# Combined chemical exposure using exposure loads on human biomonitoring data of the 4th Flemish Environment and Health Study (FLEHS-4)

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**Abstract**

To improve our understanding of simultaneous internal exposure to multiple chemicals, the concept exposure load (EL) was used on human biomonitoring (HBM) data of the 4th FLEHS (Flemish Environment and Health Study; 2017-2018). The investigated chemicals were per- and polyfluoroalkyl substances (PFASs), bisphenols, phthalates and alternative plasticizers, flame retardants, pesticides, toxic metals, organochlorine compounds and polycyclic aromatic hydrocarbons (PAHs). The EL calculates "the number of chemicals to which individuals are simultaneously internally exposed above a predefined threshold". In this study, the 50th and 90th percentile of each of the 45 chemicals were applied as thresholds for the EL calculations for 387 study participants. Around 20% of the participants were exposed to > 27 chemicals above the P50 and to > 6 chemicals above the P90 level. This shows that participants can simultaneously be internally exposed to multiple chemicals in relatively high concentrations. When the chemical composition of the EL was considered, the variability between individuals was driven by some chemicals more than others. The variability of the chemical profiles at high exposure loads (EL-P90) was somewhat dominated by e.g. organochlorine chemicals, PFASs, phthalates, PAHs, organophosphate flame retardants, bisphenols (A & F), pesticides, metals, but to a lesser extent by brominated flame retardants, the organophosphorus flame retardants TCIPP & TBOEP, naphthalene and benzene, bisphenols S, B & Z, the pesticide 2,4-D, the phthalate DEP and alternative plasticizer DINCH. Associations between the EL and exposure determinants suggested determinants formerly associated with fat soluble chemicals, PFASs, bisphenols, and PAHs. This information adds to the knowledge needed to reduce the exposure by policymakers and citizens. However, a more in depth study is necessary to explore in detail the causes for the higher EL in some individuals. Some limitations in the EL concept are that a binary number is used for exposure above or below a threshold, while toxicity and residence time in the body are not accounted for and the sequence of exposure in different life stages is unknown. However, EL is a first useful step to get more insight in simultaneous chemical exposure in higher exposed subpopulations (relative to the rest of the sampled population).

Keywords: Exposure load; HBM; FLEHS; combined chemical exposure; relatively high exposed subpopulation; exposure determinants

## Highlights

- **People are exposed to a mixture of chemicals**
- **The exposure load (EL) calculates the number of chemicals to which individuals are simultaneously internally exposed above a predefined threshold**
- **20% of the FLEHS-4 participants were exposed to > 6 chemicals out of 45 above the P90 level**
- **A cluster analysis showed that some persons are more exposed to persistent organic pollutants such as PFAS and PCBs**
- **This was also observed in associations between the EL and exposure determinants**

## 1. Introduction

There are few data on combined human internal exposure for the majority of the >100,000 chemicals available on the European market (ECHA, 2020). A considerable fraction of these chemicals is found in personal care products, electronics, food packaging, pharmaceuticals, building materials and home furnishings which leads to widespread human exposure (UN, 2020). There is also chemical exposure of humans via the environment, e.g. chemicals emitted during burning processes and through contamination of water and soil. People are typically not exposed to one chemical at a time, but to a mixture of chemicals and, due to the long half-life of many chemicals in humans, past external exposure can still be detected in the internal exposure. A paradigm shift from the chemical-by-chemical assessment towards an assessment of combined exposure to multiple chemicals is therefore imperiously necessary, together with an expansion of the exposure concepts towards mixtures. Unfortunately, the number of mixtures that can be formed from the thousands of environmental chemicals is enormous. It can be assumed that simultaneous exposure to multiple chemicals in the environment is often not random, but related to e.g. identical sources or exposure pathways, comparable personal characteristics or lifestyle factors.

To investigate the uniqueness of the combination of chemicals to which a person is internally exposed at a given point in time and whether we can distinguish subpopulations that are highly exposed to many chemicals, the concept of exposure load (EL) was used. It was slightly adapted from the Canadian Health Measurement Survey (CHMS) (St-Amand, 2019; Willey et al., 2021) and applied on HBM data from adolescents monitored in the 4$^{th}$ Flemish Environment and Health Study (FLEHS-4). Human biomonitoring studies measure concentrations of chemicals or their metabolites in body fluids or tissues (Angerer et al., 2007). Measurements of different exposure biomarkers in individual urine and/or blood samples provide an aggregated picture of the chemical internal exposure of an individual resulting from different exposure routes and from various sources.

The EL is based as first on establishing whether a person is exposed (assigned a value of 1) or non-exposed (assigned a value of 0) above a predefined concentration threshold of a given biomonitored chemical, and then summing the exposure counts. Yet, EL does not take toxicity into account. The technique finds its origin in frequent itemset mining (FIM), initially developed by marketing researchers to identify items that are frequently purchased together (Borgelt, 2016). It was already applied by Kapraun et al. (2017) to the 2009-2010 NHANES (National Health And Nutrition Examination Survey) dataset. FIM is also used to identify relationships between chemicals, health biomarkers and disease (Bell and Edwards, 2015). Other applications of FIM are the evaluation of the

presence of chemicals in food (Krishan et al., 2017) and the identification and quantification of associations between environmental and social stressors (Huang et al., 2017). This adapted approach of the CHMS exposure load was then tested on HBM data of Flemish adolescents (Belgium) participating in the 4th FLEHS campaign.

The study had three goals: a) calculate the EL and study the distribution across the FLEHS-4 population, b) study the chemical composition of the EL and c) identify determinants of EL variability, which could lead to the identification of disproportionally exposed subpopulations.

The present study was a proof of concept for the H2020 HBM4EU (Human Biomonitoring for Europe) project, which aims to develop a sustainable European wide HBM network (2017-2021). HBM4EU will also provide better evidence of the actual exposure of citizens to chemicals and the possible health effects to support policy making (https://www.hbm4eu.eu/about-hbm4eu/).

## 2. Methodology

### 2.1 Population

The 4th cycle of the Flemish Environment and Health study, FLEHS-4, gives a snapshot of exposure to chemicals in a general population of adolescents (14-15y) in the period 2017-2018 when samples were taken. Adolescents are not occupationally exposed and serve as a sentinel for the environment where they grew up in. Details of the recruitment protocols have been reported before (Den Hond et al., 2009; Baeyens et al., 2014; De Craemer et al., 2016). In FLEHS-4, a Flemish study population of 428 participants background exposed was recruited. The aim was to enrol equal numbers of girls and boys and to reflect the proportion of Flemish adolescents in all educational levels. In order to obtain a geographically representative sample, adolescents were recruited through schools in the five Flemish provinces, proportional to the number of inhabitants per province. To account for seasonal variation, recruitment was spread over one year with no recruitment during examination periods and summer holidays (June, July, August, September). Inclusion criteria were: informed consent signed by participants and parents (no cases where legal guardians needed to sign), living in Flanders for at least 5 years, the ability to fill out extensive questionnaires in Dutch. Exclusion criteria were: pregnancy, more than 1 out of 3 questionnaires missing, blood and urine sample missing, being held back in school for more than 1 year, attending boarding school.
Study participants and their parents filled in questionnaires in Dutch with information needed for interpretation of biomarkers of exposure and of effect. The questionnaires covered information on health status, dietary habits, home environment, lifestyle and socio-economic status (SES). The HBM study was approved by the Ethical committee of the Antwerp University Hospital (registration number B300201732753).

### 2.2 Chemicals and exposure biomarkers

Chemicals of interest to measure during the FLEHS-4 campaign were selected in a transparent and participatory way involving scientists, policy makers and other stakeholders, based on technical criteria, health and exposure-related criteria and policy relevance (Schoeters et al., 2012b). The involved laboratories had to fulfil standard quality assurance and quality control (QA/QC). Validation dossiers were required and participation in international ring tests was desired (Esteban López et al., 2021). A broad range of chemicals from various potential sources were included in the EL analysis including several emerging chemicals: polyaromatic hydrocarbons (PAHs), benzene (Bz), metals,

pesticides, organochlorine compounds (OC), brominated – and organophosphate flame retardants, bisphenols, per- and polyfluoroalkyl substances (PFASs) and phthalates and their alternatives. An overview is given in
Table 1.

For the EL analysis, concentrations in blood were expressed per volume unit (µg/L) for PFASs and lead and were normalized by blood fat for organochlorine compounds and brominated diphenyl ethers, while urinary concentrations were standardised by specific gravity. Only those biomarkers for which at least 30% of the values was above the LOD or LOQ reported by the laboratories were considered for the EL calculation. The value of 30% was chosen as cut-off (or threshold), as it was not the intention to focus on chemicals detected only in a small part of the studied population (<30%). The total number of chemicals considered for the EL analysis was 45.

*Table 1. List of chemicals & biomarkers considered in the EL (exposure load) analysis*

| **Chemical group** | **Nr** | **Chemical** | **Biomarker** | **Grouping*** |
|---|---|---|---|---|
| PAHs in urine | 1 | Pyrene (PYR) | 1-Hydroxypyrene (1-OH-PYR) | |
| | 2 | Naphthalene (NAP) | 2-Hydroxynaphthalene (2-OH-NAP) | |
| | 3 | Fluorene (FLU) | Sum of 2- and 3-Hydroxyfluorene (2&3-OH-FLU) | |
| | 4 | Phenanthrene (PHE) | 2-Hydroxyphenanthrene (2-OH-PHE) | Considered as group in analysis |
| | | | 3-Hydroxyphenanthrene (3-OH-PHE) | |
| | | | Sum of 1- and 9-Hydroxyphenanthrene (1&9-OH-PHE) | |
| Benzene in urine | 5 | Benzene (Bz) | T,t'-muconic acid (t,t'-MA) | |
| Metals in urine** | 6 | Cadmium (Cd) | Cadmium (Cd) | |
| | 7 | Thallium (TI) | Thallium (TI) | |
| Metals in blood | 8 | Lead (Pb) | Lead (Pb) | |
| Pesticides in urine | 9 | Pyrethroïd pesticides 3-PBA | 3-Phenoxybenzoic acid (3-PBA) | |
| | 10 | Chlorpyrifos (CPS) | 3,5,6-Trichloro-2-pyridinol (TCPY) | |
| | 11 | Phenoxy herbicide 2,4-D | 2,4-dichlorophenoxy acetic acid (2,4-D) | |
| | 12 | Glyphosate herbicide (GLY) | Glyphosate (GLY) | Considered as group in analysis |
| | | | Aminomethylphosphonic acid (AMPA) | |
| Persistent Organic Pollutants (POPs) in serum: Organochlorine Compounds (OC) | 13 | Sum polychlorinated biphenyls (Sum PCBs) (138,153,180) | Sum PCBs 138,153,180 | |
| | 14 | Hexachlorobenzene (HCB) | Hexachlorobenzene (HCB) | |
| | 15 | Dichloro-diphenyl-trichloroethane (DDT) | DDT | Considered as group in analysis |
| | | | DDT metabolite: p,p'-DDE | |
| | 16 | Oxychlordane (OXC) | Oxychlordane (OXC) | |
| | 17 | Trans-nonachlor (TN) | Trans-nonachlor (TN) | |
| | 18 | Beta-hexachlorocyclohexane (HCH) | Beta-hexachlorocyclohexane (HCH) | |
| POPs: Brominated diphenyl ethers (BDEs) in serum | 19 | Brominated diphenyl ether (BDE)47 | BDE47 | |
| | 20 | BDE99 | BDE99 | |
| | 21 | BDE154 | BDE154 | |
| Organophosphate flame retardants in urine | 22 | Diphenyl phosphate (DPHP) | Diphenyl phosphate (DPHP) | |
| | 23 | 2-Ethylhexyl diphenyl phosphate (EHDPHP) | 2-Ethylhexyl phenyl phosphate (EHPHP) | Considered as group in analysis |
| | | | 2-Ethyl-5-hydroxyhexyl diphenyl phosphate (5-OH-EHDPHP) | |
| | 24 | Tris(2-chloroisopropyl) phosphate (TCIPP) | 1-Hydroxy-2-propyl bis(1-chloro-2-propyl) phosphate (BCIPHIPP) | |
| | 25 | Tris(2-butoxyethyl) phosphate (TBOEP) | 2-Hydroxyethyl bis(2-butoxyethyl) phosphate (BBOEHEP) | |
| | 26 | Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) | Bis(1,3-dichloro-2-propyl) phosphate (BDCIPP) | |
| | 27 | Perfluorooctane sulfonate (PFOS) | Perfluorooctane sulfonate (PFOS) | |

| Chemical group | Nr | Chemical | Biomarker | Grouping* |
|---|---|---|---|---|
| POPs: Per- and polyfluoroalkyl substances (PFAS) in serum | 28 | Perfluorohexane sulfonate (PFHxS) | Perfluorohexane sulfonate (PFHxS) | |
| | 29 | Perfluorodecanoic acid (PFDA) | Perfluorodecanoic acid (PFDA) | |
| | 30 | Perfluorononanoic acid (PFNA) | Perfluorononanoic acid (PFNA) | |
| | 31 | Perfluorooctanoic acid (PFOA) | Perfluorooctanoic acid (PFOA) | |
| Bisphenols (BP) in urine | 32 | Bisphenol-Z (BPZ) | Bisphenol-Z (BPZ) | |
| | 33 | Bisphenol-B (BPB) | Bisphenol-B (BPB) | |
| | 34 | Bisphenol-S (BPS) | Bisphenol-S (BPS) | |
| | 35 | Bisphenol-F (BPF) | Bisphenol-F (BPF) | |
| | 36 | Bisphenol-A (BPA) | Bisphenol-A (BPA) | |
| Phthalates and alternatives in urine | 37 | Diisodecyl phthalate (DIDP) | Mono-oxo-isodecyl phthalate (OXO-MiDP) | Considered as group in analysis |
| | | | Mono-carboxy-isononyl phthalate (CX-MiDP) | |
| | | | Mono-hydroxy-isodecyl phthalate (OH-MiDP) | |
| | 38 | 1,2-Cyclohexane dicarboxylic acid, diisononyl ester (DINCH) | Cyclohexane-1,2-dicarboxylic acid, mono(carboxyoctyl) ester (MCOCH) | Considered as group in analysis |
| | | | Cyclohexane-1,2-dicarboxylic acid, mono(cis-hydroxy-isononyl) ester (MHNCH) | |
| | 39 | Di-isononyl phthalate (DINP) | Monocarboxyoctyl phthalate (MCOP) | Considered as group in analysis |
| | | | Mono-hydroxy-isononyl phthalate (MHNP) | |
| | 40 | di-2-ethylhexyl terephthalate (DEHTP) | mono(2-ethyl-5-hydroxyhexyl) terephthalate (OH-MEHTP) | |
| | 41 | Di-2-ethylhexyl phthalate (DEHP) | Mono(2-Ethylhexyl) phthalate (MEHP) | Considered as group in analysis |
| | | | Mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) | |
| | | | Mono(2-ethyl-5-oxohexyl) phthalate (MEOHP) | |
| | | | Mono-(2-ethyl-5-carboxypentyl) phthalate (CX-MEPP) | |
| | 42 | Benzylbutyl phthalate (BzBP) | Monobenzyl phthalate (MBzP) | |
| | 43 | Di-*n*-butyl phthalate (DBP) | Monobutyl phthalate (MBP) | |
| | 44 | Di-isobutyl phthalate (DiBP) | Monoisobutyl phthalate (MiBP) | |
| | 45 | Diethyl phthalate (DEP) | Monoethyl phthalate (MEP) | |

* Grouping process applied in exposure load analysis is explained further (see section 2.3.1).

** Arsenic was only measured in half of the participants and is thus not considered here.

Biomarkers for which less than 30% of measurements was above LOD or LOQ were: 4-hydroxyphenanthrene (4-OH-PHE), perfluoroheptane sulfonate (PFHpS), perfluorobutane sulfonate (PFBS), perfluorododecanoic acid (PFDoDA), perfluoroundecanoic acid (PFUnDA), perfluorohexanesulfonic acid (PFHxA), perfluoropentanoic acid (PFPeA), perfluoroheptanoic acid (PFHPA), bisphenol-AF (BP-AF), mono-isononyl-cyclohexane-1,2-dicarboxylate (MINCH), mono-2-ethylhexyl terephthalate (MEHTP), mono(2-ethylhexyl) adipate (MEHA), mono(2-ethyl-5-hydroxyhexyl) adipate (OH-MEHA), 1,2-di(2-ethylhexyl) trimellitate (DEHTM), BDE28, BDE100, BDE153, BDE183, gamma-hexachlorocyclohexane (γ-HCH), 4-hydroxyphenyl diphenyl phosphate (4-OH-TPHP), 4-hydroxyphenyl phenyl phosphate (4-OH-DPHP), bis(1-chloro-2-propyl) phosphate (BCIPP), tris(chloroethyl) phosphate (TCEP), bis(2-butoxyethyl) phosphate (BBOEP), bis(2-butoxyethyl) 3'-hydroxy-2-butoxyethyl phosphate (3-OH-TBOEP), di-n-butyl phosphate (DNBP).

Details of the sampling and an overview of biomarkers measured in previous FLEHS campaigns have been previously reported (Schoeters et al., 2012a; Schoeters et al., 2012b; Schoeters et al., 2017; Steunpunt Milieu en Gezondheid, 2020). Organophosphate flame retardants were measured extensively for the first time in FLEHS-4 (Bastiaensen et al., 2021b).

In short, metals were measured in urine and blood by high resolution ICP-MS (Baeyens et al., 2014). The benzene metabolite, t,t'-muconic acid, was measured according to Ducos et al. (1990). Urine was purified with solid-phase extraction (SPE) using a strong anionic-exchange cartridge and retained components were eluted with acetic acid. Analysis was with an ultra-performance liquid

chromatography (UPLC) system coupled with a (Photodiode Array) PDA detector (Waters USA). Metabolites of polyaromatic hydrocarbons (PAHs) were analysed according to Onyemauwa et al. (2009) and Ramsauer et al. (2011). They were enzymatically released overnight, followed by a ultra-performance liquid chromatography tandem mass spectrometry analysis (UPLC-MS/MS) (Waters Xevo TQ-S). POPs (organochlorine compounds and PBDEs) were measured in serum using SPE and gas chromatography-electron capture negative ionization mass spectrometry (Dirtu et al., 2013). Metabolites of organophosphate flame retardants were extracted from urine by SPE on C18 cartridges and eluted with methanol. The analytes were separated by liquid chromatography on a biphenyl column and detected by triple quadrupole mass spectrometry (Bastiaensen et al., 2018; Bastiaensen et al., 2021b). The herbicide glyphosate and its main metabolite aminomethylphosphonic acid (AMPA) were analysed by gas chromatography with tandem mass spectrometry (GC-MS-MS), according to the procedure of Alferness and Iwata (1994) with some modifications (Hoppe, 2013). TCPY, a metabolite of the organophosphorus pesticide chlorpyrifos (CPS), 3-PBA, a shared metabolite of several synthetic pyrethroid pesticides, and the herbicide 2,4-Dichlorophenoxyacetic acid (2,4-D) were measured in urine. The analytical method comprised an SPE extraction of the deconjugated urine sample and analysis by liquid chromatography and triple-quadrupole mass spectrometry (Davis et al., 2013). Phthalates and alternative metabolites were extracted from urine by SPE on Oasis Max cartridges and thereafter eluted and concentrated. The analytes were separated by liquid chromatography on a biphenyl column and detected by triple quadrupole mass spectrometry (Bastiaensen et al., 2021a). PFAS were analysed via a dilute-and-shoot technique, measured with LC-MS/MS. Bisphenols were extracted from urine by SPE on Oasis Wax cartridges and thereafter eluted and concentrated. Analytes were further separated by gas chromatography using a DB-5MS capillary column and analysed by triple quadrupole mass spectrometry (Gys et al., 2021).

### 2.3 Exposure load (EL)

Detailed information about FIM, which serves as a as basis for the EL, can be found in Kapraun et al. (2017). Briefly, for "biomarker b", the concentration distribution is generated and descriptive statistics P50 ($50^{th}$ percentile) and P90 were derived. These values serve as discretization threshold. To further illustrate this approach, P50 is used. For each participant, it was checked whether the concentration for biomarker b was higher, equal or lower than the P50 value. If the concentration was higher or equal, the participant was assigned a value of 1 for biomarker b, in case the concentration was lower a value of 0 was assigned. In case the P50 was lower than the LOD or LOQ, then LOD or LOQ was used as threshold (this was the case for 4 chemicals). This discretization was repeated for all biomarkers considered (actually a binary 0 1 matrix was made). For some biomarkers, a grouping process was first performed before applying the discretization process (see next paragraph). Eventually, the total sum of all values (0 and 1) was taken as the exposure load for each participant (Willey et al., 2021). For the analysis, a valid value (0 or 1) for every single chemical involved was required for all participants.
Participants with missing data for one of the chemicals were excluded from the analysis. This means that all participants have theoretically the same maximal EL. A similar calculation can be made for discretization thresholds other than the P50. In this study, we applied the P50 and the P90 as thresholds. Exposure load were abbreviated as EL-P50 when P50 was used as threshold and EL-P90 when P90 was used as threshold.

#### 2.3.1 Grouping process prior to EL determination

Some chemicals in the analysis were assessed by more than one biomarker (see

Table 1): phenanthrene, DDT, glyphosate, 2-ethylhexyl diphenyl phosphate (EHDPHP), diisodecyl phthalate (DIDP), 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH), di-isononyl phthalate (DINP) and di-2-ethylhexyl phthalate (DEHP). For calculating the EL, biomarkers representing the same chemical were first grouped, i.e. biomarkers were expressed as molar mass and summed. In this way, each chemical involved can have a 0 or 1 value, which brings the maximum exposure load for a participant equal to 45.
This grouping process deviates somewhat from the one of CHMS. They considered groups of chemicals and for chemical groups with more than 1 biomarker (e.g. for the benzene chemical group: benzene in blood, S-phenylmercapturic acid (S-PMA) in urine, t,t'-muconic acid (t,t'-MA) in urine), if one or more biomarker had a concentration > predefined threshold, then +1 was assigned for that chemical group. More information can be found in the publication of Willey et al. 2021).

### 2.4 Statistical analysis

In a first step, to assess how strong each internal exposure to a chemical was associated with the EL, Pearson biserial correlation coefficients were calculated between the EL and the scores for each chemical (0 or 1) by which the EL was formulated. To analyse the chemical composition further into detail, a dendrogram was created with the heatmap function in R statistical analysis software package using default settings (Euclidian distance; clustering = complete linkage method) (R Core Team, 2018). Clusters were generated based on similarities between the chemical internal exposure of the individuals (based on binary 0 1 matrix).

Determinants of variability in EL were analysed. Negative binomial regression analysis (no fixed effects) was performed with SPSS Statistics 26. Variables considered were among others personal factors as sex and blood fat, questions related to the living environment as exposure to groundwater and use of a heating stove inside, questions related to food consumption, use of consumer products, information on socio-economic status (SES; categories for equivalent household income and highest education in household), information on degree of urbanization at the home address and lifestyle factors (e.g. sports). The Benjamini-Hochberg method was used to check for false discovery rates (FDR). A backward negative binomial multiple regression analysis was performed starting from variables having a significant association ($p<0.05$) in the univariate analysis.

## 3. Results and discussion

The simultaneous exposure to chemicals from various sources is a major concern, and up to now there are few attempts to describe and deal with the diversity of environmental chemicals in humans. However questions such as whether there are subpopulations that are highly exposed to a lot of chemicals, whether these are the same chemicals or chemical clusters and whether these are related to specific lifestyle and environmental factors are key questions for prevention. The exposure load concept is a unique way to identify individuals that are higher exposed to a combination of chemicals.

### 3.1 Adolescents

Of the 428 adolescents in the FLEHS-4 study population background exposed, 387 participants had no missing data for all chemicals involved. This means 387 participants (Male: 185; Female: 202) were included in the exposure load analysis.

### 3.2 Exposure load (EL)

Exposure load is reported for discretization thresholds P50 in Figure 1 and P90 in Figure 2. It is shown that 80% of the participants are simultaneously exposed to ≤ 27 out of 45 chemicals above the P50 and ≤ 6 chemicals above the P90. Ten percent of the participants are simultaneously exposed to ≤ 15 out of 45 chemicals above the P50 and ≤1 chemical above the P90 level.
Looking from another perspective, it also means for example that 20% of the participants are exposed to > 27 chemicals out of 45 above the P50 and to > 6 chemicals above the P90 level. This shows that participants can simultaneously be internally exposed to multiple chemicals in relatively high concentrations. Keep in mind that an equal EL value does not necessarily imply exactly the same composition of chemicals present, neither the same concentration levels for these chemicals. More information on the composition is described below.

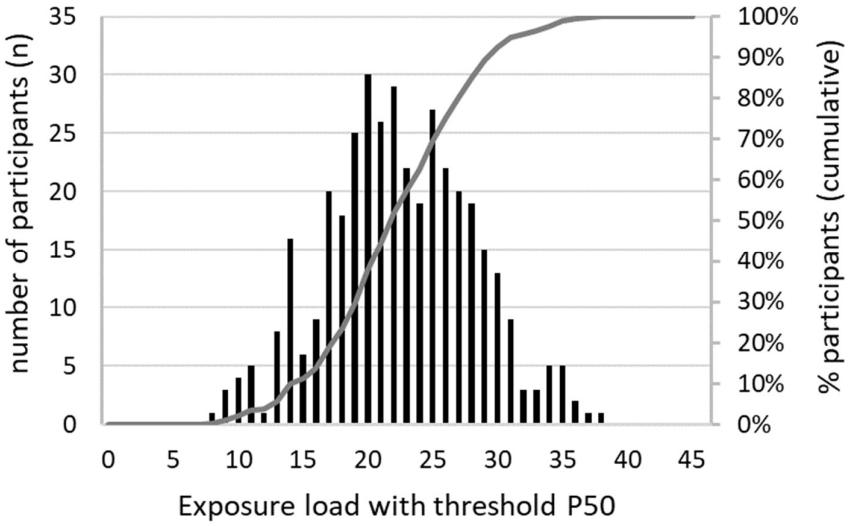


*Figure 1. Distribution of exposure load (EL) with discretization threshold P50-value. Maximum level of exposure load is 45. The line in the figure present the % of participants (cumulative).*

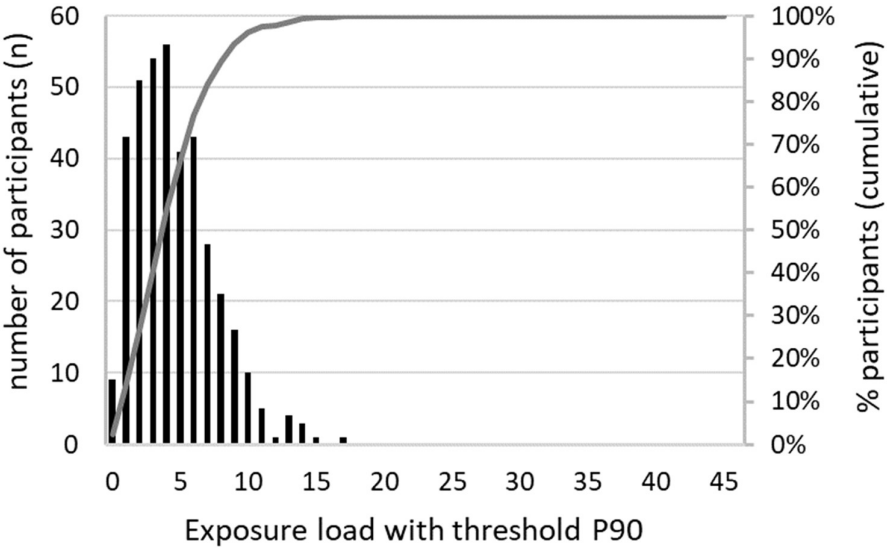


*Figure 2. Distribution of exposure load (EL) with discretization threshold P90-value. Maximum value of exposure load is 45. The line in the figure present the % of participants (cumulative).*

### 3.3 Chemical composition EL

A biserial correlation analysis between the EL and its constituents lead to following results (Table 2). Largest significant ($p<0.001$) Pearson biserial correlation coefficients between EL-P90 and chemical scores (0 or 1) were observed for TN ($r$=0.38), DBP ($r$=0.35), sum PCBs ($r$=0.31), OXC ($r$=0.30) and EHDPHP ($r$=0.30). Remaining coefficients can be found in Table 2. For the EL with threshold P50, more chemicals had a significant correlation coefficient above 0.30. In general, coefficients were larger for the EL-P50. Also DBP, sum PCBs, DEHP and DiBP are ranked relatively high for both EL-P50 and EL-P90. Chemicals which did not significantly contributed to the EL-P50 were NAP and BDE47. For BDE154, there was a significant negative correlation with the EL-P50. For the EL-P90, there was no significant correlation with Bz, NAP, DEP, BPB and BPZ.

*Table 2. Biserial correlation between the exposure loads and their constituents*

| EL-P50 | | | EL-P90 | | |
|---|---|---|---|---|---|
| **Chemical** | **Biserial Pearson correlation coefficient(r)[a]** | **p** | **Chemical** | **Biserial Pearson correlation coefficient (r)[a]** | **p** |
| DBP | 0.44 | *** | TN | 0.38 | *** |
| DiBP | 0.35 | *** | DBP | 0.35 | *** |
| FLU | 0.35 | *** | SumPCBs | 0.31 | *** |
| PFOA | 0.34 | *** | OXC | 0.30 | *** |
| DPHP | 0.34 | *** | EHDPHP | 0.30 | *** |
| BzBP | 0.34 | *** | PFOS | 0.29 | *** |
| DEHP | 0.34 | *** | DEHP | 0.29 | *** |
| SumPCBs | 0.34 | *** | DiBP | 0.28 | *** |
| PFDA | 0.33 | *** | PYR | 0.28 | *** |
| HCB | 0.33 | *** | PFNA | 0.28 | *** |

| | | | | | |
|---|---|---|---|---|---|
| EHDPHP | 0.32 | *** | HCB | 0.27 | *** |
| DDT | 0.32 | *** | PHE | 0.27 | *** |
| 3-PBA | 0.32 | *** | PFOA | 0.27 | *** |
| BPA | 0.31 | *** | DIDP | 0.26 | *** |
| PYR | 0.31 | *** | DPHP | 0.26 | *** |
| PFNA | 0.31 | *** | Pb | 0.26 | *** |
| PHE | 0.30 | *** | DDT | 0.25 | *** |
| DINCH | 0.30 | *** | BzBP | 0.24 | *** |
| DEHTP | 0.29 | *** | FLU | 0.24 | *** |
| TN | 0.29 | *** | Cd | 0.23 | *** |
| PFOS | 0.29 | *** | DINP | 0.23 | *** |
| OXC | 0.28 | *** | PFDA | 0.23 | *** |
| TCIPP | 0.28 | *** | 3-PBA | 0.22 | *** |
| DINP | 0.28 | *** | TDCIPP | 0.22 | *** |
| Pb | 0.27 | *** | PFHxS | 0.21 | *** |
| GLY | 0.27 | *** | DEHTP | 0.21 | *** |
| PFHxS | 0.26 | *** | HCH | 0.21 | *** |
| TDCIPP | 0.26 | *** | CPS | 0.20 | *** |
| CPS | 0.25 | *** | BPA | 0.20 | *** |
| HCH | 0.25 | *** | TI | 0.19 | *** |
| Cd | 0.24 | *** | BPF | 0.19 | *** |
| DIDP | 0.23 | *** | GLY | 0.18 | *** |
| BPB | 0.22 | *** | TCIPP | 0.17 | ** |
| 2,4-D | 0.21 | *** | BDE99 | 0.17 | ** |
| TBOEP | 0.21 | *** | 2,4-D | 0.17 | ** |
| DEP | 0.19 | *** | BDE47 | 0.17 | ** |
| BDE99 | 0.18 | *** | DINCH | 0.16 | ** |
| BPF | 0.17 | ** | BDE154 | 0.15 | ** |
| TI | 0.16 | ** | TBOEP | 0.15 | ** |
| BPS | 0.16 | ** | BPS | 0.12 | * |
| Bz | 0.15 | ** | Bz | 0.10 | NS |
| BPZ | 0.15 | ** | NAP | 0.10 | NS |
| BDE47 | 0.06 | NS | DEP | 0.09 | NS |
| NAP | -0.04 | NS | BPB | 0.08 | NS |
| BDE154 | -0.10 | * | BPZ | 0.06 | NS |

[a]: ranked by value of correlation coefficient

*:0.01<p≤0.05; **:0.001<p≤0.01; ***: p≤0.001

From a participants' individual point of view, the combination of chemicals or itemsets (combination of 0 and 1) may be almost unique. A cluster analysis was performed and dendrograms were created to assess possible similar clustering of chemicals within individuals (see Figure 3 and Figure 4). For 4 chemicals (BPZ, PFDA, BDE99, BDE154), the P50 was equal to the LOD or LOQ. Therefore LOD or LOQ values were used instead of the P50 as threshold and slightly less than the half of the 387participants got a score of +1 for these chemicals.

From the dendrograms for both EL-P50 and EL-P90, it can be observed that similar clusters occur. Indeed, several organochlorine compounds (TN, OXC, sum PCBs, HCB, HCH) measured in serum were clustered close to PFAS. Both PFAS and PCBs bind to e.g. albumin, but PCBs also to lipids (Guo et al., 1987; Jones et al., 2003). They are persistent and have a longer half-life than most of the short-term biomarkers measured in urine in this study.

Furthermore, similar clusters based on EL-P50 as well as EL-P90 were observed, e.g. pesticides 3-PBA, 2,4-D, CPS and GLY were closely related (GLY only based on EL-P50 and not on EL-P90). Polyaromatic hydrocarbons NAP, PYR, FLU and PHE were clustered for both EL-P50 and EL-P90. Organophosphorus flame retardants TCIPP, TDCIPP, TBOEP, DPHP, EHDPHP were clustered (DPHP and EHDPHP not in the cluster based on EL-P90 data). Brominated flame retardants BDE47 & BDE99 were clustered. Bisphenols BPB and BPZ were clustered (BPA and BPS were clustered only for the EL-P50). Phthalates and alternatives DEHP, DiBP, DBP, BzBP, DINP, DEHTP, DINCH and DIDP were clustered for the EL-P50. For the EL-P90, this group fell into two separate groups: (a) DEHP, DEHTP, DINP and DIDP and (b) DiBP, DBP, BzBP, DINCH. The phthalate DEP was not aggregated with other phthalates or alternatives. The phthalate DEP is mainly used in cosmetics, whereas the other phthalates mainly occur in clothing, household products, food or food contact materials (Tranfo et al., 2018).

The dendrogram EL-P50 (Figure 3) shows that some individuals are more exposed to POPs, such as PFAS and PCBs, which are displayed in right side in the x-axis and upper part in Y-axis, and they are less exposed to non-persistent compounds (left side). And the other way around, some individuals who are displayed on the left side in the x-axis and bottom part in y-axis, are more exposed to non-persistent compounds and less exposed to POPs.
From the dendrogram EL-P90 (Figure 4), it can be seen that TN, OXC, sum PCBs and PFAS are marginally present at EL ≤2 (green), while they are more present at EL between 2 and 5 (blue) and at EL >5 (purple). This is observed also for other biomarkers like PAHs (FLU, PYR, PHE).

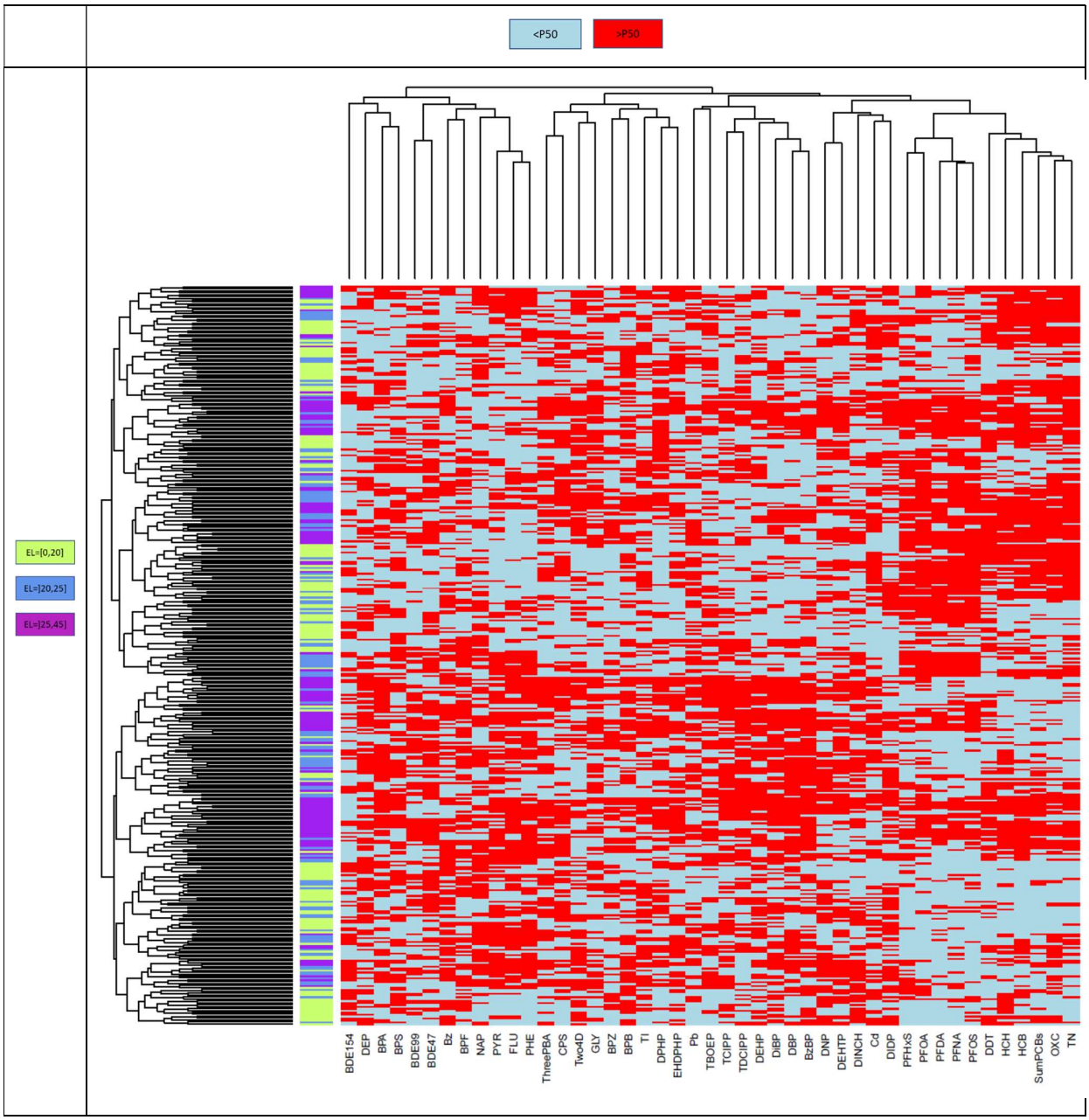


*Figure 3. Dendrogram for internal chemical exposure of 387 individuals using a threshold of P50. Each row presents an individual and each column represents a chemical. Red colour means that for the considered chemical the concentration was equal to or above the threshold. The colours of the bar at the left represent the value of the EL (green ≤20, blue >20 and ≤25, purple >25). ThreePBA=3-PBA; Two4D= 2,4D.*

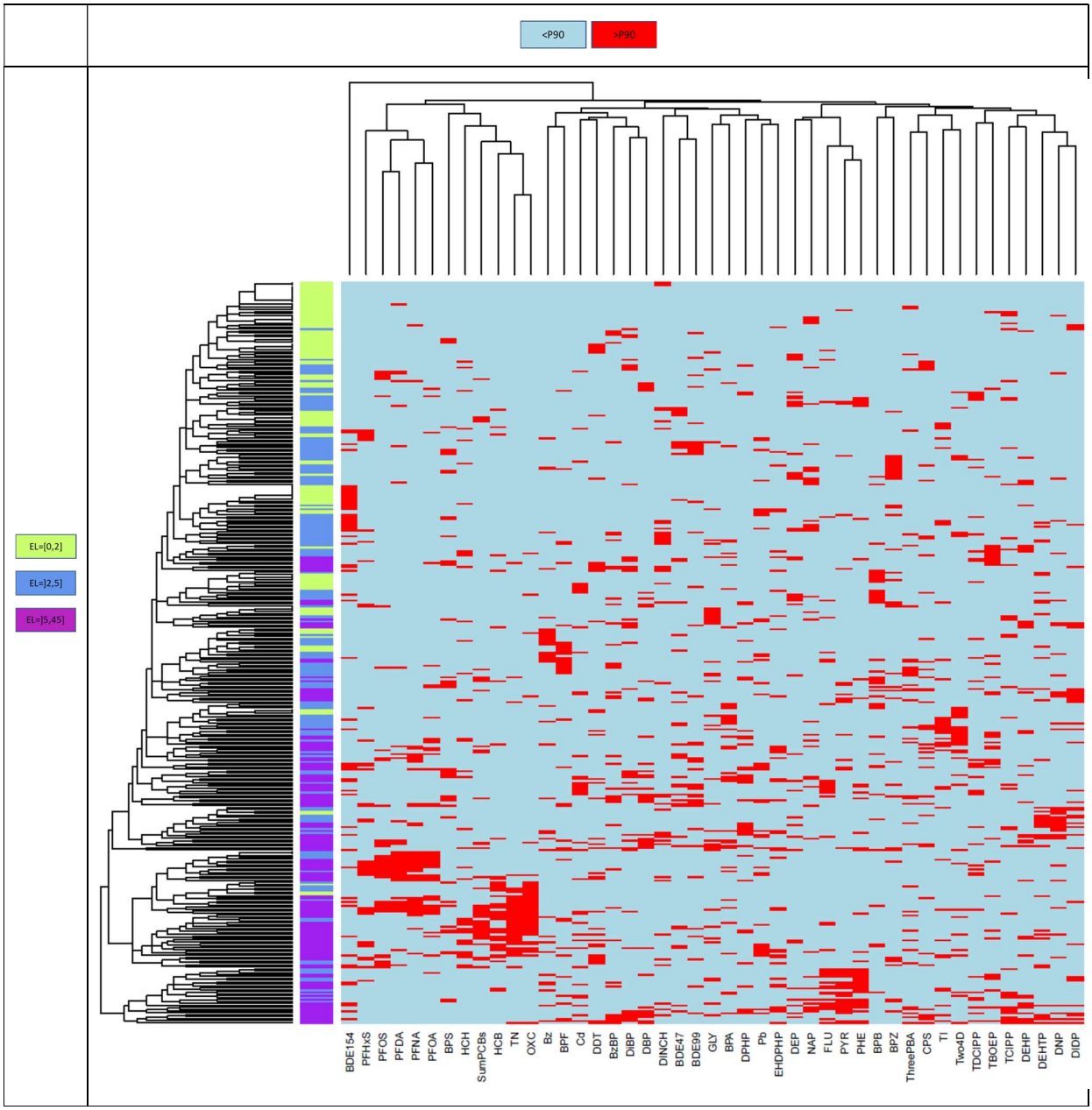


*Figure 4. Dendrogram for internal chemical exposure of 387 individuals using a threshold of P90. Each row presents an individual. Each column a chemical. Red colour means that for the considered chemical the concentration was equal to or above the threshold. The colours of the bar at the left represent the value of the EL (green ≤2, blue >2 and ≤5, purple >5). ThreePBA=3-PBA; Two4D= 2,4D.*

### 3.4 Determinants of variability in EL

We included some common determinants of exposure in the analysis of determinants in EL variability. The determinants considered here are only a fraction of the questions in the questionnaires or a fraction of the gathered information. Results of the univariate analysis are given in detail in Appendix (Table A1). Based on univariate regression results, the following observations can be reported. Mean EL values were significantly ($p<0.05$) higher for boys compared to girls, certainly for the EL-P50 (boys: 23.37 vs girls: 21.66). A possible explanation is the inverse association between EL and BMI (underweight: 24.16, normal weight: 22.70, overweight: 20.63) and the fact that girls usually have higher BMI than boys (Agentschap Zorg en Gezondheid, 2016) (data FLEHS-4). Associations between biomarker concentrations and BMI were earlier observed for fat soluble chemicals, e.g. PCBs (Agudo et al., 2009; Dirinck et al., 2011). A possible explanation for this observation (lower EL with higher BMI) can be found in the dilution capabilities of these chemicals: as these contaminants are preferably stored in adipose tissue, a higher percentage of body fat leads to faster and more efficient storage of these compounds, with lower serum concentrations as a consequence (Dirinck et al., 2011). Having lower serum concentrations of fat soluble chemicals does not mean that the total amount of fat soluble chemicals in the body is lower. In addition to the difference in BMI between girls and boys, also other variables may influence the difference in EL, such as hormonal differences which may influence toxicokinetics, different hobbies, use of cosmetics, which may result in differences in exposure etc.

A positive association between mean EL-P50 and playing sports was observed (never or seldom: 20.39, 1-2 times per week: 22.46, > 3 times per week: 23.14). This was not found for the EL-P90. It was checked if BMI could influence this association with the EL-P50, but there was no significant trend between the BMI class and playing sports. During sport activities, higher ventilation rates could lead to a higher intake of some volatile chemicals (Dong et al., 2018), however there is only a very limited amount of volatile chemicals considered here so main reasons for this observation of a higher EL-P50 with increased sport activities remain unclear.

Having been breastfed in infancy was significantly positive associated with the EL-P50 (breastmilk no: 21.07, yes: 23.19) and EL-P90 (breastmilk no: 4.06, yes: 4.87). Human milk as a source of exposure for children has been reported earlier for POPs (a.o. PCBs) (Lancz et al., 2015) and for PFAS (Mogensen et al., 2015).

Indoor use of a heating stove was significantly positive associated with the EL-P50 (stove no: 21.96, yes: 23.35). Emissions of residential woodstoves are primarily related to PAHs, but to some extent also to dioxins and PCBs (Gullett et al., 2003). The construction year of the house was significantly associated with the EL-P90 with higher values for the EL with relatively older houses (<1960: 5.07, 1961-1980: 4.96, 1981-2000: 4.31, 2001-2006: 4.22, >2006: 3.77). In the US NHANES, higher urinary cadmium, cobalt, platinum, mercury, 2,5-dichlorophenol and 2,4-dichlorophenol concentrations and mono-cyclohexyl phthalate and mono-isobutyl phthalate metabolites were shown in occupants of houses built before 1990 (Shiue and Bramley, 2015). Also increased concentrations of blood lead were found in persons of relatively older houses (Dixon et al., 2008).

Other significant determinants for the variability in EL were the use of compost in the vegetable garden, consumption of locally-produced eggs and, to some extent, consumption of locally-produced vegetables, fruit and smoked fish. EL values varied significantly for the use of compost (EL-P50: never: 22.18, sometimes: 21.73, often: 24.72; EL-P90: never: 4.43, sometimes: 4.56, often: 5.87), the consumption of locally-produced eggs (EL-P50: never: 21.57, 1 egg/month: 22, 1-4 eggs/month: 22.15, 1 egg/week: 24.74; EL-P90: never: 4.48, 1 egg/month: 4.28, 1-4 eggs/month: 4.36, 1 egg/week: 5.47), consumption of locally produced vegetables (EL-P50: never: 21.84, < once/week: 22.46, ≥ once/week: 23.70; EL-P90: never: 4.38, < once/week: 4.33, ≥ once/week: 5.39), consumption of locally produced fruit (EL-P50: never: 22.23, < once/week: 22, ≥ once/week: 24.35) and the consumption of smoked fish (EL-P50: never: 21.49, < once/week: 23.18, ≥ once/week: 22.70).

The consumption of locally-produced eggs and use of compost in the vegetable garden are determinants for increased serum levels of organochlorine compounds (such as PCBs, DDT), but also PFAS like PFOS and PFNA (data not shown). The consumption of locally-produced fruit and vegetables is also associated with increased PFAS levels (e.g. PFNA; data not shown). For PFASs, similar results were found in FLEHS-3 (Colles et al., 2020). The association with smoked fish may be related to the possible presence of PAHs, however this depends on the smoking process. Finally, the consumption of canned food in the past 3 days was associated here with an increase in the EL-P50 (no: 22.17 vs yes:23.76). Bisphenols are typically used in the lining (Russo et al., 2019) but also appear along the food production chain (González et al., 2020).

For educational level based on highest education within the household, significantly higher mean EL-P50 values were found with higher education categories (ISCED), indicating exposure to more chemicals above P50-levels with increasing level of attained education (ISCED0-2: 20.83, ISCED3-4: 21.79; ISCED≥5: 23.11). Analysis for the EL-P90 showed no clear results. Previous FLEHS studies reported social inequalities in exposure in both directions: for some chemicals (POPs, PFASs) higher exposure levels were observed with increasing ISCED scores, while an opposite trend was observed for other chemicals (e.g. metals) (Morrens et al., 2012; Buekers et al., 2018; Buekers et al., 2019). It should be kept in mind, that education level is a proxy for many variables, that cannot always easily be captured using a questionnaire.

In general, there were more associations with determinants for the EL-P50 than for the EL-P90 and they were more strongly pronounced for the EL-P50 than for the EL-P90 except for smoking and construction year of the house where there was only an association for the EL-P90. The EL-P90 increased substantially with smoking but the total of smokers in the studied population of young teenagers was limited. For all determinants, trends for the EL-P50 and EL-P90 go in the same direction. A reason why in general associations were stronger with the EL-P50 than the EL-P90 is that the distribution was wider for the EL-P50 whereas for the EL-P90 it became increasingly tightly packed, and as such less variability to be explained by possible determinants. For the EL-P50, the following associations remained significant after correction for FDR using the Benjamini-Hochberg method (BH): sex, BMI, received breastmilk, use of compost in the vegetable garden, consumption of local eggs and sports. These are also the determinants that were detected in the negative binomial multiple regression analysis (see next paragraph). For the EL-P90 no univariate significant regressions were found following the BH method. However, for the multiple regression analysis the raw data (without BH correction) were applied.

The negative binomial multiple regression analysis showed for the EL-P50 that sex, BMI, having been breastfed, local egg consumption, use of compost in the vegetable garden and playing sports were significant in the final model ($p<0.05$) (Table 3). For the EL-P90, sex, local egg consumption, use of compost in vegetable garden and smoking remained significant in the model. Next to sex, the consumption of local eggs and use of compost in the vegetable garden were found back in both EL-P50 and EL-P90. Pseudo regressions coefficients were low, i.e. 0.09 for the EL-P50 and 0.04 for the EL-P90, however these should be interpreted with caution. Take into account that for the univariate analysis, no significant associations were found for the EL-P90 according to the BH method.

*Table 3. Negative binomial multiple regression analyses*

| **Parameter** | | **ELP50** | | | | **ELP90** | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **Exp(B)** | **95% CI** | | **p** | **Exp(B)** | **95% CI** | | **p** |
| | | | **LL** | **UL** | | | **LL** | **UL** | |
| Intercept | | 24.966 | 22.719 | 27.480 | <0.001 | 9.516 | 6.708 | 13.498 | <0.001 |
| Sex | M | 1.058 | 1.008 | 1.110 | 0.021 | 1.189 | 1.044 | 1.1.353 | 0.009 |
| | F | Ref | | | | Ref | | | |
| BMI | Underweight | 1.174 | 1.063 | 1.296 | 0.002 | | | | |
| | Normal weight | 1.094 | 1.025 | 1.167 | 0.006 | | | | |
| | Overweight | Ref | | | | | | | |
| Received breastmilk | No | 0.928 | 0.881 | 0.976 | 0.004 | | | | |
| | Yes | Ref | | | | | | | |
| Consumption of local eggs | Never | 0.872 | 0.819 | 0.928 | <0.001 | 0.736 | 0.621 | 0.873 | <0.001 |
| | 1 egg/ month | 0.894 | 0.829 | 0.963 | 0.003 | 0.794 | 0.651 | 0.969 | 0.023 |
| | 1-4 eggs/month | 0.901 | 0.839 | 0.967 | 0.004 | 0.789 | 0.653 | 0.953 | 0.014 |
| | > 1 egg/week | Ref | | | | Ref | | | |
| Use of compost in vegetable garden | Never | 0.917 | 0.857 | 0.981 | 0.012 | 0.748 | 0.626 | 0.895 | 0.001 |
| | Sometimes | 0.857 | 0.783 | 0.938 | 0.001 | 0.739 | 0.586 | 0.933 | 0.011 |
| | Often | Ref | | | | Ref | | | |
| Sports (sweating or breathless) | Never or seldom | 0.882 | 0.818 | 0.950 | 0.001 | | | | |
| | 1-2 times per week | 1.019 | 0.966 | 1.074 | 0.489 | | | | |
| | ≥ 3 times per week | Ref | | | | | | | |
| Smoking | Never or once | | | | | 0.671 | 0.501 | 0.899 | 0.008 |
| | Yes | | | | | Ref | | | |
| Model Pseudo R2 (=1-L1/L0) | | 0.09 | | | <0.001 | 0.04 | | | <0.001 |

LL: lower limit; UL: upper limit; Ref: reference or Exp(B)=1

L1: likelihood model

L0: likelihood intercept

Interpretation exp(B): for example for sex the ELP50 is 5.8% higher for males than for females. Exponent is taken seeing the negative binomial multiple regression analysis.

These results clearly illustrate the usefulness of the EL-approach to identify variables associated with high exposure load. Changes in these variables, where possible, can result in lowering exposure to multiple pollutants. This can help policymakers to gather knowledge about possibilities to reduce the exposure and make citizens more aware of their possibilities to act themselves. Examples are: reduce smoking, gather more info on the chemical composition of the soil in the chicken run, check which

type of compost is used in the vegetable garden. Based on other studies in Flanders, similar suggestions are already in place (https://www.gezonduiteigengrond.be/home).

Current analysis included a biserial correlation test between EL and its biomarkers, cluster analysis of biomarkers and individuals with indication of the EL and regression analysis between EL and determinants of variability based on questionnaire data. Information was thus gathered on variation of the chemical composition by the EL value and separately on determinants of variability of the EL based on questionnaire data. Combining data of the cluster analysis directly with data of the questionnaire to discern patterns and identify chemicals associated with the determinants would also be an option.

### 3.5 Limitations

The ultimate goal for applying techniques taking into account exposure to multiple chemicals is advancing our understanding of main drivers for the health impact. For the moment, this study focused solely on exposure and the toxicity of the chemicals was not taken into account. Also the itemsets are limited by the available chemicals measured in the study. Not all chemicals to which a person is exposed to, are measured. Further, to truly understand the impact of chemical mixtures itself on health, one needs to account for the chemical concentration, the toxic potential of the chemical, the residence time in the body but also the sequence of the exposure. Therefore, the exposome concept defined as the totality of all human environmental exposures from conception to death is more appropriate (Wild, 2005), although more complicated. To tackle the toxic potential of chemicals and bring it into the EL concept, instead of choosing a threshold within the data itself (i.e. P50 or P90), the threshold can be set equal to HBM guidance values like the biomonitoring equivalents (BE), German HBM-I values or guidance values derived within the HBM4EU project. These values do not exists for all chemicals.

The exposure load (EL) combines simultaneous exposure to multiple chemicals in one measure. Groups which are disproportionally exposed can be revealed. It would be worth to see how the EL concept may be applied in health outcome studies as it is a different exposure metric that may rank individuals by aggregating their exposure levels to multiple chemicals. In addition, an EL can be calculated for a large series of chemicals, but also for a component based-approach, in which chemicals are grouped by e.g. functionality or based on adverse outcome pathways, and for which several different ELs are calculated. Associations between these ELs and biomarkers of effect or health outcomes can then be examined. As discussed above toxicity can be brought into the EL by setting the threshold to HBM guidance values or take into account potency factors when considering a component based-approach.

The exposure load concept is just one part of the puzzle of combined exposure. While it is a simple concept, it can help pushing our understanding on combined exposure to multiple chemicals. On the other hand, information about the concentration levels is lost due to the discretization process. Comparison of ELs between different studies is difficult seeing differences in studied chemicals, population characteristics, differences in the established cut-offs, etc.

For the calculation of the EL, a threshold (cut-off concentration value) was used. Persons exposed just above the threshold get a value of 1, while persons just below, a zero, however the exposure does not

differ that much between these persons. A more correct way to address the EL in future could be to use a continuous variable, e.g. distance function instead of a cut-off or threshold value.

For the associations reported here between variability in EL and possible determinants, no statements can be made about causality in this context. Also other determinants for the relatively higher EL of some participants should be added to the considered questionnaire data in our dataset.

## 4. Conclusions

We found that 20% of the study population had for 27 out of 45 chemicals biomarker levels above the 50$^{th}$ percentile. The exact profile of biomarkers in these exposed individuals was rather unique. We found also that 20 % of the 387 Flemish adolescents had for 6 out of 45 chemicals, exposure biomarkers with levels above the 90$^{th}$ percentile. Chemical profiles showed some dominance of organochlorine chemicals, PFASs, phthalates, PAHs, organophosphate flame retardants, bisphenols (A & F), pesticides, metals, but to a lesser extent brominated flame retardants, the organophosphorus flame retardants TCIPP & TBOEP, naphthalene and benzene, bisphenols S, B & Z, the pesticide 2,4-D, phthalate DEP and alternative plasticizer DINCH. Associations between the EL and exposure determinants pointed in the direction of determinants formerly associated with fat soluble chemicals, PFASs, bisphenols and PAHs. This analysis adds information on possibilities to reduce exposure and is helpful for policymakers and citizens themselves e.g. reduce smoking, gather information on chemical composition of soil in chicken run and check which type of compost is used in the vegetable garden etc.

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