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Liquid Chromatography – Tandem Mass Spectrometry Analysis of Biomarkers of Exposure to Phosphorus Flame Retardants in Wastewater to Monitor Community-Wide Exposure

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Abstract

Phosphorous flame retardants and plasticizers (PFRs) are increasingly used in consumer goods, from which they can leach and pose potential threats to human health. Monitoring human exposure to these compounds is thus highly relevant. Current assessment of exposure through analysis of biological matrices is however tedious as well as logistically and financially demanding. Analysis of selected biomarkers of exposure to PFRs in wastewater could be a simple and complementary approach to monitor, over space and time, exposure at the population level. An analytical procedure, based on solid-phase extraction (SPE) and liquid chromatography coupled to tandem mass spectrometry was developed and validated to monitor the occurrence in wastewater of human exposure biomarkers of 2-ethylhexyldiphenyl phosphate (EHDPHP), tris(2-butoxyethyl) phosphate (TBOEP), triphenyl phosphate (TPHP), tris(2-chloroisopropyl) phosphate (TCIPP), and tris(2-chloroethyl) phosphate (TCEP). Various SPE sorbents and extraction protocols were evaluated and for the optimized method, the absolute extraction recoveries ranged between 46% and 100%. Accuracy and precision were satisfactory for the selected compounds. Method detection limits ranged from 1.6 to 19 ng L⁻¹. Biomarkers of exposure to PFRs were measured for the first time in influent wastewater. Concentrations in samples collected in Belgium ranged from below limit of quantitation to 1072 ng L⁻¹, with 2-ethylhexyl phenyl phosphate (EHPPHP) and TCEP being the most abundant. Per capita loads of target biomarkers varied greatly, suggesting differences in exposure between the investigated communities. The developed method allowed to implement the concepts of human biomonitoring at the community scale, opening the possibility to assess population-wide exposure to PFRs.

Introduction

Humans and the environment are constantly exposed to an ever increasing number of potentially harmful contaminants. Recent estimates indicate that approximately 40% of human deaths, corresponding to 64 million in terms of absolute figures worldwide, were due to diseases linked to the exposure to various contaminants¹. Phosphorus flame retardants and plasticizers (PFRs) have been commonly added to consumer products to meet flammability standards for many years². Their use has increased in recent years since they were introduced as an alternative to the more persistent and bioaccumulative halogenated chemicals, such as brominated flame retardants (BFRs)². Nowadays, PFRs are mainly used for two purposes: halogenated PFRs as flame retardants and non-halogenated as plasticizers³. Products that contain PFRs include furniture, textile, floor polish, lacquers, resins, paints, electronics, polyvinyl chloride (PVC) plastics, lubricants, and hydraulic fluids⁴. More specifically, 2-ethylhexyldiphenyl phosphate (EHDPHP) is used, among others, in food packaging, tris(2-butoxyethyl) phosphate (TBOEP) in floor wax and vinyl plastics, tri-n-butyl phosphate (TNBP) in hydraulic fluids and triphenyl phosphate (TPHP) in resins and PVC. The most common chlorinated PFRs (i.e. tris(chloroethyl) phosphate (TCEP), tris(2-chloroisopropyl) phosphate (TCIPP) and tris(2,3-dichloropropyl) phosphate (TDCPP)) are generally applied in polyurethane foams⁴. Since PFRs are not chemically bound to these materials, they may be easily released into the environment⁵. PFRs have been detected in environmental matrices, such as house dust, indoor air, water, sediment, soil and biota^{2,4}. Due to their ubiquitous presence in the environment, PFRs may pose a threat to human health through different exposure routes, such as dermal contact, dust ingestion, inhalation and dietary intake. In particular, TCIPP, TDCPP, and TBOEP are suspected carcinogens² whilst neurotoxic effects have been reported after exposure to TCEP, TNBP, and TPHP^{6,7}. Other reported adverse effects related to humans include the Sick Building Syndrome from exposure to TNBP and TBOEP⁸, reduced thyroid hormone levels from TDCPP⁹ and atopic dermatitis from the presence of TCIPP and TDCPP in floor dust¹⁰.

Human exposure to PFRs is generally assessed through the analysis of specific biomarkers (i.e. parent compounds or, where appropriate, phase I and/or phase II metabolites) in biological matrices. This approach, referred to as human biomonitoring (HBM), offers an estimation of exposure at an individual level. Urine is the most commonly target matrix because it can be easily collected, it is available in large volumes and it is more suitable to measure biomarkers of exposure to PFRs compared to other matrices¹¹. In recent years, HBM studies on PFR exposure, including those by our research group, have been carried out in different countries^{12–14}. However, HBM is subject to various limitations. In particular, it requires the collection of numerous samples from multiple individuals, which can be expensive to organize. Furthermore, it often lacks of a temporal dimension (i.e., individuals being sampled only once or, at best, over a 24h period), suffers from selection bias and requires ethical approval. These issues limit the attempt to assess exposure to chemicals in the general

population¹⁵. In a sewer catchment, human excretions, e.g. urine, are conveyed through sewer networks to wastewater treatment plants (WWTPs). Mining the chemical information contained in raw wastewater to deliver epidemiologically-relevant information is an innovative approach, referred to as “wastewater-based epidemiology” (WBE)¹⁶. This approach was initially implemented to estimate illicit drug use at the population level^{17,18}. Yet, from a broader perspective, wastewater represents a pooled sample of human excretions, encompassing many chemicals and their exposure biomarkers¹⁶. Promising results were recently obtained for phthalates¹⁹ and pesticides²⁰, where the authors used WBE to monitor population-wide exposure to these chemicals.

Thus, the objectives of this study consisted of (1) developing an analytical procedure to detect and quantify biomarkers of exposure to selected PFRs in wastewater, (2) conducting preliminary experiments to investigate the stability of the target PFR biomarkers in wastewater and (3) apply the developed method to analyse wastewater samples collected in different locations in Flanders (Belgium).

Materials and methods

Target compounds

The present work focused on the development and validation of an analytical method to detect and quantify selected biomarkers of exposure to PFRs in wastewater (see Table 1 for abbreviations used). These were identified and measured in previous studies by our group^{12,21}. Except for TCEP, the corresponding parent compounds were used only to investigate their potential influence on the levels of metabolites in wastewater (see section *Stability*), but were not studied and analysed.

Chemicals

HO-TBOEP, EHDPHP, HO-TPHP, HO-DPHP, BBOEHEP, BCIPP, BCIPHIPP, EHPHP, HO-EHDPHP, BCEP, TCEP-D12, BBOEHEP-D₄, BDCIPP-D₁₀, and TBOEP-D₆ were custom synthesized by Dr. Vladimir Belov (Max Planck Institute, Göttingen, Germany). Purity was more than 98% as measured by MS and NMR techniques. TCEP was purchased from Chiron AS (Trondheim, Norway). TBOEP, DPHP and DPHP-D₁₀ were purchased from Sigma-Aldrich (Bornem, Belgium). TPHP standard was purchased from Chiron AS (Trondheim, Norway). TCIPP standard was acquired from Pfalz & Bauer (Waterbury, USA). Methanol was LC-grade and was purchased from Merck (LiChrosolv®, Merck, Darmstadt, Germany), whilst hydrochloric acid (37%), formic acid (99-100%) and ammonium acetate were of analytical-grade and were purchased from Sigma Aldrich (Bornem, Belgium). Ultrapure water (UPW) was obtained from a PURELAB Flex system ($\rho = 18.2 \text{ M}\Omega/\text{cm}$, Elga Veolia, Tienen, Belgium). β -glucuronidase enzyme solution was purchased from Sigma Aldrich (lyophilized powder from *E. coli*, >10 000 000 unit/g). Solid-phase extraction (SPE) was performed using a Visiprep SPE vacuum manifold with 24 ports (Sigma Aldrich). Filtration of wastewater samples was carried out using glass microfiber filters (GF/A, 1.6 μm , Whatman, Sigma-Aldrich).

Wastewater samples

Wastewater samples were collected at the influent of four WWTPs in Flanders (Belgium), namely Ninove (36,200 inhabitants, NIN), Ostend and surroundings (160,000 inhabitants, OST), Geraardsbergen (29,000 inhabitants, GER) and Lier (31,500 inhabitants, LI). Population figures for each location were provided by the WWTP personnel (i.e., census based). For each location, influent wastewater samples were collected on two consecutive days during 2015 and 2016. These consisted of 24 h composite samples collected using refrigerated (4 °C) autosamplers operated in a time-proportional manner with sampling intervals of 10 min. After collection, wastewater samples were immediately frozen (-20 °C) until analysis.

Sample preparation

For sample preparation, 100 mL samples were spiked with mass labelled reference standards (IS, 50 ng L⁻¹) and centrifuged at 3000 RCF for 20 min at room temperature (20 °C). The supernatant was then filtered through glass microfiber filters (1.6 µm) and acidified to pH 4-5 using HCl (37%), based on a previously established protocol for the analysis of PFRs metabolites in urine²². Subsequently, target analytes were extracted using SPE. In the optimised method, samples were loaded onto Bond-Elut C18 cartridges (3 mL, 200 mg, Agilent, Santa Clara, USA). These were pre-conditioned using 3 mL of methanol followed by 2 mL of acidified (pH 4-5) UPW. After sample loading, cartridges were washed using 5 mL of acidified (pH 4-5) UPW and dried under vacuum for 30 min. Analytes were then eluted using 5 mL of MeOH. The eluate was then evaporated to dryness under a gentle stream of nitrogen. Dry residues were then reconstituted in 200 µL of a UPW:methanol (50/50, v/v) mixture, filtered with 0.2 µm centrifugal filters (nylon membrane, VWR International, Leuven, Belgium) and transferred to amber glass vials for LC-MS/MS analysis.

Liquid chromatography – Tandem mass spectrometry

Instrumental analysis of wastewater extracts was carried out using a slightly modified version of the method previously developed and applied by Van den Eede et al.¹². Specifically, analyses were carried out on an Agilent 1290 Infinity liquid chromatography system coupled to an Agilent 6460 Triple Quadrupole mass spectrometer (LC-MS/MS, Santa Clara, CA, USA) with an electrospray ionization (ESI) source. Separation of the metabolites was performed on a Phenomenex Kinetex Biphenyl reversed phase column (2.1 x 100 mm, 2.6 µm; Torrance, CA, USA), at a column temperature of 40 °C. The mobile phase consisted of (A): UPW with 2% methanol and 5 mM ammonium acetate, and (B): methanol with 2% UPW and 5 mM ammonium acetate. The mobile phase gradient was as follows: initial gradient 5% (B) increased to 50% at 3.5 min, to 65% at 7.5 min, reached 97% at 9.5 min, hold for 4 min and 3.5 min to equilibrate at 5%. Injection volume was set at

5 μL and flow rate at 0.35 mL/min. The mass spectrometer was operated in dynamic multiple reaction monitoring (dMRM) in positive to negative switching ionization mode. Time segments were set at 1 minute for each compound. Quantifier and qualifiers of MRM transitions of target analytes are presented in Table 2. The drying gas temperature was set at 325 °C, the gas flow at 10 L/min, the nebulizer at 30 psi, sheath gas temperature 275 °C, sheath gas flow 10 L/min, capillary voltage 3500 V and nozzle voltage 0 V.

Method validation

Calibration was performed using a 10-point calibration curve ranging from 0.08 to 100 ng mL⁻¹, except for BCEP, BCIPP and HO-DPHP for which the calibration ranged from 0.4 to 500 ng mL⁻¹ (IS at 50 ng mL⁻¹ for all target analytes). DPHP-D₁₀ was used as IS for HO-DPHP and DPHP; TCEP-D₁₂ was used as IS for TCEP, BCIPHIPP and EHPHP; BBOEHEP-D₄ was used as IS for BBOEHEP and HO-TBOEP and TBOEP-D₆ was used as IS for HO-EHDHPHP. Calibration standards were prepared in UPW:methanol (50/50, v/v). Instrument detection and quantification limits (IDL and IQL) were estimated from a low concentration standard giving a (S/N) ratio of 3 and 10, respectively, and for which the ratio between quantifier and qualifier transitions was $\leq 20\%$ compared to the ratio obtained for calibration standards. Coefficients of determination (R^2) were determined from triplicate analysis of a complete calibration curve where the accuracy of the estimated concentration for each calibration point was within 15% or 20% (for the lowest level). Carry-overs were assessed by injecting calibration blanks immediately after the analysis of the highest calibration point.

Sample preparation (i.e., SPE) and instrumental analysis were validated based on the guidelines on bioanalytical method validation provided by the European Medicines Agency²³. Within-run and between-run precision (expressed as the relative standard deviation (RSD) from the mean quantified level) and accuracy (or bias, expressed as the deviation from the nominal spiking value) were determined using spiked UPW since it is not possible to obtain blank wastewater samples. Validation was performed across three days. The first validation batch consisted of five UPW samples spiked at low concentrations (i.e., 5 ng L⁻¹), one at mid (i.e., 50 ng L⁻¹) and one at high concentration (i.e., 500 ng L⁻¹), all spiked with IS (i.e., 50 ng L⁻¹) and extracted using the optimised procedure (see point 2.4). For validation days two and three, one UPW sample per concentration level was extracted and processed as described above. Procedural blanks spiked only with IS were included in each batch. Within-run precision and accuracy were determined using the low level UPW samples from the first batch ($n = 5$). Between-run precision and accuracy were determined using low, mid and high levels processed and analysed over the three days. Furthermore, on each validation day, an aliquot (i.e., 100 mL) of wastewater was extracted and analysed to assess the between-run precision using actual matrix. Acceptance criteria, for both precision and accuracy, were set at 20% for low levels and 15% for mid and high levels.

Extraction recoveries were estimated based on the ratio of native analytes responses in wastewater samples spiked before (pre-extraction) and after (post-extraction) processing²⁴. This approach was selected because blank wastewater and matched mass labelled reference standards for all target compounds were not available. Matrix effects were estimated as the ratio of IS response recorded in post-extraction samples to standards prepared in solvent. Thus, matrix effects could only be estimated for compounds for which mass labelled reference standards were available.

Method detection and quantification limits (MDL and MQL) were estimated from spiked or low concentration wastewater samples^{25,26} giving a signal-to-noise (S/N) ratio of 3 and 10, respectively, and for which the ratio between quantifier and qualifier transitions is $\leq 20\%$ compared to standards prepared in solvent.

Enzymatic deconjugation

To measure the levels of free and conjugated forms of target metabolites¹², experiments using enzymatic deconjugation were carried out. Specifically, four aliquots of wastewater (100 mL) were spiked with IS (50 ng L⁻¹) and adjusted to pH 6 using HCl (37%). In two aliquots, 100 μ L of β -glucuronidase (2 mg mL⁻¹ dissolved in phosphate buffer at pH 6) were added. The latter aliquots were then incubated in a water bath at 37 °C for 2 h. The remaining two aliquots were kept in the dark at room temperature during the whole procedure. Subsequently, all samples were extracted using the optimised protocol described previously. The deconjugated and non-deconjugated samples were compared based on the relative response of each analyte to its IS.

Blanks

To assess the presence of potential background contaminations of target analytes, procedural and calibration blanks were prepared and analysed and quantified throughout all experiments. Procedural blanks consisted of UPW spiked with mass labelled reference standards processed as real wastewater samples (i.e., centrifugation, acidification, filtration and SPE). Calibration blanks consisted of neat mass labelled reference standards prepared as calibration levels. If target analytes measured in blanks were above the IQL or MQL, measured background concentrations were subtracted from samples.

Stability

The stability of target analytes was assessed in real wastewater samples stored in the dark at room temperature (20 °C) and refrigerated (4 °C). Specifically, 7 mL wastewater aliquots were transferred to pre-conditioned (i.e., rinsed with acetone and baked overnight at 300 °C) glass tubes to ensure the absence of background contamination. For each considered temperature, two aliquots were prepared: the first aliquot was spiked with 10 ng mL⁻¹ of target exposure biomarkers, whilst the second aliquot was spiked with 10 ng mL⁻¹ of both exposure biomarkers and the corresponding parent compounds

(i.e., EHDPHP, TBOEP, TPHP, and TCIPP). From each batch, triplicate samples (250 μL) were collected immediately after spiking (T_0) and after 0.5, 1, 2, 4, 6 and 24 h. These aliquots were transferred to 0.2 μm centrifugal filters, spiked with mass labelled reference standards (10 ng mL^{-1}) and centrifuged for 5 min at 10,000 rpm. The samples were then analysed without further treatment using the described LC-MS/MS method. Relative responses of target analytes to their corresponding IS were calculated and changes across time were expressed as percentages relative to the signal at T_0 .

Results and discussion

Method validation

Instrumental performances were determined through triplicate analysis of calibration curves. For BCEP, HO-TPHP and BCIPP, the obtained results were not satisfying and it was thus decided to exclude these compounds from further optimization. Technical details for the remaining compounds are reported in Table 2 and an example of a chromatogram is shown in Figure S-1. Coefficients of determination (i.e., R^2) for all analytes were above 0.99 based on triplicate analysis of calibration curves. Linear or quadratic regression lines with $1/x$ weighing were used. The estimated concentration for the calibrators was within 20% (or 25% close to IQL) of the nominal value²³. IDLs ranged from 0.003 ng mL^{-1} for HO-EHDPHP to 0.04 ng mL^{-1} for BCIPHIPP, whilst IQLs ranged from 0.01 ng mL^{-1} for HO-EHDPHP to 0.08 ng mL^{-1} for HO-DPHP, BCIPHIPP and EHPHP. No carry-over was detected in calibration blanks injected immediately after the analysis of the highest calibration point. Based on the considered guidelines, the instrumental performances were satisfactory for all compounds.

Method performance was assessed using spiked UPW, as well as by repeated extraction of a wastewater sample. Results are reported in Table 3. Within-run precision, determined by repeated extraction ($n = 5$) of UPW spiked at low concentration (i.e., 5 ng L^{-1}) ranged from 1.5% to 20.0% and was thus satisfactory for all compounds. Between-run precisions at the three concentration levels (i.e., 5, 50 and 500 ng L^{-1}) were all below 15% or 20% (for low levels), except for HO-DPHP (i.e., 23.9%). Within-run bias at low concentration ranged from 2.8% to 20.4% (TCEP), whilst between-run bias was below the 15% or 20% threshold, except for TBOEP-OH (15.3% at mid-level) and HO-EHDPHP (16.7% at high-level). Between-run precision obtained from the repeated extraction of a real wastewater sample ranged from 2.0% to 14.0% and were satisfactory based on the defined criteria. For some compounds, performance was slightly outside the defined acceptance criteria. However, method validation guidelines used in this study were originally defined for bioanalytical methods²³. In such cases, mass labelled reference standards are mostly available and analyte concentrations are generally higher. Thus, it was decided to consider the obtained results as satisfactory.

Method detection and quantitation limits were defined as analyte responses giving a S/N ratio of 3 and 10, respectively, and were estimated from low concentration wastewater samples. MDLs ranged from 0.5 ng L⁻¹ for HO-EHDPHP to 19 ng L⁻¹ for EHPHP. MQLs ranged from 1.6 ng L⁻¹ for HO-EHDPHP to 65 ng L⁻¹ for EHPHP. In the particular case of DPHP, TCEP and EHPHP, background concentrations of approximately 3.0, 2.0 and 1.5 ng L⁻¹, respectively, were measured in procedural blanks.

Sample preparation

Different SPE sorbents and procedures were evaluated based on extraction recoveries and matrix effects. These parameters were determined by spiking wastewater aliquots (100 mL) with native reference compounds and IS (50 ng L⁻¹) before centrifugation, filtration, acidification and SPE (pre-extraction) and after sample processing (post-extraction). The ratio between native analytes responses in pre- and post-extraction aliquots was used to estimate extraction recoveries, whilst the ratio between responses of IS in post-extraction aliquots and a standard at the same concentration was used to determine matrix effects²⁴. Three different sorbents were investigated, namely Bond-Elut C18 cartridges (3 mL, 200 mg), Oasis HLB (6 mL, 200 mg, Waters, New Bedford, MA, USA) and Oasis MAX (3 mL, 60 mg, Waters). Extraction recoveries are reported in the left pane of Figure 1. Overall, recoveries for Bond-Elut C18 cartridges ranged between 80% and 100%, except for HO-DPHP which showed a recovery of 46%. For Oasis HLB, all compounds exhibited recoveries above 100% (range 106% to 129%). Finally, Oasis MAX provided good results for BBOEHP, TBOEP-OH and TCEP, whilst recoveries were lower for the remaining compounds, particularly for DPHP, HO-DPHP and EHPHP.

Matrix suppression, ranging between -60% and -95% was generally observed, as shown in the left pane of Figure 2. Only DPHP-D₁₀ showed positive matrix effects when Oasis MAX cartridges were used. It should be noted that matrix effects could be assessed only for those compounds for which mass labelled reference standards were available. Based on the obtained results, particularly the low recoveries obtained for three out of eight target compounds, Oasis MAX was excluded from further experiments.

To determine if extraction recoveries and matrix effects could be improved with Bond-Elut C18 and Oasis HLB cartridges, conditioning and washing of cartridges with neutral UPW was considered. Recoveries are shown on the right pane in Figure 1. For Bond-Elut C18 cartridges, recoveries above 100% were observed for some compounds. However, the recovery of HO-DPHP decreased substantially (i.e., 17% and 44% with Bond-Elut C18 and Oasis HLB cartridges, respectively) compared to acidic conditions. Similarly, a substantial decrease in recoveries for various compounds was observed using HLB cartridges. Furthermore, two compounds (DPHP and EHPHP) showed recoveries above 125%.

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3 A slight decrease in matrix effects was observed for BBOEHEP-d4 and DPHP-D₁₀ with Oasis HLB
4 cartridges, whilst results remained comparable for all other compounds (right pane of Figure 2).
5 Based on the obtained results, in particular the good compromise between extraction efficiency and
6 matrix effects, Bond-Elut C18 cartridges with acidic conditioning and washing were selected for
7 further validation as they provided more consistent results compared to Oasis HLB and MAX. These
8 findings are in agreement with results obtained with the extraction of the target biomarkers in urine
9 samples²⁷.
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14 Enzymatic deconjugation

15 To determine the levels of free and conjugated PFRs exposure biomarkers in wastewater, aliquots of
16 wastewater samples were processed and analysed with and without enzymatic deconjugation to
17 determine if differences in analyte response could be detected. The addition of β -glucuronidase and
18 incubation for 2 h at 37 °C did not substantially modify the analyte responses (Figure S-2). Similar
19 results have been obtained with several illicit drug metabolites, which appear to deconjugate in
20 wastewater^{28–30}. Consequently, enzymatic deconjugation prior to sample preparation was not further
21 contemplated.
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26 Stability

27 Stability of PFR metabolites was assessed at room temperature (20 °C) and refrigerated (4 °C) over
28 24 h to evaluate their potential degradation during in-sewer transportation and sample collection
29 (refrigerated autosamplers), respectively. Experiments were carried out with real wastewater samples
30 spiked with (i) metabolites only and with (ii) both metabolites and parent compounds. These
31 experiments were carried out to determine (i) the stability of metabolites in wastewater, as well as (ii)
32 potential changes in metabolite concentrations in wastewater in presence of parent compounds.
33 Results are reported in Figure 3.
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39 At room temperature, most target compounds appeared to be relatively stable over the considered
40 period. This is the first time that data about the stability of biomarkers of exposure to PFRs in
41 wastewater is reported. For TCEP, results are in agreement with previous studies showing that
42 chlorinated aliphatic esters are resistant to wastewater treatment processes³¹. A slight decrease, of
43 approximately 20% from the initial response, was observed only for HO-DPHP. On the contrary, an
44 increase of approximately 25% was observed for HO-EHDPHP at the end of the 24 h period. The
45 addition of parent compounds did not seem to modify the stability profile of the target compounds.
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49 At 4 °C, most PFR metabolites showed a stability profile similar to the one obtained at room
50 temperature. A rapid increase in the signal of HO-EHDPHP was again observed (+25% after 24 h). In
51 the particular case of HO-DPHP, a more pronounced decrease in analyte response was observed
52 compared to 20°C. A possible explanation for this observation could be linked to a decreased water
53 solubility at lower temperatures and a potential adsorption onto the walls of the glass tubes used. The
54 addition of parent compounds did not modify the profile of the target compounds.
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The results suggest that PFR metabolites are relatively stable in the tested conditions. In particular, the addition of parent PFR compounds did not have substantial effects on concentrations of PFR metabolites. This suggests that at time scales relevant for WBE applications (i.e., residence time of wastewater ranging from a few minutes to 15 h³²), PFR metabolites are not extensively formed by microorganisms present in wastewater. These findings are in line with previous studies showing that degradation of parent PFRs in wastewater requires longer periods of time (i.e., days)³³. The results are highly relevant as they support the hypothesis that levels of PFR metabolites measured in wastewater can be related to human exposure. Although preliminary, these experiments represent the first step commonly used to address the analyte stability in WBE approaches³⁰. Further experiments, contemplating the effect of biofilms, as well as aerobic and anaerobic conditions should be carried out in future since these parameters have been shown to have potential impacts on the overall stability of analytes in sewers^{34–36}.

Monitoring community-wide exposure to PFRs

The occurrence of selected PFR metabolites was monitored in wastewater samples collected from four WWTPs in Flanders (Belgium). Measured concentrations are reported in Table 4. HO-DPHP could not be detected in any sample, which is in agreement with findings from analysis of urine^{12,27}. High concentrations were measured for DPHP (range 71 to 628 ng L⁻¹), TCEP (range 211 to 389 ng L⁻¹) and EHPHP (range 168 to 1100 ng L⁻¹). In the case of DPHP, various sources not linked to human exposure could contribute to the measured levels. Firstly, it has been reported to be used as plasticizer, although production is substantially lower compared to TPHP³⁷. Furthermore, microbial hydrolysis of TPHP released from household appliances (e.g., washing machines) and/or industrial activities could also be a source of DPHP³³. However, preliminary stability tests performed in this context suggested that DPHP is not readily formed from TPHP within 24 h. DPHP can also be a biomarker of exposure to EHDPHP, yet its formation rate in serum has been shown to be significantly lower compared to the formation of DPHP from TPHP³⁸. Moreover, preliminary stability tests performed here did not show changes in DPHP levels after addition of EHDPHP. Nonetheless, other PFRs not investigated in this context, such as bisphenol-A bis(diphenyl phosphate) (BDP) and resorcinol bis(diphenyl)phosphate (RDP) formulations could also be potential sources of DPHP. For instance, RDP can contain DPHP as impurity and/or undergo spontaneous hydrolysis in physiological conditions to form the latter compound³⁹. Thus, DPHP should be used as a biomarker of exposure to aryl-PFRs, rather than only TPHP³⁸. Nevertheless, further experiments to assess the formation potential in wastewater of DPHP in presence of other PFRs will have to be carried out. TCEP concentrations measured here are in line with results from previous findings (i.e., 180 – 290 ng L⁻¹ in influents from Germany³¹). Being itself a flame retardant, TCEP levels measured in wastewater may not be exclusively due to human exposure, but could also occur through leakage from consumer goods. Surprisingly high concentrations of EHPHP (i.e., up to 1 µg L⁻¹ range) were found in LI and

GER, whilst HO-EHDPHP was found at substantially lower levels (i.e., 3.5 – 7.3 ng L⁻¹). In humans, EHPHP was shown to be formed from EHDPHP³⁸, apparently the main PFR found in food samples^{40,41}. In influent wastewater samples, EHDPHP was either not detected or found only at low levels (i.e., ≤ 100 ng L⁻¹)⁴², whilst it is among the most abundant PFRs in sludge (i.e., 0.32 – 4.6 µg g⁻¹)⁴³ due to its low solubility. Hence, considering the abundance of EHDPHP in the environment⁴¹, measured levels of EHPHP could reflect the likely high levels of human exposure to EHDPHP. Urine analysis of both EHPHP and HO-EHDPHP, specifically the ratio between the two biomarkers, will eventually allow to better understand whether measured EHPHP levels arise only from human exposure or also from other sources. HO-TBOEP and BBOEHEP, both exposure biomarkers of TBOEP, were measured at levels ranging from 29 to 94 ng L⁻¹ and 43 to 165 ng L⁻¹, respectively. Interestingly, HO-TBOEP could not be detected in urine samples from an Australian cohort¹², whilst it was found in approximately 50% of samples from a Japanese cohort²⁷. On the contrary, BBOEHEP could be detected in all samples from both cohorts, although it could not be quantified in samples from Australia because reference standards were not available at that time^{12,27}. Concentrations of BCIPHIPP, a metabolite of TCIPP, ranged from 5.3 to 13 ng L⁻¹. In pooled urine samples from Australia, the compound was always detected and ranged from 0.37 to 9.43 ng mL⁻¹.

Mass loads of target analytes were calculated by multiplying concentrations by flows measured by the WWTP personnel. Loads (in mg day⁻¹) were then further divided by the size of the population served by each WWTP (data provided by the WWTP operators) to obtain the so-called per capita loads (in mg day⁻¹ 1000 inhabitants⁻¹), as reported in Table 4. These loads are particularly useful to compare catchments with different number of inhabitants. Substantial differences in per capita loads of DPHP were observed. In particular, GER and LI (170 - 175 and 57 - 83 mg day⁻¹ 1000 inhab⁻¹, respectively) had three to seven time higher loads compared to OST and NIN (21 – 23 and 18 - 17 mg day⁻¹ 1000 inhab⁻¹, respectively). GER and LI also exhibited the highest per capita loads for both TCEP and EHPHP. For TCEP, loads in OST and NIN were half compared to the previous two locations (i.e., 50 - 48 and 52 - 58 mg day⁻¹ 1000 inhab⁻¹, respectively), whilst per capita loads of EHPHP were approximately 4 to 8 times lower (i.e., 38 - 49 and 89 - 74 mg day⁻¹ 1000 inhab⁻¹). Per capita loads of BCIPHIPP and HO-EHDPHP were however relatively homogenous across all considered locations (i.e., 4 to 10 ng L⁻¹ range). Whilst for HO-TBOEP, per capita loads measured in LI were substantially higher compared to the other locations (i.e., 25 – 61 *versus* approximately 13 mg day⁻¹ 1000 inhab⁻¹). The obtained results indicate that substantial differences exist between locations, suggesting potential differences in human exposure to the targeted PFRs. In particular, per capita loads of some PFR metabolites were substantially higher in GER and LI compared to OST and NIN. Whilst one could have expected more homogenous results due to the ubiquitous exposure to PFRs, the observed differences could be linked to the typology of the sampled locations, such as the degree of urbanisation (i.e., urban versus rural/countryside) or the proximity to industrial facilities where PFRs are incorporated in consumer products. However, the differences observed between locations should

be interpreted carefully as they arise from the analysis of only two daily composite samples per location. Additional and more long-term sampling campaigns are necessary to obtain more detailed and solid insights about the existence of geographical and temporal features in population-wide exposure to PFRs. Nonetheless, this is the first time that biomarkers of exposure to PFRs were measured in wastewater to assess community-wide exposure. Although preliminary, the obtained results illustrate the potential of WBE to monitor population-wide exposure to contaminants.

Conclusions

Monitoring human exposure to emerging contaminants is highly compelling taking into account the ever growing number of potentially harmful chemicals used in consumer goods. Analysis of exposure biomarkers in wastewater could be a simple and quick approach to acquire pertinent information about exposure at the population level. The developed method allowed for the first time to detect and quantify selected biomarkers of exposure to an important class of flame retardants (e.g., PFRs) in wastewater samples collected in Belgium. Preliminary experiments suggest that PFR metabolites are stable and are not extensively formed from the corresponding parent PFR compounds in wastewater. For some of the targeted analytes, significant differences in both concentrations and per capita loads were highlighted between sampled locations. The obtained results are highly relevant as they suggest that potential differences in exposure levels between communities exist and could be detected through wastewater-based epidemiology. Additional long-term sampling campaigns will provide a more detailed image of population-wide exposure to PFRs.

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Supporting information

Supporting information containing Table S-1, Figures S-1 and S-2 is available free of charge via the Internet at <http://pubs.acs.org>.

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Figures

Figure 1: Absolute extraction recoveries [%] of target analytes obtained using three different sorbents and two different conditioning and washing conditions.

Figure 2: Matrix effects [%] of mass labelled reference standards of target compounds obtained using three different sorbents and two different conditioning and washing conditions.

Figure 3: Stability of target analytes in wastewater spiked with PFR metabolites only (left) and with both PFR metabolites and PFR parent compounds (right) at room temperature (20 °C) and refrigerated (4 °C). The y-axis represents the variance in response relative to T₀.

Tables

Table 1: Overview of PFR parent compounds and their respective metabolites considered in this study. (*) For biomonitoring studies, it is recommended to include the parent compound TCEP as a target since *in vitro* liver metabolism studies suggest a low clearance of TCEP (Dodson et al 2014; Van den Eede et al 2013). *Italic*: analytes targeted in this study. See Table S-1 for the structures of the listed compounds.

Parent compound	Exposure biomarker
2-ethylhexyldiphenyl phosphate (EHDPHP)	<i>2-ethyl-5-hydroxyhexyl diphenyl phosphate (HO-EHDPHP)</i>
	<i>2-ethylhexyl phenyl phosphate (EHPHP)</i>
	<i>diphenyl phosphate (DHPH)</i>
tris(2-butoxyethyl) phosphate (TBOEP)	<i>bis(2-butoxyethyl) 3'-hydroxy-2-butoxyethyl phosphate (HO-TBOEP)</i>
	<i>2-hydroxyethyl bis(2-butoxyethyl) phosphate (BBOEHEP)</i>
tris(2-chloroisopropyl) phosphate (TCIPP)	<i>1-hydroxy-2-propyl bis(1-chloro-2-propyl) phosphate (BCIPHIPP)</i>
	<i>bis(1-chloro-2-propyl) phosphate (BCIPP)</i>
triphenyl phosphate (TPHP)	<i>diphenyl phosphate (DHPH)</i>
	<i>4-hydroxyphenyl phenyl phosphate (HO-DHPH)</i>
	<i>4-hydroxyphenyl diphenyl phosphate (HO-TPHP)</i>
tris(chloroethyl) phosphate (TCEP)	<i>tris(chloroethyl) phosphate (TCEP)*</i>
	<i>bis(chloroethyl) phosphate (BCEP)</i>

Table 2: Instrumental parameters used for the analysis of target PFRs metabolites. Rt = retention time; CE = collision energy; FV = fragmentor voltage; CAV = cell accelerator voltage.

Compound	Rt	IS	Precursor (m/z)	Product (m/z) (Q1;Q2)	CE	FV	CAV	R ²	IDL [ng mL ⁻¹]	IQL [ng mL ⁻¹]
HO-DPHP	2.95	DPHP-D ₁₀	265.0	93.0 108.0	-30 -50	-140	4	0.9930	0.02	0.08
DPHP	4.07	DPHP-D ₁₀	249.0	93.0 155.0	-30 -15	-146	4	0.9996	0.02	0.06
BCIPHIPP	5.87	TCEP-D ₁₂	309.0	99.0 175.0	17 4	59	4	0.9993	0.04	0.08
TCEP	6.23	TCEP-D ₁₂	285.0	63.0 99.0	29 21	110	4	0.9993	0.01	0.02
EHPHP	6.24	TCEP-D ₁₂	285.0	93.0 79.0	-40 -20	-152	4	0.9916	0.03	0.08
BBOEHEP	7.97	BBOEHEP-D ₄	343.0	45.0 243.0	17 4	88	4	0.9996	0.004	0.01
HO-TBOEP	9.09	BBOEHEP-D ₄	415.0	45.0 99.0	25 33	123	4	0.9930	0.02	0.05
HO-EHDPHP	9.58	TBOEP-D ₆	379.0	251.0 153.0	5 30	99	4	0.9980	0.003	0.01

Table 3: Performance of the validated method.

Compound	UPW								Wastewater			
	Precision (RSD %)				Accuracy (Bias %)				Blank [ng L ⁻¹]	MDL [ng L ⁻¹]	MQL [ng L ⁻¹]	Between-run precision (RSD %)
	Within-run	Between-run			Within-run	Between-run						
		Low	Low	Mid		High	Low	Low				
HO-DPHP	20	23.9	6.2	10.8	8.2	0.9	1.2	12	-	2.3	8	8.2
DPHP	18.1	15.4	1.2	4.4	2.8	3.5	2.2	1.4	3	7.4	25	4
BCIPHIPP	7.7	7.1	2.8	3.9	15.3	14.9	3	7.6	-	1.3	4.4	14
TCEP	3	3	2.9	2.8	20.4	19.8	6.9	5.2	2	1.1	3.7	5.6
EHPHP	5.8	8.8	8.8	6.8	2.9	1.2	3.3	13.6	1.5	19	65	3.9
BBOEHEP	1.5	1.6	1.1	3.9	9.2	8.6	2.3	4.1	-	1.5	5	3.3
HO-TBOEP	3.2	3	2.7	4.2	7.7	7.2	15.3	12	-	11.7	39	2
HO-EHDPHP	6.8	6.3	3.8	1.5	6.9	5.3	9.7	16.7	-	0.5	1.6	8.6

Table 4: Concentrations [ng L^{-1}] and per capita loads [mg day^{-1} 1000 inhabitants $^{-1}$] of target analytes measured in wastewater samples collected from 4 locations across Flanders (Belgium).

Compound	Measured concentrations [ng L^{-1}] (n = 2)			
	OST	LI	GER	NIN
HO-DPHP	< MQL	< MQL	< MQL	< MQL
DPHP	93-101	170-226	593-628	71-74
BCIPHIPP	9.2-13	5.3-8.9	11-11	10-11
TCEP	212-219	244-316	328-389	211-237
EHPHP	168-216	938-1072	1066-1100	301-357
BBOEHEP	37-47	58-94	24-27	29-33
TBOEP-OH	54-67	75-165	43-51	49-49
HO-EHDPHP	2.6-5.2	3.8-7.3	3.5-5.3	2.7-6.6
Compound	Per capita loads [mg day^{-1} 1000 inhabitants $^{-1}$]			
	OST	LI	GER	NIN
HO-DPHP	-	-	-	-
DPHP	21-23	57-83	170-175	18-17
BCIPHIPP	2.9-2.1	1.8-3.3	3.2-3.1	2.7-2.5
TCEP	50-48	106-90	94-108	53-58
EHPHP	38-49	315-396	306-307	89-74
BBOEHEP	8.4-11	19-35	6.9-7.5	8.2-7.1
TBOEP-OH	12-15	25-61	12-14	12-12
HO-EHDPHP	1.2-0.6	1.3-2.7	1.0-1.5	1.6-0.7

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