,2,3,7,8,9 - H6CDF								76									97	
2,3,4,6,7,8 - H6CDF	0.02	0.05	0.22	0.26	1.06	0.02–1.30	0.34	58	0.10[a]								97	
2,3,4,7,8 - PeCDF	1.40	2.15	1.91	2.50	3.00	0.02–3.20	0.82	6	0.90[a]								97	
2,3,7,8 - TCDD	0.05	0.13	0.24	0.37	0.77	0.02–1.20	0.28	42	0.80[a]								97	
2,3,7,8 - TCDF	0.02	0.05	0.13	0.14	0.54	0.02–0.68	0.18	64	0.05[a]								97	
PCB 105	483	565	722	760	1775	100–3600	563	0	0.05[a]	2.50	3.30	3.34	4.50	5.02	0.24–6.70	2.31	0	0.0002[a]
PCB 114	110	130	174	178	568	32.0–790	151	0	0.02[a]	0.20	0.29	0.36	0.42	0.83	0.11–1.10	0.23	0	0.00003[a]
PCB 123	32.3	37.5	43.2	52.0	79.0	5.30–140	23.7	0	0.003[a]	0.29	0.35	0.38	0.50	0.61	0.20–0.70	0.13	0	0.00002[a]
PCB 126	7.43	11.0	11.7	15.8	20.6	1.90–23.0	5.23	0	2.00[a]								97	
PCB 156	985	1200	1402	1575	3110	210–4200	732	0	0.10[a]	1.80	2.40	3.01	3.30	7.24	0.10–11.0	2.59	3	0.0002[a]
PCB 157	170	205	258	270	721	51.0–810	166	0	0.02[a]	0.35	0.46	0.63	0.69	1.50	0.10–2.90	0.54	3	0.00005[a]
PCB 167	293	390	412	470	766	59.0–840	170	0	0.02[a]	0.65	0.84	0.99	1.10	2.20	0.17–3.40	3.07	0	0.00007[a]
PCB 169	4.68	7.05	9.00	11.0	20.6	1.40–25.0	5.88	0	0.60[a]								100	0.003[a]
PCB 189	86.3	110	110	140	166	26.0–220	42.3	0	0.005[a]	0.10	0.15	0.26	0.29	0.82	0.10–1.30	1.28	30	0.00003[a]
PCB 77	4.93	6.80	8.76	9.93	20.9	1.90–35.0	6.51	0	0.002[a]	0.10	0.15	0.48	0.50	1.98	0.10–2.50	0.65	33	0.0002[a]
PCB 81	0.10	0.42	0.60	0.98	1.61	0.10–1.90	0.55	28	0.0005[a]								91	0.00005[a]
PCB 118	2300	2700	3361	3575	8395	470–14000	2316	0	0.25[a]	8.30	9.80	9.63	11.0	13.4	4.30–20.0	1.84	0	0.0004[a]
BDE 100	94.0	130	160	178	290	46.0–860	141	0	300	0.52	0.63	0.83	0.97	1.86	0.10–2.40	0.54	3	2.00
BDE 153	190	265	371	378	779	8.00–2400	448	0	800	0.10	0.25	0.42	0.41	1.68	0.05–1.80	0.47	21	1.70
BDE 154	6.65	12.0	19.7	16.8	50.0	3.20–160	29.3	0	50								82	
BDE 183	11.3	21.0	23.2	31.0	52.6	0.10–72.0	18.0	12	55								70	
BDE 28	43.3	54.5	74.2	69.5	174	19.0–470	79.3	0	180	0.15	0.28	0.48	0.55	1.58	0.10–2.50	0.53	18	1.60
BDE 47	383	505	660	688	1155	191–6600	891	0	1200	4.70	6.00	7.25	8.50	15.5	2.00–20.8	2.94	0	16.0
BDE 99	86.3	116	166	160	417	30.0–1100	182	0	450	0.86	1.40	1.91	2.20	5.04	0.41–7.40	2.56	0	5.00
ΣPBDE	800	1076	1234	1492	2667	470–3750	679	0	2700	6.80	9.20	11.2	13.0	25.6	2.90–32.5	2.76	0	26.0
	Individual samples maternal milk (ng/g fat)									Individual samples men's serum (ng/g)								
PCB 138	10	10	10	10	30	10–200	10	57	30								99	
PCB 153	10	10	20	20	50	10–200	200	52	50								98	
PCB 180	10	10	10	10	20	10–90	10	49	20								100	
PCB 28								100									100	
PCB 52								100									100	
PCB 101								100									100	
ΣPCB	5	20	40	50	130	0–510	70	5	130	0.30	0.30	0.31	0.30	0.30	0.15–1.70	0.11	32	0.30
HCB	5	5	6	5	10	5–20	3	78	10								100	
p,p' - DDT								99									100	
o,p' - DDT								100									100	
o,p' - DDE								100									100	
p,p'-DDE	30	50	60	70	120	20–500	40	2	120	0.10	0.25	0.28	0.33	0.61	0.10–1.30	0.17	21	0.60

(continued on next page)

Table 2 (*continued*)

	Pooled samples maternal milk (pg/g fat)									Pooled samples men's plasma (pg/g)								
	P25	Median	A. mean	P75	P95	range	SD	< LOD %	RV	P25	Median	A. mean	P75	P95	Range	SD	< LOD %	RV
p,p'- DDD								100									100	
o,p' - DDD								100									100	
ΣDDT	40	60	80	90	190	20–500	60	0	190									
Aldrin								100									100	
Endrin								100									100	
Dieldrin								99									100	
Isodrin																	100	
α HCH								100									100	
β HCH								97									100	
δ HCH								100										
γ HCH								100									99	
Heptachlor								100									100	
cis-HCE								100									100	
trans-HCE								100									100	
cis Chlordane								100									100	
trans chlordane								100									100	
α Endosulfan								100										
β endosulfan								100										

[a] pg/g TEQ.

3.4. Correlations among analytes

3.4.1. Intra- and interclass associations in samples of maternal milk (PMM + MM)

The results for correlations among analytes within the same class are presented in the supplementary Tables A.8.1 – A.10. In MM samples, the strongest correlations were observed between ΣDDT and p,p'–DDE (rho = 1, p-value < 0.0001), and between ΣPCBs and PCB 153 (rho = 0.98, p-value < 0.0001).

In PMM, we observed strong correlations within PCBs and within PBDE congeners, whereas PCDD/Fs had a weaker association with one another. 2,3,4,7,8 – PeCDF and 1,2,3,6,7,8 – H6CDD were associated with the most PCDD/Fs and 2,3,7,8 – TCDF with the least. Among PCBs, PCB 77 was not correlated with any other, whereas all other PCBs seemed strongly related. All PBDE congeners were correlated with at least one of the others, except for BDE 154, which did not appear to have any relationship with other analytes.

The strongest interclass correlations were observed between PBDE congeners and PCBs, whereas PCDD/Fs exhibited much weaker correlations with other contaminants (supplementary Tables A.8.1, A.8.2, and A.10). BDE 183 and 153 were correlated with almost all PCB analytes in this study. Among PCDD/Fs, only 1,2,3,4,6,7,8,9-OCDD was correlated with almost all PCBs and PBDEs, whereas correlations among other analytes were sporadic. As Fig. 2 shows, PCA results support the correlation outcomes. PBDEs were grouped closely together as were PCBs, whereas PCDD/Fs were more scattered. Some interclass associations were suggested, such as between 1,2,3,4,6,7,8,9 – OCDD and BDE 183. Principal component 1 (PC1) accounts for only 17% of the variation, however.

Cluster analysis (Fig. 2) indicates the presence of five clusters (determined by the elbow method) containing (1) PCDD/Fs, (2) PCB 77, 126, and 168, (3) 1,2,3,4,6,7,8,9- OCDD and BDE 183, (4) BDE 100, 99, 28, and PCB 189, and (5) PCB 167, 114, and 157.

In MM samples, all analytes showed positive correlations among each other. Only PCB 180 and HCB did not seem to have a significant relationship.

3.4.2. Associations in pooled men's plasma (PP)

In PP samples, PCBs and PBDE congeners were strongly correlated within their respective groups (Table A.9), and strong correlations were observed between PCB 114, 123, and 156 and most PBDE congeners (supplementary Table A.9). The two PCDD/Fs detected in men's plasma were significantly correlated with each other, but not with any of the other analytes.

Cluster analysis resulted in two clusters, as determined by the elbow method (Fig. 2) indicating a cluster containing PCB 118, BDE 47, and ΣPBDE and a second group containing other contaminants. In the PCA plot, PCB 118 and 105 are separated from the other compounds on the right side of the graph, whereas other analytes seemed more closely related to one another. PC1 accounted for 24% of the variation.

3.5. Age trends

Age trends investigated using linear regression showed significant positive trends for p,p'-DDE, ΣDDT, PCB 153, PCB 180, PCB 138, and HCB in MM (p-values < 0.0001, 0.01, <0.0001, 0.007, 0.05, and <0.0001, respectively). P,p'–DDE levels in men's S samples were significantly correlated with age as well (p-value < 0.0001). Furthermore, we observed decreasing p,p'-DDT/p,p'-DDE ratios with age in MM samples (p-values < 0.0001). Importantly, levels of p,p'-DDT were mostly below LOQ (LOQ/2); therefore this trend should be taken as indicatory.

3.6. BMI

The geometric mean of BMI in this population was 24.3 with a

Table 3
Presentation of PCDD/Fs and dl-PCBs TEQ (pg/g), PBDE (ng/g), ndl-PCB (PCB 138, 153, 180, ng/g), and selected OCP (ng/g) levels as reported in the literature (P = primiparae).

Reference	Country	Sample	n	Sampling period	TEF year	ΣPCDD/ Fs	ΣPCBs	ΣTEQ	ΣPBDE	Σndl-PCBs	HCB	p,p'-DDE	ΣDDT
Present study	**Slovenia – All study areas**	**pooled, P**		**2008–2014**	**2005**	**1.57a**	**1.47a**	**3.04a**	**1.08a**				
	Bela krajina					**1.77a**	**2.99a**	**4.76a**					
	Celje					**2.07a**	**1.30a**	**3.37a**					
	Posočje and Idrija					**1.80a**	**1.20a**	**2.99a**					
	Jesenice					**1.52a**	**1.28a**	**2.80a**					
	Kočevje and Cerknica					**1.31a**	**1.67a**	**2.99a**					
	Koper					**1.80a**	**1.96a**	**3.75a**					
	Ljubljana					**2.23a**	**2.61a**	**4.84a**					
	Maribor					**0.72a**	**1.14a**	**1.86a**					
	Pomurje					**2.15a**	**1.46a**	**3.61a**					
	Mežica Valley					**1.22a**	**1.16a**	**2.37a**					
	Savinjsko - Posavska					**2.89a**	**1.01a**	**3.9a**					
	Zasavje					**0.29a**	**1.25a**	**1.54a**					
Abballe et al. (2008)	Italy	pooled, P		1998–2001		12.4	15.7	28.2					
Bake et al. (2007)	Latvia	pooled		2002/2004	1998	7.69b	14.3b	22.0b		142b			
						7.63b	5.84b	13.5b		111b			
Croes et al. (2013)	Belgium	pooled		2009–2010	2005	6.90b	3.70b	10.6b					
Fängström et al. (2005)	Faroe Islands	pooled, P		1987					1.95				
				1994/95					1.80				
				1999					2.55				
Harden et al. (2007)	Australia	pooled, P		2002/2003	1998	5.60	2.60	8.90					
Ingelido et al. (2007)	Italy	pooled		2000/2001	2005				2.75				
Li et al. (2009)	China	pooled		2007	2005	3.12	1.46	4.58					
Polder et al. (2008a)	Russia	pooled, P		2000–2002	1998	9.00	17.0	26.0	1.29		51.3a	782a	884a
Pratt et al. (2012)	Ireland	pooled, P		2010	2008	6.32	3.34	9.66					
Ryan and Rawn (2014)	Canada	pooled		2005	2005	6.6a	2.2a	8,8a					
Wong et al. (2013)	Hong Kong	pooled, P		2009	2005	7.48b	3.79b	11.3b					
Zhang et al. (2011)	China	pooled		2007					1.49a				
Zhang et al. (2017)	China	pooled, P		2011					1.47b				
Thomsen et al. (2010)	Norway	pooled		2003–2005					2.10a				
Toms et al. (2007)	Australia	pooled, P		2002–2003					11.0a				
Wasser et al. (2015)	Israel	pooled, P		2011–2012					6.77	6.87		1.47	168
De Felip et al. (2014)	Italy (Campania)	pooled, P		2008–2009		6.82a	5.55a	12.4a	2.53				
Fång et al. (2013)	Sweden	pooled, P		2008	2005	2.95	1.60	4.55					
				2009		2.90	2.00	4.95					
				2010		3.15	2.60	5.70					
				2011		2.90	2.15	5.05					
Pacheco Ferreira and Rabello Alves (2015)	Brazil	pooled, P		2012–2014	2005	15.4	6.96	22.3					
Present study	**Slovenia**	**Individual, P**	**448**	**2008–2014**						**30a**	**5a**	**50a**	**60a**
Alivernini et al. (2011)	Italy (Rome)	Individual, P	13	2005–2007						118b			
Colles et al. (2008)	Belgium	Individual, P	197	2006						98.4b	15.5b	95.9b	
Raab et al. (2008)	Germany	Individual, P	85	2005						200a	21a	87a	99a
Polder et al. (2008b)	Norway	Individual, P	29	2000–2001						37a	19a	99a	109a
Lignell et al. (2009)	Sweden	Individual, P	335	1996–2006						34.3a			
Kalantzi et al. (2004)	UK	Individual	54	2001–2003						111a	20.0b	220b	
Çok et al. (2009)	Turkey	Individual	51	2007						14.9b			
	South Africa	Individual	28	2004						8.40	1.80	3550a	4920a

(continued on next page)

Table 3 (*continued*)

Reference	Country	Sample	n	Sampling period	TEF year	ΣPCDD/Fs	ΣPCBs	ΣTEQ	ΣPBDE	Σndl-PCBs	HCB	p,p'-DDE	ΣDDT
Darnerud et al. (2011)													
Kim et al. (2013)	Korea	Individual, P	50							18.0 (PCB 138 only)	20.6a	150a	187a
Zietz et al. (2008)	Germany	Individual	523	2006						183a	23a		81a
Chao et al. (2006)	Taiwan	Individual	36	2000–2001								310b	333b
Kunisue et al. (2006)	Japan	Individual, P	38	2001–2004						140b	14.0b	330b	340b
Rodas-Ortíz et al. (2008)	Mexico	Individual	38	2006						1541b	92.0b	3041b	3065b
Ennaceur et al. (2008)	Tunisia	Individual	237	2003–2005						196b	85.0b	676b	1931b
Mannetje et al. (2013)	New Zealand	Individual, P	39	2007–2010			1.54b			13.3b		378b	
Polder et al. (2009)	Norway	Individual	423	2002–2006							11.0a	41.0a	
Rawn et al. (2017)	Canada	Individual	298	2008–2011						5.67a			
She et al. (2007)	Pacific Northwest (USA, Canada)	Individual, P	40	2003						40.2a			

*a = median, b = mean.

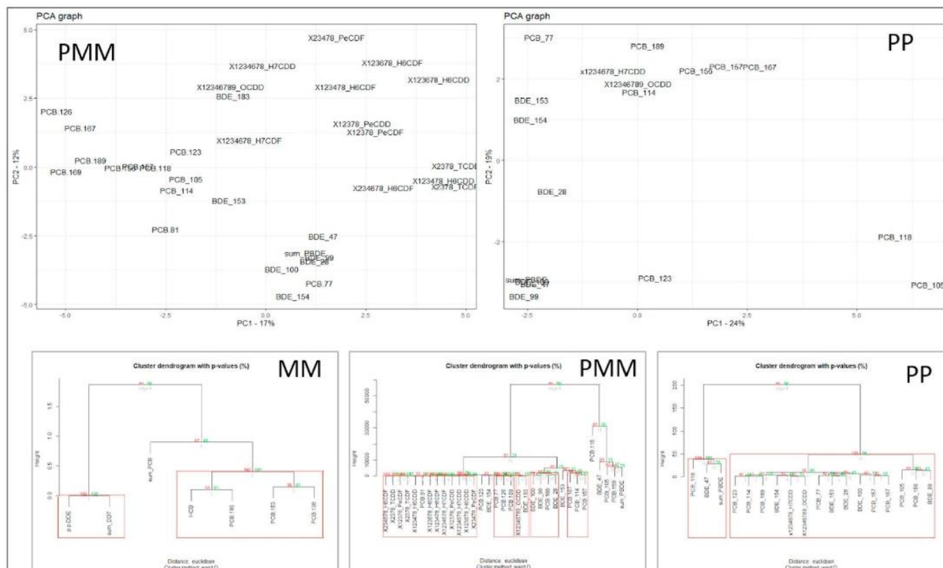

Fig. 2. Results of principal component analysis and cluster analysis in pooled samples of maternal milk (PMM), pooled samples of men's plasma (PP), and individual samples of maternal milk (MM).

standard deviation of 3.8 (supplementary Table A.1). Contaminant concentrations in MM samples were significantly inversely correlated for PCB 153 and PCB 180, as well as for p,p'-DDE in S samples after age correction.

3.7. Smoking

Non-smokers had significantly higher concentrations of p,p'-DDE (15–500 ng/g fat), ΣDDT (20–500 ng/g fat), and (marginally) significantly higher concentrations of HCB (5–20 ng/g fat) in MM samples (p-values = 0.03, 0.02, and 0.06, respectively).

3.8. Diet and food origin

A full overview of obtained data and answer frequencies is provided in the supplementary Table A.6. We observed significantly higher concentrations of p,p'–DDE, PCB 153, and PCB 180 (p-values = 0.01, 0.002, 0.0001, respectively) in MM of participants who reported higher consumption of seafood (>1x/month) and higher concentrations of p,p'–DDE in men's S samples in the same consumption group (p-value = 0.07). Regular intake of freshwater fish was significantly associated with higher concentrations of ΣPCBs in MM. The frequent consumption of eggs (>5x/week) was positively associated with elevated levels of ΣDDT

metabolites in MM. Domestic poultry was positively associated with elevated levels of p,p'-DDE in men's S as well as consumption of domestic (undefined) meat. Significant positive associations were observed between vegetable intake and p,p'-DDE in MM and men's S. In general, analyte concentrations in both matrices did not differ between vegetarians and nonvegetarians. Participants who reported frequent alcohol consumption (>1/week) had significantly higher concentrations of p, p'–DDE, ΣDDT, and PCB 153 in MM. The significance of this relationship disappeared when the source of alcohol (homemade or bought) was introduced into the model. P,p'-DDE in men's S was significantly correlated with homemade alcohol.

3.9. Sociodemographic characteristics

Concentrations of p,p'-DDE, HCB, PCB 153, PCB 180, and PCB 138 in MM samples were positively associated with the age of the building. Proximity to local roads was associated with elevated concentrations of PCB 180 in MM. Participants working in industrial production had significantly higher concentrations of PCB 153, PCB 138, and ΣPCBs in milk (p-values = 0.004, 0.001, and 0.0003, respectively). ΣPCBs in MM were significantly (p-value < 0.0001) associated with private water supplies. The percentage of participants using private water supplies in different regions was between 0% and 14%.

3.10. Regional differences

We observed significant differences among the 12 regions in Slovenia. The largest variations could be observed among PBDEs. Elevated concentrations were detected in PMM samples from Kočevje and Cerknica (BDE 154, 28, p-values = 0.02, 0.04), Bela krajina (BDE 153, p-values < 0.0001), and Zasavje (BDE 154 (PMM), 153, 154, 28, 47, ΣPBDE (PP), p-values < 0.0001, 0.01, <0.0001, 0.02, 0.003, 0.004). Significantly lower levels of BDE 183 were present in PMM from Maribor (p-value < 0.0001). The highest concentrations of p,p' – DDE in MM were detected in Ljubljana (p-value < 0.0001), and those of PCB 153 in Ljubljana and Bela krajina (p-value < 0.0001). Significantly elevated levels of p,p' – DDE were observed in men's S samples from Ljubljana (p-value = 0.0009).

ΣPCDD/Fs + dl-PCBs in PMM was 3.04 pg/gTEQ with regional differences. The highest TEQ values were obtained in the polluted region of Bela krajina (4.76 pg/g) and Ljubljana (4.84 pg/g) and the lowest in Zasavje (1.54 pg/g). ΣPCDD/FsTEQ was highest in Savinjsko-Posavska (2.89 pg/g) and lowest in Zasavje (0.29 pg/g). Σdl-PCBsTEQ was highest in Bela krajina (2.99 pg/g) and lowest in Savinjsko-Posavska (1.01 pg/g). Presenting individual compounds, 1,2,3,7,8,9 – H6CDD was significantly higher in Pomurje (PMM), and TEQ-levels of 2,3,4,7,8 – PeCDF were significantly higher in Ljubljana and Celje (PMM). TEQs of 2,3,4,7,8 – PeCDF were significantly lower in PMM and higher in PP samples from Zasavje. Concentrations of 1,2,3,6,7,8 – H6CDF, 1,2,3,7,8 – PeCDF, 1,2,3,4,7,8,9 – H7CDF, and 1,2,3,4,6,7,8 – H7CDF were significantly higher in PP samples from Zasavje, but none of these trends could be confirmed in PMM samples. Bela krajina had significantly higher TEQ values for PCB 105, 114, 123, 126, 156, 157, 167, 169, 81, and 118 in PMM. TEQs were significantly higher also in Ljubljana for PCB 126 and 169 (PMM), in Kočevje and Cerknica for PCB 169 (PMM), and in Zasavje for PCB 169 (PMM), 114, 156, 157, 189, and 77 (PP). TEQs of PCB 105 were significantly lower for PP samples from Zasavje, and PCB 126 TEQs were significantly elevated in Pomurje (PP). The impact of dl-PCBs on ΣTEQ values differed between the regions and between the sample matrices. In PMM samples, the contribution of PCBs to ΣPCDD/Fs + PCBs TEQ ranged from 26% (Savinjsko-Posavska) to 81% (Zasavje), whereas the range of percentages in PP samples was 5% (Zasavje) to 20% (Pomurje).

3.11. Results for POP (PCB, PCDD/Fs, HCB) air emissions for Slovenia between 1990 (base year) and 2014

For Slovenia, the trend of POP emissions declined from 1990 to 2014 as follows: for PCB (−75.4%), PCDD/Fs (−26.1%), and HCB (−98.9%) (Logar et al., 2016). The main sources for PCB emissions are industrial process and product use with a share of more than 99% that have been reduced due to best available techniques (BAT), industrial use of high temperature fuel combustion, and reductions in the solvent and product use subsector. A temporary increase of PCDD/Fs emissions between 2009 and 2014 could be attributed to larger consumptions of wood biomass in the residential sector as a result of the global economic crisis. Small combustion and industrial processes contribute a share of 70% and 17% to the total emissions, respectively. Emissions of HCB in Slovenia have dropped considerably in 2002 due to abatement of hexachloroethane (HCE) tablets as degassing agents in aluminium production (Rode et al., 2010).

3.12. Literature comparison

We compared the PMM and MM concentrations observed in the present study with those found in the literature for similar time periods and populations (2000–2014) (Fig. 3, Table 3). Comparable time periods and populations are essential for reliable comparisons of POP body burdens, as POPs are known to increase with age group and decrease over time (Li et al., 2019). As information on POP exposure of specific populations is limited, not many studies were available that fit the study population as well as the time period. Population matched comparisons are especially important in our population, as parity and lactation increase the elimination of POPs from the body as compared to child-free women of the same age (Dennis et al., 2017; Nøst et al., 2019). The highest TEQ values obtained in PMM samples were from Italy (Abballe et al., 2008; Ingelido et al., 2007), followed by Russia (Polder et al., 2008a), Brazil (Pacheco Ferreira and Rabello Alves, 2015), and Latvia (Bake et al., 2007), whereas Slovenia ranked among the lowest, with similar numbers being reported from China (Li et al., 2009) and Sweden (Fång et al., 2013). ΣTEQ levels from Bela krajina and Ljubljana were higher than the Slovenian average, but still among the lowest values reported. The highest levels of ΣPBDE were reported from Australia (Toms et al., 2007) and Israel (Wasser et al., 2015), whereas other countries reported values between 1 and 2 ng/g. Concentrations in Slovenian samples were among the lowest.

4. Discussion

4.1. Internal exposure to persistent organic pollutants

As described in Section 3, compared to the RVs for PCDD/Fs set for unpolluted regions (24 pg TEQ/g PMM [Solomon and Weiss, 2002], 7.4 pg TEQ/g PP [Umweltbundesamt, 2002], the levels determined in the Slovenian population are notably low (3.04 pg TEQ/g PMM, 0.09 pg TEQ/g PP), however it should be noted that the values obtained for lactating women are not representative of the general population. The large contribution of 1,2,3,4,6,7,8,9 – OCDD, 1,2,3,4,6,7,8 – H7CDD, and 2,3,4,7,8 – PeCDF to ΣPCDD/Fs (pg/g) has been reported in many studies, as have high concentrations of PCB 156 and 118 (Fromme et al., 2015). These results may be due to the half-lives of individual contaminants. This explanation finds support in the low number of correlations among PCDD/Fs (Section 3.5) as well as the large standard deviation among samples (Table 2). Among PCBs, the weakest correlations with other compounds were observed for analytes with the shortest half-life (PCB 77 and 81), whereas the pentachlorinated congeners PCB 105 and 118 were measured at high levels and were associated with many other analytes. A previous study examining the PCB burden in sediments and cave salamanders from the polluted Krupa River reported high concentrations of PCB 118 in the environment (Pezdirc et al.,

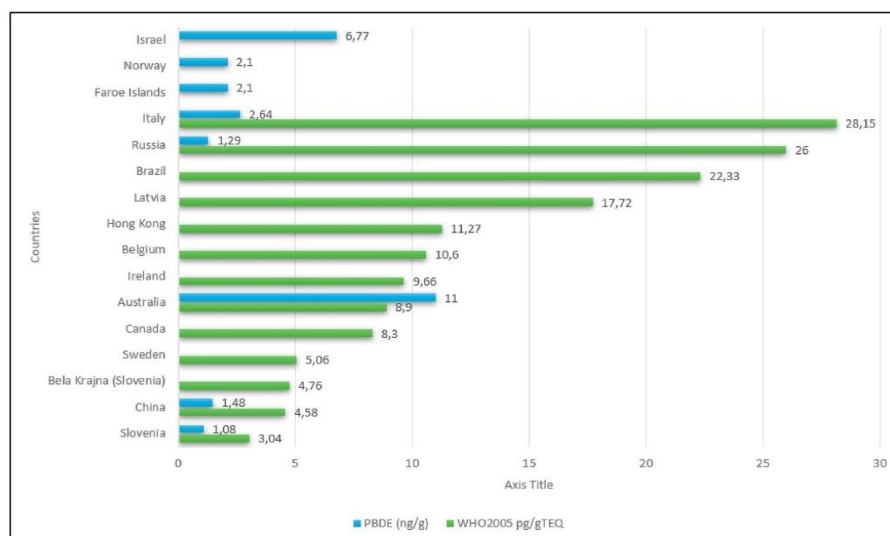

Fig. 3. Presentation of WHO 2005 TEQ and PBDE levels obtained from the literature presented in Table 3. If multiple studies on comparable populations were available for the same country, the average of values obtained from the literature was used.

2011). BDE 153, 100, and 47 have been observed to be dominant contributors to the PBDE burden of humans (Fromme et al., 2015), but exposure exceeded the RVs set for PBDE in PMM and men's PP (3210 pg/g [Main et al., 2007] and 2100 pg/g [EPA, 2008], respectively) in only one sample of PMM. The weak correlation of BDE 28 with other analytes might be due to its faster degradation in the environment. However, PCA analysis does not support any differences among analytes depending on degree of halogenation (Fig. 2). We therefore urge researchers to conduct more studies on differences in metabolism, enzyme activity, and/or genetic predisposition with respect to the elimination of pollutants from the body.

In individual samples, as described in detail in Section 3.3, p,p'- DDE could be measured above the LOQ in both matrices, but not above the RV of 6000 ng/g for ΣDDT (Solomon and Weiss, 2002) in MM and with 3% of samples above the RV in men's S (0.7 ng/g) (Linhardt, 2005; Wilhelm et al., 2003). The low ratio (0.1, Section 3.3) between p,p'-DDT and p,p'-DDE observed in our study indicates legacy exposure from historical applications of DDT, as described by Fång et al. (2015), whereas a ratio > 0.5 would hint at recent release of DDT into the environment. The decreasing p,p'-DDT:p,p'-DDE ratio with age indicates higher exposure of p,p'-DDE in the younger participants compared to the older ones. The RVs of 2.2, 3.3, and 2.4 ng/g in S (PCB 138, 153, 180) (Wilhelm et al., 2003), 1430 ng/g for ΣPCB in MM (Solomon and Weiss, 2002), 0.3 ng/g HCB in S (Wilhelm et al., 2003), and 10 ng/g HCB in MM (Solomon and Weiss, 2002) have not been reached in most cases. In MM samples, 12% were above the reference limit for HCB. The dominant contribution of the ndl-PCB congeners 138, 153, and 180 in human samples has been reported widely in the literature (Glynn et al., 2011; Rawn et al., 2012).

Among our study regions, the contribution of dl-PCBs to ΣPCDD/Fs + PCB TEQ varies, making the hypothesis of larger PCDD/Fs contribution highly geographically dependent, as previously reported in the literature (Bake et al., 2007; Croes et al., 2013; Harden et al., 2007; Polder et al., 2008a; Rawn et al., 2012). Concentrations of POPs reported from different countries reveal large differences in the chemical burden of these chemicals (Aylward et al., 2014; Fromme et al., 2015; Glynn et al., 2011; Rawn et al., 2012). Our results indicate much lower exposure in Slovenia by comparison, although regional differences still apply. The highest values were obtained in samples from Bela krajina and Ljubljana and are comparable with values from other countries that report low exposure. ΣPBDE exposure is equally low in Slovenia and comparable with Norway, the Faroe Islands, Russia, and China. In the absence of immediate sources, the spread of POPs in the environment is driven mainly by environmental factors such as wind, ocean currents, and atmospheric pressure cells. We propose that Slovenia's location between the Julian Alps and the Adriatic Sea provides shelter from the long-range transport of POPs via westerly winds, which is discussed in the following section.

4.2. Geographical and environmental determinants of exposure

In Slovenia, industrial regions are found in Zasavje (thermal power plant [1966–2014], cement industry [1876–2015], waste incineration), Jesenice (iron and steel industry), and Celje (steel industry and chemical companies). The Zasavje region is located in deep valleys with frequently occurring meteorological inversions. Indeed, we observed significantly elevated (TEQ) concentrations of some PCBs (PP), PBDEs (PP, MM), and PCDD/Fs (PP) in this region. Jesenice is home to iron and steel manufacturing companies located in a narrow valley, and it is polluted mainly with sulphur oxides and solid particles (Smrekar et al., 2020). We did not observe higher levels of POPs in these regions, though, which could be explained by the type of industry and the application of modern filter systems. It should be emphasized that POP emissions in Slovenia have been decreasing since 1990 (Section 3.12). In Celje, we observed higher concentrations of PCBs and p,p'-DDE.

It is commonly assumed that urban areas have higher air concentrations of POPs due to the presence of older buildings in immediate proximity to one another and meteorological conditions that limit air exchange. Studies investigating POP concentrations in aerosol samples have confirmed a high burden of these pollutants associated with fine particulate matter because of its higher organic matter content (Odabasi et al., 2015). The urban regions included in this study are Ljubljana, Maribor, and Koper, as well as Celje and Jesenice which are located in industrial areas. Indeed, we observed significantly higher

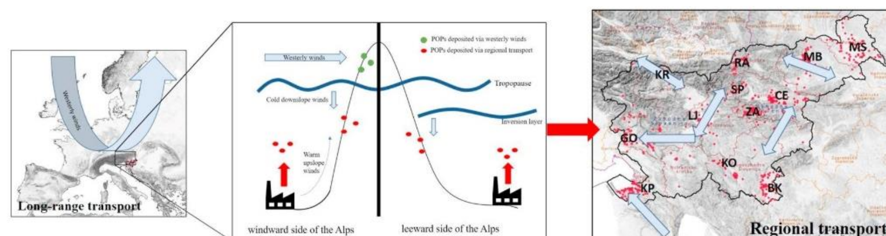

Fig. 4. Sources of POPs in the Alps. Figure adapted from Hageman et al. (2015). The map displaying regional transport contains the averaged wind directions between 2008 and 2014 according to the Slovenian Environment Agency (Republika Slovenije, 2008, 2009, 2010, 2011, 2012, 2013, 2014).

concentrations of some POPs (PCBs, p,p'-DDE) in Ljubljana. Participants from Maribor seemed less exposed (Section 3.11), which could be explained by the presence of fresh air channels, less dense construction, and multiple green areas as well as by the absence of any active emitters. Other urban locations did not reveal any significant trends.

The regions Kočevje and Cerknica, Pomurje, and Savinjsko-Posavska were chosen as unpolluted rural areas. Interestingly, PMM samples from Kočevje and Cerknica contained significantly higher concentrations of two PBDE congeners (28, 154) compared to the other regions. Its location in the wind channel from the nearby city Novo Mesto (Fig. 4) is a possible explanation, but reports of environmental monitoring of water quality have stated that the area exhibits no chemical contamination (Ambrožič et al., 2008). Besides environmental background contamination, PBDEs are present in consumer products and indoor air, although at lower concentrations than, for instance, in North America (Frederiksen et al., 2009).

The regions Posočje and Idrija, Mežica valley, and Bela krajina were chosen as areas polluted with different contaminants. Idrija is the location of a former mercury mine. Mežica valley is traditionally a lead mining and steel manufacturing region. None of the POPs included in our study has been observed in these locations in elevated concentrations. The area Bela krajina, on the other hand, stood out since 1983, when the dimensions of PCB pollution caused by illegal waste deposition were revealed. Between 1962 and 1983, a capacitor manufacturer had been disposing an assumed total of 60 t of PCB-contaminated waste. In hollow spaces underground, elevated levels of PCBs have been identified above the groundwater level, and they are assumed to be the cause of increasing concentrations of PCBs in river water after longer precipitation events (Polič et al., 2000). All PCB congeners (except PCB 77) were identified at significantly higher concentrations (TEQ) in PMM as was BDE 153. The contribution of PCBs to ΣTEQ is higher than in most other study regions. Interestingly, the addition of homemade alcohol as a confounder eliminates the significance of PCBs in MM samples, but we cannot confirm this for other matrices. Environmental data (soil and water) support elevated PCB levels (Hrvatin et al., 2020; Zupan et al., 2008). This confirms the hypothesis that – on a national level – the legacy of POPs contamination is still visible in Slovenia.

Geographical differences in POP burden do not follow a comparable trend in milk and blood samples. We attribute these results to two main factors. Firstly, maternal milk is an excretion matrix that is known to have higher fat and POP contents than blood. Secondly, POPs in PMM and MM were determined per gram fat while concentrations in blood samples are unadjusted for lipid content.

As mentioned in Section 4.1, the concentrations observed in this study are notably low. In women, the temporary physiological state is known to enhance metabolism of drugs and pollutants (Koh et al., 2014) and is, thus, a potential explanation. However, this study lacks the data to conclusively evaluate this and the low concentrations observed in men cannot be explained by our dataset. We hypothesise that the underlying cause might be Slovenia's geographical location. As a result of their low temperatures, high precipitation, and barrier effects, mountain ecosystems hinder atmospheric transport of POPs (Belis et al., 2009). The general principle is presented in Fig. 4. Studies of Central Europe have suggested that pollutants are subject to atmospheric transport via westerly winds (Weiss and Moche, 2015). Thus, as reported by the MONAPROP study, they accumulate in needles of *Picea abies* L. and in forest soils on the northern side of the Alps, but transport over the Alps is limited (Belis et al., 2009; Hageman et al., 2015; Offenthaler et al., 2009). According to this hypothesis, local sources and interchanging wind directions (Republika Slovenije, 2008, 2009, 2010, 2011, 2012, 2013, 2014) would have a larger impact on the POP burden of the country, as illustrated in Fig. 4. This finds further support in the up to seven times lower concentrations of polyaromatic hydrocarbons (PAHs) in Slovenian moss compared with neighbouring countries (Milojković, 2013) and low (<4 ng/g) PCB concentrations in sediments of the Sava River (Heath et al., 2010). Additionally, the Slovenian Environment Agency (ARSO) confirms low average annual emissions of PCDD/Fs, PCBs, and PBDEs in Slovenia (European Environment Agency, 2016). According to this report, Slovenia is emitting an average of 162 kg PCB/year and 15 kg PCDD/Fs/year (Supplementary, Figure A.1) which is below the reported international median for PCDD/Fs (36 kg PCDD/Fs/year) but above the median for PCBs (37 kg PCB/year).

4.3. *Socioeconomic characteristics and lifestyle as determinants of exposure*

We observed significant differences among regions that can be attributed only partially to environmental background levels. Naturally, socioeconomic characteristics and individual lifestyles determine the degree to which an individual comes into contact with pollutants. The observed correlations with age have been widely reported in the literature and have been found to be the consequence of long-term exposure to persistent, lipophilic compounds (Arrebola et al., 2010). Additionally, it has been reported that P450 enzyme activity decreases with age (Grandjean et al., 2008), slowing down the elimination of these compounds. The decreasing ratio between p,p'-DDT and p,p'-DDE with age additionally supports the hypothesis of decreasing exposure to the parent compound DDT, while exposure to the metabolite DDE increases. The same trend has been observed in other studies (Porta et al, 2010, 2012).

The inverse relationship between BMI and the levels of two ndl-PCBs observed in this study has been observed in other studies as well (Ingelido et al., 2017), but the results throughout the literature are highly inconsistent (Arrebola et al., 2010; Tsukino et al., 2006). It has been proposed that a larger lipid compartment size could cause a dilution effect, leading to participants with a higher BMI seemingly having lower lipid-based concentrations (Ingelido et al., 2017). Other studies suggest, longer half-lives in the human body of higher chlorinated PCBs compared to lesser chlorinated congeners (Bjerregaard-Olesen et al., 2017). Henríquez-Hernández et al. (2021) point out that elevated concentrations of PCB 153 and 180 could be expected in the adipose tissue rather than in blood and milk.

We observed an inverse relationship between smoking and contaminant concentrations that has previously been reported resulting in higher POP concentrations in non-smokers (Flesch-Janys, 1996; Ingelido et al., 2017; Miyashita et al., 2015). Smoking was found to induce P450 enzymes, which are known to be responsible for compound degradation (Flesch-Janys, 1996; Miyashita et al., 2015). Especially, CYP1B1, CYP1A1, CYP1A2, CYP2B6, CYP2C9, CYP2C19, and CYP3A4 that have been reported to metabolize chlorinated pollutants (Abass et al., 2012; Docea et al., 2017), are known to be induced by smoking, leading to faster elimination of POPs from the body (Klomp et al., 2020). Oliveira et al. (2017) advice in their extensive review to personalize drug treatment of smokers, due to pharmacokinetic interactions that influence treatment efficiency. However, associations with smoking are not consisting throughout the literature as studies report positive associations or no associations as well (Arrebola et al., 2010; Deutch et al, 2003, 2007). Alcohol consumption, on the other hand, seemed to be positively correlated with POP concentrations as other studies have shown (Miyashita et al., 2015). A plausible cause is the potential effect of alcohol intake on hepatic drug-metabolising enzymes that would decrease the elimination rate of POPs within the body (Miyashita et al., 2015). It should be noted that we observed an additional positive association between POP concentrations and the source of alcohol, namely domestic, as well as a significant correlation between alcohol-intake frequency and homemade alcohol, suggesting that the source plays a more important role than consumption frequency itself. The contribution of domestic alcohol to total alcohol consumption is the highest in Bela krajina (50%), which may contribute to the higher POP burden in this region.

It has repeatedly been reported that meat, fish, and seafood are sources of POP exposure (Bjerregaard-Olesen et al., 2017). Miklavčič et al. (2011) measured PCB levels in fresh and canned fish available in Slovenia and found levels ranging from $<$ LOD to 0.039 μg/g dw. Compared to the safety level for fish set by the U.S. Food and Drug Administration (2 μg/g ww = ~10 μg/g dw), these values are very low, which could add to the explanation of generally low levels in the Slovenian population. The reason we did not observe any significant difference between vegetarians and nonvegetarians is most likely the difference in sample size (3% vegetarians). Observed associations with egg intake might be the result of the chicken's feeding behaviour (DiGangi and Petrlík, 2005).

Products present in buildings can be sources of industrial POPs (Flores-Ramírez et al., 2017). Our findings point to old building materials, roads/traffic, and industrial production as potential sources of exposure. Furthermore, we did observe an association between POP levels and private water supplies. Because water from private supplies generally lacks both the treatment applied to tap or bottled water and the monitoring of potential contamination, it represents a potential source of POPs.

5. Study limitations and lessons learned

This is the first national human biomonitoring study in Slovenia to determine regional differences in population exposure. This study has overcome several challenges. The variety of sample matrices and different sample treatments (individual and pooled) provided opportunities, but also limitations in terms of making comparisons between sexes. Differences in sample matrix characteristics between milk and blood samples might be the underlying driver of differences in concentration, detection rates, and associations. Such characteristics include milk as a pathway of excretion and a higher fat content compared to human blood. Furthermore, the pooling method needs optimization to achieve an equal number of samples per pool for each region. Furthermore, the obtained results are not completely representative for Slovenia. Lactating women represent merely a subpopulation especially vulnerable to harmful chemicals, whereas the included men represent the general population. As such, all drawn conclusions are in need of verification via follow-up studies.

6. Conclusions

Despite strict regulations, the general population is still exposed to a wide range of POPs. In this study, we detected almost all analytes in PMM samples, whereas concentrations were mostly below the LOQ in men's PP samples. In individual samples of MM and men's S, only certain PCBs, DDT derivatives, and HCB could be detected. We observed geographical differences in POP distribution among the 12 regions that reveal histories of pollution. Even though levels in the PCB-polluted region of Bela krajina were higher than the national average, exposure was low on an international level. Using individual samples, we were able to confirm some known sources (diet, private water supply, proximity to roads, old building materials, etc.) of POPs even at low levels of exposure. We conclude that the low POP concentrations in the population are a result of decreasing local emissions of POPs and of the Alpine barrier effect, which hinders long-range transport of POPs to Slovenia. Further, the study represents the POP burden in Slovenia between 2008 and 2014, which will be useful for future biomonitoring studies. For future work, in addition to human samples, environmental samples of air, water, soil, and sediment should be included to estimate the effect of Slovenia's geography on POP distribution in the environment.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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3.3 Manuscript 3: Contaminants of Emerging Concern in Urine: A Systematic Review of Analytical Methods for Determining Diisocyanates, Benzotriazoles, Benzothiazoles, UV Filters, Isothiazolinones, Musks, and Non-Phthalate Plasticizers

In preparation: Runkel AA. & Tkalec Ž., Kosjek T., Horvat M., Heath E., (2022)

This manuscript has been prepared as a shared work project together with Žiga Tkalec to evaluate and summarize the existing analytical methods for the determination of CECs. Therefore, the candidate and Žiga Tkalec share the first authorship in this manuscript. All the tasks included (database search, compound selection, literature search, extraction of information, and writing of the manuscript) were split equally among the candidate and Žiga Tkalec.

CECs are contaminants that are to date not included in any routine HBM schemes, such as NHANES, GerES, or HBM4EU, but can be expected to be included in the near future due to increasing amounts of toxicity data and associations with adverse health outcomes (Yadav et al., 2021). Due to the availability of large sample volumes and unproblematic sample collection, compounds were selected that can be analyzed in urine, which is a suitable matrix for extensive HBM campaigns (Esteban & Castaño, 2009). After reviewing the literature for CECs and comparing the obtained list with the lists of priority substances from established HBM frameworks (NHANES, GerES, HBM4EU), 7 compound groups have been selected for review; isocyanates, benzotriazoles, pyrrolidones, UV-filters, isothiazolinones, fragrances, and non-phthalate plasticizers.

We were able to extract a suitable selection of quantitative methods for each of the listed compound groups. The most methods were available for non-phthalate plasticizers (n=9), whereas the least methods were available for isothiazolinones (n=2). From the literature, we can summarize that liquid liquid extraction (LLE) and SPE are the most frequently applied extraction procedures for all of the selected compound groups. Following extraction, the analytes are analyzed with either GCMS or LCMS, depending on the physicochemical properties of the target group. Most of the available methods use tandem mass spectrometry (MS/MS) detection and negative ionization of the compounds.

We can conclude that all of the evaluated methods use well established sample preparation, separation, and detection procedures that do not require special laboratory set-ups. This makes them suitable for the application within wider HBM frames, where large amounts of data are generated in short time using quantitative methods. Therefore, this review presents a useful asset to future HBM studies by giving a conclusive overview for the determination of selected CECs in urine.

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1 **Contaminants of emerging concern in urine: a systematic review of analytical methods**
2 **for determining diisocyanates, benzotriazoles, benzothiazoles, UV-filters,**
3 **isothiazolinones, musks and non-phthalate plasticizers**

4

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9 KEYWORDS: human biomonitoring, exposure, biomarker, exposome, mass spectrometry

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22 **Abstract**

23 The exposure of humans to harmful chemicals has become a topic of high interest over the last
24 decades. To assess the exposure load of populations to harmful substances several human
25 biomonitoring (HBM) frameworks have emerged over the years. Those programs commonly
26 include a list of priority substances, however, those are usually compounds that have been
27 researched and come with a known hazard. Contaminants of emerging concern (CEC) are
28 pollutants not currently included in routine monitoring schemes and which, based on their
29 toxicity and potential health effects, may be candidates for future regulation.

30 We reviewed the literature on currently under-researched CEC (isocyanates, benzotriazoles,
31 pyrrolidones, ultra-violet (UV) filters, the antimicrobials methylchloroisothiazolinone and
32 methylisothiazolinone (MCI, MI), fragrances, and non-phthalate plasticizers) emphasizing the
33 need for new analytical methods for these compounds. To fill the analytical gap between
34 established HBM frameworks and CEC we reviewed sample preparation and analytical
35 methods for monitoring of selected CEC in urine as a readily available, non-invasive matrix
36 suitable for large-cohort HBM campaigns.

37 Based on the literature review it is evident that following enzymatic deconjugation, liquid-
38 liquid extraction (LLE) and solid-phase extraction (SPE) are most applied sample preparation
39 procedures and the best results were achieved using online-SPE. However, for the analysis of
40 BTRs and BTHs, LLE was suitable.

41 CEC are chemically distinct, with a variety of physicochemical properties, according to which
42 different separation techniques prior to analysis are applied. For highly volatile compounds,
43 such as polycyclic musks, gas chromatography (GC) was successfully utilized, liquid
44 chromatography (LC), especially UPLC, was the instrument of choice as it generally achieved
45 lower LOQs. The majority of studies used tandem mass spectrometry (MS/MS) detection with
46 electrospray ionization (ESI) that is sufficient for most of the analytes. However, APCI(+)-

47 MS2-sMRM detection is a preferable option for the detection of diisocyanates and the UV filter
48 MBC. MBC was also successfully analyzed using UV detection.
49
50
51 **Abbreviations**

52 1-DEHTM - 1,4-di-(2-ethylhexyl) trimellitate, 1H-BTR - 1H-benzotriazole, 1-MEHTM - 1-
53 mono-(2-ethylhexyl) trimellitate, 1OH-BTR - 1-hydroxybenzotriazole, 2-amino-BTH - 2-
54 amino benzothiazole, 2cx-1-MMHTM - 1-mono-(2-carboxymethylhexyl) trimellitate, 2cx-2-
55 MMHTM - 2-mono-(2-carboxymethylhexyl) trimellitate, 2cx-MMHTP - mono-(2-ethyl-2-
56 carboxymethylhexyl) terephthalate, 2-DEHTM - 2,4-di-(2-ethylhexyl) trimellitate, 2-HESI - 2-
57 hydroxy-N-ethylsuccinimine, 2-MEHTM - 2-mono-(2-ethylhexyl) trimellitate, 2-MeS-BTH -
58 2-methylthio benzothiazole, 2-NMSI - 2-hydroxy-N-methylsuccinime, 2-OH-BTH - 2-hydroxy
59 benzothiazole, 2-SCNMeS-BTH - 2-thiocyanomethylthio benzothiazole, 2-SH-BTH - 2-
60 mercapto benzothiazole, 3cx-MnPrA - mono-3-carboxy-n-propyl adipate,
61 3-NPA - 3-nitrophthalic acid anhydride, 4-Me-1H-BTR - 4-methyl-1H-benzotriazole, 4-
62 MEHTM - 4-mono-(2-ethylhexyl) trimellitate, 4TDA - 2,4-toluenediamine, 5,6-diMe-1H-BTr
63 - 5,6-dimethyl-1H-benzotriazole, 5-Cl-1H-BTR - 5-chloro-1H-benzotriazole, 5cx-1-MEPTM -
64 1-mono-(2-ethyl-carboxypentyl) trimellitate, 5cx-2-MEPTM - 2-mono-(2-ethyl-
65 carboxypentyl) trimellitate, 5cx-MEHTP - 1-mono-(2-ethyl-5-carboxyhexyl) terephthalate,
66 5cx-MEPA - mono-5-carboxy-2-ethylpentyl adipate,
67 5-HNEP - 5-hydroxy-N-ethyl-2-pyrrolidone, 5-HNMP - 5-hydroxy-N-methyl-2-pyrrolidone,
68 5-Me-1H-BTR - 5-methyl-1h-benzotriazole, 5OH-1-MEHTM - 1-mono-(2-ethyl-5-
69 hydroxyhexyl) trimellitate, 5OH-2-MEHTM - 2-mono-(2-ethyl-5-hydroxyhexyl) trimellitate,
70 5OH-MEHA - mono-2-ethyl-5-hydroxyhexyl adipate, 5OH-MEHTP - 1-mono-(2-ethyl-5-
71 hydroxyhexyl) terephthalate,

72 5oxo-1-MEHTM - 1-mono-(2-ethyl-5-oxohexyl) trimellitate, 5oxo-2-MEHTM - 2-mono-(2-
73 ethyl-5-oxohexyl) trimellitate, 5oxo-MEHA - mono-2-ethyl-5-oxohexyl adipate, 5oxo-MEHTP
74 - 1-mono-(2-ethyl-5-oxohexyl) terephthalate, 6TDA - 2,6-toluenediamine, 7-HC - 7-
75 hydroxycitronellal,
76 7-HCA - 7-hydroxycitronellic acid, AA - acetic acid, ACN - acetonitrile, ADBI - celestolide,
77 AHTN - tonalide, AT - acetone, ATBC - acetyltributyl citrate, ATII - traesolide, CBC - 3-(4'-
78 carboxybenzylidene) camphor, DEE - diethyl ether, DEHA - di-2-ethylhexyl adipate, DEHTP
79 - di-(2ethylhexyl) terephthalate, DMAP - 4-dimethylaminopyridine, DMSO - dimethyl
80 sulfoxide,
81 DnBA - di-n-butyl adipate, FA - formic acid, FD - fluorescence detection, FR - flow rate, HDI
82 - 1,6-hexamethylene diisocyanate, HESI - heated electrospray ionization, HHCB - galaxolide,
83 IPA - isopropanol, MA - musk ambrette, MBC - 3-(4-methylbenzylidene) camphor,
84 MCI - methylchloroisothiazolinone, MDA - methylene diphenyldianiline, MDI - methylene
85 diphenyl diisocyanate, MEHA - mono-2-ethylhexyl adipate, MEHTP - mono-(2ethylhexyl)
86 terephthalate, MI - methylisothiazolinone, MK - musk ketone, MnBPA - mono-n-butyl adipate,
87 MX - musk xylene,
88 NDA - 1,5-naphthalenediamine, NEP - N-ethyl-2-pyrrolidone, NMMA - N-methylmalonamic
89 acid, NMP - N-methyl-2-pyrrolidone, PBC - 4-(1-pyrene)butanoyl chloride, PLE - Pressurized
90 liquid extraction,
91 PP - polypropylene, RT - room temperature, SPE - solid phase extraction, TAHI -
92 trisaminohexyl isocyanurate, TBBA - tert-butyl benzoic acid, TBHA - tert-butyl hippuric acid,
93 TEHTM - tri-(2-ethylhexyl) trimellitate, TTR - tolyl benzotriazole, XTR - xylyl benzotriazole,
94

95 ## 1. Introduction

96 Human biomonitoring (HBM) aims to measure potentially harmful chemicals and their
97 metabolites in response to exposure, identifying exposure sources, helping to understand
98 chemical risk, and ensuring policy informing and efficiency of reduction strategies (Ganzleben
99 et al., 2017). It also measures internal exposure that reflects the actual chemical uptake from
100 oral, dermal and inhalation exposure pathways (Vorkamp et al., 2021) and blood and urine are
101 the most commonly investigated matrices. Blood is an optimal matrix, as is in contact with all
102 tissues in the body, allowing the partitioning of compounds into every organ. However, a
103 downside of blood is the invasiveness of sample acquisition, making it less suitable for specific
104 populations, such as children and infants. Urine is easily available and can be non-invasively
105 collected as a 24-hour composite or more commonly, a spot sample. The main disadvantage is
106 varying dilution due to individual hydration status, but adjustment methods like creatinine,
107 specific gravity, and osmolarity can circumvent this issue (Esteban and Castaño, 2009).
108 With the development of new materials, consumer, and personal care products, new chemicals
109 are emerging on the market and in the environment. Contaminants of emerging concern (CECs)
110 are pollutants not currently included in routine monitoring schemes, such as the National Health
111 and Nutrition Examination Survey (NHANES), human biomonitoring for Europe (HBM4EU),
112 and the German Environmental Survey (GerES) and which, based on their toxicity and potential
113 health effects, may be candidates for future regulation. (Yadav et al., 2021).In this review we
114 included isocyanates, benzotriazoles, benzothiazoles, pyrrolidones, ultra-violet (UV) filters,
115 the antimicrobials, (MCI, MI), fragrances, and non-phthalate plasticizers.
116

117 **2. Groups of under researched CECs**

118 **Isocyanates**, MDI, 4TDI, 6TDI, NDI, PPDI, HDI are chemicals used in the production of
119 polyurethane foams, adhesives, and in paints (Lépine et al., 2020; Sabbioni et al., 2010),
120 materials commonly used in everyday life. When entering the body, isocyanates are hydrolyzed
121 to corresponding amines, MDA, 4TDA, 6TDA, NDA, PPDA, HDA, which are also followed

122 as their biomarkers of exposure (BoE). Due to their high reactivity, they can form protein
123 adducts that, after protein degradation, are excreted in urine as conjugates, particularly with
124 glutathione (GSH) (Sabbioni et al., 2010).

125 Several acute and chronic effects have been reported connected to isocyanate exposure, such as
126 irritation of eyes, skin, mucous membranes and respiratory system, asthma and reduced lung
127 function (Lépine et al., 2020). A study on the long-term effects of one-time high-dose exposure
128 reported the occurrence of diseases such as diabetes, hypertension, cancer, reproductive
129 outcomes and respiratory/orthopedic/general morbidity in the exposed population (Ganguly et
130 al., 2018).

131 **Benzotriazoles** (BTRs) are high production volume chemicals used as corrosion inhibitors, UV
132 stabilizers for various applications such as photography, and cooling liquids. **Benzothiazoles**
133 (BTHs) are similarly used for corrosion inhibition, while also finding use as industrial biocides
134 and as vulcanization accelerators in the production of rubber (Asimakopoulos et al., 2013b;
135 Maceira et al., 2018; Naccarato et al., 2014). Multiple studies found BTRs and BTHs in many
136 environmental samples (Naccarato et al., 2014) listing them as CECs. Data on toxicological
137 effects are limited, with evidence that BTR adversely affects the liver and kidney (Naccarato et
138 al., 2014), while BTH is a suspected carcinogen (X. Li et al., 2018).

139 **Pyrrolidones**, N-methylpyrrolidone (NMP) and N-ethylpyrrolidone (NEP) are aprotic polar
140 solvents used in the petrochemical and microelectronics industries, dyes, biocides, and
141 cosmetics (Schmied-Tobies et al., 2021). Due to their volatility, the main route of exposure is
142 inhalation although ingestion and dermal absorption have also been reported (Suzuki et al.,
143 2009). Their main metabolites are 5-HNMP, 2-NMSI, 5-HNEP, 2-HESI, which are used as
144 BoE. Pyrrolidones have known effects on reproduction and are skin, eye and respiratory tract
145 irritants (1-methyl-2-pyrrolidone - Substance Information - ECHA (europa.eu)).

146 As phthalate-based plasticizers have long been under criticism, **phthalate alternatives** have
147 entered the market with a constantly increasing share (Bui et al., 2016). Although many have
148 been well-studied (e.g. diisononyl ester of cyclohexane-1,2-dicarboxylic acid, DINCH) others
149 are less present in the literature. Included in this review are phthalate alternatives that have an
150 increasing share on the market, but are not frequently monitored: tri-(2-ethylhexyl) trimellitate
151 (TEHTM), di-2-ethylhexyl adipate (DEHA), di-n-butyl adipate (DnBA), and di-(2-ethylhexyl)
152 terephthalate (DEHTP).

153 All of the above-listed phthalate alternatives fall under the high molecular weight compounds
154 (> 6 C atoms in the side chain) and undergo similar metabolic pathways in humans. The tri-
155 ester compound TEHTM can form three diester isomers during hydrolysis, followed by further
156 hydrolysis to monoesters. TEHTM, DEHA, DnBA, and DEHTP undergo biotransformation to
157 secondary metabolites containing hydroxy, oxo, or carboxy groups on the side chain.

158 Most of these are alternatives for the traditional phthalate plasticizer di-2-ethylhexyl phthalate
159 (DEHP); DnBA is an alternative for di-n-butyl phthalate (DnBP). Their toxicity and migration
160 rates are significantly lower than traditional phthalates; however, leaching into the surrounding
161 media still occurs with DnBA being restricted to low temperature applications due to high
162 migration rates. This compound is often used in high concentrations in personal care products
163 (PCPs), solvents, and cleaning products. It exhibits very low toxicity to humans with a set no-
164 observed-effect level (NOEL) of 300 mg/kg bw (Ringbeck et al., 2020), however, estimating
165 human exposure to DnBA is a challenge due to unavailability of exposure data, data on intake
166 rate estimates and data on health effects. For instance, no data exists to date on any potential
167 endocrine disruptive capacity of DnBA (Bui et al., 2016). Monitoring is therefore needed as a
168 preventive measure.

169 Furthermore, animal studies suggest adverse health outcomes in rats following exposure to high
170 doses of DEHA (Silva et al., 2013). DEHA is a high production volume adipate and human

171 exposure is continuously increasing. DEHTP is a structural isomer of DEHP that has not shown
172 any of the reported adverse health effects of DEHP with a reported NOEL of 500 mg/kg bw/d.
173 It is produced in the same quantities as DINCH in the European Union (>10 000 t/year). It has
174 been frequently detected in household dust and indoor air and humans are widely exposed to
175 this compound (Bui et al., 2016). TEHTM is a tri-ester structurally similar to DEHP, but with
176 lower toxicity in comparison. It is, however, classified as a GHS Category 2 reproductive
177 toxicant, a Category 3 specific target organ toxicant, and is of concern due to its relative
178 environmental persistency (half-life 16-60 days). As such, TEHTM has a set derived no effect
179 level (DNEL) of 1.13 mg/kg bw/d within the ECHA registration (Höllerer et al., 2018a). Due
180 to their expected increasing market share, limited exposure data, and limited toxicological data,
181 we included the listed phthalate alternatives in this review.

182 **Organic UV filters** are a group of compounds capable of filtering UV radiation due to their
183 degree of conjugation. They can be roughly classified into BP, cinnamate, crylene, camphor,
184 and salicylate derivatives. The latter consists of other organic UV-filters that include
185 compounds that do not fall into the abovementioned groups (Huang et al., 2021). The majority
186 of these groups are highly studied, however, there is currently a lack of studies on camphor
187 derivatives. We, therefore, included those in this review. 4-Methylbenzylidene camphor (MBC)
188 is currently detected at rather low concentrations as well as frequencies and most of the studies
189 focus on aquatic systems (Huang et al., 2021). MBC is highly lipophilic and accumulates in the
190 fatty tissue of biota. The metabolic pathway of MBC in humans has been studied after dermal
191 application. 3-(4′-carboxybenzylidene)camphor (CBC) and four isomers of 3-(4′-
192 carboxybenzylidene)hydroxycamphor (CBC-OH) were identified as the main metabolites of
193 MBC (León-González et al., 2013) and are followed as BoE to MBC.

194 Health concerns have been rising due to the suspected endocrine activity of this compound.
195 Human exposure can result from either direct application or ingestion of contaminated food and

196 drinks, for instance fish consumption or tap water (Li et al., 2019). In vitro studies suggest that
197 MBC can enhance the process of apoptosis and hence directly affect nerve cells (Broniowska
198 et al., 2016). Other studies report an effect of MBC on the immune system (Ao et al., 2018a)
199 and it has been suggested that MBC facilitates the migration of human breast cancer cells
200 (Alamer and Darbre, 2018). Studies investigating the toxicological profile of MCB report mild
201 endocrine disruptive effects on the thyroid gland and the potential to delay tissue growth and
202 placenta formation during early pregnancy (https://www.hbm4eu.eu/wp-
203 content/uploads/2021/02/HBM4EU_D4.9_Scoping_Documents_HBM4EU_priority_substanc
204 es_v1.0-UF-filters.pdf).

205 Exposure to **fragrances** is widespread as they find applications in personal care products,
206 household and other scented products. Fragrances can be persistent and non-persistent and the
207 main pathways of exposure are inhalation and dermal absorption. The most common health
208 endpoint of fragrances is allergic responsed, but endocrine disruptive effects are also reported
209 (Dodson et al., 2012). ADBI, ATII, HHCB, and AHTN belong to polycyclic musks, while MX
210 and MK are nitro musks. MX is classified under REACH as a chemical of deep concern and a
211 use warning has been placed on MK. Polycyclic musks have been introduced on the market to
212 replace nitro musks (Taylor et al., 2014) and are of concern due to their persistence and toxic
213 effects in organisms. Other synthetic fragrances of concern are Lysmeral with the known
214 metabolites lysmerol, lysmerylic acid, hydroxylysmerylic acid, TBHA, and TBBA (Scherer et
215 al., 2017) and 7-HC with the main metabolite 7-HCA. Known health endpoints are skin
216 irritation, and skin sensitization (Stoeckelhuber et al., 2018), whereas endocrine disruptive
217 effects are suspected (Scherer et al., 2021)

218 Frequently used antimicrobials in products are isothiazole and its derivatives. The most
219 extensively used among them are those based on isothiazolinone, many of which are restricted
220 for applications in personal care products. Methylchloroisothiazolinone and

221 methylisothiazolinone **(MCI/MI)** is often applied in combination and find application in the
222 preservation of aqueous solutions in a range of products covering personal care products and
223 industrial products. MCI/MI are broad-spectrum antimicrobials with high efficiency at trace
224 concentrations (Silva et al., 2020). Over the years, concerns over the sensitization potential and
225 allergic contact dermatitis have been rising, leading to an interest in monitoring these
226 compounds. Other adverse health outcomes such as potential cytotoxicity, dermatitis, skin
227 burns, and eye damage have been repeatedly mentioned in the literature (Castanedo-Tardana
228 and Zug, 2013; Park and Seong, 2020). MCI is considered to have the highest toxicity (Silva et
229 al., 2020). Due to the application of MCI and MI in personal care products, human exposure is
230 mainly the result of dermal applications. Both MCI and MI undergo rapid biotransformation in
231 the human body with a mean half-life of 3.6 h. The main biomarker of MCI/MI exposure is N-
232 methylmalonamic acid (NMMA), whereas acetylamino([3-(methylamino)-1-(methylthio)-3-
233 oxopropyl]thio)acetic acid (M-21) is a minor metabolite of MCI/MI with an excretion fraction
234 between 10% and 23% in humans (Schettgen et al., 2021).

235 We reviewed the literature on CEC and excluded priority substances in major HBM
236 frameworks, such as the European HBM initiative, HBM4EU, EU's 7th Framework
237 programme project HEALS (Steckling et al., 2018), German Environmental Study (GerES) and
238 the National Health and Nutrition Examination Study (NHANES) and included recently
239 reviewed CEC by Salthammer (Salthammer, 2020) and Kolossa-Gehring (Kolossa-Gehring et
240 al., 2017) emphasizing the need for new analytical methods for CEC. To fill the analytical gap
241 between established HBM frameworks and CEC we reviewed sample preparation and
242 analytical methods for monitoring of selected CEC in urine.

243

244 3. Sample pre-treatment and clean-up for determination of CEC in urine

245 3.1 Deconjugation

246 Most CECs undergo phase I and II metabolism in the human body involving esterases, lipases,
247 and enzymes of the cytochrome P450 family (phase I). The phase II metabolism occurs mainly
248 in the liver, although these enzymes are expressed to a lower degree also in other tissues such
249 as kidneys and intestine. The main phase II reactions are glucuronidation, sulfation, and
250 glutathione conjugation (James, 2021). To measure total CECs (free + conjugated), enzymatic
251 deconjugation is usually applied, using enzymes like β-glucuronidase/arylsulfatase or acid/base
252 hydrolysis (Glauser et al., 2014). As listed in Table 1, in the case of BoEs to BTRs, BTHs,
253 lysmerol, 7-HC, pyrrolidones, UV-filters, and non-phthalate plasticizers deconjugation with β-
254 glucuronidase were used (Ao et al., 2018b; Asimakopoulos et al., 2013a; Bastiaensen et al.,
255 2020; Been et al., 2019; Gries et al., 2015; Höllerer et al., 2018b; Leng and Gries, 2017; León-
256 González et al., 2013; Li et al., 2017; X. Li et al., 2018; Nehring et al., 2019; Pluym et al., 2016;
257 Ringbeck et al., 2020; Stoeckelhuber et al., 2017; Zhou et al., 2018), whereas deconjugation
258 with mineral acids, such as sulfuric and hydrochloric acid were used for deconjugation of BoEs
259 to diisocyanates (Bhandari et al., 2016; Henriks-Eckerman et al., 2015; Lépine et al., 2020,
260 2019a; Mirmohammadi et al., 2013; Robbins et al., 2018; Sun et al., 2018). In order to determine
261 BoEs of polycyclic musks, a deconjugation step was not included.
262

263 **3.2 sample preparation**

264 Sample clean-up and extraction procedures are needed to remove unwanted matrix constituents,
265 to concentrate the compound, and to reduce matrix effects and interferences. Furthermore,
266 during extraction, the sample is usually concentrated, enabling the detection of BoEs at low
267 levels and reaching concentrations significant for HBM.
268 SPE is a commonly used method for the preparation of biological samples. Compared to LLE
269 it offers higher selectivity due to different available sorbents for the retention of analytes of
270 choice with a wide range of physico-chemical properties. The availability of SPE in 96-well
271 format offers and adaptation to high-throughput sample preparation workflows, which is of

272 great importance in HBM, where large cohorts are analyzed. However, the latter requires more
273 parameter optimization than LLE. For the analysis of CECs, a variety of sorbents were used,
274 most commonly wide polarity range hydrophilic-lipophilic balance Oasis HLB, which were
275 used for the extraction of BTRs, BTHs and MBC (Asimakopoulos et al., 2013a; Liu et al.,
276 2015), followed by cation-exchange-group containing Waters MCX and Phenomenex Strata
277 XC for diisocyanates (Lépine et al., 2019b; Sun et al., 2018), mixed mode with anion-exchange
278 Waters MAX for TEHTM (Bastiaensen et al., 2020; Been et al., 2019), and polystyrene-
279 divinylbenzene copolymer Biotage Isolute ENV+ (Schindler et al., 2012) for diisocyanates.
280 Although the majority of extractions were done off-line, in some cases extraction was
281 automated using on-line systems (Frederiksen et al., 2017; Gries et al., 2015; Höllerer et al.,
282 2018b; Krause et al., 2017; Leng and Gries, 2017; Nehring et al., 2019; Pinguet et al., 2019;
283 Ringbeck et al., 2020), which limits sample handling and contamination while limiting sample
284 preparation time.
285 Along with common extraction methods, other more specific ones, such as solid-supported
286 liquid-liquid extraction (SSLE) on microporous diatomite cartridges to extract fragrances (Liu
287 et al., 2015), ultrasound-assisted emulsification microextraction (USAEME) with
288 tetrachloromethane for the extraction of polycyclic and nitro musks (Chen et al., 2018) or solid-
289 phase microextraction (SPME) for extraction of BTRs and BTHs using polyacrylate fiber
290 (Naccarato et al., 2014) were used.
291
292 **3.2.1 Fragrances**
293 All presented methods for the extraction of fragrances include an LLE approach using between
294 0.8 and 1 mL of sample volume. Lysmeral and 7-HCA require a derivatization step, whereas
295 polycyclic musks can be analyzed via GC with no further adaptations. As only one study
296 presented a method for the determination of BoEs of lysmeral (Pluym et al., 2016) and one
297 study for the determination of the 7-HC BoE 7-HCA (Stoeckelhuber et al., 2017), these methods

298 cannot be compared with other approaches. However, both studies report low LOQs (≤ 0.5
299 ng/mL). The two methods presented for the detection of polycyclic musks in urine (Chen et al.,
300 2018; Liu et al., 2015) require no derivatization step and yield comparable results. However,
301 regarding the LOQs the method presented by Liu et al., (2015) using a solid-supported liquid-
302 liquid extraction (SSLE) approach yields slightly better results compared to the ultrasound-
303 assisted emulsification microextraction (USAEME) procedure suggested by Chen et al., (2018).
304

305 **3.2.2 Benzotriazoles and benzothiazoles**

306 The available sample preparation methods for the determination of BTRs and BTHs include
307 LLE (Asimakopoulos et al., 2013a; Li et al., 2017; Zhou et al., 2018), SPE (Asimakopoulos et
308 al., 2013a; C. Li et al., 2018; Naccarato et al., 2014), and online-SPE (Gries et al., 2015).
309 Online-SPE requires the lowest sample volume (0.5 mL), whereas the presented LLE methods
310 require between 0.6 and 1 mL of sample and the SPE methods between 1.33 and 2 mL. None
311 of the methods requires additional derivatization. The SPME method by Naccarato et al., (2014)
312 yields the highest LOQs (0.4 - 4.9 ng/mL), however, it is also the only GC approach among the
313 presented method. Therefore, the high LOQ cannot with certainty be attributed to the sample
314 preparation procedure. The other procedures yield comparable results with no clear trend
315 towards LLE or SPE. The online-SPE method included only one compound and achieved an
316 LOQ comparable to the other methods. However, this method has advantages with regards to
317 time efforts and sample handling compared to more complex offline approaches.
318

319 **3.2.3 Antimicrobials**

320 Only two methods describe the determination of BoEs of MI/MCI exposure in urine using an
321 LLE (Schettgen et al., 2017) and an SPE (Schettgen et al., 2021) approach. The LLE approach
322 includes an additional derivatization step that is omitted in the SPE procedure. However, the
323 SPE method requires 0.5 mL of urine, whereas the LLE method yields good results (LOQ 0.5

324 ng/mL for NMMA) with 0.1 mL of sample. The SPE method achieves a low LOQ as well (0.2
325 ng/mL), however, the analyte in this case is M12, which makes it impossible to compare the
326 methods. We can conclude, however, that both methods achieve low LOQs, require a small
327 sample volume, and do not require complex procedures or installations in the laboratory.
328

329 **3.2.4 Diisocyanates**

330 LLE is a conventional technique, used in an array of different applications. Using pure or
331 mixtures of non-polar solvents, BoEs are extracted from urine samples relatively easily.
332 However, the drawbacks are that the process uses large quantities of solvents and is relatively
333 time consuming. Based on the here reviewed methodological approaches, LLE is the most
334 commonly applied method for the extraction of diisocyanates (Henriks-Eckerman et al., 2015;
335 Lépine et al., 2019a; Mirmohammadi et al., 2013; Robbins et al., 2018), whereas SPE is also
336 applied (Bhandari et al., 2016; Lépine et al., 2020; Sun et al., 2018). Differences in the required
337 sample volume can be observed, that lies in the microliter range for SPE methods and between
338 1 and 2 mL for LLE. Lépine et al., (2019a), however, propose and LLE method with a required
339 sample volume of 250 µL. Among the presented methods, only two present approaches that do
340 not require a derivatization step (Bhandari et al., 2016; Lépine et al., 2019a). The by far lowest
341 LOQ (0.001 ng/mL for MDA) was achieved by Sun et al., (2018) using and SPE sample
342 preparation method with derivatization. However, there is no clear difference in LOQs between
343 LLE and SPE approaches and we cannot conclude that SPE generally yields better results.
344

345 **3.2.5 Pyrrolidones**

346 In the case of pyrrolidones, the samples were either minimally pre-treated with the dilute-and-
347 shoot approach (Bhandari et al., 2019; Haufroid et al., 2014; Suzuki et al., 2009) or extracted
348 with SPE (Schindler et al., 2012). Based on the obtained LODs/LOQs, required sample volume,
349 and overall sample preparation time, the dilute-and-shoot approach seems to yield the better

350 results. Only one study (Schindler et al., 2012) included a derivatization step in their sample
351 preparation in order to adapt the compounds for GC analysis. This procedure, however, does
352 not seem to yield advantages regarding the LOQ.
353
354 **3.2.6 UV-filters**
355 The most commonly applied sample preparation method is offline SPE (Ao et al., 2018b; León-
356 González et al., 2013) followed by online extraction (Frederiksen et al., 2017; Krause et al.,
357 2017; Leng and Gries, 2017). Janjua et al. used urine lyophilization with reconstitution before
358 analysis (Janjua et al., 2008). Among those methods, online SPE requires the lowest sample
359 volume (500 µL) for analysis, whereas other methods require a minimum of 2 mL. Additionally,
360 the method presented by Leng and Gries, (2017) yields the lowest LOQ (0.15 ng/mL for CBC),
361 whereas an LOQ of 0.9 ng/mL (Frederiksen et al., 2017; Krause et al., 2017) is the second
362 lowest achieved. We, therefore, conclude that online-SPE is the most suitable sample
363 preparation method for the determination of these compounds. Only one study (Ao et al.,
364 2018b) included a derivatization step that is necessary for the instrumental analysis (GC),
365 however this does not seem to yield advantages regarding the LOQ.
366
367 **3.2.7 non-phthalate plasticizers**
368 The methods presented for the determination of BoEs of **TEHTM** exposure all include SPE
369 sample preparation techniques and no derivatization is required in any of them. The methods
370 by Höllerer et al., (2018b) and Pinguet et al., (2019) include online-SPE, whereas Bastiaensen
371 et al., (2020) and Been et al., (2019) describe and offline procedure. Only Pinguet et al., (2019)
372 use 50µL of sample, whereas the other methods require 1mL. None of the approaches requires
373 additional derivatization, though. Based on the obtained LOQs, the online-SPE based method
374 by Pinguet et al., (2019) yields the most-desirable results for the determination of mono-esters.
375 Bastiaensen et al., (2020) and Been et al., (2019) determined di-esters in their method at trace

376 concentrations in urine (LOQ 0.1 ng/mL). The online-SPE method by Höllerer et al., (2018b)
377 achieved the highest LOQs, however, they also included the largest number of analytes (n =
378 11) in their method, which is of value in HBM studies. From that we can conclude, that online-
379 SPE achieves the best results for the determination of TEHTM metabolites, however, the
380 inclusion of too many compounds can raise the LOQ and researchers have to carefully evaluate
381 the costs and benefits of each method. The presented procedure for the determination of di-
382 esters yields satisfactory results.
383 Three online-SPE methods and one offline SPE method are presented for the determination of
384 BoEs of **DEHA** exposure. None of the presented methods requires a derivatization step. The
385 offline procedure (Bastiaensen et al., 2020; Been et al., 2019) requires a much higher sample
386 volume compared to the online-SPE methods (1mL vs. 50-300µL) and it yields the highest
387 LOQ (0.15 ng/mL for MEHA and OH-MEHA). The online-SPE methods by Nehring et al.,
388 (2019) and Pinguet et al., (2019) yield very comparable results regarding LOQs, however,
389 Pinguet et al., (2019) determined only MEHA, whereas Nehring et al., (2019) included OH-
390 MEHA, oxo-MEHA, and cx-MEHA in their method. Ringbeck et al., (2020) describe a method
391 for the determination of DnBA BoEs (MnBA, 3OH-MnBA, and cx-MnPrA) using online-SPE
392 and achieved low LOQs (0.05 - 0.5 ng/mL). We conclude that all of the presented methods are
393 suitable for implementation in HBM studies, however, the method presented by Nehring et al.,
394 (2019) has clear advantages in terms of preparation procedure (online), LOQ (comparable with
395 others), and the number of analytes included (n=3). The presented method for the determination
396 of DnBA BoEs is suitable for HBM in terms of sample volume, achieved LOQs, and the number
397 of included analytes.
398 For the determination of terephthalates, namely **DEHTP** BoEs, an online-SPE based method
399 (Pinguet et al., 2019) and an offline-SPE based method (Bastiaensen et al., 2020; Been et al.,
400 2019) are included in this review. The offline method requires a much larger amount of sample
401 (1mL) compared to the online method (50µL), but none of the procedures requires

402 derivatization. There are large differences in sample preparation efforts, however, as the online
403 method requires very little sample handling. The benefits of this method are clearly visible also
404 in terms of LOQs; the online-SPE based LOQ for MEHTP is 0.018 ng/mL, whereas the offline
405 method achieved an LOQ of 0.1 ng/mL for the same analyte.

406 *Table 1: Sample preparation procedures and analytical methods for the selected CECs*

Analyte family	CEC	BoE	Sample preparation	Analysis		LOQ (ng/mL)	Ref.
				Separation	Detection		
Fragrances	Polycyclic musks: ADBI, ATII, HHCB, AHTN	ADBI, ATII, HHCB, AHTN	SSLE: 0.8 mL sample loaded on cartridge, equilibrated 10 min, extracted with 8 ml HEX. Dried under N_2 and reconstituted in 80 µL of HEX.	GC: TG-5HT column (15 m × 0.25 mm × 0.1 µm), program: 70 °C 5 min, 70-190 °C at 30 °C/min, 190 °C 3 min, 190-300 °C at 30 °C/min, 300 °C 3 min	EI-MS2-MRM: TSQ Quantum (Thermo Fischer), EI 40 eV	0.107, 0.143, 0.250, 0.017, 0.103	(Liu et al., 2015)
	Polycyclic musks and nitro musks: ADBI, AHMI, ATII, HHCB, AHTN, MX, MK	ADBI, AHMI, ATII, HHCB, AHTN, MX, MK	USAEME: 1 mL mixed with 0.1 g NaCl and 50 µL of CCl_4, ultrasonicated for 1 min at 40 °C. Then centrifuged at 7000 rpm for 3 min. Extract collected and 10 µL analysed.	GC: Instrument Varian 450 (Walnut Creek, CA, USA), column: DB-5MS (30 m × 0.25 mm × 1.0 µm), Injection: 10 µL, 80 °C for 2 min then 200 °C/min to 280 °C Program: 70 °C, 4 min, 70-190 °C, 30 °C/min, 190-196 °C, 1 °C/min, 196-280 °C, 120 °C/min, 120 °C, 2.3 min	EI-MS2-MRM: Varian 220 (Walnut Creek, CA, USA)	0.25, 0.1, 0.5, 0.1, 0.1, 0.1, 0.25	(Chen et al., 2018)

	Lysmeral	TBBA, lysmerol, lysmerylic acid, hydroxy lysmerylic acid , TBHA	LLE: 1 mL of deconjugated urine (37 °C, 16 h) acidified and extracted with DCM. Dried and derivatized with 3-NPA in WA (80 °C, 30 min).	UPLC: Waters Acquity UPLC I-Class Column: Waters Acquity BEH C18 (100 mm × 2.1 mm, 1.7 µm) Mobile phases: A (MeOH), B (5 mM ammonium acetate with 0.025 % ammonium hydroxide, pH 9.2), flow 0.35 mL/min Gradient elution: 80 % B, 0-4 min, 80-55 % B, 4-10 min, 55-35 % B, 10-11 min, 35-0 % B, 11-11.1 min, 0 % B, 11.1-13 min, 0-80 % B, 13-13.1 min, 80 % B, 13.1-15 min	ESI(-)-MS2-MRM: Waters TQ-S Triple Quadrupole	0.42, 0.1, 0.36, 0.45, 0.39	(Pluym et al., 2016)
	7-HC	7-HCA	LLE: 1 mL urine deconjugated (37 °C, 3h), acidified and extracted with DCM. Dried and derivatized with 3-NPA (80 °C, 30 min).	UPLC: Waters Acquity UPLC I-Class Column: Waters Acquity BEH C18 (100 mm × 2.1 mm, 1.7 µm) Mobile phases: A (0.1 % FA / W), B (0.1 % FA / ACN), flow 0.5 mL/min Gradient elution: 80 % A, 0-1 min, 80-20 % a, 1-5 min, 20 % A, 5-5.5 min, 20-80 % A, 5.5-7 min	ESI(-)-MS2-MRM: Waters TQ-S Triple Quadrupole	0.5	(Stoeckelhuber et al., 2017)

Benzotriazoles and benzothiazoles	1H-BTR , 1OH-BTR, TTR, XTR, 5-Cl-1H-BTR, BTH, 2-OH-BTH, 2-MeS-BTH, 2-amino-BTH, 2-SH-BTH, 2-SCNMeS-BTH	1H-BTR , 1OH-BTR, TTR, XTR, 5-Cl-1H-BTR, BTH, 2-OH-BTH, 2-MeS-BTH, 2-amino-BTH, 2-SH-BTH, 2-SCNMeS-BTH	SPE: 2 mL, deconjugation with β-glucuronidase (37 °C, 24 h), diluted, pH adjusted to 3, extracted on Oasis HLB 6 cc/200 mg with MeOH/ACN (1:1), dried and reconstituted in 200 µL MeOH/ACN (1:1) LLE: 600 µL extracted with 6 mL ACN/DCM (1:1), extract dried and reconstituted in 150 µL MeOH/ACN	HPLC: Agilent 1100 HPLC Column: Zorbax SB aq (150 × 2.1 mm, 3,5 µm), Mobile phases: A (0.1 % FA / W), B (ACN), flow 0.25 mL/min Gradient elution: 10-40 % B, 4.5 min, 40-100 % B, 11.5 min, 100 % B, 6.1 min, 10 % B, 7.1 min	ESI(+)-MS2-MRM:Applied Biosystems triple quadrupole	SPE: 0.50, 2.00, 0.20, 0.20, 0.50, 5.00, 2.50, 0.20, 0.20, 0.50 LLE: 1.70, 0.70, 0.70, 1.70, 0.70. 17.00, 3.50,	(Asimakopoulos et al., 2013a)

						1.70, 0.70, 1.70	
	1H-BTR , 1OH-BTR, TTR, XTR, 5-Cl-1H-BTR, BTH, 2-OH-BTH, 2-MeS-BTH, 2-amino-BTH, 2-SCNMeS-BTH	1H-BTR , 1OH-BTR, TTR, XTR, 5-Cl-1H-BTR, BTH, 2-OH-BTH, 2-MeS-BTH, 2-amino-BTH, 2-SCNMeS-BTH	LLE: 1 mL deconjugated with β-glucuronidase (37 °C, 24 h), extracted with 3 mL MTBE/EA (5/1), dried and reconstituted in 200 µL ACN/W (6/4)	UPLC: Dionex Ultimate 3000 UHPLC Column: Thermo Hypersil GOLD (100 × 2.1 mm, 1.9 µm) Mobile phases: A (0.01 % FA / W), B (ACN), flow 0.25 mL/min Gradient elution: 10-83 % B, 9.5 min, 83-100 %, 100 % B, 3 min, 10 % B	ESI(+)-MS2-MRM: Thermo Scientific TSQ Quantiva Triple Quadrupole	0.2, 11, 2, 7, 4, 9, 6, 11, 4, 14	(Li et al., 2017)
	1H-BTR , 1OH-BTR, TTR, XTR, 5-Cl-1H-BTR, BTH, 2-OH-	1H-BTR , 1OH-BTR, TTR, XTR, 5-Cl-1H-BTR, BTH, 2-OH-	LLE: 1 mL deconjugated with β-glucuronidase (37 °C, 24 h), extracted with 3 mL MTBE/EA (5/1), repeated	UPLC: Waters Column: Acquity BEH C18 (100 × 2.1 mm, 1.7 µm) Mobile phases: A (0.01 % FA / W), B (ACN), flow 0.25 mL/min	ESI(+)-MS2-MSM: Waters Xevo TQ-XS Triple Quadrupole	0.03, 0.12, 0.06, 0.03, 0.03,	(Zhou et al., 2018)

	BTH, 2-MeS-BTH, 2-amino-BTH, 2-SCNMeS-BTH	BTH, 2-MeS-BTH, 2-amino-BTH, 2-SCNMeS-BTH	twice, dried and reconstituted in 200 µL ACN/W (6/4)	Gradient elution: 10 % B, 0.5 min, 10 % B, 9.5 min, 10-83 % B, 2 min, 100 % B, 2.8 min, 10 % B		0.06, 0.03, 0.06, 0.03, 0.24	
	1H-BTR , 1OH-BTR, TTR, XTR, 5-Cl-1H-BTR, BTH, 2-OH-BTH, 2-MeS-BTH, 2-amino-BTH, 2-SCNMeS-BTH	1H-BTR , 1OH-BTR, TTR, XTR, 5-Cl-1H-BTR, BTH, 2-OH-BTH, 2-MeS-BTH, 2-amino-BTH, 2-SCNMeS-BTH	SPE: 2 mL deconjugated with β-glucuronidase (37 °C, 24 h) and extracted on Oasis HLB 6cc/200 mg with MeOH/ACN (1:1), dried and reconstituted in 200 µL MeOH/ACN (1:1)	UPLC: Waters Acquity Column: Waters Acquity BEH Shield RP18 (100 × 3 mm, 1.7 µm) Mobile phases: A (0.1 % FA / W), B (MeOH / ACN, 1:1), flow 0.4 mL/min Gradient elution: 10 % B, 0.5 min, 10-40 % B, 0.5-1.0 min, 40-100 % B, 1.0-2.0 min, 100 % B, 2.0-6.0 min, 100-10 %, 6.0-6.1 min, 10 % B, 6.0-7.0 min	ESI(+)-MS2-MSM: Waters Xevo TQ-S Triple Quadrupole	0.005-0.51	(X. Li et al., 2018)
	1H-BTR ,	1H-BTR ,	SPME: to 1.33 mL urine 0.5 g NaCl added, diluted, extraction	GC: Thermo Fisher TSQ Quantum	EI-MS2-MRM: Thermo Fisher Triple	4.9, 3.1, 1.8, 2.7,	(Naccarato et al., 2014)

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	5,6-MeMeBTR, 4-Me-BTR, 5-Me-BTR, 5-Cl-1H-BTR, BTH, 2-Me-BTH, 2-OH-BTH, 2-MeS-BTH, 2-amino-BTH, 2-SH-BTH	5,6-MeMeBTR, 4-Me-BTR, 5-Me-BTR, 5-Cl-1H-BTR, BTH, 2-Me-BTH, 2-OH-BTH, 2-MeS-BTH, 2-amino-BTH, 2-SH-BTH	by 85 µm polyacrylate fiber, direct immersion, 40 min, room T. Thermal desorption at 290 °C for 10 min	Column: Thermo TR-5MS (30 m × 0.25 mm i.d., 0.25 µm) Program: 65 °C, 1 min, 65-240 °C, 10 °C/min, 240-290 °C, 50 °C/min, 290 °C, 3 min	Quadrupole Quantum	4.9, 0.91, 0.80, 0.45, 0.006, 0.41, 3.11	
	SH-BTH	SH-BTH	Online SPE: 0.5 mL of urine deconjugated with β-glucuronidase (37 °C, over night) and extracted on Waters Oasis HLB column (20 mm × 2.1 mm, 25 µm)	HPLC: Waters Alliance 2695 Column: Agilent Zorbax Eclipse XDB-C8 (50 mm × 4.6 mm, 5 µm) Mobile phases: A (W), B (1 % FA / W), C (ACN), flow rate 0.2 mL/min Gradient elution: 70 % A, 10 % B, 20 % C, 0-4 min, 70-0 % A, 10 % B, 20-90 % C, 4-5 min, 0 % A, 10	ESI(+)-MS2-MRM: Waters Quattro Ultima Triple Quadrupole	1	(Gries et al., 2015)

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				% B, 90 % C, 5-8 min, 0-70 % A, 10 % B, 90-20 % C, 8-8.5 min, 70 % A, 10 % B, 20 % C, 8.5-13 min			
Antimicrobials	MI, MCI	NMMA	LLE: 100 µL of urine freeze dried and redisolved in 1 mL of ACN. Derivatized with PFBBr (K_2CO_3, 60 °C, 16h). Extracted with 2x 1 mL of HEX. Dried and reconstituted in MePh.	GC: Agilent 7890 A Column: HP-5-MS (60 m × 0.25 mm i.d., 0.25 µm) Program: 90 °C, 1 min, 90-120 °C, 30 °C/min, 120 °C, 1min, 120-240 °C, 10 °C/min, 240-310 °C, 25 °C, 310 °C, 5 min	EI-MS2-MRM: Agilent 7000 Triple Quadrupole	0.5	(Schettgen et al., 2017)
	MI, MCI	M-12	SPE: 500 µL of urine mixed with 500 µL of 100 mM ammonium formate buffer (pH 2.5). Addition of 10 µL formic acid and 10 µL of D3-M-12 (1 µg/mL) in water) as internal standard, Injection of 100 µL of this solution in the LC/MS/MS-system. Analyte enrichment and clean with Phenomenex Strata-X-column	LC system: Agilent Technologies 1200 Infinity series), column: Phenomenex C18(2), 150 4.6 mm, 3 mm, 100 °, Mobile phases: A: water pH 2.5 (adjusted with formic acid), B: MeOH, C: ACN, flow rate 0.5 mL/min, column switching technique: 90% A for 5 min for analyte enrichment, valve switching to analytical column: 75% A, 25% C at flow rate 0.3 mL/min	Sciex API 5500 LC/MS/MS, ESI+, detection mode: MRM	0.2	(Schettgen et al., 2021)

			(20 × 2 mm; 20 µm) using water (pH 2.5) and methanol (90:10, v:v), flow rate of 0.5 ml/min. Backflush on the analytical column [Phenomenex C18(2), 150 × 4.6 mm, 3 µm, 100 A], separation from interferences using a gradient of water (pH 2.5) and acetonitrile.				
Diisocyanates: MDI, 4TDI, 6TDI, NDI, PPDI, HDI	MDI	MDA	LLE: 2 mL of urine, deconjugated by sulfuric acid (100 °C, 90 min), neutralized, extracted with 4 mL DEE. Dried and reconstituted in 500 µL of MePh, derivatized by HFBA (55 °C, 60 min). Dried and reconstituted in 100 µL of MePh.	GC: type not given Column: BP-5 (dimensions not reported, 1 µm) Program: 150 °C, 1 min, 150-280 °C, 10 °C/min, 280 °C, 1.5 min	NCI-SIM: type not reported	Not given	(Mirmohammadi et al., 2013)

	MDI, 4TDI, 6TDI, NDI, PPDI	MDA, 4TDA, 6TDA, NDA, PPDA	SPE: 250 µL of urine deconjugated by HCl (80 °C, 4h), neutralized, extracted on Phenomenex Strata XC 30 mg/3mL with MeOH/IPA/NH₄OH 75/20/5. Evaporated and reconstituted in 250 µL of mixed buffer	UPLC: Waters Acquity UPLC Column: ACE Excel2 SuperC18 (dimensions not reported) Mobile phases: A (100 mM Ammonium acetate / w), B (100 mM ammonium acetate / ACN, 5/95), flow: 0.5 mL/min Gradient elution: 10 % B, 0-1 min, 10-30 % B, 1-2 min, 30-90 %, 2-2.5 min, 90-15 % B, 2.5-4 min	APCI(+)-MS2-sMRM: Sciex 5500 Triple Quadrupole	0.03, 0.10, 0.10, 0.10, 0.33	(Bhandari et al., 2016)
	MDI, 4TDI, 6TDI	MDA, 4TDA, 6TDA	SPE: 250 µL of urine deconjugated by HCl (80 °C, 4h), neutralized, extracted with Phenomenex Strata XC 30 mg/3mL. Evaporated and reconstituted in 250 µL of ACN. Derivatized with PBC (DMAP, 80 °C, 20 min), neutralized with HCl.	HPLC: Agilent HP 1100 Column: Agilent SB C18 (4.6 × 150 mm, 3.5 µm), flow 1.0 ml/min Mobile phases: A (0.1 % FA in 5 % ACN/W), B (0.1 % FA in ACN) Gradient elution: 70-90 % B, 0-15 min, 90 % B, 15-20 min	FLD: Agilent HP 1000, excitation 330 nm, emission 475 nm	0.00105-0.00163	(Sun et al., 2018)
	HDI	TAHI	LLE: 1 mL of urine deconjugated with 100 µL	Nano-UPLC: Type not reported	ESI(+)-MS2-MRM: Type not reported	0.03 (LOD)	(Robbins et al., 2018)

			sulfuric acid (100 °C, 16 h), neutralized and extracted with DCM. Derivatized with acetic anhydride (55 °C, 16 h). Dried and deconstituted in 200 µL of ACN (0.1 % FA)	Column: Waters Symmetry C18 (100 mm × 100 µm, 3 µm) Mobile phases: A (0.1 % FA/W), B (0.1 % FA / ACN), flow: 0.6 µL/min Gradient elution: 95-10 % A, 17 min			
	MDI	MDA	LLE: 190 µL of urine deconjugated with sulfuric acid (100 °C, 60 min). Neutralised and extracted with MePh. Dried and reconstituted in 200 µL of water.	UPLC: Waters Acquity Column: Waters Acquity HSS T3 (50 mm × 2.1 mm, 1.8 µm) Mobile phases: A (0.1 % ammonium acetate / W), B(0.1 % ammonium acetate / MeOH), flow 0.6 mL/min Gradient elution: 5 % B, 0.5 min, 5-90 %, 3 min, 90 %, 1 min	ESI(+)-MS2-MRM: Waters Xevo Triple Quadrupole	0.535	(Lépine et al., 2019a)
	MDI	MDA	LLE: 1 mL of urine deconjugated with sulfuric acid (100 °C, 16 h), neutralised and extracted with MePh. Dried and derivatised by	GC: Agilent 6890 A Column: HP-5 (25 m × 0.32 mm × 0.17 µm) Program: 50 °C, 1 min, 50-145 °C, 10 °C/min, 145-165 °C, 5 °C/min, 165-300 °C, 25 °C/min, 300 °C, 10 min	NCI-MS-SIM: Agilent 5973N	0.1	(Henriks-Eckerman et al., 2015)

			HFBA. Dried and reconstituted in EA.				
	MDI, 4TDI, 6TDI, HDI	MDA, 4TDA, 6TDA, HDA	SPE: 190 µL of urine deconjugated with sulfuric acid (100 °C, 60 min). Neutralised and extracted with Waters MCX 30 mg / 1 ml. Evaporated and reconstituted in borate buffer. Derivatized with acetic anhydride (instantly, room T).	UPLC: Waters Acquity Column: Waters Acquity HSS T3 (50 mm × 2.1 mm, 1.8 µm) Mobile phases: A (0.1 % FA / W), B(0.1 % FA / MeOH), flow 0.6 mL/min Gradient elution: 2 % B, 1 min, 2-17 % B, 3 min, 17-40 % B, 1.5 min, 40-90 %, 0.5 min, 90 %, 1 min	ESI(+)-MS2-MRM: Waters Xevo Triple Quadrupole	1.33, 0.76, 1.24, 2.04	(Lépine et al., 2020)
Pyrrolidones: NMP, NEP	NMP	5-HNMP, 2-NMSI	SPE: 600 µL urine extracted on Biotage Isolute ENV+ 100 mg / 1ml, eluted with EA/MeOH (4:1). Evaporated and derivatized with MTBSTFA (110 °C, 60 min). Diluted with EA.	Cooled injection GC: Agilent 7890 GC Column; DB-35MS (60 m × 0.25 mm i.d., 0.25 µm) Injector temperature program: 40 °C, 0.5 min, 40-240 °C, 120 °C/min, 240-260 °C, 600 °C/min, 600 °C, 10 min Column temperature program: 50 °C, 4 min, 50-90 °C, 25 °C/min, 90 °C, 1 min, 90-190 in, 190 min, 190-280 °C /min, 280 °C, 10 min	EI-MS: Agilent 5975	20, 5, 15, 5 (LOD)	(Schindler et al., 2012)
	NEP	5-HNEP, 2-HESI					

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	NMP	5-HNMP, 2-NMSI	Dilute and shoot: 100 µL of urine diluted 10-fold with water	HPLC: Agilent Column: Agilent Zorbax Eclipse Plus C18 (100 mm × 4.6 mm, 3.5 µm) Mobile phases: A (0.1 % FA / W), B (ACN), 0.2 mL/min Gradient elution: 0-30 % B, 0-9 min, 30-100 % B, 9-9.5 min. 100 % B, 9.5-10.5 min	ESI(+)-MS2-MRM:Agilent 6460 Triple Quadrupole	0.2	(Haufroid et al., 2014), (Suzuki et al., 2009)
	NMP	5-HNMP	Dilute and shoot: Urine diluted 10-fold with 5 mM ammonium formate buffer	UPLC: Waters Acquity I-Class Column; Waters HSS-PFP (100 mm × 2.1 mm, 1.8 µm) Mobile phases: A (5 mM ammonium formate / w), B (MeOH), flow 0.4 ml/min Gradient elution: 2.5 % B, 0-0.6 min, 2.5-35 % B, 0.6-2.8 min, 35-80 %, 2.8-4.5 min, 80-2.5 % B, 4.5-5.5 min	ESI(+)-MS-MRM: Sciex Triple Quadrupole 5500	0.274 (LOD)	(Bhandari et al., 2019)
UV filters	MBC	MBC, CBC	SPE: 4 mL of deconjugated urine (β-glucuronidase, 37 °C, overnight) extracted on C18 (brand not reported) with	UPLC: Waters Acquity Column: Waters Acquity BEH C18 (50 mm × 2.1 mm, 1.7 µm)	ESI(±)-MS2-MRM: Waters Acquity TQD Triple Quadrupole MBC esi +, CBC esi-	6, 6 (LOD)	(León-González et al., 2013)

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			acetone. Dried and reconstituted in W:MeOH:ACN (2:1:1)	Mobile phases: A (0.1 % FA / w), B (0.1 % FA / ACN:MeOH 1:1), flow 0.3 mL/min Gradient elution: 40 % B, 0-1 min, 40-100 % B, 1-1.1min, 100 % B, 2 min			
	MBC	MBC	SPE: 2 mL of deconjugated urine (β-glucuronidase, 37 °C, 12 h), centrifuged, diluted to 30 mL, extracted on Oasis HLB (200 mg, 6 mL) with EA. Dried and derivatized with BSTFA-TMCS (1 %) at (room T, 30 m).	GC: Thermo Scictific TSQ Quantum XLS Column: RTX-5 (30 m × 0.25 mm, 0.25 µm) Program: 40-200 °C, 15 °C/min, 200-280 °C, 8 °C/min, 280-320°C, 10 °C/min	EI-MS2-MRM: Thermo Scientific TSQ Quantum XLS	3.454	(Ao et al., 2018b)
	MBC	MBC	On-line	HPLC: Aria TLX-1 Columns: TurboFlow on TurboFlow Cyclone P (50 × 0.5 mm) and Hypersil Gold aQ columns (50 × 4 mm) Mobile phases: A, B, C (not reported), flow 0.7 mL/min	APCI(+)-MS2-MRM: Thermo Scientific TSQ Triple Quadrupole	0.87 (LOD)	(Krause et al., 2017), (Frederiksen et al., 2017)

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				Gradient elution: 100 % A, 0-1.5 min, 41-32 % A, 59-68 % B, 2-5 min, 32-5 % A, 68-95 B, 5-6 min, 100 % C, 6-7.5 min, 0-5 % A, 0-95 % B, 7.5-8.5 min, 100 % A, 8.5-9.5 min			
	MBC	MBC	2 mL of urine lyophilized, resuspended in 1 mL of 90 % MeOH/W, centrifuged. Supernatant injected.	HPLC: Type not reported Column: Sephasil Peptide C18 (250 mm × 4.60 mm, 5 µm) Isocratic elution: 88:12 MeOH/W, flow 0.5 mL/min	UV: SPD-6 UVD	2.9 ng/mL (LOD)	(Janjua et al., 2008)
	MBC	CBC, CBC-OH	Online SPE: 0.5 mL urine deconjugated (β-glucuronidase, T and t not reported) and extracted on X Bridge C8 direct Connect HP (30 mm × 2.1 mm, 10 µm).	UPLC: Waters Acquity Column: Waters Acquity HHS C18 (150 mm × 2.1 mm, 1.8 µm) Mobile phases: Not reported Elution: Not reported	ESI (Polarity not reported)-MS2-MRM: Waters Xevo TSQ	0.15, 0.30	(Leng and Gries, 2017)
Non-phthalate plasticizers Trimellitate	TEHTM	1-MEHTM, 2-MEHTM, 4-MEHTM, 5OH-1-	Online: 1 mL of urine deconjugated (β-glucuronidase, 37 °C, 2 h), centrifuged and injected on a	HPLC: Agilent 1100 Column: Restek Core-Shell Raptor Biphenyl (100 mm × 2.1 mm, 2.7 µm)	ESI(-)-MS2-MRM: Sciex API 200 Triple Quadrupole	4.6, 1.0, 0.7, 2.6, 1.5, 1.1, 2.6, 1.6,	(Höllerer et al., 2018b)

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		MEHTM, 5OH-2-MEHTM, 5oxo-1-MEHTM, 5oxo-2-MEHTM, 5cx-1-MEPTM, 5cx-2-MEPTM, 2cx-2-MMHTM, 2cx-1-MMHTM	restricted access material phase (Merck LiChrospher RP-18 ADS, 4 mm × 25 mm, 25 µm) with W/MeOH/FA 80/20/0.1 at 0.7 mL/min. Enrichment for 4 min.	Mobile phases: A (0.1 % FA / W), B (0.1 % FA / ACN), flow rate 0.3 mL/min Gradient elution: 0-2 % b, 0-2 min, 2-35 % B, 2-3 min, 35 % B, 3-8 min, 35-55 % B, 8-9 min, 55 % B, 9-12 min, 55-80 % B, 12-16 min, 80 % B, 16-19 min, 80-2 % B, 19-20 min, 2 % B, 20-21 min		2.6, 1.7, 2.4	
	TEHTM	1-MEHTM, 2-MEHTM, 4-MEHTM	Online SPE: 50 µL of urine extracted on TurboFlow Cyclone column (50 mm × 0.5	UFLC: Shimadzu Prominence UFLC Column: Thermo Scientific Betasil phenyl/hexyl (100 mm × 3 mm, 3 µm)	ESI(-)-MS2-MRM: Sciex QTrap 5500	0.012, 0.044, 0.012	(Pinguet et al., 2019)

			mm) with 0.1 % AA / W and 0.1 % AA / ACN	Mobile phases: A (0.1 % AA/W), B (0.1 % AA / ACN), flow 0.5 mL/min Gradient elution: 25 % B, 0-5 min, 25-55 % B, 5-9 min, 55-75 % B, 9-14 min, 75-99 % B, 14-15 min, 99 % B, 15-24 min, 99-25 % B, 24-24.1 min, 25 % B, 24.1-25 min			
	TEHTM	1-DEHTM, 2-DEHTM	SPE: 1 mL of deconjugated urine (β-glucuronidase, 37 °C, 90 min) extracted on Waters Oasis MAX 30 mg/3 mL with MeOH. Evaporated and reconstituted in ACN/W 1:1.	HPLC: Agilent 1290 LC Column: Phenomenex Kinetex biphenyl RP (100 mm × 2.1 mm, 2.6 µm) Mobile phases: A (0.1 % AA / W), B (0.1 % AA/ACN), flow rate 0.2 mL/min Gradient elution: 15-45 % B, 4 min, 15-100 % B, 10 min, 100-15 % B, 20 min, 15 % B, 10 min	ESI(-)-MS-dMRM: Agilent 6460 Triple Quadrupole	0.10, 0.10	(Bastiaensen et al., 2020), (Been et al., 2019)
Adipates	DEHA	MEHA	Online SPE: 50 µL of urine extracted on TurboFlow Cyclone column (50 mm × 0.5 mm) with 0.1 % AA / W and 0.1 % AA / ACN	UFLC: Shimadzu Prominence UFLC Column: Thermo Scientific Betasil phenyl/hexyl (100 mm × 3 mm, 3 µm) Mobile phases: A (0.1 % AA/W), B (0.1 % AA / ACN), flow 0.5 mL/min	ESI(-)-MS2-MRM: Sciex QTrap 5500	0.044	(Pinguet et al., 2019)

				Gradient elution: 25 % B, 0-5 min, 25-55 % B, 5-9 min, 55-75 % B, 9-14 min, 75-99 % B, 14-15 min, 99 % B, 15-24 min, 99-25 % B, 24-24.1 min, 25 % B, 24.1-25 min			
	DEHA	MEHA, OH-MEHA	SPE: 1 mL of deconjugated urine (β-glucuronidase, 37 °C, 90 min) extracted on Waters Oasis MAX 30 mg/3 mL with MeOH. Evaporated and reconstituted in ACN/W 1:1.	HPLC: Agilent 1290 LC Column: Phenomenex Kinetex biphenyl RP (100 mm × 2.1 mm, 2.6 µm) Mobile phases: A (0.1 % AA / W), B (0.1 % AA/ACN), flow rate 0.2 mL/min Gradient elution: 15-45 % B, 4 min, 15-100 % B, 10 min, 100-15 % B, 20 min, 15 % B, 10 min	ESI(-)-MS-dMRM: Agilent 6460 Triple Quadrupole	0.15, 0.15	(Bastiaensen et al., 2020), (Been et al., 2019)
	DEHA	5OH-MEHA, 5oxo-MEHA, 5cx-MEPA	Online SPE: 300 µL of urine deconjugated (β-glucuronidase, 37 °C, 3 h) and extracted on Thermo Scientific TurboFlow Phenyl (50 mm × 0.5 mm).	HPLC: Agilent LC 1200 Column: Thermo Scientific Accucore Phenyl-X (150 × 3 mm, 2.6 µm) Mobile phases: A (0.05 % AA / W), B (0.05 % AA / ACN), flow rate 0.3 mL/min Gradient elution: 30 % B, 0-2.5 min, 30-40 % B, 2.5-4 min, 40-55 % B, 4-19 min, 55-95 % B, 19-20	ESI(-)-MS2-MRM: AB Sciex Qtrap 5500	0.05, 0.1, 0.05	(Nehring et al., 2019)

				min, 95 % B, 20-22 min, 95-30 % B, 22-23 min, 30 % B, 23-27 min			
	DnBA	MnBA, 3OH-MnBA, 3cx-MnPrA	Online SPE: 300 µL of urine deconjugated (β-glucuronidase, 37 °C, 2 h). Frozen and centrifuged. Samples extracted on Thermo Scientific TurboFlow Cyclone-P column (50 mm × 0.5 mm).	HPLC: Agilent 1260 Infinity II HPLC Column: Phenomenex Kinetex C18 (150 × 3 mm, 2.6 µm) Mobile phases: A (0.05 % AA / W), B (0.05 % AA / ACN), flow rate 0.4 mL/min Gradient elution: 12 % B, 0-3.5 min, 12-45 % B, 3.5-14 min, 45-95 % B, 14-15 min, 95 % B, 15-21 min, 95-12 %, 21-21.5 min, 12 % B, 21.5-29.5 min	ESI(-)-MS2-scheduled MRM: AB Sciex Triple Quadrupole 5500	0.1, 0.05, 0.5	(Ringbeck et al., 2020)
Terephthalates	DEHTP	MEHTP	Online SPE: 50 µL of urine extracted on TurboFlow Cyclone column (50 mm × 0.5 mm) with 0.1 % AA / W and 0.1 % AA / ACN	UFLC: Shimadzu Prominence UFLC Column: Thermo Scientific Betasil phenyl/hexyl (100 mm × 3 mm, 3 µm) Mobile phases: A (0.1 % AA/W), B (0.1 % AA / ACN), flow 0.5 mL/min Gradient elution: 25 % B, 0-5 min, 25-55 % B, 5-9 min, 55-75 % B, 9-14 min, 75-99 % B, 14-15 min,	ESI(-)-MS2-MRM: Sciex QTrap 5500	0.018,	(Pinguet et al., 2019)

				99 % B, 15-24 min, 99-25 % B, 24-24.1 min, 25 % B, 24.1-25 min			
	DEHTP	MEHTP, OH-MEHTP	SPE: 1 mL of deconjugated urine (β-glucuronidase, 37 °C, 90 min) extracted on Waters Oasis MAX 30 mg/3 mL with MeOH. Evaporated and reconstituted in ACN/W 1:1.	HPLC: Agilent 1290 LC Column: Phenomenex Kinetex biphenyl RP (100 mm × 2.1 mm, 2.6 μm) Mobile phases: A (0.1 % AA / W), B (0.1 % AA/ACN), flow rate 0.2 mL/min Gradient elution: 15-45 % B, 4 min, 15-100 % B, 10 min, 100-15 % B, 20 min, 15 % B, 10 min	ESI(-)-MS-dMRM: Agilent 6460 Triple Quadrupole	0.10, 0.10	(Bastiaensen et al., 2020), (Been et al., 2019)

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408

409 **4. Instrumental analysis of CEC in urine**

410 After exposure, BoEs of organic contaminants are present in urine at low levels, most often at
411 µg/L concentrations. Given that, sensitive analytical methods for their determination are
412 required. The most commonly applied methods here were LC or GC based, however also other
413 approaches were used as discussed in detail below.

414

415 **4.1 LC-based detection**

416 Due to the high degree of molecular functionalization and suitable polarity, reversed-phase
417 liquid chromatography (LC) was used for separation of the majority of compounds. High and
418 ultra-performance LC (HPLC and UPLC) do not require high volatility and thermal stability
419 and are suitable for the analysis of polar matrices, such as urine. It is evident from Table 1, that
420 MS2 detection in multiple reaction monitoring (MRM) was used in all cases where LC-MS was
421 used.

422

423 **4.1.1 Fragrances**

424 For the determination of lysmeral and 7-HC BoEs in urine, two UPLC-based methods using
425 electrospray ionization and MS2-MRM detection are presented (Pluym et al., 2016;
426 Stoeckelhuber et al., 2017). Separation in both cases was achieved on a 100 mm C18 analytical
427 column using binary flow. For the detection of lysmeral BoEs, MeOH was used as the organic
428 phase, whereas 5mM ammonium acetate with 0.025% ammonium hydroxide at a pH of 9.2 was
429 used as the water phase (Pluym et al., 2016). The method for the determination of 7-HCA
430 included 0.1% FA in ACN and 0.1% FA in water as the mobile phase. Additives such as FA or
431 ammonium acetate can be used to improve separation and ionization.

432

433 **4.1.2 Benzotriazoles and benzothiazoles**

434 Four out of five methods used an LC approach combined with electrospray ionization and MS2-
435 MRM detection for the determination of BTRs and BTHs in urine. Differences among the
436 methods can be found in the applied column. Asimakopoulos et al., (2013a) achieved separation
437 on a 150 mm Zorbax SB aq column, whereas Zhou et al., (2018) and Li et al., (2017) used a
438 100 mm C18 column, Gries et al., (2015) used a 50 mm C8, and Li et al., (2018) used a BEH
439 shield RP18 column. All presented methods use a binary flow of FA in water in a concentration
440 range of 0.01 - 1% FA as water phase and ACN as the organic phase. All of the presented
441 methods achieved good separation and reliable detection at low LOQs. Due to the similarity of
442 the separation and detection methods, we can conclude that the slight differences in LOQs can
443 most likely be attributed to the differences in sample preparation.
444

445 **4.1.3 Antimicrobials**

446 Schettgen et al., (2021) present in their publication an LC-based method for the determination
447 of the MI/MCI BoE M-12 using tandem mass spectrometry, electrospray ionization and MRM
448 detection. Separation is achieved on a 150 mm C18 column using a tertiary system consisting
449 of water (pH 2.5, adjusted with FA), MeOH and ACN as mobile phase. The method together
450 with SPE sample preparation achieved an LOQ of 0.2 ng/mL, which is suitable for HBM
451 studies.
452

453 **4.1.4 Diisocyanates**

454 Among the included LC methods, three are UPLC, one is HPLC, and one is nano-UPLC.
455 Differences occur based on the detector of choice. Three methods apply electrospray ionization
456 with MS2-MRM detection (Lépine et al., 2020, 2019a; Robbins et al., 2018), whereas Sun et
457 al., (2018) applied FLD detection and Bhandari et al., (2016) used APCI(+)-MS2-MRM
458 detection. Three methods describe separation on a 100mm or 150mm C18 column (Bhandari et
459 al., 2016; Robbins et al., 2018; Sun et al., 2018), whereas two studies used a 50 mm TSS T3

460 column (Lépine et al., 2020, 2019a). Among these methods, Sun et al., (2018) achieved the
461 lowest LOQ (0.001 ng/mL) for MDA. However, this method requires a derivatization step that
462 is omitted in the method by Bhandari et al., (2016), while achieving a low LOQ as well (0.03
463 ng/mL) for MDA. The other LC based methods either require a derivatization step as well,
464 while achieving higher LOQs (Lépine et al., 2020) or achieve a higher LOQ while omitting the
465 derivatization (Lépine et al., 2019a). The method presented by Robbins et al., (2018) does not
466 include MDA, but achieves an LOQ of 0.03 ng/mL for TAHI, which is suitable for HBM as
467 well.
468
469 **4.1.5 Pyrrolidones**
470 Two methods are presented for the determination of NMP BoEs using LC with electrospray
471 ionization and MS2-MRM detection. Haufroid et al., (2014)and Suzuki et al., (2009) achieved
472 compound separation on a 100 mm C18 column using 0.1% FA in water and ACN as mobile
473 phase. The second method utilized an 100mm HSS-PFP column for separation and 5mM
474 ammonium formate in water and MeOH as mobile phase (Bhandari et al., 2019). Both methods
475 achieved comparable LOQs (0.2 and 0.274 ng/mL (LOD), respectively) with the same sample
476 preparation procedure (dilute-and-shoot). Therefore, both methods are suitable for HBM.
477
478 **4.1.6 UV filters**
479 Four methods describe the detection of MBC and/or BoEs of MBC in urine using LC. While
480 most studies achieve separation on a 50 - 250 mm C18 column (Janjua et al., 2008; Leng and
481 Gries, 2017; León-González et al., 2013), Frederiksen et al., (2017) and Krause et al., (2017)
482 used a 50mm TurboFlow Cyclone P and a 50mm hypersil Gold aQ column for their purposes.
483 Most of the studies utilizes a different detection approach. The classical electrospray ionization
484 together with MS2-MRM detection is used by León-González et al., (2013) and Leng and Gries,
485 (2017), even though the latter does not specify the polarity of ionization. A UV detection

486 approach was selected in another study (Janjua et al., 2008), specifically SPD-6 UVD, whereas
487 Frederiksen et al., (2017) and Krause et al., (2017) chose atmospheric pressure chemical
488 ionization (APCI) MS2-MRM. Different mobile phase compositions are presented in the
489 included studies. A binary flow of 0.1%FA in water and 0.1% FA in ACN:MeOH (1:1) is used
490 in one study (León-González et al., 2013), whereas Frederiksen et al., (2017) and Krause et al.,
491 (2017) use a tertiary system without specifying the solvents. Isocratic elution with MeOH/water
492 (88:12) is used in one study (Janjua et al., 2008), whereas Leng and Gries, (2017) do not specify
493 their mobile phase.
494 The method by Frederiksen et al., (2017) Krause et al., (2017) achieved by far the lowest LOQ
495 for MBC (0.9 ng/mL), whereas the LOQs of the other studies range between 2.9 and 6 ng/mL
496 for the same compound. Leng and Gries, (2017) achieved low LOQs as well, however their
497 focus lay on CBC (0.15 ng/mL) and CBC-OH (0.3 ng/mL) and can, thereby, not be compared
498 with the other approaches. As the procedure presented by Leng and Gries, (2017) involves time-
499 saving online extraction with minimal sample handling and no derivatization, this method
500 seems to be the most efficient one for the determination of MBC.
501

502 **4.1.7 Non-phthalate plasticizers**

503 **TEHTM** BoEs were determined exclusively with LC-based methods using electrospray
504 ionization and MS2-MRM detection (Bastiaensen et al., 2020; Been et al., 2019; Höllerer et al.,
505 2018b; Pinguet et al., 2019). While two of the methods chose a more traditional HPLC approach
506 for the determination of BoEs of TEHTM exposure (Bastiaensen et al., 2020; Been et al., 2019;
507 Höllerer et al., 2018b), Pinguet et al., (2019) utilized and UFLC instrumental set up for analysis.
508 Additionally, differences between the methods lie in choice of the analytical column and the
509 mobile phase composition. Biphenyl columns are used in two methods (Bastiaensen et al.,
510 2020; Been et al., 2019; Höllerer et al., 2018b), whereas Pinguet et al., (2019) utilizes a phenyl
511 hexyl column with a binary mobile phase system (0.1 % AA/water, 0.1 % AA / ACN). The

512 other studies report a binary system as well, however differences lie in the composition of
513 choice. One study achieves separation with 0.1% FA in water and 0.1% FA in ACN (Höllerer
514 et al., 2018b), whereas another study uses 0.1% AA in water and 0.1% AA in ACN (Bastiaensen
515 et al., 2020; Been et al., 2019). Among the presented methods, UFLC seems to yield the best
516 results in terms of LOQ (0.01 - 0.04 ng/mL) and the required sample volume (50 µL). The
517 HPLC-based method by Bastiaensen et al., (2020) and Been et al., (2019) achieved promising
518 results as well (LOQ 0.1 ng/mL), however were different BoEs targeted in this approach.
519 For the determination of BoEs of **adipates**, four LC-based methods are available for
520 comparison, of which three targeted DEHA and one DnBA. All of the listed method utilize an
521 electrospray ionization approach with MS2-MRM detection, but one study chose UFLC as the
522 instrumental set-up (Pinguet et al., 2019), whereas the other studies chose HPLC (Bastiaensen
523 et al., 2020; Been et al., 2019; Nehring et al., 2019; Ringbeck et al., 2020). Three different types
524 of columns, namely biphenyl (Bastiaensen et al., 2020; Been et al., 2019), phenyl hexyl (Pinguet
525 et al., 2019), phenyl-X (Nehring et al., 2019), and C18 (Ringbeck et al., 2020) were used. All
526 methods use the same mobile phase consisting of a binary system of AA in water and AA in
527 ACN, but Nehring et al., (2019) and Ringbeck et al., (2020) use 0.05% AA, whereas the other
528 studies use 0.1% AA (Bastiaensen et al., 2020; Been et al., 2019; Pinguet et al., 2019). All of
529 the methods achieve comparable results for DEHA BoEs (LOQs 0.04 - 0.15) and are suitable
530 for HBM. The lowest LOQ, however, was achieved with only one analyte (MEHA) in the
531 method (Pinguet et al., 2019), which makes the method presented by Nehring et al., (2019)
532 more appealing for implementation in HBM. The method for the determination of DnBA BoEs
533 achieved low LOQs (0.05 - 0.5 ng/mL), which is suitable for HBM as well (Ringbeck et al.,
534 2020).
535 Two studies presented their method for the determination of **DEHTP** BoEs in urine, using an
536 LC approach. One method utilized UFLC with electrospray ionization and MS2-MRM
537 detection (Pinguet et al., 2019), whereas the other method chose and HPLC approach with

538 electro spray ionization and MS-dMRM detection (Bastiaensen et al., 2020; Been et al., 2019).
539 The methods differ in their choice of column as well. Pinguet et al., (2019) utilized a phenyl
540 hexyl column, whereas a biphenyl column was used in the other study (Bastiaensen et al., 2020;
541 Been et al., 2019). Both studies, however, use the same binary system as mobile phase (0.1%
542 AA in water and 0.1% AA in ACN). The method presented by Pinguet et al., (2019) achieved
543 a lower LOQ for MEHTP (0.018 ng/mL), whereas 0.1 ng/mL was achieved in the other method
544 (Bastiaensen et al., 2020; Been et al., 2019). The latter includes another BoE to DEHTP
545 exposure, though, (OH-MEHTP), which is more appealing to HBM. On the other hand, the
546 UFLC approach requires a very small amount of sample and minimal sample handling through
547 online SPE, which is an important aspect in high throughput studies as well (Pinguet et al.,
548 2019). Both studies, however, are suitable for implementation in HBM.
549
550 **4.2 GC-based detection**
551 **4.2.1 Fragrances**
552 Due to their high volatility, BoEs to polycyclic musks were separated using GC with electron
553 impact (EI) ionization and detected by MS2 acquisition in MRM mode.
554
555 **4.2.2 Benzotriazoles and benzothiazoles**
556 Only one method presented a GC-based approach for the determination of BTRs and BTHs in
557 urine. The analytes were separated using GC with electron impact (EI) ionization and detected
558 by MS2 acquisition in MRM mode. Separation was achieved on a 30m TR-5MS column. The
559 results of this method prove that it is possible to detect BTRs and BTHs using GC, however,
560 the more complex sample preparation procedure as well as the relatively high LOQs compared
561 to the LC-based methods, suggest that GC should be the method of choice only if an LC method
562 is not possible.
563

564 **4.2.3 Antimicrobials**

565 Schettgen et al., (2017) presented a GC-based method with electron impact (EI) ionization and
566 MS2-MRM detection for the determination of the MI/MCI BoE NMMA in urine. The used
567 column for separation was a HP-5-MS 60m column. The presented method achieved - together
568 with LLE sample preparation and derivatization - an LOQ of 0.5 ng/mL, which is suitable for
569 HBM studies.
570

571 **4.2.4 Diisocyanates**

572 Two methods are presented that determined MDA in urine using a GC-based approach.
573 Mirmohammadi et al., (2013) only briefly describe their method using NCI-SIM detection and
574 separation on a BP-5 column. However, their achieved LOQ is not given in the publication,
575 whereas Henriks-Eckerman et al., (2015) achieved a low LOQ of 0.1 ng/mL using a 25m HP-
576 5 column for separation and NCI-MS-SIM detection. This method is suitable for HBM,
577 however, it requires 1mL of sample and derivatization while achieving a higher LOQ than the
578 LC based methods described earlier. Therefore, GC should be the method of choice for MDA
579 detection only if an LC-based method is not possible.
580

581 **4.2.5 Pyrrolidones**

582 Schindler et al., (2012) present a method for the determination of NMP and NEP BoEs in urine
583 using cooled injection GC. Separation is achieved on a 60m DB-35MS column and detection
584 with electron impact ionization. The presented method, however, achieves relatively high
585 LOQs (5 - 20 ng/mL) that are considerably higher than those achieved with the above described
586 LC-based methods (Bhandari et al., 2019; Haufroid et al., 2014; Suzuki et al., 2009). The LOQs
587 obtained in this method might be too high for application in HBM, where the analytes are often
588 present only at trace concentrations. Therefore, LC seems to be the better approach for the
589 determination of these compounds.

590

591 **4.2.6 UV filters**

592 One study presented their method on the detection of MBC using GC-EI-MS2_MRM detection
593 (Ao et al., 2018a). The compound separation was achieved on a 30m TSQ Quantum XLS
594 Column. This method requires an additional derivatization step during sample preparation in
595 order to make the compound suitable for GC detection. The obtained LOQ (3.5 ng/mL) is higher
596 than what is possible using an LC approach (0.9 ng/mL) (Frederiksen et al., 2017; Krause et al.,
597 2017) and therefore, GC should be considered for these compounds only if and LC-based
598 approach is not feasible.

599 When separated using GC, a variety of columns were used. The majority of studies used low
600 polarity 5 % diphenyl / 95 % dimethylsiloxane based columns or equivalent for separation of
601 BoEs to fragrances (Chen et al., 2018; Liu et al., 2015), MI, MCI (Schettgen et al., 2017),
602 diisocyanates (Henriks-Eckerman et al., 2015; Mirmohammadi et al., 2013) and MBC (Ao et
603 al., 2018b). Other column chemistries, such as non-polar 5 % phenyl polysilphenylene-siloxane
604 and mid polarity 35 % phenyl/methylpolysiloxane were used for the separation of BTRs and
605 BTHs (Naccarato et al., 2014) and pyrrolidones (Schindler et al., 2012), respectively. The
606 column dimensions were usually 30 m × 0.25 mm i.d., 0.25 µm. Longer, 60 m columns were
607 used in two instances (Schettgen et al., 2017; Schindler et al., 2012).

608 The compounds were ionised using electron impact (EI) ionisation and detected by MS2
609 acquisition in MRM mode, while in two cases, negative chemical ionisation (NCI) and MS
610 acquisition in single ion monitoring (SIM) was used, both times for analysis of BoEs to
611 diisocyanates (Henriks-Eckerman et al., 2015; Mirmohammadi et al., 2013). Prior to GC
612 separation, BoEs to MI, MCI, MBC and diisocyanates were derivatized to achieve sufficient
613 volatility and separation. Silylation, forming trimethylsilyl ethers is a common derivatization
614 method, however here it was used in only one instance. Ao et al. used N, O-
615 bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1 % trimethylchlorosilane (TMCS) to

616 derivatize MBC (Ao et al., 2018b). Schettgen et al. used pentafluorobenzoyl chloride (PFBBr)
617 to derivatize BoEs to MI and MCI (Schettgen et al., 2017), while heptafluorobutyric acid
618 (HFBA) was used to derivatize BoEs to diisocyanates (Henriks-Eckerman et al., 2015;
619 Mirmohammadi et al., 2013).
620

621 **4.3 Summary of alternative detection methods**

622 As evident from Table 1, separation techniques coupled to mass spectrometry (MS) are the
623 main analytical platforms for analyzing CECs after exposure, however, other approaches are
624 suitable for some compounds as well. In one case, HPLC with fluorescence detection was used
625 for the detection of BoEs to diisocyanates (Sun et al., 2018). This required fluorescence
626 derivatization using 4-(1-pyrene)butanoyl chloride (PBC) (Sun et al., 2018). Compared to the
627 other methods using GC NCI-SIM (Mirmohammadi et al., 2013), UPLC with MRM detection
628 (Bhandari et al., 2016; Lépine et al., 2020, 2019a), and nano-UPLC with MRM detection
629 (Robbins et al., 2018) described for the detection of MDA, this approach yields a ten times
630 lower LOQ (0.001 ng/mL), while not requiring any complex sample preparation procedures
631 (offline-SPE).
632

633 **5. Occurrence of selected CECs in urine**

634 Most of the publications included in this manuscript verified their respective method by
635 implementation in HBM studies of varying scales, thereby confirming the occurrence of CECs
636 in human urine (Table 2). Most of the CECs (fragrances, BTR and BTH, antimicrobials,
637 pyrrolidones, and diisocyanates) could be detected frequently in urine, whereas the UV filter
638 MBC was detected at very low maximum concentrations, if detected at all (Ao et al., 2018b;
639 Frederiksen et al., 2017; Leng and Gries, 2017). The included non-phthalate plasticizers, on the
640 other hand, have been detected at often high frequencies and (partially) high concentrations,
641 with a maximum concentration of 1165 ng/mL of OH-MEHTP (Bastiaensen et al., 2020). We

642 can, thereby, conclude that the presumably non-exposed general population is frequently
643 exposed to most of the included CECs, which highlights the need for the inclusion of these
644 compounds in routing HBM studies.
645
646
647 *Table 2: Occurrence of CECs in urine*

Compound group	Analyte	Median (ng/ml)	Mean (ng/mL)	Range (ng/mL)	population	Reference
Fragrances	ADBI			n.d.		(Chen et al., 2018)
	AHMI			1.38 - 2.65		
	ATII			n.d.		
	HHCB			0.48 - 2.09		
	AHTN			0.14 - 0.57		
	MX			n.d.		
	MK			n.d. - 1.49		
	Lysmerol	0.8	2.25 ±6.14	0.1 - 38.7		(Pluym et al., 2016)*
	Lysmerylic acid	0.8	1.25 ± 1.59	0.02 - 9.2		
	TBBA	19.6	28.7 ± 34.3	5.3 - 188.9		
	OH-lysmerylic acid	0.06	0.76 ± 1.46	0.01 - 8.2		
	7-HCA	15.1	34.4 ± 85.2	4.9 - 557.0		(Stoeckelhuber et al., 2017)*
BTRs & BTHs	1H-BTR		1.23	<LLOQ - 5.67	males	(Asimakopoulos et al., 2013a)*
			1.63	0.85–3.16	females	
	1-OH-BTR		-	-	males	
			-	-	females	
	TTR		0.70	<LLOQ - 2.14	males	
			0.72	<LLOQ–3.37	females	
	XTR		0.39	<LLOQ - 2.39	males	
			-	3.69	females	
	5-Cl-1H-BTR		-	-	males	

		-	-	females	
BTH		3.51	<LLOQ - 16.7	males	
		5.59	<LLOQ–34.3	females	
2-OH-BTH		1.55	<LLOQ	males	
		2.40	<LLOQ–6.00	females	
2-Me-S-BTH		-	-	males	
		-	-	females	
2-amino-BTH		0.11	<LLOQ - 0.30	males	
		0.17	<LLOQ–0.25	females	
2-SCNMeS-BTH		-	-	males	
		-	-	females	
MTB (total)	<1	<1	<1 - 10.8	Non-exposed	(Gries et al., 2015)
	4527	3958	567 - 6210	exposed	
MTB (free)	70	69	<1–137	exposed	
1-OH-BTR	0.78	1.13	<LOQ-1.13		(Li et al., 2017)
1H-BTR	2.45	2.4	<LOQ-4.12		
TTR	2.38	2.46	<LOQ-4.79		
5-Cl-BTR	n.d.	n.d.	n.d.		
XTR	2.38	2.46	<LOQ-4.79		
BTH	-	-	1.74		
2-OH-BTH	-	-	0.38		
2-amino-BTH	0.26	0.28	<LOQ-0.68		
2-MeS-BTH	n.d.	n.d.	n.d.		
2-SCNMeS-BTH	n.d.	n.d.	n.d.		
1H-BTR	1.09	1.31	<LOD - 3.86	Pregnant women	(X. Li et al., 2018)
1-OH-BTR	0.21	0.25	<LOD - 1.39		
TTR	0.22	0.27	<LOD - 0.90		
5-Cl-1H-BTR	0.015	0.023	<LOD - 0.096		
BTH	1.13	1.49	0.016 - 3.91		
2-OH-BTH	<LOD	<LOD	<LOD		
1-H-BTR		0.10	? - 36		

	1-OH-BTR		0.32	? - 330		(Zhou et al., 2018)#
	XTR		0.04	? - 18		
	TTR		0.036	? - 6.1		
	BTH		1.5	? - 53		
	2-OH-BTH		0.28	? - 160		
	2-MeS-BTH		0.30	? - 15		
	2-NH_2-BTH		0.017	? - 8.3		
	2-SCNMeS-BTH		<LOD	? - 0.66		
	5-Cl-H-BTR		<LOD	? - 0.53		
Antimicrobials	NMMA	3.6		? - 7.5	Non-exposed males	(Schettgen et al., 2017)*
		2.9		? - 11.8	Non-exposed females	
	M-12	0.57*		0.20–1.79*	Non-exposed	(Schettgen et al., 2021)
	NMMA	2.7		0.9–9.6		
Diisocyanates	MDA			0.1 - 0.2*		(Henriks-Eckerman et al., 2015)
	MDA		3.30	2.0 - 4.0	Factory workers	(Mirmohammadi et al., 2013)*
	HDA			0.06 - 5.96		(Robbins et al., 2018)
	TAHI			<LOD - 1.99		
	MDA			<LOD - 15.18		(Sun et al., 2018)
	6TDA			<LOD - 8.46		
	4TDA			<LOD - 2.35		
Pyrrolidones	5-HNMP	0.39			exposed	(Haufroid et al., 2014)*
	2-HMSI	0.56				
	5-HNMP	0.09			Non-exposed	
	2-HMSI	0.23				

	5-HNMP	69.5		? - 620.0	Non-exposed	(Schindler et al., 2012)
	2-HMSI	63.5		? - 256.2		
	5-HNEP	<15.0		? - 769.3		
	2-HESI	<5.0		? - 310.8		
UV filters	MBC	2.46	0.65	<LOD - 13.93		(Ao et al., 2018b)*
	MBC	-		<LOD	Children/adolescents	(Frederiksen et al., 2017)
	CBC			<LOD	Non-exposed	(Leng and Gries, 2017)
	CBC-OH			<LOD		
Non-phthalate plasticizers	1-MEHTM	0.10	0.52	<LOQ - 13.1		(Pinguet et al., 2019)
	2-MEHTM	0.47	25.6	<LOQ - 925		
	4-MEHTM	0.04	0.19	<LOQ - 4.14		
	1-MEHTM		455			(Höllerer et al., 2018b)
	2-MEHTM		1629			
	4-MEHTM		16.0			
	5OH-1-MEHTM		308			
	2OH-2-MEHTM		256			
	5oxo-1-MEHTM		81.4			
	5oxo-2-MEHTM		60.7			
	5cx-1-MEPTM		338			
	5cx-2-MEPTM		46.6			
	2cx-2-MMHTM		<LOD			
	2cx-1-MMHTM		14.6			

1-DEHTM	<LOQ		<LOQ - 0.89		(Bastiaensen et al., 2020)
2-DEHTM	<LOQ		<LOQ - 0.91		
MEHA	0.08	0.38	<LOQ - 7.92		(Pinguet et al., 2019)
5OH-MEHA	<LOQ		<LOQ - 0.07	Pregnant women	(Nehring et al., 2019)
5oxo-MEHA	<LOQ		<LOQ - 0.13		
5cx-MEHA	<LOQ		<LOQ - 0.24		
5OH-MEHA	<LOQ		<LOQ	adults	
5oxo-MEHA	<LOQ		<LOQ		
5cx-MEHA	<LOQ		<LOQ - 0.24		
MEHA	0.02		0.02 - 3.18		(Bastiaensen et al., 2020)
OH-MEHA	0.92		0.07 - 2.94		
MnBA	<LOQ		? - 0.18		(Ringbeck et al., 2020)
3OH-MnBA	<LOQ		<LOQ		
3cx-MnBA	2.54		? - 78.3		
MEHTP	<LOQ	0.21	<LOQ - 9.90		(Pinguet et al., 2019)
MEHTP	0.02		0.02 - 50.8		(Bastiaensen et al., 2020)
OH-MEHTP	0.51		0.07 - 1165		

**creatinine adjustment*

#SG adjustment

6. Conclusions and future trends

HBM follows exposure of populations to potentially hazardous chemicals. With the emergence of new chemicals on the market, there is a need for new sample preparation procedures and analytical methods to enable rapid monitoring of CEC. In this literature review we reviewed sample preparation procedures and instrumental methods for the determination of CEC in

656 human urine. From the present review, we can, first of all, conclude that all of the CECs are
657 suitable candidates for inclusion in HBM studies, based on either their suspected toxicity,
658 expected increasing market share, and/or their occurrence in urine. While some of the included
659 CECs are classical GC compounds (polycyclic musks), others can be equally detected by GC
660 and by LC (antimicrobials). For most of the compounds, however, an LC-based approach
661 achieved the best results in terms of the detection limit, which is a crucial aspect in HBM, where
662 compounds are often present in trace concentrations. Especially for the analysis of BTRs and
663 BTHs, pyrrolidones, and UV filters, the GC-based methods achieved worse results and LC
664 should clearly be favored for these compounds. Most of the studies chose a traditional HPLC
665 approach coupled to ESI-M2-MRM detection, however, for some compounds other
666 instrumental set-ups achieved better results. For the detection of lysmeral and 7-HC
667 metabolites, UPLC achieved low detection limits. UPLC was also the most suitable set-up for
668 the detection of BTRs and BTHs. In the case of diisocyanates, pyrrolidones, and UV filters no
669 clear difference could be seen between HPLC and UPLC with both approaches being suitable.
670 UFLC, however, stood out for the analysis of non-phthalate plasticizers by achieving the lowest
671 LOQs. Among the detection method, ESI-MS2-MRM is suitable for most compounds,
672 however, good results were achieved for the analysis of diisocyanate suing APCI(+)-MS2-
673 sMRM detection and FLD detection. APCI(+)-MS2-sMRM detection is also an efficient
674 approach for the analysis of the UV filter MBC. The analysis of MBC using UV detection
675 achieved unsatisfactory results regarding the LOQ.

676 Among the LC methods, online-SPE was a sample preparation step that stood out in terms of
677 the low sample volume required, limited sample handling, and achieved LOQs. However, also
678 traditional SPE approaches and in the case of BTR and BTHs also LLE were suitable. Prior to
679 extraction, enzymatic hydrolysis was most commonly used to degrade phase II metabolites,

680 which is a sufficient approach for most of the analytes. Diisocyanates, however, were mostly
681 deconjugated using acids, which achieved satisfactory results as well.
682

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936

3.4 Manuscript 4: Exposure of Men and Lactating Women to Environmental Phenols, Phthalates, and DINCH

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As indicated in the introduction of this dissertation, exposure assessment can be led a step further, namely to the evaluation if the observed real-life exposure scenarios pose any risk for the population. This question has been addressed within this manuscript by assessing the exposure of Slovenian men and lactating women (HBM I) to environmental phenols, PHs, and DINCH (Runkel et al., 2022).

The study consisted of an assessment of exposure followed by a simplified risk assessment approach using established guidance values. The urinary concentrations of methyl paraben (MP), ethyl paraben (EtP), propyl paraben (PrP), iso-propyl paraben (iPrP), butyl paraben (BuP), iso-butyl paraben (iBuP), benzyl paraben (BzP), triclosan (TCS), bisphenol A (BPA), bisphenol S (BPS), and bisphenol F (BPF) were determined by the candidate using an SPE-based sample preparation method followed by analysis by an Agilent 7890 B GC coupled to a triple quadrupole mass analyzer Agilent 7000, where the utilized separation column was a DB5-MS UI column (30m, 0.25 mm, 0.25 µm).

Aliquotes of urine samples were sent to the VITO NV laboratory in Belgium, where individual PH metabolites, namely, MEHP, 5oxo-MEHP, 5OH-MEHP, 5cx-MEPP, MEP, MiBP, MnBP, MBzP, cx-MINP, OH-MIDP, OH-MINCH, oxo-MINCH, and OH-MINP, were measured using ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) following direct injection.

All statistical analyses were carried out in RStudio Version 3.6.2 by the candidate using spearman's rank correlation, analysis of variance (ANOVA), multiple linear regression and ordinal logistic regression analysis.

For the simplified risk assessment performed by the candidate, we followed the generally recommended procedure by comparing the obtained concentrations to existing guidance values (here: BE, ADI, NOAEL).

The highest detection frequencies (>76%) were observed for BPA, MP, EtP, and all of the PH and DINCH metabolites, whereas the other phenols could be detected in a minimum of 2% of the samples (*i*BuP). We observed significant differences in exposure between men and women. Whereas men had significantly higher levels of BPs, DINCH and PH metabolites, the concentrations of PBs were higher in women. Using questionnaire data, we were able to associate most of the obtained concentrations with the time of sampling and the residential environment (urban/rural). Physiological and demographic factors, such as age, BMI, smoking, and the level of education were associated with individual compounds. The results from the risk assessment approach indicate that no risks can be expected for the population at current exposure levels. The obtained HQ values (0.74 and 0.76 for women and men, respectively) are, however, close to the threshold of 1 above which adverse health outcomes cannot be excluded anymore. Thus, we urge to repeat the presented analysis within the frame of upcoming HBM campaigns and in consideration of a wider selection of compounds.

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Exposure of men and lactating women to environmental phenols, phthalates, and DINCH

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HIGHLIGHTS

- Endocrine disrupting chemicals were detected at high frequencies.
- Concentrations were low compared to the literature.
- The combined hazard quotient is near 1.
- Concentrations of phthalates and phenols are decreasing between 2008 and 2014.
- Concentrations of DINCH are increasing between 2008 and 2014.

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ABSTRACT

Phthalates and 1,2-Cyclohexane dicarboxylic acid diisononyl ester (DINCH), bisphenols (BPs), parabens (PBs), and triclosan (TCS) are high-production-volume chemicals of pseudo-persistence that are concerning for the environment and human health. This study aims to assess the exposure to 10 phthalates, DINCH, and environmental phenols (3 BPs, 7 PBs, and TCS) of Slovenian men (n = 548) and lactating primiparous women (n = 536). We observed urinary concentrations comparable to studies from other countries and significant differences among the sub-populations. In our study, men had significantly higher levels of phthalates, DINCH, and BPs, whereas the concentrations of PBs in urine were significantly higher in women. The most significant determinant of exposure was the area of residence and the year of sampling (2008–2014) that mirrors trends in the market. Participants from urban or industrialized sampling locations had higher levels of almost all monitored analytes compared to rural locations. In an attempt to assess the risk of the population, hazard quotient (HQ) values were calculated for individual compounds and the chemical mixture. Individual analytes do not seem to pose a risk to the studied population at current exposure levels, whereas the HQ value of the chemical mixture is near the threshold of 1 which would indicate a higher risk. We conclude that greater emphasis on the risk resulting from cumulative exposure to chemical mixtures and additional studies are needed to estimate the exposure of susceptible populations, such as children.

Abbreviations: ACN, acetonitrile; ADI, acceptable daily intake; BBP, Butylbenzyl phthalate; BE, biomonitoring equivalent; BPA, bisphenol A; BPF, bisphenol F; BPS, bisphenol S; BuP, butyl paraben; BzP, benzyl paraben; cx-MINP, monocarboxy-isononyl phthalate; DBP, Di-n-butyl phthalate; DCM, dichloromethane; DIBP, Diisobutyl phthalate; DINCH, 1,2-Cyclohexane dicarboxylic acid diisononyl ester; DiNP, di-*iso*-nonyl phthalate; EDI, estimated daily intake; EtAc, ethyl acetate; EtP, ethyl paraben; HQ, hazard quotient; iBuP, iso-butyl paraben; iPrP, iso-propyl paraben; MBzP, mono-benzyl phthalate; MCHP, mono-cyclohexyl phthalate; McxEPP, mono (2-ethyl-5- carboxypentyl) phthalate; MEHP, mono (2-ethylhexyl) phthalate; MeOH, methanol; MEP, monoethyl phthalate; MiBP, mono-isobutyl phthalate; MnBP, mono-n-butyl phthalate; MnOP, mono-n-octoyl phthalate; MnPeP, mono-n-pentyl phthalate; MOE, margin of exposure; MP, methyl paraben; MSTFA, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide; NOAEL, no adverse effect level; OH-MEHP, mono (2-ethyl-5-hydroxyhexyl) phthalate; OH-MIDP, monohydroxy isodecyl phthalate; OH-MINCH, cyclohexane-1,2-dicarboxylic acid-mono (hydroxyl – isononyl) ester; OH-MINP, monohydroxy-isononyl phthalate; oxo-MEHP, mono (2-ethyl-5-oxohexyl) phthalate; oxo-MINCH, cyclohexane-1,2-dicarboxylic acid-mono (oxo-isononyl) ester; PrP, propyl paraben; P95, 95th percentile; TCS, triclosan; TDI, tolerable daily intake.

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1. Introduction

Bisphenols (BPs), parabens (PBs), triclosan (TCS), phthalates, and DINCH are synthetic chemicals that are employed in many consumer products and widely measured in environmental samples. Exposure to these contaminants is causing increasing global concern due to their ubiquitous presence in biological and environmental matrices, suspected adverse health effects, and the inevitable contact between individuals and these chemicals (Husøy et al., 2019). Despite their short half-life (3–18h) and fluctuating concentrations in the body, these chemicals can be measured at high detection rates in urine of the general population at any given time (Koch and Calafat, 2009).

BPs, PBs, and TCS can be categorized as "environmental phenols" due to their structural similarities. BPs are employed in the manufacture of polycarbonate plastics and epoxy resins and are found in many products, such as cans, food packaging, dental fillings, and thermal paper (Koch and Calafat, 2009; Lehmler et al., 2018). Among them, bisphenol A (BPA) is produced and applied in the largest volumes but finds increasing replacement by related BPs with assumed lesser endocrine activity, such as bisphenol S (BPS) and bisphenol F (BPF). However, recent studies evaluating the endocrine disruptive effects of alternative BPs report comparable or exceeding effects in humans (Pelch et al., 2019). For humans, dietary ingestion is the most important route of exposure (Lehmler et al., 2018). In the body, BPs undergo rapid metabolism and excretion mainly as the more hydrophilic sulphates and glucuronides. PBs are utilized as preservatives in dietary products, personal care products (PCPs), and medical products. Chemicals such as methyl paraben (MP), ethyl paraben (EtP), propyl paraben (PrP), and butyl paraben (BuP) are the most commonly employed representatives of these chemicals, while chemicals such as iso-propyl paraben (iPrP), isobutyl paraben (iBuP), and benzyl paraben (BzP) are less applied (Honda et al., 2018; Moos et al., 2015). Due to the variety of applications, PBs can enter the human body via ingestion, inhalation, and dermal absorption, where they are rapidly metabolized and mainly excreted as β-D-glucuronide and sulphate within 1–7h (Frederiksen et al., 2014). Although no acute toxicity is observed, studies suggest potential endocrine-disrupting properties of these chemicals (Boberg et al., 2010; Nowak et al., 2018; Witorsch and Thomas, 2010). TCS is a synthetic broad-spectrum antibacterial agent with various applications in PCPs (Weatherly and Gosse, 2017). Accordingly, human exposure occurs mainly via dermal absorption and ingestion. Despite its lipophilic character, the bioaccumulative potential of TCS is assumed to be insignificant due to its rapid metabolism and excretion as glucuronide and sulphate conjugates via urine within 48 h after exposure (Frederiksen et al., 2014). Studies investigating the potential health effects of TCS often report conflicting results that often suggest estrogenic and androgenic activity in mammals as well as the potential to trigger antibiotic resistance (Goodman et al., 2018; McNamara and Levy, 2016; Wang and Tian, 2015).

Phthalates are high-production-volume synthetic chemicals with numerous applications. High molecular weight (HMW) phthalates are widely employed as softening agents in plastics, such as polyvinyl chloride (PVC) and food packaging materials, while low-molecular-weight (LMW) phthalates find additional applications in PCPs, solvents, pesticide formulations, paints, and lubricants (Berger et al., 2019). Due to their presence in food contact materials, the main pathway of exposure to HMW phthalates is via ingestion, while LMW phthalates often enter the body via dermal absorption and inhalation (Wormuth et al., 2006). The metabolic fate of phthalates is highly dependent on the chain length. Hydrolytic monoesters of the parent diesters are rapidly formed, and HMW phthalates can be further oxidized to secondary metabolites followed by excretion as mainly glucuronide conjugates via urine within 48 h after exposure. In 2015, four phthalates (DEHP, DBP, BBP, DIBP) have been restricted in the European Union because of their suspected endocrine activity (Koch et al., 2017). Whether other phthalates have negative health impacts in humans remains a controversial topic in the literature, but accumulating evidence suggest endocrine activity with endpoints, such as male fertility impairment, adverse child neurodevelopment, and increased levels of follicle-stimulating hormone (FSH) (Koch et al, 2007a, 2017; Koch and Calafat, 2009). As such, alternatives to traditional phthalate plasticizers have been introduced to the market, among which, DINCH had the largest market share in 2012 (Bui et al., 2016). DINCH was introduced to the market in 2002 and is structurally similar to di-*iso*--nonyl phthalate (DiNP) (Bui et al., 2016; Urbancova et al., 2019). Therefore, it finds the most applications in medical devices, food packaging materials, and toys. As are phthalates, DINCH is not bound to the matrix in which it is applied and can therefore leach into the environment. Upon entering the human body mainly via ingestion, but also inhalation, DINCH undergoes rapid metabolism and is excreted either free or in the form of conjugates with glucuronic acid (Urbancova et al., 2019; Völkel et al., 2016). To date, it is assumed that neither DINCH nor its metabolites cause adverse health effects in humans and frequent monitoring is carried out mainly in response to the increased market share and to obtain more data on its behaviour in humans (Schütze et al., 2017; Urbancova et al., 2019). Therefore, we included DINCH in this monitoring study to provide information of DINCH exposure of the Slovenian population.

Human biomonitoring (HBM) is an important tool in estimating the exposure of populations to potentially harmful chemicals. The human body is especially sensitive to the effects of endocrine-disrupting chemicals during critical developmental stages. Pregnant and lactating women are among the most susceptible groups (Koch and Calafat, 2009). Within the first national HBM project, 536 primiparous women and 548 men were recruited from 12 regions in Slovenia during two sampling campaigns (2008–2009 and 2011–2014) to assess the exposure of men and lactating women to harmful chemicals (Runkel et al., 2021; Snoj Tratnik et al., 2019a). Exposure to trace elements and persistent organic pollutants has been estimated in our previous studies and is described elsewhere (Runkel et al., 2021; Snoj Tratnik et al., 2019a). The present study exploited available samples and data for further assessment of exposure, including the above-mentioned chemicals.

2. Material and methods

2.1. Study population and design

A detailed description of the study population and design have been previously published (Snoj Tratnik et al., 2019b). In the original study, a total of 536 lactating women and 548 men between the ages of 18 and 49 have been recruited from the 12 statistical regions of Slovenia covering rural, urban, and industrial environments. Out of all participants, data on phthalate and DINCH metabolites, BPs, PBs, and TCS was obtained for 304 women and 299 men. As such, the population included in this study represents a subset of the original parent study. The sampling regions are visualized in Fig. 1. We categorized the regions based on air pollution levels as a source of phthalate exposure (Quintana--Belmares et al., 2018), which deviates from the original study design presented by Snoj Tratnik et al. (2019a). Urban areas (Ljubljana, Maribor, and Koper) and regions with particulate matter (PM_{10}) values exceeding 50 μg/m^3 for more than 35 days per year (Jesenice, Celje, and Zasavje) were categorized as "polluted", while rural areas not exceeding this threshold were categorized as "rural". Data on air pollution for 2010 were obtained from annually published reports by the Slovenian Environmental Agency (Republika Slovenije, 2010). The regions Ljubljana, Kočevje and Cerknica, and Bela Krajina were selected within the pilot study (2008–2009), whereas other regions were included at a subsequent stage during the follow-up project (2011–2014). Participants were recruited via maternity hospitals, maternity classes, and gynaecologists and could withdraw from the study at any time. Each participant provided a random spot urine sample collected in a previously distributed

100 mL polypropylene (PP) urine collection cup. The samples were further aliquoted into 2 or 5 mL PP cryovials and stored at −20 °C from the sampling period (2008–2014) until analysis (2019–2020). A general questionnaire that covers information on lifestyle, diet, residence, occupation, and health was included in the sampling campaign. Population characteristics are provided in Table S1. The National Medical Ethics Committee of the Republic of Slovenia granted approval of the pilot study (number of accordance 42/12/07) and the follow-up study (number of accordance 53/07/09). Additional ethical approval was obtained for the use of biobanked samples (number of accordance 0120–431/2018/4). All participants were asked to provide informed written consent to allow the use of biobanked samples. Positive answers were obtained from 56% of the participants in the initial study, which revealed some demographic trends. The average age of those that gave permission was slightly higher (GM 29.9) than the age of those who did not (GM 28.9). Among the participants with a university degree, 66 % gave permission, whereas among those with a lower level of education only 49 % gave permission. Among participants from the rural sampling locations, 56 % gave permission, whereas 57 % from the urban/-industrialized sampling locations gave permission.

SG was measured on a PAL-10 S refractometer, and calculation of SG-adjusted concentrations was based on the method described by (Suwazono et al., (2005)).

2.2. Laboratory analysis of bisphenols, parabens, and triclosan

A detailed description of the analytical methods has been published elsewhere (Tkalec et al., 2021). Briefly, metabolites were deconjugated over night at 37 °C using β-glucuronidase/arylsulfatase (*Helix Pomatia*, type H-2) at a concentration of 200 U/mL. Next, 1 mL of sample spiked with internal standard was extracted on an Oasis HLB (60 mg/1 mL) 96-well plate that was previously conditioned and equilibrated with 1 mL DCM, 1 mL EtAc, 1 mL MeOH, and 1 mL water. The sample was washed with 1 mL of 20 % MeOH in water and eluted with 1.8 mL of EtAc/MeOH (1:1). Eluates were evaporated under nitrogen stream until dryness and reconstituted in 100 µL ACN. Samples were derivatized with MSTFA at 50 °C for 1 h. Afterwards, samples were cleaned on two Strata Si (100 mg/1 mL) 96-well plates that were previously conditioned with 1 mL DCM and 2 mL EtAc. To achieve sample cleaning through two plates, one Strata Si plate was mounted on top of the other plate, and loaded samples were eluted with DCM. Eluates were concentrated under nitrogen stream to an approximate volume of 100 µL and transferred to inserts for analysis.

Samples were analysed on an Agilent 7890 B gas chromatograph coupled to a triple quadrupole mass analyzer Agilent 7000. Chromatographic separation was achieved on a DB5-MS UI column (30 m, 0.25 mm, 0.25 µm) using helium as a carrier gas (1 mL/min). The temperature program is described as follows: 120 °C (2 min), ramped at 16 °C/min until 300 °C, and 300 °C (12 min). The injector temperature was set to 270 °C. Injections were performed in splitless mode at an injection volume of 2 µL. Nitrogen was utilized as collision gas at 1.5 mL/min. Three transitions per molecule were employed in multiple reaction monitoring mode for compound detection. The most intensive transitions were used for quantification, and the second transition was used for qualification.

Method validation was performed using method linearity, range, accuracy error, repeatability, reproducibility, limit of detection, and limit of quantification (LOQ). Reproducibility was evaluated using the relative standard deviation of spiked urine (quality control, QC) samples (5 ng/mL). We did observe fluctuations in QC samples for iPrP that exceed the acceptable 20 % of deviation. The presented results should be regarded as indicatory. Other compounds did not exceed the deviation of 20 %. The LOQs are listed as follows BPA: 0.25 ng/mL, BPF: 0.25 ng/mL, BPS: 0.5 ng/mL, MP: 0.5 ng/mL, EtP: 0.5 ng/mL, iPrP: 0.5 ng/mL, PrP: 1 ng/mL, iBuP: 1 ng/mL, BuP: 1 ng/mL, TCS: 0.25 ng/mL, and BzP: 0.25 ng/mL.

The laboratory for organic analysis at the Jožef Stefan Institute is a successful participant of the ICI-GEQUAS rounds (inter-laboratory comparison investigation) organized within the European project HBM4EU.

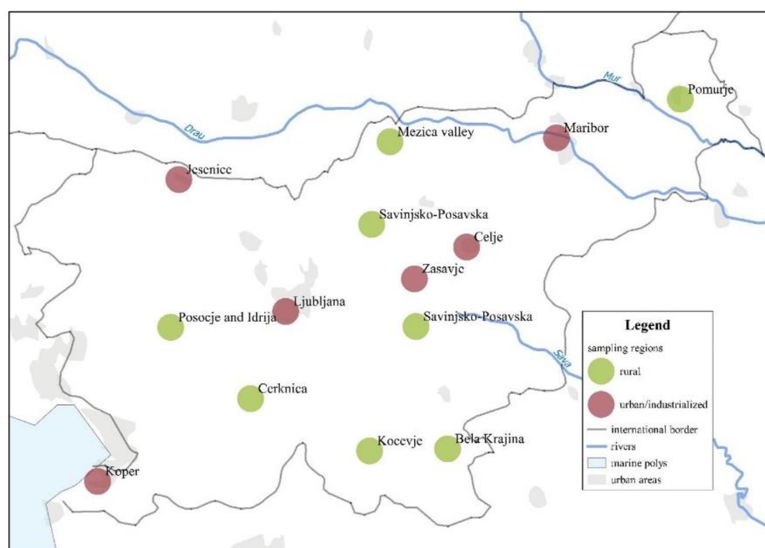

Fig. 1. Sampling regions (Natural Earth quick start for QGIS). Green circles represent rural sampling locations. Urban and/or industrialized areas are indicated by red circles. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.3. Laboratory analysis of phthalate metabolites

Urine samples were sent to the VITO NV laboratory in Belgium for analysis. A total of 13 phthalate metabolites (MEP, MBzP, MiBP, MnBP, MCHP, MnPeP, MEHP, OH-MEHP, oxo-MEHP, McxEPP, MnOP, cx-MINP, OH-MIDP, and OH-MINP) and 2 metabolites of DINCH (OH-MINCH and oxo-MINCH) were analysed. Briefly, 1 mL of urine sample were deconjugated using β-glucuronidase in ammonium acetate buffer solution and analysed after direct injection with ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Samples were analysed in batches of 20, each batch containing one procedure blank, one QC sample (water), one spiked QC sample (urine), one sample analysed in duplicate, and one independent control sample (G-EQUAS). Recovery of the QC samples in water and urine were within 20 % of deviation for all analytes. Repeatability of all analytes, as controlled by duplicate samples, was within 20 % of variation. The reported LOQs are listed as follows: oxo-MEHP, cx-MINP, McxEPP, MnOP, MnPeP, OH-MEHP, OH-MiDP, OH-MINCH, OH-MINP, and oxo-MINCH: 0.1 ng/mL; MBzP and MCHP: 0.2 ng/mL; MEP, MiBP, and MnBP: 0.5 ng/mL; and MEHP: 0.8 ng/mL.

Vito is a successful participant of the ICI-GEQUAS rounds (inter-laboratory comparison investigation) organized within the European project HBM4EU.

2.4. Statistical analyses

Statistical analysis was performed on specific gravity (SG)-adjusted concentrations to overcome the urine dilution effect. Concentrations < LOQ were replaced with the value of LOQ/2 for each analyte. All statistical analyses have been carried out in the statistical software R version 3.6.2, and only analytes with detection frequencies greater than 30% were included in the advanced statistical analysis and modelling. The correlations among the analytes were investigated in a correlation matrix using Spearman's rank correlation coefficient and Benjamini-Hochberg adjustment using ggstatsplot (Patil, 2018) after the Shapiro-Wilk test revealed a non-normal distribution of data. Questionnaire data on dietary habits were categorized into 7 categories representing increasing consumption frequency averaged for one year. In response to the uneven distribution over the categories, we re-categorized as consumers and non-consumers and individually explored associations for all analytes. The correlations between continuous variables, such as age and body mass index (BMI) and analyte concentrations were investigated using Spearman's rank correlation coefficient. Following this approach, significantly associated confounders were included in the regression modelling and Akaike information criterion (AIC) analysis was performed to optimize the model parameters. Challenges occurring in this step of the data analysis could be attributed to the unreliability (diet) and unavailability (personal care products) of questionnaire data, which was originally optimized for persistent pollutants and trace elements. An attempt to utilize linear regression modelling (LRM) resulted in unsatisfactory diagnostic plots in response to a lack of appropriate confounders. We attempted to overcome these limitations by categorizing analyte concentrations into quartiles and performed ordinal logistic regression (OLR) analysis. The utilization of quartiles in contrast to continuous variables reduces the effect of extreme values and, thus, decreases the uncertainty related to the model outcome. Therefore, as confounders were used only parameters that can either influence physiology (age, BMI, intake of alcohol, smoking, and intake of supplements) or exposure (residential environment, level of education, year of sampling, and occupational exposure). Directed Acyclic Graphs (DAGs) analysis (Textor et al., 2016) confirmed the inclusion of age, alcohol consumption, education, and smoking into the model (data not presented). However, as the AIC analysis highlighted the importance of year of sampling, smoking, intake of supplements, occupation, these variables were chosen as confounders as well. All OLRs were confirmed in LRM that showed the same trends, however under unsatisfactory model performance. Therefore, the results from LRM are not presented or discussed in this study.

The models were separately applied to men and women as metabolism differs between the sexes and can additionally be influenced by pregnancy (Koh et al., 2014; Waxman and Holloway, 2009). The dataset was split into train data (70%) and test data (30%). The train data were employed to build the models for each analyte, and model performance was evaluated using the test data set. Model parameters were optimized based on the p-values for each confounder and the overall prediction error obtained when predicting the results for the test dataset. High prediction errors can be attributed to a lack of sufficient confounders. The trends of the obtained results were confirmed with linear regression modelling, but as the errors are high, any conclusions based on these results have large uncertainties. The authors are aware of this limitation.

2.5. Exposure and risk assessment

To assess the risk of the target population to the selected compounds, we closely followed the approach presented by Sanchis et al., (2020). Risk assessment was attempted only for compounds with detection frequencies greater than 30 %. We estimated the risk of the population by calculating the hazard quotient (HQ) using reference values available in the literature, such as biomonitoring equivalents (BEs) and no-observed-adverse-effect levels (NOAEL), or by calculating the estimated daily intake (EDI) of the respective compound. An HQ > 1 indicates risk and is calculated with Equation (1) (EFSA, 2013):

$$HQ = (P95)/(BE) \tag{1}$$

To assess the hazard to mixtures, the HQ can be calculated for combined exposure if BE values are available (Equation (2)) (Porras et al., 2020):

$$HQ_{combined} = \left(\frac{P95_{compound\ 1}}{BE_{compound\ 1}}\right) + \left(\frac{P95_{compound\ 2}}{BE_{compound\ 2}}\right) + \left(\frac{P95_{compound\ 3}}{BE_{compound\ 3}}\right) + compound\ n \tag{2}$$

In the absence of BE values, the ratio between the calculated EDI and the acceptable daily intake (ADI) can be utilized to estimate the HQ using Equation (3):

$$HQ = (EDI)/(ADI) \tag{3}$$

The EDI of phenols can hereby be calculated as follows (Equation (4)):

$$EDI\left(\frac{\frac{mg}{kgbw}}{day}\right) = \left(P95\left(\frac{mg}{L}\right) \times V_{urine}\left(\frac{L}{day}\right)\right) \Big/ (F \times BW\ (kg)) \tag{4}$$

whereas the EDI calculation of phthalates should follow Equation (5):

$$EDI\left(\frac{\frac{mg}{kgbw}}{day}\right) = \left[\left(\frac{P95(\mu g/L)}{MW_m\left(\frac{g}{mol}\right)} \times \frac{MW_P\left(\frac{g}{mol}\right) \times V_{urine}\left(\frac{L}{day}\right)}{F \times BW\ (kg)}\right)\right] \Big/ 1000 \tag{5}$$

where P95 is the 95th percentile of each compound; V_{urine} is the total urine volume excreted in 24h (1.7 L/day for men and 1.6 L/day for women (Aylward et al., 2009a)); F is the urinary excretion factor of the

compound; MW_m and MW_p represent the molecular weight of the metabolite and parent compound, respectively, and BW is the mean body weight of the population in kg (women: 63.7; men: 83.4).

For DEHP, we estimated the EDI by including the sum of all measured metabolites (MEHP (m1), OH-MEHP (m2), oxo-MEHP (m3), and McxEPP (m4)) as follows (Equation (6)):

$$EDI\left(\frac{\frac{mg}{Kg_{BW}}}{day}\right)=\left[\left(\left(\frac{P95_{m1}\left(\frac{ug}{L}\right)}{MW_{m1}\left(\frac{g}{mol}\right)}\right)+\left(\frac{P95_{m2}\left(\frac{ug}{L}\right)}{MW_{m2}\left(\frac{g}{mol}\right)}\right)+(m3)+(m4)\right)\times\left(\frac{MW_{P}\left(\frac{g}{mol}\right)\times V_{urine}\left(\frac{L}{day}\right)}{\Sigma F_{m1,\ m2,\ m3,\ m4}\times BW\ (Kg)}\right)\right]\Big/1000 \quad (6)$$

Apel et al., (2020) suggest $\Sigma F = 0.471$ for all four included metabolites.

For compounds with no available BE or ADI in the literature, it is possible to estimate the risk using the margin of exposure (MOE), which indicates low risk if the value exceeds 10,000 (EFSA, 2005). The MOE can be calculated as follows (Equation (7)):

$$MOE=(NOAEL)/(EDI) \quad (7)$$

An overview over the applied parameters for different analytes is presented in Table 1. The excretion factor for BPS and BPF is assumed to be the same as that for BPA, as the metabolic pathway of all three compounds is assumed to be very similar. HQ calculation is performed on a worst-case-scenario basis, meaning that the highest values of a given range of EDI or ADI were included (applied to ΣEtP + MP and BzP) to obtain the HQ.

3. Results and discussion

3.1. Levels of phenols and metabolites of phthalates and DINCH in urine samples

We analysed a total of 11 environmental phenols, 14 phthalate metabolites, and 2 metabolites of the alternative plasticizer DINCH in urine

Table 1
Parameters for the calculation of estimated daily intake (EDI), hazard quotient (HQ), and margin of exposure (MOE). No parameters are available for BPF, OH-MIDP, and OH-MINP (italic).

Compounds	MW (g/mol)	P95 F (mg/L)	P95 M (mg/L)	F		BE (mg/L)		ADI ($mg/kg_{bw}/d$)		NOAEL ($mg/kg_{bw}/d$)	
BPA	228.3	0.011	0.017	100	Krishnan et al. (2010a)	2	Krishnan et al. (2010a)				
BPS	250.3	0.002	0.0008	100						10	Khmiri et al., (2020)
BPF	350.4	0.002	0.008	100							
MP	152.2	0.185	0.294	17.4	Moos et al., (2017)[b]			10[b]	EFSA, (2004)		
EtP	166.2	0.068	0.058	13.7	Moos et al. (2017)			10[b]	EFSA (2004)		
iPrP	180.2	0.002	0.001	10.2	Moos et al. (2017)			0.02	Moos et al., (2017)		
PrP	180.2	0.029	0.042	9.7	Moos et al. (2017)			0.1	Honda et al., (2018)		
iBuP	194.2	0.001	0.0004	6.8	Moos et al. (2017)			0.02	Moos et al., (2017)		
BuP	194.2	0.003	0.003	5.6	Moos et al. (2017)			0.02	Moos et al. (2017)		
BzP	228.2	0.0004	0.0004	100–1							
TCS	289.5	0.002	0.003	54	Apel et al., (2017)	2.6	Krishnan et al. (2010b)				
MEHP	278.3	0.009	0.016	47.1[c]	Apel et al., (2020)	1[c]	Aylward et al. (2009a)				
Oxo-MEHP	292.3	0.016	0.021	47.1[c]	Apel et al. (2020)	1[c]	Aylward et al. (2009a)				
OH-MEHP	294.3	0.024	0.035	47.1[c]	Apel et al. (2020)	1[c]	Aylward et al. (2009a)				
McxEPP	308.3	0.034	0.035	47.1[c]	Apel et al. (2020)	1[c]	Aylward et al. (2009a)				
MEP	194.2	0.227	0.947	69	Koch and Calafat, (2009)	18	Aylward et al. (2009b)				
MBzP	256.3	0.015	0.021	73	Koch and Calafat (2009)	12	Aylward et al. (2009b)				
MnBP	222.2	0.043	0.071	69	Koch and Calafat (2009)	0.2	Aylward et al. (2009b)				
MiBP	222.2	0.088	0.124	69	Koch and Calafat (2009)	0.19	Porras et al., (2020)				
cx-MINP	322.6	0.008	0.013	9.1	Koch and Calafat (2009)	0.03	Porras et al. (2020)				
OH-MIDP	322.4	0.002	0.004	100–1							
OH-MINP	308.4			18.4	Koch and Calafat, (2009)						
OH-MINCH	314.4	0.013	0.022	10.73	Kasper-Sonnenberg et al., (2019)			1[a]	Kasper-Sonnenberg et al., (2019)		
Oxo-MINCH	312	0.006	0.012	2.03	Kasper-Sonnenberg et al. (2019)			1[a]	Kasper-Sonnenberg et al. (2019)		

[a] TDI.
[b] Sum of EtP and MP.
[c] Sum of DEHP metabolites

Table 2
Descriptive statistics of specific gravity corrected concentrations, sample size, and LOQs. Lines in italic represent analytes with detection rates <30 %, which were excluded from the statistical analysis.

		<LOQ %	GM	Median	Range	LOQ (ng/mL)
BPF	*women*	*73*	*<LOQ*	*<LOQ*	*<LOQ-47.7*	0.25
	men	59	0.37	0.25	0.08–129	
BPA	women	8	2.41	2.52	0.12–60.6	0.25
	men	2	3.81	3.70	0.17–59.8	
BPS	*women*	*90*	*<LOQ*	*<LOQ*	*<LOQ-30.7*	0.5
	men	*86*	*<LOQ*	*<LOQ*	*<LOQ-1.93*	
MP	women	2	20.7	21.5	0.18–1523	0.5
	men	6	12.5	13.6	0.19–1086	
EtP	women	16	5.86	6.71	0.13–145	0.5
	men	20	4.32	5.26	0.15–564	
iPrP	*women*	*79*	*<LOQ*	*<LOQ*	*<LOQ-76.1*	0.5
	men	*84*	*<LOQ*	*<LOQ*	*<LOQ-30.5*	
PrP	women	24	1.73	1.75	0.07–327	0.25
	men	40	0.97	0.58	0.07–286	
iBuP	*women*	*95*	*<LOQ*	*<LOQ*	*<LOQ-5.76*	1
	men	*98*	*<LOQ*	*<LOQ*	*<LOQ-2.47*	
BuP	women	46	0.41	0.37	0.06–9.21	0.25
	men	59	0.29	0.22	0.08–43.2	
BzP	women	49	0.17	0.17	0.05–0.76	0.25
	men	*71*	*<LOQ*	*<LOQ*	*<LOQ-1.44*	
TCS	*women*	*70*	*<LOQ*	*<LOQ*	*<LOQ-15.9*	0.1
	men	*73*	*<LOQ*	*<LOQ*	*<LOQ-43.7*	
MEHP	women	25	1.98	2.05	0.26–33.5	0.8
	men	5	4.29	4.34	0.49–85.3	
Oxo-MEHP	women	1	3.89	3.80	0.20–35.3	0.1
	men	1	5.23	5.48	0.08–71.3	
OH-MEHP	women	0	5.82	5.53	0.20–51.5	0.1
	men	0	9.11	8.72	0.82–115	
McxEPP	women	1	7.75	7.07	0.12–97.6	0.1
	men	0	9.34	9.43	1.42–112	
MEP	women	0	29.9	27.9	1.02–1062	0.5
	men	0	65.0	55.7	1.21–5033	
MBzP	women	12	2.23	2.67	0.09–66.4	0.2
	men	4	3.6	3.76	0.10–256	
MIBP	women	2	19.8	19.7	0.34–266	0.5
	men	1	26.0	25.4	0.40–363	
MnBP	women	0	11.4	11.4	1.05–106	0.5
	men	1	16.3	15.0	0.40–268	
cx-MINP	women	1	1.99	1.85	0.15–44.2	0.1
	men	0	2.90	2.82	0.52–94.3	
OH_MidP	women	23	0.37	0.37	0.04–72.3	0.1
	men	8	0.81	0.77	0.04–83.5	
OH-MINCH	women	20	0.66	0.49	0.04–297	0.1
	men	10	1.08	0.87	0.04–720	
Oxo-MINCH	women	39	0.32	0.23	0.03–84.5	0.1
	men	21	0.56	0.48	0.04–254	
OH-MINP	women	0	2.21	2.26	0.67–11.7	0.1
	men	0	3.66	3.03	0.54–114	

samples of men and lactating women. Among the phenols, BPS, iPrP, iBuP, and TCS were detected in <30 % of all samples, with the detection rate as low as 3% for iBuP. Other phenols could be detected at rates ranging from 96% (MP) to 34% (BPF). Detection rates were generally higher for phthalate and DINCH metabolites. OH-MEHP, McxEPP, MEP, MnBP, and OH-MINP could be detected in 100% of all samples, whereas detection rates of other metabolites ranged between 99% (oxo-MEHP, MIBP, cx-MINP) and 70% (oxo-MINCH). MnOP, MnPeP, and MCHP were detected in <2% of all samples. Descriptive statistics for SG-corrected urinary concentrations and LOQs are presented in Table 2, whereas information on unadjusted and creatine-adjusted levels are provided in the supplementary material (Table S2 and S3). Results for MnOP, MCHP, and MnPeP are not presented due to their low detection rates. Men have significantly ($p \leq 0.01$) higher levels of BPA and BPF, as well as all phthalate and DINCH metabolites, while urinary concentrations of MP, EtP, iPrP, PrP, and BuP were significantly higher in women ($p \leq 0.04$). Similar trends have been observed in other studies (Husøy et al., 2019), while a study on phthalate exposure of the general Slovene population (DEMOCOPHES) did not observe large differences (Runkel et al., 2020). The authors attributed higher paraben levels in women to a larger number of applications and a higher diversity of PCPs compared to men, but our study lacks the data to confirm or reject this hypothesis.

Furthermore, there is increasing evidence that the activity of drug metabolizing enzymes is significantly altered during pregnancy (Isoherranen and Thummel, 2013; Koh et al., 2014). As our female study population consists of primiparous women, it cannot be excluded that the observed differences could at least partially be attributed to the physiological state. The levels of BzP did not differ significantly between genders, but the lower detection rate in men (29%) versus women (51%) might bias the results. The urinary concentrations of phenols and phthalates and the differences between men and women are illustrated in Fig. 2.

Fig. 3 presents the associations among analytes in urine of men and women. All observed correlations were positive; however more correlations were significant in the samples of women. For both genders, the associations among phthalates were the strongest, with Spearman's rho mostly greater than 0.5, whereas among BPs rho was mostly less than 0.4. Lesser correlations among BPs and PBs have been reported by other studies (Husøy et al., 2019; Sakhi et al., 2018) and attributed to differences in the exposure pathway. Exposure to PBs occurs mostly via PCPs, while the main pathway of exposure to BPs is diet. The same route is shared with HMW phthalates, which explains the observed correlation patterns in both our study and other studies. The results show a relatively strong correlation between MP and PrP (rho of 0.74 and 0.41 in women and men, respectively), suggesting a common exposure source. To increase the preservative effect, different PBs are often applied in combination, and strong correlations between PrP and MP have been observed in similar studies from Slovenia and other countries (Sakhi et al., 2018; Tkalec et al., 2021). Less correlations were significant in men, but the observed trends are similar between men and women. MEP was correlated weaker with other phthalates compared to other metabolites, suggesting different industrial applications for this compound. Metabolites of the same parent compound were correlated the strongest, confirming their relationship. Similar conclusions have been derived in other studies (Porras et al., 2020).

3.2. Comparison with the literature

Concentrations of phthalate metabolites vary substantially throughout the literature (Wang et al., 2019), with very low concentrations for **MEP** being reported in a study on men and women from Germany (13.5 ng/mL) (Koch et al., 2017) and concentrations of 246 ng/mL being reported in pregnant women from Spain (Valvi et al., 2015); the latter exceeding concentrations observed in an occupationally exposed population (201 ng/mL) from Slovakia (Kolena et al., 2017). The concentrations observed in this study (median: 27.9 and 55.7 ng/mL SG for women and men, respectively) are among the lower values reported in the literature and are comparable to concentrations measured in Japan (21.4 ng/mL) (Itoh et al., 2009), Italy (61.0 ng/mL and 73.2 ng/mL for women and men, respectively) (Tranfo et al., 2013), Belgium (37.6 ng/mL, adults) (Dewalque et al., 2014), China (28.2 ng/mL, men) (Zhang et al., 2016), and the Czech Republic (56.7 ng/mL, women) (Černá et al., 2015). DEP is a LMW phthalate that does not underlie any regulations and finds wide applications in e.g. fragrances.

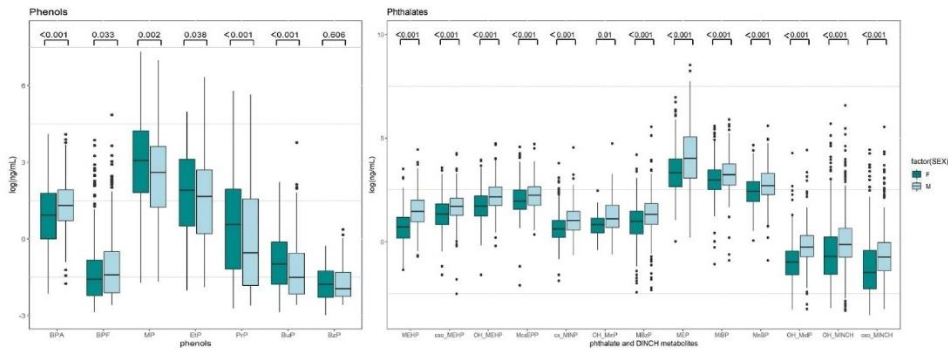

Fig. 2. Log transformed urinary concentrations of phenols, phthalates, DINCH (ng/mL) grouped by sex (F = women and M = men). Significance obtained using Wilcoxon test and indicated by p-values.

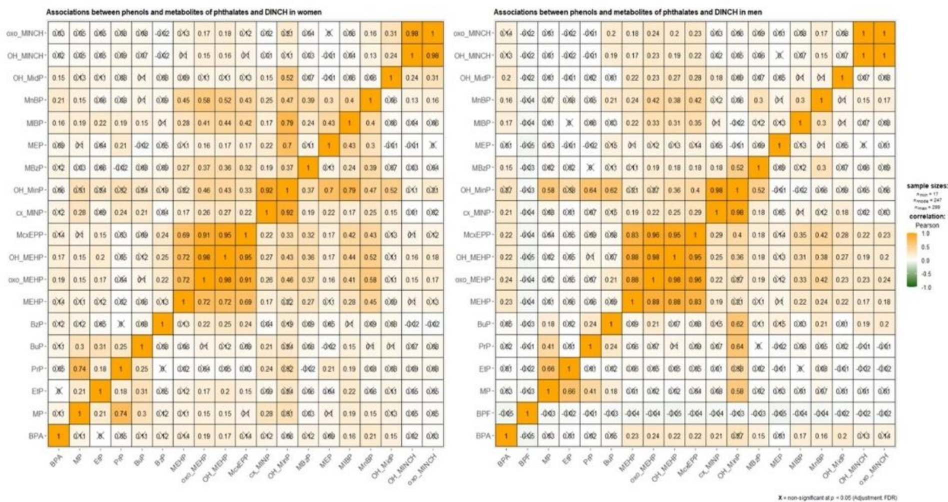

Fig. 3. Spearman's rank correlation of phthalate and DINCH metabolites and environmental phenols in women (left) and men (right). Non-significant correlations (p value < 0.05) are crossed out.

As such, exposure largely depends on personal lifestyles. In a study from 2011 including Slovenian women and men the observed concentrations of MEP in urine were more than twice as high in women (median: 50.2 ng/mL SG) and slightly higher in men (median: 39.7 ng/mL SG) (Runkel et al., 2020) compared to the present study. Differences among women from other countries as well as from Slovenia could be attributed to the temporarily altered physiological stage during pregnancy and lactation or to altered behaviour. Unfortunately, our dataset does not allow the confirmation or rejection of this hypothesis. The determined concentrations in men are comparable to those observed in the DEMOCOPHES study. As such, differences to other countries can most likely be attributed to differences in lifestyle and/or the respective market. Other metabolites, such as **MnBP**, exhibit less variation among populations, but differences still apply even within one country. In the DEMOCOPHES study, urinary levels were 25.3 ng/mL SG for women and 29.4 ng/mL SG for men (median) (Runkel et al., 2020). However, in the present study, the respective values were 11.4 ng/mL SG and 15 ng/mL SG. Similarly, three studies from Germany report MnBP concentrations of 181 ng/mL, 19.6 ng/mL, 8.0 ng/mL, and 16.4 ng/mL for adults (Göen et al., 2011; Koch et al, 2003, 2017bib_Koch_et_al_2017bib_Koch_et_al_2003), whereas values of 11.8 ng/mL, 31.3 ng/mL, 49.6 ng/mL, 27.1 ng/mL, and 13.7 ng/mL were reported from Australia (Heffernan et al., 2016), Belgium (Dewalque et al., 2014), China (Guo et al., 2011a), Spain (Valvi et al., 2015), and the USA (Wenzel et al., 2018). The wide range among MnBP concentrations furthermore reflects the importance of adequate questionnaire data. The included studies were selected based on the time period in order not to bias the results. As such, we can only hypothesize about lifestyle differences or physiological alterations that can influence urinary metabolite levels. Levels of **MiBP** in urine from Belgium (24.3 ng/mL) (Dewalque et al., 2014) and Germany (25.5 ng/mL and 20.3 ng/mL) (Göen et al., 2011; Koch et al., 2017) in 2011 are comparable to this study (19.7 ng/mL SG for women and 25.4 ng/mL SG for men), whereas three studies from the USA (Colacino et al., 2010; Ferguson et al., 2015; Wenzel et al., 2018) observe lower concentrations in their populations (4.9 ng/mL, 7.57 ng/mL, and 11.3 ng/mL, respectively) Other countries, however, observe higher concentrations. As

such, studies from China report median concentrations of 55.6 ng/mL (young adults) (Gao et al., 2016) and 51.7 ng/mL and 70.2 ng/mL for women and men, respectively (Guo et al., 2011b) and a study from Germany reports urinary MiBP levels in women of 43.0 ng/mL (Kasper-Sonnenberg et al., 2012) Kolena et al., (2017) included occupationally exposed hairdressers (mostly female) and a non-occupationally exposed control group from Slovakia in their study. The hairdressers had median concentrations of 40.3 ng/mL MiBP in their urine, whereas the values for the control group were 24.2 ng/mL. These results highlight that exposure to phthalates is highly influenced by behaviour and obtained metabolite concentrations can vary among countries, but also within a state. The latter is demonstrated by the MiBP levels for women and men that were determined in the DEMOCOPHES study (38.9 ng/mL SG and 47.3 ng/mL SG, respectively) that were twice as high as in the here presented population. The observed concentrations of **ΣDEHP** metabolites in Slovenia are low compared with other studies (24.3 ng/mL SG and 36.9 ng/mL SG for women and men, respectively); in comparison, the values for women and men from the DEMOCOPHES study were 24.3 ng/mL SG and 36.9 ng/mL SG, respectively (calculated based on the results published by Runkel et al., (2020)). Values of a magnitude comparable to those observed in this study were only reported by two studies from Germany (Koch et al., 2017), Belgium (Dewalque et al., 2014), and Slovakia (Kolena et al., 2017) with calculated ΣDEHP levels of 29.2 ng/mL, 26.3 ng/mL, and 26.9 ng/mL. However, a study from Kuwait reports a median of 180.4 ng/mL (Guo et al., 2011a) and the Slovakian study (Kolena et al., 2017) included occupationally exposed hairdressers with urinary levels of 31.4 ng/mL that were only slightly above the here observed levels. These results demonstrate the differences in the market, with DEHP being widely restricted in Europe, as well as the application of the chemical in plastic materials rather than PCPs; the latter being seen by the concentrations obtained by Kolena et al., (2017). Levels of DINCH metabolites in Slovenia are comparable to those obtained by a German study (Schütze et al., 2012) that determined **oxo-MINCH** and **OH-MINCH** at concentrations of 0.22 ng/mL and 0.36 ng/mL in non-occupationally exposed adults. In this study, urinary concentrations of both metabolites in women were 0.23 ng/mL SG and 0.49 ng/mL SG, respectively, whereas the levels for men were 0.48 ng/mL SG and 0.87 ng/mL SG. Unfortunately, the levels of DINCH metabolites were not determined in the DEMOCOPHES study and also other studies rarely report these concentrations for samples obtained between 2008 and 2014. However, DINCH is becoming a more frequently applied plasticizer (Bui et al., 2016) and more and more studies include its metabolites in their analyses. Metabolites of DINP were observed at comparable concentrations by two German studies that report comparable median values of 2.5 ng/mL and 5.0 ng/mL (Koch et al., 2007b) for **OH-MINP** and **cx-MINP**, respectively and 5.6 ng/mL for OH-MINP (Fromme et al., 2007). However, the geometric means reported by (Koch et al., (2007b)) are up to 5 times higher than in our study. The comparable levels observed among the studies could suggest that exposure to DINP underlies less variations than other compounds. A possible reason for this might be the restriction of this compound und REACH (ECHA, 2012), but our dataset lacks the necessary information to evaluate this hypothesis. Similarly, concentrations of the BBzP metabolite **MBzP** observed by (Koch et al., (2007b)) are higher (5.6 ng/mL) than those observed in this study (2.67 ng/mL SG for women and 3.76 ng/mL SG for men). However, the results from the DEMOCOPHES study (5.5 ng/mL SG for women and 5.95 ng/mL SG for men) are comparable to the German study, whereas (Campbell et al., 2018) report slightly higher values (8.0 ng/mL for women and 6.4 ng/mL for men). Higher levels of MBzP in women compared to men have been often reported in the literature and studies suggest a higher exposure of women through PCPs (Berger et al., 2019; Wormuth et al., 2006).

TCS is generally detected in urine at low frequencies and in varying concentrations (Wang and Tian, 2015). Wang and Tian (2015) summarize the results from 10 studies on TCS exposure and report detection frequencies between 0 % in Belgium and 76 % in China, which illustrates the differences in TCS exposure among populations. As such, the reported maximum values for adults of 2580 ng/mL from Greece (Asimakopoulos et al., 2014), of 3790 ng/mL from USA (Calafat et al., 2008), of 232 ng/mL (women) and 109 ng/mL (men) from China (Engel et al., 2014), of 1586 ng/mL from Denmark (Frederiksen et al., 2013), of 3158 ng/mL from Korea (Kim et al., 2011), of 2388 ng/mL, 2780 ng/mL and 2690 ng/mL from USA (Koeppe et al., 2013; Meeker et al., 2013), of 1630 ng/mL from Germany, and 599 ng/mL from Belgium (Pirard et al., 2012), are higher than those observed in Slovenia (15.9 ng/mL SG for women and 43.7 ng/mL SG for men). The detection frequencies of 30 % (women) and 27 % (men) fall in the range of those summarized by Wang and Tian (2015). TCS is used as an antimicrobial in PCPs, such as soaps and tooth paste. However, its application varies among products and countries and the intensity and frequency of application is highly consumer dependent. As such, exposure underlies large variations and our dataset lacks the necessary data to determine the sources of exposure in this population. Among frequently monitored **PBs**, MP, EtP, and PrP are detected at the highest frequencies and concentrations. Moos et al., (2015) report median MP levels of 32–50.8 ng/mL, 1–2.2 ng/mL EtP, and 2.2–5.7 ng/mL PrP, as well as iPrP and iBuP medians < LOQ for the German population in the years 2008, 2009, and 2012. MP and PrP concentrations are higher than in the present population (MP: 21.5 ng/mL SG for women and 13.6 ng/mL SG for men; PrP: 1.75 ng/mL SG for women and 0.58 ng/mL SG for men), whereas EtP, iPrP, and iBuP are comparable (EtP: 6.71 ng/mL SG for women and 5.26 ng/mL SG for men; iPrP and iBuP: < LOQ for men and women). We found that the values observed for MP and PrP in the present study are widely comparable to concentrations reported from several Asian countries (Honda et al., 2018) and generally much lower than levels observed in the USA (MP: 39.0 ng/mL; PrP: 4.70 ng/mL) (CDC, 2017), Korea (MP: 112 ng/mL and PrP: 47.4 ng/mL) (Honda et al., 2018), and Germany (MP: 42.6 ng/mL and PrP: 2.2 ng/mL) (Moos et al., 2015). However, concentrations of EtP in Slovenia are higher in comparison to China (2.74 ng/mL), India (0.25 ng/mL), Japan (0.53 ng/mL), Kuwait (0.68 ng/mL), Saudi Arabia (0.19 ng/mL), Vietnam (0.26 ng/mL), Greece (0.76 ng/mL), and USA (0.30 ng/mL) (Honda et al., 2018). BzP is one of the lesser monitored PBs, and studies observe generally low detection frequencies (Frederiksen et al., 2014) and low concentrations that are comparable with the present study (0.17 ng/mL SG in women and < LOQ in men). The results from other studies demonstrate how differences in the market and culture can influence exposure, especially if it occurs through PCPs. PBs are common additives in many products and the individual lifestyle largely determines how much a person is exposed. However, this hypothesis is in need of confirmation from other studies, as our questionnaire did not cover data on PCPs. Among the **BPs** in this study, BPA is detected at the highest frequencies and concentrations (2.52 ng/mL SG in women and 3.70 ng/mL SG in men), whereas the alternatives BPF and BPS are present in urine samples at much lower levels (BPF: < LOQ in women and 0.25 ng/mL SG in men; BPS: < LOQ in women and men). Similar trends have been observed in women from other studies from Greece (BPA: 1.2 ng/mL) (Myridakis et al., 2015) and Norway (BPA: 3.08 ng/mL SG; BPF: <LOQ; BPS: 0.13 ng/mL SG). It is known that BPA as a high-production-volume chemical is ubiquitous in the environment and that humans are widely exposed to it (Lehmler et al., 2018). However, its reputation as an endocrine disrupting chemical lead to the replacement of BPA with alternatives such as BPS and BPF that are currently produced and applied in much lesser quantities compared to BPA (Pelch et al., 2019). Therefore, the trends observed in this study and by others can be explained by the dynamics of the global market.

3.3. *Determinants of exposure to phthalates, DINCH, and phenols*

The demographic characteristics of the study population that were included in the models are presented in Table S1. As we observed

Table 3
Results of ordinal logistic regression modelling and suggested determinants of exposure. Model results were separately obtained for men (M) and women (F). Listed are the results for each confounder included in the separate models. Blank values indicate that this confounder was not included in this model. Included confounders: occupational exposure, education, age, year of sampling, BMI, supplements, smoking, alcohol, and residence; training dataset: 70 % and test dataset: 30 %. The values before brackets represent beta coefficients, whereas the p value is given after the slash and significant p values are indicated in bold (beta/p-value).

	Age	BMI	Residence/R	Education/U	Year	Exposure/Yes	Supplements	Alcohol	Smoking	error
					F					
MEHP		−0.8/0.3			−316/**<0.0001**		−0.4/0.2			**0.64**
Oxo_MEHP	−1.8/0.07				−569/**<0.0001**	0.2/0.58				**0.64**
OH_MEHP			−1.0/**0.0002**	−0.4/0.14	−692/**<0.0001**		−0.3/0.3	−0.09/0.6		**0.61**
McxEPP	−1.1/0.3		−0.6/**0.02**		−860/**<0.0001**			−0.3/0.07		**0.64**
Cx_MINP		1.2/0.12			−366/**<0.0001**	−0.3/0.4				**0.72**
MBzP	−2.8/**0.007**	3.0/**0.0005**	−0.6/**0.04**		−518/**<0.0001**					**0.64**
MEP		1.4/0.08	−0.6/**0.02**		−569/**<0.0001**					**0.67**
MIBP			−0.6/**0.01**		−567/**<0.0001**	−0.3/0.6				**0.65**
MnBP		1.0/0.3	−7.1/**0.01**		−1396/**<0.0001**	−0.6/0.1	−2.8/0.4			**0.63**
OH_MidP			−0.5/**0.05**	−0.7/**0.01**	−126/**<0.0001**		−0.5/0.1			**0.67**
OH_MINCH			−0.7/**0.01**		59.4/**<0.0001**		−0.7/**0.03**			**0.65**
OH_MinP	−5.0/**0.03**				210/**<0.0001**					**0.64**
Oxo_MINCH			−0.7/**0.008**		94.4/**<0.0001**	0.3/0.5		−0.3/0.1		**0.65**
BPA		2.0/**0.03**	−1.0/**0.0009**	−0.3/0.3	495/**<0.0001**			−0.2/0.3		**0.59**
MP					−542/**<0.0001**			0.5/**0.01**		**0.67**
EtP			−0.3/0.3	0.5/0.07	−303/**<0.0001**			0.3/0.1		**0.66**
PrP				−0.6/0.07	−586/**<0.0001**		0.7/0.06	0.3/0.2		**0.70**
BuP			−0.6/**0.05**	0.7/**0.02**	−640/**<0.0001**					**0.59**
BzP				1.0/**0.001**	181/**<0.0001**					**0.61**
					M					
MEHP		−2.3/**0.02**			−78.6/**<0.0001**				−1.1/0.07	**0.70**
Oxo_MEHP					−261/**<0.0001**		0.5/0.09			**0.68**
OH_MEHP		−1.0/0.4			−359/**<0.0001**				−1.5/**0.01**	**0.62**
McxEPP					−567/**<0.0001**	0.2/0.5	−0.1/0.7	−0.3/0.2	−1.8/**0.004**	**0.62**
Cx_MINP	1.4/0.06			−0.2/0.49	- 408/**<0.0001**	0.5/0.13		−0.3/0.12	−2.1/**0.0005**	**0.67**
MBzP					−318/**<0.0001**				−7.4/0.2	**0.68**
MEP		−1.0/0.3			−51.9/**<0.0001**	0.4/0.3		0.2/0.4		**0.64**
MIBP		0.6/0.6		0.5/0.09	−59.6/**<0.0001**				−0.5/0.4	**0.69**
MnBP		−2.4/**0.02**	−0.3/0.38		−332/**<0.0001**		−0.2/0.59			**0.61**
OH_MidP	1.1/0.1				−298/**<0.0001**				−1.0/0.08	**0.68**
OH_MINCH	−1.0/0.2			0.5/**0.05**	184/**<0.0001**					**0.67**
Oxo_MINCH	−1.2/0.1			0.5/**0.04**	273/**<0.0001**		0.3/0.3			**0.69**
BPF		−0.9/0.4		−0.09/0.7	53.1/**<0.0001**					**0.68**
BPA			−1.5/**<0.0001**		12.8/**<0.0001**					**0.53**
MP	2.2/**0.02**	−1.8/0.09	−0.4/0.2		−380/**<0.0001**			0.2/0.3		**0.65**
EtP		−3.6/**0.002**	0.04/0.9		−18.4/**<0.0001**			0.6/**0.02**		**0.62**
PrP	1.2/0.2	−2.5/**0.03**	0.6/**0.05**	−0.3/0.4	−569/**<0.0001**	0.9/**0.02**				**0.63**
BuP			−1.1/**0.0003**		373/**<0.0001**	−0.4/0.3	0.6/0.06			**0.58**

significant differences between men and women, OLR models were presented separately for each gender (Table 3).

Interesting time trends in exposure could be observed. While the concentrations in traditional phthalate plasticizers are significantly decreasing between 2008 and 2014 in this population (M = men and F = women), levels of DINCH metabolites are increasing significantly, mirroring trends in production (Bui et al., 2016). Concentrations of BPF and BPA follow an increasing trend, whereas concentrations of PBs are mostly decreasing, with the exception of BuP in men and BzP in women. All observed time trends in the model are significant ($p < 0.0001$). HBM studies from Europe report a decline in urinary phthalate metabolite concentrations between 2011 and 2016 (Tranfo et al., 2018) and a 67% decline in DEHP metabolites in the USA (Wang et al., 2019). Similar trends were previously observed for phenols, which has been linked to the negative image of these chemicals in the media (Sakhi et al., 2018). MP concentrations in men were positively associated with age (coef: 2.2, $p = 0.02$), whereas other PBs and BPs did not show any significant trend with age. Among phthalate metabolites, we observed significantly decreasing concentrations (F:MBzP and F:OH-MINP F) with age (coef: 2.8, $p = 0.007$ and coef: 5.0, $p = 0.03$, respectively). As this study lacks application frequencies of PCPs and detailed data on dietary habits as a confounder, these results should be carefully considered. The model results are conflicting regarding the influence of BMI on contaminant concentrations. Despite an often significant impact on the model, no distinct trend is visible and more information on the participants' lifestyles would be needed to increase the certainty of the results. Significantly negative associations were observed between BMI and MEHP, MnBP, EtP, and PrP in men (coef: 2.3, $p = 0.02$; coef: 2.4, $p = 0.02$; coef: 3.6, $p = 0.002$; coef: 2.5, $p = 0.03$, respectively), whereas positive correlations were observed between BMI and MBzP, and between BMI and BPA in women (coef: 3.0, $p = 0.0005$ and coef: 2.0, $p = 0.03$, respectively). Participants from urban/industrialized sampling regions had significantly higher concentrations of almost all phthalate metabolites, BPs, and PBs in urine with the exception of PrP in men's urine, which were significantly higher in rural locations (coef from −0.6 to −7.1, $p < 0.05$). The lack of sufficient data limits the possibilities of discussion here, but reported higher concentrations of these compounds in urban air (Rakkestad et al., 2007; Rudel and Perovich, 2009) are a possible explanation. Participants with a university degree had significantly higher ($0.05 > p > 0.001$) levels of some compounds (M: OH-MINCH, coef: 0.5; M: oxo-MINCH, coef: 0.5; F: BuP, coef: 0.7; F:BzP, coef: 1.0), whereas concentrations of OH-MIDP were significantly higher in women with lower education (coef: 0.7, $p = 0.01$). Negative associations between metabolite concentrations of HMW phthalates and the level of education was observed also in the DEMOCOPHES study that included Slovenian men and women (Runkel et al., 2020), whereas the LMW phthalates (excluding MEP) were significantly higher in participants with a university degree. It was discussed that higher education might be associated with a higher awareness towards harmful food contaminants, such as HMW phthalates, while the occupation associated

with a higher level of education might require more a frequent application of PCPs, such as make-up. However, the results in this study are inconclusive and cannot confirm the trends observed in DEMOCOPHES. The inclusion of occupational exposure (self-reported exposure to solvents, lubricants, plasticizers, adhesives, and paints) and dietary supplement intake was beneficial for lowering the prediction error, but the results were mostly non-significant. Men that reported occupational exposure had significantly higher concentrations of PrP (coef: 0.9, p = 0.02) and the intake of supplements was significantly negatively associated with concentrations of OH-MINCH in women (coef: 0.7, p = 0.03). However, the trend was not repeatable for oxo-MINCH. Alcohol intake was significantly associated with elevated levels of MP in women (coef: 0.5, p = 0.01) and with elevated levels of EtP in men (coef: 0.6, p = 0.02). In men, smoking was associated with lower levels of cx-MINP (coef: 2.1, p = 0.0005), McxEPP (coef: 1.8, p = 0.004), and OH-MEHP (coef: 1.5, p = 0.01). Alcohol has been associated with a negative impact on hepatic drug-metabolizing enzymes that would decrease the elimination rates of contaminants in the human body (Miyashita et al., 2015). Smoking, on the other hand, has been found to induce cytochrome P450 enzymes involved in phase I metabolism of phthalates, which would lead to faster elimination of pollutants from the body (Flesch-Janys, 1996; Miyashita et al., 2015). It needs to be highlighted that the obtained prediction errors in this study are very high. Therefore, the results are in urgent need of verification and should be considered indicatory.

3.4. *Estimated daily intake and risk assessment for men and lactating women*

The calculated EDIs for men and women are presented in Table 4. As the calculation of HQs is based on the 95th percentile, so are the EDIs. Therefore, this should be considered when comparing the EDIs from this study with other publications. To allow an overview, a few studies from Europe (Norway and Germany) with supposed comparable exposure and Asia (Taiwan) with supposed higher exposure were selected and presented here. The EDIs in Slovenia are comparable or slightly lower than those reported in other studies. Sakhi et al., (2018) report EDIs of 0.056, 0.024, 0.032, 0.00027, and 0.0076 mg/kg$_{bw}$/day for MP, EtP, PrP, BPA, and TCS, respectively, in Norwegian mothers. With the exception that the EDIs of BPA in Slovenian women from this study are slightly higher (0.0007 mg/kg$_{bw}$/day), the intakes of other compounds are lower than those reported by Sakhi et al., (2018). We obtained an EDI of 0.006 mg/kg$_{bw}$/day for the sum of DEHP metabolites for men and women, which is slightly lower than the value reported for a population in Taiwan (0.008 μg/kg$_{bw}$/day) and substantially lower than the EDI of 0.021 mg/kg$_{bw}$/day reported for the adult general population in Germany (95th percentile) (Koch et al., 2006). At this point it is worth noting that different urinary adjustment methods might introduce uncertainty to the exposure assessment as observed and discussed by Runkel et al., (2020). Other reasons for variations in EDIs among countries might lie in the exposure itself (lifestyle and market) or differences in the calculation of intake values, e.g. the use of different excretion factors or by presenting the 95th percentile instead of the geometric mean as it was done in this study.

The results of the HQ calculation are presented in Table 4. No analyte exceeds the value of 1 in men or women, and the MOE obtained for BPS exceeds 10,000, which corresponds to a no-risk scenario. The highest HQ was obtained for PrP (0.08 and 0.09) for women and men, respectively. To assess the risk of cumulative exposure to chemical mixtures, the HQ for all analytes was estimated. While the value for phenols is near 0.6 in both men and women, the risk resulting from phthalate exposure is lower (HQs: 0.15 and 0.19 for women and men, respectively). The combined HQ of phenols and phthalates does not exceed but approaches the limit of 1 in both populations (0.74 and 0.76 for women and men, respectively), which might cause concern due to the unknown impact of other chemicals not addressed in this study. Similar results were reported for BPA, BPS, BPF, and PBs in the framework of a Norwegian HBM study. Sanchis et al., (2020) report EDIs and HQs of comparable magnitude for lactating women ($EDI_{EtP + MP}$ 0.01–0.04 mg/kg$_{bw}$/day, HQ_{BPA} 0.005 and $HQ_{EtP + MP}$ 0.004). The authors conclude that current exposure levels do not imply a risk for mothers; however, HQs were evaluated only for individual analytes, while the HQ for the mixture has not been evaluated. A Finnish study evaluating a non-occupationally exposed population reports individual HQs higher than those obtained

Table 4
Presentation of Estimated Daily Intakes (EDI), Hazard Quotients (HQ), and Margins of Exposure (MOE) for women (F) and men (M). Cells were left blank if the calculation of HQs was not possible (BPS, BPF, BzP, OH-MIDP) because no biological equivalent (BE) values were available for these compounds. The availability of a no-adverse-effect-level (NOAEL) value for BPS allowed the calculation of the MOE for this compound.

Compounds	EDI F (mg/kg$_{bw}$/d)	EDI M (mg/kg$_{bw}$/d)	HQ F	HQ M	MOE F	MOE M
BPA	0.0007	0.0008	0.006	0.009		
BPS	0.0001–0.01	0.00004–0.004			199063	613235
BPF	*0.0002–0.02*	*0.0006–0.06*				
MP	0.04	0.05	0.004	0.004		
EtP	0.02	0.01				
iPrP	0.0009	0.0004	0.03	0.01		
PrP	0.01	0.02	0.08	0.09		
iBuP	0.0008	0.0002	0.02	0.006		
BuP	0.002	0.002	0.07	0.06		
BzP	0.00002–0.002	0.00001–0.002				
TCS	0.002	0.003	0.001	0.001		
ΣDEHP	0.006	0.006	0.006	0.006		
MEP	0.009	0.03	0.0005	0.002		
MBzP	0.0006	0.001	0.00005	0.00006		
MnBP	0.002	0.003	0.01	0.01		
MiBP	0.004	0.005	0.021	0.02		
cx-MINP	0.003	0.004	0.10	0.13		
OH-MIDP	*0.0001–0.07*	*0.0001–0.11*				
OH-MINCH	0.004	0.006	0.004	0.006		
Oxo-MINCH	*0.010*	*0.02*	0.01	0.02		
phenols			**0.59**	**0.57**		
phthalates			**0.15**	**0.19**		
total			**0.74**	**0.76**		

in the present study, and the HQ of the mixture exceeds the threshold of 1 (1.41–1.46) (Porras et al., 2020). Relatively high concentrations of MnBP (0.059 mg/g creatinine) compared to other European and US populations (CDC, 2019; Tranfo et al., 2018) as well as the present population (0,047 mg/g creatinine) substantially contributed to the high HQ. The monitoring of HQs in populations exposed to endocrine disruptors is an important measure in risk assessment. Exposure to mixtures is a controversial topic, as they can act either synergistic or antagonistic and the dose-response relationship in the human body is rarely linear (Siroux et al., 2016). Studies report that exposure to phthalates at a critical developmental stage can lead to an impaired development of psychomotoric skills (Polanska et al., 2014) and neurological disorders, such as attention deficit syndrome, hyperactivity or lower intelligent quotients (Ejaredar et al., 2015). A recent study by Papaioannou et al., (2021) estimated by utilization of multi-omics analyses how exposure to phthalate mixtures disturbs the urea cycle and choline metabolism. Findings like this highlight the need of frequent risk monitoring of populations as for instance through the calculation of HQs.

4. Study limitations

This study is part of the first national HBM program in Slovenia, which was originally designed for the monitoring of trace elements and persistent organic pollutants. The included questionnaire was optimized accordingly and lacks many details necessary for exposure assessment to non-persistent chemicals. This represents the largest limitation of this study. In an attempt to overcome this limitation, ordinal logistic regression modelling was performed, and the results were validated using prediction modelling. The obtained high prediction errors highlight the need for an adapted questionnaire, and the results should be considered indicatory. In future studies, first morning urine samples should be used instead of random spot samples as this limits the inter-individual variation between exposure and sampling.

5. Conclusion

This study evaluated the exposure of men and lactating women in Slovenia to phthalates, BPs, PBs, and TCS. We observed significantly higher concentrations of phthalates, DINCH, and BPs in men, whereas the levels of most PBs were significantly higher in women. We observed differences in exposure based on the area of residence (urban/industrial > rural) and urinary concentrations that were comparable to several other studies. Additionally, we assessed the risk of the population via HQs and conclude that while HQ values are sufficiently low, the HQ of the chemical mixture is near the threshold value of 1. Considering that many compounds were not included in this study, it is likely that the HQ of a larger chemical mixture will exceed 1. Additional studies that address a wider range of chemicals and potentially other susceptible populations, such as children, are needed.

Credit author statement

Agneta Runkel: Conceptualization, Methodology, Formal analysis, Investigation, Writing-Original draft, visualization, Tina Kosjek: supervision, writing - review & editing, Žiga Tkalec: laboratory supervision, writing - review & editing, Darja Mazej: project administration, writing – review & editing, Janja Snoj Tratnik: project administration, writing – review & editing, Milena Horvat: project administration, supervision, funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2021.131858.

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3.5 Manuscript 5: Assessment of Susceptibility to Phthalate and DINCH Exposure Through CYP and UGT Single Nucleotide Polymorphisms

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As hinted at in the introduction, risk assessment so far neglects the influence of individual susceptibilities on resilience. Therefore, this study investigated – for the first time – the role of SNPs coding for enzymes involved in the biotransformation of PHs and DINCH using HBM data (Stajnko et al., 2022). PHs and DINCH are xenobiotics that undergo compound-dependent biotransformation in the liver and partly in the gut that is largely dependent on CYPs and UGTs (Benjamin et al., 2017; Domínguez-Romero & Scheringer, 2019; Frederiksen et al., 2007; Lyche, 2011). Enzymes belonging to this group are involved in the oxidative biotransformation of many known xenobiotics; CYP2D6, for instance, is known to be catalyzing for 20 – 30% of all biotransformation processes of pharmaceuticals while being highly polymorphic (Horn, 2012); an in vitro study by Choi et al., (2012) has demonstrated its active role in the biotransformation of DEHP. In the same study, Choi et al., (2012) identified 5 more enzymes besides CYP2D6 that are involved in the production of secondary metabolites of DEHP, namely CYP2C9, CYP2C19, CYP3A4, CYP3A5, and CYP3A7. UGTs, on the other hand, are involved in the glucuronidation of PH and DINCH metabolites, which facilitates their excretion in urine. In vitro studies suggest the involvement of UGT1A1, UGT2B7, and UGT2B15 in this process (Luo et al., 2020).

Functional polymorphisms can alter the gene expression and, consequently, enzyme functioning. Therefore, functional SNPs in the genes of the afore-mentioned enzymes can influence the biotransformation of phthalates and DINCH in the human body (Choi et al., 2012). This can potentially make the carrier more susceptible to the adverse health effects caused by these compounds, as a slower metabolism leads to a longer latency in the body and a thereby caused increase of the biologically effective dose. As the biotransformation products of some phthalates such as DEHP are more potent than the parent compound, a delay in elimination caused by slow metabolism increases the exposure to these toxicants (Choi et al., 2012). Thus, the presence of SNPs with a known negative effect on enzyme performance can be read as a biomarker of susceptibility (Choi et al., 2012).

A total of seven functional SNPs (rs1799853 (*CYP2C9*2*), rs1057910 (*CYP2C9*3*), rs4244285 (*CYP2C19*2*), rs12248560 (*CYP2C19*17*), rs38920979 (*CYP2D6*4*), rs1902023 (*UGT2B15*2*), and rs11692021 (*UGT1A7*3*) were selected based on available information in the literature, reported SNP's minor allele frequencies (MAFs), and the availability of pre-designed hydrolysis probe assays. The effect of the selected SNPs was evaluated by associating the ratios between the metabolites (5OH-MEHP/MEHP, 5oxo-MEHP/MEHP, 5cx-MEPP/MEHP, 5oxo-MEHP/5OH-MEHP, and oxo-MINCH/OH-MINCH) as a proxy of biotransformation efficiency with the respective SNP. All associations were obtained using multiple linear regression modeling and ordinal logistic regression as a confirmation. This is the first study to investigate and observe the effect of selected SNPs in genes coding for CYPs and UGTs on the biotransformation of PHs and DINCH.

We observed a reduction of the biotransformation of DEHP in carriers of *CYP2C9*2* and *CYP2C9*2* and an increased excretion in carriers of *CYP2C19*17*. The observed effect of the two CYP2C9 SNPs was even more pronounced in carriers of both variant alleles.

Among the SNPs in genes of UGTs, only *UGT1A7*2* was associated with elevated levels of selected PH metabolites (MBzP, MiBP) in women and of DINCH metabolites in men.

The results obtained in this study demonstrate that the above-mentioned SNPs could be utilized as biomarkers of susceptibility to PH and DINCH exposure.

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Assessment of susceptibility to phthalate and DINCH exposure through CYP and UGT single nucleotide polymorphisms

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ABSTRACT

Single nucleotide polymorphisms (SNPs) of cytochrome P450 (CYPs) and UDP-glucuronosyltransferase (UGTs) genes have been proposed to influence phthalates and 1,2-*cyclo*-hexanedicarboxylic acid diisononyl ester (DINCH) biotransformation but have not been investigated on a populational level.

We investigated the role of SNPs in *CYP2C9, CYP2C19, CYP2D6, UGT2B15,* and *UGT1A7* genes in the biotransformation of phthalates (DEHP, DEP, DiBP, DnBP, BBzP, DiNP, DidP) and DINCH by determining their urine metabolites.

From the Slovenian study population of 274 men and 289 lactating primiparous women we obtained data on phthalate and DINCH urine metabolite levels (MEHP, 5OH-MEHP, 5oxo-MEHP, 5cx-MEPP, MEP, MiBP, MnBP, MBzP, cx-MINP, OH-MiDP, MCHP, MnPeP, MnOP, 5OH-MINCH, 5oxo-MINCH), SNP genotypes (rs1057910 = *CYP2C9*3*, rs1799853 = *CYP2C9*2*, rs4244285 = *CYP2C19*2*, rs12248560 = *CYP2C19*17*, rs3892097 = *CYP2D6*4*, rs1902023 = *UGT2B15*2*, and rs11692021 = *UGT1A7*3*) and questionnaires. Associations of SNPs with levels of metabolites and their ratios were assessed by multiple linear regression and ordinary logistic regression analyses.

Significant associations were observed for *CYP2C9*2, CYP2C9*3, CYP2C19*17,* and *UGT1A7*3* SNPs. The most pronounced was the influence of *CYP2C9*2* and *3 on the reduced DEHP biotransformation, with lower levels of metabolites and their ratios in men and women. In contrast, carriers of *CYP2C19*17* showed higher urine levels of DEHP metabolites in both genders, and in women also in higher DiNP, DiDP, and DINCH metabolite levels. The presence of *UGT1A7*3* was associated with increased metabolite levels of DINCH in men and of DiBP and DBzP in women. Statistical models explained up to 27% of variability in metabolite levels or their ratios.

Our observations confirm the effect of *CYP2C9*2* and *3 SNPs towards reduced DEHP biotransformation. We show that *CYP2C9*2, CYP2C9*3, CYP2C19*17,* and *UGT1A7*3* SNPs might represent biomarkers of susceptibility or resilience in phthalates and DINCH exposure that have been so far unrecognised.

1. Introduction

Phthalates (PHs) are diesters of phthalic acid commonly classified as high molecular weight (HMW: 7–13 carbon atoms) or low molecular weight (LMW: 3–6 carbon atoms) PHs. HMW PHs are primarily used in plastics, while LMW PHs are additives in solvents and personal care products from where they migrate into the environment (Wittassek et al., 2011; Berger et al., 2019; Wang et al., 2019). The general population is frequently exposed via ingestion, inhalation, and dermal absorption (Benjamin et al., 2017; Wang et al., 2019). Exposure to PHs has been associated with various health issues, (Benjamin et al., 2017; Wang et al., 2019; Giuliani et al., 2020), therefore, the presumably less toxic substitute plasticizer 1,2-*cyclo*-hexanedicarboxylic acid diisononyl ester (Hexamoll® DINCH) has been introduced (Bui et al., 2016; Wang et al., 2019). DINCH and PHs are listed as a priority substance group within the pan-European HBM4EU project (Schoeters and Lange, 2020).

In the human body, PHs and DINCH undergo compound-dependent biotransformation in the liver and partly in the gut. Firstly, they are

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hydrolysed by esterase or lipases into the corresponding monoesters (phase I biotransformation) followed by oxidation of the monoester side chain by the cytochrome P450 enzymes (CYPs; phase I biotransformation) resulting into secondary metabolites – mainly with hydroxy, oxo, and carboxy functional groups. Most of the metabolites further undergo conjugation (phase II biotransformation), which is catalysed mainly by UDP-glucuronyl transferases (UGTs), forming hydrophilic conjugates that are easily excreted in within 48 h in urine (major pathway) or faeces (minor pathway) (Frederiksen et al., 2007; Benjamin et al., 2017; Lyche, 2017; Domínguez-Romero and Scheringer, 2019).

Accordingly, exposure to PHs and DINCH is assessed by the measurement of the primary and secondary metabolites in the urine; mostly without distinguishing between conjugated and non-conjugated forms. The patterns of urine metabolites show inter-individual variability, which can be attributed to the exposure as well as to differences in physiology; the latter influencing enzyme activity and, consequently, biotransformation capacity (Frederiksen et al., 2007; Yaghjyan et al., 2016; ATSDR, 2019; Domínguez-Romero and Scheringer, 2019). Important contributors to the inter-individual variability might also be single nucleotide polymorphisms (SNPs) in genes coding for the main metabolizing enzymes, such as CYPs and UGTs (Choi et al., 2012, 2013; Stein et al., 2013; Ito et al., 2014; Yaghjyan et al., 2016; Hanioka et al., 2017; ATSDR, 2019). This can consequently result in higher susceptibility towards the toxic effects of PHs and DINCH on human health. However, information on specific genes or enzyme isoforms involved in PH or DINCH biotransformation is scarce. The most extensively studied is the biotransformation of HMW diethylhexyl phthalate (DEHP) in animals and humans (ATSDR, 2019); as shown in Fig. 1.

Choi et al. (2012) assessed DEHP biotransformation by subcellular fractions of various human tissues and human CYPs. They identified CYP2C9, CYP2C19, CYP2D6, CYP3A4, CYP3A5, and CYP3A7 as the major CYP isoforms producing hydroxy, oxo, and carboxy secondary metabolites (5OH-MEHP, 5oxo-MEHP, and 5cx-MEPP, respectively). Moreover, the in vitro assessed effect of different CYP2C9 enzyme subisoforms defined by the SNPs rs1799853 (*CYP2C9*2*) and rs1057910 (*CYP2C9*3*) indicated reduced enzyme activity (Choi et al., 2012, 2013). Also, the rs743572 polymorphism in *CYP17A1* has been suggested to influence the effect of PHs exposure (DEP, DEHP, DnBP) on the development of leiomyoma (Huang et al., 2014). Although, in *CYP2C19* several functional SNPs have been reported – with rs4244285 (*CYP2C19*2*) and rs12248560 (*CYP2C19*17*) being the most common among Caucasians (Rosemary and Adithan, 2007; Hirota et al., 2013; Hiratsuka, 2016) – none have been yet investigated in the relation to PHs or DINCH biotransformation. Furthermore, around 85% of the applied DEHP dose is excreted in urine in the form of glucuronidated metabolites (Koch et al., 2006; Frederiksen et al., 2007; ATSDR, 2019). According to an in vitro study by Hanioka et al. (2017), the main UGT enzyme isoforms involved in MEHP glucuronidation in isolated human liver and intestine microsomes were UGT2B7, UGT1A9, and UGT1A7. Although, various SNPs resulting in induction or suppression of various UGT enzymes have been determined (Guillemette, 2003; UGT Nomenclature Commitee, 2005; Hanioka et al., 2017), their association with biotransformation and negative health effects of PHs or DINCH have been poorly investigated. The assessment of rs4148323 (*UGT1A1*6*), rs7439366 (*UGT2B7*2*), and rs1902023 (*UGT2B15*2*) polymorphisms has revealed a significant association of the latter two with total serum PHs (undefined) levels in patients with polycystic ovary syndrome (Luo et al., 2020).

The investigation of CYP and UGT polymorphisms' influence on urine levels of PHs or DINCH metabolites on a population level has been proposed (Choi et al., 2012; Ito et al., 2014; Yaghjyan et al., 2016; Hanioka et al., 2017) but, to our knowledge never performed. Therefore, the purpose of the present study was to test the possible role of selected SNPs in three CYP (*CYP2C9*, *CYP2C19*, *CYP2D6*) and two UGT (*UGT2B15*, and *UGT1A7*) genes in the biotransformation of PHs (DEHP, DEP, DiBP, DnBP, BBzP, DiNP, and DiDP) and DINCH in the Slovenian population of men and lactating women.

2. Material and methods

2.1. Study population

In this study, a subset of unrelated subjects was selected from a wider Slovenian Human Biomonitoring programme carried out between 2008 and 2014. In the original programme, 1084 participants – consisting of men and lactating *primiparous* women – were recruited from across Slovenia, with the aim to estimate trace elements' levels and persistent organic pollutants in a childbearing population and to estimate babies' exposure via maternal milk. Due to the existence of archived samples, levels of PHs and DINCH metabolites in urine were obtained in 2019–2020 for 603 participants, and Runkel et al. (2022) describe their exposure assessment in detail. Among these, genetic material was

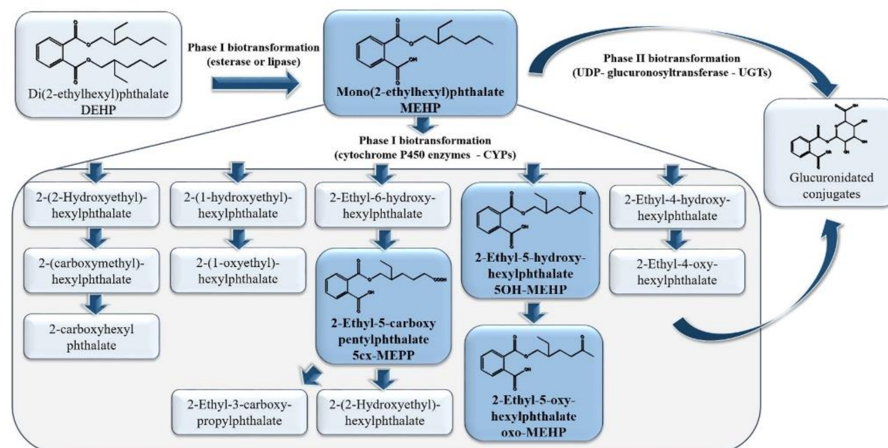

Fig. 1. Biotransformation of DEHP (adapted with permission from Koch et al., 2005). Highlighted are the major metabolites, which are determined in the present study.

obtained for 572 participants (298 lactating *primiparous* women and 274 men), who's data was used in order to test the present study's aim.

Stajnko et al. (2017) and Snoj Tratnik et al. (2019) describe the detailed recruitment and sampling procedures. Briefly, all participants provided a random spot urine sample and a sample of whole blood, and they completed questionnaires covering their general characteristics, socio-economic status, life-style, and dietary habits. The samples were aliquoted and stored at −20 °C prior to analyses. All participants signed an informed consent form, and the study protocol was approved by the Republic of Slovenia National Medical Ethics Committee, with numbers of accordance 42/12/07, 53/07/09 and 70/02/11. To be able to use biobanked samples, we obtained additional ethical approval (number of accordance 0120-431/2018/4), and all participants provided informed written consent.

2.2. Analyses of PHs and DINCH metabolites

The spot urine samples were sent to the VITO NV laboratory in Belgium for analysis of 13 PHs primary and secondary metabolites (MEP, MBzP, MiBP, MnBP, MCHP, MnPeP, MEHP, 5OH-MEHP, 5oxo-MEHP, 5cx-MEPP, MnOP, cx-MiNP, and OH-MiDP) and 2 secondary metabolites of DINCH (OH-MINCH and oxo-MINCH) (Table 1). The C or D labelled standards of phthalate or DINCH metabolites were supplied by Cambridge Isotope Laboratories (Andover, USA). VITO has proved its excellence by successfully participating in the ICI-EQUAS rounds (inter-laboratory comparison investigation) organised within the European project HBM4EU (Elbers and Mol, 2019). The laboratory measured the total content (conjugated and free form) of urinary PH and DINCH metabolites.

A brief description of the measurement process is as follows. First, β-glucuronidase in an ammonium acetate buffer solution was added to 1 mL of each sample and analysed using ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) after direct injection. Separation was achieved on an Acquity UPLC BEH PHENYL 1.7 μm, 2.1 × 100 mm column with mobile phase A: water + 0.1% acetic acid and B: acetonitrile (ACN) + 0.1% acetic acid and a total run time of 8 min. The analysis was performed in negative ionization mode with MRM detection. Contamination control was assured via the inclusion of procedural blanks and parallels as well as spiked quality control samples in water and urine. One independent quality control sample (G-EQUAS) was included to assure the repeatability and reproducibility of the results. Only linear weighted (1/X) calibration curves with squared regression coefficients >0.995 and residuals <10% were accepted. The inter-day repeatability for all metabolites was <10%. A deviation of 20% in recovery of quality control samples was considered acceptable and was obtained for all analytes. The method trueness was assessed via spiking experiments to overcome the lack of certified reference materials. The relative recovery (%) calculated as the ratio between experimentally observed concentrations and nominal concentrations was taken as an approximation of trueness and ranged between 80% and 120%.

The obtained LOQs were as follows: 0.1 ng/mL for 5oxo-MEHP, 5OH-MEHP, 5cx-MEPP, cx-MiNP, MnOP, MnPeP, OH-MiDP, OH-MINCH, and oxo-MINCH; 0.2 ng/mL for MBzP and MCHP; 0.5 ng/mL for MEP, MiBP, and MnBP; and 0.8 ng/mL for MEHP.

All results were adjusted to specific gravity (SG) to overcome the effects of urinary dilution. SG was measured on a PAL-10 S refractometer, closely following the method for SG correction described by Suwazono et al. (2005).

2.3. Analyses of selenium in blood

Aliquot of venous blood (0.3 mL) was analysed for selenium (Se) by Octopole Reaction System (ORS) Inductively Coupled Plasma Mass Spectrometry (ICP-MS; 7500ce, Agilent Technologies) equipped with an ASX-510 autosampler (Cetac). The LOD was 8 ng/g. The procedure was

Table 1
Primary and secondary metabolites of PHs and DINCH measured in the present study.

			Metabolites measured in the present study:	
Parent compound	**LMW**	**HMW**	**Primary metabolite**	**Secondary metabolites**
DEHP Di(2-ethylhexyl) phthalate		X	MEHP Mono(2-ethylhexyl) phthalate	5OH-MEHP (MEHHP[a]) Mono(2-ethyl-5-hydroxyhexyl) phthalate 5oxo-MEHP (MEOHP[a]) Mono(2-ethyl-5-oxyhexyl) phthalate 5cx-MEPP (MECPP[a]) Mono(2-ehtyl-5carboxypentyl) phthalate
DEP Di-ethyl phthalate	X		MEP Mono-ethyl phthalate	
DiBP Di-isobutyl phthalate	X		MiBP mono-isobutyl phthalate	
DnBP Di-n-butyl phthalate	X		MnBP mono-n-butyl phthalate	
BBzP Benzyl butyl phthalate	X		MBzP Mono-benzyl phthalate	
DiNP Diisononyl phthalate		X		cx-MiNP Monocarboxy-isononyl phthalate
DiDP Diisodecyl phthalate		X		OH-MiDP Monohydroxy-isodecyl phthalate
DCHP Dicyclohexyl phthalate	X		MCHP* Mono-cyclohexyl phthalate	
DnPeP Di-n-pentyl phthalate	X		MnPeP* Mono-n-pentyl phthalate	
DnOP Di-n-octyl phthalate		X	MnOP* Mono-n-octyl phthalate	
DINCH Di-(isononyl)-cyclohexane-1,2-dicarboxylate		X		OH-MINCH (MHNCH[a]) Cyclohexane-1,2-dicarboxylic acid-mono (hydroxyl-isononyl) oxo-MINCH (MONCH[a]) Cyclohexane-1,2-dicarboxylic acid-mono (oxo-isononyl)

* Could not be detected in >95% of samples and were as such excluded from the statistical analysis in the present study; LMW: low molecular weight compound, HMW: high molecular weight compound.

[a] Alternative commonly used metabolite nomenclature.

previously described by (Miklavčič et al., 2013).

2.4. Genotyping of selected SNPs

2.4.1. SNP nomenclature and selection

Within clinical studies, where CYPs and UGTs are mostly studied, the most widely adopted SNP nomenclature is the "star" (*) nomenclature, with *1 mainly defining reference or wild-type (fully functional) allele, while further numbers (e.g. *2, *3, *4, …) correspond to variant alleles of different SNPs (https://www.pharmvar.org/; https://www.pharmacogenomics.pha.ulaval.ca/ugt-alleles-nomenclature). To be consistent and transparent with the current literature, in this manuscript, we follow the recommendations by Kalman et al. (2016) and in case of each tested SNP reported both rs ID from dbSNP and its corresponding * allele nomenclature, as presented in Table 2 (e.g. *CYP2C9* SNP rs1057910: A > C allele change, resulting genotypes AA, AC, and CC; corresponding * nomenclature is *1 > *3 and *1/*1, *1/*3, and *3/*3).

Specific genes and corresponding SNPs were selected based on the following criteria:

(i) literature data on specific genes predominantly involved in the biotransformation of PHs – mainly DEHP – (Choi et al., 2012; Hanioka et al., 2017), and the reported functional influence of SNP on enzyme activity (Guillemette, 2003; UGT Nomenclature Commitee, 2005; Di et al., 2009; Hanioka et al., 2011; Choi et al., 2012, 2013; Cao et al., 2019; PharmVar: Pharmacogene Variation Consortium, 2021);
(ii) reported SNP's minor allele frequency (MAF) of ≥7% for the European population;
(iii) the availability of pre-designed hydrolysis probe assays (https://www.thermofisher.com/order/genome-database/).

Seven functional SNPs: rs1799853 (*CYP2C9*2*), rs1057910 (*CYP2C9*3*), rs4244285 (*CYP2C19*2*), rs12248560 (*CYP2C19*17*), rs38920979 (*CYP2D6*4*), rs1902023 (*UGT2B15*2*), and rs11692021 (*UGT1A7*3*) were selected. Their general information is presented in Table 2.

2.4.2. DNA isolation and genotyping

Genomic DNA was isolated from archived venous whole blood (0.5 mL) using the FlexiGene® DNA kit (Qiagen, Germany) following the manufacturer's instructions. The quality and quantity of DNA were evaluated by UV–VIS spectrophotometer NanoDrop 2000c (Thermo-Fisher Scientific, USA). DNA isolates were stored at −80 °C prior to genotyping.

Selected SNPs were genotyped using pre-designed TaqMan SNP Genotyping Assays (Applied Biosystems, USA; Table 2). The 5 µL reaction consisted of 2.5 µL of FastStart Essential DNA Probes Master (Roche, Germany), 1.875 µL of ultrapure nuclease-free water (Life Technologies, USA), 0.125 µL of 44X TaqMan probe/primer mix, and 0.5 µL of genomic DNA. LightCycler® 480 Instrument II and LightCycler480® Software version 1.5.1 (Roche, Germany) were used for the amplification and fluorescence detection. PCR cycling included the following steps: pre-PCR step (1 cycle: 50 °C for 2 min), activation step (1 cycle: 95 °C for 10 min), annealing and amplification step (50 cycles: 95 °C for 15 s and 61 °C for 1 min), and post-PCR step (1 cycle: 40 °C for 30 s). For each SNP, a subset of randomly selected samples was repeated as a control (~30%).

2.5. Statistical analyses

All statistical analyses were performed separately for the groups of men and lactating women, due to significant physiological differences between men and women that are intensified by the temporary physiological state (i.e. lactation) of the participating women (Waxman and Holloway, 2009; Moya et al., 2014). Descriptive statistics were used to assess general characteristics of the study population (age, BMI, education and smoking), levels of metabolites in urine (exposure biomarkers), and genotype and allele frequency distribution of SNPs. The descriptive statistics of metabolite levels are presented unadjusted and with SG adjusted data, while all further statistics were performed using only SG adjusted data.

To assess the efficiency of DEHP and DINCH oxidative biotransformation, the following metabolite ratios were calculated: 5OH-MEHP/MEHP, 5oxo-MEHP/MEHP, 5cx-MEPP/MEHP, and 5oxo-MEHP/5OH-MEHP for DEHP and oxo-MINCH/OH-MINCH for DINCH.

Statistical differences between groups were assessed using the Mann-Whitney *U* test, the Kruskal Wallis test with Dunn post hoc test, or Pearson's chi-squared test. The associations of seven SNPs with PHs and DINCH urine metabolite levels and their ratios were tested by multiple linear regression analyses (MLR) with levels of metabolites or ratio values as the dependent variable and SNPs as the independent variable. Each association of SNP with urinary metabolite level and ratio was tested in a separate model with adjustments for age, BMI, education, year of sampling, blood selenium, and smoking. The latter was, due to there being only one female smoker, tested in men only. The confounders were chosen based on their previously reported association with PHs or DINCH biotransformation, their influences on enzyme activity and their general physiological relevance (Bui et al., 2016; Yaghjyan et al., 2016; Klomp et al., 2020; Runkel et al., 2020). Selenium in whole blood was added as a rough estimate of selenium nutritional status, which can influence cytochrome P450 enzyme's activity (Burk, 1983; Jiang et al., 2020) and possibly, consequently, the biotransformation of PHs and DINCH. Moreover, in the case of DEHP metabolites, the models were additionally adjusted by the 5cx-MEPP/5OH-MEHP ratio as a rough approximation of the time between DEHP exposure

Table 2
Information on studied SNPs.

Gene	dbSNP ID	Variant allele nomenclature[a]	Chr/ location	nt change	Amino acid change	MAF EU	TaqMan assay ID	Reported effect on enzyme activity[b]
CYP2C9	rs1057910	*CYP2C9*3*	10/exon	A > C	Ile > Leu	7	C_27104892_10	C or *3: reduced
	rs1799853	*CYP2C9*2*	10/exon	C > T	Arg > Cys	12	C_25625805_10	T or *2: reduced
CYP2C19	rs4244285	*CYP2C19*2*	10/exon	G > A	Pro > Pro	15	C_25986767_70	A or *2: reduced
	rs12248560	*CYP2C19*17*	10/ promoter	C > T		22	C__469857_10	T or *17: increased
CYP2D6	rs3892097	*CYP2D6*4*	22/intron	C > T		19	C_27102431_D0	T or *4: reduced
UGT2B15	rs1902023	*UGT2B15*2*	4/exon	T > G	Asp > Tyr	52	C_27028164_10	A or *2: inconsistent
UGT1A7	rs11692021	*UGT1A7*3*	2/exon	T > C	Trp > Arg	36	C__287260_10	C or *3: reduced

Chr: chromosome; nt: nucleotide, MAF EU: minor allele frequency in populations with European ancestry (NCBI, 2021).

[a] "star" (*) nomenclature for the variant alleles based on the nomenclature consortium (https://www.pharmvar.org/, https://www.pharmacogenomics.pha.ulaval.ca/ugt-alleles-nomenclature).

[b] Based on the measured enzyme activity in various in vitro and pharmacogenomical studies (https://www.pharmvar.org/, https://www.pharmacogenomics.pha.ulaval.ca/ugt-alleles-nomenclature; Guillemette et al., 2000).

and urine spot sampling. This is based on the longer estimated elimination half-life of 5cx-MEPP (12–15 h) compared with 5OH-MEHP (~10 h) (Lorber et al., 2011; Meeker et al., 2012). As hypothesized by Lorber et al. (2011) "initially after exposure, the ratio of 5cx-MEPP to 5OH-MEHP in a spot urine sample is <1.0, but after 10 h or so, this ratio exceeds 1.0". Furthermore, to assess the MLR performance, diagnostic analyses were carried out to test for linearity, normality, homoscedasticity and multicollinearity.

The available personalized questionnaire data unfortunately do not include information on possible exposure, such as the use of personal care products or food packaging within the last days prior to sampling. This might, however, influence the assessment of the SNPs role in PHs or DINCH biotransformation. Therefore, to test for possible influences of such un-identified individual exposure sources (i.e. outliers), beside the Cook's distance test in MLR, the models described above were additionally tested with ordinal logistic regression analyses (OLR).For this purpose, the levels of each metabolite or their ratios were split into quartiles (1st: ≤25th perc.; 2nd: >25th perc. and ≤50th perc.; 3rd: >50th perc. and ≤75th perc.; and 4th: >75th perc.), and then used as categorical dependent variables. With this approach – commonly used in the epidemiological studies – we believe that the effect of outliers on the tested associations is reduced.

In the case of CYP enzymes – mainly responsible for reactions resulting in secondary metabolites – the corresponding SNPs were tested for associations with urine levels and/or ratios of secondary metabolites of DEHP, DiNP, DiDP, and DINCH. SNPs in UGTs were tested for associations with urine levels of all metabolites. Moreover, due to the sufficient number of subjects in certain SNP groups, namely, rs1902023 (*UGT2B15*2*), rs11692021 (*UGT1A7*3*), and rs12248560 (*CYP2C19*17*), analyses were performed based on allele (e.g. **2(*1/*2+*2/*2)* vs **1/*1*) and genotype stratification (e.g. **2/*2* vs. **1/*2* vs. **1/*1*), while in the case of other SNPs, we used only stratification by alleles.

The level of statistical significance (p-value) was set to ≤0.05. Values below the LOQ were substituted with a value of LOQ/2, and when appropriate, non-normally distributed data was log transformed to approximate normal distribution. Statistical analyses and visualisations of the results were carried out in statistical software R version 3.6.0 with RStudio version 1.2.1335 using the packages ggplot2 (Wicklam, 2016), stargazer (Hlavac, 2018), stats (R Core Team, 2019), and MASS (Venables and Ripley, 2002) and in OriginPro® version 2020b (OriginLab Corporation, USA).

3. Results and discussion

To date, the literature widely focusses on the effect of CYP and UGT SNPs on the biotransformation of pharmaceuticals, while studies on PHs and DINCH mostly investigate distribution, levels of exposure and its health outcomes. Their gene-environment interaction on a populational level has previously been pointed out, but no studies examining this issue exist to date. Therefore, in the present study, SNPs in the genes of *CYP2C9*, *CYP2C19*, *CYP2D6*, *UGT2B15*, and *UGT1A7* were tested for possible associations with PHs and DINCH metabolite levels and their ratios. For six tested SNP variant alleles, it was reported that they have an inhibitory effect, and for one (rs12248560 or *CYP2C19*17*), a stimulating effect on enzyme activity (Table 2). Here we observe similar trends regarding their impact on metabolite levels and/or ratios.

3.1. Study population and biomarkers of exposure and biotransformation

The participants' samples were obtained between 2008 and 2014, with the highest acquisition in the years 2012 and 2013 (71% and 61% for women and men, respectively). Thus, all subjects were recruited prior to the EU-wide restriction of DiBP, DEHP, BBzP, and DMP in 2015 (Tranfo et al., 2018). The general characteristics of the studied men and lactating women are presented in Table 3. Among the women and men,

Table 3
General characteristics of the studied population.

	Lactating women	Men
N (%)	289 (51)	274 (49)
Age (years)	29 ± 4	31 ± 6
Weight (kg)	65 ± 11	84 ± 13
Height (cm)	168 ± 6	180 ± 7
BMI (kg/m^2)	23 ± 4	26 ± 4
Current smokers (N (%))	1 (0.3)	13 (2)
Education (N (%))		
<University	129 (46)	162 (61)
≥University	153 (54)	102 (38)
Year of sampling N		
2008	10	10
2009	41	22
2010	10	16
2011	13	6
2012	140	87
2013	65	79
2014	8	49
Selenium in blood (ng/g)	96 (54–176)	117 (77–226)

Age, weight, height, and BMI are presented as arithmetic mean ± SD, and Selenium in blood as GM (min–max)

with average ages of 29 and 31, respectively, we noticed that 54% and 38% of women and men, respectively, hold at least a university degree. As such, the education level of the present population is skewed towards higher levels compared to the average education level of the Slovenian population (SURS, 2017).

Concentration levels of metabolites of seven phthalates (DEHP, DEP, DiBP, DnBP, BBzP, DiNP, DiDP) and DINCH are presented in Table 4, while primary metabolites of DCHP, DnPeP, and DnOP could not be detected in >95% of samples and were thus excluded from the statistical analysis. The numbers of samples < LOQ ranged between 0% and 21% and between 0% and 38% for all other metabolites in men and women, respectively. In both men and women, the highest concentrations found were for MEP, followed by MiBP, MnBP, 5cx-MEPP, 5OH-MEHP, and 5oxo-MEHP, whereas the lowest concentrations were obtained for oxo-MINCH, followed by OH-MiDP and OH-MINCH. Despite different time frames of sampling, similar trends have been observed in other studies (Wang et al., 2019). Women had significantly lower concentrations of all metabolites compared with men ($p < 0.001$). This trend was not generally observed in our study from 2011, in which non-lactating women ($n = 155$) and men ($n = 177$) of similar ages from Slovenian urban and rural areas, with similar exposure levels among men and higher among women, were involved (Runkel et al., 2020). Therefore, the observed sex differences in the present study could be at least partially related to the generally upregulated drug biotransformation in women during pregnancy, which remains elevated after birth, or to the altered life style during lactation (Meeker et al., 2012; Moya et al., 2014; Zhao et al., 2018; Domínguez-Romero and Scheringer, 2019). This observation can be further supported by the DEHP metabolite ratios (5OH-MEHP/MEHP, 5oxo-MEHP/MEHP, 5cx-MEPP/MEHP, and 5oxo-MEHP/5OH-MEHP), which were significantly higher in women than in men (Table 4), indicating a higher DEHP biotransformation efficiency in women (if biotransformation is not concentration dependent).

The ratios' ranking order of 5cx-MEPP/MEHP > 5OH-MEHP/MEHP > 5oxo-MEHP/MEHP is the same in men and women. This aligns with the literature stating that the majority of MEHP is further metabolized to secondary metabolites (Frederiksen et al., 2007). The lower ratio between the oxo and hydroxy (oxo/OH) metabolites of DEHP and DINCH indicates a higher proportion of the OH metabolite as compared with oxo, which agrees with the current state of knowledge (Bolt et al., 2004; Koch et al., 2005, 2017; Völkel et al., 2016; Schütze et al., 2017).

Table 4
Descriptive statistics of primary and/or secondary PHs and DINCH metabolite levels in urine and their ratios in lactating women and men.[a]

		LACTATING WOMEN								MEN								p-Value*
		% <LOQ	N	GM	Min	P25	P50	P75	Max	% <LOQ	N	GM	Min	P25	P50	P75	Max	
Parent compound	**Metabolite levels**	**Unadjusted data (μg/L)**																
DEHP	MEHP	24.6	289	1.67	0.40	0.81	1.89	3.40	35.1	4.4	274	3.96	0.40	2.23	4.18	6.93	127	<0.001
	5OH-MEHP	0.3	289	4.95	0.05	2.21	5.25	10.3	77.1	0.0	270	8.18	0.48	4.29	8.63	15.3	161	<0.001
	5oxo-MEHP	0.7	289	3.32	0.05	1.54	3.61	6.99	66.7	0.8	266	4.66	0.05	2.62	4.99	8.67	101	<0.001
	5cx-MEPP	0.7	289	6.62	0.05	3.20	6.89	13.6	152	0.0	271	8.40	0.78	4.57	8.91	15.4	134	0.006
DEP	MEP	0.4	273	24.2	0.25	10.68	21.4	52.7	2354	0.4	226	54.1	0.25	17.2	48.7	159	4926	<0.001
DiBP	MiBP	1.7	288	16.7	0.25	8.08	17.6	35.8	524	1.1	268	23.3	0.25	11.8	25.1	47.1	371	<0.001
DnBP	MnBP	0.0	289	9.69	0.69	4.50	10.7	20.3	200	0.8	259	14.2	0.25	8.07	14.8	27.0	345	<0.001
BBzP	MBzP	11.8	288	1.89	0.10	0.97	2.23	4.96	109	4.0	274	3.18	0.10	1.63	3.28	6.27	317	<0.001
DiNP	cx-MiNP	0.7	289	1.71	0.05	0.89	1.70	3.28	105	0.0	273	2.65	0.20	1.61	2.65	4.38	107	<0.001
DiDP	OH-MiDP	22.8	289	0.32	0.05	0.17	0.37	0.70	107	7.3	274	0.73	0.05	0.41	0.73	1.45	33.9	<0.001
DINCH	OH-MINCH	21.1	289	0.56	0.05	0.19	0.47	1.51	220	9.6	271	0.97	0.05	0.34	0.83	2.27	268	<0.001
	oxo-MINCH	37.8	289	0.27	0.05	0.05	0.24	0.77	93.0	21.0	272	0.49	0.05	0.20	0.46	1.19	87.3	<0.001
										SG adjusted data (μg/L SG)								
DEHP	MEHP		287	1.95	0.25	1.16	2.01	3.20	33.0		271	4.45	0.50	2.87	4.50	7.57	87.8	<0.001
	5OH-MEHP		287	5.79	0.20	3.42	5.46	9.02	50.7		267	9.22	0.85	5.88	8.88	14.4	118	<0.001
	5oxo-MEHP		287	3.87	0.20	2.27	3.75	6.22	34.8		263	5.29	0.08	3.57	5.37	8.48	73.5	<0.001
	5cx-MEPP		287	7.71	0.12	4.68	7.00	12.2	96.1		269	9.44	1.46	5.69	9.41	14.5	115	<0.001
DEP	MEP		271	29.5	1.00	13.9	27.7	55.7	1046		224	65.3	1.25	22.3	55.9	161	5185	<0.001
DiBP	MiBP		286	19.7	0.33	12.3	19.6	32.4	262		265	26.2	0.42	15.5	25.8	42.7	374	<0.001
DnBP	MnBP		287	11.4	1.04	6.89	11.2	18.1	104		256	16.3	0.42	10.0	15.1	26.9	276	<0.001
BBzP	MBzP		286	2.21	0.09	1.44	2.65	4.28	65.4		271	3.57	0.10	2.17	3.78	6.09	264	<0.001
DiNP	cx-MiNP		287	1.99	0.15	1.22	1.87	2.79	43.6		270	3.00	0.54	1.74	2.90	4.57	97.1	<0.001
DiDP	OH-MiDP		287	0.37	0.04	0.20	0.37	0.64	71.2		271	0.83	0.04	0.48	0.79	1.39	86.0	<0.001
DINCH	OH-MINCH		287	0.66	0.04	0.20	0.50	1.25	292		268	1.12	0.04	0.49	0.91	2.16	244	<0.001
	oxo-MINCH		287	0.32	0.03	0.10	0.23	0.64	83.2		269	0.57	0.04	0.25	0.49	1.02	79.4	<0.001
	Ratios																	
DEHP	5OH-MEHP/MEHP		289	2.95	0.13	1.98	2.97	4.62	21.3		270	2.09	0.28	1.40	2.07	3.00	21.2	<0.001
	5oxo-MEHP/MEHP		289	1.98	0.13	1.30	1.97	3.12	15.6		266	1.20	0.13	0.83	1.20	1.73	13.1	<0.001
	5cx-MEPP/MEHP		289	3.94	0.13	2.49	4.01	6.18	33.4		271	2.14	0.29	1.29	2.23	3.21	21.2	<0.001
	5oxo-MEHP/5OH-MEHP		289	0.67	0.09	0.61	0.67	0.74	1.00		264	0.58	0.10	0.53	0.59	0.66	0.85	<0.001
	5cx-MEPP/5OH-MEHP		289	1.34	0.06	1.13	1.37	1.63	12.2		267	1.03	0.22	0.83	1.02	1.27	2.55	<0.001
DINCH	oxo-MINCH/OH-MINCH		289	0.49	0.09	0.36	0.49	0.81	4.02		270	0.50	0.14	0.40	0.51	0.66	3.50	0.649

[*] Difference between men and lactating women tested by Mann-Whitney *U* test.
[a] The exposure data (metabolites levels) for the wider set of participants (including those without available genetic material; n = 304 women and 299 men) is presented by Runkel et al. (2022).

Table 5
Genotype and allele frequencies of studied SNPs (N (%)).

Gene	SNP ID	Genotype	All	Lactating women	Men	HWE p-value	% of genotyped individuals
CYP2C9	rs1057910	AA or *1/*1	578 (85)	253 (88)	225 (83)	0.859	99.6
	or	AC or *1/*3	80 (14)	35 (12)	45 (17)		
	*CYP2C9*3*	CC or *3/* 3	3 (0.5)	1 (0.4)	2 (0.7)		
		MAF %	8	6	9		
	rs1799853	CC or *1/*1	433 (77)	225 (78)	208 (77)	0.100	99.5
	or	CT or *1/*2	114 (21)	57 (20)	57 (21)		
	*CYP2C9*2*	TT or *2/*2	13 (2)	7 (2)	6 (2)		
		MAF %	13	12	13		
CYP2C19	rs4244285	GG or *1/*1	436 (78)	227 (79)	209 (77)	0.525	99.5
	or	GA or *1/*2	118 (21)	59 (20)	59 (22)		
	*CYP2C19*2*	AA or *2/*2	6 (1)	3 (1)	3 (1)		
		MAF %	12	11	12		
	rs12248560 or	CC or *1/*1	297 (54)	149 (53)	148 (55)	0.188	97.9
	*CYP2C19*17*	CT or *1/*17	204 (37)	106 (38)	98 (36)		
		TT or *17/*17	50 (9)	26 (9)	24 (9)		
		MAF %	28	28	27		
CYP2D6	rs3892097	CC or *1/*1	374 (70)	195 (71)	179 (68)		
	or	CT or *1/*4	146 (27)	70 (26)	76 (29)		
	*CYP2D6*4*	TT or *4/*4	18 (3)	9 (3)	9 (3)	0.223	99.1
		MAF %	17	16	18		
UGT2B15	rs1902023	CC or *1/*1	139 (25)	74 (26)	65 (24)	0.997	99.6
	or	CA or *1/*2	280 (50)	147 (51)	133 (49)		
	*UGT2B15*2*	AA or *2/*2	142 (25)	66 (23)	76 (28)		
		MAF %	50	49	52		
UGT1A7	rs11692021 or	TT or *1/*1	193 (35)	100 (35)	93 (34)		
	*UGT1A7*3*	TC or *1/*3	282 (51)	150 (52)	132 (49)		
		CC or *3/*3	83 (15)	38 (13)	45 (17)	0.997	99.6
		MAF %	40	39	41		

HWE: Hardy-Weinberg Equilibrium; MAF: minor allele frequency.

3.2. *Allele frequencies of the studied SNPs in the Slovenian population*

Table 5 presents the distribution of genotypes and alleles for all selected SNPs of the whole study population and separately for men and lactating women. Each SNP was successfully genotyped in at least 95.6% of the study population and was in accordance with the Hardy-Weinberg equilibrium. The minor allele frequencies (MAF) were between 8% and 50% (for rs1057910 (*CYP2C9*3*) and rs1902023 (*UGT2B15*2*), respectively) and were, in the case of each SNP, similar to those reported for populations with European ancestry (Table 2). Moreover, there were no significant differences in the MAFs of SNPs between men and women.

3.3. *SNPs influence on metabolite levels and biotransformation*

The estimation coefficients for specific SNP genotypes and/or alleles from MLR analyses (confirmed also in OLR) are summarised in Fig. 2 (CYPs) and Fig. 3 (UGTs). In general, associations with p-values ≤0.05 or ≤0.1 – based on both MLR and OLR – are discussed. The supplements present additional summary statistics (Supplementary material: subgroup comparisons for urine levels (Tables SP1–SP5) and for metabolite ratios (Tables SP6 and SP7), and results of the regression models (Tables SP8–SP10)).

3.3.1. *Cytochrome P450 enzymes – CYPs*

An in vitro study on human and rat tissues identified isoforms ranked as CYP2C9 > CYP2C19 > CYP2D6 as the most efficient among the six major isoforms mainly responsible for the production of DEHP secondary metabolites (Choi et al., 2012). *CYP2C9*, CYP2*C19*, and *CYP2D6* are highly polymorphic with several functional SNPs, resulting in enzyme isoforms with decreased or increased activity when compared with their respective wild-type enzymes (Pelkonen et al., 2008; Hiratsuka, 2016; PharmVar: Pharmacogene Variation Consortium, 2021).

3.3.1.1. *CYP2C9 SNPs: rs1799853 (CYP2C9*2), rs1057910 (CYP2C*3).* In the present study, the results indicated a reduced oxidative biotransformation of DEHP in both lactating women and, even more evidently, in men carriers of the variant alleles of both SNPs (Fig. 2; Tables SP1, SP6, and SP8).

The presence of the **rs1799853 (*CYP2C9*2*) variant allele** was associated with lower urine levels of all three DEHP secondary metabolites in men, and in a lower 5cx-MEPP/MEHP ratio in men and women. Differences between variant allele carriers and non-carriers were as follows: 5OH-MEHP (P50: 7.35 vs. 8.99 μg/L SG; coef:-0.26), 5oxo-MEHP (P50: 4.20 vs 5.65 μg/L SG; coef:-0.28), and 5cx-MEPP (P50: 7.07 vs 10.3 μg/L SG; coef:-0.35) in men, and 5cx-MEPP/MEHP in men (P50: 1.73 vs 3.57, coef: −0.24) and in women (coef: −0.23; P50: 3.57 vs. 4.15).

Similarly, the presence of the **rs1057910 (*CYP2C9*3*) variant allele** resulted in three lower DEHP metabolite ratios in male carriers and in lower levels of 5cx-MEPP and 5cx-MEPP/MEHP ratios in female carriers. Results for carriers versus non-carriers were as follows: 5oxo-MEHP/MEHP (P50: 1.04 vs. 1.24; coef: −0.19), 5cx-MEPP/MEHP (P50: 1.46 vs. 2.33; coef: −0.38) and 5oxo-MEHP/5OH-MEHP (P50: 0.45 vs. 0.61; coef: −0.13) in men, and 5cx-MEPP (P50: 6.18 vs 7.38 μg/L SG; coef: −0.16) and 5cx-MEPP/MEHP (P50: 3.22 vs 6.18; coef: −0.46) in women.

Our results are aligned with in vitro studies by Choi et al. (2012, 2013), that report negative effects of these variant alleles on the catalytic activity of the enzyme, resulting in a lower production of DEHP-derived secondary metabolites. Moreover, the presumed negative impact of both SNPs was most noticeable on the production of 5cx-MEPP in both men and women (Fig. 2). Similar observations were also reported by Choi et al. (2012, 2013), who found that rs1057910 (*CYP2C9*3*) resulted in minor production of OH- and oxo-MEHP and a complete loss of 5cx-MEPP formation. Additionally, 5cx-MEPP also shows higher binding affinity compared with other metabolites (Choi et al., 2013), which could highlight the influence of genetic variations on its production.

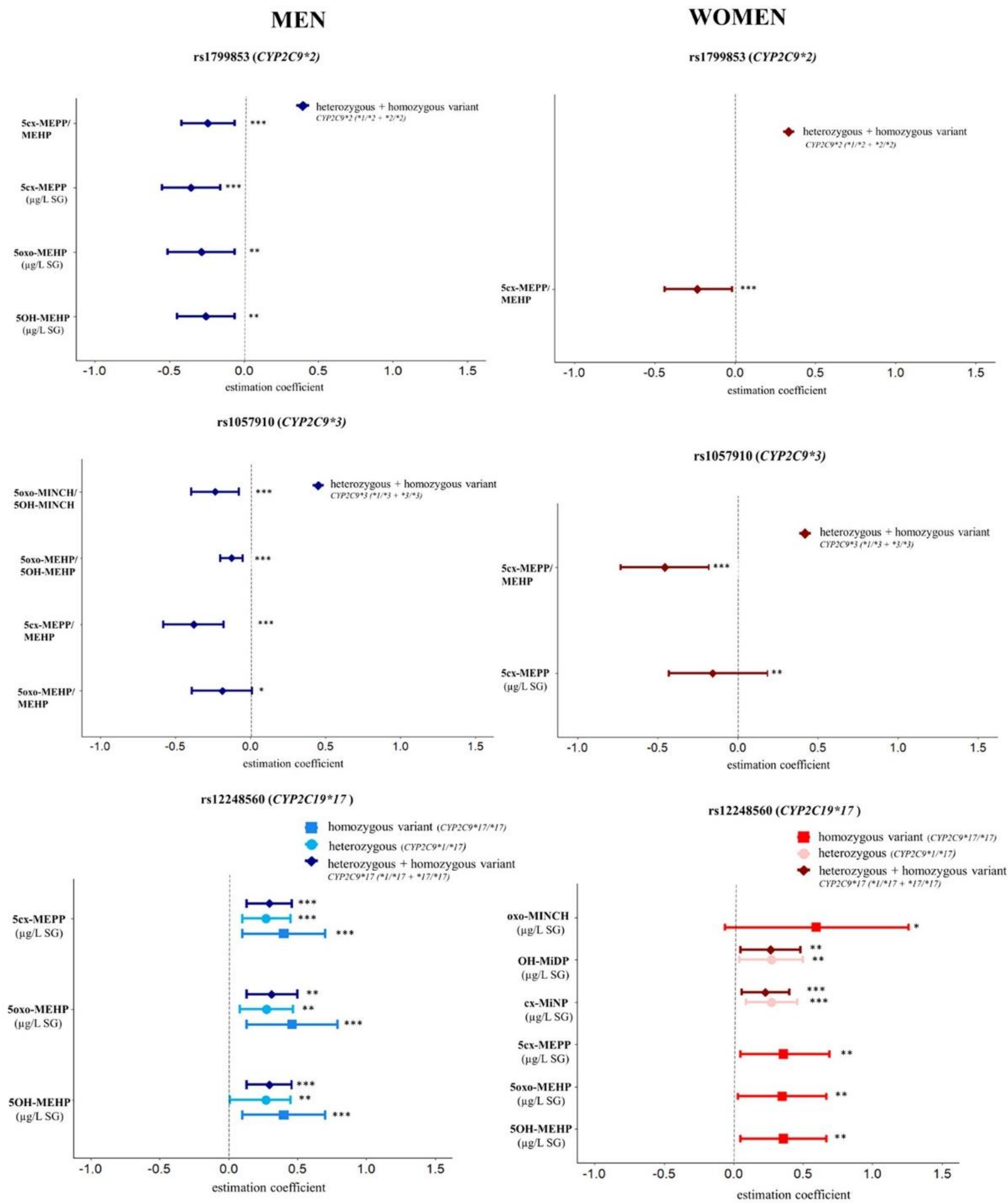

Fig. 2. Associations with p-values ≤0.05 or ≤0.1 for rs1799853 (*CYP2C9*2*), rs1057910 (*CYP2C9*3*), and rs12248560 (*CYP2C19*17*) SNPs with DEHP, DiDP, DiNP, and/or DINCH metabolite levels and/or ratios in men (left; blue) and lactating women (right; red). Presented are estimation coefficients of MLR analyses with a 95% confidence interval for heterozygote + variant homozygote, for heterozygote, and/or for homozygous variant when compared with homozygous wild-type (*1/*1); (***$p < 0.01$, **$p < 0.05$, *$p < 0.1$).

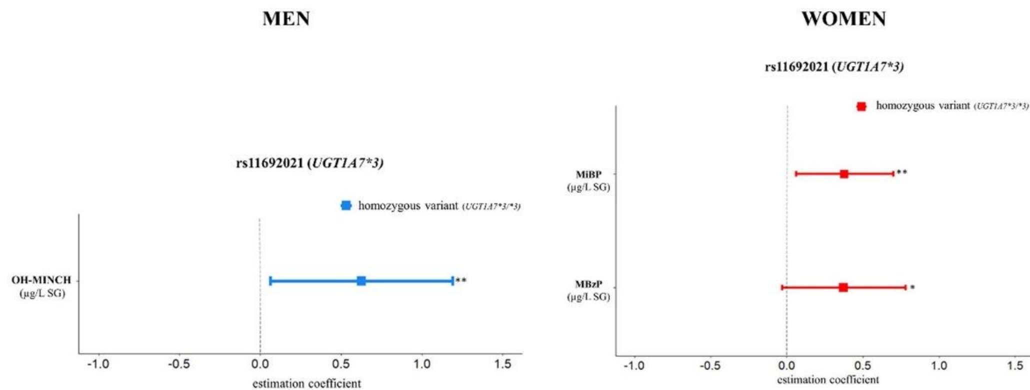

Fig. 3. Associations with p-values ≤0.05 or ≤0.1 for rs11692021 (*UGT1A7**3) with DiBP, BBzP, or DINCH metabolite levels in men (left; blue) and lactating women (right; red). Presented are estimation coefficients of MLR analyses with a 95% confidence interval for homozygous variant compared with homozygous wild-type (*1/*1); (***p < 0.01, **p < 0.05, *p < 0.1).

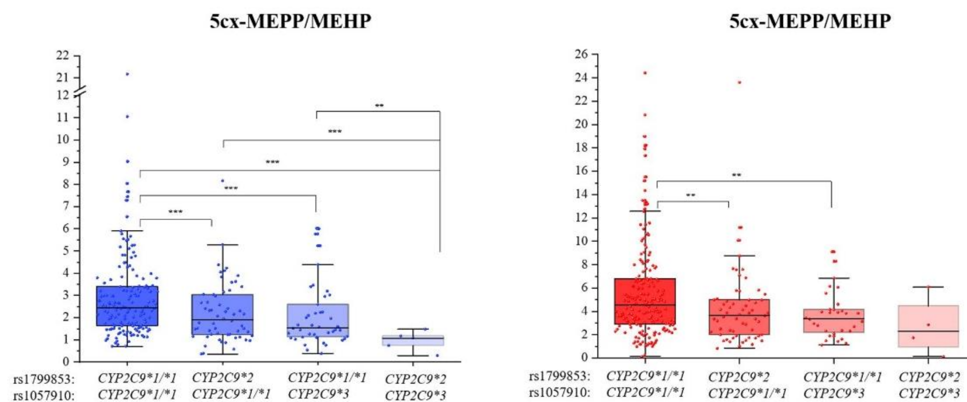

Fig. 4. 5cx-MEPP/MEHP ratio in men (left; blue) and women (right; red) based on the combination of the presence or absence of rs1799853 (*CYP2C9*2*) and/or rs1057910 (*CYP2C9*3*) variant alleles (***p < 0.01; **p < 0.05; *1/*1 represents wild-type genotype).

The association (linkage disequilibrium) between the variant alleles of rs1799853 (*CYP2C9*2*) and rs1057910 (*CYP2C9*3*) is very low. In our study population, only nine individuals (four women and five men) were identified as carriers of both variant alleles. However, as expected, those individuals show an even greater reduction in DEHP biotransformation, with the production of 5cx-MEPP being lower for 50% or more compared with wild-type carriers, as presented in Fig. 4. Furthermore, comparing both variant alleles, generally a slightly higher impact on DEHP biotransformation was observed in the case of rs1057910 (*CYPC9*3*) then rs1799853 (CYP2C9*2) (Fig. 2, Table SP1, SP6, and SP8), most evidently based on the 5cx-MEPP/MEHP ratio (Fig. 4). This could be explained by the rs1057910 (*CYPC9*3*) location that affects the catalytic unit of the enzyme influencing substrate recognition, which could lead to a higher reduction in activity than the rs1799853 (*CYP2C9*2)* variant allele (Rosemary and Adithan, 2007; Wang et al., 2009; Hirota et al., 2013).

Altogether, individuals with the presence of at least one of the discussed variant alleles – and especially those with the presence of both – might be more susceptible to the toxic effects of DEHP due to the reduced biotransformation of bioactive primary metabolite MEHP into less toxic secondary metabolites with better water solubility and faster excretion (Fig. 1) (Frederiksen et al., 2007; Yaghjyan et al., 2016). Moreover, % of MEHP (calculated based on the sum of all measured DEHP metabolites) – which is considered a possible indicator of susceptibility to PH exposure (Meeker et al., 2012) – was significantly higher for variant allele carriers (in the case of both SNPs) than for non-carriers (P50 MEHP% in carriers was for ~14% higher than in non-carriers; coef.: 0.11–0.22; data not presented).

For the other two HMW PHs evaluated in the present study – which also undergo oxidative biotransformation (DiNP and DiDP) –, we did not observe any associations of either variant allele with urine levels of their corresponding secondary metabolites (cx-MiNP and OH-MiDP, respectively). On the contrary, for DINCH, we observed a slight, although significant, reduction in the oxo-MINCH/OH-MINCH ratio in men for rs1057910 (*CYP2C9*3*) variant allele carriers compared with non-carriers (P50: 0.40 versus 0.5; coef: −0.24) (Fig. 2, Tables SP6 and SP9). As seen in the case of DEHP, the effect of variant alleles can be reflected by metabolite ratios and/or urinary levels; however, in our opinion, ratios better reflect biotransformation or enzyme activity than urinary levels alone do. From this viewpoint, examining additional primary or secondary metabolites of DiNP, DiDP, DiBP, DnBP, and DINCH, allowing for the assessment of their respective ratios, could improve the assessment of *CYP2C9* SNPs with those chemicals.

*3.3.1.2. CYP2C19 SNPs: rs4244285 (CYP2C19*2), rs12248560 (CYP2C19*17).* The **rs4244285 (*CYP2C19*2*) variant allele** results in an aberrant splicing site and, consequently, an altered mRNA reading frame, leading to reduced metabolic activity (Hirota et al., 2013). However, in the present study, we did not observe any significant associations of the SNP with PHs or DINCH metabolite urine levels or their ratios in men or women (Tables SP3, SP6 and SP8). To eliminate possible confounding by the influence of *CYP2C9* SNP variant alleles, the associations of rs4244285 (*CYP2C19*2*) were investigated only in wild-type homozygotes for both *CYP2C9* SNPs (n = 166 men and 193 women). However, results did not yield any significant associations (data not presented).

By contrast, the **rs12248560 (*CYP2C19*17*) variant allele,** located in the promotor region, leads to induced enzyme expression and, presumably, enhanced activity (Rosemary & Adithan 2007; Hirota et al., 2013). Indeed, in the present study, its presence was associated with higher levels of HMW PH and DINCH secondary metabolites in urine (Fig. 2 and Tables SP2 and SP8). Among men, carriers of at least one variant allele had significantly higher urine levels of all three DEHP secondary metabolites. Among women, the trends were similar but significant only in the case of variant allele homozygotes (*CYPC2C19*17/*17*).

The differences between male variant allele carriers and non-carriers were as follows: 5OH-MEHP (P50: 9.02 vs 8.84 µg/L SG; coef: 0.30), 5oxo-MEHP (P50: 5.88 vs. 4.91 µg/L SG; coef: 0.31), and 5cx-MEPP (P50: 10.4 vs. 8.42 µg/L SG; coef: 0.30); for variant homozygotes (*C19*17/*17*) the differences were even higher (coef: 0.40–0.46).

The differences between female variant homozygous carriers and wild-type carriers were as follows: 5OH-MEHP (P50: 6.63 vs 5.35 µg/L SG; coef: 0.36), 5oxo-MEHP (P50: 4.77 vs. 3.53 µg/L SG; coef: 0.35), and 5cx-MEPP (P50: 9.23 vs 6.67 µg/L SG; coef: 0.36). Furthermore, *C19*17/*17* women also had significantly higher levels of oxo-MINCH (P50: 0.45 vs. 0.20 µg/L SG; coef: 0.60), however, model performance did not pass the diagnostic analyses (Table SP8).

With respect to other PHs, the presence of the rs12248560 (*CYP2C19*17*) variant allele among women was significantly associated with slightly higher levels of cx-MiNP (P50: 1.97 vs. 1.65 µg/L SG; coef: 0.23) and OH-MiDP (P50: 0.40 vs. 0.32 µg/L SG; coef: 0.27).

Unlike CYP2C9, in the case of CYP2C19 no significant or consistent influence of the SNP on metabolite ratios was observed (Table SP8). Similarly, the *CYP2C19*17* allele was previously associated with faster clearance of certain drugs (e.g. escitalopram, sertraline) from patients' serum but did not show a significant effect on pharmacokinetic parameters when compared with the wild-type enzyme (Li-Wan-Po et al., 2010; Hirota et al., 2013). Therefore, for a better interpretation of the role of *CYP2C19*17* in PHs and DINCH biotransformation, further studies are needed.

*3.3.1.3. CYP2D6 SNP: rs39892097 (CYP2D6*4).* Finally, in the case of ***CYP2D6***, its most common SNP in Caucasians is **rs3892097 (*CYP2D6*4*)**. The presence of its variant allele was previously reported to result in reduced enzyme activity or, in the case of homozygous carriers, in an inactive enzyme (Ingelman-Sundberg, 2005; He et al., 2015).

The presence of the rs3892097 (*CYP2D6*4*) variant allele in the present study showed a tendency towards lower urine levels of PHs and DINCH metabolites and some of their ratios compared with wild-type carriers, but none of the associations were significant (Table SP3, SP6, and SP8). However, when considering only homozygous variant allele carriers (*CYP2D6*4/*4*), men indeed expressed significantly lower urine levels of all measured DEHP, DiNP and DiDP secondary metabolites compared with *CYP2D6*1/*4* and *CYP2D6*1/*1* carriers, while among women, such associations were not observed (data not shown). The low number of homozygous variant allele carriers (*CYP2D6*4 /*4*; 9 men and 9 women; Table 5) did not present sufficient statistical power to test associations in models; therefore, such associations should be further studied on a larger population size. Nevertheless, our observed results are in line with the statement by He et al. (2015) that homozygous variant allele carriers are most commonly associated with the phenotype of poor metabolizers.

3.3.2. UDP-glucuronosyl transferases – UGTs

According to previous studies, that have measured free and conjugated forms of PHs and DINCH primary and secondary metabolites in human urine, the majority of metabolites studied in the present work (Table 1) are excreted predominantly in glucuronidated form (>70%); only MEP and cx-MINP metabolites of DEP and DiNP, respectively, are excreted mainly in free form (Silva et al., 2003, 2013; Frederiksen et al., 2007; Seckin et al., 2009; Saravanabhavan and Murray, 2012).

*3.3.2.1. UGT2B15 SNP: rs1902023 (UGT2B15*2).* In the present study, we did not observe any significant associations between the SNP **rs1902023 (*UGT2B15*2*)** variant allele and urine levels or ratios of pH and DINCH metabolites, regardless of sex (Tables SP4, SP7, and SP9). For DEHP such results are in line with the in vitro study reporting negligible activity of the UGT2B15 recombinant enzyme in the glucuronidation of MEHP (Hanioka et al., 2017). Moreover, the influence of rs1902023 on its enzyme activity has been reported inconsistently; it has been associated with with a higher clearance of total (undefined) PHs in the serum of homozygous variant allele carriers (Luo et al., 2020) and by contrast, with decreased glucuronidation capacity for some anxiolytic pharmaceuticals (e.g. oxazepam, lorazepam) in kidney cells (HK293) (Guillemette, 2003; Clarke and Jones, 2009) and bisphenol A (Hanioka et al., 2011).

*3.3.2.2. UGT1A7 SNP: rs11692921 (UGT1A7*3).* **UGT1A7** is an extrahepatic enzyme expressed mainly in the small intestines (also in oesophagus, stomach, lungs and pancreas), and its common SNP **rs11692021 (*UGT1A7*3*)** was reported to lead to reduced activity (Guillemette et al., 2000; Miners et al., 2002; Guillemette, 2003; Clarke and Jones, 2009). However, in the present study, carriers of the rs11692021 *CYP1A7*3* variant allele showed a tendency towards higher excretion of all DEHP metabolites when compared with wild-type carriers (Table SP5), but no associations were statistically significant (Table SP9). Moreover, homozygous variant allele carriers (*UGT1A7*3/*3*) compared to wild-type carriers show significantly higher urine levels of MBzP (P50: 3.87 vs 2.26 µg/L SG; coef: 0.37) and MiBP (P50: 28.8 vs. 16.3 µg/L SG; coef: 0.38) among women, and among men higher urine levels of OH-MINCH (P50: 1.14 vs. 0.88 µg/L SG; coef: 0.63) (Fig. 3, Tables SP5 and SP9).

Interpreting the observed higher levels of metabolites in the urine of individuals with expected reduced UGT1A7 activity is challenging, but it could be related to either interaction with other undefined UGTs or their induced activity in the liver; glucuronidation can be compensated across UGT isoforms (Gao et al., 2021). For instance, UGT2B7 and UGT1A9 enzymes in the liver have shown the highest activity towards MEHP glucuronidation (Hanioka et al., 2016). However, their polymorphisms rs7439366 (*UGT2B7*2*) and rs72551330 (*UGT1A9*3*), respectively, unfortunately were not investigated in the present study due to their low occurrence in European populations or the unavailability of TaqMan genotyping assays (ThermoFisher Scientific, 2021). The role of their polymorphisms in PHs and DINCH biotransformation should be investigated in the future using a larger population size and alternative genotyping methods.

3.4. Evaluation of statistical models, predictors and study limitations

In the present study, predictors used in multiple linear regression models explained 1–27% variability of pH and DINCH metabolite levels in urine or their ratios – the highest for DEHP secondary metabolites levels (5OH-MEHP, 5oxo-MEHP, and 5cx-MEHP) – and only 2–11%

variability of DEHP and DINCH metabolite ratios (Table SP10). Among the predictors used, a significant influence was observed for year of sampling, age, current smoking in men, and, in the case of DEHP metabolites, the 5cx-MEPP/5OH-MEHP ratio (Table SP10). The model results suggest that urinary PHs metabolite concentrations decreased significantly over the sampling period from 2008 to 2014. As studies observed these compounds to be stable in urine at −70 °C for several years (Silva et al., 2008; Samandar et al., 2009), these trends can be attributed rather to the general utilization patterns of PHs in the European market(Tranfo et al., 2018; Wang et al., 2019) than to compound degradation. The obtained results for age and BMI are inconsistent, which agrees with the literature, in which to date, the effect of neither could be determined with certainty (Goodman et al., 2014; Chiang et al., 2016; Koch et al., 2017). The negative associations of current smoking among men with urinary metabolite levels (mainly DEHP-derived) is in need of re-evaluation on a population with a larger number of active smokers. The inclusion of 5cx-MEPP/5OH-MEHP as a model predictor indicated its potential to approximate for the time of DEHP exposure. According to the hypothesis by Lorber et al. (2011) the ratios in the present study with geometric means ≥1 in women and men (Table 4), indicate that exposure in general occurred 10 h or more before the spot urine sampling. However, one should keep in mind that this ratio might also be influenced by the differences in metabolism (Meeker et al., 2012), therefore, its use should be confirmed in future studies including also data on DEHP exposure. Selenium in blood was positively associated with DINCH metabolite levels in women, while no significant influence was observed for PH metabolite levels in either men or women; the observed selenium levels in our study population are within the reference range for adult populations (58–243 ng/mL) (Roberts et al., 2012).

Low coverage of the variability in the models could be explained by the missing information on individual's exposure (e.g. usage of personal care products or food packaging within the last days prior to sampling) as well as introduced uncertainty by the use of random spot urine samples, both of which represent a limitation of the present study. Therefore, in future studies, first morning urine samples should be obtained to limit the variation in time between exposure and measurement (Bastiaensen et al., 2020). As the number of measurable PH and DINCH primary and secondary metabolites in urine is continuously increasing (Schütze et al., 2017; Wang et al., 2019), more of them should be included in our future studies to more adequately estimate the influence of studied SNPs on PHs and DINCH biotransformation. Furthermore, especially in the case of UGT SNPs, the information on percentage of glucuronide-conjugated versus un-conjugated (free) forms of metabolites would be of great importance, as it would give more relevant insight into the biotransformation II pathway.

4. Conclusions

The present study investigated, for the first time, the possible influence of SNPs in *CYP2C9, CYP2C19, CYP2D6, UGT2B15* and *UGT1A7* genes on the biotransformation of phthalates and DINCH using human biomonitoring data on men and lactating women.

Our results confirm the previously only in vitro observed influence of rs1799853 (*CYP2C9*2*) and rs1057910 (*CYP2C9*3*) on the reduced biotransformation of DEHP and suggest a negative influence of rs1057910 (*CYP2C9*3*) on DINCH biotransformation. The latter was observed only in men. Moreover, rs12248560 (*CYP2C19*17*) was associated with a higher excretion of secondary metabolites of DEHP (men and women), DiNP and DiDP (women), while the rs11692021 (*UGT1A7*2*) resulted in higher urine levels of BBzP, DiBP (women) and DINCH metabolites (men).

Although most of the variance in phthalates and DINCH metabolites urinary levels and ratios remains unexplained, we demonstrate that the above-mentioned SNPs could represent important biomarkers of susceptibility to phthalates and DINCH exposure that have been so far unrecognised.

As genes studied in the present study were selected based on the DEHP biotransformation, in future, more attention should be directed into the identification of possible specific CYP and UGT isoforms and their SNPs, which are the most active in the biotransformation of DINCH and other phthalates.

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CRediT authorship contribution statement

Anja Stajnko: Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Agneta Annika Runkel:** Conceptualization, Investigation, Writing – review & editing, Resources. **Tina Kosjek:** Writing – review & editing. **Janja Snoj Tratnik:** Writing – review & editing. **Darja Mazej:** Writing – review & editing. **Ingrid Falnoga:** Writing – review & editing, Resources. **Milena Horvat:** Supervision, Project administration, Funding acquisition, Writing – review & editing, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.107046.

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Chapter 4

Conclusions

The presented dissertation aimed at assessing the exposure of the Slovenian general population as well as susceptible groups to harmful persistent and non-persistent contaminants, to identify the determinants of exposure, to evaluate existing analytical methods and to develop new ones for the determination of such chemicals in urine, to evaluate if the current exposure levels exceed the respective guidance values, and to address the influence of individual susceptibility on the biotransformation of pollutants.

We can conclude that the evaluated populations are exposed to multiple non-persistent and persistent chemicals and that these compounds can be detected in human matrices at varying concentrations and often high detection frequencies.

The candidate's PhD research work is gathered in five manuscripts:

- **Manuscript 1:** Within the frame of the first hypothesis, we aimed at assessing the exposure of Slovenian men, women, and children to five PHs, to assess potential differences in exposure, to identify determinants of exposure, and to compare the observed levels with other studies. We can confirm that Slovenian women, men, and especially children are exposed to DEHP, DEP, BBzP, DnBP, and DiBP. We observed higher concentrations in the urine of children compared to adults, in the rural sampling location compared to Ljubljana, and we observed differences based on the level of education. Via available questionnaire data, we identified multiple dietary, industrial, and personal care product sources, as well as specific lifestyles as determinants of exposure, including some sources that are applicable for family units. Overall, exposure in Slovenia falls within the European average. The results summarized in this manuscript confirm the first hypothesis.
- **Manuscript 2:** From the assessment of exposure to POPs (PCDD/Fs, PCBs, PBDEs, and OCPs) of Slovenian men and lactating women, we can conclude that the obtained levels are low compared to other studies. We hypothesize that the underlying cause for this is Slovenia's geographic location on the Southern side of the Alps that provide shelter from the westerly winds. The determined concentrations could still be associated with some dietary and industrial sources. Within a national comparison, Bela krajina and Ljubljana stood out with the highest POP burden of the population. Therefore, we can confirm the second hypothesis.

Complementing the association between exposure and questionnaire data, in the third manuscript, we focused on the analytical side of exposure assessment. We, therein, evaluated and summarized the available methods for the determination of selected contaminants of emerging concern.

- **Manuscript 3:** From the review of analytical methods for the determination of CECs in urine, we can conclude that the available methods mostly use approaches that can be applied in HBM laboratories without further modifications, such as LLE/SPE extraction followed by GC or LC analysis. This makes them suitable for the inclusion in large-scale HBM studies. The highest number of available methods, however, was 9, therefore, we conclude that these compounds are so far understudied and more literature on analytical procedures is necessary in order to prepare for the inclusion of these compounds in wide-scale HBM studies. This confirms the third hypothesis.

After having evaluated the methodological perspective of exposure assessment as well as the associations between concentrations of chemicals in different matrices with questionnaire data, the fourth hypothesis was addressed in the fourth manuscript, where we evaluated the exposure of Slovenian men and lactating women to PHs, DINCH, and environmental phenols, and calculated the HQ to preliminarily assess if at current exposure levels, adverse health effects are possible.

- **Manuscript 4:** We can conclude that women are more exposed to parabens, compared to men, but men have higher urinary concentrations of PHs, DINCH, and BPs. The HQ is below 1 for both populations, however the limited number of analytes might bias the results. As such, we suggest to repeat the assessment using a larger number of compounds and different populations, such as non-lactating women and children. The fourth hypothesis can, therefore, be preliminarily confirmed, but is in need of further verification.

This simplified approach of risk assessment is based on a number of assumptions, such as the appropriateness of guidance values, and neglects the influence of individual susceptibility. Thus, the obtained conclusions should be taken as an indicatory preliminary result that is in need of further elaborations. One step to address these limitations was undertaken within the frame of the fifth manuscript, where the fifth hypothesis is being addressed. This hypothesis states that genetic predisposition can influence the response of individuals to harmful chemicals. This was evaluated on the example of phthalates and DINCH and selected enzymes (CYPs and UGTs) that are involved in their biotransformation.

- **Manuscript 5:** The results suggest that selected SNPs in genes of *CYP2C9, CYP2C19, CYP2D6, UGT2B15* and *UGT1A7* can influence the biotransformation of PHs and DINCH. We can confirm the previously only in vitro observed negative effect of rs1799853 (*CYP2C9*2*) and rs1057910 (*CYP2C9*3*) on PH and DINCH biotransformation and associated rs12248560 (*CYP2C19*17*) and rs11692021 (*UGT1A7*2*) with higher excretion of some metabolites. The observed negative effect of both CYP2C9 genes on the biotransformation of DEHP was even more pronounced in carriers of both variant alleles. We propose these SNPs to be regarded as novel biomarkers of susceptibility towards PH and DINCH exposure. The results presented in this manuscript confirm the fifth hypothesis.

Overall, this dissertation presents an overview over the exposure of the Slovenian population to POPs, PHs, DINCH, PBs, BPs, and TCS and identifies determinants of exposure. Furthermore, it addresses the methodological side of exposure assessment via the review of analytical methods for the measurement of CECs in urine. By calculation of the

HQ, we obtained the preliminary result that at current exposure levels adverse health effects are not assumed to occur. This conclusion, however, is in need of a more detailed analysis including more compounds, different populations, and an evaluation of the appropriateness of the applied guidance values. Thus, it should be regarded with caution. Additionally, we evaluated differences in individual resilience towards exposure to PHs and DINCH and identified four SNPs in three genes of CYPs and UGTs as potential biomarkers of susceptibility.

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Awards

Best young presentation recognition, 2019 poster award: Runkel, A. A., Snoj Tratnik, J., Mazej, D., Horvat, M. (2019) Exposure to endocrine disruptors: urinary phthalate metabolite concentrations in the Slovenian general population. *BIONANOTOX 2019, 10th international conference "Biomaterials and nanobiomaterials: Recent Advances Safety-Toxicology And Ecology Issues", Including Russian-Hellenic Workshop and School of Young Scientists, 05th - 12th May. Heraklion. Crete - Greece.*

3rd winner of poster presentation: Runkel, A. A., Snoj Tratnik, J., Mazej, D., & Horvat, M. (2021). Exposure to Phthalates of Women, Men, and Children in Slovenia. *HBM4EU 5th consortium meeting, 21st - 22nd September. Berlin - Germany.*

Biography

The author of this thesis Agneta Annika Runkel was born on October 15th, 1991 in Frechen, Germany. She attended the Janusz-Korczak primary school in Erftstadt Erp, Germany, from 1998 until 2002 and finished her high school education at Gymnasium Marienschule Euskirchen, Germany, in 2011. In October 2011, she enrolled in the 2-major Bachelor's program "Biology/English Studies" at the University of Bonn, Germany, within which she completed a semester at the National University of Ireland, Maynooth, as part of the Erasmus exchange program. In 2014, she changed the subject "English Studies" to "Geography" and obtained the degree "Bachelor of Science" in the subjects "Biology" and "Geography" in 2015. The same year, she enrolled into the international Master's program "Global Change Ecology" at the University of Bayreuth, Germany, that is part of the Elite Network of Bavaria. In March 2016, she successfully attended the science school "Disturbance Driven Island Ecology, La Palma, Canary Islands" under the supervision of Prof. Dr. Anke Jentsch. From August 2016 until January 2017, she joined the Department of Analytical and Environmental Chemistry (ACES) at the University of Stockholm, Sweden, for a 6-month training in analytical chemistry, specifically in the sample preparation techniques for the multi-elemental analysis of fish muscle and owl liver samples as part of the Erasmus exchange program. From January 2017 until April 2017, she received further training in analytical chemistry, specifically in the multi-elemental analysis of hair samples at the Department of Environmental Sciences at the Jožef Stefan Institute, Ljubljana, Slovenia. In April 2017, she joined the group of atmospheric chemistry at the Bayeuth Center of Ecology and Environmental Research (BayCEER) that is part of the University of Bayreuth, Germany. Here she conducted her master thesis work under the supervision of Prof. Dr. Andreas Held and Dr. Sarmite Kernchen with the title "Organic Acids in the Atmosphere: An HPLC Analysis of Fog and Precipitation". She successfully completed her Master studies in 2018 with the degree "Master of Science" and the grade 1.4 (very good). In October the same year, she started her doctoral studies at the Jožef Stefan International Postgraduate School, Ljubljana, Slovenia, under the supervision of Prof. Dr. Milena Horvat and Assoc. Prof. Dr. Tina Kosjek.

The aim of her PhD is to assess the exposure of the Slovenian population to selected persistent and non-persistent contaminants, identify the determinants of exposure, develop a sensitive and robust analytical method for the determination of phthalate and DINCH metabolites in urine, to evaluate if the determined exposure levels exceed common guidance values, and to identify novel biomarkers of susceptibility for phthalate and DINCH exposure. During her studies, she attended workshops and seminars as well as (inter-) national conferences to broaden her knowledge and to present her work.