Comprehensive screening of polar emerging organic contaminants including PFASs and evaluation of the trophic transfer behavior in a freshwater food web

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ABSTRACT

Bioaccumulation and trophic transfer of persistent legacy contaminants have been intensively characterized, but little is known on the contaminants of emerging concern (CECs) in freshwater food webs. Herein, we comprehensively screened CECs with a focus on polar substances and further evaluated their trophic transfer behavior in selected items from the food web of Lake Templin, Germany. We selected one plankton, two mussel, and nine fish samples covering three trophic levels. With an effective multi-residue sample preparation method and high-resolution mass spectrometry-based target, suspect, and non-target screening, we characterized 477 targets and further screened unknown features in complex biota matrices. Of the 477 targets, 145 were detected and quantified in at least one species (0.02–3640 ng/g, dry weight). Additionally, the suspect and non-target analysis with experimental mass spectra libraries and in silico techniques (MetFrag and SIRIUS4/CSE:FingerID) enabled further identification of 27 unknown compounds with 19 confirmed by reference standards. Overall, the detected compounds belong to a diverse group of chemicals, including 71 pharmaceuticals, 27 metabolites, 26 pesticides, 16 per- and polyfluoroalkyl substances (PFASs), 4 plasticizers, 3 flame retardants, 11 other industrial chemicals and 14 others. Moreover, we determined the trophic magnification factor (TMF) of 34 polar CECs with >80% detection frequency, among which 6 PFASs including perfluorooctanoic acid (PFOS), perfluorodecanoic acid (PFDA), perfluorohexane sulfonic acid (PFHxS), perfluorotridecanoic acid (PFTrA), perfluorotetradecanoic acid (PFPeA), and perfluoroundecanoic acid (PFUnA), exhibited biomagnification potential (TMF = 1.8 - 4.2, p < 0.05), whereas 5 pharmaceuticals (phenazone, progesterone, venlafaxine, levamisole, and lidocaine) and 1 personal care product metabolite (galaxolidone) showed biodilution potential (TMF = 0.4 - 0.6, p < 0.05).

1. Introduction

Contaminants of emerging concern (CECs) include pharmaceuticals and personal care products (PPCPs), per- and polyfluoroalkyl substances (PFASs), endocrine-disrupting compounds, and others (Dulio et al., 2018; US EPA, 2019). These chemicals and their metabolites are currently not well regulated. CECs are new challenges for human health and environmental quality. Indeed, many CECs and their metabolites have been ubiquitously detected in the environment, particularly in surface water (Arinaitwe et al., 2021; Munz et al., 2017; Peng et al., 2018), groundwater (Burke et al., 2016; Kiefer et al., 2021; Reemtsma et al., 2013), soil and sediment (Chiaia-Hernandez et al., 2020; Zhang et al., 2016), with concentrations in the ng/L to μg/L range. Despite trace levels in the surrounding environment, they can accumulate in aquatic organisms (de Solla et al., 2016; Munz et al., 2018; Pico et al., 2019; Valdes et al., 2014). For example, Munz et al. detected 63 CECs in the invertebrate Gammarus sp. from wastewater-impacted streams (Munz et al., 2018). Pico et al. (2019) found 76 CECs in fish from four Spanish rivers. CECs may pose potential risks to the environment and human health. Once CECs enter an organism, they may induce adverse...
effects and further undergo trophic transfer along the food web. For example, the accumulation of endocrine disruptors in aquatic wild life such as mollusk and alligators could lead to reproductive abnormalities, resulting in a substantial population decline (Sumpter and Johnson, 2005).

Therefore, it is essential to evaluate the bioaccumulation potential and trophic transfer behavior of organic chemicals along the food web. The trophic transfer risks are usually determined by trophic magnification factors (TMFs), which are calculated from the slope of logarithmically transformed concentrations of chemicals versus the trophic level of organisms in the food web (Borgå et al., 2012; Conder et al., 2012; Kidd et al., 2018). Current trophic transfer research has mainly focused on non-polar legacy compounds such as polychlorinated biphenyls (PCBs) (Figueiredo et al., 2014; Kofsd et al., 2021; Kwon et al., 2006; Maul et al., 2006), polybrominated diphenyl ethers (PBDEs) (Lapointe et al., 2020; Ma et al., 2013), and dichlorodiphenyltrichloroethane (DDT) (Blair et al., 2015) due to their high hydrophobicity and known biomagnification potential. In contrast, less research has been conducted on the trophic transfer of polar CECs in freshwater ecosystems. Compared to non-polar compounds, polar CECs should be considered equivalent in terms of toxicological significance due to their higher bioavailability in water systems. Polar CECs are often hydrophilic and comprise various functional moieties that diversify their uptake and bioavailability in water systems. Therefore, it is challenging to predict the trophic transfer potential of polar CECs based on past knowledge of hydrophobic legacy compounds. Thus, it is needed to investigate polar CECs along the food web in a natural environmental scenario. Recent studies have reported the trophic transfer of some CECs, including antibiotics, PPCPs, and PFASs (Fang et al., 2014; Houde et al., 2008; Jartun et al., 2019; Liu et al., 2018a; Loi et al., 2011; Martin et al., 2004; Simonet-Laprade et al., 2019; Yang et al., 2020; Zhou et al., 2020). For example, Zhou et al. investigated the trophic transfer of 24 antibiotics in the freshwater food web in Lake Taihu, China (Zhou et al., 2020). Yang et al. studied the trophic transfer of 30 PPCPs in urbanized rivers (Yang et al., 2020). Other studies focused on the trophic transfer of up to 30 PFASs in aquatic food webs (Chen et al., 2018; Fang et al., 2014; Houde et al., 2008; Jartun et al., 2019; Loi et al., 2011; Martin et al., 2004; Mazzoni et al., 2020; Simonet-Laprade et al., 2019; Xu et al., 2014). These studies are informative but mostly focus on specific chemical classes through target screening. The lack of an analytical approach has hampered the comprehensive screening of CECs to further explore the trophic transfer behavior under field conditions. High-resolution mass spectrometry (HRMS)-based suspect and non-target screening offers a promising approach to simultaneously identify a broad range of CECs (Hollender et al., 2017). It has been applied in abiotic environmental samples such as wastewater, surface water (Hollender et al., 2017), groundwater (Kiefer et al., 2021), soil, and sediment (Chíaña-Hernández et al., 2020), but rarely for complex biota matrices, especially for multiple species. It is challenging to develop an analytical approach, which covers a broad range of polarity and diversity of chemical structures in various biota matrices.

The present study aimed to comprehensively screen potential polar CECs in freshwater biota and evaluate their trophic transfer behavior in a freshwater food web. Various species at three trophic levels were selected, including one plankton, two mussels, and nine fish samples from the food web of Lake Templin near Potsdam in Germany. The derived food web samples were evaluated and proven to successfully capture the trophic magnification potential of POPs with well-known biomagnification properties (e.g., PCBs, mercury/mercury) in a previous investigation (Kosfeld et al., 2021), supporting the use of these samples in this study to characterize the trophic transfer of other substances, such as CECs. After confirming the suitability of the food web samples, we optimized a sample preparation method and an analytical workflow to comprehensively screen polar CECs in the different biota types through an integrated target, suspect, and non-target approach. We then quantified the known polar CECs and identified unknowns in different species. Finally, we characterized the trophic transfer potential of the identified polar CECs. This is the first study of an integrated target, suspect, and non-target approach to thoroughly evaluate the trophic transfer of various polar CECs in a freshwater food web.

2. Materials and methods

Fig. 1 outlines the overall workflow for this study.

2.1. Chemicals and solvents

Details on chemicals and solvents used in this study are given in the first Supporting Information document SI1st. A, Table S1. Details on the target substances, internal substances, suspect lists are provided in the second Supporting Information document SI2nd. A–D.

2.2. Aquatic biota samples and passive samplers

Biota samples, including plankton, mussels and fish, were collected in Lake Templin near Potsdam, Germany in 2018 (Kosfeld et al., 2021) (SI1st. B, Fig. S1). After collection, the plankton fraction (<200 µm) was freeze-dried. Zebra mussels were transferred into rearing aquaria for 24 to 48 h for gut clearance, followed by immediate freezing, sorting (small size: <2 cm, big size: >2 cm), shelling and subsequent cryo-storage using liquid nitrogen. Fish (white bream, roach, bleak, perch, asp, pike, and pikeperch) were dissected into fillet and carcass and then frozen with liquid nitrogen. Details on the sampling site selection and the food web sample information are provided in SI1st. B and SI1st. C (Table S2 and Figs. S1 and S2).

All biota samples were cryo-stored (<−150 °C) before sample preparation. Frozen biota samples were manually crushed, cryo-milled and freeze-dried. Composite whole-body tissue samples were grouped according to their pre-determined species and sizes. Different to a previous analysis of the same sample set for legacy POPs from Lake Templin (Kosfeld et al., 2021), we combined perch and perch 1 into one perch sample, pike and pike 1 into one pike sample, as well as pikeperch A and B into one pikeperch sample in this study due to the limited biomass. Accordingly, we used mean values of δ15N to analyze the trophic position of perch, pike and pikeperch, respectively.

To reduce the analytical challenges that come along with complex biota matrices, we preliminarily screened chemicals in passive samplers to get a first overview of CECs in the lake. Empore® styrenevinylbenzene (SDB) passive sampler disks covered by a polyethersulfone (PES) membrane, were placed in the northern, middle, and southern parts of the lake, respectively. Then the list of the masses detected in the passive sampler extracts was used as a suspect list to support the suspect and non-target analysis of contaminants in complex biota samples.

2.3. Water, lipid, and protein content determination of biota

The water content was gravimetrically determined by freeze-drying the biota samples. Lipids of biota samples were extracted with a mixture of isopropanol-cyclohexane-water (8:10:11, v:v:v) according to established protocols (Schlechtriem et al., 2019; Smedes, 1999). The protein content was determined using the Tissue Protein Extraction Reagent (Thermo Scientific). Further details are described in a previous publication (Kosfeld et al., 2021).

2.4. Sample preparation

We optimized and compared two different sample extraction methods in terms of absolute recovery, relative recovery, matrix factor, and limit of quantification (LOQ) for comprehensive screening of chemicals in biota, i.e., a sequential solvent extraction (SSE) method, as
well as a quick, easy, cheap, effective, rugged, and safe approach (QuEChERS) (Anastassiades et al., 2003). Each freeze-dried biota sample (0.14 - 0.2 g, three replicates) was weighted into a 5-mL Eppendorf tube and then mixed with 100 μL of isotopic labeled internal standards (ISTDs) solution (1 mg/L), followed by extraction using SSE or QuEChERS methods and a cleanup described below. SDB disks of the passive samplers were extracted with methanol and acetone according to the method described in a previous publication (Moschet et al., 2015).

For the SSE method, 2.5 mL of an acetonitrile/methanol/water mixture (2:2:1, v:v:v, pH 7.0) were added into the tube containing the biota sample and ISTDs, followed by vortexing for 60 s and ultrasonication for 10 min in a water bath at 25 °C. The suspension was centrifuged (6 min, 20 000 g, 4 °C), and the supernatant was decanted into a new clean 5-mL tube. The same extraction step was repeated once, and the supernatants were pooled. After that, the residual was sequentially extracted with an acidified acetonitrile/methanol/water mixture (0.15% formic acid, pH 3.0) and a basified acetonitrile/methanol/water mixture (0.5% of a 25% aqueous ammonia solution, pH 8.5). After centrifugation (6 min, 20 000 g, 4 °C), the acidic and basic supernatants were pooled to neutralize each other (pH 7.0).

For the QuEChERS method, 2.5 mL of an acetonitrile/methanol/water mixture (1:1, v:v, pH 7.0) was added, followed by vortexing for 60 s and ultrasonication for 10 min in a water bath at 25 °C. The suspension was centrifuged (6 min, 20 000 g, 4 °C), and the supernatant was decanted into a clean 5-mL tube. The same extraction step was repeated once, and the supernatants were pooled.

After extraction, all samples were frozen at –20 °C for 48 h and then centrifuged to remove the precipitates. The supernatants were concentrated under nitrogen to about 0.5 mL using a vacuum concentrator (Eppendorf Concentrator plus) at 30 °C. The extracts were reconstituted to 1 mL by adding a mixture of acetonitrile/methanol (1:1).

2.5. LC-HRMS/MS analysis

All sample extracts were preliminarily cleaned up and enriched with an automated online - solid phase extraction (SPE) method followed by liquid chromatography coupled to a high resolution mass spectrometry (LC-HRMS/MS) analysis using a Q Exactive or Q Exactive Plus mass spectrometer, equipped with an electrospray ionization (ESI) interface (Thermo Scientific). Briefly, an aliquot of 200 μL of the extract was added to 20-mL of headspace amber glass vials and filled up to 20 mL with ultrapure water. After online SPE enrichment of the 20 mL, the chromatographic separation was achieved on a reversed-phase C18 column at 30 °C (Atlantis® T3, 3 μm, 3.0 × 150 mm, Waters). The HPLC system comprised a PAL autosampler (CTC Analytics) and a Dionex UltiMate3000 RS pump (Thermo Scientific). For positive mode, water (with 0.1% formic acid) and methanol (with 0.1% formic acid) were used as mobile phases A and B, respectively. For negative mode, 100% water and 95% methanol/5% water (both with 5 mM ammonium formate) were used as mobile phases A and B, respectively. The flow rate was 300 μL/min.

Mass spectra were acquired using ESI positive and negative mode separately. Full scan MS1 acquisition (m/z: 100–1000) with a mass resolution of 140 000 (full-width half-maximum (FWHM) at 200 m/z) was performed, followed by data-dependent MS/MS scans (top 5 highest intensity) with a resolution of 17500 (FWHM at 200 m/z) using higher energy collision-induced dissociation and an isolation window of 1 Da. More details about the analytical procedure and parameters of LC–HRMS/MS are available in SI1st D (Tables S3–S5).

2.6. Target quantification

Target quantification was performed with the software TraceFinder...
4.1 or 5.1 (Thermo Scientific). Target analytes were quantified based on the retention time (RT), MS/MS fragments, and a linear calibration curve (weighting 1/x) of the reference standards (n = 477) using the peak area ratio of the respective reference standard and ISTD of the analyte. For targets without their own ISTD, an alternative ISTD was selected based on a similar elution RT and concentrations were further corrected based on the corresponding relative recovery. Details on the quantification method and quality control are given in SI1st. F, Fig. S3. The target and internal standard chemicals are listed in SI2nd, A and B.

2.7. Suspect and non-target screening

2.7.1. Raw data pre-processing

Compound Discoverer 3.1 (CD, Thermo Scientific) was used for the suspect and non-target screening. Three suspect lists of the NORMAN Suspect List Mass Exchange Database (~40,000 substances from https://www.norman-network.com/?q=suspect-list-exchange), a suspect mass list from the initial screening of passive samplers (~5000 substances), and an in-house Eawag database with reference standards (~500 substances) were imported into the CD workflow. In addition, MS/MS spectra databases including mzCloud, EU MassBank, MassBank of North America (MoNA), and Eawag MassBank were added to the workflow for structural identification. Before analyzing the data from the biota samples, we processed the HRMS/MS raw data of a calibration standard solution containing about 200 target substances to optimize the parameters of the CD workflow. After the workflow validation, the acquired HRMS/MS raw data of biota samples were pre-processed (peak picking, retention time alignment, grouping of isotopologues and adducts, and compounds across samples). Finally, a feature matrix table containing compound IDs, molecular formula, ion intensities, sample IDs, and groups was generated as a result of the performed workflow. The workflow chart and detailed parameter settings for each step are provided in SI1st. F (Fig. S4 and Tables S6–22). The suspect chemicals are listed in SI2nd, C and D.

2.7.2. Feature prioritization strategies

The features obtained from raw data pre-processing were prioritized based on the following steps. (1) High-intensity peaks were selected based on the inclusion criteria: (i) 5 min < RT < 30 min, (ii) peak intensity >10^5, (iii) ratio of samples and all controls/blanks > 5. Then, multiple filtering strategies were applied to pick out features in parallel as described in the following steps (2a, 2b, and 2c). (2a) The resulting features from step (1) were compared with suspect list databases, and the matched features opted in; (2b) the features with characteristic mass spectra of halogens (i.e., Cl, Br, F) were selected; and (2c) the features with an increasing intensity trend along the food web were considered indicative of biomagnification and therefore selected. (3) The matched features from (2a), (2b), and (2c) were manually checked to filter out bad peak shapes. (4) Finally, the resulting features were stored for further structural elucidation and identification. The schematic representation and the number of prioritized features at each step are given in SI1st. G.

2.7.3. Structural elucidation and confirmation

The final prioritized features were annotated based on matching MS/MS spectra of mzCloud and MassBank libraries in CD3.1. For features that did not match a library spectrum, structure proposals were assigned using in silico fragmentation spectra of PubChem Lite compounds with MetFrag CL2.4.5 (from R package ReSOLUTION) (Ruttkies et al., 2016) or the machine learning-based tool SIRIUS4/CSF/FingerID (4.0.1) (Hübner et al., 2015). The top 10 candidates were checked manually and an initial confidence level was assigned. Finally, if commercially available, the standards were purchased to confirm or reject the identification. Confirmed structures (level 1) were achieved by matching RT and MS/MS with reference standards (Schymanski et al., 2014). Probable structures (level 2) were assigned and achieved by matching major fragments with MS/MS libraries, while tentative structures (level 3) were proposed based on the MS/MS interpretation. Prioritized features with exact mass and unequivocal molecular formula were assigned to unknowns as level 4. Finally, features with no structure and no unequivocal molecular formula were assigned to unknowns of interest as level 5.

2.8. Trophic level characterization and trophic magnification factors

The trophic levels of aquatic biota were determined from stable isotope ratios of carbon (δ^{13}C) and nitrogen (δ^{15}N). The δ^{13}C and δ^{15}N values of all of the pretreated samples were determined by Agroisolab GmbH (Jülich, Germany) after lipid extraction. The detailed analysis of δ^{13}C and δ^{15}N values can be found elsewhere (Kosfeld et al., 2021). The trophic level of one organism was calculated based on the previously established method (Post, 2002) (Eq. (1)).

\[
\text{Trophic level} = \left( \frac{\delta^{15}N_{\text{baseline}} - \delta^{15}N_{\text{baseline}}}{\Delta^{15}N} \right) + \lambda
\]

Where \( \delta^{15}N_{\text{baseline}} \) is the δ^{15}N value of the baseline organism (mussels in this study), \( \delta^{15}N_{\text{predator}} \) is the δ^{15}N value of each consumer. The \( \lambda \) represents the trophic level of the baseline organisms (\( \lambda = 2 \) in this study) (Kosfeld et al., 2021). \( \Delta^{15}N \) is a nitrogen isotope trophic increment, which was set to 2.3‰ according to the analysis by McCutchan et al. (2003).

The trophic magnification factor (TMF) was calculated based on the trophic level of a given organism Eqs. (2) and (3)

\[
\text{TMF} = 10^{b \times \text{trophic level} - a}
\]

Where \( C_{b} \) is the chemical concentration in the organisms of a given trophic level; \( b \) is the slope of the linear regression of the log-transformed %C; \( a \) is the constant of the linear regression. A TMF > 1 suggests biomagnification along the food web.

To account for differences in lipid or protein content for the different species, we further normalized the biota concentrations based on their defined lipid or protein contents in the organisms and then determined their TMF_{lip} or TMF_{prot} respectively.

3. Results and discussion

3.1. Comparison and performance of analytical methods

Sample preparation is a crucial step for the comprehensive screening of chemicals in complex matrices. We optimized and compared two extraction methods, SSE and QuEChERS, to achieve an optimal performance for a broad coverage of chemicals in plankton, mussel, and fish samples. The recoveries and LOQs were not significantly different between SSE and QuEChERS. Finally, we choose the SSE method for all the field samples because more interferences for the mass spectrometric detection were expected from the salts used in the QuEChERS method. The detailed method performance in terms of the relative recovery, absolute recovery, matrix factor, and LOQ are given in SI1st. E, Fig. S3 and SI2nd. A.

The SSE method was successfully applied for the field biota samples to quantify 477 target compounds with a wide range of physicochemical properties (log D_{ow} ranging from ~3.9 to 6.9, at pH = 7, SI2nd. A and B). The average absolute recoveries in plankton, mussels, and fish were similar and about 52% (Fig. 2). The average matrix factors in plankton, mussels, and fish was 0.52, 0.58, and 0.66. The matrix factor of most compounds (406 of 477 substances) was below 1, indicating ion suppression. Therefore, the matrix should be considered as a major factor influencing the analytical performance. Nevertheless, we used the isotope-labeled internal standards to account for this effect and achieved acceptable relative recoveries (70%–130%) for the quantification of major
over 80% of the target compounds (Fig. 2), indicating a broad coverage of substances. The concentration of the remaining target compounds with a relative recovery of less than 70% or over 130% have higher uncertainty, and should be more carefully interpreted. This uncertainty might be due to the lack of an own isotope-labeled internal standard, although the matrix factor was used to correct the relative recovery. LOQs ranging from 0.05 to 5.70 ng/g (dry weight, dw) (equivalent to 0.23 to 28.5 ng/g, wet weight based on the average water content of 78%) were achieved for 477 compounds in the three different biota matrices, showing excellent sensitivity for a diverse set of chemicals in

Fig. 2. Analytical method performance of the sequential solvent extraction (SSE) for 477 target substances in different biota: A: absolute recovery; B: matrix factor; C: relative recovery; D: limit of quantification (LOQ). *we used biofilm as an alternative material for method performance validation due to the limited plankton amount collected from the field. Note: the spiked amounts of standards were 10 ng/g for PFASs and 100 ng/g for other chemicals.

Fig. 3. The trophic levels of biota and concentrations of organic substance classes in biota (ng/g, dry weight).
Table 1
Identified compounds via the suspect and non-target screening sorted according to the chemical group. Note: Different gray shades indicate the confidence level of identification. a: the ions were ordered by descending intensity. b: the initial level refers to the identification based on spectra libraries or in silico prediction. The final level refers to the identification with reference standards.

| Substance class | Chemical name | Initial to final confidence level a | CAS-No | Molecular formula | Monoisotopic Mass (g/mol) | Retention Time (min) | Adduct and CE (eV) for MS/MS | MS/MS confirmatory ions b |
|-----------------|---------------|-------------------------------------|--------|-------------------|--------------------------|---------------------|----------------------------|--------------------------|
| Pharmaceutical  | Nicotine      | 2a-1                                 | 54-11-5| C_{10}H_{14}N_{2}  | 162.1157                 | 6.7                 | [M + H]^{+} 95              | 117.0574, 130.0652, 106.0652 |
|                 | Haloperidol   | 2a-1                                 | 52-86-8| C_{18}H_{23}ClFNO_{3} | 375.1401                | 16.7                | [M + H]^{+} 15              | 376.1477, 165.0711, 368.1360 |
|                 | Pyrimethamine (Daraclor) | 3-1 | 58-14-0 | C_{10}H_{14}CIN_{5} | 248.0829                | 15.3                | [M + H]^{+} 60              | 249.0897, 233.0589, 177.0217 |
| Pharmaceutical metabolite | Desacetyl diltinamin | 2a-1 | 75472-41-2 | C_{10}H_{12}CIN_{4}O_{3}S | 372.1508 | 16.0 | [M + H]^{+} 15              | 373.1576, 178.0320, 328.1010 |
|                 | Dibhydro bupropion | 3-1 | 92264-42-9 | C_{10}H_{14}CINO | 241.1233                | 15.5                | [M + H]^{+} 60              | 158.0576, 57.0702, 114.0621 |
| 2,2,4-Trimethyl-1,3-pentanediol dlisobutyrate | 2a-1 | 6845-50-0 | C_{10}H_{14}O_{6} | 286.2144 | 23.9 | [M + H]^{+} 45             | 69.0699, 111.1168, 83.0854 |
|                 | Acetyl tributyl citrate (Citroflex A-4) | 2a-1 | 77-90-7 | C_{32}H_{48}O_{3} | 402.2254 | 23.6 | [M + H]^{+} 20             | 185.0110, 157.0133, 129.1430 |
| Plasticizer     | Benzy1 butyl phthalate | 2a-1 | 85-68-7 | C_{10}H_{14}O_{5} | 312.1362 | 23.1 | [M + H]^{+} 30             | 91.0543, 149.0233 |
|                 | Dibutyl phthalate | 2a-1 | 94-74-2 | C_{10}H_{14}O_{4} | 278.1518 | 23.1 | [M + H]^{+} 50             | 149.0224, 57.0702, 121.0285 |
|                 | Dimethyl phthalate | 2a-1 | 131-11-3 | C_{9}H_{12}O_{3} | 194.0579 | 18.0 | [M + H]^{+} 80              | 79.0541, 77.0384, 105.0466 |
|                 | Triisohexyl phosphate (TIEP) | 2a-1 | 126-71-6 | C_{20}H_{36}O_{5}P | 266.1647 | 23.0 | [M + H]^{+} 50             | 98.9843, 57.0702 |
|                 | Tri(2-butoxyethyl) phosphate (TBEYP) | 2a-1 | 78-51-3 | C_{12}H_{20}O_{5}P | 398.2433 | 23.4 | [M + H]^{+} 15             | 299.1619, 199.0750, 399.2500 |
|                 | Tri(2-chloroethyl) phosphate (TCEP) | 2a-1 | 115-96-8 | C_{10}H_{12}Cl_{2}O_{5}P | 283.9539 | 16.6 | [M + H]^{+} 45             | 62.9998, 98.9842, 124.9999 |
| Antioxidant     | Diphénylamines | 2a-1 | 122-39-4 | C_{9}H_{14}N | 169.089 | 21.1 | [M + H]^{+} 95             | 93.0575, 65.0388, 92.6497 |

(continued on next page)
complex matrices. The detailed parameters of method performance including LOQs, absolute recoveries, relative recoveries, and matrix factors of 477 target substances in three different biota matrices, are provided in the Second Supporting Information document SI2nd. A. Overall, the LOQs were better or comparable to those reported in other studies, which determined 84 substances with LOQs ranging from 0.1 to 380 ng/g (wet weight) in aquatic amphipods (Munz et al., 2018) and 217 substances with LOQs ranging from 0.2 to 1100 ng/g (wet weight) in fish and sea eagles (Dürig et al., 2020).

3.2. Internal concentrations of target substances in the biota

Combining stable isotope analyses and chemical analyses of biota can reveal the bioaccumulation and biomagnification of contaminants within the food web. The detailed value of $\delta^{15}$N and $\delta^{13}$C are provided in SI1st, Table S2 and Fig. S2. Based on $\delta^{15}$N values from 14.7 ± 0.1% to 22.0 ± 0.1% (Fig. 3A), the trophic levels of all organisms followed the ascending order of plankton, mussel, small mussel (size: <2 cm), white bream, roach (size: 35–36 cm), small roach (size: 9–11 cm), bleak, small perch (size: 7–10 cm), asp, pike, perch (size: 28–32 cm), and pikeperch (Fig. 3A). To obtain realistic trophic positions, we applied the increment of 2.3‰ proposed by Kosfeld et al. (2021), which is comparable to that ($\Delta^{15}$N: 2.0‰) in stream ecosystems (Jardine et al., 2013) but lower than the conventional value ($\Delta^{15}$N: 3.4‰) for aquatic organisms in freshwater systems. Because trophic positions directly influence the calculation of TMFs, the applied increment has to be considered when interpreting TMFs in Section 3.4 below.

Of 477 target compounds, 145 were detected and quantified above Table 1 (continued)

| Flavoring agent | Sulfural | 3/1 | 137.20-8 | C$_{18}$NOS | 143.9405 | 12.8 | [M + H]$^+$ 100 | 113.032, 112.025, 126.041 |
|-----------------|----------|-----|---------|------------|---------|-----|----------------|-----------------------------|
| Halogenated tryptophan | 6-Bromo-2,3-tryptophan | 3/1 | 33592-41-0 | C$_{14}$H$_{12}$BrN$_{2}$O$_{3}$ | 282.0094 | 16.3 | [M + H]$^+$ 45 | 78.91487, 193.91114, 219.97687 |
| Surfactant | N,N-Dimethyldodecylamine N-oxide | 2a/2a | 1643-26-5 | C$_{18}$H$_{35}$NO | 229.2406 | 19.8 | [M + H]$^+$ 60 | 58.0655, 62.0603, 212.2382 |
| Other industrial chemical | 2-nitro-2-butylphenolet | 2a/1 | 85-72-5 | C$_{6}$H$_{8}$O | 150.1045 | 17.1 | [M + H]$^+$ 100 | 93.0698, 95.0490, 53.0399 |
| | Benzamidine | 2a/1 | 55-21-0 | C$_{9}$H$_{9}$NO | 121.0528 | 12.9 | [M + H]$^+$ 60 | 105.0353, 79.0542, 122.0560 |
| | Valerozone | 2a/1 | 1068-14-9 | C$_{14}$H$_{10}$O | 162.1045 | 18.2 | [M + H]$^+$ 100 | 53.0390, 51.0234, 95.0492 |
| Unknowns | N$_{2}$-[4-(4-Chlorophenyl)-1,2,4-oxadiazol-5-y]phenyl| | 3/3 | - | C$_{18}$H$_{12}$CIN$_{2}$O$_{3}$ | 365.0567 | 22.7 | [M + Na]$^+$ 20 | 388.0471, 194.0811, 164.0705 |
| | 7-chloro-2,1,7-dithyrozoxyl-1,7-| | 3/3 | - | C$_{18}$H$_{18}$ClO$_{3}$ | 410.1880 | 21.5 | [M + H]$^+$ 20 | 113.0480, 409.2067, 125.0607 |
| | 2-thiophenyl-2,1,7-dithyrozoxyl-1,7-| | | - | C$_{18}$H$_{18}$ClO$_{3}$ | 412.2380 | 26.5 | [M + H]$^+$ 20 | 411.3155, 129.0195, 85.0295 |
| | 5-((1-carboxy-2-(1H-imidazol-5-yl)methyl)mino)-5-methyl-5-enoic acid | | 3/3 | - | C$_{18}$H$_{18}$N$_{2}$O$_{3}$ | 281.1376 | 15.2 | [M + H]$^+$ 45 | 110.0713, 156.0768, 95.0604 |
| Unknowns (initially identified as 4-| 3/5 | - | - | 196.9476 | 16.5 | [M + H]$^+$ 95 | - |
| Unknowns (initially identified as Chloroderex| 3/5 | - | - | 319.1815 | 11.4 | [M + H]$^+$ 30 | - |
| Unknowns (initially identified as Camphor | 3a/5 | - | - | 152.1280 | 20.2 | [M + H]$^+$ 100 | - |
LOQ in at least one species. These chemicals include 68 pharmaceuticals, 25 pesticides, 16 PFASs, 25 metabolites, and 11 others. The concentrations of all detected compounds in different biota samples are given in SI2nd. In total, 13 of 145 detected chemicals were found across all biota, including 4-formylaminoantipyrine, methyl-1H-benzotriazole, benzotriazole, fosinopril, lidocaine, perfluorodecanoic acid (PFDA), perfluorohexane sulfonic acid (PFHxS), perfluorooctane sulfonic acid (PFOS), perfluorotetradecanoic acid (PFTeA), perfluorotridecanoic acid (PFTrA), pregabalin, progesterone and terbutryn. It is worth noting that illicit drugs and sweeteners were mostly found in plankton and mussels and rarely detected in fish.

Among these chemical categories, PFASs showed the highest total concentrations (9401 ng/g, dw) followed by pharmaceuticals (6578 ng/g, dw), metabolites (1527 ng/g, dw), pesticides (960 ng/g, dw), and others (330 ng/g, dw) (Fig. 3B). The 25 metabolites include 12 pharmaceutical metabolites, 12 pesticide metabolites, and 1 illicit drug metabolite. These results emphasize the importance of monitoring contaminant metabolites, which can be present at higher concentrations than their parent compounds and may be bioactive or even more toxic than parent compounds (Cwiertny et al., 2014; Fu et al., 2020; Qu et al., 2013).

The different groups of chemicals enter the freshwater system through different pathways. A large number of pharmaceuticals (n = 68) were detected in biota, probably due to the effluent discharge from the largest WWTP of the city of Berlin upstream of the Lake Templin. Indeed, an earlier study on streams influenced by WWTP discharge in

![Fig. 4. Linear regressions between the trophic levels of biota in the aquatic food web and log-transformed concentrations of detected (A) PFASs (biomagnification, TMF > 1, p < 0.05); (B) pharmaceuticals (biodilution, TMF < 1, p < 0.05); and (C) a personal care product metabolite (biodilution, TMF < 1, p < 0.05). Note: gray areas display the 95% confidence intervals. The respective 95% confidence intervals of TMFs are displayed in brackets. The p-value is the probability for the observed linear regression, dw in the unit of the Y-axis means dry weight. The trophic level on the X-axis is given in SI1st, Table S2. Phenazone and levamisole were detected in > 80% samples, but only in 10 biota samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image-url)
Switzerland has found higher concentrations of pharmaceuticals in gammarids caught downstream of the WWTPs than upstream (Munz et al., 2018). In contrast, the presence of pesticides, including herbicides, fungicides, insecticides, and biocides, in biota across different trophic levels was likely due to emission from agricultural or urbanized areas (Wittmer et al., 2010). PFASs may come from different pathways, such as WWTP effluents, industrial emissions, or agricultural runoff. The widespread occurrence of PFAS has been reported in many environmental compartments, including sediment, soil, aquatic vertebrates and invertebrates (Giesy and Kannan, 2001; Koch et al., 2019; Liu et al., 2018; Teunen et al., 2021; Tian et al., 2020). The metabolites in the biota samples could either be attributed to uptake from water or biotransformation in the organisms or both. 5 of the 25 detected metabolites (including diuron-desdimethyl, flufenacet OXA, metazachlor OXA, methiocarb sulfone phenol, and N-2,4-Dimethylphenyl-N'-methylformamidine) were detected in the organisms but not in the passive sampler samples, indicating biotransformation in the organisms. The remaining 20 metabolites were also detected in passive sampler samples, suggesting that they most probably were assimilated from the water phase.

3.3. Identification of unknown features in the biota via suspect and non-target screening

Beyond target analysis, the suspect and non-target screening allowed further identification of 27 compounds. We applied rigorous prioritization strategies to focus on the most relevant features from about one million unknown features in 12 aquatic species (36 extract samples, 3 replicates) (details of feature prioritization and filtering in SI1st). Of the 27 features, 17 were initially assigned to the confidence level 2a based on matches with library spectra, and 10 were assigned to the confidence level 3 based on in silico predictions from at least one algorithm (MetFrag or SIRIUS/CSI:FingerID). Next, commercially available reference standards (n = 22, no available standards for the other 5 features) were purchased to confirm the initial identifications. Finally, 15 features initially at level 2a were confirmed and assigned to confidence level 1, and one feature at level 2a was rejected (Table 1), resulting in a 94% successful confirmation rate. In addition, 4 features initially at level 3 were confirmed as level 1 by reference standards, and 2 features were rejected (Table 1), i.e. a 67% of successful confirmation rate. Overall, 19 out of the 22 newly purchased reference standards were confirmed (and three rejected). The detailed information for each confirmed substance and their MS/MS spectra are provided in SI1st, H and SI1st, I (Figs. S6–S53). Despite the complex matrices and broad analyte coverage of the target analysis (477 compounds), our results present a very high success rate (86%) of structural confirmation of contaminants at level 1. Besides the rigorous prioritization strategies, the experimental MS/MS spectral databases such as Massbank are crucial for initial identification since 94% of the database-matched features were further successfully confirmed using reference standards. Also, 67% of the features, initially assigned to level 3 based on the in silico prediction techniques MetFrag and CSI:FingerID were successfully confirmed as level 1 using reference standards. These results highlight how the high-quality MS/MS spectral libraries and in silico prediction techniques play a crucial role in identification of unknown organic substances. Nevertheless, the use of reference standards remains the gold standard for unambiguous final structural confirmation.

The identified non-targets mainly include plasticizers, flame retardants, other industrial chemicals and pharmaceutical metabolites. This observation could be attributed to the enormous use of plasticizers and flame-retardants in plastics, toys, textiles, furniture, buildings, and other materials. Plasticizers and flame-retardants are released to the environment through leaching, abrasion, and evaporation. Hence, they have been previously detected in various environmental matrices, including wastewater, surface water, groundwater, air, and particulate material (Reemtsma et al., 2008). Additionally, plasticizers and flame-retardants are found to accumulate in biota. In fact, the chemicals triisobutyl phosphate (TIBP), tris(2-butoxyethyl) phosphate (TBP), and tris(2-chloroethyl) phosphate (TCP) found in the freshwater biota analyzed in this study (Table 1), were previously detected in arctic fish, birds, and mammals from diverse habitats (Hallanger et al., 2015), as well as tropical fishes from the Philippines (Kim et al., 2011). Collection of samples close to WWTP discharge resulted in higher concentrations of flame retardants and plasticizers (e.g., TBP) in both marine and freshwater biota (Sundkvist et al., 2010). In addition, this study also identified other polar CECs through suspect and non-target screening, such as industrial chemicals, a flavoring agent, and pharmaceutical metabolites. For instance, to our knowledge, the flavoring agent sulfuroil was detected for the first time in freshwater organisms.

3.4. Biomagnification and biodilution of the identified substances in the food web

To evaluate the biomagnification or biodilution potential of the confirmed chemicals from the target, suspect, and non-target approaches, we calculated the TMFs of the chemicals in the food web. To ensure minimal uncertainty in the food web, we only calculated the TMFs of chemicals (n = 34, 29 targets and 5 non-targets) with a detection frequency greater than 80% across all samples (SI2st, Table S23). Among these 34 contaminants, 12 (including 6 PFASs, 5 pharmaceuticals, and 1 metabolite) showed significant biomagnification or biodilution patterns (p < 0.05, Fig. 4). The remaining compounds showed no significant pattern (p > 0.05, SI2st, Table S23). The TMFs of six PFASs, i.e., PFOS, PFDA, PFHxS, PFTrA, PFPeA, and per-fluoroundecanoic acid (PFUnA), ranged from 1.8 to 4.2 (Fig. 4A), indicating a biomagnification potential from plankton to fish in the selected freshwater food web. In contrast to the well-known lipophilic bioaccumulation mechanism of hydrophobic compounds, PFASs are suggested to enter the food web by binding to proteins, especially water-soluble proteins such as serum proteins (e.g., serum albumin) (Forsthuber et al., 2020; Zhang et al., 2009). Therefore, we further calculated the protein-based TMFs based on the protein normalized concentrations of PFASs in the organisms (SI2st, Tables S24 and S25). We found that the protein-based TMFs of PFASs are similar to the dry weight-based TMFs, indicating a consistent calculation of bioaccumulation potential.

The biomagnification of long-chain PFASs (≥ C8) in the food web is in agreement with previous observations (Fang et al., 2014; Houdé et al., 2008; Kosfeld et al., 2021) and PFOS can even be used as a benchmark chemical (Franklin, 2016; Kosfeld et al., 2021). For example, Miranda et al. found biomagnification of PFOS, EtFOSA, and PFNA in a tropical estuarine food web of bivalve, crustacean, and fish (Miranda et al., 2021). Similarly, Gao et al., found biomagnification of PFHxS and PFOS in an Antarctic food web (Gao et al., 2020). As PFASs have different isomers in linear and branched forms, these isomers may have distinct bioaccumulation and biomagnification potential due to different hydrophobicity, persistence and molecular structure. For example, the linear PFOS isomer was found to be more strongly biomagnified than the branched isomers probably due to the preferential interaction of linear isomers with cell membranes (Fang et al., 2014; Houdé et al., 2008). In the current study, for the total of linear and branched PFOS a dry weight-based TMF of 4.2 (2.3–7.5) was calculated. The TMF of the linear PFOS was determined to be 3.0 (1.8–4.8, dry weight), equivalent to 5.4 (2.6–11.3, wet weight) in a previous analysis of the same sample set from Lake Templin (Kosfeld et al., 2021). The TMF of PFOS in the examined food web is slightly higher than previously reported (2.9 ± 1.6 wet weight) (Chen et al., 2018; Fang et al., 2014; Houdé et al., 2008; Jartun et al., 2019; Loi et al., 2011; Martin et al., 2004; Mazzoni et al., 2020; Simonnet-Laprade et al., 2019a; Simonnet-Laprade et al., 2019b; Xu et al., 2014), which might be attributed to biotransformation of precursors of PFOS (Simonnet-Laprade et al., 2019b). Conversion of other PFASs such as perfluorooctanesulfonamide (FOSA) to PFOS has
been reported in rainbow trout (Brandsma et al., 2011). In fact, FOSA has been shown to be transformed to PFOS in freshwater fish (Babut et al., 2017; Simmonet-Laprade et al., 2019a) and presumably also in other animals (Galatius et al., 2013; Ross et al., 2012). Concurrently, the PFOS tissue concentration increased from mussel to fish, which may be not only due to biomagnification but also to the phylogenetic difference in the ability to biotransform FOSA to PFOS. Therefore, FOSA may serve as another source of PFOS in the food web, leading to a higher TMF of PFOS.

In contrast to the PFASs, the TMFs of five pharmaceuticals (phenozone, progesterone, venlafaxine, levamisole, and lidocaine) and one personal care product metabolite (galaxolidone) ranged from 0.4 to 0.6 (Fig. 4B and 4C), suggesting a biodilution potential in the food web. The observed trophic dilution (TMF < 1.0) of pharmaceuticals in the food web is in agreement with previous reports covering different levels of complexity of the food web at various locations (Haddad et al., 2018; Xie et al., 2017). For example, Xie et al. detected eight pharmaceuticals in 14 aquatic species, including plankton, invertebrates, and fish collected in Taihu Lake, China, and found a biodilution pattern for all detected pharmaceuticals, i.e., roxithromycin, propranolol, diclofenac, ibuprofen, ofloxacin, norfloxacin, ciprofloxacin, and tetracycline (Xie et al., 2017). Similarly, biodilution was observed for four other pharmaceuticals (sertraline, cilostazol, caffeine, and roxithromycin) in a food web consisting of plankton, three invertebrate species, and nine fish species (Yang et al., 2020). The trophic biodilution of pharmaceuticals might be attributed to a series of factors, including accumulation mechanisms, chemical properties, and metabolic capacity. In general, aquatic species at a higher trophic level have metabolic enzymes similar to that of humans. The enzymatic activities of cytochrome P450 oxidases (CYPs) and conjugate transferases are widely shared between humans and aquatic animals including fish and invertebrates (Katagi, 2010; Kosfeld et al., 2020).

4. Conclusions

- The targeted approach enabled the quantification of a broad range of CECs (n = 145) in various freshwater food web species, revealing an effective target analysis strategy to simultaneously monitor many polar CECs including PFASs in various complex biota matrices.
- The suspect and non-target screening identified 27 unknown compounds, with 19 confirmed by reference standards (level 1). This finding highlights the successful application of suspect and non-target screening in identifying unknown compounds in aquatic biota.
- Biomagnification was observed mostly for PFASs, while biodilution mostly occurred for pharmaceuticals, indicating the varying trophic transfer of polar CECs in a freshwater food web.
- These results call for more detailed analyses to further elucidate the possible emissions of PFASs into Lake Templin and their ecological risks.

Declaration of Competing Interest

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

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Supplementary materials

Supplementary material associated with this article can be found in the online version, at doi:10.1016/j.watres.2022.118514.

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