

IDENTIFICATION OF POLAR ORGANIC CHEMICALS IN THE AQUATIC FOODWEB: COMBINING HIGH-RESOLUTION MASS SPECTROMETRY AND TREND ANALYSIS

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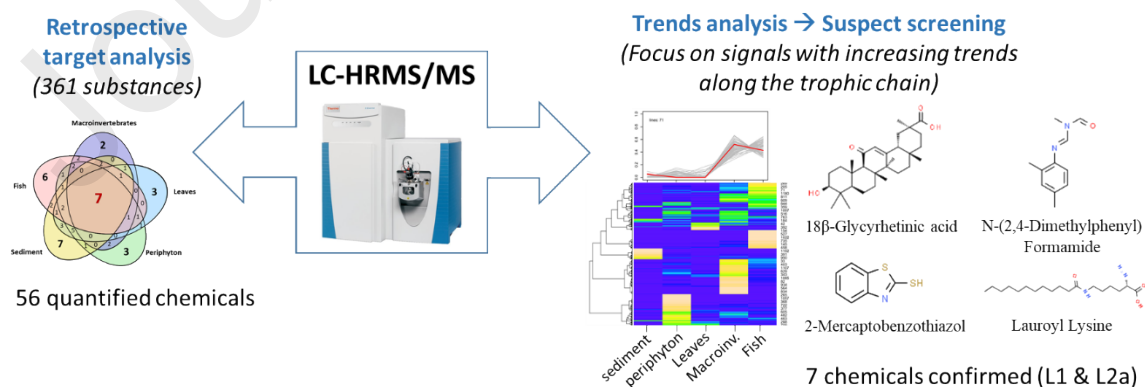
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Graphical abstract



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Highlights :

An aquatic foodweb in a small urban river was characterized using isotopic analysis.

56 of 361 polar target chemicals were detected in various organisms using retrospective analysis of LC-HRMS/MS data archives

Only 7 target compounds were detected in all compartments (sediment, periphyton leaves, macroinvertebrates, and fish)

Suspect-screening with trends analyses allowed to identify additional chemicals with biomagnification potential.

Suspect screening resulted in tentative identification of 12 xenobiotic and natural compounds.

Abstract

Environmental risk assessment of chemical contaminants requires prioritizing of substances taken up by biota as it is a starting point for potential adverse effects. Although knowledge about the occurrence of known chemical pollutants in aquatic organisms has significantly improved during the last decade, there is still a poor understanding for a broad range of more polar compounds. To tackle this issue, we proposed an approach that identifies bioaccumulative and biomagnifiable polar chemicals using liquid chromatography coupled with electrospray ionization to high resolution tandem mass spectrometry (LC-HRMS/MS) and combine it with trend analysis using hierarchical clustering. As a proof-of-concept, this approach was implemented on various organisms and compartments (sediment, litter leaves, periphytic biofilm, invertebrates and fish) collected from a small urban river. HRMS/MS data measured via data-independent acquisition mode were retrospectively analysed using two analytical strategies: (1) retrospective target and (2) suspect/non-target screening. In the retrospective target analysis, 56 of 361 substances spanning a broad range of contaminant classes were detected (i.e. 26 in fish, 18 in macroinvertebrates, 28 in leaves, 29 in periphyton and 32 in sediments, with only 7 common to all compartments), among which 49 could be quantified using reference standards. The suspect screening approach based on two suspect lists (in-house, Norman SusDat) led to the confirmation of 5 compounds with standards (three xenobiotics at level 1 and two lipids at level 2) and tentative identification of seven industrial or natural chemicals at level 2 and 3 through a mass spectra library match. Overall, this proof-of-concept study provided a more comprehensive picture of the exposure of biota to emerging contaminants (i.e., the internal chemical exposome) and potential bioaccumulation or biomagnification of polar compounds along the trophic chain.

1. INTRODUCTION

Worldwide aquatic ecosystems are contaminated by thousands of organic chemicals from natural and anthropogenic origins that may adversely impact exposed organisms including wildlife and humans [1][2]. As a result, regulations and guidelines have been established around the globe (e.g., Water Framework Directive, Convention for the Protection of the Marine Environment of the North-East Atlantic-OSPAR convention) to monitor environmental conditions and ultimately propose remediation actions to reduce the human environmental footprint and ensure sustainability of aquatic ecosystems and associated services. To date, this monitoring includes a restricted list of pollutants in surface water and in sediments mostly based on spot or grab sampling [3]. Such strategy gives only a partial view of the actual/true exposure of aquatic organisms since the spatio-temporal variability of the contamination, the toxicokinetics (TK) (uptake/metabolism/distribution/depuration) that defines the actual dose in the target tissue, and finally, the toxicodynamics (TD), are not considered. Therefore, a better characterization and/or prediction of concentration and overall bioaccumulation potential (i.e., accumulation and enrichment of contaminants in organisms, relative to that in the environment) in aquatic biota is needed to accurately define exposure and address associated risks. At the very least, low trophic levels should be considered in addition to surface water or sediment monitoring. Furthermore, some chemicals can be metabolized into more hazardous chemicals or persist and/or biomagnify (i.e. increasing concentration with increasing trophic level) along the trophic chain, which can potentially trigger unexpected adverse effect at high trophic levels [4],[5].

The number of studies regarding the contamination of aquatic organisms by organic contaminants increased during the last decade. These studies have mainly focused on contamination of fish or top predators (including humans) with hydrophobic, often biomagnifying chemicals such as persistent organic pollutants (POPs), including

polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins/dibenzofurans (PCDD/DFs) and polybrominated diphenyl ethers (PBDEs) flame retardants [4; 6-8]. In contrast, polar chemicals with functional groups such as carboxyl acids and amines detectable by electrospray ionization and usually $\log K_{ow} < 4$ have been poorly investigated although there is a growing evidence on the bioaccumulation of pharmaceuticals and personal care products (PPCPs), polar pesticides and/or perfluorinated chemicals in aquatic organisms [9]. For instance, Richmond et al. recently highlighted the occurrence of PPCPs and pesticides in platypus and spider in riparian ecosystems [10]. Also, Pico et al. showed the occurrence of emerging (semi)polar pollutants (pharmaceuticals, pesticides, plasticizers and UV-filters) in fish from Spanish rivers [11]. Since fish collection and vertebrate sampling raise ethical concerns, additional studies have additionally investigated the occurrence of organic contaminants in invertebrates such as gammarids [12], chironomids [13], copepods [14] or snails [15]. For instance, Munz et al. recently reported the occurrence of pesticides in gammarids and highlighted that risk assessment based on internal concentrations provide a different picture of the actual risk than that based on surface water concentrations alone [16]. Although there is growing knowledge on the actual exposure of aquatic organism to organic chemicals, most studies so far focused on a subset of pollutants via targeted analysis of known harmful chemicals. Given that this approach only provides a partial view of the exposure, there is a clear need to improve the knowledge about potential bioaccumulative and biomagnifiable chemicals in aquatic ecosystems. To tackle this issue, high resolution mass spectrometry (HRMS) is a relevant and cutting-edge technique through sensitive full scan detection that provides a more comprehensive picture of the chemicals present in environmental matrices, further allowing the identification of chemicals of concern [17; 18]. This technique, together with appropriate data evaluation workflows, is increasingly used in the field of environmental monitoring of chemicals in surface water, wastewaters, etc. In particular, HRMS has been used

for the prioritization of signals regarding their persistence and overall fluctuation along spatial and temporal domains [19; 20]. However, to the best of our knowledge, such methods has been only used in a few studies to investigate the actual exposure of aquatic organisms [21] [22].

The aim of this study was to evaluate an HRMS based workflow to improve our knowledge about the actual contamination of aquatic biota by organic polar chemicals (xenobiotics and their (bio)transformation products) that potentially bioaccumulate and biomagnify along the trophic chain. By doing so, this approach provided a streamlined approach to prioritize a list of chemicals for which their TK/TD can be subsequently investigated. Indeed, through its capacity to provide a comprehensive picture of the chemical landscape of biota, the implementation of LC-HRMS/MS analysis on exposed organisms combined with relevant chemometrics tools would identify chemicals of concern for aquatic ecosystems. Here, a retrospective target analysis and a suspect screening were implemented on HRMS data acquired from a set of samples representative for the trophic chain collected at one site with high expected anthropogenic chemical contamination based on land use. Then, a hierarchical cluster analysis (HCA) was then applied to the processed data to identify chemicals/features of interest along the trophic chain.

2. MATERIAL AND METHODS

2.1. Study site and sampling

The samples were collected in the Chamberonne River, which is located in the west of Lausanne, Switzerland (Figure S1). The river flows into the lake of Geneva, and is approximately 12 km long. The catchment of about 40 km² consists of an urbanized (43%) and agricultural (40%) area with roads, buildings (e.g., University of Lausanne), and a highway nearby, while the remaining land is occupied by forest (17%).

Various types of samples were collected during the campaign, including sediment, biofilm, leaves, macroinvertebrates, and fish. As described in [23; 24] sediment was collected with a stainless steel shovel from the upper layer (8 cm), while periphyton was sampled by scraping approximately 10-12 rocks per sample with a scalpel. The leaves were comprised of highly decomposed fallen leaves (detrital organic particles/organic matter), which were collected from the bottom of the river (i.e., on top of the sediment) with a stainless steel shovel. Macroinvertebrates were collected with a kicknet, and included: gammarids (*Gammaridae*), oligochaetes (*Oligochaeta*), rhyacophilids (*Rhyacophilidae*), and baetids (*Baetidae*). They were chosen due to their widespread occurrence and their possibility of providing information on the food web since they are consumed by fish. The fish were represented by the Riverine Brown Trout (*Salmo trutta fario*), the most prominent species in the river, and the Common Minnow (*Phoxinus phoxinus*). Sampling was performed with an electro-fishing device by biologists from La Maison de la Rivière (Tolochenaz, Switzerland). All fish and macroinvertebrates were collected after the authorization by the Canton of Vaud in accordance with the Animal Welfare Act (Article 15) and Animal Welfare Ordinance (Annexe 2), [25; 26]. All directives and procedures were fulfilled for the entire sampling campaign.

2.2. Sample preparation

For isotopic analysis, the samples must be free from inorganic carbon (both carbonates and skeleton) and lipid-free (or with a low-lipid content). To this end, following 1-2 hours after the sampling, all the biota samples were rinsed with HCl (10%) overnight followed by several rinsing with ultrapure water (MilliQ) to fully remove the inorganic carbon. They were then freeze-dried and homogenized using mortar and pestle prior their storage at -20°C. For the sediments, they were freeze-dried, rinsed overnight in HCl (10%) and then with MilliQ water and finally dried at 40°C during several hours, as described in [23; 24].

For carbon analysis in fish, lipids (with poor ^{13}C content) were first removed from homogenized samples (i.e. powder) through a soxhlet extraction. Then 70 mg of this lipid-free powder was fumigated overnight using concentrated HCl solution (37%) to remove inner inorganic carbon (fish skeleton), as described in details in Rammarine et al [27]. The dorsal exoskeleton of some macroinvertebrates (especially the gammarids) was not considered as a source of inorganic content.

For chemical analysis, all samples (except sediment) were rinsed with nanopure water to eliminate any possible residue present on the surface. Then, they were freeze-dried and stored at -20°C until further sample preparation. For the biota, one sample was composed of 30-40 organisms for the gammarids, 10 for the baetids and rhyacophilids, and 5 for the oligochaets. Five fish were dissected into different tissues: brain, gills, muscle, stomach, spleen, liver, and heart, while from the remaining seven only the muscle was taken. It should be noted that the brain sample is from a pool of five fish since the extracted tissue amount was otherwise not of sufficient quantity.

Biota samples (macroinvertebrates, fish, biofilm, and leaves) were all prepared according to the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method, which was optimized for the different matrices, as previously reported [28]. The sediments were extracted and purified by using pressurized liquid extraction (PLE), as previously described in Chiai-Hernandez (2020)[29]. Both methods are detailed in the supporting information.

2.3. Fish stomach content

Stomach content analysis gives information about feeding immediately prior to capture [30] contrary to isotopic signatures which depicts long-term diet. Briefly, fish were cut on the field and stomachs were removed and placed in 50% ethanol for storage. Stomachs were carefully incised and the preys were removed one by one and put on a petri dish for further

characterization. The abundance, occurrence and prey-specific abundance were computed according to the literature [31; 32].

2.4. Isotopic analysis and further data processing

The identification of organisms in the trophic chain is based on the principle that consumers feeding on prey from lower trophic level show an increase of $\delta^{13}\text{C}$ content ($+0.8 \pm 1.1 \text{ ‰}$) and $\delta^{15}\text{N}$ ($+3.0 \pm 2.6 \text{ ‰}$) per trophic level. In particular, the values of $\delta^{13}\text{C}$ can identify the primary producer(s) (i.e. source of carbon) while the $\delta^{15}\text{N}$ values define the trophic levels.

The isotopic composition was obtained by measuring the samples using Isotope Ratio Mass Spectrometry (IRMS). Isotopic ratios $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were quantified through the measurement of analytical standards (carbon analysis: glycine, urea, graphite-24, pyridine; nitrogen analysis: glycine, USGS-40, IAEA-600) via calibration curves. As a control of the lipid content that could alter the isotopic signature, the molar C:N ratio for each sample was computed by using the Total Organic Carbon (TOC) (%C w.w.) and Total Nitrogen (TN) (%N w./w.) and their conversion in molar quantity.

The trophic level was calculated from the nitrogen isotopic composition of predators (hence fish) by using the following equation:

$$\text{TL}_{\text{cons}} = \lambda + \frac{(\delta^{15}\text{N}_{\text{cons}} - \delta^{15}\text{N}_{\text{base}})}{\Delta\text{N}} \quad \text{Eq (1)}$$

TL_{cons} is the trophic level of the consumer; λ is the level of the organism at the basis of the chain, $\delta^{15}\text{N}_{\text{cons}}$ is the nitrogen isotopic ratio of the consumer; $\delta^{15}\text{N}_{\text{base}}$ is the nitrogen isotopic ratio in the organism at the basis; ΔN is the trophic enrichment of nitrogen 3.4 ‰ per trophic level[33]. As the baseline was assigned with a trophic level λ of 2, this value has to be added to calculate the trophic level of the predator.

2.5. LC-HRMS analysis

Following injection of 50 μ L of sample with a CTC PAL auto sampler (CTC analytics, Zwingen, Switzerland), chromatographic separation was performed on a Waters X-Bridge C18 column (2.1 x 50 mm, 3.5 μ m particle size) connected to a C18 security guard cartridge (2.1 x 10 mm). After electrospray ionization, detection was carried out on a Q-Exactive HRMS (Thermo Fisher Scientific, USA) coupled to a RHEOS 2200 pump with degasser (Flux instruments, Switzerland), or on a Q-Exactive plus (Thermo Fisher Scientific, USA) coupled to a RHEOS 2200, a RHEOS 2000, (both from Flux Instruments), or an Ultimate 3000 (Thermo Scientific Fisher) pump (Section SI 1.3, Table S1). Moreover, both instruments were equipped with electrospray ionization (ESI) sources that were operated in the positive ionization mode (4kV) with nitrogen as nebulizer gas (Table S2). The samples were analysed using data independent acquisition (DIA) and data dependant acquisition (DDA). DIA consisted in a full-scan with a mass to charge (m/z) range of 100-800 with a resolution of 140,000 followed by MS2 acquisition of nine different mass scan ranges (Table S3) with a resolution of 17,500 and corresponding mass-dependent high-energy collisional dissociation (HCD) energies. DDA measurements consisted of a full-scan (100-800 m/z), followed by MS2 acquisition based on an mass inclusion list from analysis of the DIA measurement and if these masses were not detected, the five most intense peaks of each scan. Details on mass spectrometer acquisition parameters are provided in the supporting information (section 1.3, Table S2-S3).

2.6. Data processing workflows for HRMS analysis

The collected data were submitted to a retrospective analysis and a suspect screening (Figure 1).

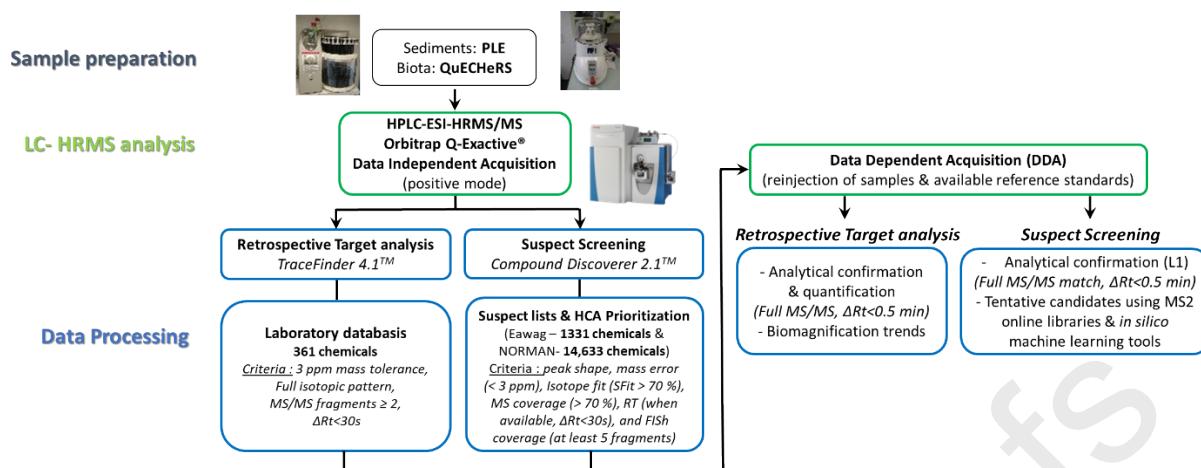


Figure 1. HRMS-based workflow for the identification of potentially bioaccumulative chemicals

For the **retrospective target analysis**, all the 361 chemicals (e.g. 81 pharmaceuticals, 208 pesticides) present in the calibration mixes were investigated. Detailed information on the compounds are summarized in Table S4. For detection and quantification, a TraceFinder (TF) 4.1 (Thermo Fisher Scientific, USA) in-house database that contained information on the isotopic pattern, retention time (RT), and fragments was used. The criteria for the detection included mass tolerance window (3ppm), an evaluation of the isotopic pattern, MS2 fragments (≥ 2 fragments), and RT ($\Delta R_t < 30s$), when available. The DIA data were first analysed to establish a list of candidates that were further confirmed by DDA acquisition to obtain better fragmentation spectra. Additionally, for compounds lacking MS2 data in the TF database, reference standards were injected to obtain fragmentation patterns. The confirmed chemicals were then quantified through internal standard calibration (SI section 1.4 and 2.2). Lastly, in order to identify biomagnification trends for the quantified compounds, hierarchical clustering analysis (HCA) on average concentrations was performed with the use of an in-house R script [19].

The **suspect screening** was performed in compounds discoverer (CD) 3.1 (Thermo Fisher Scientific, USA). Details on CD workflow and processing parameters are provided in the supporting information (Section 2.3. in the SI, Figure S2). The DIA measurements were first

processed and then filtered with the following criteria: background removal, single matches with two different suspect lists, no values in the calibrations (to exclude compounds previously investigated in the retrospective target screening), intensity $> 1 \times 10^5$ in at least five samples, and a full match for the predicted composition (Figure S3). The two suspect lists consisted of a list from the "Network of reference laboratories, research centers and related organization for monitoring of emerging environmental substances" (NORMAN) containing 14,633 compounds (i.e. Merged NORMAN Suspect List, S0-SUSDAT 2018 transferable to CD format), and an internal Eawag suspect list including 1,331 substances that correspond to all the standards available in the laboratory (mainly pesticides, pharmaceuticals, industrial compounds). Depending on the suspect list employed, two different approaches were utilized. The first approach was to use the matched candidates from the Eawag suspect list, and to manually check hits for the peak shape, mass error (< 3 ppm), Isotope fit (SFit > 70 %), MS coverage (> 70 %), RT (when available, $\Delta Rt < 30$ s), and FISh coverage (at least 5 fragments) (see supporting information for explanation of these parameters). The resulting tentative candidates were then measured in DDA, and compared to reference standards, if available. Moreover, prediction of the RTs by linear correlation to the log Kow values of reference standards (Figure S6), and comparison of the fragmentation pattern to databases (e.g., MASSBANK [34], mzCloud®) and *in-silico* identification tools (e.g., MetFrag [35], SIRIUS 5 [36]) were used. The second approach differed slightly from the previous one, as after the filtration step with the NORMAN suspect list a high number of candidates was present (higher than 1000), and manual checking would have been time-consuming. It was thus decided to focus on candidates displaying biomagnification trends. For this purpose, prioritization by HCA was performed as described below. The resulting candidates were then manually checked, and confirmed analogously to the procedure described above for the matches with the Eawag suspect list.

2.6. Statistical analysis: HCA-based trend analysis

HCA analyses were performed in order to identify biomagnifying target chemicals or suspect candidates. This was done by using an R script from Schollee et al. [19] with slight modification [20]. The settings are detailed in the SI. In the case of the suspect screening, prior clustering the area of the signals from CD were corrected by using the average absolute recovery of chemicals (from the retrospective target analysis) with similar retention time in order to take into account the different extraction recoveries and ion suppressions/enhancements of the different matrices. It was assumed that chemicals with similar retention times share similar properties but high uncertainties have to be expected. However, the identified trends based on the analysis of non-corrected data did not show remarkable differences with the corrected ones (data not shown).

2.7. Quality control/ Quality assurance

Before the field work, all sampling containers were either rinsed with acetone and hexane, or cleaned for 4h by calcination at 450°C to avoid any contamination. In the laboratory, all glassware was also cleaned by calcination. During the injection, instrument blanks containing only the solvents (50% methanol and 50% nanopure water) were used to check for carry-over and contamination, while matrix blanks were employed to detect the presence of contamination during the sample preparation procedure. Additionally, every 20 samples a duplicate sample and a quality control (QC) standard (at 2.5 or 5 µg/L) were measured to check the precision of the method. Relative percent difference (RPD), relative standard deviation (RSD), limits of quantification/detection (LOQ/LOD), absolute, relative and extraction recoveries were calculated as described in the supporting information (section 2.4).

3. RESULTS AND DISCUSSION

3.1. Characterization of the trophic chain in the Chamberonne River

The isotopic composition showed an expected ascending alignment from the leaves, to macroinvertebrates and fish, as depicted in Figure 2a.

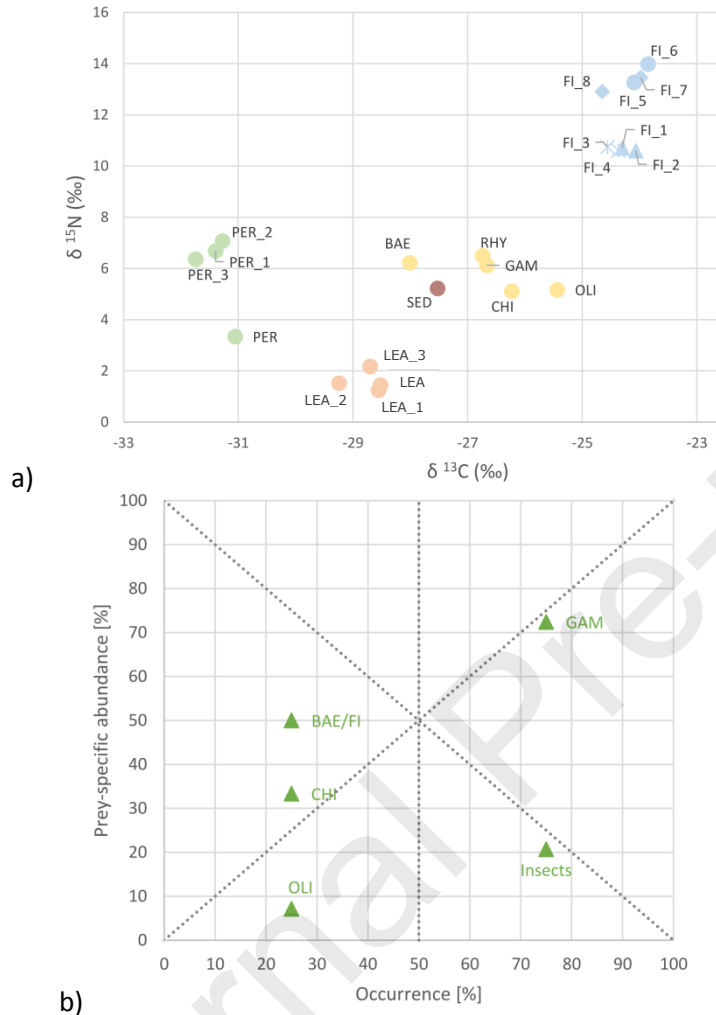


Figure 2. Isotopic composition of substrates and organisms (a) and fish stomach content (b) in March 2017. PER : periphyton; LEA : Leaves ; SED : sediments; GAM : gammarids; BAE : baetidae; RHY : rhyacophilids; CHI : chironomids; OLI : oligochaetes ; FI: Fish ; Insects : various remaining parts of insects (probably ants, spiders and wasps). Prey-specific abundance were computed according to the literature ^{26, 27}.

Overall, the mean values of nitrogen (1.6 ‰ for DOP, 5.8 ‰ for macroinvertebrates and 12.0 ‰ for fish) and carbon (-28.8 ‰ for DOP, -26.6 ‰ for macroinvertebrates and -24.2 ‰ for fish) are in accordance with the theoretical partitioning of $+ 3.0 \pm 2.6$ ‰ for nitrogen and of $+ 0.8 \pm 1.1$ ‰ for carbon, respectively, between a consumer and its diet [37]. Since periphyton shared a similar nitrogen composition with the macroinvertebrates, but has a lower carbon

composition, it is unlikely that it constituted a significant food source neither for macroinvertebrates (due to nitrogen composition) nor for fish (due to carbon composition). On the other hand, leaves appeared as the main food source for macroinvertebrates as illustrated by its position in Figure 2a. Their isotopic composition was specific to C3 plants [37]. All macroinvertebrates shared a similar position regarding their nitrogen composition. Nevertheless, the variability of carbon ($\Delta 2.7\text{ ‰}$) suggested several food sources. For instance, the baetidae seemed to feed also on periphyton because of their lower carbon content while oligochaetes could use a third source of food as illustrated by their high carbon content. In central position, gammarids were representative of the primary consumers while the stomach content analysis showed that they were the dominant prey for fish (Figure 2b) among chironomids, oligochaetes, rhyacophilidae, limnephilids, baetidae, various insect (pieces of ants, spiders, wasps), cocoons of unidentified insects, juveniles and fish eggs. Finally, fish had a high variability in nitrogen isotopic composition, suggesting that they may range over more than one trophic level. Such results might highlight cannibalism between fish due to limited amount of prey in winter, in accordance with the presence of fish eggs in the stomach content (Figure 2b).

3.2. Retrospective identification of bioaccumulative and biomagnifying target compounds

As a first step, DIA measurements of all samples were retrospectively screened against an in-house target list of 361 chemicals including mainly pharmaceuticals and pesticides that were used in the standard mixture (Table S4). Overall, a total of 145 chemicals were tentatively identified by checking the RT, isotopic pattern, and MS/MS data. Generally, at least the isotopic pattern and RT had to match for a compound with a clear peak shape to be assigned as tentatively identified. Some exceptions were accepted if the fragmentation pattern was a match but one of the other criteria was not. Confirmation of the tentative identifications was

then performed with DDA measurements as it usually provides better fragmentation spectra, and thus helped in the unambiguous confirmation of the substances. Finally, 56 compounds could be confirmed (Table S7-S9) while 305 were rejected due to MS/MS mismatch (216 based on DIA measurements and 89 based on additional DDA measurements). According to the confidence system of Schymanski et al. [38], the identified chemicals were confirmed to the highest level (i.e. level 1 – reference standard).

These chemicals belong to different classes (pharmaceuticals, insecticides, fungicides, herbicides, biocides, corrosion inhibitors, transformation products of all classes) from which the pharmaceuticals (18 chemicals) and the fungicides (18 chemicals) were the most prominent. This is in agreement with the urban (43%) and agricultural (40%) land use in the catchment where typical urban hydrophobic contaminants (i.e. PAHs, PBDEs, PCBs), pharmaceuticals (e.g. carbamazepine, sulfamethoxazole) and pesticides (propiconazole, mecoprop) have been previously detected in the water[39],[40]. The distribution of the concentrations among the different compartments was explored by calculating the average and standard deviation of the different classes (Figure 3). To this end, we normalized the concentration in fish organs by the percentage of their weight relative to the whole organism weight in order to provide an estimated total body residue (section 2.3 in supporting information).

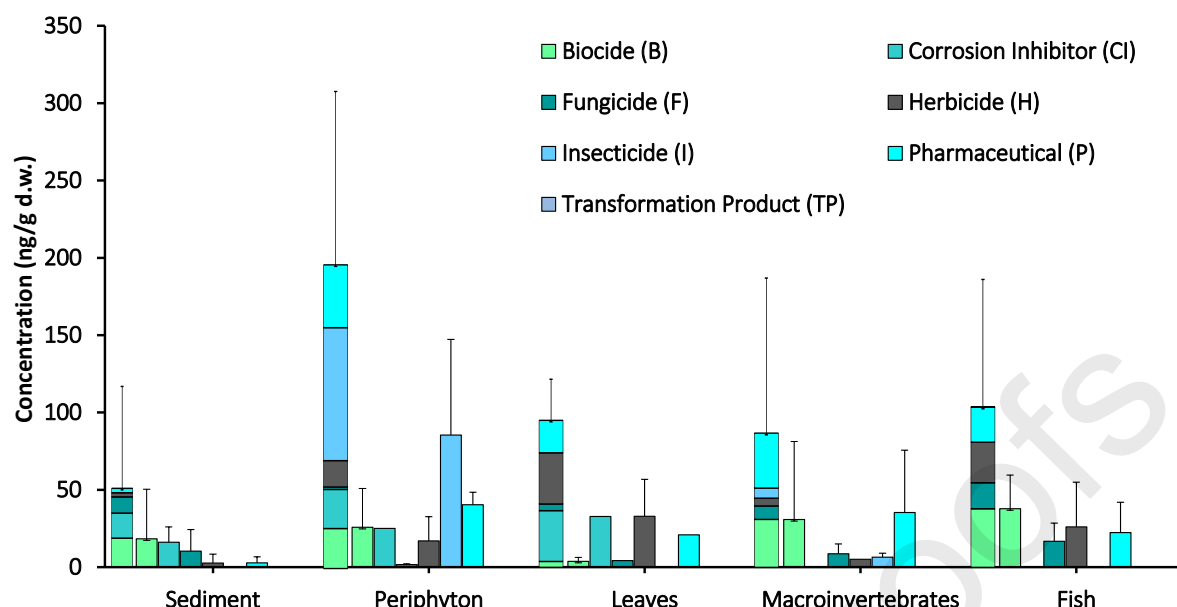


Figure 3. Average of the total concentration per individual in each compartment, grouped per contaminant class. The error bars depict the standard deviation among the samples, and the stacked bar plot shows a summary of the total concentration of the contaminants in the different compartments.

Overall, pharmaceuticals were at relatively high concentrations in all compartments (25-100 ng/g d.w.). The average concentration of biocides were around 50 ng/g d.w. in all the compartments, except in the leaves. Corrosion inhibitors were below LOQ in the macroinvertebrates. Fungicides were only present at very low level (3.3 ng/g d.w.) in the periphyton whereas all the other classes were high in this compartment. In particular, insecticides were very abundant in periphyton but very low in the other compartments. As a consequence, the highest average total concentration of contaminants was found in the periphyton (195 ± 111 ng/g d.w.) (Figure 3). Even if not significant, this higher concentration is plausible since they have a large surface area exposed directly to the water[41]. Further, periphyton is known to accumulate a high number of compounds from water likely because of the diversity of binding/trapping site for both hydrophilic and hydrophobic chemicals -- a reason why periphyton was proposed as sentinel species in river monitoring programs[42]. The higher concentration of xenobiotics could be influenced by the sorption capacity of the extracellular polymeric substances (EPS) of the periphyton, which are mainly composed of polysaccharides, nucleic acids, proteins, and lipids. These EPS play a pivotal role in the

bioaccumulation potential by limiting the bioavailability of the contaminant[41]. Finally, a last explanation might be related to the LOQs that differ between the matrices although, except for sediment, LOQs are quite similar between the organisms investigated. Apart from the periphyton, the total average concentration increase along the identified trophic chain (i.e. from the leaves to the fish). This could be an indication that compounds possibly bioaccumulate and also biomagnify along the trophic chain.

A more detailed look at the distribution of the confirmed substances among the compartments was taken by using a Venn diagram, and highlighted several differences (Figure 4). To the best of our knowledge, this study is one of the first to show such a broad range of polar contaminants in different compartments including biota.

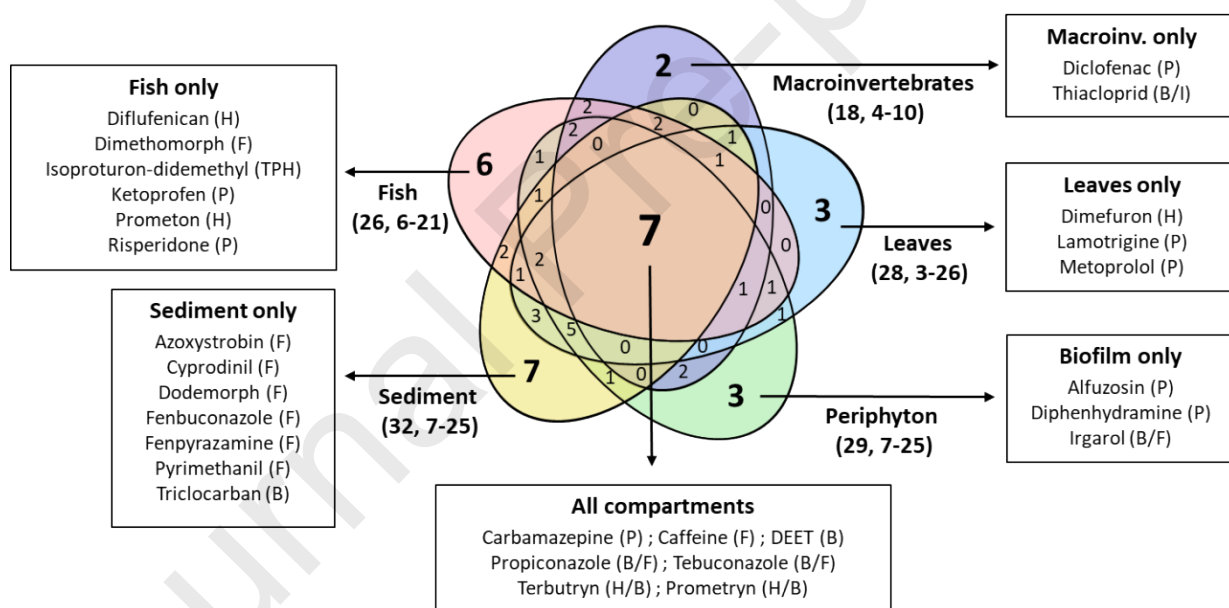


Figure 4. Venn diagram showing the distribution of the 56 target chemicals among the different compartments. Compounds exclusively found in one, or in all compartments are listed. Moreover, in brackets the total number of compounds, and the range of the number of chemicals detected per sample are depicted (B: Biocides H: Herbicides; F: Fungicides; P: Pharmaceuticals; TP: Transformation Products)

Many pollutants were only identified in one of the compartments, with the highest number of such compounds detected in the sediment (7), closely followed by the fish (6). The high number of compounds detected in the sediment could be due to the lower LOQs, which may be the

result of the larger initial amount of material used (i.e., 5 g d.w.) when compared to other matrices (i.e., 100 mg d.w.). The total number of compounds detected per compartment was similar for the sediment (32), fish (26), biofilm (29), and leaves (28), while it was slightly lower for the macroinvertebrates (18). The number of compounds detected in a specific matrix varied substantially (Figure 3). This highlights that even in the same compartment at the same site, there were differences between the various samples raising the need to increase the number of replicates to give a meaningful picture.

Among the compounds only found in one compartment, interestingly the transformation product isoproturon-didemethyl was only detected in the fish (10.3 ± 3.3 ng/g d.w.), with its parent compound (isoproturon) also observed but at very low concentration (1.9 ± 0.3 ng/g d.w.). While isoproturon was detected in other compartments, such as the sediment (below LOQ), the biofilm, and the leaves (below LOQ), the transformation product was not found. In the literature, the above-mentioned degradation product has been reported in soil [43; 44], and although the uptake and elimination of isoproturon was studied in fish [45], to the best of our knowledge the transformation product isoproturon-didemethyl has not yet been reported. To clarify whether the isoproturon-didemethyl is formed in the fish, further experiments e.g. with fish S9 extracts would be needed.

Seven compounds were detected in all compartments, three of which were found at values above the LOQ in all of them (carbamazepine, DEET, and tebuconazole). These contaminants have been previously reported in biota and sediment, corroborating these results [16; 46]. Specifically, carbamazepine, a psychoactive drug, was detected in water, sediment, fish, mussels, and gammarids, showing the wide occurrence of this compound [16; 46]. DEET is the most common active ingredient in insect repellents, and is known to be pervasive in water [47; 48] but is only marginally toxic to fish (e.g., rainbow trout) and invertebrates [49]. Tebuconazole, a triazole fungicide, is listed as a possible carcinogen and potential endocrine

disruptor [50; 51] and was recently detected in gammarids and sediment [16; 28; 52]. The log Kow values are 2.2, 2.5, and 3.7 (predicted from EpiSuite) for DEET, carbamazepine, and tebuconazole, respectively, which are similar to the other substances detected in the samples. Therefore, the hydrophobicity cannot solely account for the ubiquity of these compounds in all compartments, and other factors such as the metabolism, exposure time, and especially exposure concentration in the water, are likely contributing factors [53].

The relation of the average concentrations of all detected compounds to predicted physico-chemical properties from EPI suite (v4.11, [54]) were investigated for the different compartments (Figure S6). The log Kow range (-1.75 to 7.5) highlighted that a broad range of chemicals with different hydrophobicity can be “bioaccumulated”. In the same way, interestingly, most of the 56 chemicals have a predicted BAF lower than 2000 L/kg which is the REACH threshold for bioaccumulative chemicals [55]. Most of the chemicals had a predicted biotransformation half-life (BCFBFAF module in EPIsuite) below 6 days whereas it would be expected that bioaccumulative chemicals have longer half-life in the organisms. Overall, no common trend could be observed, corroborating the above-mentioned statement that other parameters such as the continuous exposure are more important for the (bio-) accumulation of these compounds.

Since the average total concentration of contaminants in the samples suggested that biomagnification of the compounds could be possible, an HCA of quantified chemicals was performed for the better visualization of trends and classifications. (Figure 5, Figure S7).

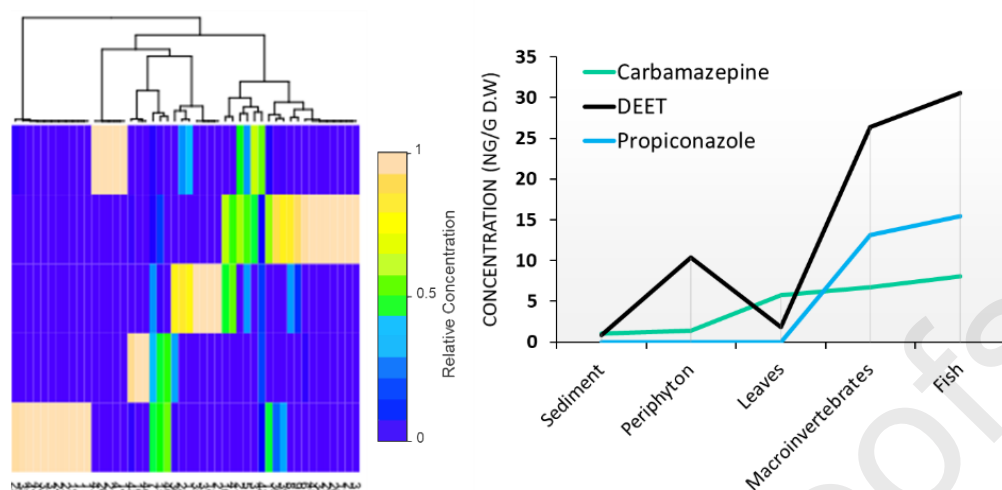


Figure 5. Hierarchical clustering and associated biomagnification trend for target compounds (cluster 5).

Thirteen clusters were chosen by visual inspection, selecting the minimum number of clusters needed to separate the desired biomagnification trend (i.e., leaves, macroinvertebrates, and fish) from others without losing any data (Figure S7). The cluster number 5, which contains DEET, carbamazepine and propiconazole, was chosen for further inspection (Figure 4). Unlike DEET and carbamazepine, propiconazole was not detected in all compartments but showed an increase along the trophic chain. Propiconazole is a triazole fungicide with a log Kow (3.7) that is frequently detected in surface water and previously found in sediment [28; 56].

Since organic contaminants can be bioaccumulated in lipids, concentration in biota are often expressed relative to lipid content. In the present study, normalization by using literature values for the fish (brown trout) and gammarids (4% w.w. and 2.7% w.w., respectively) [16; 57] resulted for all the identified chemicals in higher concentration in macroinvertebrates than in fish (data not shown). However, such lipid normalization might be not so relevant for polar chemicals investigated in the present study since they can also interact preferably with proteins in various tissues (e.g. blood, muscle, liver), as previously reported [58-60].

Although most of the identified chemicals were previously detected in water [61; 62] and sediment [63], this study highlight their presence along the trophic chain. Our results are in accordance with increasing reporting of the occurrence of anthropogenic chemicals in aquatic organisms [10; 11; 16; 46; 64-66].

3.3. Prioritization of bioaccumulative and biomagnifying suspects / non-targets

The second part of this study used a suspect/non-target screening approach for data processing in CD in order to cover an even broader range of contaminants. The analysis was focused on the detection of candidates from two suspect lists (Eawag, Norman) on the basis of DIA raw data. Initially, a total of 503,915 potential substances were found in CD for all the samples with less restrictive parameters settings. By applying different filtering steps (see section 2.6), this number was narrowed down to 64 candidates with the Eawag suspect list. These candidates were then manually evaluated (i.e. peak shape, isotopic pattern), and resulted in 26 tentative identifications corresponding to 24 chemicals (Table S11). Confirmation of the tentative candidates was performed with the injection of the samples for DDA acquisition and comparison with MS2 libraries (m/zCloud, MassBank) and *in silico* fragmentation (MetFrag, MassFrontier). If available, the reference standard was also injected. In total, 3 compounds were confirmed at level 1 (i.e. with injection of the analytical standard) and 2 compounds at level 2a (m/z cloud and Mass bank library match) and level 2b (MetFrag and Sirius *in silico* prediction high match), according to the confidence level system from Schymanski et al. [33]) (Table 1, Figure S8-S13). The remaining 20 chemicals were rejected due to no or low MS2 match and an overall low ranking.

None of the tentative candidate showed a biomagnification trend along the trophic chain (Figure 6A). Among the confirmed chemicals (L1), 18 β -glycyrrhetic acid, also known as enoxolone, is a nonsteroidal anti-inflammatory and anti-cancer drug obtained from the

hydrolysis of glycyrrhic acid contained in the herb liquorice. It also has biocidal activities (antiviral, antifungal, antiprotozoal, and antibacterial)[67]. Although its environmental occurrence has been recently reported in sediment[68], this study is the first to report its detection in aquatic biota, surprisingly, since its physico-chemical properties (log Kow: 6.9; log BAF:6.4; Biotransformation Half-Life: 92.4 days, EPI-Suite) makes it of particular concern in terms of bioaccumulation and biomagnification potential, even if it did not show a biomagnification trend here. Conversely, the 2-mercaptobenzothiazol and the N-(2,4-dimethylphenyl)formamide have lower Log Kow (1.8 and 2.2 respectively EPI suite) and log BAF (0.77 and 0.95 respectively, EPI suite) making their occurrence in aquatic biota more surprising. Overall the detection of these chemicals raise the question on their effect in these organisms. 2-Mercaptobenzothiazol is described as highly toxic for aquatic organisms [69] and N-(2,4-dimethylphenyl)formamide is used as an insecticide and likely toxic for aquatic invertebrates.



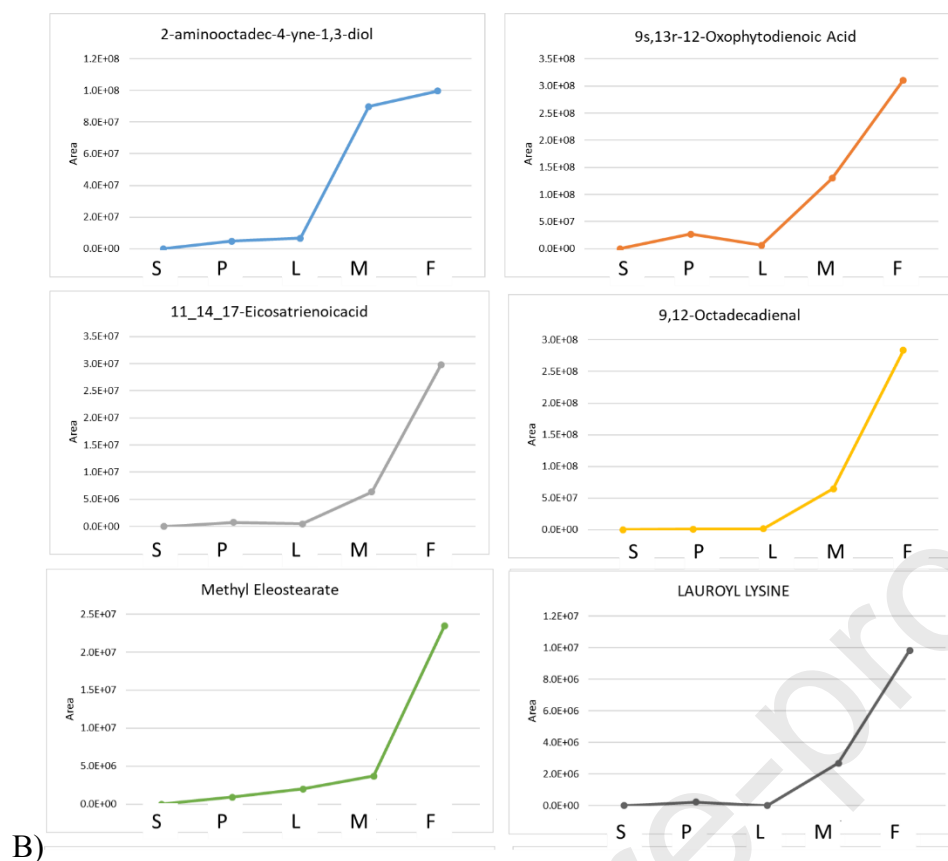


Figure 6: Trends of the tentative and confirmed candidates from the Eawag suspect list (A) and NORMAN suspect list (B). *S*, sediment; *P*, periphyton; *L*, leaves; *M*, macroinvertebrates; *F*, fish

Table 1. Identified and putative candidates (level 1, 2 or 3) from the Eawag suspect list.

Candidates	Structure	InchiKey	Pubchem	ΔR_t pred. (min)	ΔR_t std. (min)	Matrices /Blanks	CD (Fishcov.)	m/z Cloud (Score)	MassBank Score	MetfragScore (Rank, Peaks)	Sirius Score (Rank, peaks)	Id Level	Class/ Use
18 β -Glycyrrhetic acid C ₃₀ H ₄₆ O ₄ ; 470.684 Da		MPDGHEJMBKOTSU-UHFFFAOYSA-N	CID 3230	1.9	0.2	L, M/ n.d.	26.7 (25/94)	91.2	67	7.1/8 (1/564, 57/126)	80.41 (10/77, 57/58)	L1	Terpenoids/ Pharma
2-Mercaptobenzothiazol C ₇ H ₅ NS ₂ ; 167.251 Da		YXIWHUQXZSMYRE-UHFFFAOYSA-N	CID 697993	0.2	0.5	P, L, M, F/ n.d.	22.4 (8/36)	94.9	68	5.8/8 (1/58, 14/49)	100 (1/1, 9/22)	L1	Benzene derivative/ Industrial.
N-(2,4-Dimethylphenyl) Formamide C ₉ H ₁₁ N ₂ O ; 149.084 Da		JOFDPSBOUCXJCC-UHFFFAOYSA-N	CID 92363	-1.3	0.4	S, P, L, M, F/ n.d.	31.3 (10/32)	90.6	71.2	5.6/8 (2/2160, 15/40)	79.7 (1/97, 15/25)	L1	Formamidine/ Insecticides
Benzenesulfonamide C ₆ H ₇ NO ₂ S ; 157.190 Da		KHBQMWCZKVMBLN-UHFFFAOYSA-N	CID 7370	4.3	-	P, L, M, F/ n.d.	17.8 (4/20)	91.4	67	6.2/8 (1/533, 7/31)	100 (1/27, 8/12)	L2a	Benzene derivative/ Pharma./Ind.
Benzothiazol C ₇ H ₅ NS ; 135.014 Da		IOJUPLGTWVMSFF-UHFFFAOYSA-N	CID 7222	-1.1	-	S, P, M, F/ n.d.	16.2 (3/13)	90.4	68.5	5.7/8 (1/51, 4/30)	98.8 (1/11, 4/19)	L2a	Benzene derivative/ Industrial
Azelaic acid C ₉ H ₁₆ O ₄ ; 188.221 Da		BDJRBEYXGGNYIS-UHFFFAOYSA-N	CID 2266	1.6	-	P, L, M, F/ n.d.	56.3 (40/71)	n.m	n.m	5.2/8 (1/2412, 17/89)	34.45 (1/77, 42/58)	L3	dicarboxylic acid/ Pharma

Matrices : S: sediment; P: periphyton; L:leaves; M: macroinvertebrates, F: fish
 ΔR_t pred, (min) difference in retention time between the sample and the prediction
 ΔR_t std.(min) difference in retention time between the sample and the standard
Id Level. Identification Level [33]
n.m : no match

Table 2. Tentative candidates (level 2 to 3) from the Norman suspect list following DDA.

Candidates	Structure	InchiKey	Pubchem	ΔR_t pred. (min)	ΔR_t std. (min)	Matrices /Blanks	CD 2.1 Fish cov. (match)	mzCloud Score	Mass bank Score	MetFrag Score (Rank, Peaks)	SIRIUS Score* (Rank, Peaks)	Id Level	Class/ Use
2-Aminooctadec-4-yne-1,3-diol $C_{18}H_{35}NO_2$; 297.476 Da		YJXGFSAPYAXAY-UHFFFAOYSA-N	CID 2802958	4.5	-	P, L, M, F/ n.d.	77.4 (41/53)	91	n.m	5.6 (2/8 45/75)	70.0 (4/1450, 53/58)	L3	Ceramides-sphingosines (lipids)
9s,13r-12-Oxophytodienoic Acid $C_{18}H_{28}O_3$; 292.4 Da		PMTMAFAPLCGXGK-TTXFDSJOSA-N	CID 14037063	2.3	-	P, L, M, F/ n.d.	70.0 (55/90)	94.4	n.m	4.5/6 (2/20, 67/114)	86.3 (2/2553, 59/59)	L3	Oxilipins (lipids)
11,14,17-Eicosatrienoic acid $C_{20}H_{34}O_2$; 306.483		AHANXAKGNAKFSK-IUQGRGSQSA-N	CID 5282827	2.7	0.4	P, L, M, F/ n.d.	78.3 (40/50)	93.4	n.m	3.1/6 (15/20, 26/72)	99.0 (1/1580, 35/47)	L2a	Lipids (unstaturated fatty acid) /Pharma.
9,12-Octadecadienal $C_{18}H_{32}O$; 264.446 Da		HXLZULGRVFOIDK-AVQMFATSA-N	CID 5283383	5.7	-	P, L, M, F/<LOQ	86.7 (33/40)	n.m	n.m	4.4/6 (8/11, 28/64)	63.3 (3/73 35/57)	L3	Lipids (Fatty Aldehydes)/Sex-Pheromones
Methyl Eleostearate $C_{19}H_{32}O_2$; 292.456 Da		KOJYENXGDXRGDK-ZUGARUELSA-N	CID 21718552	3.6	-	P, L, M, F/ n.d.	79.0 (44/55)	94.1	n.m	4.1/8 (400/687, 38/67)	69.7 (9/1300, 41/50)	L3	Lipids (conjugated linolenic acid)/ Pharma
Lauroyl Lysine $C_{18}H_{36}N_2O_3$; 328.490 Da		GYDYJUZYBRGMCC-INIZCTEOSA-N	CID 104151	3.6	1.4	P, M, F / n.d.	43.8 (21/48)	n.m	n.m	6.1/8 (1/681, 19/70)	86.1 (1/6, 24/27)	L2a	Lipopeptides/Cosmetics

*, CSI Finger ID score; n.m: no match

Matrices : S: sediment; P: periphyton; L:leaves; M: macroinvertebrates, F: fish

ΔR_t pred, (min) difference in retention time between the sample and the prediction

ΔR_t std.(min) difference in retention time between the sample and the standard

Id Level. Identification Level [33]

From the 503,915 features, filtration based on the NORMAN suspect list allowed the list reduction to 1,182 chemicals. Since the number of candidates (1,182) was too high to be manually checked, an HCA based trend analysis was first performed in order to focus on candidates with a biomagnification trend. The corresponding heat map and 13 normalized clusters are depicted in Figures S14. The desired biomagnification trend (i.e. increase between leaves and fish) was seen in clusters 3, 4, 9 that contain 312 chemicals in total. Among them, only 52 were actually detected at all the trophic levels and increased along the trophic chain. After manual checking of the peak shape and isotopic pattern, 30 tentative candidates were kept for further investigation (Table S12). Additional DDA acquired spectra were compared with spectral libraries (i.e., MassBank and mzCloud), to *in-silico* fragmentation spectra (i.e. MetFrag and FISH coverage), structure predictions (SIRIUS) and the plausibility of the RT was checked. Finally, 6 lipid structures were tentatively identified but unambiguous structure assignment is difficult for these mostly CHO compounds (Table 2, Figure S15-S19). Although there was partially a good match with MS2 libraries, four of the candidates (2-aminooctadec-4-yne-1,3-diol; 9s,13r-12-oxophytodienoic acid; 9,12-octadecadienal; methyl eleostearate) can maximally be assigned a level 3 as it was impossible to distinguish between isomeric structures. Two other structures were assigned to Level 2a based on the MS2 library spectra match. Reference standards were purchased to confirm them, but the standards were injected long time after the sample, which was no more available. Despite good match between the MS2 spectra of sample and reference standard (Figure S19), there was a retention time shift for 11,14,17-eicosatrienoic acid (0.4 min) and lauroyl-lysine (1.45 min). Several isomeric structures might have similar MS2 spectra, so we hesitated to upgrade them to level 1.

Among the putative chemicals following a biomagnification trend (Figure S6B), there is only a paucity of knowledge about the environmental occurrence of lauroyl-lysine, a personal care product most often used as hair and skin conditioning agent prepared from the combination of

the fatty acid lauric acid and the essential amino-acid L-Lysine. Beyond this synthetic chemical, the other candidate chemicals following a biomagnification trend are lipids. Among them, some are used as pharmaceuticals (i.e. 11,14,17-Eicosatrienoic acid and methyl elostearate) and could come from urban activities while the others were likely produced by primary producers or degraders and biomagnified through feeding along the trophic chain. Contrary to the lauroyl-lysine, their physico-chemical properties (log Kow: 5.1-8.3 ; log BAF: 2.35-5.78) make their occurrence in biota more probable. Among them 12-oxophytodienoic acid is a specific plant oxylipin produced in the chloroplast and involved in stress acclimation and development[70]. Also, the 9,12-octadecadienal was recently described as a fungi secondary metabolite[71]. Overall, this biomagnification of lipids seems contradictory to recent evidence about the decline of omega-3 and -6 poly unsaturated fatty acids with increasing trophic position[72].

Environmental significance

Our results show that even chemicals with relatively low hydrophobicity can be present at the surface or into aquatic organisms if they occur continuously in relevant concentrations in the environment and therefore should get more attention. Although many might not be bioaccumulative according to REACH criteria (i.e. BCF < 2000) the risk should be characterized even though this remains challenging with lacking hazard data based on internal dose.

Overall, as previously reported in the literature [18; 28; 73-75], our results confirm that retrospective target screening as well as suspect screening based on HRMS/MS-data are promising and a relevant approach to increase our knowledge and understanding of the chemical exposome. Our results highlight that there is the need to extend existing MS2 online libraries to facilitate and improve retrospective screening since only few of the detected signal

could actually be annotated/confirmed. The use of HRMS/MS-based trend analysis seems a good prioritization strategy to focus structure identification efforts on potentially bioaccumulative and/or biomagnifiable chemicals. These chemicals can then be further characterised regarding their toxicokinetics in order to predict internal concentration from the external concentration or ideally the actual dose at the target tissue, and also their toxicodynamic to improve knowledge on associated hazard.

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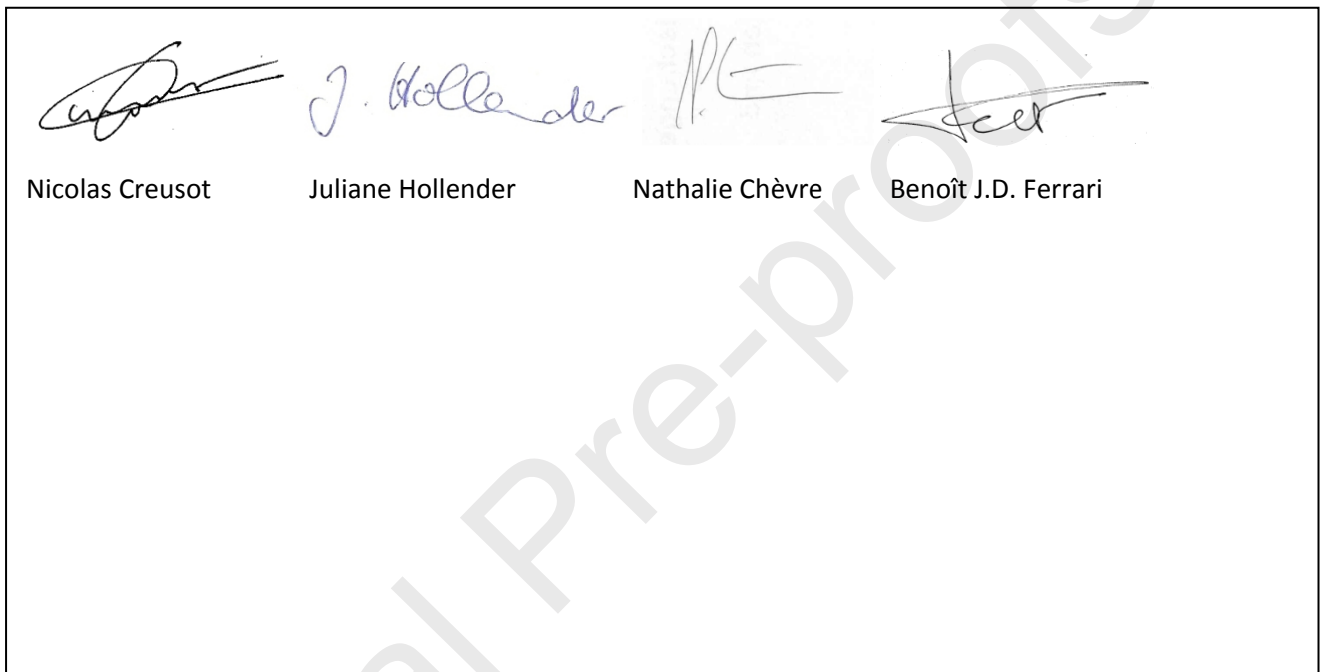
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Declaration of interests

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

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

**Credit Author Statement**

Nicolas Creusot (Conceptualization, Methodology, Formal analysis, Writing - Original Draft , Visualization, Project administration, Funding acquisition), **Kristina Huba** (Methodology, Formal analysis, Writing), **Christophe Borel** (Resources, Writing, Funding acquisition), **Benoît J.D. Ferrari** (Resources, Writing - Review & Editing, Funding acquisition), **Nathalie Chèvre** (Resources, Writing - Review & Editing, Funding acquisition) and **Juliane Hollender** (Conceptualization, Methodology, Resources, Writing - Original Draft, Writing - Review & Editing, Supervision Funding acquisition).

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