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Effect-directed analysis and suspect screening to identify potential toxic drivers in water

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Abstract

A method for effect-directed analysis (EDA) was developed using the novel technique of high-throughput fractionation in combination with ultra-high pressure liquid chromatography high-resolution mass spectrometry (UPLC-HRMS) and a bioassay battery targeting four different endpoints. The endpoints included the estrogen receptor (ER), androgen receptor (AR), aryl hydrocarbon receptor (AhR) and nuclear factor erythroid 2-related factor 2 (Nrf2). The EDA method was applied to wastewater treatment plant (WWTP) effluent, urban snow, and commercial bottled water to identify potential toxic drivers. Additionally, the currently achievable harmonisation of suspect screening and non-target screening of fish biota was investigated in an interlaboratory study across 16 laboratories using both liquid chromatography (LC)-HRMS and gas chromatography (GC)-HRMS. Estrogenic activity was detected for WWTP effluent and one urban snow sample originating from an artificial football field. The sources of the activity could only be suggested with limited certainty, underscoring the need for higher sensitivity of HRMS methods in EDA. AhR activity was detected for the WWTP effluent and two urban snow samples, and Nrf2 activity was found only in the WWTP effluent. However, upon fractionation both the AhR and Nrf2 activities were lost. Of compounds identified with greater confidence in WWTP effluent, 60 % were detected downstream of the effluent discharge point, while <1 % were detected upstream, highlighting the substantial impact of WWTP effluent on the micropollutant load of receiving waters.

The interlaboratory study on fish biota showed that there was a large variation between target, suspect and non-target screening results across participating laboratories. The differences seemed to be unrelated to sample preparation method, but instead be due to differences in HRMS workflow strategies. Further harmonisation efforts are necessary to improve comparability and reliability in future screenings.

Keywords: Effect-directed analysis; high-resolution mass spectrometry; high-throughput fractionation; suspect screening; wastewater treatment plant effluent; urban snow; bottled water; whole fish tissue

Effektdriven analys och suspect screening för att identifiera potentiellt toxicitetdrivande ämnen i vatten

Abstract

En metod för effektdriven analys (EDA), baserad på den nya tekniken högkapacitetsfraktionering, ultra-högtrycks vätskekromatografi högupplöst masspektrometri (UPLC-HRMS), och ett bioanalysbatteri riktat mot fyra olika biologiska targets utvecklades. Bioanalyserna använde sig av östrogenreceptor (ER), androgen receptor (AR), arylkolvätereceptor (AR) och nukleär faktor erytroid 2-relaterad faktor 2 (Nrf2) aktivering. EDA metoden applicerades sedan för att hitta ämnen som potentiellt driver toxicitet i effluent från avloppsreningsverk (WWTP), urban snö, och kommersiellt flaskvatten. Utöver det så undersöktes den för närvarande uppnådda graden av harmonisering inom suspect screening och non-target screening av hel fiskvävnad genom en interlaboratoriestudie med 16 olika deltagare, baserat på både vätsktekromatografi (LC)-HRMS och gaskromatografi (GC)-HRMS. Östrogen aktivitet detekterades i WWTP-effluenten, och det urbana snöprovet som härstammade från en fotbollsplan med konstrgräs. Ursprunget till aktiviteten kunde bara föreslås med osäkerhet, och ett behov av ökad känslighet hos HRMS metoder i EDA framlyfts. AhR-aktivitet detekterades i WWTP-effluenten och i de två urbana snöproverna, och Nrf2-aktivitet i WWTP-effluenten. Både AhR- och Nrf2-aktiviteten försvann dock när proven fraktionerades. Av de ämnen som identifierades med högre säkerhet i WWTP-effluenten kunde 60 % detekteras även i åvattnet hos den mottagande ån nedströms effluentutsläppet, medan <1 % detekterades uppströms. Detta tyder på att WWTP-effluenten bidrar signifikant till mikroföroreningsbelastningen av ån.

Interlaboratoriestudien på fiskvävnad visade att det fanns en stor variation mellan target-, suspect- och non-target screeningar som utförts av olika laboratorier. Skillnaden verkade inte bero på olika metoder för provupparbetning utan på andra faktorer. Fortsatta ansträngningar för förbättrad harmonisering är nödvändiga för att uppnå mer jämförbara studier i framtiden.

Keywords: Effektdriven analys; högupplöst masspektrometri; högkapacitetsfraktionering; suspect screening; effluent från avloppsreningsverk; urban snö; flaskvatten; hel fiskvävnad

Preface

“The impediment to action advances action, what stands in the way becomes the way”
− *Marcus Aurelius*

Dedication

To all my loved ones. You know who you are.

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Lindblad S**., Frieberg K., Wiberg K., Lundqvist J., Ahrens L. Effect-directed analysis with high-throughput fractionation and a 4-tiered bioassay battery for identification of toxic drivers in WWTP effluent. (submitted)

II. **Lindblad S.**, Mandava G., Frieberg K., Wiberg K., Lundqvist J., Ahrens L. Application of high-throughput effect-directed analysis on urban snow samples with potential impact on drinking water quality. (manuscript)

III. **Lindblad S.**, Mandava G., Wiberg K., Lundqvist J., Ahrens L. Is high-throughput effect directed analysis suitable for bottled water? (manuscript)

IV. Dürig W.*, **Lindblad S**.*, Golovko O., Gkotsis G., Aalizadeh R., Nika M-C., Thomaidis N., Alygizakis N., Plassmann M., Haglund P., Fu Q., Hollender J., Chaker J., David A., Kunkel U., Macherius A., Belova L., Poma G., Preud'Homme H., Munschy C., Aminot Y., Jaeger C., Lisec J., Hansen M., Vorkamp K., Zhu L., Cappelli F., Zuloaga O., Gil-Solsona R., Gago-Ferrero P., Rodrigez-Mozaz S., Budzinski H., Devier M-H., Dierkes G., Boulard L., Jacobs G., Voorspoels S., Rüdel H., Ahrens L. (2023). What is in the fish? Collaborative trial in suspect and non-target screening of organic micropollutants using LC-HRMS and GC-HRMS. Environment International, 181, 108288. https://doi.org/10.1016/j.envint.2023.108288

* Shared first authorship
All published papers are published open access.

The contribution of Sofia Lindblad to the papers included in this thesis was as follows:

I. Everything (e.g. planning, sample preparation, HRMS analysis, fractionation, data analysis, synthesis of results, making of figures, writing of first draft, writing corrections etc.) apart from performing the bioanalytical analyses.

II. Everything (e.g. planning, sample preparation, HRMS analysis, fractionation, data analysis, synthesis of results, making of figures, writing of first draft, writing corrections etc.) apart from performing the bioanalytical analyses.

III. Everything (e.g. planning, sample preparation, HRMS analysis, fractionation, data analysis, synthesis of results, making of figures, writing of first draft, writing corrections etc.) apart from performing the bioanalytical analyses.

IV. Everything (e.g. data collection from participants, interpreting data, data analysis, synthesising results, making figures, writing of first draft, writing corrections etc.) apart from the laboratory work.

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1. Introduction

1.1 Organic micropollutants

1.1.1 Pollution of the aquatic environment

An organic compound classifies as a contaminant if it is present in an environment in a concentration that would not occur naturally. If the contaminant can cause adverse effects in living organisms, it is also considered a pollutant. If a pollutant is present, and potent enough to cause adverse effects, in very low concentrations (ranging from ng/L to µg/L[1]), then it can be referred as an organic micropollutant (OMP)[2].

The widespread use of anthropogenic chemicals is causing pollution of the environment by OMPs, including our aquatic environment[3]. Groups of chemicals of concern are, among others, industrial chemicals, pesticides, pharmaceuticals, and personal care products[3, 4]. OMPs being present in the aquatic environment can pose a threat to the ecosystem itself, e.g. through acute and chronic toxicity, endocrine disruption, and antibiotic resistance, as well as to us humans[1]. The pollutants can spread to recreational waters, or drinking water source areas and ultimately end up in our drinking water, where potential human toxicity becomes a public concern[1]. Many of the anthropogenic chemicals, e.g. pharmaceuticals or pesticides, were originally designed to have biological effects and are therefore likely to display toxicity at relatively low concentrations. The fact that they are present in a complex mixture might also affect their potential toxicity, making it challenging to predict the overall risk[1].

The main source of OMPs in the aquatic environment is waste water treatment plants (WWTPs)[5]. Typically they do not utilize purification methods specifically designed to remove OMPs and, because of the diversity of compounds, it is difficult to find methods to remove them completely, resulting in varying removal efficiencies in WWTPs[1, 6]. The inefficient removal of anthropogenic chemicals during wastewater treatment along with other point sources such as leachate from landfills, contributes to OMP pollution[1, 3]. Additionally, diffuse sources such as storm water runoff resulting from heavy rain or wind events represent a significant pathway for

introducing pollutants into the aquatic environment[7-9]. Runoff from agriculture introduces OMPs into the aquatic environment such as pesticides, herbicides and animal pharmaceutical residues from manure[7, 10]. Release of process water from industries to surface waters is also contributing to OMPs in the environment[7, 11].

1.1.2 Contaminants of emerging concern

Contaminant of emerging concern (CEC) is a term used to describe chemicals that have been discovered recently, or with increasing frequency, in the environment[12, 13]. The CECs can have unknown effects, or be known to cause adverse effects. CECs also include previously known chemicals, for which new toxicological effects have been recently discovered[13]. The common thread is that CECs are currently not sufficiently monitored or regulated, and thereby posing a potential threat to humans and ecosystems[12-14].

The NORMAN network, dedicated to CECs, was established in 2005 by the European Commission[13]. The aim of this network is to facilitate collaboration, exchange of data, harmonisation of methods and practices, and increase transparency and data quality[13]. Among other things, NORMAN has established a database of the currently most discussed CECs, based on member and external contributions[13]. The NORMAN Substance Database, or NORMAN SusDat, contains over 100 000 compounds (January 2023) and is updated continously[15]. Categories of compounds currently included in the database are biocides, drinking water chemicals, drugs of abuse, flame retardants, food additives, food contact materials, human metabolites, human neurotoxins, indoor environment substances, industrial chemicals, metals and their compounds, natural toxins, per- and polyfluoroalkyl substances, persistent mobile and toxic substances, personal care products, pharmaceuticals, plant protection products, plastic additives, REACH chemicals, smoke compounds, and surfactants[15].

A recent review[16] prioritized CECs in aquatic environments based on risk intensity and detection frequency. The top ten included several pharmaceuticals and personal care products: sulfamethoxazole (antibiotic), diclofenac (analgesic and anti-inflammatory drug), acetaminophen (analgesic and antipyretic drug), caffeine, ofloxacin (antibiotic), triclosan

(antibiotic), ibuprofen (analgesic and anti-inflammatory drug), erythromycin (antibiotic), clarithromycin (antibiotic), and carbamazepine (anticonvulsant for epilepsy and nerve pain, and treatment of alcohol abstinence). However, it can be questioned whether publication frequencies of certain compounds accurately reflect the presence of compounds, or rather represent research interest and ability of detection[17].

CECs do pose a great potential threat to humans and ecosystems. However, a potentially larger threat are contaminants present in our environment that we have not yet identified, or whose toxicity is not yet understood[17, 18]. Perhaps they do not appear hazardous on their own, but contribute to overall toxicity through complex mixtures in the environment[18-20]. Improving analytical techniques and methods to detect unknown compounds is therefore of high importance[20-22]. Without awareness of their presence, effective regulation and mitigation become impossible[17]. By the increasing use and number of chemicals around the world[20], identification of unknown, toxic chemicals in the environment is of emerging importance.

As of today (January 2023), the CAS RegistrySM contains 204 million compounds[23], and over 26 000 compounds are registered in REACH[24]. Of these compounds registered in REACH, 102 are classified as persistent, bioaccumulative and toxic (PBT) or very persistent and bioaccumulative (vPvB), but only 7 349 compounds are registered as not PBT/vPvB, and 1 492 have the status that PBT assessment does not apply. This leaves approximately 17 000 compounds, for which information appears to be lacking. In addition, the chemical classification persistent, mobile and toxic (PMT) and very persistent and very mobile (vPvM) has only recently been added to the CLP regulation, identifying an even greater lack of data for commercially available compounds[25]. Taken into account the number of degradation products or metabolites possibly formed from these compounds, it is intuitive to realize the vastness of compounds lacking toxicity evaluations, or even proper identification. Examples of this are studies[18, 26] showing that detected chemicals in water could only explain as little as 0.1 % of the response in bioassays of specific toxicity pathways. However, according to EU's drinking water directive EU 2020/218428, Article 4 Paragraph 1, drinking water has to be "*wholesome and clean*", and a condition for this is for the water to be free "*from any substances which, in*

numbers or concentrations, constitute a potential danger to human health". To ensure that we fulfil this requirement, ongoing investigation for the presence of unknown, potentially toxic compounds in our waters, especially in relation to drinking water, is essential.

1.2 Suspect and non-target screening

1.2.1 Overview

Suspect screening and non-target screening (NTS), along with target analysis, are analytical strategies in environmental analysis. They are usually applied using high resolution mass spectrometry (HRMS) in combination with liquid chromatography (LC-HRMS) or gas chromatography (GC-HRMS).

1.2.2 Liquid chromatography

Liquid-solid chromatography (LC) is a technique utilizing a solid phase – a column packed with solid material – and a liquid phase – a solvent system – to separate compounds with different properties[27]. In high-pressure or ultra-pressure liquid chromatography (HPLC/UPLC) a pump drives the solvent through the column. After sample injection, compounds travel through the column, where they interact differently with the solid phase vs the mobile phase. The more it is interacting with the solid phase, the longer it will take before the compound exits the column with the solvent[27]. The retention time (RT) refers to the interval between sample injection until the compound exits the column and reaches the detector. All molecules of the same compound will have similar RT. The RT can therefore be used to aid in the identification of a compound, however, only if compared to a RT generated from the same chromatographic setup; with the same type of column and the same solvent system[27].

1.2.3 Gas chromatography

Gas chromatography (GC) is a technique that utilises gas as the mobile phase, instead of liquid(s)[28]. The injected sample is vaporized, after which the carrier gas transport the sample along a column packed with solid material[28]. The same principle for separation as for the LC applies; the molecules from the sample will interact and be retained by the solid phase to

different degrees depending on the its properties, obtaining separation of different types of molecules[28].

1.2.4 High resolution mass spectrometry

High resolution mass spectrometry (HRMS), often used as a detector connected to an LC or GC system, generates a signal based on the mass-to-charge ratio (*m/z*) of the analyte. A prerequisite for detection in a mass spectrometer is that the molecule must have a charge[29]. For small molecules, the charge is often 1, and the *m/z* therefore equals the mass. However, for instance in the world of proteomics, several charges might be present on the same molecule, and the *m/z* will then be proportionate to, but not equal to, the mass[29].

Upon entering the mass spectrometer from the LC or GC, analytes are ionized into charged molecules in gas-phase by the ion source[29]. There are many different types of techniques for this, one of which is electrospray ionization (ESI), common for LC-HRMS systems. With ESI, the analyte solution is passing through a needle, to which high voltage is applied. This results in droplets being sprayed from the needle, containing electrical charges on their surface. As solvent molecules evaporate from the droplets, they reduce in size until the charge repulsion overcomes the surface tension, and the droplet ejects smaller droplets. At some point, when very small droplets with a very high charge has been formed, ions of the analytes are repulsed into gas phase[29]. The ions are then ready to be transferred to the detector. In instruments such as an Orbitrap mass spectrometer (e.g. Q Exactive Focus, Thermo Fisher Scientific)[30] (*Figure 1*), the ions are being transferred through a mass filter that only allows analytes with certain *m/z* to pass through it, into a C-trap. The C-trap acts as a storage of ions, and when enough ions have been accumulated it can either send the ions into the Orbitrap mass analyser for detection or into the higher-energy collisional dissociation (HCD) cell for fragmentation before being returned to the Orbitrap mass analyser. In the Orbitrap mass analyser, ions are influenced by an electric field generated by three electrodes at high voltage. As they orbit and oscillate between the electrodes, the ions cause a current detected by a differential amplifier. The signal is converted into *m/z* signals through Fourier transformation, a mathematical model[30].

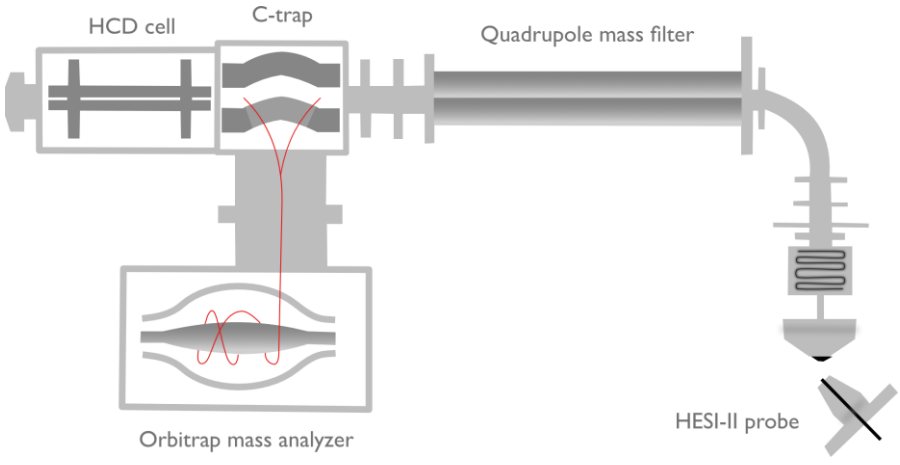

Figure 1. A schematic overview of the parts of an Orbitrap mass spectrometer (e.g. Q Exactive Focus, Thermo Fisher Scientific). The HESI-II probe is performing heated electrospray ionization. The quadrupole mass filter determines which ions reaches the C-trap, a storage for ions. From there, the ions can be directed into the HCD cell for fragmentation, or to the Orbitrap mass analyser for detection.

1.2.5 Target analysis

In target analysis, only predefined specific compounds referred as targets are recorded. A set of compounds with specified *m/z* and RTs are selected before running the analysis and the presence and signal intensity of matching compounds are recorded[31]. To assure correct identification and quantification, reference standards of the compounds must be analysed using the same system and method, confirming accurate *m/z* values, RTs, and potentially MS2 features[31, 32]. Target analysis is reliable and fast, but limited due to the fact that only information about the specific target compounds is obtained.

1.2.6 Suspect screening

In suspect screening, all data within a specified *m/z* range (e.g. 120-1 000 *m/z*) is recorded. This dataset is then compared to a suspect list, which may contain thousands of compounds with *m/z* and potentially MS2 features, although these may have been generated under different analytical conditions[31, 32]. The data is typically filtered with the data from blanks by removing features which are below the blank (e.g. less than 5 times the intensity of the blank). Recording all the data requires more resources in

terms of time and storage, but the advantage is that one does not have to know in advance what one expects to find, and one can revisit the dataset at any point to investigate for the presence of compounds of emerging concern, even years after its recording[33].

1.2.7 Non-target screening

NTS is usually performed alongside suspect screening, for the peaks that could not be confidently matched with the suspect list. Identification is attempted using *m/z* values, calculated molecular formula, and fragmentation pattern[31, 32]. When a compound is tentatively identified using NTS or suspect screening, its reference standard may be purchased and analysed under identical conditions to confirm the match and increase confidence (discussed further in section *1.3.6. Identification of chemical hazards*)[31]. The advantage of NTS is the ability to detect compounds without prior knowledge or reliance on a suspect list[33]. Thus, new, unknown compounds can be identified. The drawback is that NTS is extremely time consuming, which might limit the amount of compounds that can be identified from a spectrum in a given time. NTS is therefore most effective when combined with suspect screening to ensure analytical resources are used efficiently and only directed toward truly unknown features [33].

1.3 Effect-directed analysis

1.3.1 Overview

One way of focusing the efforts of suspect screening and NTS on the identification of relevant compounds is through the application of effect-directed analysis (EDA)[34, 35]. EDA is a method, utilized since the early 1980s, that combines bioassays, fractionation and chemical analysis to identify compounds with toxicity (*Figure 2*). After fractionation of sample extracts, the results of bioassays are directing where the efforts of chemical analysis should be applied, saving resources and time while still generating relevant results. The preliminary result of the chemical analysis is then confirmed through performing further bioassays, an important step in case the active toxicant failed to be identified from the initial complex mixture, perhaps due to being present in too low concentration[34]. An example of an EDA setup can be found in *Figure 3*.

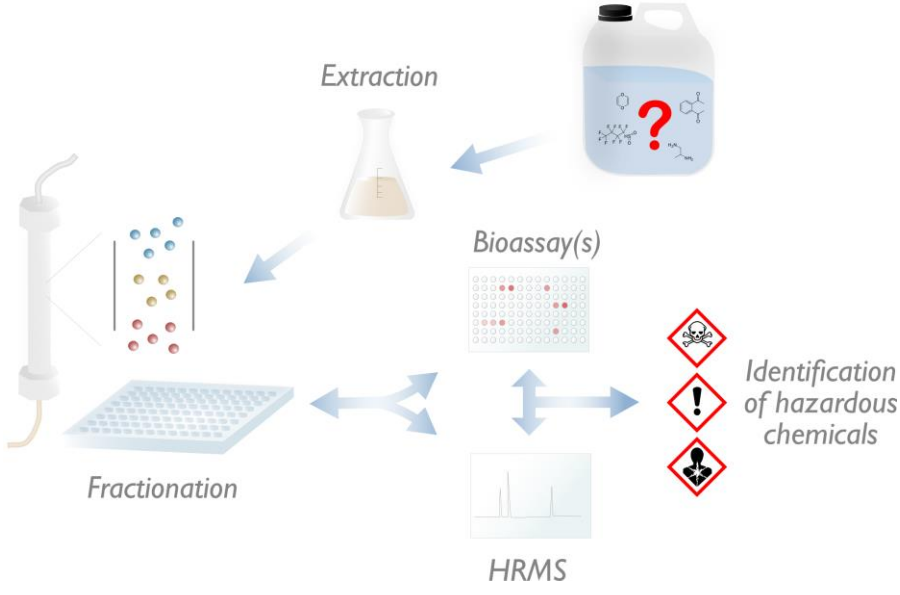

Figure 2. A simplified scheme of effect-directed analysis (EDA), from water sample to identified toxic compounds, using HRMS for the chemical analysis.

Figure 3. An example of a set up for running EDA based on HPLC (top left), HRMS (top right), and fractionation into 96-well plates using a FractioMate™ (bottom). The well plates are then used for bioanalytical analysis.

1.3.2 Extraction

Extraction of environmental samples vary depending on the sample matrix as well as the type of compounds of interest. No method is able to extract all anthropogenic compounds present in a complex mixture sample, so compromises are necessary[34].

Historically, the focus when extracting aqueous samples was mostly on lipophilic organic compounds. During those early times, sample preparation evolved from liquid/liquid extraction, to solid-phase extraction (SPE) with non-polar C18 or XAD resins[34]. Further developments used passive samplers with e.g. semi-permeable membrane devices (SPMDs), which not only account for compound bioavailability but also reduce the amount of water needed to be collected and shipped. They also correspond to longer time periods, especially relevant for compounds prone to bioaccumulation[34]. To detect polar or ionic compounds, which has gained interest since the late 1990s, a different strategy for sample preparation is required. Poly(styrene-divinylbenzene) phases at different pH has become common to be used for SPE. To cover a broader range of polarities, sequential SPE with C18 and poly(styrene-divinylbenzene) at different pH has been adopted[34]. Specific interest in e.g. mutagenic compounds or estrogenic compounds has also lead to more selective and specialised extraction methods[34, 36].

1.3.3 Fractionation

Fractionation can be performed based on different properties, such as polarity, molecular size, shape and presence of specific structural moieties. Sequential SPE can give different initial fractions. After extraction, size-exclusion chromatography (SEC) such as gel permeation chromatography (GPC) with Sephadex has been used for further fractionation[34, 37, 38]. Alternatively, following SPE, HPLC or UPLC is commonly utilized[34, 39, 40]. Thin-layer liquid chromatography (TLC) is another option, but due to lower resolution it is rarely used[34].

1.3.4 Bioassays

Bioassays play a crucial role in EDA since they determine which compounds will be analysed. In many cases, it is necessary to use a battery of bioassays to perform a comprehensive analysis[34]. Depending on whether the aim is to simply detect toxic fractions and compounds, or to assess environmental hazards, the requirements on the bioassays differ. If the goal

is detection of toxic compounds, key requirements include rapid test execution, high throughput, small sample volume, reproducibility, sensitivity, and quantifiability. If hazard assessment is the goal, a further requirement is that the detected effects as well as cells/organisms used, are of relevance to the specific environment in question. It is also important that the acute or chronic effects can be detected already at low doses[34]. In the past, bioassays for EDA was mostly performed with invertebrates such as different species of daphnia, or the fish species *Pimephales promelas*[34, 41]. In the early 2000s, the bacteria *Vibrio fischeri* was predominantly used for EDA, in acute bioluminescence inhibition bioassays[34, 39, 42]. However, this test also measures nonspecific effects (e.g. narcosis, uncoupling) caused by all the components in the mixture based on concentration and hydrophobicity, making it less suitable for identifying individual compounds with specific toxicicity[34]. During the 2000s the interest for bioassays in water quality testing grew, and the focus shifted from ecosystems to human health, which increased the use of mammalian cell-based bioassays[18]. In general, such bioassays can be classified as either Category 1 or Category 2, depending on the number of different chemicals that trigger a response[18]. Category 1 bioassays are generally activated by a limited number of chemicals that are potent and mostly well known (e.g. assays based on the estrogen or androgen receptor). Category 2 on the other hand is activated by a wider range of different chemicals, many of which have lower potency (e.g. assays based on nuclear factor erythroid 2-related factor 2, Nrf2, or the aryl hydrocarbon receptor, AhR). This means it is often more difficult to recover toxicity upon fractionation, since the compounds responsible are often spread out over many fractions.

Estrogenic bioassays

Bioassays indicative of compounds with estrogenic activity is of high relevance in water testing. It is one of the most common assay types for endocrine disrupting chemicals, as well as for water testing in general[18]. One reason is residues in wastewater due to use of contraceptive pharmaceuticals, but also industrial chemicals and pesticides can interfere with hormone signalling. In these bioassays the estrogen receptors (ER), ERα and/or ERβ, are connected to a system of detection. Mammalian reporter gene assays, such as ERα CALUX or T47D-KBluc, have been found more sensitive than the yeast reporter gene assay Yeast Estrogen Screen (YES)[18]. 17β-Estradiol is an endogenic compound that activates the ER, and is often used as the

reference compound for bioassays[18]. It is an important hormone that is vital for many parts of and functions in the body, such as the cardiovascular system, bones, sexual development, and growth and homeostasis of uterus and mammary glands[18]. Estrogenic activity has implication in several types of cancers as well, for example uterine cancer[43, 44].

Androgenic bioassays

Bioassays indicative of compounds with androgenic activity is also of high relevance in water testing. Mammalian reporter gene systems, such as AR CALUX and GeneBLAzer, are more sensitive than the yeast reporter gene assay Yeast Androgen Screen (YAS)[18]. Dihydrotestosterone (DHT) is an endogenic compound that activates the androgen receptor (AR), and is often used as the reference compound for bioassays[18]. The AR plays many roles in different tissues including reproduction, immune, cardiovascular and skeletal muscle systems[18]. It has also been implicated in different types of cancers, such as testicular cancer[45].

Nrf2 bioassays

Nuclear factor erythroid 2-related factor 2 (Nrf2) bioassays are used to indicate oxidative stress response. Reactive oxygen species (ROS) and electrophilic chemicals activate the transcriptional regulatory element called Antioxidant Response Element (ARE) through Nrf2[18, 46]. Mammalian reporter gene assays that utilize this are, e.g., ARE GeneBLAzer and Nrf2 CALUX[18]. The compound *tert*-butylhydroquinone (*t*BHQ) is commonly used as the reference compound[18, 46]. ROS and electrophilic chemicals are reactive compounds, capable of interacting with and damaging structures of the cells including DNA, which is relevant to understanding potential carcinogenicity[47, 48].

Aryl hydrocarbon receptor bioassays

The aryl hydrocarbon receptor (AhR) is responsible for mediating toxicity of halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons (PAHs)[18]. Upon activation, it upregulates the transcription of CYP1A1, CYP1B1 and NADPH-quinone oxidoreductase. The cytochrome P450 monooxygenases are enzymes, for example, part of the phase I metabolism of the liver, and their substrates can often be transformed into carcinogenic intermediates capable of interaction with and damaging of DNA[18]. AhR activation is therefore also associated with cancer risk.

Crosstalk between the AhR and ER[49], as well as between AhR and Nrf2, has also been observed[18]. Cell-based reporter gene systems used for water analysis are e.g. AhR CAFLUX and AhR CALUX. The compound commonly used as a reference compound is 2,3,7,8-tetrachloro-*p*-dibenzodioxin (TCDD)[18].

1.3.5 Chemical analysis

The most common method of chemical analysis in EDA is HRMS, coupled to LC (for hydrophilic compounds) or GC (for hydrophobic compounds)[34, 40]. Alternative or complementary methods that has been used is nuclear magnetic resonance (NMR) spectroscopy, ultraviolet (UV) absorption spectroscopy, and infrared (IR) absorption spectroscopy[34].

1.3.6 Identification of chemical hazards

Confirmation of the toxic compounds identified through EDA is made on several different levels. The first level is analytical confirmation, where the identity of the compounds is confirmed or strengthened[50]. The second level is effect confirmation, where it is investigated whether the identified compounds cause effects in the utilized bioassay(s), and whether all of the initially observed effect can be explained by their presence[50]. The last level is hazard confirmation where the potential hazard to ecosystems posed by the identified compounds is investigated[50].

The identity of a compound can be considered analytically confirmed if the mass spectrum and RT is matching those of pure reference standards, though this alone may not guarantee accurate identification. Structural elucidation via NMR strengthens confidence[50]. In cases where neat standards are unavailable, tentative identifications may be based on comparisons with spectral libraries[50]. If also the RT, or retention index can be matched with those of published standards under similar conditions, the structural assignment can be considered confident[50]. For unknown compounds, or compounds not present in libraries, *in silico* generation of structures that match a molecular formula can be utilized, and prediction tools for mass spectra and retention indices can narrow down the results[50].

To communicate the strength of evidence for the structures obtained through HRMS, confidence levels based on the Schymanski confidence

levels are often being used[51]. Level 1 means that the structure has been confirmed using pure standards, and both the MS1, MS2 and RT is in agreement[51]. Level 2 means that the structure is probable. For level 2a, the spectrum is unambiguously matching the data from a library, while for level 2b no library or literature information is available, but there is still enough information (parent compound, diagnostic MS2 fragments etc.) to not cause any ambiguity in which structure it is[51]. Level 3 signifies that the structure is tentative. The exact structure is unsure, but there is evidence pointing at possible structure(s)[51]. Level 4 means that one can assign only one molecular formula, but the evidence is not strong enough to propose a structure[51]. Lastly, level 5 is when the exact mass can be measured, but it is not possible to assign it a molecular formula[51]. Target screening always lead to level 1 confirmation. For suspect screening and NTS where the result is being confirmed by pure standards, level 1 confirmation is possible. Without the use of standards, suspect screening can at best lead to level 2a confirmation, and NTS to level 2b confirmation.

Using prediction, such as quantitative structure-activity relationships (QSAR) or structural alerts, can help identifying which compounds of a fraction that might be responsible for bioassay effect[50]. However, definitive effect confirmation requires pure standards[50]. To quantitate and compare toxicity of different compounds, Toxicity Equivalent Quantities (TEQs) are often used, which is the equivalent concentration of a reference compound that would give the observed effect[50]. If combined effect of a mixture is used as the equivalent concentration of the reference compound, this is often called bioanalytical equivalent concentration (BEQ)[18]. In this way the toxicity of mixtures can be compared, when effects are exerted through a common mechanism, and an effect concentration (EC) causing less than 30 % effect ((EC)y with y <30 %) is being used[18]. The BEQ is calculated as ECy(reference)/ECy(sample), with the ECy(sample) being expressed in the unitless relative extraction factor (REF). REF is a measure of the enrichment (or dilution) compared to the original sample[18].

If the sample extract is analysed in the bioassays before as well as after fractionation, the effect of the fractions can be added and compared to that of the whole extract, to tentatively determine whether the full effects have been recovered. If the concentrations of the identified chemicals are known,

and they either have known ECy values or these are measured with the help of standards, this can be used to calculate the expected BEQ, which is usually called BECchem. If this is compared to the measured BEQ of the extract, usually called BEQbio, the difference between BEQbio and BEQchem indicates how much of the total effect is not explained by the identified compounds[18]. Unexplained effects may result from individual compounds contributing to the total effect being present in concentrations below their detection limit or from substances that do not exhibit activity in isolation but contribute cumulatively within mixtures. Fractionation may also inadvertently isolate compounds below biologically relevant thresholds unless prior knowledge of their effects guides the analysis[18].

Hazard confirmation is very challenging, in part because it is difficult to extrapolate from *in vitro* effects to effects on higher biological levels, and is thus not always considered[50]. Work is however being performed to effect translation of bioassay results into, for instance, EU Environmental Quality Standards (EQS)[52]. This is a step towards making hazard confirmation more accessible.

1.3.7 Weaknesses and strengths

One challenge with EDA is that the bioassays and the chemical analysis require a threshold concentration to successfully identify the presence of toxic compounds[34]. It is therefore possible to miss compounds that contribute to the overall effect of the water, that is present at low concentrations. This is especially the case with category 2 bioassays that are activated by many different compounds rather than category 1 bioassays that are activated by a few, strongly acting compounds[18]. Also, when the samples are concentrated to enable finding compounds present in low concentrations, there is a risk that compounds are lost during the sample preparation process[53].

Near known sources of pollution, or where specific toxic compounds have already been identified, EDA is most effective. However, with increasing distance from the source the dilution of toxic compounds will increase, making them harder to detect[34]. Furthermore, compounds acting through nonspecific pathways will constitute a greater proportion of the complex mixture, making interfering cytotoxicity a problem. Both of these effects

contribute to making EDA more difficult[34] and have led to the view that EDA is unsuitable for screening purposes[34].

Another challenge is that many EDA procedures disregards bioavailability of the detected toxic compounds, which possibly cause overestimation of their toxicity[34]. Strategies to address this include partitioning-based passive sampling, where polar organic pollutants from the water is enriched, and bioaccessibility-directed extraction, where only relevant compounds are investigated[54]. Another way to investigate chemical pollution in water through a bioavailability and bioaccumulating perspective is to analyse, for example, fish tissue from fish that has lived in, or been exposed to, the water in question[55]. It is however common to regard all compounds that are dissolved in water as available to biota[50], which does not always hold true.

Overall, given the incomprehensibly large amount of chemicals possibly present in the aquatic environment, EDA is a great tool since it allows focusing the efforts of identification on those chemicals that are of potential concern, even if the chemical is previously unknown[35]. This is saving both resources and time, while contributing to increased safety, both for humans and ecosystems. EDA also provides a useful methodology for assessment of mixture effects, which is highly relevant given the complexity of the chemical cocktail present in the environment[18, 19, 52]. With time and further development, the current challenges of EDA might be diminished, creating an even more powerful tool for identifying potential hazards posed by anthropogenic chemicals in our environment.

2. Objectives

The objective of my PhD project was to identify toxic drivers in the aqueous environment, with special focus on water with drinking-water relevance, using EDA, suspect and NTS (*Figure 4*).

- Paper I: The objective was to develop and validate a method for EDA, and apply it to effluent and influent water of a WWTP, and the recipient river. The goal was to investigate the presence of potentially toxic compounds as well as to evaluate the impact of the WWTP effluent on the micropollutant load in the river which ultimately flows into a lake used for drinking water production.
- Papers II and III: The objective was to apply the method on other matrices that also had potential impact on drinking water quality; urban snow and commercially available bottled water and tap water. The goal was to identify potential toxic drivers.
- Paper IV: The study adopted a different perspective by examining water pollution through fish tissue analysis. The objective was to investigate the current level of harmonisation within target, suspect and NTS of whole-fish samples, to determine how different studies within the field are comparable.

The following main research questions were asked:

Papers I-III

- Does the WWTP effluent contain detectable toxic drivers?
- Does the urban snow contain detectable toxic drivers?
- Does bottled and tap water contain detectable toxic drivers?
- What toxic drivers can be identified in the WWTP effluent, urban snow and bottled and tap water?
- Does WWTP effluent impact the organic micropollutant load of river water used for drinking water production?
- Is bottled and tap water suitable for EDA studies?

Paper IV

- Are different methods using target, suspect- and NTS in fish comparable?

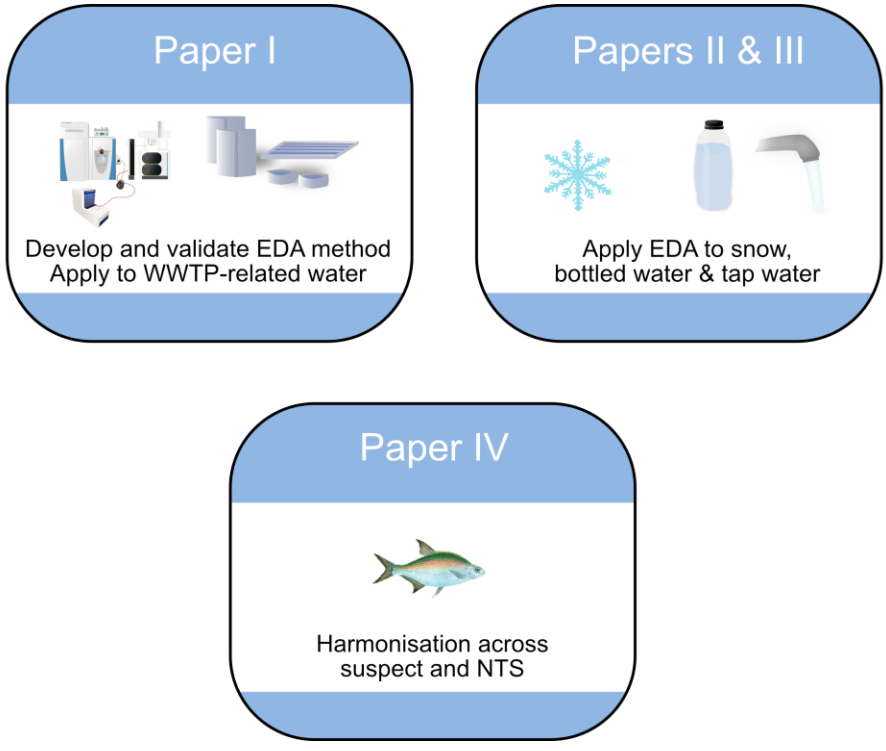

Figure 4. Overview of the project objectives.

3. Material and Methods

3.1 Papers I-III

3.1.1 Materials, samples and sample collection

Solvents for sample preparation and chemical analysis was purchased from VWR. Reference compounds for MS (estradiol (E2), dihydrotestosterone (DHT), *tert*-butylhydroquinone (*t*BHQ), 6-formylindolo[3,2-b]carbazole (FICZ), ethinylestradiol (EE2), bisphenol-A (BPA), androstenedione, resveratrol, epigallocatehin-3-gallate, omeprazole, nimodipine) and bioassays (E2, DHT, *t*BHQ, 2,3,7,8-tetrachlorodibenzodioxin (TCDD)) was purchased from Sigma Aldrich.

Water samples for Paper I were collected from the effluent and influent water of a WWTP (24 h composite, flow compensated samples), as well as down- and upstream from the effluent-recipient river (grab samples, ~30 cm below the surface) in mid-November 2023. For the method validation, effluent and influent samples were collected from a different WWTP in July 2023. Samples were collected in PP-bottles that had been pre-washed with methanol three times.

Snow samples for Paper II (*Figure 5*) were collected from an Uppsala school's artificial football field (sample A) using granulates of ethylene propylene diene monomer (EPDM) and styrene butadiene rubber (SBR), as well as from Uppsala city's snow dump site (sample B), early February 2024. To use as reference, snow from a forest on the countryside around Uppsala (sample C) was also collected on the same day. Snow was collected in 10 L metal buckets, that had been pre-cleaned with methanol three times. The snow was allowed to melt in the buckets, covered with aluminium foil, in a fume hood. Snowmelt was then transferred to PP-bottles (pre-washed with methanol three times).

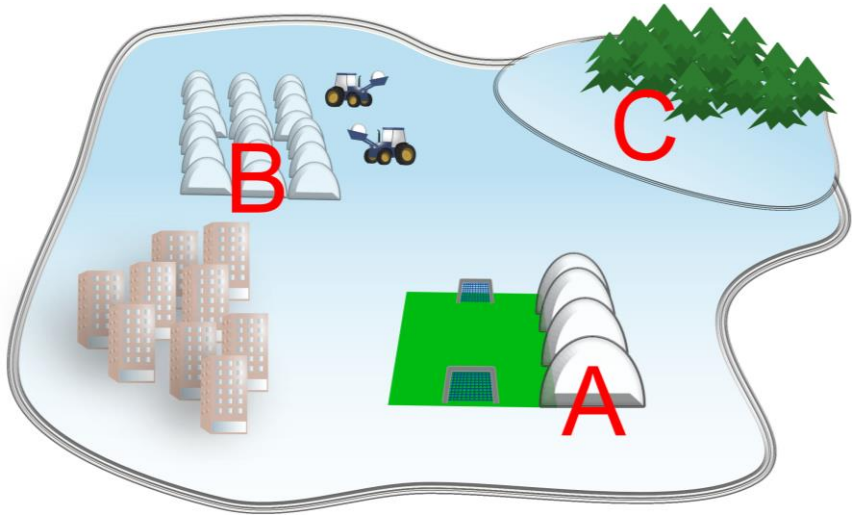

Figure 5. Depiction of the sampling locations of snow for Paper II. A: an urban, artificial football field, B: an urban snow dump site, and C: forest on the countryside outside of Uppsala.

Bottled water for Paper III was purchased from a supermarket in Uppsala in mid-January 2025, and was analysed together with tap water from 21st of January 2025 from the SLU laboratory. The larger bottles (1.5 L) had expiration date 2028-12-10, while the small bottles (0.5 L) had expiration date 2026-10-30.

For each sampling site/sample type, three technical replicates were made. For Papers I and II, each replicate originated from 1 L water/snowmelt, while for Paper III each replicate was made from 5 L water. MQ-water samples were prepared and analysed along with the samples to be used as blanks. Pre-spiked samples for quality assurance were prepared for WWTP influent, WWTP effluent, downstream river water, snowmelt from the snow dump site, and bottled water through addition of a solution containing the reference compounds for MS prior to sample preparation. Post-spiked samples for method validation in Paper I were also prepared, through addition of the same solution to the extracts from WWTP influent, WWTP effluent, and downstream river water after sample preparation. Side-spikes (reference compounds in methanol) were also prepared for each Paper I-III.

3.1.2 Sample preparation

Water sample preparation was performed by first filtering (Atlantic® fast flow sediment pre-filter, 1 micron fine, Horizon Technology) and extracting (47 mm HLB-H disc, Atlantic® Disk, Biotage) the water using an automated solid-phase extraction system (SPE-DEX® 4790). The conditioning cycles (soaking time: 30 s, air dry time: 10 s) were performed with 1x MQ, 2x methanol, 2x MQ, followed by sample loading. The wash cycles (soaking time: 10 s, air dry time: 10 s) were performed with 2x 5 % methanol in MQ, followed by the sample air dry cycle (30 min). The rinse cycle (soaking time: 1 min, air dry time: 2x 30 s, 1x 45 s) was performed with 3x methanol.

The extracts (~50 mL) obtained from the extraction was collected in E-flasks, in which they were concentrated (N_2, 30 °C) to approximately <10 mL using a RATEX concentrator. After transferring to Falcon® tubes, the concentration was continued to approximately 0.5 mL. Then, the extract was transferred to an HPLC vial, and the volume was adjusted to exactly 1.0 mL using methanol. The extraction factor (EF) was therefore 1 000 for Papers I-II, and 5 000 for Paper III.

Prior to fractionation into 96-well plates for bioanalytical analysis, the extracts of technical replicates were pooled for simplicity. For MS analysis, all the technical replicates were analysed individually.

3.1.3 Liquid chromatography high resolution (LC-HRMS) analysis and fractionation

A Vanquish Horizon UPLC system with a TriPlus RSH autosampler (Thermo Fischer Scientific, Bremen, Germany) was used for separation. An Acquity UPLC C18 1.7 µm guard column on an Acquity UPLC BEH C18 1.7 µm (50 x 2.1 mm) column, kept at 40 °C, was used. The injection volume was set to 10 µL, and flow rate to 0.3 mL/min. MQ was used as solvent A and methanol as solvent B, both with the same additive. For analysis in negative ionisation mode, 0.01 M pyrrolidine was used as the additive, while for analysis in positive mode 0.1 % formic acid was used. The gradient program is illustrated in *Figure 6*.

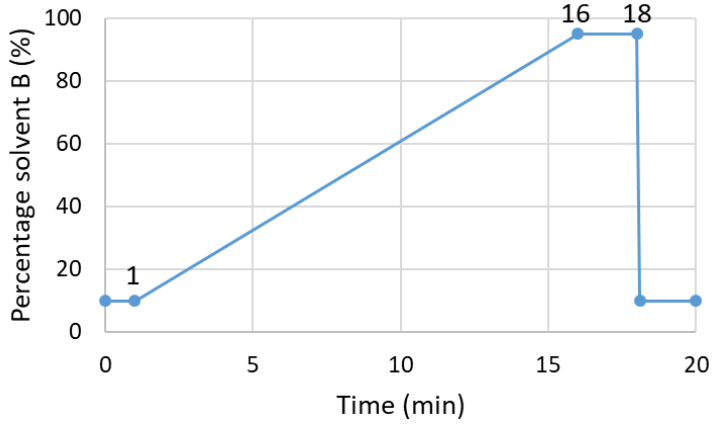

Figure 6. The gradient used for the LC method. Percent solvent B is on the y-axis, and min of the program on the x-axis.

For HRMS analysis, the UPLC system was connected to an Orbitrap Q Exactive Focus, equipped with an Ion Max heated electrospray ionisation source (HESI-II). Ion source settings are shown in *Table 1*.

Table 1. Ion source settings for the HRMS analysis

Setting	Positive ionisation mode	Negative ionisation mode
Sheath gas flow rate	35	35
Auxiliary gas flow rate	10	5
Spray voltage	2.5 kV	3.70 kV
Capillary temperature	350 °C	350 °C
S-lens RF level	55.0	70.0
Auxiliary gas heater temperature	350 °C	400 °C

Data-dependent acquisition (top N DDA, $n = 3$) was used, and the MS analysis was performed in discovery mode. Resolutions were 70 000 for full range (120-1 000 *m/z*) and 35 000 for MS2 at 200 *m/z*. CE (stepped absolute collision energy) was set to 20 and 60 eV. Full details are found in the supporting material to Papers I, II and III. Each technical replicate was analysed three times in the HRMS.

For fraction collection, the UPLC system was connected to a FractioMate™ [56]. Transparent polystyrol 96-well plates were used, that had been prepared through addition of a keeper solution (4 µL 10 % dimethyl sulfoxide in MQ) prior to fraction collection. FractioMate™ solvent blanks were added to the plates through running a methanol blank sample using a short (3 min) program, with the same gradient profile as the sample program, and collecting into 6 wells on the plate (*Figure 7*).

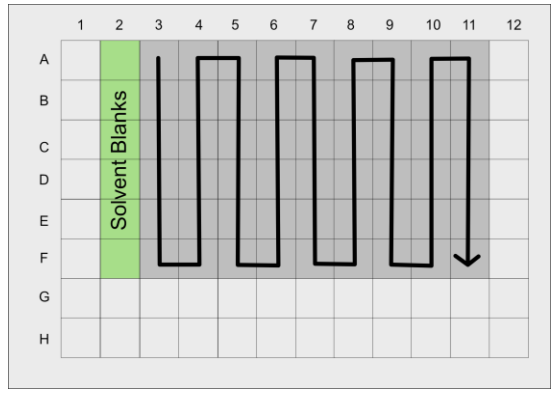

Figure 7. Spotting pattern of the sample fractions (A3-F11) and the FractioMate™ solvent blanks (A2-F2) on the 96-well plates.

For each bioassay, sample, and ionisation mode, one 96-well plate was required. For the AR, AhR and Nrf2 bioassays in Paper I, each plate was spotted 1 time, while for all the other bioassays (i.e. ER in Paper I, and all four in Papers II and III), each plate was spotted 3 times. After fraction collection, the plates were dried (N_2, 30 °C) using a RATEK concentrator, and frozen (−20 °C) awaiting bioanalytical analysis.

3.1.4 Bioanalytical analyses

Samples were analysed using previously established bioassays for ER, AR, AhR and Nrf2-activity[57]. The assays are cell and receptor based, and utilizes detection of binding through luciferase activation. The responses were measured on a luminescence reader (TECAN Infinite M1000 for Paper I, Tecan Spark for Papers II-III). Full methodological details can be found in the supporting information of each corresponding paper. In addition to the aforementioned bioassays, cytotoxicity was evaluated for each sample to

ensure data reliability[57]. If less than 80 % cell viability (compared to solvent control) was measured, the sample was considered cytotoxic.

Unfractionated samples were analysed prior to fraction analysis. A calibration curve of a positive control compound (E2 for ER, DHT for AR, TCDD for AhR and *t*BHQ for Nrf2) was analysed together with each sample, as well as negative controls such as ethanol solvent blanks. Cut off values for activity was >20 % of maximum response for ER, AR, and AhR bioassays, and >1.5 fold change (compared to solvent control) for the Nrf2 bioassay due to the lack of a true maximum response[46, 57].

3.1.5 Method validation and confirmation

Method confirmation as performed in Papers II and III was done through establishment of accuracy though relative recoveries (%) and precision via coefficient of variations (CV) for the MS reference compounds, as well as analysis of blanks to evaluate background contamination. In Paper I, a more comprehensive method validation was performed, including instrument detection limits (IDL), method detection limits (MDL), matrix effects and sample preparation recoveries (%) (*Figure 8*).

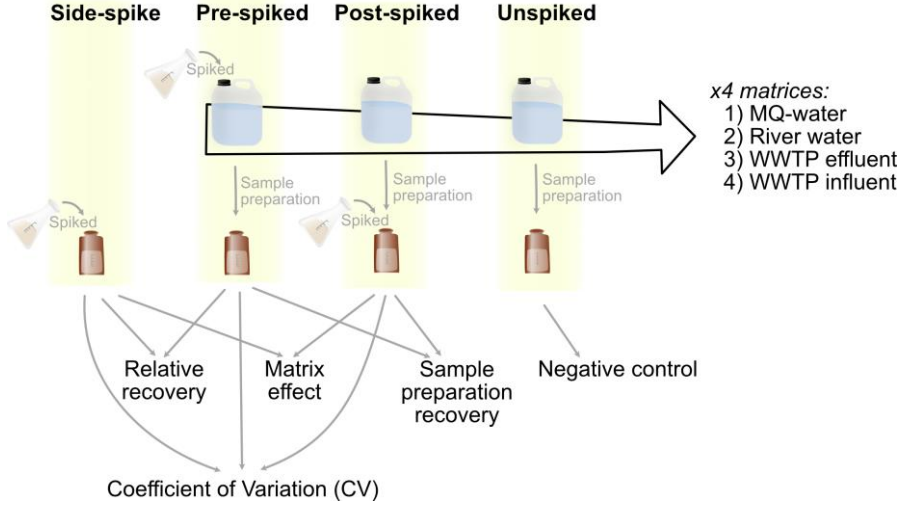

Figure 8. Overview of the experimental design for method validation in different water matrices in Paper I.

Relative recovery

Relative recovery is a measure of how well the measured signal in an analysis represents the true concentration when compared to a calibration curve. It was determined through comparison of pre-spiked samples to the side-spike samples. The difference in signal strength are mainly attributed to sample preparation method and the matrix effect.

Coefficient of variation (CV)

CV is a measurement of how much a signal from a compound varies between technical replicates and replicate runs within the same sample type. It was calculated by dividing the standard deviation of the group by the average area-under-the-curve.

Matrix effect

Matrix effect is a measure of how much a compounds signal is influenced by the matrix the it is present in, where 0 % signifies no change and 100 % signifies complete signal suppression. It was determined by comparing the post-spiked samples to the side-spike samples.

Sample preparation recovery

Sample preparation recovery is a measure of how much of the compound is recovered during the extraction process. This was determined by comparing the pre-spiked and post-spiked samples, which eliminates any potential matrix effect from the recovery result.

Instrument detection limit (IDL)

For determination of IDLs of the HRMS method, calibration curves of 14 concentrations (0.1-100 ppb) were prepared in triplicates. Each triplicate was then analysed three times. The IDLs were determined visually as the lowest concentration where acceptable peaks were obtained with signal-to-noise (S/N) $\approx$ 3.

Method detection limit (MDL)

Method detection limits (MDLs), taking matrix effects and sample preparation recovery into account, were calculated from the IDLs and relative recoveries (IDL divided with relative recovery not converted to percent).

Retention time (RT) and fraction matching

The RT and fractions on the plate were matched by running a side-spike sample. A 96-well plate was spotted three times, after which the fractions were injected directly into the HRMS. Predicted compound fractions (based on RT) were compared to the fractions where the compounds were actually detected, guiding the RT-window investigated around an active fraction (RT ±1 fraction).

3.1.6 Data analysis

FreeStyle 1.8 SP2 QF1 (Thermo Fischer Scientific, Bremen, Germany) was used to visualise MS data. Compound Discoverer 3.3 was used for performing suspect screening. CompTox Chemicals Dashboard v.2.5.3 (https://comptox.epa.gov/dashboard/)[58], ECOSAR in EPI Suite 4.1, or VEGA GUI 1.2.4[59] was used for toxicity predictions.

Compound Discoverer

To perform peak-picking and data convolution, signal intensity (>10 000 counts), S/N (>3), and blank comparison (>5 times intense signal in sample compared to blank) requirements were implemented. Norman SusDat[15] (mass ready SMILES, downloaded 2022-01-18, n = 70 575), as well as mzCloud and ChemSpider (EPA, DSSTox, EPA Toxcast, MassBank, MolBank, PubMed, Royal Society of Chemistry, Sigma Aldrich) was used as suspect lists. After peak-picking, filters (background subtraction, peak-shape rating ≥6) were applied. FISh scoring was performed for features with MS2 data with suggested matches (all suggestions for Paper I, suggestions with SFit or Match >50 for Papers II and III). FISh-scoring is a way of comparing the recorded MS2 data for a feature with the predicted MS2 data for a suggested structure. Full details on the Compound Discoverer method can be found in the supporting informations for Papers I-III.

A confidence level was assigned each feature based on an adaptation of the Schymanski[51] system. Level 5 (exact mass) was assigned if no chemical formula could be calculated. Level 4 (one formula) was assigned if no matches could be made, but one formula could be determined. Level 3.3 (no MS2) was assigned if matches were made, but no MS2 data had been recorded for the feature. Level 3.2 (tentative candidates) was assigned if FISh scoring was <50 (Paper I), <70 (Paper II) or <60 (Paper III). Level 3.1

(multiple viable candidates) was assigned if FISh scoring was >50, >70 or >60 (for the respective Papers I, II and III) for more than one structure suggestion. Level 2 (probable structure) was assigned if only one structure suggestion had FISh scoring >50, >70, >60. Level 1 was assigned if a feature matched (MS1, MS2 and RT) with a reference compound.

ER activity prediction

Compounds in Paper I and II that were identified at level 2 or 3.1 in ER-active fractions (±1 fraction) were subjected to ER activity prediction. In Paper I, binding was predicted (Yes/No, as well as relative to β-estradiol) using CompTox Chemicals Dashboard v.2.5.3 (https://comptox.epa.gov/dashboard/)[58]. The predictions were performed using 5 different models: consensus, hierarchical clustering, nearest neighbour, single model and group contribution. In Paper II, ER mediated effect was predicted using VEGA GUI 1.2.4, Estrogen receptor-mediated effect (IRFMN-CERAPP) 1.0.1.[59] The levels assigned were NON-active, Possible NON-active, Possible active, and Active. Not predicted was assigned if the compound was outside the applicability domain of the model.

Ecotoxicity prediction

Compounds that were identified at levels 2 or 3.1 in Paper I were submitted for prediction of LC50-values in fish, with 96-hrs exposure time, to broaden the potential to identify potentially toxic compounds. If several values were predicted, the lowest were chosen. Ecotoxicity predictions were performed using ECOSAR in EPI Suite 4.1.

Broad range toxicity predictions

In Paper III, a broader range of toxicity predictions were performed for compounds identified at level 2 and 3.1. In total 79 prediction models from the program VEGA GUI 1.2.4[59] were used, including endocrine disruption endpoints, mutagenicity/carcinogenicity, developmental/reproductive toxicity, skin and eye irritation, metabolic endpoints, and aquatic toxicity. All the tests are listed in the supporting information to Paper III (*Table S2*).

3.2 Paper IV

3.2.1 Samples and experimental design

Homogenised, freeze-dried whole-fish samples from bream (*Abramis brama*) were obtained from Lake Stechlin (longitude 13.0278 N, latitude 53.1514E) and Teltow Canal (longitude 13.1900 N, latitude 52.3983E) through provision from the Fraunhofer IME (Germany). Teltow Canal was selected as a contaminated site due to the discharge of multiple WWTPs into it, and Lake Stechlin was used as a reference site since it is relatively clean.

A portion of the whole-fish samples was used for preparation of reference extracts (extracts prepared through the reference sample preparation protocols) for LC-HRMS and GC-HRMS, respectively (labelled as "ref provided").

Some of the prepared reference extracts from Lake Stechlin were pre-spiked, with 32 compounds for LC-HRMS (0.5 mg/L in methanol) and 19 compounds for GC-HRMS analysis (1 mg/L in hexane). More details are found in the paper and supporting material to Paper IV. Only some of the spiked compounds were revealed to the participants prior to the investigation (known compounds, for target analysis), while the identity of most compounds remained undisclosed (unknown compounds, for suspect and NTS analysis).

Along with the reference extracts (spiked and non-spiked), the remaining whole-fish samples were shipped to the participants, and were then prepared by the participants for LC-HRMS and/or GC-HRMS analysis. Participants could decide to prepare extracts through the reference sample preparation protocols (ref pcp), or through their own in-house preparation protocols (in-house, varying between participants). The participants were also provided with solutions containing the spiking compounds for LC- and GC-HRMS analysis, to allow them to create their own spiked samples using the reference sample preparation protocols, and/or in-house sample protocols. The experimental design is summarised in *Figure 9*.

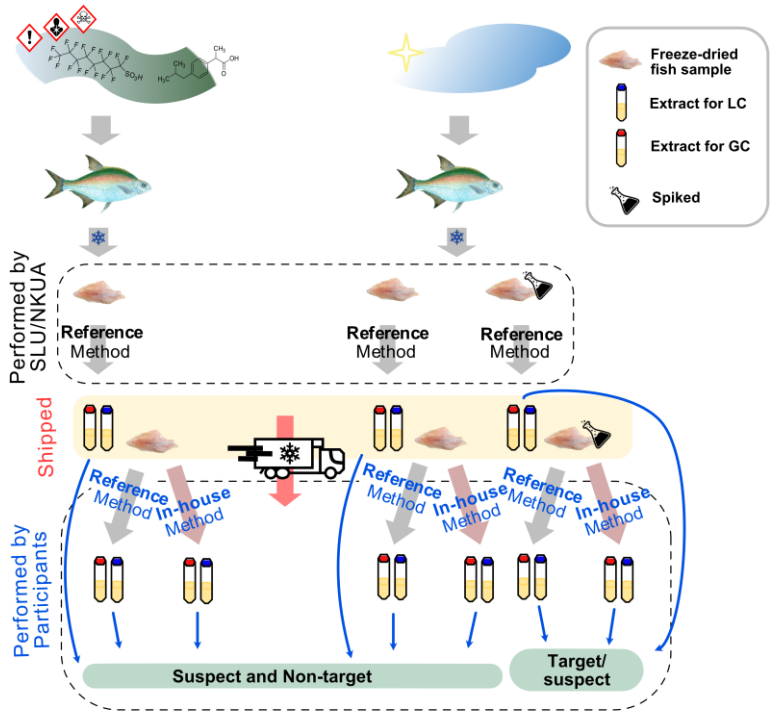

Figure 9. A schematic overview of the experimental design, adapted from Paper IV[60].

Spiked samples were analysed through target, suspect- and/or NTS screening to assess the number of correctly identified compounds, while non-spiked sample extracts were used for suspect and/or NTS analysis to assess the conformity of identified compounds, between participants and sample preparation methods.

3.2.2 Participants and method choices

Overall, there were 16 laboratories (labelled A-P) participating in the study, all from within the NORMAN network. Fourteen of them performed LC-HRMS analyses, whereof 6 prepared their own reference extracts and 10 prepared extracts following their own in-house sample preparation methods.

Five of the laboratories performed GC-HRMS analyses, whereof one prepared their own reference extract, and one prepared an extract following their own in-house sample preparation method.

3.2.3 Sample preparation

Reference methods

For analysis by LC-HRMS, the reference sample preparation followed an established protocol[61]. Freeze-dried fish homogenate (0.5 g) (spiked or non-spiked) was extracted using acetonitrile with 0.1 % formic acid (3 mL), and homogenized with a bead mixer (5000 rpm, 2 x 40 s). After centrifugation (3900 rpm, 15 min, 20 °C), the sample was filtered through a regenerated cellulose syringe filter (0.2-0.45 µm). The extract was then frozen (−20 °C, 16+ h), centrifuged (10 000 rpm, 3 min 20 °C), and 200 µL aliquots were taken for analysis.

For analysis by GC-HRMS, the reference sample preparation followed another established protocol[62]. Freeze-dried fish homogenate (3 g) was mixed with Na_2SO_4 (12 g). To create spiked samples a solution of spiking compounds was then added. Pressurized solvent extraction (100 °C, hexane:dicholormethane 2:1, x3) was performed. Isooctane (50 µL) was added, and the extract was concentrated to 10 mL using a rotary evaporator (30 °C). Clean-up was performed on a florisil column (170 µm, 80 Å) conditioned with 1) 10 % isopropyl alcohol in dichloromethane (20 mL), and 2) hexane (30 mL), eluting with 1) hexane:dichloromethane 1:1 (20 mL), and 2) hexane (20 mL). To the extract, isooctane (50 µL) was added, prior to concentration to 50 µL on a rotary evaporator. The sample was reconstituted in hexane (1 mL), filtered through a regenerated cellulose syringe filter (0.2 µm), after which 500 µL aliquots were taken for analysis.

Extracts created by the organisers and provided to the participants for analysis are labelled “Ref (provided)”, while extracts created by the participants through following the reference sample preparation protocols are labelled “Ref (pcp)”.

In-house methods

The in-house sample preparation methods used by the participants are described in detail in the supporting information to Paper IV. For LC-HRMS, 12 in-house sample preparation methods were applied (by 10 participants) to create extracts for analysis, while for GC-HRMS 1 in-house sample preparation method was applied. Extracts created by the participants through

following their own in-house sample preparation method are labelled "in-house".

3.2.4 Data curation and reporting

All the participants used their own protocols for data analysis, detailed in the supporting information to Paper IV. The data was reported using a data collection template (DCT) that had been used before in similar studies within the NORMAN network (see supporting information to Paper IV online). For NTS, there were three conditions made; the signal should be at least 10-fold higher in the sample from Teltow Canal than in that from Lake Stechlin, the compounds were to be anthropogenic, and only the 10 most intense signals needed to be investigated.

3.2.5 Data analysis

Comparability and overall performance was assessed through the spiked samples. The correctly identified known and unknown compounds (%) for each participant, using the three different options for sample preparation, was compared (paired Wilcoxon tests, confidence level 0.95). Medians, means, standard deviation and number of reported "extra" compounds were also assessed. This was performed in R[63] (version 4.1.2, external packages Tidyverse[64] and rcompanion[65]).

The range of physiochemical properties of the detected compounds was assessed through predicted log K_{OW} values (predicted from SMILES using EPI Suite 4.1, KOWWIN v.1.68.) and molecular masses. For spiked compounds, detected vs undetected compounds were compared, while for the non-spiked samples these properties were plotted against each other to detect potential trends.

Lastly, the suspect and NTS results from each participant was compared to determine how many compounds were reported from at least two different participants within each of the three sample preparation categories (to prevent comparing results from the same participant using multiple sample preparation methods).

4. Results and Discussion

4.1 Paper I

4.1.1 Method development and validation of EDA

Blanks and non-spiked samples

The blank samples made from MQ-water with the sample preparation method during method development showed no contamination with the reference compounds (E2, DHT, *t*BHQ, FICZ, EE2, BPA, androstenedione, resveratrol, epigallocatechin-3-gallate, omeprazole, and nimodipine), nor any activity upon analysis in the four bioassays (ER, AhR, Nrf2, and AR).

However, analysis of the non-spiked sample waters used for method development showed that the effluent water contained BPA (estimated 0.152 ppb), EE2 (estimated 0.010 ppb), and nimodipine (estimated 0.002 ppb), and that the influent water contained BPA (estimated 0.150 ppb), and androstenedione (estimated 0.090 ppb). The concentrations of these compounds, confirmed at level 1, were accounted for in the validation calculations described below.

Estrogenic reference compounds in spiked samples

The estrogenic reference compounds (E2, EE2, and BPA) performed consistently well, which can be expected from a structurally similar compound group[18]. All compounds had IDLs between 3.0-6.0 ppb, suggesting good ionisation efficiency and MS detection. Likewise, the relative recovery for all three compounds was >50 % (in all matrices used for validation), suggesting minimal matrix interference and effective sample preparation. Resulting MDLs ranged from 3.8-8.6 ppb in the effluent matrix.

AhR reference compounds in spiked samples

The aryl hydrocarbon receptor agonists group (FICZ, omeprazole, and nimodipine) gave a mixed performance, with larger variation. The compound IDLs were determined to be between 0.8-13 ppb. For relative recovery, omeprazole performed well (>50 %) in all matrices, nimodipine displayed lower recoveries in MQ (34 %) and influent (43 %), while FICZ had poor performance in all matrices (2.0 % in MQ, 4.1 % in river water, and <3.0 % in effluent and influent). For nimodipine, matrix effects were large in the

influent and mainly responsible for the poor relative recovery (57 %). For FICZ, both poor sample preparation recovery (<3.0 in influent and effluent, and 4.1% in river water) and matrix effects in influent (80 %) contributed to the low performance. Due to this, MDLs were determined as 1.2 ppb for nimodipine, 22 ppb for omeprazole, and >200 ppb for FICZ in effluent matrix.

Nrf2 reference compounds in spiked samples

The Nrf2 agonist group (*t*BHQ, resveratrol, and epigallocatechin-3-gallate) performed relatively poorly. The compound IDL for resveratrol was acceptable (6.0 ppb), but for *t*BHQ it was relatively high (25 ppb). Epigallocatechin-3-gallate could not be detected. The relative recovery for resveratrol was overall poor (20 % in MQ, <3.0 % influent, effluent and river water), as it was for *t*BHQ (<13 % for all). In river water, the low recovery of resveratrol appeared to be mainly due to sample preparation (matrix effects were only 31 %), but for all other matrices for both compounds, the matrix effects suppressed compound detection.

Androgenic reference compounds in spiked samples

The androgenic reference compounds (DHT and androstenedione) performed generally well in the method. The IDLs were 0.04 ppb (androstenedione) and 0.8 ppb (DHT). Relative recoveries for all matrices except influent (<0.4 % for DHT and <22 % for androstenedione) were >50 %. Matrix effects (71 %) were determined as the main reason for the poor performance of androstenedione in influent. For DHT, matrix effects (>99.96 %) were at least a contributing factor. MDLs were 0.7 ppb (androstenedione) and 1.0 ppb (DHT) in effluent matrix.

General comment

The reference compounds had been chosen due to their toxicity, and it was at the time of selection unknown how they would perform using LC-HRMS. AhR and Nrf2 active compounds are generally recognised as structurally heterogeneous groups[18], in contrast to ER and AR agonists, and it was therefore anticipated that not all compounds would necessarily perform equally well. The priority was to establish a robust method for ER and AR agonists, and for at least partial applicability to AhR and Nrf2 compounds. For this purpose, the method establishment was successful.

Comparison of sensitivity of HRMS and bioassays

The reference compounds for bioassay activity was E2, DHT, *t*BHQ and TCDD. Comparing the IDLs from the HRMS method with the bioassays cut-off concentration-equivalents show that the bioassays were more sensitive than the HRMS method for E2 (>$1.9x10^{-4}$ ppb vs >$6x10^{-3}$ ppb) and DHT (>$4.7x10^{-6}$ ppb vs $8x10^{-4}$ ppb)), while the HRMS method was more sensitive for *t*BHQ (>50.5 ppb vs >0.25 ppb).

Retention time (RT) and fraction matching of spiked compounds

RT predictions and actual detection fractions were generally in good agreement. Most compounds were detected in the expected fractions, ±1 fraction. E2, EE2 and androstenedione were found where predicted. BPA, DHT and omeprazole were found where predicted and in one later fraction, and nimodipine was found where predicted and in two later fractions. Resveratrol was only found in the last of the three predicted fractions. More details can be found in Paper I and the supporting information to Paper I.

4.1.2 Analysis of real WWTP-related water samples

Activity detection

Activity was detected in the whole-effluent extract (unfractionated) in the ER, AhR, and Nrf2 bioassays. For AR, no activity was detected. Upon fractionation, the activity was no longer detectable in the AhR and Nrf2 bioassays, but ER activity was successfully detected in some of the fractions (negative ionisation mode fractions 24-26, RT 9.05-10.10; positive ionisation mode fractions 28-29, RT 10.45-11.15). No problem with cytotoxicity was indicated.

Feature detection

After background subtraction, analysis led to the detection of 5 663 features in negative ionisation mode and 9 089 features in positive ionisation mode. Applying the peak rating filter (≥6 in all replicates) led to 1 220 (negative) and 2 208 (positive) features. Confidence level assignment were as follows: 14 (negative) vs 24 (positive) features at level 2, 65 vs 219 at level 3.1, 195 vs 143 at level 3.2, 302 vs 863 at level 3.3, 106 vs 225 at level 4, and 536 vs 734 at level 5.

Linking activity and identity

The number of features detected within the RT of ER active fractions (±1 fraction) was 178 for the data obtained in negative ionisation mode (RT 8.70-10.45), and 206 for positive ionisation mode (RT 10.10-11.50). None of the best matches for features identified on level 2 or 3.1 were having structures strongly indicative of estrogenic activity (phenol moieties, relatively hydrophobic nature, and planar structures)[66]. However, one compound at confidence level 2 (*N*,*N*'-[1,2-di(4-pyridinyl)-1,2-ethanediyl]bis(4-methoxybenzamide), and 2 compounds at confidence level 3.1 (3-benzyl-7-methyloctanoic acid and ascr#3) were predicted to be ER binders in the CompTox prediction. To aid comparison, the respective predicted values in the consensus, hierarchical clustering, nearest neighbour, single model and group contribution method for EE2 (strong binder) were 4.995, 8.638, 1.876, 7.689 and "no value", while for BPA (weak binder) the values were 0.012, 0.010, 0.015, 0.019 and 0.007, respectively. The predicted affinity relative to *β*-estradiol for the level 3.1 compounds were therefore generally low (0.006-0.014 for consensus, nearest neighbour and hierarchical clustering vs 0.006 for hierarchical clustering). For the compound on confidence level 2, the value for predicted relative affinity was rather strong (1.995), but only in the nearest neighbour method. Since the structure of this compound contained two benzene rings with methoxy substituents, it is likely that at least some of the nearest neighbour's structures used for prediction contained phenol rings. Whether the compound itself would have estrogenic binding potential with methoxy rather than hydroxy groups remains uncertain. However, some of the alternative candidates for level 3.1 detected in positive ionisation mode may have estrogenic potential. These included e.g. chalcones[67, 68] and flavonoids[69], both known to be phytoestrogens, as well as specific compounds with known estrogenicity such as agrimonolide[70], zearalenone[71], and erianin[72]. More details are available in Paper I.

During method development, the reference compounds E2 and EE2 were recovered in fractions 24-26 in the side-spike sample analysed in negative ionisation mode, the same fractions in which activity was detected in the effluent sample. These compounds were not detected during the suspect screening, nor when searched for directly using the program FreeStyle. It is possible that either of these compounds, or a structurally very similar compound, was present, simply in a concentration that was between the

method detection limit for the HRMS (0.0071 ppb E2-equivalents) and the cut-off concentration for detection in the bioassay (0.00038 ppb E2-equivalents).

Compounds on confidence level 2

Considering the full RT range (0-20 min), in total 38 compounds were assigned level 2. Four of these were pharmaceuticals: benzonanate[73] (used for cough medicine), promethazine *N*-oxide[74] (metabolite of an antidopaminergic, antihistaminergic and anticholinergic drug), solpecainol[75] (antiarrythmic drug) and hoquizil[76] (bronchodilator for treatment of e.g. asthma). Others were surfactants used in personal care products (e.g. polyglyceryl-4-oleate[77] and myreth sulfate[78]), sugar derivatives and peptides.

Ecotoxicity prediction

All the compounds identified at level 2 or 3.1 were subjected to LC50 prediction (96-hr exposure, in fish) using EPI Suite 4.1 to estimate their ecotoxicity, and broaden the detection potential of problematic compounds. Five of the compounds at level 2, and 70 at level 3.1, were predicted to have values <10 ppm. The eight most ecotoxic compounds with values <0.1 ppm were: stearic acid (0.011 ppm), oleic acid (0.017 ppm), [(2-phenyl-1-cyclohexen-1-yl)sulfanyl]benzene (0.018 ppm), linoleic acid (0.026 ppm), {[(5*E*)-6-phenyl-1,5-hexadien-3-yl]sulfanyl}benzene (0.028 ppm), 2-octyl-1,4-benzenediol (0.051 ppm), 3-hydroxy-2-methyl-4-(3-methylbutyl)-5-(2-methyl-2-propanyl)phenyl hydrogen carbonate (0.076 ppm), and *o*-(1-methylheptyl)phenol (0.089 ppm). More details are found in Paper I and in the supporting information to Paper I.

Estimated treatment efficiency and impact on river water

To perform a rough evaluation of the treatment efficiency of the WWTP, as well as an estimation of the impact of the effluent water discharge on the river water, a subsample of detected features was investigated. All effluent features detected at level 2 (n = 38) were included, as well as those detected through analysis with negative ionisation mode at level 3.1 (n = 65). For the features at level 3.1 detected through positive ionisation mode (n = 219), a selection of compounds was made, so only compounds within the RT of the ER active fraction interval (RT 10.10-11.50) were included (n = 18), resulting in a total of 121 features in the effluent samples.

Of these, 82 % were also detected in the influent. The signal area was lower in the effluent than in the influent for 55 % of the detected compounds, indicating some degree of removal. For the rest of the compounds (45 %), that was found to have a greater signal in the effluent than in the influent, this could be due to transformation of compounds, signal suppression in the influent matrix[79], or time lags in WWTP processing.

Upstream the effluent discharge, the river water was only found to contain 1 of the compounds from the sample list, corresponding to <1 %. Downstream of the effluent discharge, however, 60 % of the compounds were found. All the signals had lower area intensities in the downstream river water compared to the effluent (1-55 % of effluent levels), as expected due to dilution effects[80], although for some compounds the dilution was less than expected. This could potentially be due to signal suppression in the effluent matrix[81]. The fact that so many compounds were detectable after, but not before, the effluent release indicates that the WWTP contributes considerably to the micropollutant load of the river.

4.2 Paper II

4.2.1 Toxic drivers in snow samples

Activity

A summary of the detected activities is depicted in *Figure 10*. ER-activity was detected in snow sample A, which consisted of rubber-granulate infused snow collected from the artificial football field. Compounds with ER activity leaching from the plastic and/or rubber particles to the snowmelt is possible[82-84], but has, to the best of my knowledge, not yet been conclusively demonstrated. Upon fractionation, ER activity was still detected (fractions 23-24 in negative mode conditions, 27-29 in positive mode conditions).

AhR-activity was detected in snow samples A, B and to a lesser extent in sample C (24 % ±1.2). Fractionation of sample A and B caused the activity to disappear, which is common for category 2 bioassays[18]. Recombination experiments[35], which could have confirmed whether the loss of activity was due to dilution across fractions rather than compound loss, were not performed due to practical challenges associated with high-throughput

fractionation where small volume fractions are significantly diluted (EF 1 000 → EF 95 during fractionation).

Nrf2 activity was just about detected for sample A (1.7 ±0.1 fold-change), but was deemed too low to justify further fractionation-based activity determination. No AR activity was detected for either sample. All samples performed well (>80 % cell viability) in the cytotoxicity tests, so unspecific toxicity was not of concern.

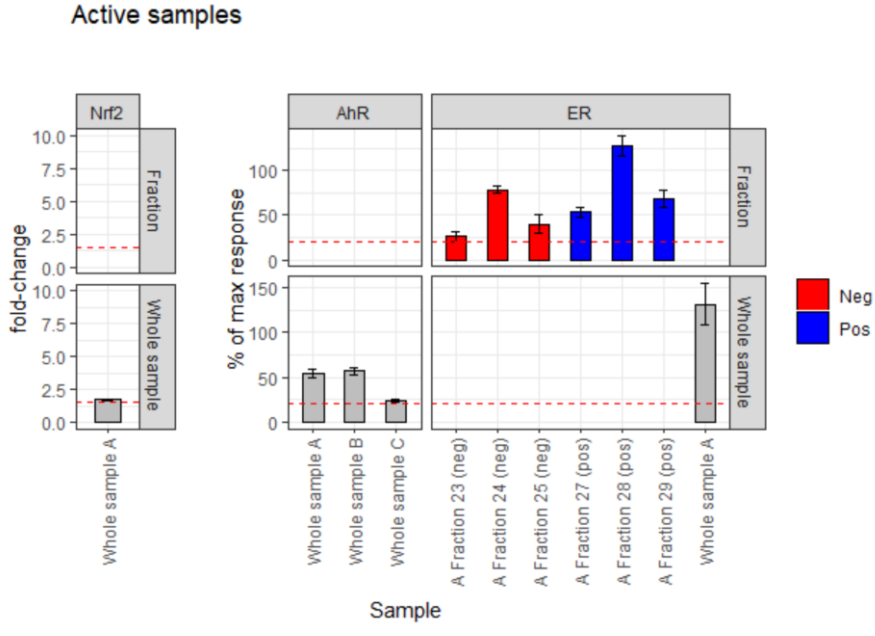

Figure 10. Summary of the detected activity in the snow whole samples and fractions using Nrf2, AhR and ER in positive and negative ionization mode.

Feature identification

The HRMS analysis of the snow samples led to initial detection of 115 131 features in negative mode and 66 771 in positive mode. Application of feature filters requiring all sample replicates of at least one sample type having peak ratings ≥6, as well as the group area of at least one sample type being at least 5 times that in the MQ-bucket blank, the number of features were reduced to 3 199 (pos) and 1 650 (neg).

Selecting only features that were present in the RT span of ER active fractions ±1 fraction (9.75-11.50 for positive and 8.35-10.10 for negative mode) further reduced the number of features to 364 (pos) and 277 (neg) (*Figure 11*). This was necessary due to the estimated time for analysis – had the full RT range been investigated – being several months longer than that of Paper I, which had still taken a few months to perform in Compound Discoverer. Confidence level assessment of the selected features resulted in a list of 37 compounds identified at level 2 (n = 4) and 3.1 (n = 33) with both ionisation modes combined. Most of these 37 compounds were detected in snow sample A (97 %), and slightly more than half of them in snow samples B (65 %) and C (54 %). The four compounds identified at level 2 were: 8-(1H-indol-1-yl)-2,5-dimethyl-6-octen-2-ol, (2*S*,4*E*)-2-(methoxymethoxy)-7-methyl-1-phenyl-4-octen-3-one, *N*-(diphenylacetyl)-*L*-leucine, and *N*-phenyl-1-piperidineacetamide. Further, 122 features were assigned level 5, 52 level 4, 225 level 3.3, and 205 level 3.2.

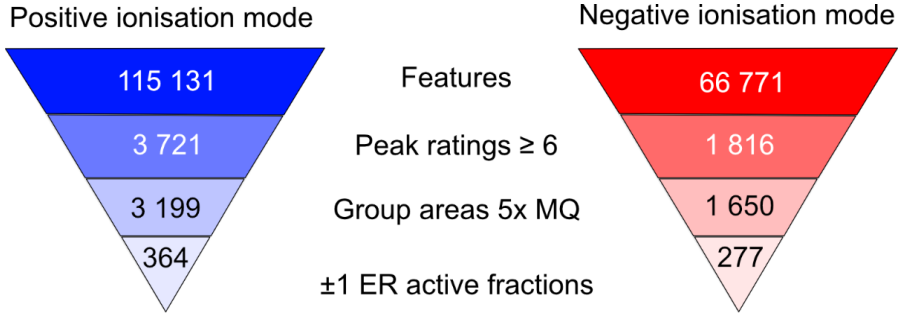

Figure 11. The identified HRMS features in positive and negative ionisation mode at the different stages of filtration: all features, peak ratings of all replicates of a sample ≥6, group area ≥5x that of the MQ-bucket reference in at least one group, and within the ER active fractions ±1 fraction.

ER activity predictions (VEGA GUI 1.2.4) were performed for the 37 compounds, yielding 1 compound predicted to be “Possible active”. Four of the 37 features (including the one with the main structure predicted “Possible active”) had viable structure alternatives that contained a phenol moiety, and those were also subjected to the ER activity prediction. Of these four features, one of them was excluded from further consideration based on the signal intensity being roughly similar in all samples A, B, and C, while activity only was detected for sample A. More precise RT matching further

excluded two other features. The remaining feature had two structure alternatives that were predicted “Possible active”, and 7 for which the outcome had been “Not predicted”. This leaves ambiguity to the identity of the feature, and since the ER prediction was not strongly suggesting activity for either of the suggestions, it is likely that the activity detected was due to a compound present in such low concentration that it was not detected by HRMS.

4.2.2 Method confirmation

The method confirmation resulted in relative recoveries >50 % for E2, DHT, EE2, BPA, androstenedione, and omeprazole, but <50 % for *t*BHQ (4.77 %), FICZ (ND), resveratrol (14 %), and nimodipine (40 %). This was deemed acceptable due to the methods aim to detect a broad range of different types of chemicals. Precision, measured by CV (%), ranged between 0.9-19 %, which is similar to Paper I. This variation suggests that concentration determination based on the HRMS method is approximate, and indicate that BEQ evaluations based on this would be somewhat unreliable. In combination with the ambiguous result, BEQ calculations were therefore not performed.

The reference compound *t*BHQ was detected in the snow sample B from the snow dump during the method validation. The concentration was estimated to be 5.4 ppb in the snowmelt, calculated using a relative recovery of 4.8 %. This falls above the IDL of the HRMS method (0.025 ppb), and below the limit of detection (LOD) for the Nrf2 bioassay (15 ppb), which explains why no activity was detected for the sample. The compound *t*BHQ is an antioxidant, often added to oils and have found usage in products such as biodiesel[85]. Exhaust from vehicles are therefore a potential source of this contamination.

4.3 Paper III

4.3.1 Bioanalytical analysis

The bioanalytical analyses of bottled and tap water led to no activity being detected in the AR, AhR or Nrf2 bioassays. Although activity was detected for the ER bioassay, it was still below the detectable activity in the MQ blank

sample. Unless originating from accidental contamination, this suggests that the sample preparation method adds ER activity that is detectable when concentrating the original water to an EF of 5 000 instead of 1 000. Cytotoxicity experiments for the four cell lines did not indicate any unspecific toxicity, which suggests that a greater EF could potentially be used to enhance sensitivity of the method for AR, AhR and Nrf2. For ER this would not be suitable unless the problem with potential addition of ER activity from the sample preparation method is solved.

4.3.2 Suspect screening

Despite the absence of bioassay activities, suspect screening based on HRMS data was performed. This was to ensure that no toxic compounds detectable by HRMS was overlooked, and to assess low contamination levels in bottled and tap water using HRMS.

After background subtraction, 41 997 features were detected. Upon addition of peak rating requirements (≥6 in all bottled water sample replicates), 121 of them remained. Categorisation into confidence levels yielded 67 at level 5, 2 at level 4, 24 at level 3.3, 24 at level 3.2, 3 at level 3.1, and 1 at level 2. The compounds identified at level 3.3 (no MS2 data recorded) and 3.2 (no viable match, FISh-score <60 for all candidates) is an indication of how well the suspect screening succeeded in obtaining HRMS data of high enough quality and provide sufficient suspects for matching. Excluding levels 5 and 4 (potential false positives), 48 out of 52 compounds remained at level 3.3 or 3.2. Many compounds have therefore not been sufficiently identified, suggesting a need for higher sensitivity and quality data of the HRMS method or a broader suspect list, however the current list already covers 70 575+ suspects which is already in the upper limit of what can be processed using Compound Discoverer. To improve the data quality over a broad range of chemical space is challenging[86]. A wiser approach for future EDA studies on low contamination-level samples could therefore be to use several optimised HRMS methods that cover complementary, but each a narrower range of, chemical spaces[87]. For instance, a method that target specifically typically estrogenic compounds[88] (since they are often structurally similar), combined with separate analyses using methods optimized for i.e. typical androgenic compounds. Or perhaps combining

different methods for more or less polar substances, or larger vs. smaller compounds based on molecular weight.

The compound confirmed at level 2 was mono-carboxy-isooctyl phthalate (MCiOP), which is a metabolite of the plasticiser diisononyl phthalate (DiNP)[89]. DiNP is used, for example, in polyvinyl chloride (PVC) products[90]. Confirmed at level 3.1 was octanoic anhydride (with 92 viable structure alternatives, hence not a reliable assignation), cumenesulfonic acid (with only two structural isomers of cumenesulfonic acid having FISh-score >60, hence a more confident identification), and 4-[(17-carboxyheptadecyl)oxy]benzoic acid (with two similar compounds as structure alternatives with FISh-score >60; 4-[(18,18-dihydroxy-17-octadecen-1-yl)oxy]benzoic acid and 6-(3-formylphenoxy)-2-hydroxyhexyl laurate).

4.3.3 Broad range toxicity predictions

The four compounds identified at level 2 and 3.1 were subjected to the broad range toxicity prediction using 79 prediction models in VEGA GUI 1.2.4[59]. Summarised, for MCiOP there appeared to be concerns due to activity in models for steroidogenesis, pregnane X receptor (PXR) activation, peroxisome proliferator-activated receptor gamma (PPARγ) activation, aquatic toxicity, and bee toxicity. For octanoic anhydride it was skin sensitisation, aquatic toxicity, and bee toxicity that was found as potentially of concern. Cumenesulfonic acid was predicted possibly active in models for skin and eye irritation, PXR activation, aquatic toxicity, and bee toxicity. On top of that it was predicted possibly not readily biodegradable. The last compound, 4-[(17-carboxyheptadecyl)oxy]benzoic acid, was found to potentially display aquatic toxicity and bee toxicity, as well as to not be readily biodegradable. Not being readily biodegradable makes the toxicity concerns for the compounds more severe. The full result from the toxicity prediction models can be found in the supporting information to Paper III (*Table S2*).

4.3.4 Method confirmation

Due to only using negative ionisation mode for the HRMS analysis in Paper III, the reference compounds were reduced to 7 (E2, *t*BHQ, FICZ, EE2, BPA, omeprazole, and nimodipine), none of which were detected in the non-spiked bottled water used for the study. For the spiked QC-samples, CV

(%) was between 0.2-20 %, consistent with Papers I and II. Relative recoveries were >50 % for E2, EE2, BPA, omeprazole and nimodipine. FICZ had a relative recovery of 3.3 %, while *t*BHQ was not detected after sample preparation. This is also in accordance with what was previously found in Papers I and II, and was accepted due to the broad chemical range that is attempted to be covered by the method.

4.4 Paper IV

4.4.1 Spiked compounds

During LC-HRMS detection, 9-69 % (average = 41 %, median = 44 %) of all the spiked compounds were correctly identified, while for GC-HRMS 20-60 % were (average = 37 %, median = 35 %) (*Figure 11*). This indicates similar results, on average, between the two detection methods, but a large interlaboratory variation. Within the sample preparation group Ref (provided), 4-56 % of the unknown compounds, and 0-100 % of the known were identified using LC-HRMS, further highlighting the large interlaboratory variation (*Figure 12*).

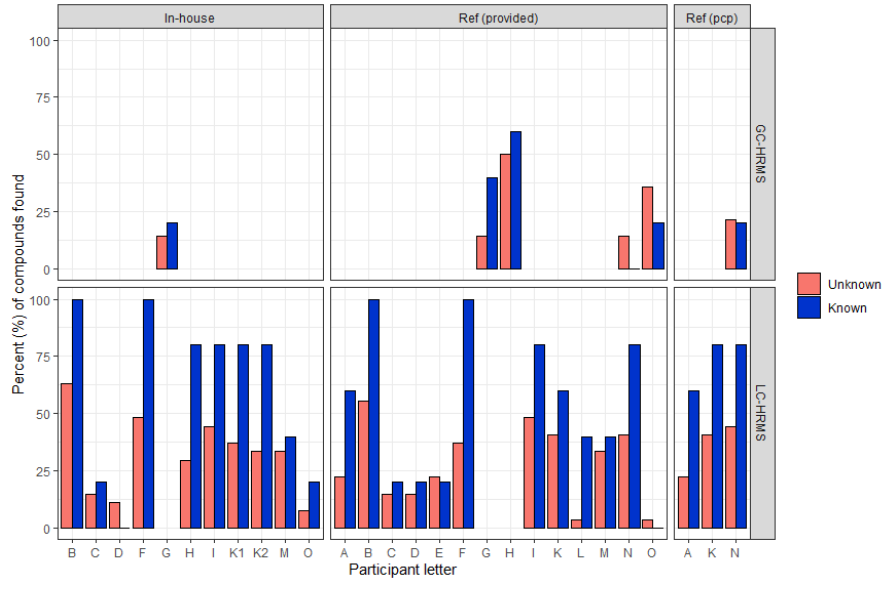

Figure 12. The percentage of successfully identified spiked compounds, divided on those whose identity was known to the participants (blue) vs. unknown to the participants (red) prior to analysis. Figure reprinted from Paper IV[60].

The results between different sample preparation methods did not seem to vary very much. For LC-HRMS analysis, the in-house group identified 32 ± 17 % correctly, the ref (provided) group identified 28 ± 17 % correctly, and the ref (pcp) group identified 36 ± 12 % correctly. This was further supported by statistical comparison (paired Wilcoxon tests) of results from participants performing analyses on both ref (provided) and in-house extracts, as well as both ref (provided) and ref (pcp) extracts. No test showed statistically significant differences ($p > 0.05$) between the sample preparation methods. Similar comparison for GC-HRMS analysis was not possible due to the low number of participants ($n = 1$) performing analyses of in-house and ref (pcp) produced extracts.

The lack of significant differences between the ref (provided) and ref (pcp) extracts supports interlaboratory reproducibility of the reference sample preparation method. However, the fact that neither the in-house methods and ref (provided) extracts performed statistically different indicates that the sample preparation method is not a main contributor to the difference in performance of participants. Probable causes include differences in instrumental settings, data analysis workflow, software, suspect library, and acceptance/rejection criteria. Upon inspection, the LC-HRMS methods that were used by different participants appear rather similar, leaving data processing or instrument specific factors (such as peak intensity, mass error, resolution etc.) to be the most likely cause of interlaboratory variation. This is in agreement with a previous study, indicating different data protocols could make the result of the same data set vary greatly, with as little as 10 % overlap of detected compounds[91].

The in-house protocols of the most well performing LC-HRMS participants (B = 22, F = 18, I = 16 correct compounds) were rather similar, which is potentially a sign of some level of harmonisation of sample preparation methods of fish tissue.

The number of reported non-spiked compounds ranged from 0-185 (LC-HRMS) and 2-34 (GC-HRMS). If the reported extra compounds had been contaminants present in the fish tissue from the start, it would have been assumed that the same compounds would have been identified by multiple laboratories. However, the reported extra compounds were not replicated

between laboratories. This suggests that they are likely false positives, a known problem within suspect screening and NTS[92, 93].

Six reference compounds (natamycin, dichlofluanid, dazomet, clopyralid, amidotrizoic acid and 1,3-dichloro-5,5-dimethylhydantoin) were not detected in the LC-HRMS analysis by any participant. The log K_{OW} values of these compounds were relatively low (−2.5-−2.7). Most participants used reverse phase LC columns, for which such polar substances are less suited. This could be an explanation to why these compounds specifically were not detected. Five reference compounds (*n*-butylbenzenesulfonamide, musk tibeten (known), diphenyl phthalate, decabromobiphenyl and 1-chlorononane) were not detected by GC-HRMS by any participant. For these compounds, the range of log K_{OW} values was wider (2.3-12), and all of them have earlier been detected in similar samples[94]. Taken together, this shows that false negatives are a risk both for LC- and GC-HRMS analyses. The trend seemed to be that compounds with K_{OW} values that were either high or low (>6 or <0), or with molecular masses that were high (>550) were not as readily detected. However, this cannot be proven statistically since the number of spiked compounds that had such properties were low.

4.4.2 Suspect-screening

During LC-HRMS analysis, suspect screening yielded a median of 21 unique features per participant and sample preparation method (on average 145 due to a few participants reporting a high number of features; the highest reported number was 886 features). Overall, approximately 1 000 unique features (removing those with only chemical formulas and/or *m/z*) were reported. Of these, approximately 420 were reported on a rather high confidence level (1 or 2), however not all laboratories reported confidence levels for their features.

The LC-HRMS analysis led to only 16 features being detected by at least two participants (within the same group of sample preparation method to not bias the result). Most of these were identified by at least one participant with a rather high confidence level (1 or 2). A list of them can be found in the supporting information to Paper IV.

For GC-HRMS analysis, the median was 10 unique features per participant and sample preparation method (on average 16, highest reported

number 33 features), but the overall number of unique features (removing those with only chemical formulas and/or *m/z*) was much lower (47) due to the low number of participants performing the analysis (n = 4, with in total 5 analyses performed). No identified features were replicated between participants.

Overall, suspect screening led to detection of features with a wide range of log K_{OW} values (−9.9-16), as well as *m/z* values (68-761). It was not expected that compounds with such high polarities (log $K_{OW} < 0$) would be detected since they usually do not bioaccumulate. This could either be due to false positives, or is indicative of very high concentrations in the water of Teltow Canal.

4.4.3 Non-target screening

Although participants were told to only identify the 10 compounds of highest intensity, many reported more (or fewer). All of the reported compounds have therefore been included in the result.

By LC-HRMS, a median of 9 unique features were reported per participant and sample preparation method (42 on average, highest reported number 178 features). By GC-HRMS, a median of 22 unique features were reported per participant and sample preparation method (34 on average, highest reported number 60 features).

The total number of unique features (removing those with only chemical formulas and/or *m/z*) was 37 for LC-HRMS and 79 for GC-HRMS.

For the LC-HRMS analysis, no reported features were replicated between participants, however for GC-HRMS one compound (*pp'*-DDMU) was reported by two out of three participants. *pp'*-DDMU is a metabolite of *pp'*-DDT, an organochloride pesticide.

Overall, NTS also led, unsuspectingly, to detection of compounds with a wide range of log K_{OW} (−7.5-14), and *m/z* values (68-714). A list of all the reported features with confidence levels ≥3 (i.e. features that were not just assigned a chemical formula and/or *m/z*), detected both through suspect screening and NTS, is found in the supporting information to Paper IV online.

5. General Conclusions and Outlook

In Papers I-III, a high-throughput EDA method was developed and applied to WWTP effluent, urban snow samples, commercial bottled water and tap water. In both the WWTP effluent and one of the urban snow samples (collected from the artificial football field), ER activity was detected and potential contenders for driving the toxicity were identified, although with limited confidence. The fact that the bioassay had higher sensitivity towards detection of the reference compound E2 than the HRMS method suggests that the main cause of the detected ER activity likely was compound(s) that were unsuccessfully detected by the HRMS analysis.

For Paper I, the potential activity contributors included *N*,*N*′-[1,2-di(4-pyridinyl)-1,2-ethanediyl]bis(4-methoxybenzamide (confidence level 2), 3-benzyl-7-methyloctanoic acid, and ascr#3 (confidence levels 3.1). Neither of them had a strong prediction of ER activity, and the latter two having several viable structural candidates further weakens their likelihood as true contributors due to uncertain identifications. For Paper II, the potential activity contributor was narrowed down to one possible feature. However, it was a compound at level 3.1, and had two viable structure alternatives that were predicted to be "Possible active" and 7 that were "Not predicted". Again, the prediction of ER activity was not strong, and the identity uncertain.

Whole extract activity was also found for the WWTP effluent sample (Paper I) in the AhR and Nrf2 bioassays, and for the urban snow samples A and B (Paper II) in the AhR bioassays. However, the activity disappeared upon fractionation, which is common for category 2 bioassays since they are often activated by a wide range of chemicals that tend to spread out to undetectable levels over several fractions[18].

In Papers I and III, suspect screening was extended beyond bioassay activity guided RT, to ensure that potentially toxic compounds were not overlooked: In Paper I since the EDA method was being developed, and in Paper III since no activity was detected in the bioassays. Suspect screening was therefore performed over the entire RT range (20 min). For Paper II, this was not possible within the time frame due to the large amount of features

detected overall in the three different snow samples, using two different ionisation modes for detection. Thus, the suspect screening was limited to the active fractions RT range (±1 fraction). This shows how quickly suspect screening becomes data-intensive and time-consuming, since scaling up from one (Paper I) to three samples (Paper II) made the estimated data processing time several months longer, highlighting the necessity of streamlining strategies such as EDA.

In Paper I, three of the reference compounds (~0.152 ppb BPA, ~0.010 ppb EE2 and ~0.002 ppb nimodipine) were found in the effluent water during method development, and two (~0.150 ppb BPA and ~0.090 ppb androstenedione) in the influent water during method development, using targeted analyses strategies. In the real sample analysis of effluent water from another WWTP, 38 compounds were identified at confidence level 2, and 284 at level 3.1. In total, 1 503 features were detected at levels 3.2 and 3.3, for which no viable matches could be achieved, or no MS2 data had been recorded. Of the compounds identified at level 2, four were identified as pharmaceuticals; benzonanate, promethazine *N*-oxide, solpecainol and hoquizil.

In Paper I, a subset of 121 compounds identified at levels 2 and 3.1 in the WWTP effluent sample (compounds at level 3.1 originating from HRMS analysis in positive ionisation mode had to be limited due to the great amount of compounds) were further investigated in the WWTP influent, the river water upstream, and the river water downstream of the WWTP effluent discharge. Of these, 82 % of the compounds were detected in the influent, of which only 55 % had a lower signal intensity in the effluent sample, suggesting a treatment efficiency over 0 %. This rather low number could, however, be due to signal suppression in the influent matrix. Downstream the effluent discharge into the river, 60 % of the compounds were detected, whereas only 1 compound was detected in the upstream sample. This indicates that the WWTP contributes significantly to the micropollutant load of the river.

In Paper II, the reference compound *t*BHQ (~5.4 ppb) was detected in the snow sample B (from the snow dump) during method confirmation. The

suspect screening, narrowed to the ER active RT, yielded 37 compounds on levels 2 and 3.1, and 430 on levels 3.2 and 3.3.

In Paper III, one compound on level 2 (MCiOP), and three on level 3.1 were identified. MCiOP is a metabolite of DiNP, a common plasticiser used for example in PVC products. In total, 48 compounds were identified at levels 3.2 and 3.3. The low amount of features detected through the HRMS analysis, in combination with no detected activity, suggests that commercial bottled water and drinking water is relatively clean. The low concentrations of potential pollutants are a challenge for the current EDA analysis, and could to some extent be attempted to be overcome by increasing the concentration of the original sample extract even further (EF >5 000).

Due to the high number of features at levels 3.2 and 3.3 (no viable match or no MS2 data) in Papers I-III, as well as the fact that the bioassays in general were more sensitive to the reference compounds than the HRMS method, improving HRMS sensitivity is a key priority for future EDA studies. Enhanced sensitivity would give better data quality to perform more secure compound matching, as well as record MS2 data for more features. If the sensitivity of the HRMS matched that of the bioassays, it would also be less ambiguous whether the features causing detected activity could be visible in the MS spectra. One potential strategy is to move away from a single, broad spectrum HRMS method in favour of multiple, specialized methods towards specific ranges of the chemical space. Especially for cleaner samples, that are predicted to have very low concentrations of potential pollutants, it seems to be a necessary focus of future efforts. This further highlights the importance of performing EDA analyses on water with drinking water relevance such as raw water, or water sources with potential of polluting the raw water, where the pollutants are more likely to be identified, to enable searching for them in the cleaner drinking water perhaps through targeted methods.

In Paper IV, investigating the current level of harmonisation of suspect screening and NTS of biota, a large interlaboratory variation was found. The sample preparation method seemed to contribute less to the variation of results than differences in analytical methods between participating laboratories. On average, 41 % (LC-HRMS) and 37 % (GC-HRMS) of the spiked compounds added to the fish tissue was correctly identified, and many

false positive hits were reported. During suspect screening and NTS of non-spiked fish tissue, only some of the total amount of features were reported by at least two different participants. For suspect screening, overall approximately 1 000 (LC-HRMS) and 47 (GC-HRMS) unique features were reported, of which only 16 (LC-HRMS) and 0 (GC-HRMS) were detected by at least two participants. For NTS, overall 37 (LC-HRMS) and 79 (GC-HRMS) unique features were reported, of which 0 (LC-HRMS) and 1 (GC-HRMS) were reported by at least two participants. The result indicates that different studies on biota are, as of the time of the study, not comparable. Further harmonisation efforts are necessary, focusing on establishing more standardised quality control procedures to reduce the risk of false positives and negatives while maintaining varying methods to detect complementary ranges of the chemical space.

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Popular science summary

Most people these days intuitively understand that our environment contains pollutants, originating from industries and traffic, but also from inside our own homes through pharmaceuticals, use of personal care products or cleaning products. The list goes on. However, fewer people are aware of the true extent of this pollution and probably do not realize that a single environmental water sample contains as much as tens of thousands of compounds. Do we know what all of them are? No. Could we find out? Probably not. The amount of compounds present in such samples are too many to be able to identify them all. Does this mean that there is nothing we can do? Fortunately, also no. There are ways that we can narrow down the amount of compounds that we need to identify, and one of them is based on the realisation that the compounds that are most pressing to identify are those that are potentially toxic. Known as effect-directed analysis (EDA), this method combines both bioanalytical analyses – to detect potential toxicity in a sample – and chemical analysis – to be able to identify the substances responsible for the toxicity. Knowing what is toxic in water samples is necessary to be able to protect both human health and the environment against it. This knowledge can inform regulators to perhaps prevent further release of such harmful compounds and guide treatment process to remove what has already been released.

In this work, a method for EDA was developed and used to find potential toxic compounds in wastewater treatment plant effluent, urban snow samples and bottled and tap water. Wastewater effluent release from wastewater treatment plants are one of the major contributors of pollutants to the aquatic environment. Pollutants from human waste such as pharmaceuticals are especially dangerous since they are designed to be able to enter into our bodies and exert effects at very low concentrations. Snow can act as a medium that carries pollutants from both air and surfaces in which it comes in contact. When the snow melts, these pollutants may be carried into surface water systems, and sometimes even groundwater. Surface water, as well as groundwater, can potentially be used as raw water in drinking water production. Their cleanliness directly affects drinking water quality, and pollutants in the raw water are a potential risk. Bottled water then faces the additional risks of having pollutants being introduced during the bottling

process, or through leeching from packaging materials. Many compounds were detected across the sample types, but bioassay activity was only detected in the wastewater effluent and in the urban snow samples. The link between the detected activities and the compounds responsible for it was weak. For the future, improving the sensitivity of the chemical analysis is desirable.

Furthermore, it is important that studies performed by different laboratories produce comparable results. Not being able to detect compounds that are actually present, or having a false indication that something is present that actually is not, can both lead to serious misinterpretations. A study involving 16 different laboratories performing chemical analysis on the same sample was therefore conducted, to assess how comparable the results would be. The sample this time was whole fish tissue rather than water, which is another strategy to narrow down the number of aquatic pollutants being looked at. Not to toxic compounds this time, but to compounds that are actually taken up into biological tissue upon exposure through the water. The outcome was that such studies on fish biota are as of today not very comparable, and further efforts of harmonisation is necessary.

Populärvetenskaplig sammanfattning

De flesta vet idag att vår miljö innehåller föroreningar. Från industrier, från trafik, men också från insidan av våra hem i form av läkemedel, personliga vårdprodukter och städmedel. Listan fortsätter. Hur som helst, färre är medvetna om omfattningen av detta och vet antagligen inte att ett enda miljövattenprov innehåller så mycket som flera tiotusentals ämnen. Vet vi vilka alla är? Nej. Kan vi ta reda på det? Troligtvis inte. Mängden ämnen närvarande i sådana prover är för stor för att vi ska kunna identifiera alla. Betyder detta att det inte finns något vi kan göra? Som tur är, också nej. Det finns sätt vi kan minska antalet ämnen som vi behöver identifiera, och en av dem är baserat på insikten att de ämnen som är viktigast att veta vilka de är, är de som potentiellt är giftiga. Kallad effektdriven analys, eller förkortat EDA, är detta en metod som kombinerar både bioanalytisk analys – for att detektera potentiell toxicitet i ett prov – med kemisk analys – för att identifiera vad det är som orsakar toxiciteten. Att veta om vad som är giftigt i ett vattenprov är nödvändigt för att vi ska kunna skydda oss, och miljön, mot det genom regleringar (för att kanske motverka framtida utsläpp av sådana ämnen) och reningsprocesser (för att kunna ta bort det som redan har släppts ut).

I det här arbetet har en metod för EDA utvecklats och använts för att hitta potentiellt giftiga ämnen i reningsverkseffluent, urbana snöprover, och flaskvatten. Effluent från avloppsreningsverk är en av de största bidragarna till föroreningar i vattenmiljön. Föroreningar från mänskligt avfall så som läkemedel är speciellt farliga då de är designade för att kunna tas upp i våra kroppar och utöva sina effekter i väldigt låga koncentrationer. Snö är ett medium som bär föroreningar från luften och ytor som den kommer i kontakt med. När snön smälter hittar den till ytvattendrag, och ibland även till grundvattnet. Ytvatten, liksom grundvatten, kan potentiellt användas som råvatten vid dricksvattenproduktion. Ju renare råvattnet är från början, desto renare kommer också det resulterande dricksvattnet bli, så föroreningar i råvatten är ett potentiellt hot mot dricksvattenkvalitet. Flaskvatten utsätts sedan för risken att förorenas genom tappningsprocessen, och genom urlakning från förpackningsmaterialet. Många ämnen detekterades i alla provtyper, men det var bara i reningsverkseffluenten och i de urbana snöproverna som aktivet detekterades. Kopplingen mellan den detekterade

aktiviteten och vad som orsakat den var dock svag. I framtiden behöver känsligheten av den kemiska analysen förbättras.

Utöver detta så är det viktigt att studier som utförs av olika laboratorium är jämförbara för att kunna lita på deras resultat. Att inte kunna detektera ämnen som faktiskt är närvarande, eller att ha en falsk indikering att något är närvarande som faktiskt inte är det, är båda problematiska. En studie som involverade att 16 olika laboratorium genomförde kemisk analys av samma prov gjordes därför, för att se hur jämförbara resultaten var. Provet var hel fiskvävnad istället för vatten, vilket representerar ett annat sätt att minska ner antalet vattenföroreningar. Inte till giftiga ämnen den här gången, utan till ämnen som faktiskt tas upp i biologisk vävnad vid exponering genom vatten. Resultatet var att den här typen av studier på fiskbiota i dagsläget inte är så jämförbara, och vidare ansträngningar som eftersträvar harmonisering är nödvändiga.

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Last but not least, to all my loved ones – thank you. You all motivate me to be the best version of myself. To finish with another quote by Marcus Aurelius: "*The universe is indifferent to our lives and deaths, but love is what makes us human*."

IV

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Full length article

What is in the fish? Collaborative trial in suspect and non-target screening of organic micropollutants using LC- and GC-HRMS

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ABSTRACT

A collaborative trial involving 16 participants from nine European countries was conducted within the NORMAN network in efforts to harmonise suspect and non-target screening of environmental contaminants in whole fish samples of bream (*Abramis brama*). Participants were provided with freeze-dried, homogenised fish samples from a contaminated and a reference site, extracts (spiked and non-spiked) and reference sample preparation protocols for liquid chromatography (LC) and gas chromatography (GC) coupled to high resolution mass spectrometry (HRMS). Participants extracted fish samples using their in-house sample preparation method and/or the protocol provided. Participants correctly identified 9–69 % of spiked compounds using LC-HRMS and 20–60 % of spiked compounds using GC-HRMS. From the contaminated site, suspect screening with participants' own suspect lists led to putative identification of on average ~145 and ~20 unique features per participant using LC-HRMS and GC-HRMS, respectively, while non-target screening identified on average ~42 and ~56 unique features per participant using LC-HRMS and GC-HRMS, respectively. Within the same sub-group of sample preparation method, only a few features were identified by at least two participants in suspect screening (16 features using LC-HRMS, 0 features using GC-HRMS) and non-target screening (0 features using LC-HRMS, 2 features using GC-HRMS). The compounds identified had log octanol/water partition coefficient (K_{OW}) values from −9.9 to 16 and mass-to-charge ratios (m/z) of 68 to 761 (LC-HRMS and GC-HRMS). A significant linear trend was found between log K_{OW} and m/z for the GC-HRMS data. Overall, these findings indicate that differences in screening results are mainly due to the data analysis workflows used by different participants. Further work is needed to harmonise the results obtained when applying suspect and non-target screening approaches to environmental biota samples.

1. Introduction

Risk assessment and management of potentially harmful chemical substances relies on environmental and health data of high quality, including indications of emerging issues (Wang et al., 2020, Dulio et al., 2018). Conventional chemical target analyses typically use liquid chromatography (LC) and gas chromatography (GC) with low resolution mass spectrometry (MS), but wide-scope target, suspect and non-target screening strategies have been developed in recent years to identify contaminants of emerging concern (CECs) in environmental samples (Chen et al. 2022). Hereby, suspect screening requires prior knowledge about the compounds of interest to screen for known compounds or suspects, whereas non-target screening does not consider a tentative structure from the start (Hollender et al., 2023). These techniques rely on high resolution mass spectrometry (HRMS) (Chen et al., 2022; Hogenboom et al., 2009; Hollender et al., 2017; Sobek et al., 2016) and have been applied to e.g. water samples (Menger et al., 2020, Wode et al., 2015, Diamanti et al., 2020, Badea et al., 2020) and biota samples (Álvarez-Ruiz and Picó, 2020, Vandermeersch et al., 2015, Rebryk and Haglund, 2021). While these approaches appear promising as complementary tools for environmental monitoring and support for chemicals management, data comparability is a challenge (Hollender et al., 2019, Alygizakis et al., 2018, Hohrenk et al., 2020, Schulze et al., 2020). Biotic samples in particular represent a complex matrix and co-extraction of abundant endogenous molecules (e.g. lipids, residual proteins) can interfere with the instrumental analysis of CECs. This "matrix effect" typically encompasses disturbances such as background increase, chromatographic alteration (retention time shifts, peak broadening) or ion suppression caused by preferential ionisation of matrix molecules (David et al., 2014, Hajeb et al., 2022, González-Gaya et al., 2021) and is sought minimised through rigorous clean-up steps in target analyses. Efforts are being made to develop generic and non-selective protocols to extract CECs with different physicochemical properties for suspect and non-target screening approaches, offering an acceptable compromise between selectivity and efficient removal of interfering matrix compounds (Dirtu et al., 2012, Fidalgo-Used et al., 2007, Knoll et al., 2020, Dürig et al., 2020, Xia et al., 2019, Baduel et al., 2015, Vitale et al., 2021, Chaker et al., 2022). Furthermore, recently developed guidelines for sampling, sample preparation, chemical analysis and data analysis will likely contribute to more harmonisation in this rapidly developing field (Hollender et al., 2023; Caballero-Casero et al., 2021).

The NORMAN network started as a European research project in 2005 and focuses on CECs and innovative techniques in analytical chemistry. Particular emphasis is placed on data quality and comparability through harmonisation efforts and structured data sharing. NORMAN has a track record of conducting collaborative trials in emerging fields. Previous examples include interlaboratory studies on passive sampling (Schulze et al., 2021), analyses of water (Schymanski et al., 2015, Bader et al., 2016), dust (Rostkowski et al., 2019) and human tissues (Pourchet et al., 2020), and quality control of screening workflows (Bastian et al., 2020, Caballero-Casero et al., 2021). Harmonisation efforts have also been performed on sampling and target analysis of biota (Crimmins et al., 2018, Fakouri Baygi et al., 2020, Badry et al., 2020).

This paper describes a collaborative trial on suspect and non-target screening in biota performed under the auspices of the NORMAN network, using fish samples from Teltow Canal and Lake Stechlin (Germany). Teltow Canal was expected to have high levels of contaminants because it receives discharge from several wastewater treatment plants, while Lake Stechlin is relatively clean and was therefore used as a reference site. The objective was to assess the currently achievable level of harmonisation in suspect and non-target screening of whole-fish tissue through comparison of sample preparation protocols and suspect and non-target screening workflows based on LC-HRMS and GC-HRMS analysis.

2. Materials and methods

2.1. Samples and experimental design

Bream (*Abramis brama*) samples from the reference site Lake Stechlin (longitude 13.0278 N, latitude 53.1514E) and the more polluted site Teltow Canal (longitude 13.1900 N, latitude 52.3983E) were kindly provided by the Fraunhofer IME, Germany. Whole fish from the lake (15 individuals, 30–50 cm, 1–2 kg fish^{-1}) and the canal (10 individuals, 40–46 cm, 0.8–1.3 kg fish^{-1}) were homogenised by cryogenic grinding (Rüdel et al., 2008), freeze-dried and shipped to the Swedish University

[1] These authors contributed equally to the work.

of Agricultural Sciences (SLU), Sweden, for preparation of extracts for LC-HRMS and the National and Kapodistrian University of Athens (NKUA), Greece, for preparation of extracts for GC-HRMS and further preparation and distribution to project participants (see section 2.3).

At SLU/NKUA, a sub-sample of freeze-dried fish material from both sites was used to prepare reference extracts for analysis by LC-HRMS and GC-HRMS. The methods used for these extractions (Dürig et al., 2020, Badry et al., 2022), referred to as the reference methods, are described in detail in section 2.3. A second sub-sample of freeze-dried fish material from the reference lake was pre-spiked with 32 compounds for analysis by LC-HRMS (c = 50 ng mL^{-1}, equivalent tissue concentration 300 ng g^{-1} dry weight (dw) for each compound) and with 19 compounds for analysis by GC-HRMS (c = 75 ng mL^{-1}, equivalent tissue concentration 25 ng g^{-1} dw for each compound). Only 10 'known compounds' among the compounds used for spiking (5 for LC-HRMS and GC-HRMS, respectively) were revealed, while the remaining 'unknown compounds' (n = 41) were not revealed to the participating laboratories prior to analysis (*Table S1* in Supplementary data 1 (SD1)). The spiked compounds were selected based on relevance, previous detection in biota (Rebryk and Haglund, 2022, Vandermeersch et al., 2015) and represented a wide range of physicochemical properties (log K_{OW} values −2.5–10 for LC-HRMS and 0.2–12 for GC-HRMS; molecular mass 162–679 Da for LC-HRMS and 162–949 Da for GC-HRMS). Some participants prepared additional extracts for LC-HRMS and GC-HRMS following their own in-house methods for sample preparation and/or the reference methods using sub-samples of freeze dried fish material that was provided to them along with the pre-made reference extracts.

The reference extracts that were prepared with the reference methods and sequentially shipped to the participants were: i) two extracts from the reference site Lake Stechlin (non-spiked), for LC-HRMS and GC-HRMS, respectively; ii) two extracts from the reference site Lake Stechlin (spiked), for LC-HRMS and GC-HRMS, respectively; and iii) two extracts from the contaminated Teltow Canal site (non-spiked), for LC-HRMS and GC-HRMS, respectively (Fig. 1). In addition to the reference extracts, sub-samples of freeze-dried fish material from both sites (non-spiked freeze-dried fish material from Teltow Canal, and both spiked and non-spiked from Lake Stechlin) were provided to the participating laboratories, to allow them to prepare corresponding fish homogenate samples with their own in-house sample preparation protocols and/or the reference methods. All participants analysed the extracts using their own HRMS instrumentation and data analysis approaches (*Tables S2-S6 in SD1*). The extracts of the spiked samples were analysed by the participants for the 10 known and the unknown compounds applying their own workflows, including their own suspect screening lists. The other extracts (non-spiked) were screened for the presence of CECs following the participants' suspect and non-target screening workflows. The difference between these approaches was that specific criteria had to be fulfilled for the non-target screening (based on abundance and origin, see section 2.5). Thus, the non-target screening was only applied to the samples from Teltow Canal with the criterion of at least a 10x difference in signal between Teltow Canal and Lake Stechlin.

In addition to the fish samples and reference extracts, two mixtures of the reference standards used for the spiked samples were provided to the participants. Retention time (RT) mixtures (two for LC-HRMS, intended for positive (n = 18 compounds) and negative (n = 18) electrospray ionisation (ESI) modes, respectively, and one for GC-HRMS (n = 24, C_7-C_{30} alkane mixture) were also distributed to the participants (see SD3), to facilitate quantitative structure-retention relationship (QSRR)-based predictions of RTs for unknown compounds (Aalizadeh et al., 2021). The spiking mixtures were prepared by mixing individual compound standards in methanol (for LC-HRMS) or hexane (for GC-HRMS), all purchased from commercial vendors (Wellington Laboratories, Sigma-Aldrich, European Pharmacopeia Reference Standard, UPS Reference Standard, and LGC Standards). The final concentration of individual compounds in the spiking mixtures was 0.5 mg/L for LC-HRMS analysis and 1 mg/L for GC-HRMS analysis (*Table S1*).

2.2. Participants and instrumental method choices

In total, 16 laboratories (allocated code letters A-P) from nine different European countries participated in the study, which had been announced within the NORMAN network (Fig. 1*b*). No previous experience of suspect or non-target screening was required, however all participants had experience with suspect or non-target screening. Fourteen participants performed analyses by LC-HRMS and five

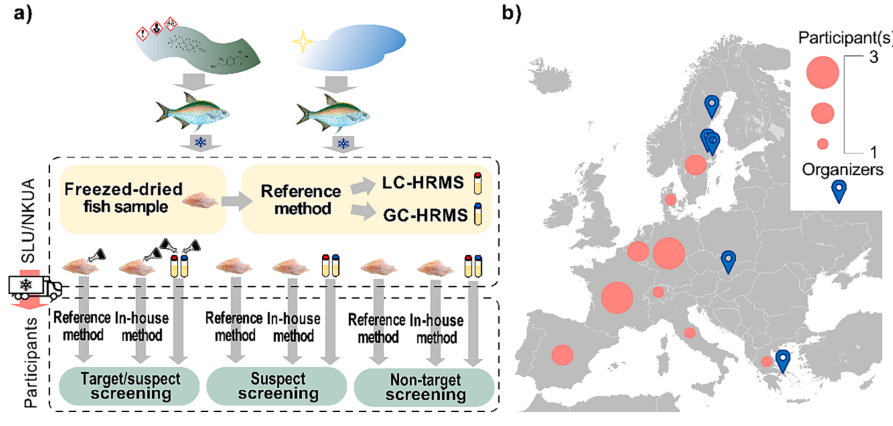

Fig. 1. Experimental design of the collaborative trial and participant map. a) Design of the study. Fish samples and reference extracts prepared with the reference methods were sent to the participants for LC-HRMS and GC-HRMS analysis. The participants analysed the extracts provided and/or prepared their own extract(s) using their in-house sample preparation method(s) and/or the reference method. Spiked samples are indicated by an Erlenmeyer flask symbol and were analysed using suspect screening, while the non-spiked samples were analysed using suspect and non-target screening. b) Distribution of the 16 laboratories participating in the study. The locations of the five organising institutes (Swedish University of Agricultural Sciences (SLU), National and Kapodistrian University of Athens (NKUA), Environmental Institute (EI), Stockholm University, Umeå University) are indicated by blue markers.

performed analyses by GC-HRMS, while three laboratories performed analyses using both methods. The participants used their own data analysis workflows for suspect and non-target screening (see *Figs. S1-S23 in SD1*). For LC-HRMS, ESI was the only ionisation source, while both electron ionisation (EI) and atmospheric pressure chemical ionisation (APCI) were used for GC-HRMS. Additional information on the analytical methods (manufacturer, instrumentation, column dimensions, mobile phases, injection volume, scan range and software) can be found in *Tables S2-S5 in SD1*.

3. Reference methods for sample preparation

Preparation of extracts for LC-HRMS analysis (Fig. 2*a*) was performed at SLU, Sweden, according to an existing protocol (Dürig et al., 2020) (for details, see *SD1*). Six of the participating laboratories used this reference method for their own sample preparation. The extracts for GC-HRMS analysis (Fig. 2*b*) were prepared at NKUA, Greece, following an existing protocol (Badry et al., 2022) with some modifications (for details, see SD1). One of the participating laboratories used the reference method for preparation of extracts for analysis. A summary of the samples analysed by the participants and the methods they used is given in Table 1.

3.1. In-house methods

Twelve in-house methods, used by 10 participating laboratories, were applied in preparation of extracts for analysis by LC-HRMS, while only one in-house method was applied in preparation of extracts for GC-HRMS analysis (Table 1) (for details, see SD1).

3.2. Data curation and reporting

Participants were requested to submit their results in a data collection template (DCT), a multi-tab spreadsheet commonly used by the NORMAN network in collaborative trial studies, to ensure sufficient and coherent information (for details, see SD3). The DCT included details relating to retention time index (RTI), the chromatographic and mass spectrometric methods and reported compounds (e.g. RT, *m/z* intensity, MS/MS data, type of workflow, proposed compound identity, molecular formula, Chemical Abstracts Service (CAS) registration number, Simplified Molecular Input Line Entry System (SMILES) notation and identification confidence level (Schymanski et al., 2014)). For the spiked samples, suspect analysis was used to screen for the known and unknown compounds, using the participants' own suspect lists for the screening of the unknown spiked compounds (see section 3.1). Furthermore, suspect screening with the participants' own suspect lists and non-target screening were performed for non-spiked fish samples from Teltow Canal and Lake Stechlin. Requirements for non-target screening of non-spiked samples were: i) a minimum 10-fold change in contaminated samples (Teltow Canal) compared with the control sample (Lake Stechlin); ii) identified compounds should be of anthropogenic origin; and iii) identification of the 10 most intense compounds.

3.3. Data analysis

For assessments of overall performance and comparability between the participants, the percentages of correctly identified known and unknown spiked compounds were compared. Specifically, the number of correctly identified compounds was compared between the three different sample preparation options, i.e. using the provided reference extract, extracting the fish sample with the reference methods or extracting the fish sample with an in-house method. These comparisons were conducted statistically through paired Wilcoxon tests, using a confidence level of 0.95. Median, mean and standard deviation for the number of correctly identified spiked compounds were also calculated. The number of reported compounds not added during spiking was additionally assessed. The data were analysed using R version 4.1.2 (R-Core-Team, 2021), with the external packages Tidyverse (Wickham et al., 2019) and rcompanion (Mangiafico, 2021).

Apart from the number of compounds detected and whether different participants identified the same compounds, the range of compounds with different physicochemical properties detectable in the fish samples was of interest. Therefore, predicted log K_{OW} and molecular mass of the identified compounds were investigated. For the spiked samples,

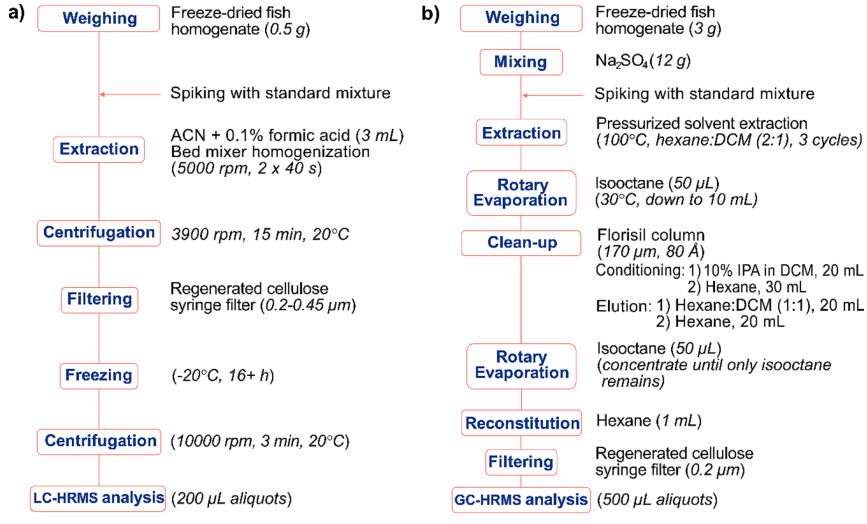

Fig. 2. Reference methods used in sample preparation for analysis by a) LC-HRMS and b) GC-HRMS. ACN: acetonitrile. DCM: dichloromethane. IPA: isopropyl alcohol.

Table 1
Overview of participants (n = 16, codes A-P). Analyses performed by the different participants (n = 16, code A-P), including types of samples (spiked samples or samples from Teltow Canal), identification method (suspect or non-target screening), instrumental analysis method (LC-HRMS or GC-HRMS) and sample preparation method (Ref (provided) = reference extract provided, Ref (pcp) = extract prepared with the reference method by the participant, in-house = extract prepared with the participant's in-house protocol). The symbol x indicates one analysis was performed, while 2x indicates that two analyses were performed in this category.

Sample	Identification method	Analysis	Sample Preparation	Participants														
				A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
Lake Stechlin (spiked)	Suspect screening	LC-HRMS	Ref (provided)	x	x	x	x	x	x			x		x	x	x	x	x	
			Ref (pcp)	x										x			x		
			In-house		x	x	x		x		x	x		2x		x		x	
		GC-HRMS	Ref (provided)							x	x						x	x	
			Ref (pcp)														x		
			In-house							x									
Teltow Canal and Lake Stechlin (non-spiked)	Suspect screening	LC-HRMS	Ref (provided)	x		x	x		x			x			x		x		
			Ref (pcp)	x	x							x		x	x		x		
			In-house		x	x	x		x		2x	x		2x					
		GC-HRMS	Ref (provided)					x		x	x							x	
			Ref (pcp)																
			In-house							x									
	Non-target screening	LC-HRMS	Ref (provided)	x		x	x					x			x		x		x
			Ref (pcp)	x	x							x			x		x		
			In-house		x	x	x	x			2x	x							
		GC-HRMS	Ref (provided)							x			x				x		
			Ref (pcp)														x		
			In-house							x									

predicted log K_{OW} and molecular mass of the detected and undetected compounds were used to support comparisons of the methods. Predicted log K_{OW} values were calculated from the SMILES of the compounds, using the program EPI Suite 4.1 with the individual model KOWWIN v.1.68.

4. Results and discussion

4.1. Known and unknown spiked compounds

Samples from Lake Stechlin were spiked with 32 and 19 compounds for LC- and GC-HRMS analysis, respectively, of which five compounds

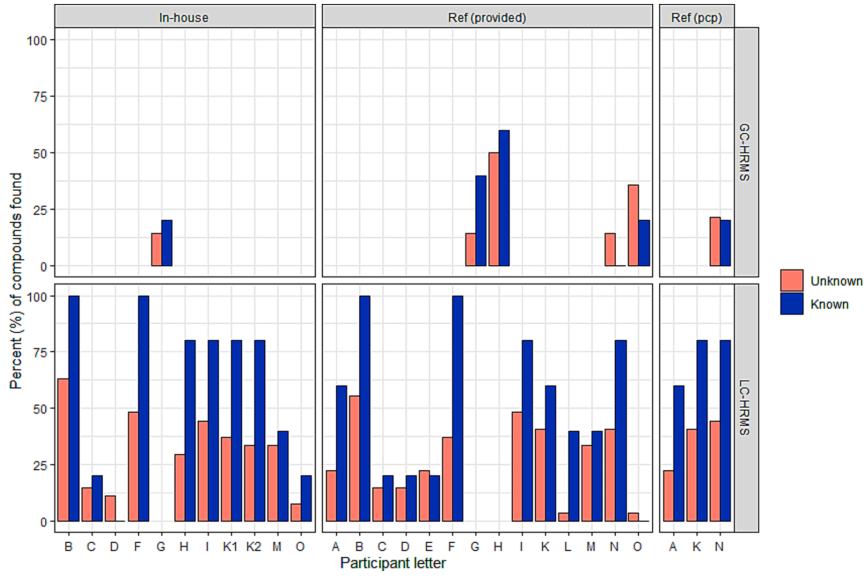

Fig. 3. Detected spiked compounds. Percentage of spiked compounds correctly identified by the different participants (n = 16, codes A-P) for unknown spiked compounds (red bars; identity not disclosed, LC-HRMS 100 % = 27; GC-HRMS 100 % = 14)) and known spiked compounds (blue bars; identity revealed to the participants, 100 % = 5 for both methods), including different methods for sample preparation (in-house = extract prepared according to the participant's in-house protocol, Ref (provided) = reference extract provided, Ref (pcp) = extract prepared by the participant using the reference method) and for analysis (LC-HRMS, GC-HRMS). If the same laboratory analysed extracts from several in-house sample preparation protocols, these were given sequential numbers following the laboratory code letter (e.g. K1, K2).

each were disclosed to the participants. This part of the study had the purpose to assess the number of correct identifications, while sample preparation and data analysis methods varied. The percentages of known (5 for LC-HRMS and GC-HRMS, respectively) and unknown (i.e. the remaining spiked compounds not disclosed to the participants) spiked compounds detected by the participating laboratories are presented in Fig. 3. For the known and unknown spiked compounds, 9–69 % (average = 41 %, median = 44 %, out of 32 compounds) were correctly identified using LC-HRMS (13 participants) and 20–60 % (average = 37 %, median = 35 %, out of 19 compounds) were correctly identified using GC-HRMS (4 participants). LC-HRMS and GC-HRMS gave similar results (mean and median) for the correctly identified compounds, but with high variation between the participating laboratories. The number of reported compounds that were not added during spiking (see *Fig. S22*) ranged from 0 to 185 for the LC-HRMS analyses (although $\leq$ 27 for all but one participant) and 2–34 for the GC-HRMS analyses. If these compounds were present in the fish from natural contamination prior to spiking, it should have led to replicate detections across participants. However, the findings were not reproducible and could be due to inter-laboratory variability and are likely false positives. Over-reporting in non-target screening was previously described in a collaborative blinded analysis (Ulrich et al., 2019), and the need for quality assurance/quality control (QA/QC) measures to keep the false positive rate as low as possible has been identified for future non-target screening studies (Schulze et al., 2020).

Visual inspection of the percentages of correctly identified compounds did not indicate major differences between the sample preparation methods, with the results appearing to vary more between participants than between methods (i.e. 4–56 % correctly identified unknown compounds and 0–100 % identified known compounds for the samples prepared using the LC-HRMS reference method). Means and medians of correctly identified unknown compounds were rather similar for the different sample preparation methods for LC-HRMS (32 $\pm$ 17 % for the in-house method, 28 $\pm$ 17 % for provided extracts, 36 $\pm$ 12 % for the reference method). A similar assessment of the GC-HRMS results was impeded by the low number of participants (14 % for the in-house method (1 participant), 29 $\pm$ 17 % for provided extracts (4 participants), 21 % for the reference method (1 participant)) (*Table S7 in SD1*). Percentages of correctly identified spiked known compounds were below 25 % for participants G, N and O for GC-HRMS and C, D, E and O for LC-HRMS indicating that better performance and harmonisation of identification methods are needed.

Statistical comparison of the number of compounds correctly identified with LC-HRMS by the same laboratory using the in-house extract and the reference extract provided (participants B, C, D, F, I, K, M and O) revealed no significant difference ($p > 0.05$, paired Wilcoxon test) for either the known ($p = 0.77$) or unknown compounds ($p = 0.40$). Likewise, the number of compounds correctly identified by participants who used the reference method to prepare their own extract and the reference extract provided (participants A, K and N) did not show a significant difference ($p > 0.05$). A paired Wilcoxon test for the known compounds resulted in $p = 1$, as did a similar test for the unknown compounds. This means that there was no statistical difference in the number of compounds correctly identified regardless of whether a participant analysed the provided reference extract, their own extract obtained through the reference method, or their own extract obtained through their in-house method. For the Ref (provided) and Ref (pcp) groups, finding no difference is desirable since it indicates reproducibility between laboratories using the same sample preparation method. The lack of significant difference in results using different methods indicates that the differences in the number of compounds correctly identified mainly originated from factors other than the sample preparation method. Other relevant factors include the workflow used for data analysis, such as software and library uses, criteria for acceptance and rejection etc., as well as instrument settings. However, the LC-HRMS methods used by the participants (Tables S2-S3 in SD1) were fairly similar, so data processing seems to be the most important source of variation (Figures S1-S19 in SD1). In general, the participants followed data processing workflows as recommended in the literature (e.g. Hollender et al., 2019, Alygizakis et al., 2018, Hohrenk et al., 2020, Schulze et al., 2020), but some factors such as peak intensity, mass error, resolution are instrument specific and can explain the differences in the compounds identified by the participants. Furthermore, if the unknown spiked compounds were not included in the participants' suspect library the participant was not able to identify them. This highlights the importance of selection criteria for the suspect libraries. It should also be noted that comparisons of multiple results from the same participant might be biased since, although the results may be technically independent if based on different methods, the laboratory's data evaluation may be influenced by an interest in consistency.

The participants who identified most spiked compounds ($n = 32$) correctly by LC-HRMS analysis were participant B (22 correct compounds through the in-house protocol) and participants F and I (18 and 16 correct compounds, respectively, with their in-house method). Since these participants performed roughly equally well applying their in-house protocol as with the extract prepared by the reference protocol, different methods may serve to achieve comparable results. Upon inspection of the sample preparation protocols (for details, see SD1), the in-house protocols were relatively similar. These findings are a step forward in harmonisation of sample preparation protocols.

The percentages of participants who correctly detected specific compounds are shown in *Figs. S20-S21*. Natamycin, dichlofluanid, dazomet, clopyralid, amidotrizoic acid and 1,3-dichloro-5,5-dimethylhydantoin were not detected by any of the participants in their LC-HRMS analyses, which could be related to the low log K_{OW} (−2.5 to –2.7) of these compounds making them less suitable for reverse phase LC columns, which were mainly used by the participants (*Table S1 in SD1*). Likewise, *n*-butylbenzenesulfonamide, musk tibeten (known), diphenyl phthalate, decabromobiphenyl and 1-chlorononane were not detected by any of the participants in their GC-HRMS analyses. These compounds have a wide range of log K_{OW} values (2.3–12) and have previously been detected in biota (Sørensen et al., 2023). Thus, both LC-HRMS and GC-HRMS analysis show a non-negligible risk of false negatives.

Compounds detected with high frequency were triadimefon, pyrimethamine, mebendazole (known), ifosfamide (known), fenpiclonil and bicalutamide by LC-HRMS analysis, and hexachlorobenzene and chlorfenviphos (known) by GC-HRMS. These results indicate that compounds with high (>6) or low (<0) log K_{OW} (Fig. 4) or high m/z (>550) value (*Fig. S23 in SD1*) were found less frequently. For LC-HRMS analysis, this can be partly explained by the separation methods since mainly hydrophobic C_{18}-type LC columns were used by the participants, which do not retain very polar compounds to any significant extend (*Table S2* in SD1). However, this can merely be taken to indicate a trend, since the number of compounds with these characteristics was very low. Thus, more work is needed to optimise sample preparation, instrumental methods and workflow strategies for suspect and non-target screening with minimal compound discrimination.

4.2. Suspect screening

LC-HRMS analysis (10 participants) led to reporting of ~1000 unique features (on average ~145, median ~21, per participant) of fish samples from Teltow Canal (Fig. 5; *Tables S9-S10 in SD1*). The high average in comparison to the median can be explained by the high number of identified unique features by a few participants. The total number of features identified through GC-HRMS analysis (4 participants) was much lower (on average ~20, median ~21, per participant) of fish samples from Teltow Canal, which could be related to the low number of participants performing these analyses. Notably, the median number of features reported by LC-HRMS and GC-HRMS participants are almost the same, which suggests that most laboratories performed at a similar level. Suspect screening with LC-HRMS analysis performed on

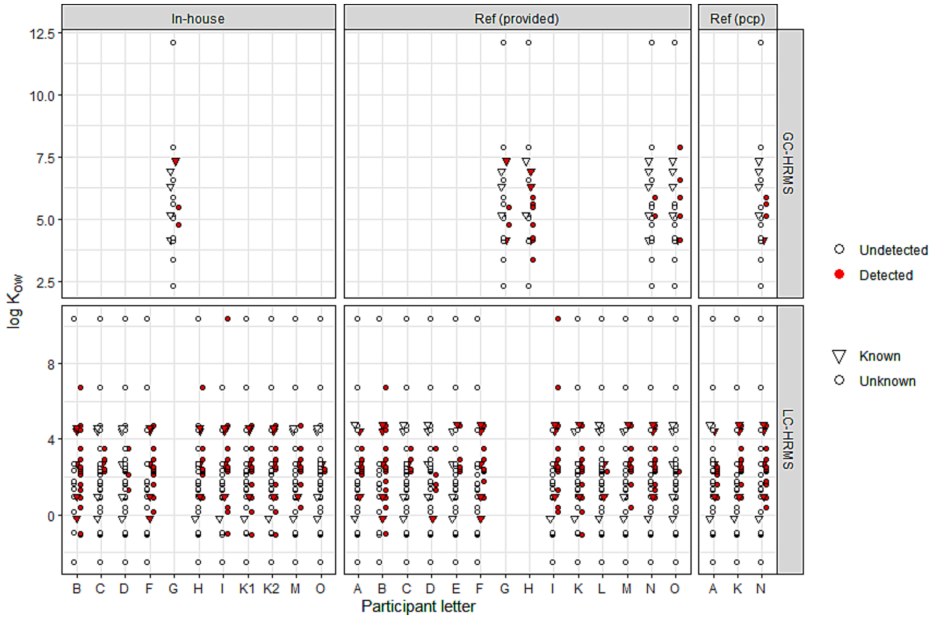

Fig. 4. Predicted log K_{OW} values of spiked compounds. Log K_{OW} values of spiked compounds predicted by participants ($n = 16$, code A-P) using EPI Suite 4.0, along with indication of positive (red) or negative (white) detection. Unknown compounds (spiked compounds whose identity was not disclosed) are indicated with a circle, while known compounds (spiked compounds whose identity was revealed to the participants) are marked with a triangle. The compounds are grouped according to sample preparation method (Ref (provided) = provided reference extract, Ref (pcp) = extract prepared by the participant using the reference method by the participant, in-house = extract prepared according to the participant's in-house protocol) and method of analysis (LC-HRMS, GC-HRMS). If the same laboratory analysed extracts from several in-house sample preparation protocols, these were given sequential numbers following the laboratory code letter (e.g. K1, K2).

fish samples from Teltow Canal led to 16 features detected by at least two participants within the same sub-group of sample preparation method (In-house, Ref (provided), Ref (pcp)). For most of the 16 identical features, at least one of the participants identified the compound at a confidence level of 1 or 2, i.e. with a probable or confirmed structure (Schymanski et al., 2014). The 16 identified features have a wide range of predicted log K_{OW} values (−0.2 to 7.9) and masses (m/z 119 to 500) (*Table S8 in SD1*). Including duplicates within and between sample preparation groups, ~420 features in total were reported at a confidence level of 1 and 2 (although these are underestimates, since not all participants reported confidence levels for the identified features). Suspect screening by GC-HRMS only led to uniquely identified features ($n = 25$) in contaminated samples from Teltow Canal, with no overlapping features between participants.

In suspect screening using LC-HRMS and GC-HRMS, the features detected had log K_{OW} values in the range of −9.9 to 16 (*Fig. S24* in SD1) and an m/z range of 68 to 761 (Fig. 5, *Figs. S26 and S33* in SD1). A significant linear trend was found between log K_{OW} (*Figs. S24-S25* in SD1) and m/z (*Figs. S26-S27* in SD1) for the GC-HRMS results ($p < 0.05$), but not for the LC-HRMS results ($p > 0.05$) in suspect and non-target screening of samples from Teltow Canal (Fig. 5). Highly polar substances (log $K_{ow} < 0$) are not likely to be bioaccumulative due to their typically high water solubility (with a few exceptions such as per- and polyfluoroalkyl substances (PFAS)), and thus their tentative identification is unexpected. The detection of such compounds could either indicate false positives, or that extremely high concentrations of these compounds were present in the water. The number of features detected by the different participants using suspect screening in fish samples from Teltow Canal showed high variations for both GC-HRMS and LC-HRMS results (Fig. 6, *Figs. S28-S32*, for reported confidence levels see *Figs. S28-S30 and S38-S40* in SD1). The variation in suspects detected by the different participants can mainly be explained by different suspect lists and data processing steps and are less likely due to differences in sample preparation. This is consistent with previous findings showing only 10 % overlap between different processing tools applied to the same data set used for non-target screening (Hohrenk et al., 2020) indicating that the compound identification depends largely on the performance of the processing tools (e.g. resolution, QA/QC). However, harmonized data processing can be challenging because often, vendor software is used and these programs are largely "black boxes".

4.3. Non-target screening

Non-target screening was different from the suspect screening approaches by introducing a set of criteria that had to be met: i) a minimum 10-fold change in contaminated samples (Teltow Canal) compared with the control sample (Lake Stechlin); ii) identified compounds should be of anthropogenic origin; and iii) identification of the 10 most intense compounds. The participants were asked to highlight the ten compounds with the highest intensity in the samples from Teltow Canal. However, some participants identified > 10 compounds and thus all compounds identified are reported here. The number of compounds detected by the different participants using non-target screening is shown in Fig. 7 (for details see *Figs. S27, S29, S30, S32, S39 and S40* in SD1). Non-target screening using LC-HRMS (10 participants) led to the detection of, on average, 42 features (median 14) per participant, with a maximum

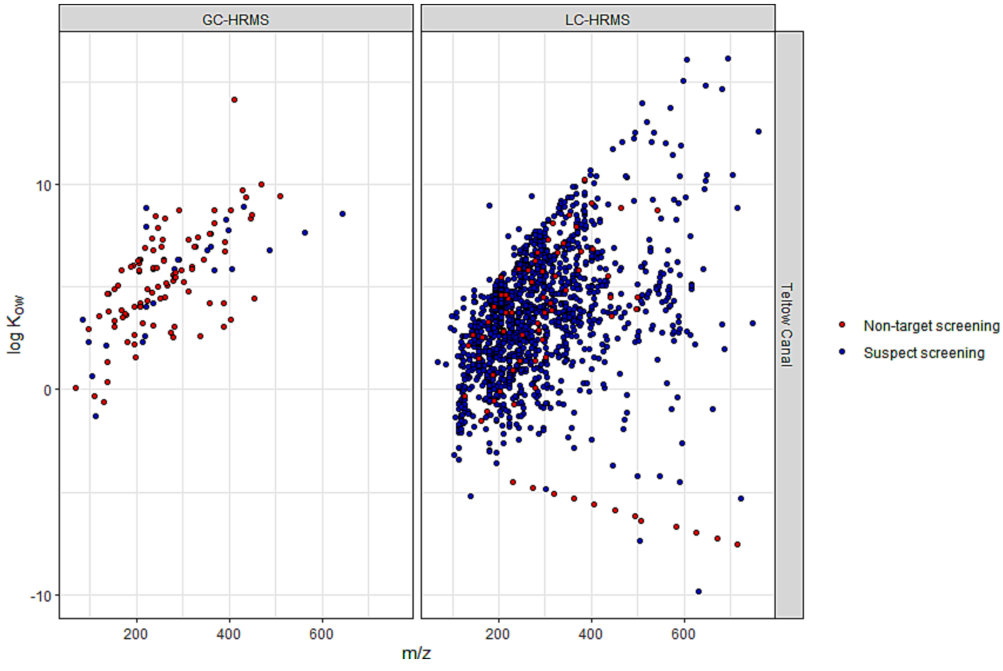

Fig. 5. Range of detected features. *m/z* values versus log K_{OW} values for the features found by all participants using suspect screening (blue) or non-target screening (red) in fish samples from Teltow Canal with (left) GC-HRMS analysis and (right) LC-HRMS analysis and all three sample preparation methods (reference extract provided, extract prepared according to the reference protocol, or extract prepared according to the participant's in-house protocol). Reported features that were ambiguously identified (not containing a name/SMILES/other identifier, or containing several ones for the same *m/z*) are excluded from the diagram since no single log K_{OW} value could be calculated.

number of 178 features (*Table S9 in SD1*). The number of features identified through GC-HRMS ($n = 3$) was, on average, 56 (median 45) per participant, with a maximum number of 60 features. Non-target screening by LC-HRMS only generated unique identified features, which is in line with findings in a previous study comparing data process software (Hohrenk et al., 2020). In GC-HRMS analysis of the samples, two out of three participants identified *pp*'-DDMU, a metabolite of the organochlorine pesticide *pp*'-DDT, from the reference extract provided.

In non-target screening using LC-HRMS and GC-HRMS, the features detected had a range of log K_{OW} values of −7.5 to 14 (*Fig. S25* in SD1) and *m/z* of 68 to 714 (Fig. 5, *Figs. S27 and S33* in SD1). A full list of the features detected and identified by suspect and non-target screening can be found in *Table S11* in SD2, where detected *m/z* values reported with molecular formula as the sole identifier (≥ level 4 without tentative name or structure) have been removed for clarity. Some participants reported naturally occurring features, despite a request that only anthropogenic compounds should be reported. In cases where such features were reported they were included, since it is challenging to discriminate between anthropogenic and natural compounds (Singh et al., 2023) and revising all reported features and removing them would have been too labour-intensive. RTI was used by 64 % and 33 % of the participants applying LC-HRMS and GC-HRMS, respectively (one participant used Kovats index instead of RTI in GC-HRMS). A previous study has shown that RTI increases the reliability of the identification (Aalizadeh et al., 2021).

5. Conclusions and recommendations

The percentage of correctly identified known and unknown spiked compounds showed high variation between the participating laboratories with, on average, 41 % (maximum 69 %) correctly identified using LC-HRMS and 37 % (maximum 60 %) correctly identified using GC-HRMS. Means and medians of correctly identified unknown compounds in LC-HRMS analysis were rather similar for the different sample preparation methods (i.e. in-house method, extracts provided, reference method) (with fewer participants, interpretation of the results obtained by GC-HRMS was limited). Thus factors such as the data analysis seemed to be a more important source of variation. False positives were also reported by all participants, indicating the need for better QA/QC steps in data curation. Suspect screening resulted in a large number of features identified in samples from the contaminated Teltow Canal (on average ~145 and ~20 unique features per participant using LC-HRMS and GC-HRMS, respectively), as did non-target screening with predefined reporting criteria (on average 42 and 56 unique features per participant using LC-HRMS and GC-HRMS, respectively). The compounds detected had log K_{OW} values ranging from −9.9 to 16 and *m/z* values from 68 to 761, with a significant linear trend between log K_{OW} and *m/z* for the GC-HRMS data. Within the same sub-group of sample preparation method, only a few features were identified by at least two participants in suspect screening.

Overall, the field of suspect and non-target screening in biota is still under development and results in different studies performed on biota are currently not fully comparable, with a high inter-laboratory

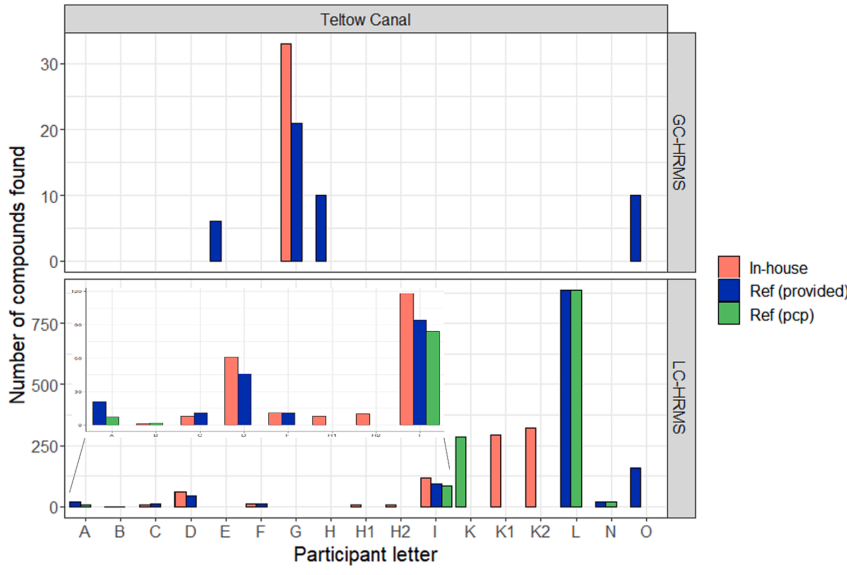

Fig. 6. Compounds detected by suspect screening. Number of compounds (at all confidence levels) in fish samples from Teltow Canal reported by the participants (n = 16, code A-P) using a suspect screening approach, for different sample preparation methods (reference extract provided = blue, extract prepared by the participant using the reference method = green, extract prepared according to the participant's in-house protocol = red), and instrumental analysis methods (LC-HRMS, GC-HRMS). If the same laboratory analysed extracts from several in-house sample preparation protocols, these were given sequential numbers following the laboratory code letter (e.g. K1, K2).

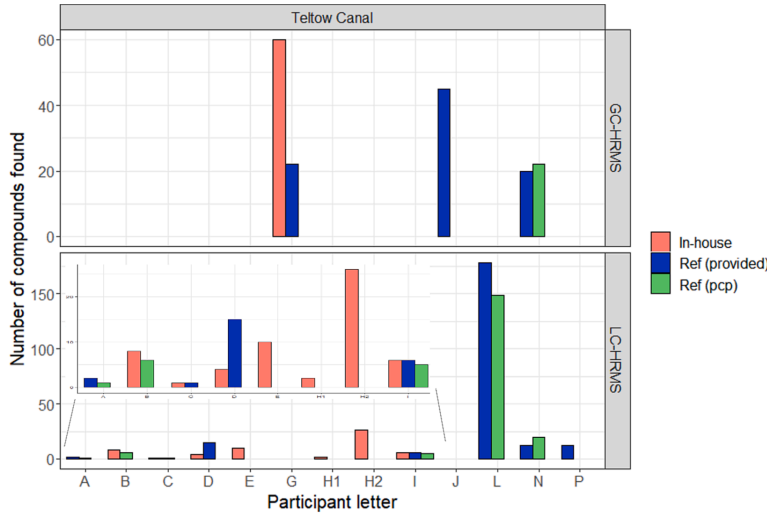

Fig. 7. Compounds detected by non-target screening. Number of compounds reported by the participants (at all confidence levels) in fish samples from Teltow Canal (n = 16, code A-P) using the non-target screening approach, for different sample preparation methods (reference extract provided = blue, extract prepared by the participant using the reference method = green, extract prepared according to the participant's in-house protocol = red), and instrumental analysis methods (LC-HRMS, GC-HRMS). If the same laboratory analysed extracts from several in-house sample preparation protocols, these were given sequential numbers following the laboratory code letter (e.g. H1, H2).

variability. Different methods were applied for sample preparation, but above all data processing contributed substantially to the overall variation observed in the present trial. Some recommendations on suitable data processing can be found in the recently published NORMAN guidance on suspect and non-target screening in environmental monitoring (Hollender et al., 2023). In addition, it is recommended to implement routine QA/QC measures for suspect and non-target screening such as blanks, internal standards, repetitions, randomization, calibration, tuning, data independent acquisition, use of multiple databases, and use of confidence levels (Schulze et al., 2020). However, the complex biota matrix also demands further work to establish sample preparation methods that provide an acceptable level of selectivity to minimise matrix effects and reduce the rate of false positive results. On the instrument side, alternative soft ionisation techniques can provide molecular ions for a wider range of GC amenable compounds, which could be useful in suspect screening workflows. Use of different sample preparation protocols and instruments is probably advantageous, as they are often complementary and therefore broaden the visible chemical space. However, there is a high risk of false positives and false negatives in suspect and non-target screening, and more standardised approaches in QA/QC are needed to manage and reduce these risks.

CRediT authorship contribution statement

Wiebke Dürig: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Sofia Lindblad:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Oksana Golovko:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Georgios Gkotsis:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Reza Aalizadeh:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Maria-Christina Nika:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Nikolaos Thomaidis:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Nikiforos A. Alygizakis:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Merle Plassmann:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Peter Haglund:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Qiuguo Fu:** Investigation, Methodology, Writing – review & editing. **Juliane Hollender:** Investigation, Methodology, Writing – review & editing. **Jade Chaker:** Investigation, Methodology, Writing – review & editing. **Arthur David:** Investigation, Methodology, Writing – review & editing. **Uwe Kunkel:** Investigation, Methodology, Writing – review & editing. **André Macherius:** Investigation, Methodology, Writing – review & editing. **Lidia Belova:** Investigation, Methodology, Writing – review & editing. **Giulia Poma:** Investigation, Methodology, Writing – review & editing. **Hugues Preud'Homme:** Investigation, Methodology, Writing – review & editing. **Catherine Munschy:** Investigation, Methodology, Writing – review & editing. **Yann Aminot:** Investigation, Methodology, Writing – review & editing. **Carsten Jaeger:** Investigation, Methodology, Writing – review & editing. **Jan Lisec:** Investigation, Methodology, Writing – review & editing. **Martin Hansen:** Investigation, Methodology, Writing – review & editing. **Katrin Vorkamp:** Investigation, Methodology, Writing – review & editing. **Linyan Zhu:** Investigation, Methodology, Writing – review & editing. **Francesca Cappelli:** Investigation, Methodology, Writing – review & editing. **Claudio Roscioli:** Investigation, Methodology, Writing – review & editing. **Sara Valsecchi:** Investigation, Methodology, Writing – review & editing. **Renzo Bagnati:** Investigation, Methodology, Writing – review & editing. **Belén González:** Investigation, Methodology, Writing – review & editing. **Ailette Prieto:** Investigation, Methodology, Writing – review & editing. **Olatz Zuloaga:** Investigation, Methodology, Writing – review & editing. **Ruben Gil-Solsona:** Investigation, Methodology, Writing – review & editing. **Pablo Gago-Ferrero:** Investigation, Methodology, Writing – review & editing. **Sara Rodriguez-Mozaz:** Investigation, Methodology, Writing – review & editing. **Hélène Budzinski:** Investigation, Methodology, Writing – review & editing. **Marie-Helene Devier:** Investigation, Methodology, Writing – review & editing. **Georg Dierkes:** Investigation, Methodology, Writing – review & editing. **Lise Boulard:** . **Griet Jacobs:** Investigation, Methodology, Writing – review & editing. **Stefan Voorspoels:** Investigation, Methodology, Writing – review & editing. **Heinz Rüdel:** Investigation, Methodology, Writing – review & editing. **Lutz Ahrens:** Project administration, Conceptualization, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary materials

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2023.108288.

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Supplementary data 1

What is in the fish? Collaborative trial in suspect and non-target screening of organic micropollutants using LC- and GC-HRMS

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Table of Contents

1. Compounds used for spiking

Table S1. Information on the compounds used to spike freeze-dried fish tissue samples from the reference lake prior to extraction with the reference method. First is indicated whether the compound was used for spiking of samples for liquid or gas chromatography, followed by compound name, CAS number, molecular formula, SMILES annotation, monoisotopic mass, InChI annotation and predicted log K_{ow} via EPI Suite. A row highlighted with light grey indicates that the compound was known to the participants prior to analysis.

LC/GC	Compound name	CAS number	Mol. formula	SMILES	Monoisotopic mass	InChI	Predicted log K_{ow}
GC	Alachlor	15972-60-8	$C_{14}H_{20}ClNO_2$	CCc1cccc(c1N(COC)C(=O)CCl)CC	269.1183	InChI=1S/C14H20ClNO2/c1-4-11-7-6-8-12(5-2)14(11)16(10-18-3)13(17)9-15/h6-8H,4-5,9-10H2,1-3H3	3.37
GC	Benzene, (1-ethyldecyl)-	2400-00-2	$C_{18}H_{30}$	CCCCCCCCCC(CC)c1ccccc1	246.2348	InChI=1S/C18H30/c1-3-5-6-7-8-9-11-14-17(4-2)18-15-12-10-13-16-18/h10,12-13,15-17H,3-9,11,14H2,1-2H3	7.87
GC	Bisphenol G	127-54-8	$C_{21}H_{28}O_2$	CC(C)c1cc(ccc1O)C(C)(C)c2ccc(c(c2)C(C)C)O	312.2089	InChI=1S/C21H28O2/c1-13(2)17-11-15(7-9-19(17)22)21(5,6)16-8-10-20(23)18(12-16)14(3)4/h7-14,22-23H,1-6H3	6.55
GC	Chlorfenvinphos	470-90-6	$C_{12}H_{14}Cl_3O_4P$	CCOP(=O)(OCC)O/C(=C\Cl)/c1ccc(cc1Cl)Cl	357.9695	InChI=1S/C12H14Cl3O4P/c1-3-17-20(16,18-4-2)19-12(8-13)10-6-5-9(14)7-11(10)15/h5-8H,3-4H2,1-2H3/b12-8-	4.15
GC	Chlorpyrifos	2921-88-2	$C_9H_{11}Cl_3NO_3PS$	CCOP(=S)(OCC)Oc1c(cc(c(n1)Cl)Cl)Cl	348.9263	InChI=1S/C9H11Cl3NO3PS/c1-3-14-17(18,15-4-2)16-9-7(11)5-6(10)8(12)13-9/h5H,3-4H2,1-2H3	5.11
GC	1-Chlorononane	2473-01-0	$C_9H_{19}Cl$	CCCCCCCCCCl	162.1175	InChI=1S/C9H19Cl/c1-2-3-4-5-6-7-8-9-10/h2-9H2,1H3	5.02
GC	1-Chloropyrene	34244-14-9	$C_{16}H_9Cl$	c1cc2ccc3ccc(c4c3c2c(c1)cc4)Cl	236.0393	InChI=1S/C16H9Cl/c17-14-9-7-12-5-4-10-2-1-3-11-6-8-13(14)16(12)15(10)11/h1-9H	5.58
GC	Decabromodiphenyl ether	1163-19-5	$C_{12}Br_{10}O$	c1(c(c(c(c(c1Br)Br)Br)Br)Br)Oc2c(c(c(c(c2Br)Br)Br)Br)Br	949.1783	InChI=1S/C12Br10O/c13-1-3(15)7(19)11(8(20)4(1)16)23-12-9(21)5(17)2(14)6(18)10(12)22	12.11
GC	Dibenzothiophene	132-65-0	$C_{12}H_8S$	c1ccc2c(c1)c3ccccc3s2	184.0347	InChI=1S/C12H8S/c1-3-7-11-9(5-1)10-6-2-4-8-12(10)13-11/h1-8H	0.17

GC	Diphenyl phthalate	84-62-8	$C_{20}H_{14}O_4$	c1ccc(cc1)OC(=O)c2ccccc2C(=O)Oc3ccccc3	318.0892	InChI=1S/C20H14O4/c21-19(23-15-9-3-1-4-10-15)17-13-7-8-14-18(17)20(22)24-16-11-5-2-6-12-16/h1-14H	4.1
GC	Hexabromobenzene	87-82-1	C_6Br_6	c1(c(c(c(c(c1Br)Br)Br)Br)Br)Br	545.5100	InChI=1S/C6Br6/c7-1-2(8)4(10)6(12)5(11)3(1)9	7.33
GC	Musk Tibeten	145-39-1	$C_{13}H_{18}N_2O_4$	Cc1c(c(c(c(c1C)[N+](=O)[O-])C(C)(C)C)[N+](=O)[O-])C	266.1267	InChI=1S/C13H18N2O4/c1-7-8(2)11(14(16)17)10(13(4,5)6)12(9(7)3)15(18)19/h1-6H3	5.18
GC	n-Butylbenzenesulfonamide	3622-84-2	$C_{10}H_{15}NO_2S$	CCCCNS(=O)(=O)c1ccccc1	213.0823	InChI=1S/C10H15NO2S/c1-2-3-9-11-14(12,13)10-7-5-4-6-8-10/h4-8,11H,2-3,9H2,1H3	2.31
GC	2,4,6-tribromoanisole	607-99-8	$C_7H_5Br_3O$	COc1c(cc(cc1Br)Br)Br	341.7891	InChI=1S/C7H5Br3O/c1-11-7-5(9)2-4(8)3-6(7)10/h2-3H,1H3	4.74
GC	Transeolide	68140-48-7	$C_{18}H_{26}O$	CC(C1=C(C)C=C(C(C)(C)C(C)C2C(C)C)C2=C1)=O	258.1984	InChI=1S/C18H26O/c1-10(2)17-12(4)18(6,7)16-8-11(3)14(13(5)19)9-15(16)17/h8-10,12,17H,1-7H3	6.31
GC	UV-327	3864-99-1	$C_{20}H_{24}ClNO_3$	CC(C)(C)c1cc(c(c(c1)n2nc3ccc(cc3n2)Cl)O)C(C)(C)C	357.1608	InChI=1S/C20H24ClN3O/c1-19(2,3)12-9-14(20(4,5)6)18(25)17(10-12)24-22-15-8-7-13(21)11-16(15)23-24/h7-11,25H,1-6H3	6.91
GC	Dieldrin	60-57-1	$C_{12}H_8Cl_6O$	C1C2C3C(C1C4C2O4)C5(C(=C(C3(C5(Cl)Cl)Cl)Cl)Cl)Cl	377.8706	InChI=1S/C12H8Cl6O/c13-8-9(14)11(16)5-3-1-2(6-7(3)19-6)4(5)10(8,15)12(11,17)18/h2-7H,1H2/t2-,3+,4+,5-,6-,7+,10+,11-	5.45
GC	β-HCH	58-89-9	$C_6H_6Cl_6$	C1(C(C(C(C(C1Cl)Cl)Cl)Cl)Cl)Cl	287.8601	InChI=1S/C6H6Cl6/c7-1-2(8)4(10)6(12)5(11)3(1)9/h1-6H	5.26
GC	Hexachlorobenzene	118-74-1	C_6Cl_6	C1(=C(C(=C(C(=C1Cl)Cl)Cl)Cl)Cl)Cl	281.8131	InChI=1S/C6Cl6/c7-1-2(8)4(10)6(12)5(11)3(1)9	5.86
LC	Natamycin	7681-93-8	$C_{33}H_{47}NO_{13}$	[H][C@@]12C[C@H](O)C[C@]3(O)C[C@H](O)[C@@H](C(O)=O)[C@]([H])(C[C@@H](O[C@]4(C)O[C@H](C)[C@@H](O)[C@H](N)[C@@H]4O)\C=C\C=C\C=C\C=C\	679.3204	InChI=1S/C34H49NO13/c1-19-11-9-7-5-4-6-8-10-12-22(47-33(3)31(40)29(35)30(39)20(2)46-33)16-26-28(32(41)42)23(37)18-34(43,48-26)17-21(36)15-25-24(45-25)13-14-27(38)44-19/h4-10,12-14,19-26,28-31,36-37,39-40,43H,11,15-18,35H2,1-3H3,(H,41,42)/b5-4+,8-	-2.51

				C[C@@H](C)OC(=O)\C=C\[C@H]1O2)O3		6+,9-7+,12-10+,14-13+/t19-,20-,21+,22+,23+,24-,25-,26+,28-,29+,30-,31+,33+,34-/m1/s1	
LC	Metronidazole-OH	4812-40-2	$C_6H_9N_3O_4$	OCCN1C(CO)=NC=C1[N+]([O-])=O	187.0593	InChI=1S/C6H9N3O4/c10-2-1-8-5(4-11)7-3-6(8)9(12)13/h3,10-11H,1-2,4H2	-1.06
LC	Benzothiazole-2-sulfonaic acid (BTSA) potassium	941-57-1	$C_7H_5NO_3S_2$	C1=CC=C2C(=C1)N=C(S2)S(=O)(=O)O	214.9711	InChI=1S/C7H5NO3S2/c9-13(10,11)7-8-5-3-1-2-4-6(5)12-7/h1-4H,(H,9,10,11)	-0.99
LC	1,3-Dichloro-5,5-dimethylhydantoin	118-52-5	$C_5H_6Cl_2N_2O_2$	CC1(C)N(Cl)C(=O)N(Cl)C1=O	195.9806	InChI=1S/C5H6Cl2N2O2/c1-5(2)3(10)8(6)4(11)9(5)7/h1-2H3	-0.94
LC	4-Amino-6-(trifluoromethyl)benzene-1,3-disulfonamide (2,4-disulfamyl-5-trifluoromethylaniline)	654-62-6	$C_7H_8F_3N_3O_4S_2$	NC1=C(C=C(C(=C1)C(F)(F)F)S(N)(=O)=O)S(N)(=O)=O	318.9908	InChI=1S/C7H8F3N3O4S2/c8-7(9,10)3-1-4(11)6(19(13,16)17)2-5(3)18(12,14)15/h1-2H,11H2,(H2,12,14,15)(H2,13,16,17)	-0.19
LC	Sparfloxacin	110871-86-8	$C_{19}H_{22}F_2N_4O_3$	C[C@H]1CN(C[C@@H](C)N1)C1=C(F)C2=C(C(N)=C1F)C(=O)C(=CN2C1CC1)C(O)=O	392.1660	InChI=1S/C19H22F2N4O3/c1-8-5-24(6-9(2)23-8)17-13(20)15(22)12-16(14(17)21)25(10-3-4-10)7-11(18(12)26)19(27)28/h7-10,23H,3-6,22H2,1-2H3,(H,27,28)/t8-,9+	0.12
LC	Sulisobenzone	4065-45-6	$C_{14}H_{12}O_6S$	COC1=C(C=C(C(=O)C2=CC=CC=C2)C(O)=C1)S(O)(=O)=O	308.0355	InChI=1S/C14H12O6S/c1-20-12-8-11(15)10(7-13(12)21(17,18)19)14(16)9-5-3-2-4-6-9/h2-8,15H,1H3,(H,17,18,19)	0.37
LC	2,6-Dichlorobenzamide (BAM)	2008-58-4	$C_7H_5Cl_2NO$	NC(=O)C1=C(Cl)C=CC=C1Cl	188.9748	InChI=1S/C7H5Cl2NO/c8-4-2-1-3-5(9)6(4)7(10)11/h1-3H,(H2,10,11)	0.9
LC	Dazomet	533-74-4	$C_5H_{10}N_2S_2$	CN1CSC(=S)N(C)C1	162.0285	InChI=1S/C5H10N2S2/c1-6-3-7(2)5(8)9-4-6/h3-4H2,1-2H3	0.94
LC	Ifosfamide	3778-73-2	$C_7H_{15}Cl_2N_2O_2P$	ClCCNP1(=O)OCCCN1CCCl	260.0248	InChI=1S/C7H15Cl2N2O2P/c8-2-4-10-14(12)11(6-3-9)5-1-7-13-14/h1-7H2,(H,10,12)	0.97
LC	4-(Trifluoromethyl) benzenesulfonamide	830-43-3	$C_7H_6F_3NO_2S$	NS(=O)(=O)C1=CC=C(C=C1)C(F)(F)F	225.0071	InChI=1S/C7H6F3NO2S/c8-7(9,10)5-1-3-6(4-2-5)14(11,12)13/h1-4H,(H2,11,12,13)	1.33
LC	Amidotrizoic acid	117-96-4	$C_{11}H_9I_3N_2O_4$	CC(=O)NC1=C(I)C(C(O)=O)=C(I)C(NC(C)=O)=C1I	613.7697	InChI=1S/C11H9I3N2O4/c1-3(17)15-9-6(12)5(11(19)20)7(13)10(8(9)14)16-4(2)18/h1-2H3,(H,15,17)(H,16,18)(H,19,20)	1.37

LC	Chlorzoxazone	95-25-0	$C_7H_4ClNO_2$	ClC1=CC=C2OC(=O)NC2=C1	168.9931	InChI=1S/C7H4ClNO2/c8-4-1-2-6-5(3-4)9-7(10)11-6/h1-3H,(H,9,10)	1.59
LC	Clopyralid	1702-17-6	$C_6H_3Cl_2NO_2$	OC(=O)C1=C(Cl)C=CC(Cl)=N1	190.9541	InChI=1S/C6H3Cl2NO2/c7-3-1-2-4(8)9-5(3)6(10)11/h1-2H,(H,10,11)	1.63
LC	Pentafluorobenzoic acid	602-94-8	$C_7HF_5O_2$	OC(=O)C1=C(F)C(F)=C(F)C(F)=C1F	211.9897	InChI=1S/C7HF5O2/c8-2-1(7(13)14)3(9)5(11)6(12)4(2)10/h(H,13,14)	1.78
LC	Perfluorobutyric acid (PFBA)	375-22-4	$C_4HF_7O_2$	OC(=O)C(F)(F)C(F)(F)C(F)(F)F	213.9865	InChI=1S/C4HF7O2/c5-2(6,1(12)13)3(7,8)4(9,10)11/h(H,12,13)	2.14
LC	Malathion	121-75-5	$C_{10}H_{19}O_6PS_2$	CCOC(=O)CC(SP(=S)(OC)OC)C(=O)OCC	330.0361	InChI=1S/C10H19O6PS2/c1-5-15-9(11)7-8(10(12)16-6-2)19-17(18,13-3)14-4/h8H,5-7H2,1-4H3	2.29
LC	Bicalutamide	90357-06-5	$C_{18}H_{14}F_4N_2O_4S$	CC(O)(CS(=O)(=O)C1=CC=C(F)C=C1)C(=O)NC1=CC(=C(C=C1)C#N)C(F)(F)F	430.0610	InChI=1S/C18H14F4N2O4S/c1-17(26,10-29(27,28)14-6-3-12(19)4-7-14)16(25)24-13-5-2-11(9-23)15(8-13)18(20,21)22/h2-8,26H,10H2,1H3,(H,24,25)	2.3
LC	Carbaryl	63-25-2	$C_{12}H_{11}NO_2$	CNC(=O)OC1=C2C=CC=CC2=CC=C1	201.0790	InChI=1S/C12H11NO2/c1-13-12(14)15-11-8-4-6-9-5-2-3-7-10(9)11/h2-8H,1H3,(H,13,14)	2.35
LC	Pyrimethamine	58-14-0	$C_{12}H_{13}ClN_4$	CCC1=NC(N)=NC(N)=C1C1=CC=C(Cl)C=C1	248.0829	InChI=1S/C12H13ClN4/c1-2-9-10(11(14)17-12(15)16-9)7-3-5-8(13)6-4-7/h3-6H,2H2,1H3,(H4,14,15,16,17)	2.41
LC	Daidzein	486-66-8	$C_{15}H_{10}O_4$	OC1=CC=C(C=C1)C1=COC2=C(C=CC(O)=C2)C1=O	254.0579	InChI=1S/C15H10O4/c16-10-3-1-9(2-4-10)13-8-19-14-7-11(17)5-6-12(14)15(13)18/h1-8,16-17H	2.55
LC	Mebendazole	31431-39-7	$C_{16}H_{13}N_3O_3$	COC(=O)NC1=NC2=C(N1)C=CC(=C2)C(=O)C1=CC=CC=C1	295.0957	InChI=1S/C16H13N3O3/c1-22-16(21)19-15-17-12-8-7-11(9-13(12)18-15)14(20)10-5-3-2-4-6-10/h2-9H,1H3,(H2,17,18,19,21)	2.71
LC	Dichlofluanid	1085-98-9	$C_9H_{11}Cl_2FN_2O_2S_2$	CN(C)S(=O)(=O)N(SC(F)(Cl)Cl)C1=CC=CC=C1	331.9623	InChI=1S/C9H11Cl2FN2O2S2/c1-13(2)18(15,16)14(17-9(10,11)12)8-6-4-3-5-7-8/h3-7H,1-2H3	2.72
LC	Triadimefon	43121-43-3	$C_{14}H_{16}ClN_3O_2$	CC(C)(C)C(=O)C(OC1=CC=C(Cl)C=C1)N1C=NC=N1	293.0931	InChI=1S/C14H16ClN3O2/c1-14(2,3)12(19)13(18-9-16-8-17-18)20-11-6-4-10(15)5-7-11/h4-9,13H,1-3H3	2.94

LC	Fenpiclonil	74738-17-3	$C_{11}H_6Cl_2N_2$	ClC1=CC=CC(C2=CNC=C2C#N)=C1Cl	235.9908	InChI=1S/C11H6Cl2N2/c12-10-3-1-2-8(11(10)13)9-6-15-5-7(9)4-14/h1-3,5-6,15H	3.48
LC	Diflufenican	83164-33-4	$C_{19}H_{11}F_5N_2O_2$	FC1=CC=C(NC(=O)C2=C(OC3=CC(=CC=C3)C(F)(F)F)N=CC=C2)C(F)=C1	394.0741	InChI=1S/C19H11F5N2O2/c20-12-6-7-16(15(21)10-12)26-17(27)14-5-2-8-25-18(14)28-13-4-1-3-11(9-13)19(22,23)24/h1-10H,(H,26,27)	3.53
LC	Ketoconazole	65277-42-1	$C_{26}H_{28}Cl_2N_4O_4$	CC(=O)N1CCN(CC1)C1=CC=C(OC[C@H]2CO[C@@](CN3C=CN=C3)(O2)C2=CC=C(Cl)C=C2Cl)C=C1	530.1488	InChI=1S/C26H28Cl2N4O4/c1-19(33)31-10-12-32(13-11-31)21-3-5-22(6-4-21)34-15-23-16-35-26(36-23,17-30-9-8-29-18-30)24-7-2-20(27)14-25(24)28/h2-9,14,18,23H,10-13,15-17H2,1H3/t23-,26-/m0/s1	4.45
LC	Chloroquine phosphate	50-63-5	$C_{18}H_{32}ClN_3O_8P_2$	CCN(CCCC(NC1=C2C=CC(Cl)=CC2=NC=C1)C)CC	319.1815	InChI=1S/C18H26ClN3/c1-4-22(5-2)12-6-7-14(3)21-17-10-11-20-18-13-15(19)8-9-16(17)18/h8-11,13-14H,4-7,12H2,1-3H3,(H,20,21)	4.5
LC	Glimepiride	93479-97-1	$C_{24}H_{34}N_4O_5S$	CCC1=C(C)CN(C(=O)NCCC2=CC=C(C=C2)S(=O)(=O)NC(=O)N[C@H]2CC[C@H](C)CC2)C1=O	490.2250	InChI=1S/C24H34N4O5S/c1-4-21-17(3)15-28(22(21)29)24(31)25-14-13-18-7-11-20(12-8-18)34(32,33)27-23(30)26-19-9-5-16(2)6-10-19/h7-8,11-12,16,19H,4-6,9-10,13-15H2,1-3H3,(H,25,31)(H2,26,27,30)/t16-,19-	4.7
LC	Glybenclamide	10238-21-8	$C_{23}H_{28}ClN_3O_5S$	COC1=C(C=C(Cl)C=C1)C(=O)NCCC1=CC=C(C=C1)S(=O)(=O)NC(=O)NC1CCCCC1	493.1438	InChI=1S/C23H28ClN3O5S/c1-32-21-12-9-17(24)15-20(21)22(28)25-14-13-16-7-10-19(11-8-16)33(30,31)27-23(29)26-18-5-3-2-4-6-18/h7-12,15,18H,2-6,13-14H2,1H3,(H,25,28)(H2,26,27,29)	4.79
LC	N-ethyl - 1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-heptadecafluoro-n-(2-hydroxytheyl)octane-1-sulphona (EtFOSA)	4151-50-2	$C_{10}H_6F_{17}NO_2S$	CCNS(=O)(=O)C(C(C(C(C(C(C(C(F)(F)F)(F)F)(F)F)(F)F)(F)F)(F)F)(F)F)(F)F	526.9848	InChI=1S/C10H6F17NO2S/c1-2-28-31(29,30)10(26,27)8(21,22)6(17,18)4(13,14)3(11,12)5(15,16)7(19,20)9(23,24)25/h28H,2H2,1H3	6.76

LC	Tris(4-tert-butylphenyl) phosphate (TBPP)	78-33-1	$C_{30}H_{39}O_4P$	CC(C)(C)C1=CC=C(C=C1)OP(=O)(OC2=CC=C(C=C2)C(C)(C)C)OC3=CC=C(C=C3)C(C)(C)C	494.2586	InChI=1S/C30H39O4P/c1-28(2,3)22-10-16-25(17-11-22)32-35(31,33-26-18-12-23(13-19-26)29(4,5)6)34-27-20-14-24(15-21-27)30(7,8)9/h10-21H,1-9H3	10.43

2. General information on methods for analysis

Table S2. Summary of liquid chromatographic methods used by the participants.

Participant	Instrument and model	Column	Dimensions (mm x mm, µm)	Mobile phase (Aqueous phase / organic phase)	Injection volume (µL)	Flow; run time (mL min^{-1}); (min)
A	Thermo Q Exactive	Agilent Zorbax Eclipse Plus C_{18}	2.1 x 150, 3.5	H_2O + 0.1% FA/ MeOH +0.1% FA	20	0.3
B	AB Sciex QToF-X500R	Acquity HSS T3	1.0 x 150, 1.8	H_2O + 0.01% FA/ ACN +0.01% FA	2	0.1
C	Thermo Q Exactive	Waters XBridge C_{18}	2.1 x 100, 3.5	H_2O + 0.05% FA/ ACN	4	0.2
D	Agilent QToF	InfinityLab Poroshell 120 EC-C_{18}	3.0 x 100, 2.7	H_2O + 0.1% FA/ MeOH + 0.1% FA (ESI+) H_2O + 5mM NH_4ac / MeOH + 5mM NH_4ac (ESI-)	2	0.3
E	Thermo Q Exactive HF	PepMap RSLC, C_{18}	0.075 x 250, 2	2% ACN + 0.1% FA/ 98% ACN +0.1% FA	1	0.0003
F	Thermo Orbitrap Q Exactive	Acquity UPLC C_{18}	2.1 x 10, 1.8	H_2O + 0.1% FA/ MeOH + 0.1% FA	5	0.3
H	Bruker UHPLC-QToF-MS	Acclaim RSLC C18 + guard column Acquity UPLC BEH C_{18} 1.7 µm, VanGuard Pre-Ccolumn	2.1 x 100, 2.2	H_2O:MeOH (90:10) with 5 mM NH_4FA + 0.01%FA/ MeOH with 5 mM NH_4 FA + 0.01% FA (ESI +) H_2O:MeOH (90:10) with 10 mM NH_4ac/ MeOH with 10 mM NH_4ac (ESI-)	5	200 µL min^{-1} at 0-3 min, 400 µL min^{-1} at 14 min, 480 µL min^{-1} at 16 min, 200 µL min^{-1} at 16.1-20 min

I	Thermo Dionex Ultimate 3000 UHPLC/Q Exactive Focus Orbitrap	Kinetex XB-C_{18}	2.1 x 150, 2.6	H_2O + 0.1% FA/ MeOH + 0.1% FA (ESI+) H_2O + 5mM NH_4ac / MeOH + 5 mM NH_4ac (ESI-)	5	0.3
K	Thermo Q Exactive Plus	Atlantis T3	3.0 x 100, 3.0	H_2O + 0.1% FA/ MeOH + 0.1% FA (ESI+) H_2O + 5mM NH_4fa / 95% MeOH/5% H_2O (5mM NH_4fa) (ESI-)	200 µL extract in 200 mL H_2O with online-SPE	0.3
L	Thermo Q Exactive HF	Thermo Hypersil Gold aQ C_{18}	2.1 x 100, 1.9	H_2O + 0.1% FA/ ACN +0.1% FA	5	0.25
M	AB Sciex Triple ToF 6600	Zorbax C_{18}	2.1 x 150, 3.5	H_2O + 0.1% FA/ ACN +0.1% FA	100	0.3
N	Agilent LC/Q-ToF 6540	Kinetex C_{18}	2.1 x 100, 1.7	H_2O + 0.1% FA/ ACN +0.1% FA (ESI+) H2O / ACN (ESI-)	3	0.3
O	AB Sciex Triple ToF 6600	Eclipse pluse C_{18}	2.1 x 50, 1.8	H_2O + 0.1% FA/ ACN	4	0.5
P	Thermo Orbitrap	Waters BEH C_{18}	2.1 x 100, 1.7	H_2O:MeOH (90:10) with 5 mM NH_4ac + 0.01%FA/ MeOH with 5 mM NH_4ac + 0.01% FA (ESI +) H_2O:MeOH (90:10) with 5 mM NH_4ac/ MeOH with 5 mM NH_4ac (ESI-)	5	0.35

ACN = acetonitrile, H_2O = water, MeOH = methanol, FA = formic acid, NH_4ac = ammonium acetate, NH_4fa = ammonium formate, SPE = solid-phase extraction. Column temperatures ranged from 30 to 50 °C.

Table S3. Summary of mass spectrometric parameters and data processing procedures used by the participants for LC-HRMS analysis.

Participant	Scan range	Ionization	Fragment method	Target software	Suspect list for suspect screening	Suspect and non-target procedure
A	100-1050	ESI ±	HCD	Trace Finder	NormanNews2, SoffIdent (both norman-network.com, EFS HRAM Compound Database (ThermoFisher))	Compound Discoverer, MS, MS/MS
B	50-1100	ESI + only	n/a	Sciex OS	PubChem, Web of Science, The Blood Exposome Database, Drugbank, exposome explorer, hmdb, Norman	MarkView, In-house annotation tool[1], PubChem, MassBank, MoNA, Metlin, MS, RT, RT prediction, Isotopic ratio, MS/MS, MetFrag, CFM-ID

C	75-950	ESI + only	CID	Compound Discoverer 3.1	Norman SusDat	Compound Discoverer 3.1, MS isotope pattern, MzCloud library, ChemSpider, mass list search, *in silico* fragmentation MS2, RT prediction
D	100-1700	ESI ±	CID	MassHunter Qualitative Analysis B.07.00; MassHunter PCDL Manager B.08.00	Norman SusDat	Profinder B.08.00, Mass Profiler Professional 15.0, MassBank, MS, RT, MS/MS, prediction of MS/MS, CFM-ID
E	75-1000	ESI ±	HCD	Compound Discoverer	n/a	Compound Discoverer, MS, MS/MS, Library comparison (MzCloud, In house, HighRes Lib., ChemSpider with in silico comparison), mzLogic, FISH
F	66.7-1000	ESI + only	HCD	Xcalibur	NORMAN EXPHRMSMSAVAL (7586 compounds)	Massbank, Metlin, MoNA, MS, fragment ions in DIA
H	50-1000	ESI ±	CID	Target Screener R package, MS, RT, MS/MS	Norman SusDat	AutoNon-Target R package, MS, RT, MS/MS, RT prediction, Exp. RTI match, Molecular Formula evaluation, MetFrag, FragPred, CMF-ID
I	70-1050	ESI ±	HCD	Trace Finder 4.1	In-house database (>29 000 compounds, sources: KEMI Market list; STOFF-ident; Uni. Athens Surfactant and Suspect list; Uni. Jaume I; Eawag; Targ_Sus_NT_WID; UFZ; PFAS Suspect list; KWR; Antibiotic list; Cosmetic products; NORMAN priority list; Swiss pesticides; Pharmaceuticals; MassBank; NormanNews; MzClouds)	Compound Discoverer 3.1, RT prediction, Massfrontiers, MetFrag, MzCloud,
K	100-1000	ESI ±	HCD	Xcalibur	In-house database (>1000 compounds), Norman SusDat mass list by KWR/Thermo	Compound Discoverer 3.1, MzCloud, MassBank, MS, RT, MS/MS, MetFrag, CSI-FingerID
L	100-1000	ESI ±	HCD	Compound Discoverer	Norman Nontarget list	Compound Discoverer, MS, MS/MS using MzCloud
M	100-1200	ESI ±	CID	In-house evaluation script (based on R with packages ggplot, shiny, xcms, and other), MetFrag, MzCloud, MoNA	In-house database (>800 compounds)	In-house evaluation script (based on R with packages ggplot, shiny, xcms, and other), MetFrag

N	70-1700	ESI ±	CID	MassHunter Qualitative B.07.00, MS, RT, MS/MS	n/a	MassHunter Qualitative B.07.00/ internal library, MassHunter PCDL Manager B.08.00 (Forensics, Metlin, Pesticides, Waters, E&L), MS, RT, MS/MS, Massbank, MetFrag
O	50-1000	ESI ±	CID	MSDial, MSFinder, R, MS, MS/MS	MS-DIAL "All public MS/MS", Norman SusDat	MS-Finder, MS, MS/MS
P	60-900	ESI ±	HCD	None	-	Compound Discoverer 3.1, MS, RT, MS/MS, RT prediction, Mass Frontier

ESI = electrospray ionisation, CID= collision-induced dissociation, HCD = higher energy CID, RT = retention time, DIA= data independent acquisition, MoNA= Massbank of North America, n/a = not available.

Table S4. Summary of gas chromatographic (GC) methods used by the participants.

Participant	Instrument and model	Column	Dimensions (mm x m, µm)	Carrier Gas	Injection volume (µL), injection mode	Temperature program
E	Thermo Q Exactive	Thermo Fisher TG-5MS	0.25 x 60, 0.25	He	2, splitless	60°C (5) – 5°C/min – 320°C (5)
G	Bruker timsToF (Scion 456-GC)	Restek Rxi-5Sil MS	0.18 x 40, 0.18	He	2, splitless	120°C (1.25) – 30°C/min – 180°C (0) – 20°C/min – 250° (0) – 9°C/min – 320°C (10.47)
H	Bruker 450 GC, QToF-MS Maxis Impact	Restek Rxi-5Sil MS	0.25 x 30, 0.25	He	1, splitless	55°C (3) - 15°C/min - 180°C (0) - 6.5°C/min - 280°C (5) - 10°C/min - 300°C (5.28)
J	Agilent 7250 GC/QToF	J&W/DB-5ms	0.25 x 30, 0.25	He	1, splitless	80°C (2) – 5°C/min – 300°C (2)
N	Agilent 7200 GC/QToF	Agilent HP-5MS	0.25 x 30, 0.25	He	1, pulsed splitless (25 psi)	50°C (2) – 10°C/min – 320°C (10)
O	Agilent 7250 GC/QToF	Agilent HP5-MS	0.25 x 30, 0.25	H_2	1, pulsed split	60°C (2) -15°C/min – 320°C(5)

Table S5. Summary of mass spectrometry and data processing procedures used by the participants for GC-HRMS analysis.

Participant	Scan range	Ionization	Target software	Suspect list for suspect screening	Suspect and nontarget procedure
E	60-900	EI	TraceFinder 4.1	Norman SusDat	NIST Library search, manual interpretation,
G	100-1000	APCI pos	none	In-house database (~ 150 compounds)	Haloseeker/in-house database + occasional manual search on SusDat (but LC amenable compounds produce numerous inaccurate hits on GC analysis), Library search, interpretation from in-source fragmentation patterns, occasional literature search
H	50-1000	APCI pos	Bruker Tasq 2.1, RT, MS, MS/MS	Norman SusDat	Mass accuracy, tR (experimental data from in-house database), MS/MS fragments (MzCloud and in-house database)
J	50-450	EI	n/a	n/a	NIST 2017, Library search, manual review, mass accuracy and RT check
N	30-980	EI	MassHunter Qualitative Analysis B.07.00	n/a	MassHunter Unknown Analysis, -NIST11 and W9N11 Libraries search, manual interpretation, mass accuracy
O	50-600	EI	MS-DIAL, R	Norman SusDat	MS-DIAL, R, Library search

EI = electron ionisation, APCI= atmospheric pressure chemical ionization, RT= retention time, n/a = not available.

3. Reference methods for extract preparation

3.1. Reference method for LC-HRMS

Freeze-dried whole fish homogenate (0.5 g) was added into homogenization tubes with ceramic beads. For the spiked extracts, LC-standard mixture (100 µL) was added, and the solvent was allowed to evaporate at room temperature for 30 min. Acetonitrile with 0.1 % formic acid (3 mL) was added, and the samples were extracted (2 x 40 s, 5000 rpm) in a Precellys tissue homogenizer (Bertin Technologies, France). After centrifugation (15 min, 20 °C, 3900 rpm) and filtration through a 0.2 µm regenerated cellulose syringe filter (Thermo Scientific, Rockwood, USA) into 2 mL Eppendorf safe-lock tubes (Eppendorf AG, Hamburg, Germany), the solution was frozen (-20 °C) for at least 16 h. The sample was then left at room temperature for 10-20 min before centrifugation (3 min, 20 °C, 10 000 rpm). Aliquots (200 µL) were transferred to auto-injector vials.

3.2. Reference method for GC-HRMS

Freeze-dried whole fish homogenate (3 g) was mixed with Na_2SO_4 (12 g). For the spiked extracts, GC-standard mix (75 µL) was added. The sample was then extracted by accelerated solvent extraction (3 cycles, 100 °C). A mixture of hexane and dichloromethane (2:1) was used as the extraction solvent. Isooctane (50 µL) was added, and the sample was concentrated on a rotary evaporator (30 °C) until 10 mL remained. A solid-phase cartridge (Strata FL-PR Florisil, 170 µm, 80 Å) was conditioned with 10 % isopropanol in dichloromethane (20 mL), followed by hexane (30 mL). The sample was loaded and eluted with dichloromethane in hexane (1:1, 20 mL), followed by hexane (20 mL). Again, isooctane (50 µL) was added, and the eluate was concentrated on a rotary evaporator (30 °C) until 10 mL remained. After adding more isooctane (50 µL), the sample was concentrated using a nitrogen stream. The extract was reconstituted in hexane (1 mL), vortex stirred for 1 min, and filtered through a 0.2 µm regenerated cellulose syringe filter. Aliquots (500 µL) were then transferred to auto-injector vials.

4. In-house methods for extract preparation

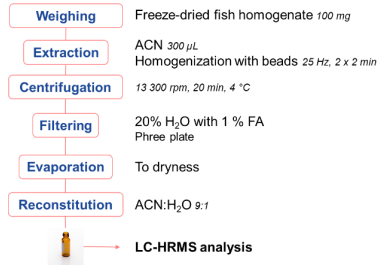
Table S6. Common steps of the in-house methods used by the participants and the reference method (Ref) for preparing extracts for LC-HRMS analysis. Each participant has been allocated a unique letter. If the same laboratory analysed extracts from several in-house sample preparation protocols they were designated additional numbers following the letter (e.g. K1, K2).

Sample preparation step	Participant(s)	% of participants
Amount freeze-dried whole fish homogenate		
1 g	H2	8
0.5 g	Ref, C, I	23
0.2 g	F, K1, K2, H1	31
100 mg	B	8
50 mg	D	8
NA	E, O, M	23
Extraction method		
Homogenization with beads	Ref, B, E, F, M	39
Ultrasonication, FUSLE	C, I, K1, K2, O, H1	46
Vortexing	D	8
Accelerated Solvent Extraction	H2	8
Extraction Solvent		
ACN with 0.1 % FA	Ref, C, D	23
ACN:Citric acid buffer 1:1	F	8
ACN	B, I	15
ACN:MeOH 1:2	H2	8
1. ACN:H_2O 1:1, 2. ACN	K2	8
1. ACN:MeOH:H_2O 2:2:1, 2. ACN:MeOH:H_2O with FA, 3. ACN:MeOH:H_2O with NH_3	K1	8
1. Sodium acetate buffer, 2. *n*-heptane, 3. ACN	M	8
ACN:MeOH:(H_2O with 0.1 % FA) 1:1:1	H1	8
Hexane:MeOH:H_2O:DCM 1:2:2:4	E	8
MeOH:methyl-*tert*-butyl ether 1:3	O	8
Additionals during extraction		
None	Ref, B, C, E, F, I, K1, O	62
$MgSO_4$:NaCl 4:1	D, K2	15
0.1 % EDTA	H1	8
Sodium sulfate	H2	8
Glucuronidase	M	8
Extraction temperature		
Not controlled/specified	Ref, B, C, D, F, H2	46
60 °C	H1	8
50 °C	H2	8
20 °C	K1, K2	15
0 °C	E, I, M	15
< 0 °C	O	8

Filtration		
Regenerated cellulose syringe filter 0.2-0.45 µm	Ref, C, H1, H2	31
Phree™ plate	B	8
Centrifugal filter 0.45 µm	D	8
Captiva ND-Lipid filters	I	8
None	E, F, K1, K2, O, M	46
Freezing		
-20 °C, ≥ 16 h	Ref, C, D	23
-20 °C, ≥ 12 h	I, H1	15
-20 °C, 48 h	K1, K2	15
None	B, E, F, O, H2, M	46
Additional clean-up		
None	Ref, B, C, D, I, K1, K2, O	62
µ-SPE	E	8
SPE, multilayer	F, H2	15
SPE, silica gel	M	8
n-hexane	H1, H2	15
RAM chromatography	M	8
Evaporation		
None	Ref, C	15
Yes, unspecified, to dryness	B, D	15
Yes, unspecified, to specific volume	M	8
In vacuo, to dryness	E, O	15
In vacuo, to specific volume	K1, K2	15
N_2 flow, to dryness	F, H1, H2	23
N_2 flow, to specific volume	F, I	15
Final solvent for analysis		
ACN with 0.1 % FA	Ref, C	15
ACN with > 0.1 % FA	M	8
ACN:H_2O 9:1	B	8
ACN:MeOH 1:1	K1, K2	15
MeOH	D, I	15
MeOH 5 %	E	8
MeOH 20 %	O	8
MeOH:H_2O 1:1	F, H1, H2	23

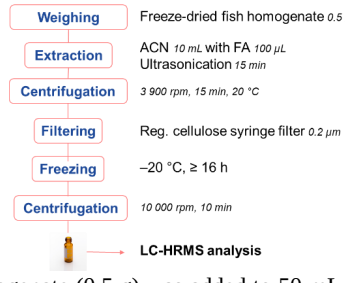
5.1. In-house methods for extracts to be analysed by LC-HRMS

In-house method B



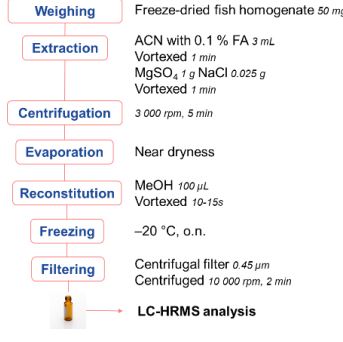
This is a modified version of a previously described sample preparation[2]. To freeze-dried whole fish homogenate (100 mg) was added acetonitrile (300 µL). The sample was homogenized with beads (25 Hz, 2 x 2 min with 20 min break), and then centrifuged (20 min, 13 300 rpm, 4 °C). After addition of 20 % ultrapure water with 1 % formic acid, the sample was filtrated on a Phree plate. The solvent was evaporated, and the sample reconstituted in acetonitrile:ultrapure water (9:1) before analysis.

In-house method C



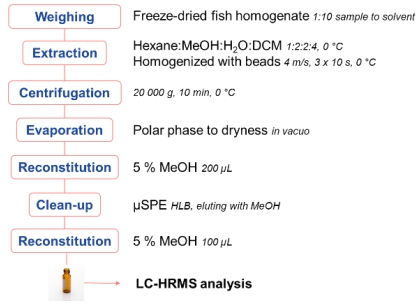
Freeze-dried whole fish homogenate (0.5 g) was added to 50 mL centrifuge tubes. For spiked samples only: LC standard mix (100 µL) was added, and the solvent was allowed to evaporate for 30 min. For all samples: acetonitrile (10 mL) and formic acid (100 µL) was added, and the tube was placed in an ultrasonic bath for 15 min. Sequentially, the sample was centrifuged (15 min, 3 900 rpm, 20 °C) and the supernatant filtered through a syringe filter (reg. cellulose, 0.2 µm) to an Eppendorf tube. The tube was stored at –20 °C for at least 16 h. Then, it was left at room temperature for 10-20 min and centrifuged (3 min, 10 000 rpm) prior to taking 200 µL of the supernatant for analysis. For blank samples, the same procedure was followed without addition of freeze-dried whole fish homogenate.

In-house method D



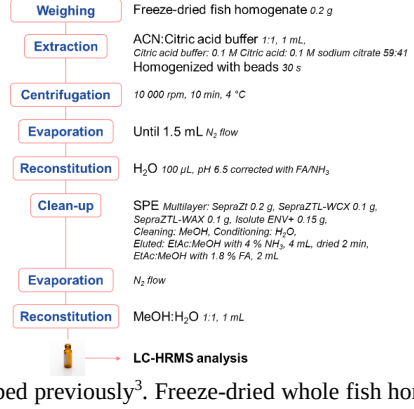
Freeze-dried whole fish homogenate (50 mg) was weighed up. For the spiked samples, provided standard mix (100 µL, 50 pg/µL) was added. Acetonitrile with 0.1 % formic acid (3 mL) was added, and the sample was vortexed for 1 min. $MgSO_4$ (1 g) and NaCl (0.25 g) was added, and the sample was vortexed again for 1 min, followed by centrifugation (5 min, 3 000 rpm). The supernatant was transferred, and concentrated near dryness. The sample was then reconstituted in methanol (100 µL), and vortexed for 10-15 s. After freezing the sample overnight (- 20 °C), the supernatant was transferred to a centrifugal filter (0.45 µm), and was centrifuged (2 min, 10 000 rpm). The extract was then transferred to a LC injection vial prior to analysis.

In-house method E



Freeze-dried whole fish homogenate was further homogenized with ice-cold hexane:methanol:water:dichloromethane (1:2:2:4) in a 1:10 sample to solvent ratio. Homogenization was performed with bead beating under liquid nitrogen cooling (3 cycles at 4 m/s and 0 °C, each cycle for 10 sec with 5 sec dwell) using 1.4 mm ceramic beads and a Bead Ruptor Elite connected to an Omni BR-Cryo cooling unit (Omni International, USA). After centrifugation (10 min, 20 000 g, 0 °C), the polar phase was collected and evaporated to dryness using a vacuum concentrator (SpeedVac SPD 1030, Thermo Scientific, Germany), and reconstituted in 200 µL of a 5% methanolic solution. Then was performed µSPE (10 mg HRP, Thermo) and elution with methanol, followed by reconstitution in 100 µL of a 5% methanolic solution.

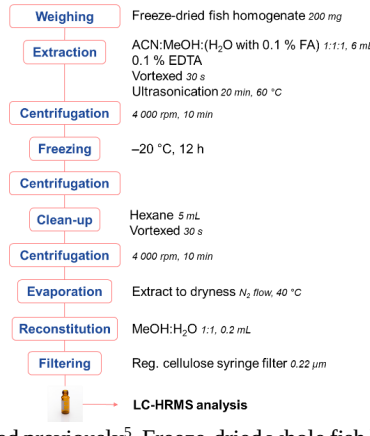
In-house method F



The method has been described previously[3]. Freeze-dried whole fish homogenate (0.2 g) was added to a 2 mL tissuelyzer tube with zirconium oxide beads (1 g). Solvent mixture (1 mL) of acetonitrile:citric acid buffer (1:1) (citric acid buffer from 0.1 M citric acid:0.1 M sodium citrate 59:41) was added. The tube was shaken (5 s) before being subjected to the tissuelyzer (30 s, power 5.5). The sample was then centrifuged (10 min, 4 °C, 10 000 rpm), and the supernatant transferred to a glass tube. More solvent mixture (1 mL) was added to the remaining pellet and the extraction process was repeated two more times. The combined supernatants were then concentrated under N_2 flow (30 min) until approximately

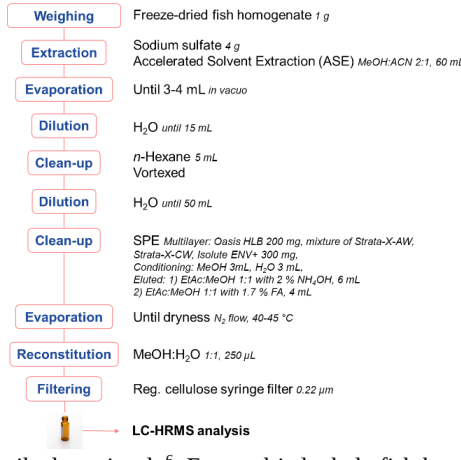
1.5 mL remained. HPLC-grade H_2O (100 mL) was added, and the pH was corrected to 6.5 using ammonia and/or formic acid. The solution was then passed through a homemade, multi-layer SPE cartridge containing Sepra ZT (0.2 g), Sepra ZTL-WCX (0.1 g), Sepra ZTL-WAX (0.1 g) and Isolute ENV+ (0.15 g) as described previously[4]. The cartridge had been cleaned with methanol and conditioned with HPLC-grade H_2O at pH 6.5. The sample was eluted with ethyl acetate:methanol with 2 % ammonia (4 mL), dried with air for 2 min, and then eluted again with ethyl acetate:methanol with 1.8 % formic acid (2 mL). The elute was dried by N_2 flow, and the sample was reconstituted in methanol:H_2O (1:1, 1 mL) prior to the analysis.

In-house method H1



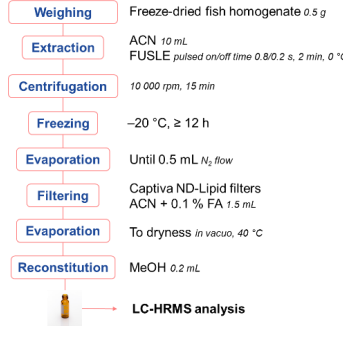
The method has been described previously[5]. Freeze-dried whole fish homogenate (200 mg) was weighed and placed into a 15 mL polypropylene centrifuge tube. The extraction of the analytes was realized by adding 2 mL of Milli-Q water containing 0.1% formic acid (v/v) and 0.1% EDTA (w/v), 2 mL of methanol and 2 mL of acetonitrile. After the addition of each solvent, the tube was vortex-mixed for 30 s. The sample set was placed in an ultrasonic bath at 60°C for 20 min, the samples were then centrifuged at 4000 rpm for 10 min, and the supernatant was decanted into a new polypropylene centrifuge tube. The tubes were then placed in the freezer, at -20 °C, for 12 h to precipitate the lipids and remaining proteins. After centrifuging and discarding the precipitate, a defatting step with hexane completed the sample clean-up. 5 mL hexane was added, and the tube was vortex-mixed for 30 s, centrifuged at 4000 rpm for 10 min, and finally the hexane layer was discarded. The extracts were collected in glass test tubes, evaporated to dryness under a gentle stream of N_2 at 40 °C, and reconstituted in 0.2 mL methanol/Milli-Q water, 50:50 (v/v). Finally, the extracts were filtered through a 0.22 µm RC syringe filter and were transferred to a glass vial for LC-HRMS analysis.

In-house method H2



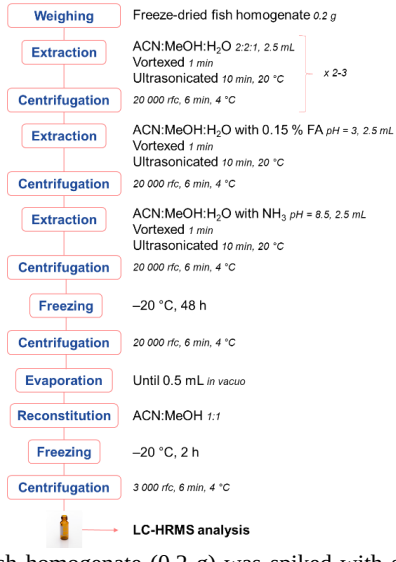
The method has been described previously[6]. Freeze-dried whole fish homogenate (1 g) was weighted and mixed with sodium sulfate (4 g) and then placed in extraction cells. The analytes were extracted by Accelerated Solvent Extractor (Dionex™ ASE™ 350, Thermo Fisher Scientific) with methanol and acetonitrile (2/1, v/v) as extraction solvents, using the following conditions: temperature: 50°C, pressure: 1500 psi, heating time: 300 s, static time: 420 s, 3 static cycles, purge time: 180 s and extraction solvents volume: 60 mL). After ASE, the extracts were pre-concentrated using a rotary evaporator (at 40°C) until reaching a final volume of 3-4 mL. Milli-Q water was added to adjust the final volume to 15 mL and 5 mL of *n*-hexane was added as defatting step. After vortex stirring, the hexane layer was discarded, and water was added until reaching a final volume of 50 mL. The samples were then cleaned-up by solid phase extraction (SPE). Layered 'mixed bed' in-house cartridges consisted of Oasis HLB (200 mg) and a mixture of Strata-X-AW (weak anion exchanger), Strata-X-CW (weak cation exchanger) and Isolute ENV+ (300 mg of total mixture) were used. Conditioning of the cartridges was performed with 3 mL methanol and 3 mL Milli-Q water. After conditioning, the samples were loaded in the SPE cartridges. The cartridges were dried and the elution of analytes from the adsorbent material was performed by a basic solution (6 mL of ethylacetate/methanol (50/50 v/v) containing 2% ammonia hydroxide (v/v)), followed by an acidic solution (4 mL of ethylacetate/methanol (50/50, v/v) containing 1.7% formic acid (v/v)). The extracts were evaporated using nitrogen stream at 40-45°C till dryness and 250 µL of methanol (LC-MS grade)/ Milli-Q water (50/50 v/v) were used for the final reconstitution of the extract. The final extract was filtered through a 0.22 µm RC syringe filter and were transferred to a glass vial for LC-HRMS analysis.

In-house method I



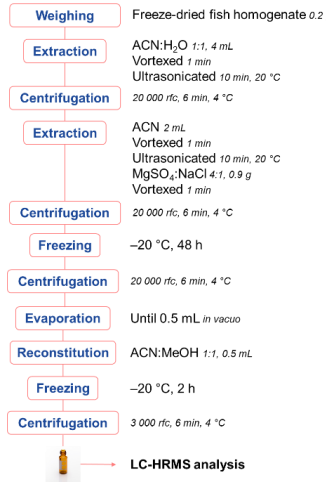
The method has been described previously[7]. To freeze-dried whole fish homogenate (0.5 g), acetonitrile (10 mL) was added, and focused ultrasound solid-liquid extraction (FUSLE) was carried out (0 °C, 2 min, pulsed on/off time of 0.8/0.2 s). The sample was centrifuged (15 min, 10 000 rpm) and the supernatant was frozen (- 20 °C) for at least 12 h, after which the new supernatant was transferred to a glass tube and concentrated using N_2 stream until 0.5 mL remained. Clean-up was performed using Captiva ND-Lipid filters. Acetonitrile containing 0.1 % formic acid (1.5 mL) was added to the cartridge, the sample was loaded and the mixture was five-fold mixed. The sample was eluted and the filter dried, after which the elute was dried *in vacuo* at 40 °C. The sample was then reconstituted in methanol (0.2 mL) prior to analysis.

In-house method K1



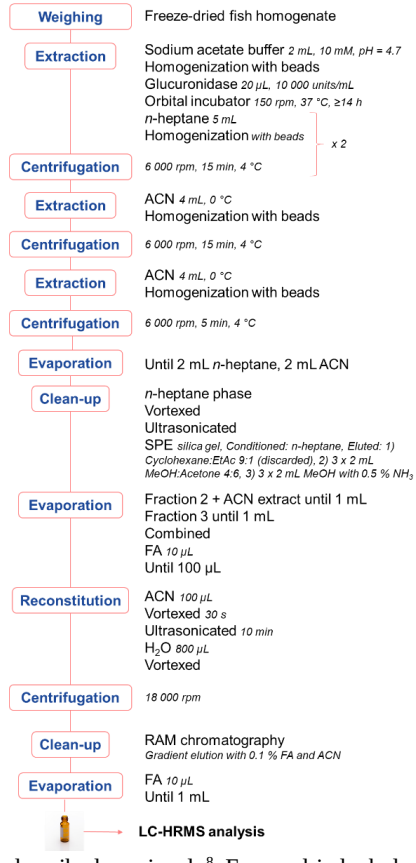
Freeze-dried whole fish homogenate (0.2 g) was spiked with standard mix and stored in the freezer overnight. Acetonitrile:methanol:H_2O (2.5 mL, 2:2:1) was added and the sample was vortexed (1 min) and sonicated (10 min, 20 °C, sonication capacity 9). After centrifugation (6 min, 4 °C, 20 000 rcf) the supernatant was transferred to an Eppendorf tube. More acetonitrile:methanol:H_2O (2.5 mL, 2:2:1) was added to the remaining pellet, and the steps were repeated until 2-3 supernatants had been transferred to the new Eppendorf tube. Acetonitrile:methanol:H_2O with 0.15 % formic acid (pH = 3) (2.5 mL) was added to the pellet, and again the extraction and centrifugation was repeated. The supernatant was transferred to a new Eppendorf tube. Then acetonitrile:methanol:H_2O with ammonia (pH = 8.5) (2.5 mL) was added to the pellet, the extraction and centrifugation was added, and the basic supernatant was transferred to the previous Eppendorf tube with the acidic supernatant. The extracts were frozen (-20 °C) for 48 h, and then centrifuged (6 min, 4 °C, 20 000 rcf). The supernatants were transferred to new Eppendorf tubes, and a Speed Vac was used to evaporate the samples until 0.5 mL. Acetonitrile:methanol (1:1) was added and the samples were frozen (-20 °C) for 2 h. After centrifugation (6 min, 4 °C, 3 000 rcf) the supernatant was transferred to 1.5 mL LC glass vials prior to analysis.

In-house method K2



Freeze-dried whole fish homogenate (0.2 g) was spiked with standard mix and stored in the freezer overnight. Acetonitrile:H_2O (4 mL, 1:1) was added and the sample was vortexed (1 min) and sonicated (10 min, 20 °C, sonication capacity 9). Quenchers salt (0.9 g, $MgSO_4$:NaCl 4:1) was added, and the sample was vortexed (1 min). After centrifugation (6 min, 4 °C, 20 000 rcf) the supernatant was transferred to an Eppendorf tube. Acetonitrile (2 mL) was added to the remaining pellet, and the extraction and centrifugation was repeated as above. The supernatants were combined, and frozen (-20 °C) for 48 h. After centrifugation (6 min, 4 °C, 20 000 rcf), the supernatant was transferred to a new Eppendorf tube and evaporated on a Speed Vac until 0.5 mL remained. Acetonitrile:methanol (0.5 mL, 1:1) was added, and the sample was frozen (-20 °C) for 2 h. After centrifugation (6 min, 4 °C, 3 000 rcf) the supernatant was transferred to a 1.5 mL LC glass vial prior to analysis.

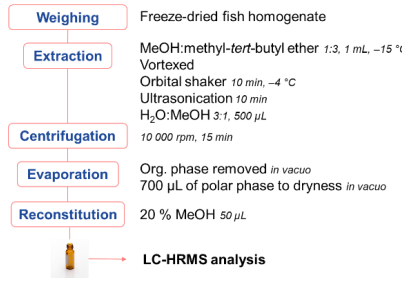
In-house method M



The method has been described previously[8]. Freeze-dried whole fish homogenate was mixed with garnet matrix A (500 mg, MP Biomedicals, Illkirch-Graffenstaden, France) and lysing matrix D (150 mg, MP Biomedicals). Sodium acetate buffer (2 mL, 10 mM, pH 4.7) and internal standard solution (100 µL, 0.01 mg/L) was added. The cells were disrupted by a FastPrep-24™ 5G (MP Biomedicals) with a CoolTeenPrep™ adapter (40 s, 4.0 m/s). β-Glucuronidase (20 µL, 10 000 units/mL) was added, and the samples were left in an orbital incubator SI500 (Stuart, Staffordshire, United Kingdom) for at least 14 h (150 rpm, 37 °C). *n*-Heptane (5 mL) was added, and the cells were disrupted by a FastPrep-24TM 5G (40 s, 4.0 m/s) before centrifugation (5 min, 6 000 rpm) with a Hettich Mikro 200R (Tuttlingen, Germany). The organic phase was removed, before more *n*-heptane was added and the extraction was repeated. After removing the *n*-heptane phase, the sample was further extracted through cell disruption with acetonitrile (4 mL, 0 °C) followed by centrifugation (15 min, 6 000 rpm, 4 °C). The supernatant was removed and the pellet was again extracted with acetonitrile through cell disruption as described and centrifugation (5 min, 6 000 rpm, 4 °C). The two *n*-heptane phases were combined, as were the two acetonitrile phases. The two resulting extracts were concentrated to 2 mL each. The *n*-heptane phase was vortexed and exposed to ultrasonication (10 min), before running silica gel SPE. The silica gel cartridge (6 mL, 1 000 mg, Chromabond, Machery-Nagel, Düren, Germany) was dried (85 °C, 3 h) and conditioned (3 x 2 mL *n*-heptane) before loading the *n*-heptane extract and eluting in three steps. 1) 3 x 2 mL cyclohexane:ethyl acetate (9:1, v/v) that was discarded, 2) 3 x 2 mL methanol:acetone (4:6, v/v) eluted directly into the previous acetonitrile extract, and 3) 3 x 2 mL

methanol with 0.5 $\%_v$ NH_3. Fractions 2 and 3 were concentrated to 1 mL and then combined. Formic acid (10 µL) was added. Further concentration until 100 µL was performed, and acetonitrile (100 µL) was added. The sample was vortexed (30 s) and exposed to ultrasonication (10 min). Milli-Q water (800 µL) was added, before vortexing and centrifugation (18 000 rpm). The supernatant was transferred, and subjected to RAM chromatography (Agilent 1260 system, G1364C fraction collector) with lichrospher RP-8 ADS (injection volume 500 µL, solvent A: 0.1 $\%_v$ formic acid, solvent B: acetonitrile, flow rate 1 mL/min, gradient: 0-3 min 2 % B, 3-3.5 min 2-60 % B, 3.5-8.5 min 60 % B, 8.5-9 min 60-98 % B, 9-14 min 98 % B, 14-14.5 min 98-2 % B, 14.5-20 min 2 % B). Collection of the elute was performed between 3-13 min. Formic acid (10 µL) was added, and concentration to 1 mL was conducted prior to analysis.

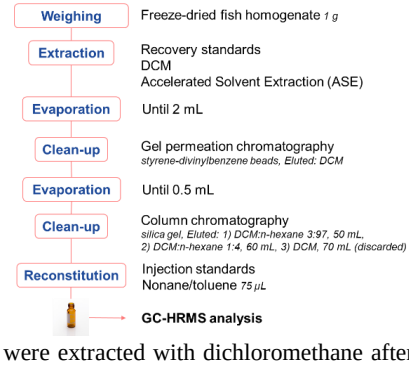
In-house method O



Pre-cooled extraction mixture (1 mL, - 15 °C, methanol:methyl-*tert*-butyl ether 1:3) was added to freeze-dried whole fish homogenate, and the sample was vortexed until fully re-suspended. The sample was incubated on an orbital shaker (10 min, - 4 °C), followed by an ultra-sonication bath (10 min). A mixture of water:methanol (500 µL, 3:1) was added, and the sample was mixed. After centrifugation, a portion of the upper organic phase was set aside for lipid analysis (not performed in this study), and the remaining organic phase was removed *in vacuo*. Of the remaining polar phase, a portion (700 µL) was transferred and dried *in vacuo*, before being reconstituted in 20 % methanol (50 µL) prior to analysis.

5.2. In-house methods for extracts to be analysed by GC-HRMS

In-house method G



Freeze-dried samples (1 g) were extracted with dichloromethane after addition of recovery standards (^{13}C or D-labelled: 17 ^{13}C-labelled organochlorine pesticides, 18 ^{13}C-labelled PCBs, 11 ^{13}C-labelled PBDEs, 1 ^{13}C-labelled MeO-PBDE, 1 ^{13}C-labelled OH-PBDE, 1 ^{13}C-labelled dichlorocarbazole, 5 ^{13}C-labelled bromophenols, ^{13}C-labelled triclosan and ^{13}C-labelled methyltriclosan) by accelerated solvent extraction (ASE, Dionex). The extract was concentrated to 2 mL and purified by gel permeation chromatography in a glass column (460 × 26 mm) filled with styrene-divinylbenzene beads (65 g of Bio-Beads S-X3) and eluted with a 5 mL/min flow of dichloromethane (175 mL first discarded, followed by the collection of a 175 mL fraction). After changing the solvent to hexane and concentrating to 0.5 mL, the extracts were fractionated on a 5 g silica column (5% H_2O) into 3 successive fractions of increasing polarity: [F1] 50 mL dichloromethane:*n*-hexane 3:97 (v/v), [F2] 60 mL dichloromethane:*n*-hexane 20:80 (v/v), and [F3] 70 mL dichloromethane, using an adapted version of an established method[9]. As previous work indicated that the vast majority of GC amenable halogenated compounds eluted in F1 and F2, F3 was not processed further. The extracts were finally spiked with injection standards (3 ^{13}C-labelled PCBs, 4 ^{13}C-labelled PBDE, 2 D-labelled DDT derivatives, 1 ^{13}C-labelled MeO-PBDE, 1 ^{13}C-labelled OH-PBDE and 1 ^{13}C-labelled tetrachlorocarbazole) and reconstituted in nonane/toluene (75 µL).

6. Workflows for suspect and non-target screening

Common steps for the LC-HRMS workflows were:

a) Peak picking
b) Filtration criteria (examples:
    i. mass error < 10 ppm or 5 ppm or 3 ppm
    ii. fold-change > 10
    iii. minimum peak intensity > 1000
    iv. Minimum peak area > 50 000 (HESI+) and > 1000000 (HESI-)
    v. Minimum peak height > 300
    vi. RSD of triplicates < 20% or 30%
    vii. Predicted RT
    viii. Molecular formula containing Cl, Br, S
    ix. Suspect masslist match
c) MS^2 available for comparison in e.g., MzCloud

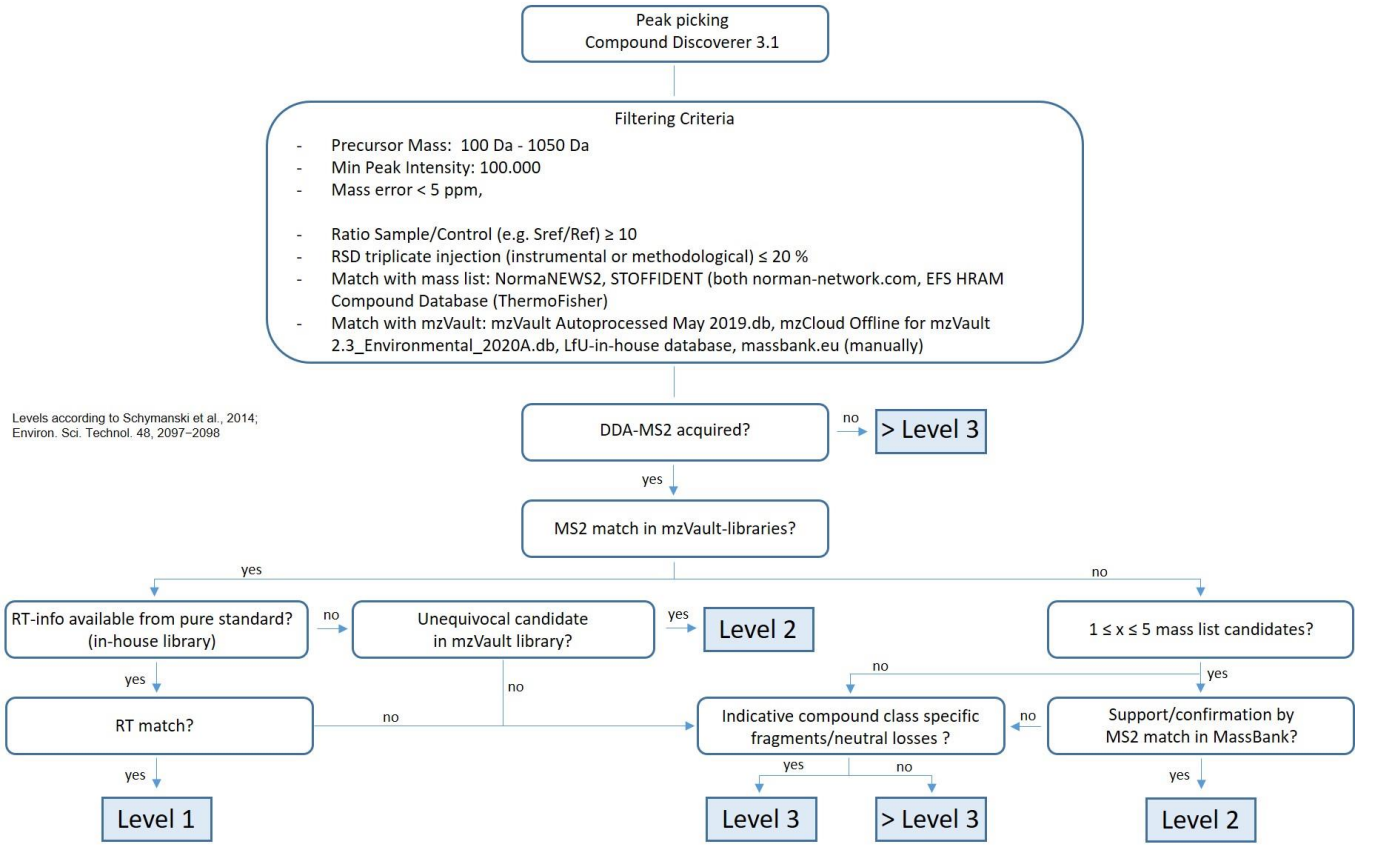


Figure S1. Suspect and non-target screening workflow for LC-HRMS analysis of the participating laboratory A.

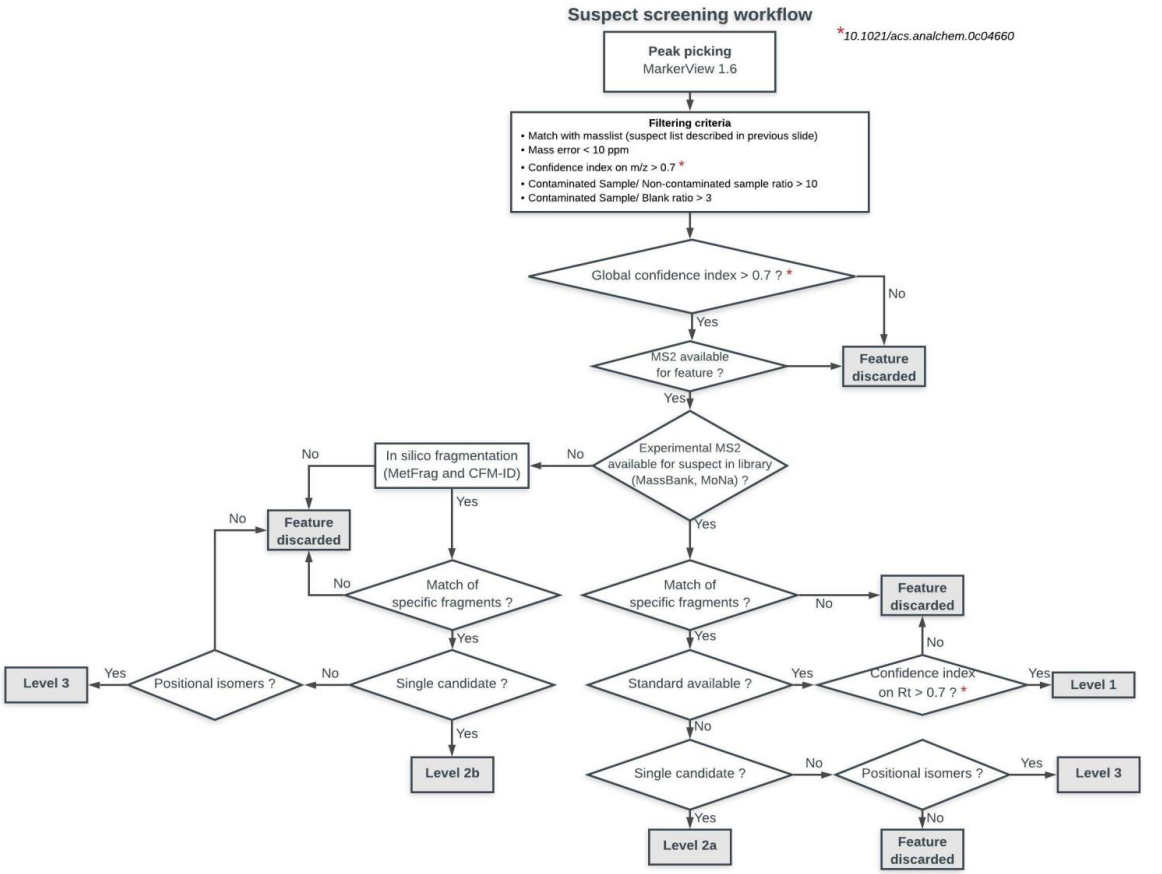


Figure S2. Suspect screening workflow for LC-HRMS analysis of the participating laboratory B according to Chaker et al (2021)[1].

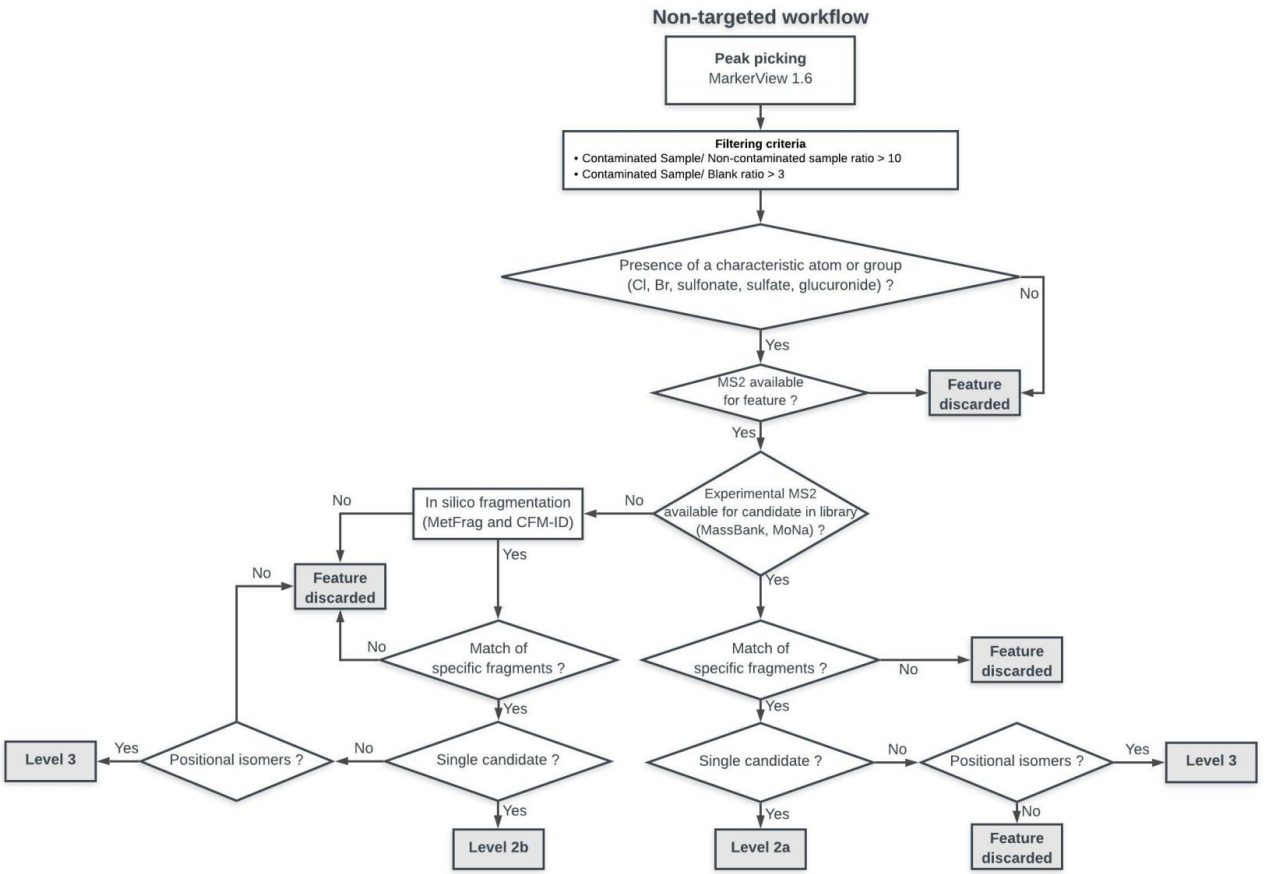


Figure S3. Non-target screening workflow for LC-HRMS analysis of the participating laboratory B.

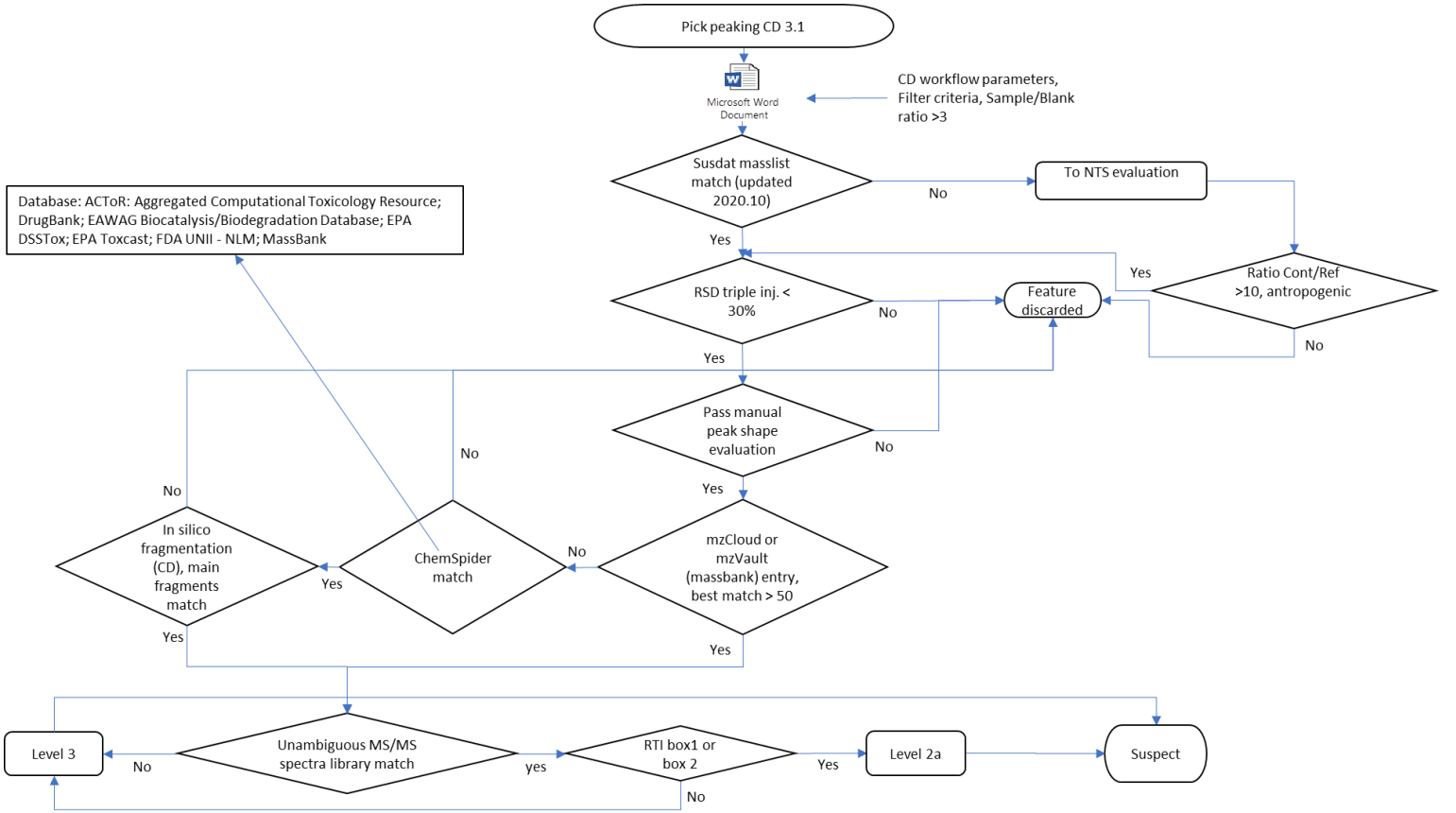


Figure S4. Suspect and non-target screening workflow for LC-HRMS analysis of the participating laboratory C.

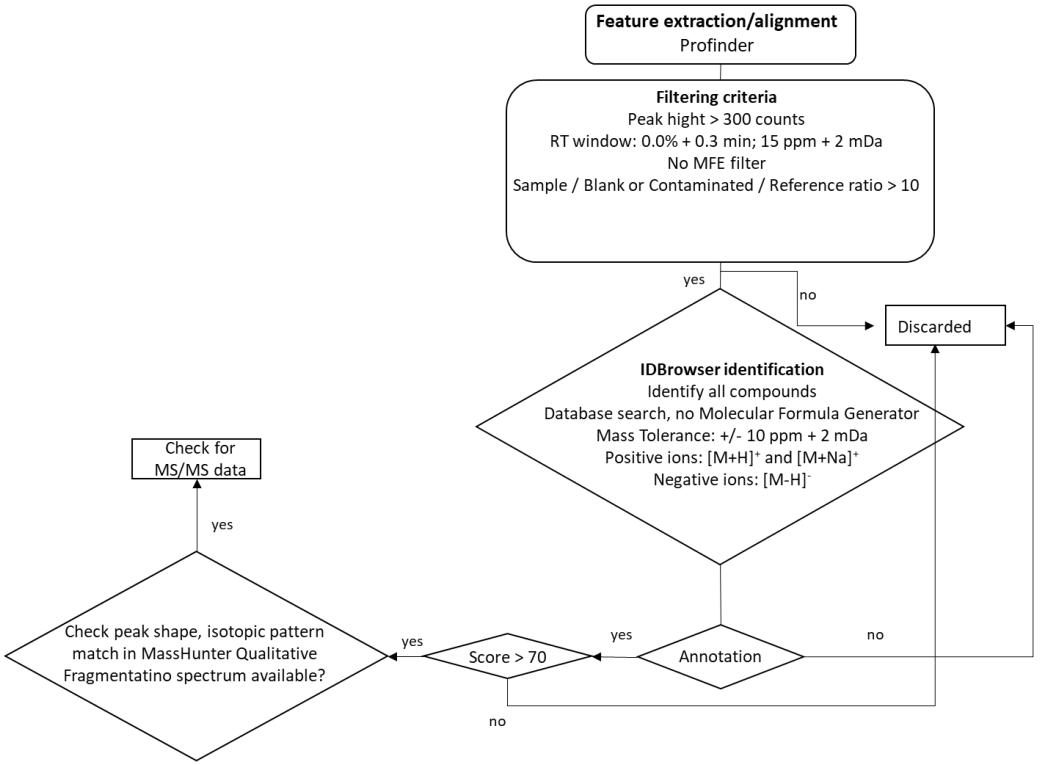


Figure S5. Suspect and non-target screening workflow for LC-HRMS analysis of the participating laboratory D.

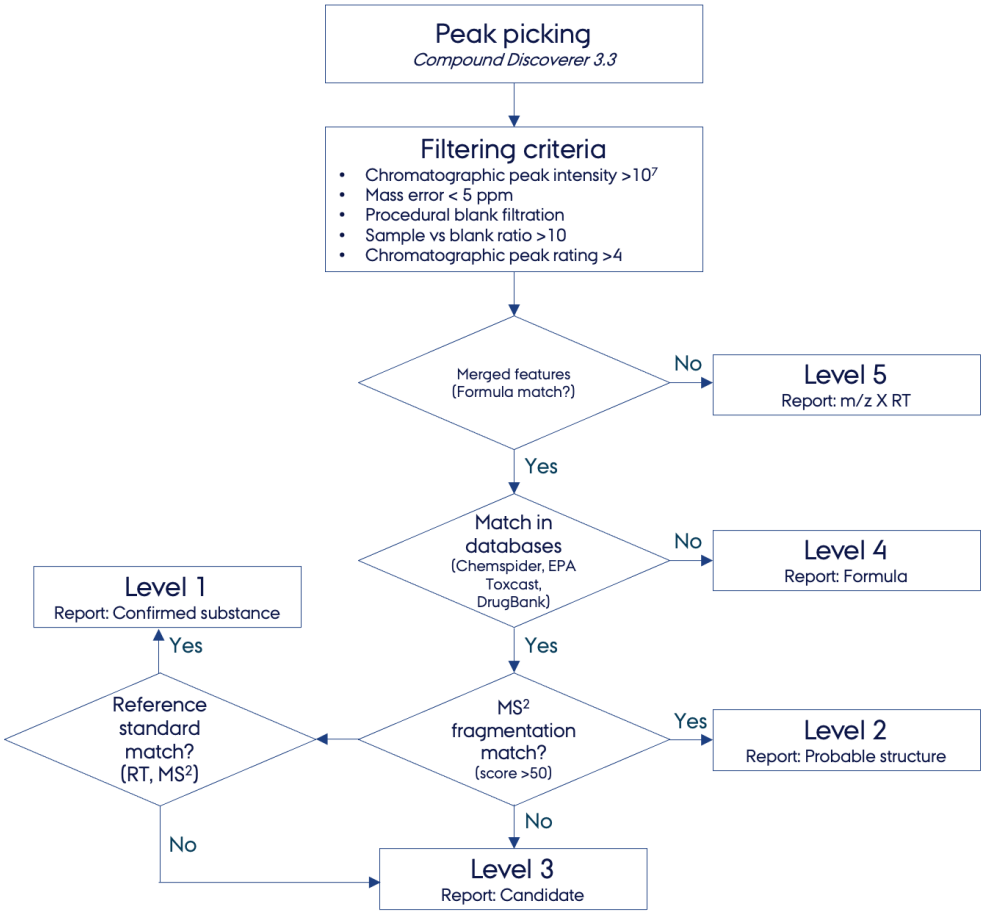


Figure S6. Suspect and non-target screening workflow for LC-HRMS analysis of the participating laboratory E.

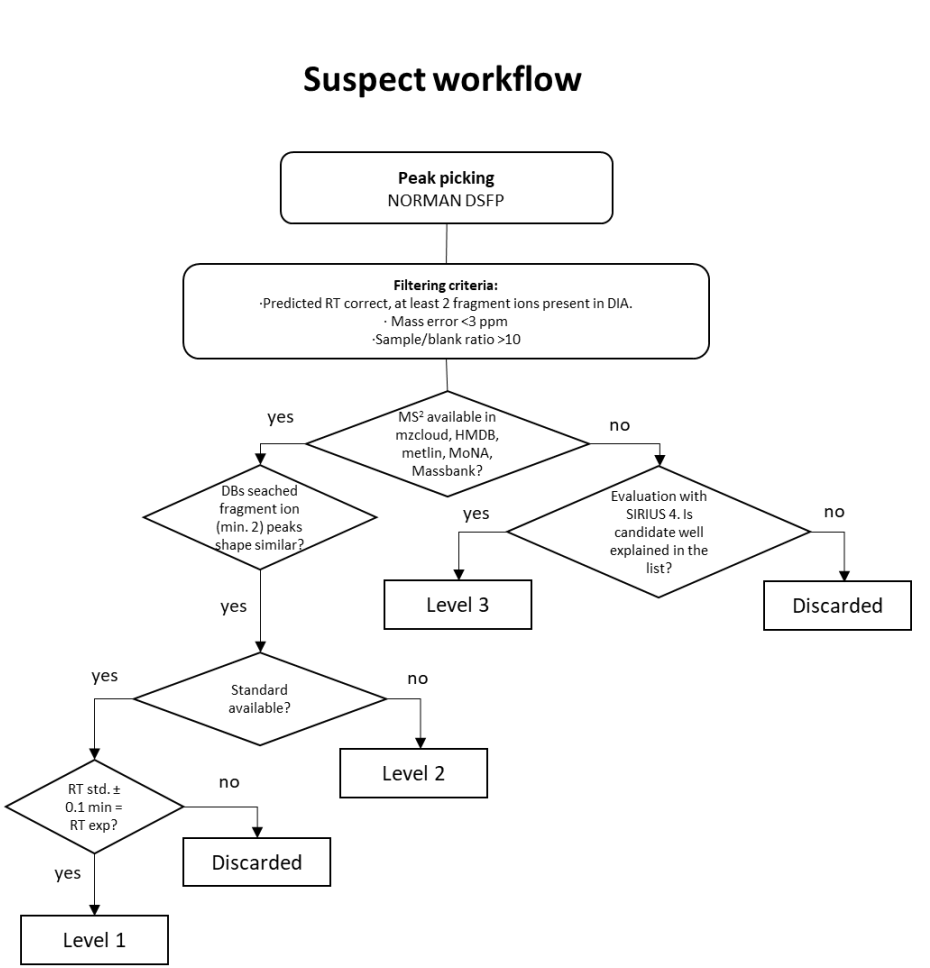


Figure S7. Suspect screening workflow for LC-HRMS analysis of the participating laboratory F.

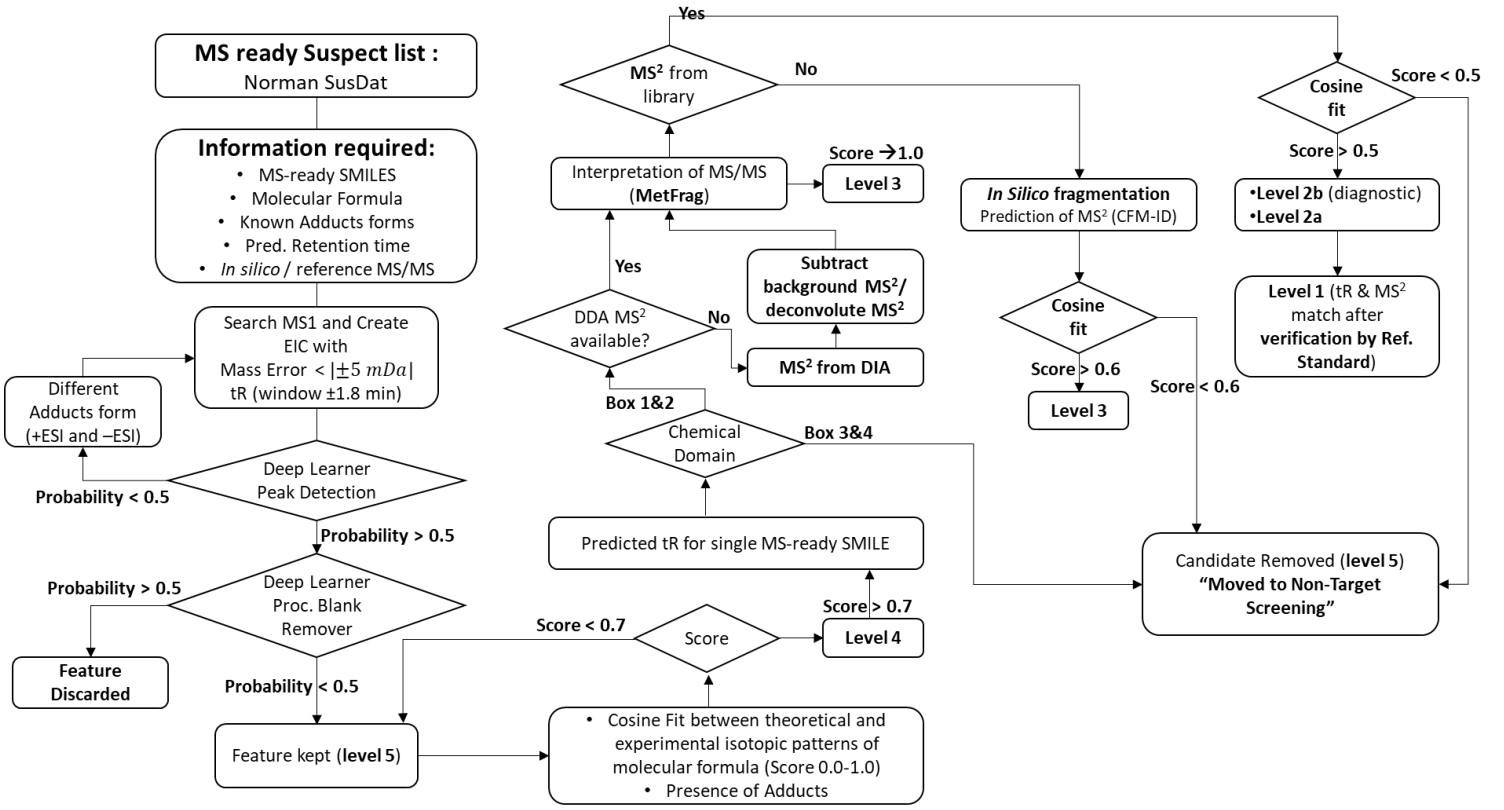


Figure S8. Suspect screening workflow for LC-HRMS analysis of the participating laboratory H.

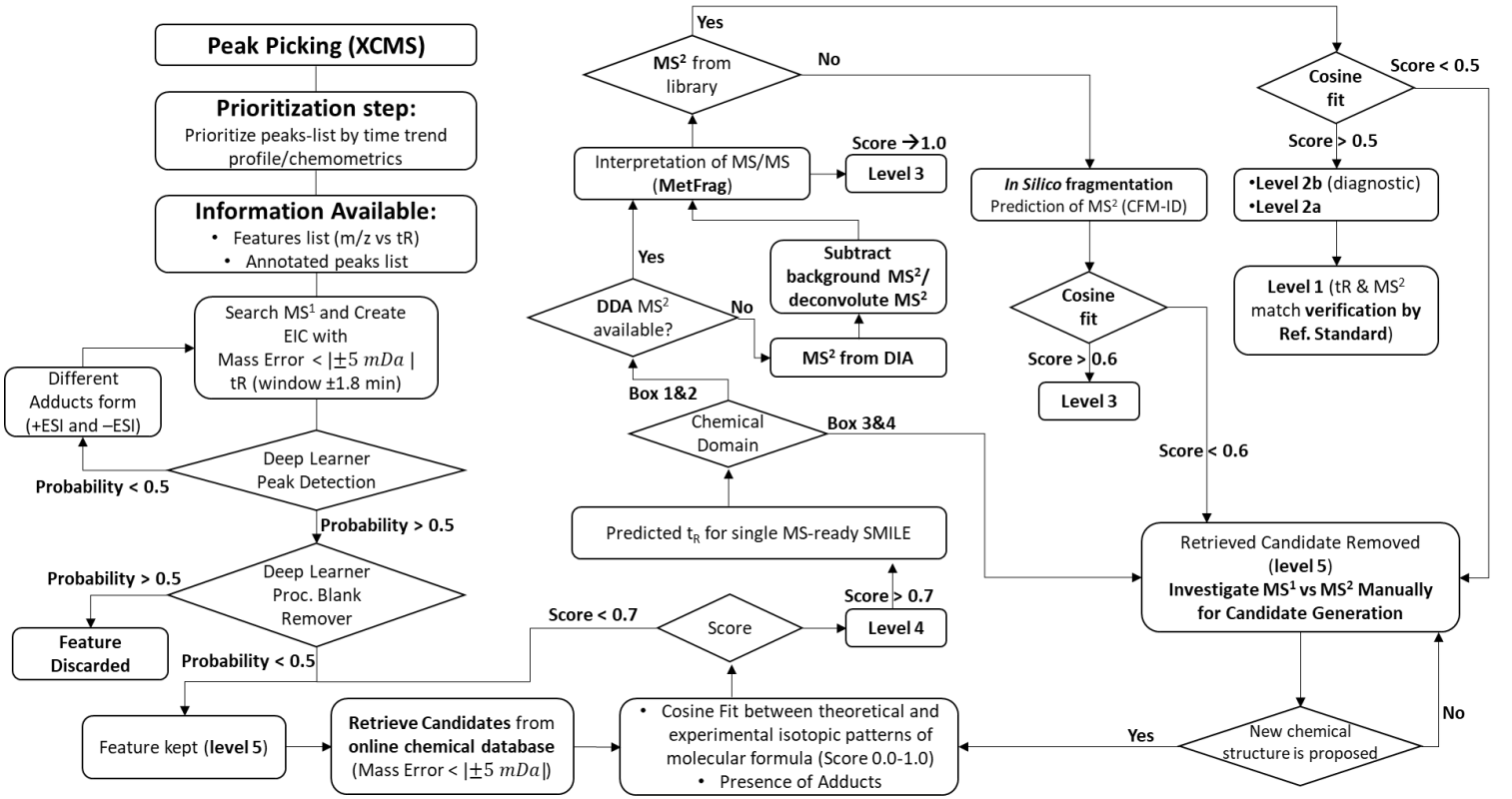


Figure S9. Non-target screening workflow[10] for LC-HRMS analysis of the participating laboratory H.

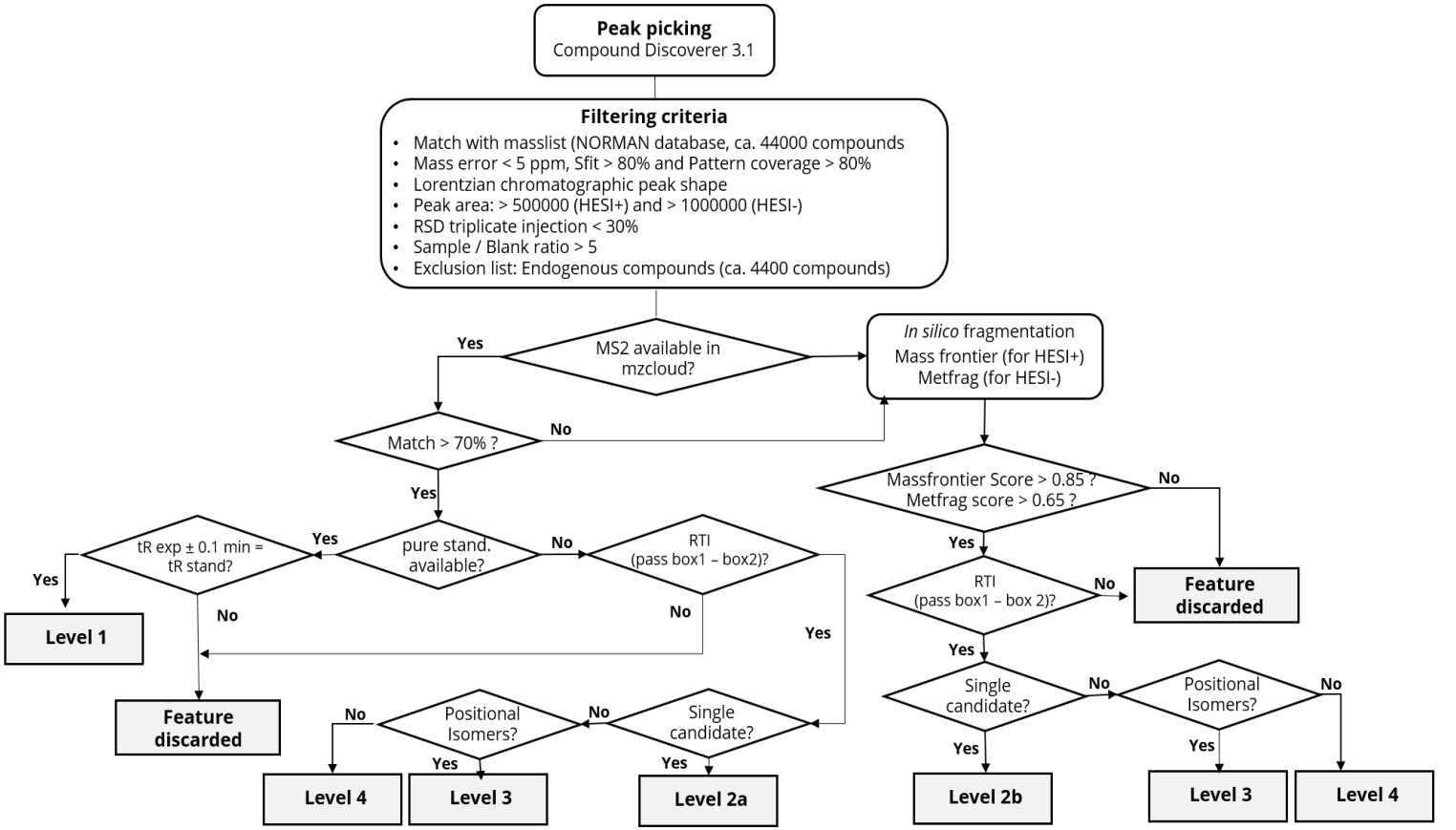


Figure S10. Suspect screening workflow for LC-HRMS analysis of the participating laboratory I.

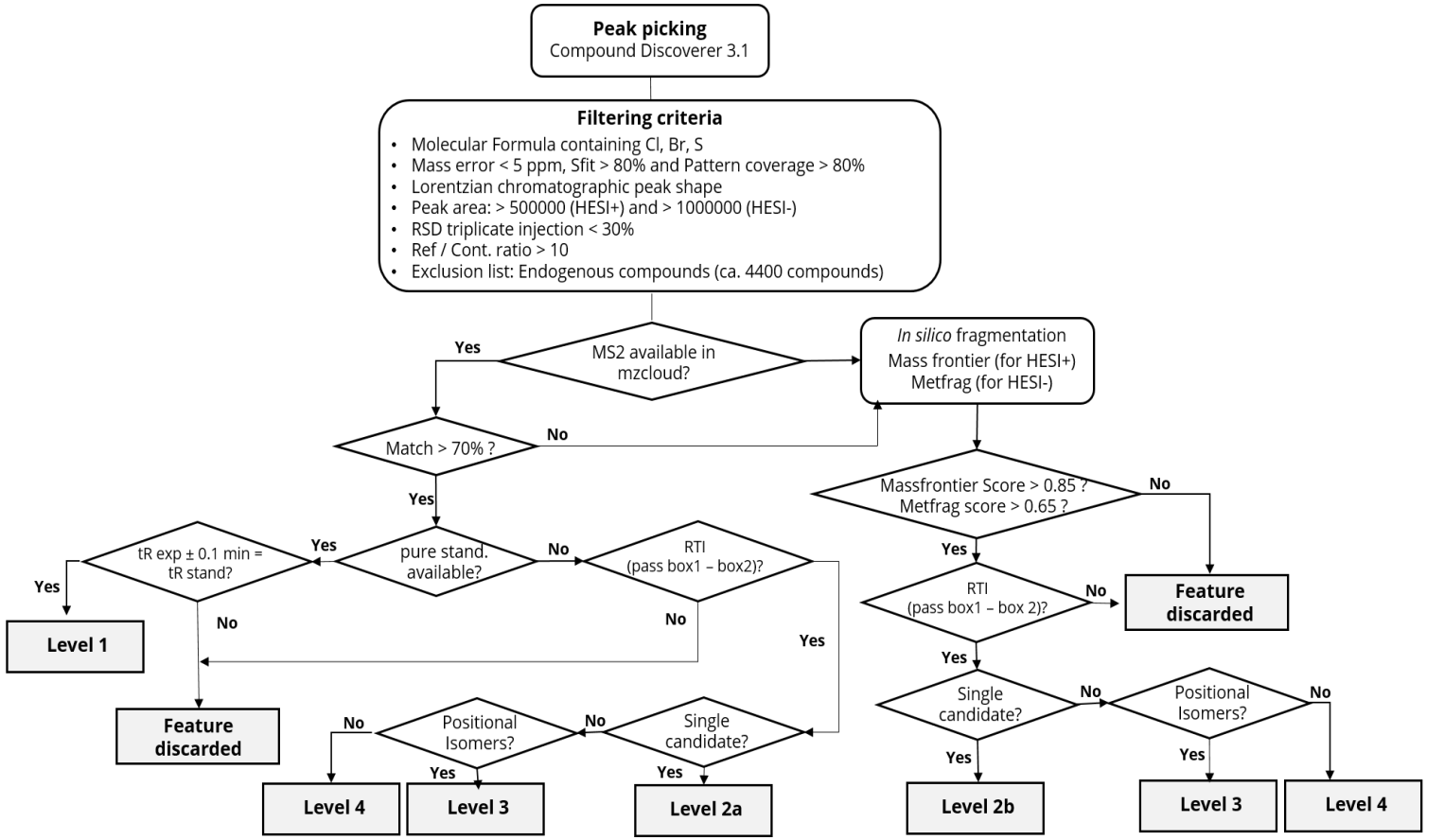


Figure S11. Non-target screening workflow for LC-HRMS analysis of the participating laboratory I.

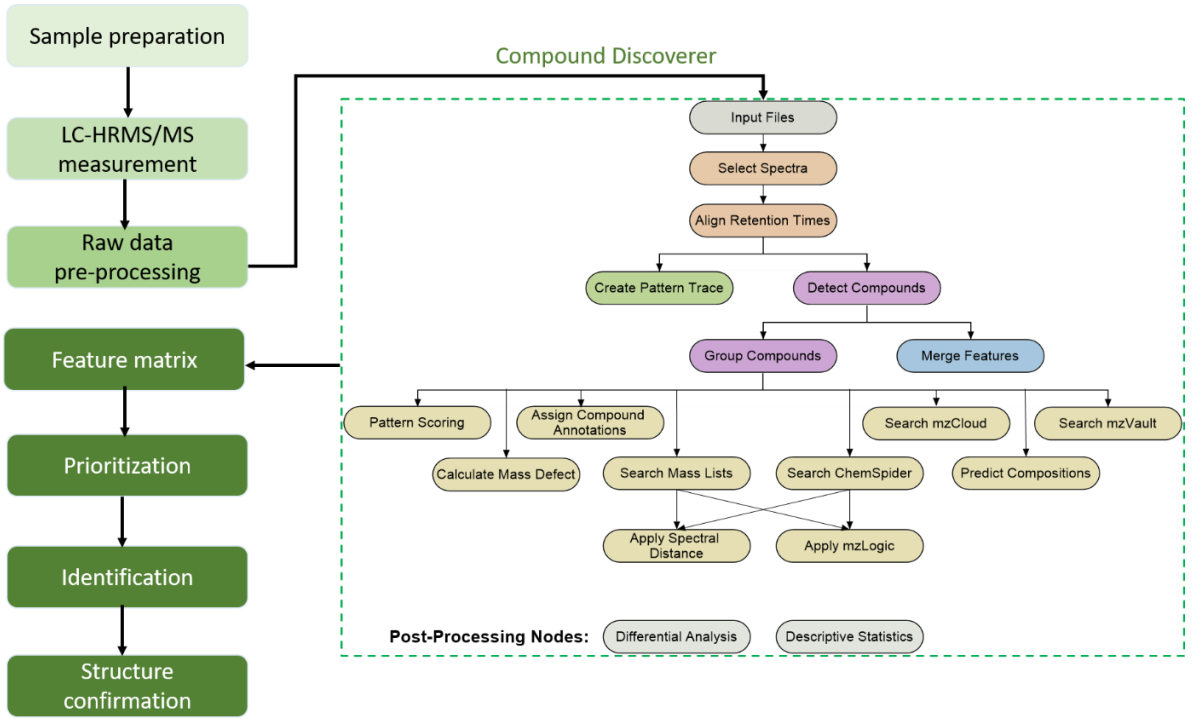


Figure S12. Suspect screening workflow for LC-HRMS analysis of the participating laboratory K.

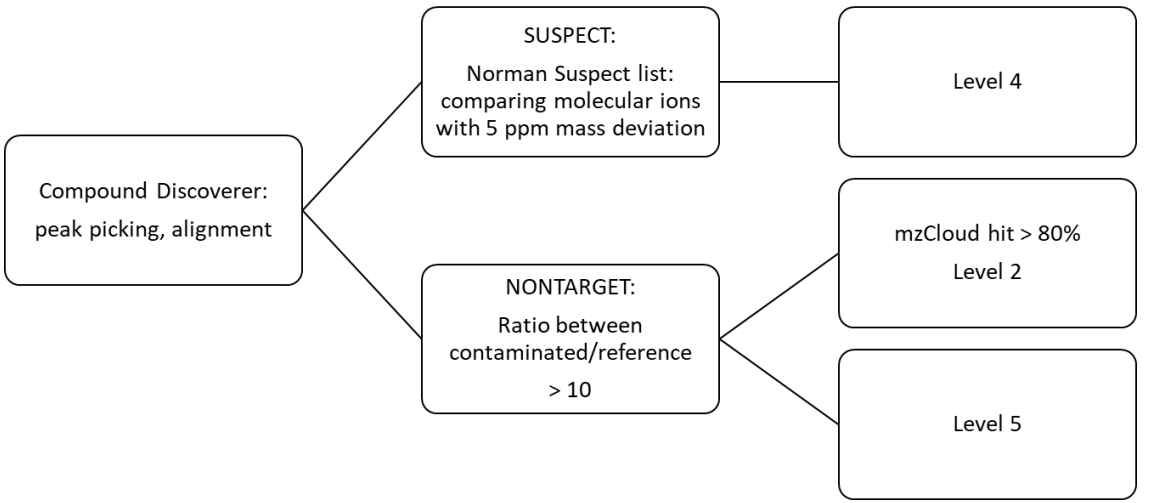


Figure S13. Suspect and non-target screening workflow for LC-HRMS analysis of the participating laboratory L.

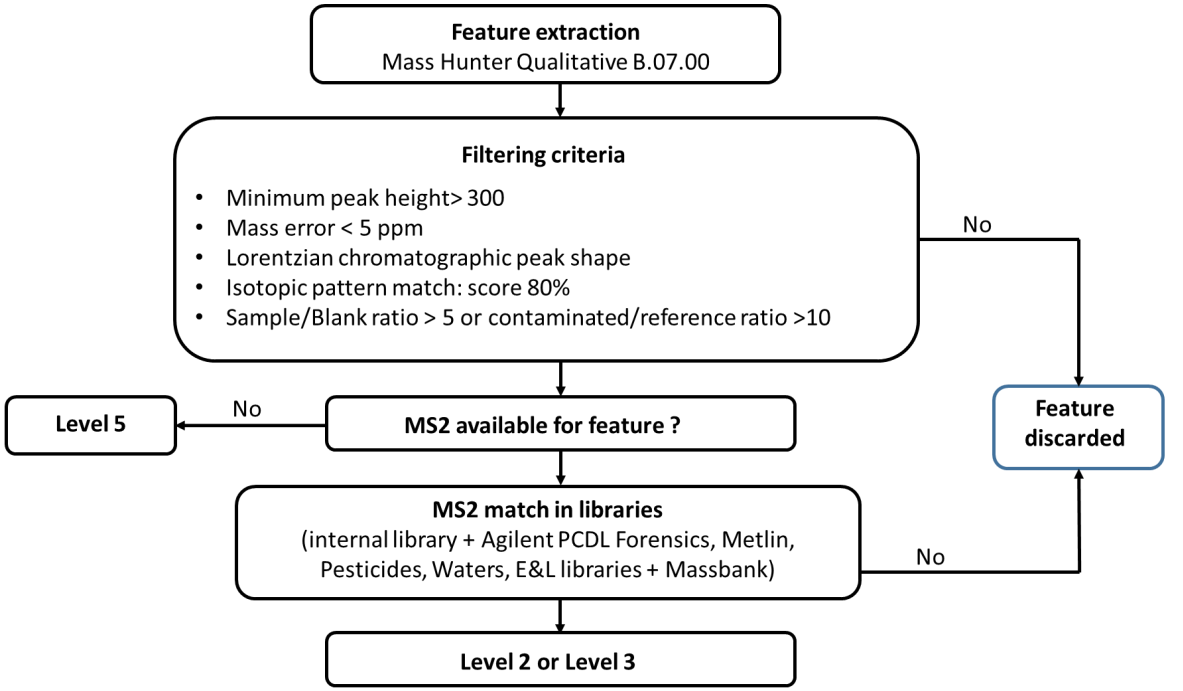


Figure S14. Suspect and non-target screening workflow for LC-HRMS analysis of the participating laboratory N.

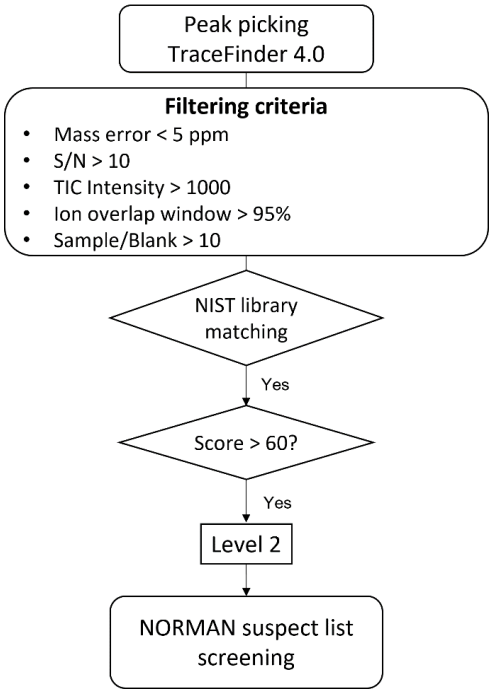


Figure S15. Suspect screening workflow for GC-HRMS analysis of the participating laboratory E.

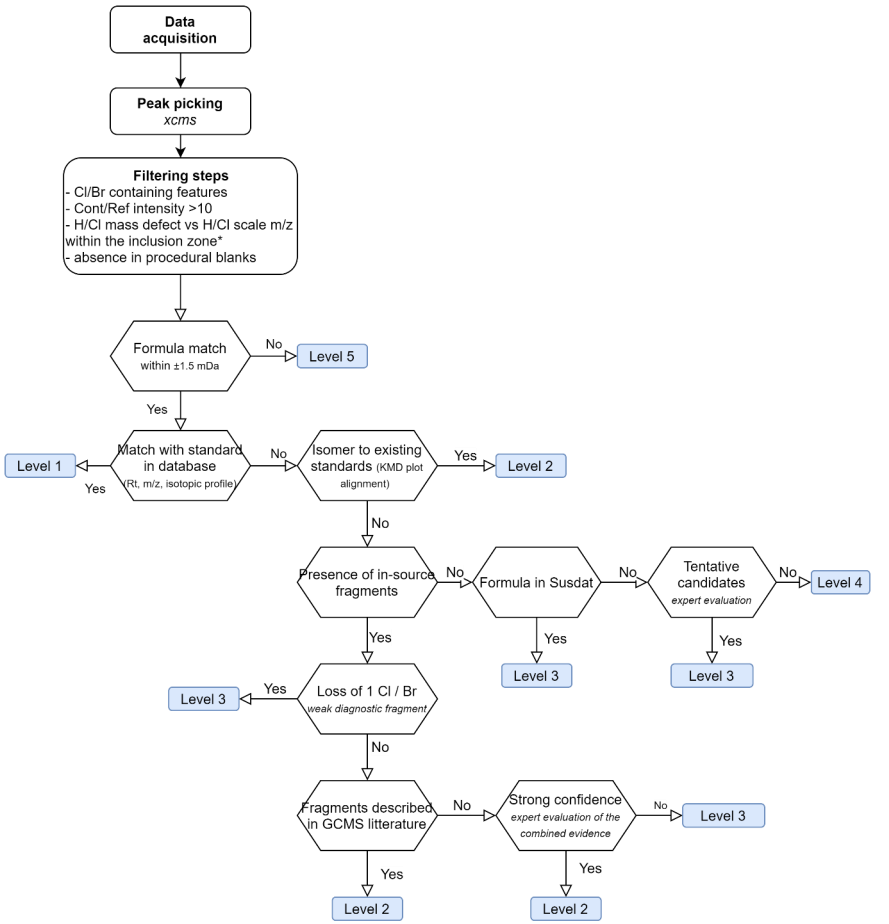


Figure S16. Suspect and non-target screening workflow for GC-HRMS analysis of the participating laboratory G.

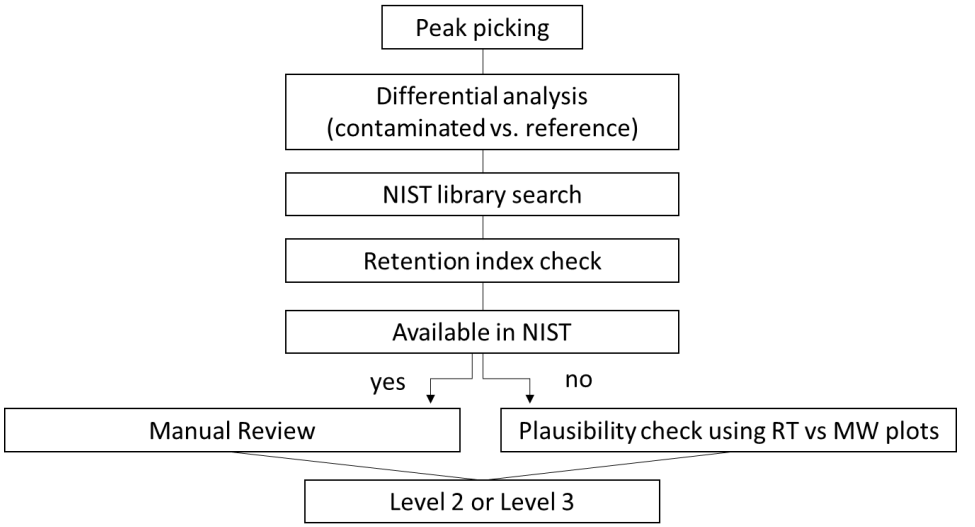


Figure S17. Non-target screening workflow for GC-HRMS analysis of the participating laboratory J.

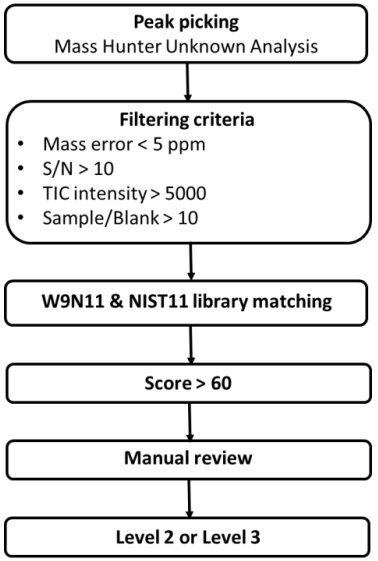


Figure S18. Non-target screening workflow for GC-HRMS analysis of the participating laboratory N.

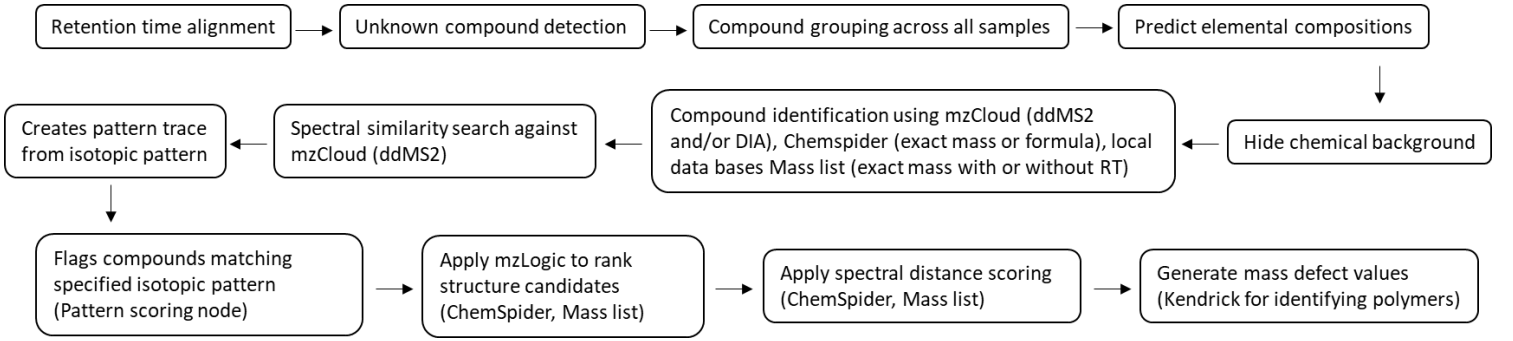


Figure S19. Non-target screening workflow for LC-HRMS analysis of the participating laboratory P.

7. Detection of compounds from spiked samples

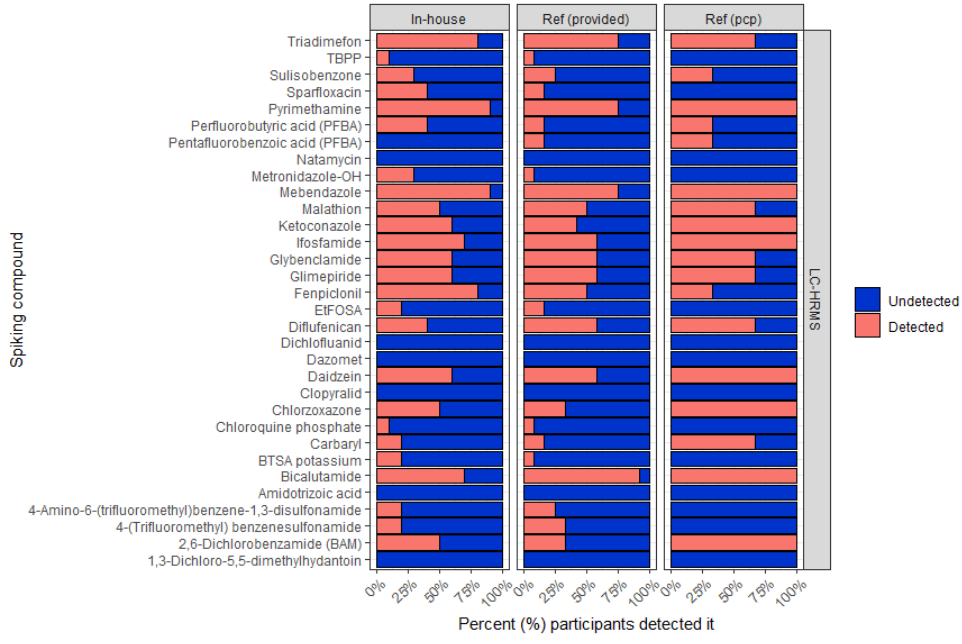


Figure S20. The percentage of participants (n = 10, 12 and 3 for In-house, Ref(provided) and Ref(pcp), respectively) detecting the compounds added during the spiking of fish samples for LC-HRMS analysis, divided on the different sample preparation methods.

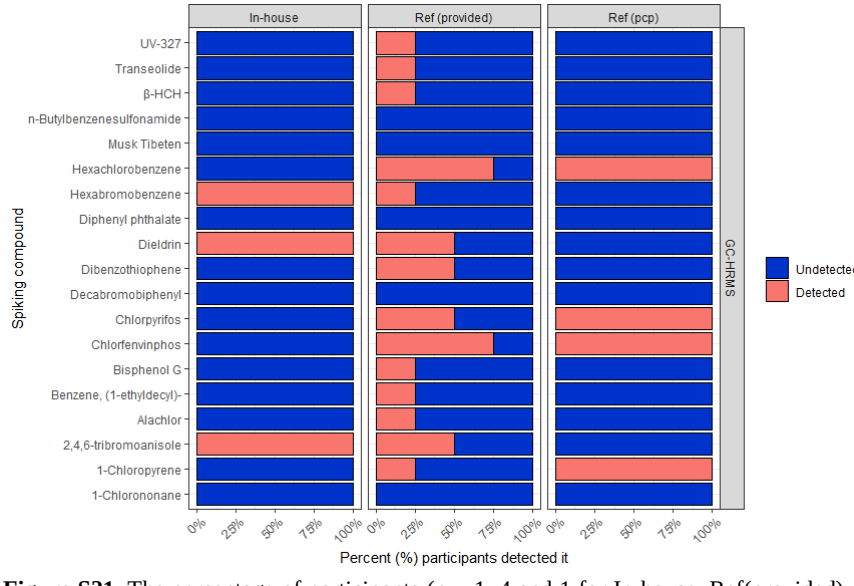


Figure S21. The percentage of participants (n = 1, 4 and 1 for In-house, Ref(provided) and Ref(pcp), respectively) detecting the compounds added during the spiking of fish samples for GC-HRMS analysis, divided on the different sample preparation methods.

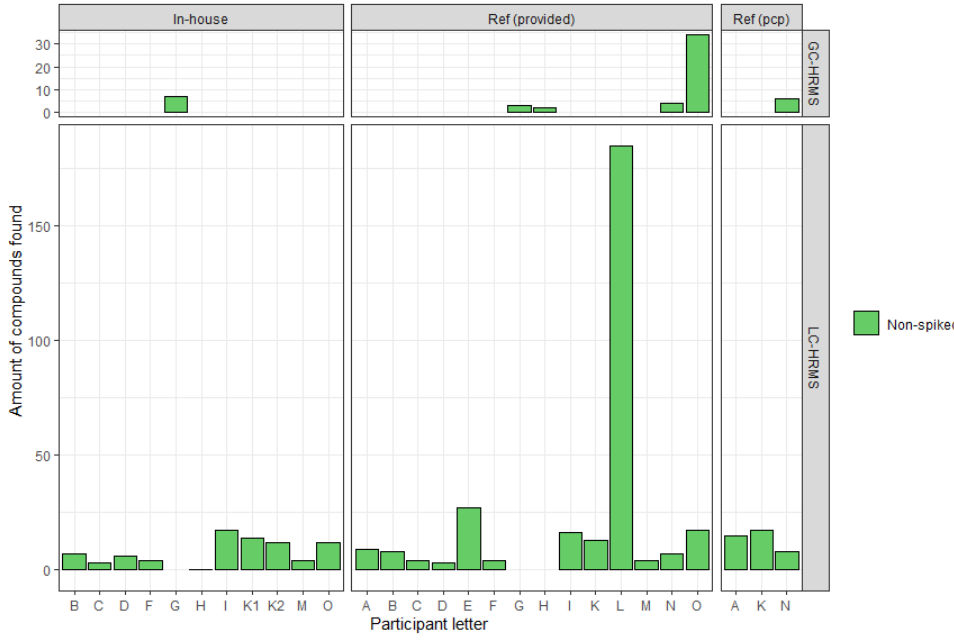


Figure S22. The number of compounds that were not added during spiking, detected from the spiked samples by the different participants.

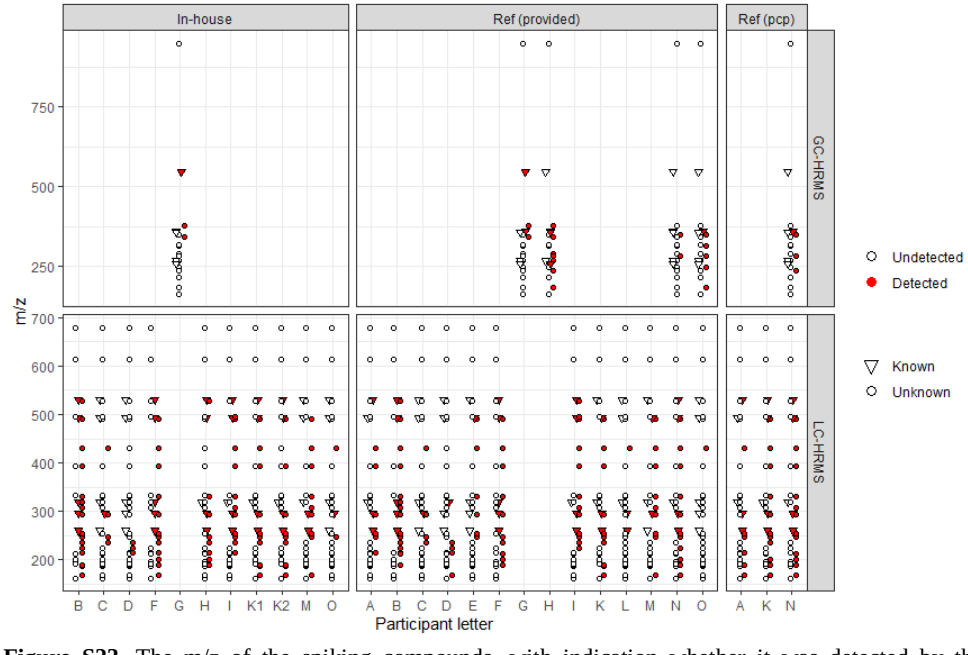


Figure S23. The m/z of the spiking compounds, with indication whether it was detected by the participant (red fill) or not (white fill). The compounds known to the participants are depicted by a triangle, and the unknown compounds by circles.

Table S7. Overall medians and means with standard deviations of the percentages indicating how many spiked compounds were correctly identified by the participants of each group (divided on sample preparation method, method for analysis and whether the compound was known or unknown).

Method of analysis	Method of sample preparation	Type of compound	Mean and standard deviation (%)	Median (%)	# Participants
LC-HRMS	In-house	Known	60 ± 37	80	10
		Unknown	32 ± 17	33	
	Ref (provided)	Known	51 ± 34	50	12
		Unknown	28 ± 17	28	
	Ref (pcp)	Known	73 ± 12	80	3
		Unknown	36 ± 12	41	
GC-HRMS	In-house	Known	20	20	1
		Unknown	14	14	
	Ref (provided)	Known	30 ± 26	30	4
		Unknown	29 ± 17	25	
	Ref (pcp)	Known	20	20	1
		Unknown	21	21	

8. Detection of compounds from samples of Teltow Canal

Table S8. A list of the compounds, with predicted log K_{OW} from EPI Suite 4.0 and m/z, from bream in the Teltow Canal that was identified through LC-HRMS and suspect screening by at least two of the participating laboratories with the same sample preparation method. Ref (provided) corresponds to the obtained extract, Ref (pcp) to the extract prepared by the participants themselves following the reference method, and in-house to the extract prepared by the participants through their own protocols.

Compound	Log K_{OW}	m/z	Sample preparation method(s)	No. of times found
N-(6-Aminohexyl)-4-hydroxybutyramide	-0.19	202.17	Ref (provided)	2
			Ref (pcp)	2
1-Naphthol	2.69	144.06	Ref (provided)	2
			Ref (pcp)	2
2-(Decylsulfanyl)ethan-1-ol	4.37	218.17	In-house	3
			Ref (provided)	2
			Ref (pcp)	3
Acridine	3.32	179.07	In-house	2
			Ref (provided)	2
			Ref (pcp)	2
4-(1,1,3,3-Tetramethylbutyl)phenol	5.28	206.17	Ref (provided)	2
			Ref (pcp)	2
Alachlor-OXA	1.55	265.13	In-house	2
Galaxolidone	5.26	272.18	In-house	3
			Ref (provided)	3
			Ref (pcp)	3
Estradiol	3.94	273.18	Ref (provided)	2
Fenuron	1.38	164.09	In-house	2
			Ref (provided)	3
Eicosapentaenoic acid (Icosapent)	7.85	303.22	In-house	2
			Ref (provided)	2
Amorolfine	6.00	317.27	In-house	3
			Ref (provided)	2
Megestrol	3.41	342.22	Ref (provided)	2
			Ref (pcp)	2
1,2,3-Benzotriazole	1.17	119.13	In-house	3
			Ref (provided)	2
2-(Methylthio)benzothiazol	3.22	181.00	Ref (provided)	2
			Ref (pcp)	2
Ibuprofen	3.79	206.13	In-house	4
			Ref (provided)	2
			Ref (pcp)	2
Perfluorooctanesulfonic acid (PFOS)	4.49	500.13	In-house	2
			Ref (provided)	4
			Ref (pcp)	2

Table S9. The ranges of log K_{OW}'s and m/z detected by the different methods for identification, analysis and sample preparation of fish samples from Teltow Canal. The number of identified compounds refer to unique chemicals within the specified group, not counting reports of m/z with a molecular formula as sole identifier.

Method of identification	Method of analysis	Method of sample preparation	Min log K_{OW}	Max log K_{OW}	Min m/z	Max m/z	# Identified compounds
Suspect	LC-HRMS	Ref (provided)	-5.16	16.13	82.04	760.58	1109
Suspect	LC-HRMS	In-house	-9.85	16.11	68.03	748.51	750
Suspect	LC-HRMS	Ref (pcp)	-9.85	16.13	98.12	760.59	1260
Suspect	GC-HRMS	Ref (provided)	-1.27	14.12	83.05	485.71	47
Suspect	GC-HRMS	In-house	5.22	8.91	235.01	643.53	32
Suspect	GC-HRMS	Ref (pcp)	-	-	-	-	0
Non-target	LC-HRMS	Ref (provided)	-0.73	8.87	126.04	497.66	29
Non-target	LC-HRMS	In-house	-7.52	8.76	161.12	714.33	37
Non-target	LC-HRMS	Ref (pcp)	-0.73	10.24	135.01	499.94	22
Non-target	GC-HRMS	Ref (provided)	-0.59	14.12	96.17	452.94	79
Non-target	GC-HRMS	In-house	2.57	10.03	208.95	509.73	26
Non-target	GC-HRMS	Ref (pcp)	0.06	14.12	68.08	448.75	22

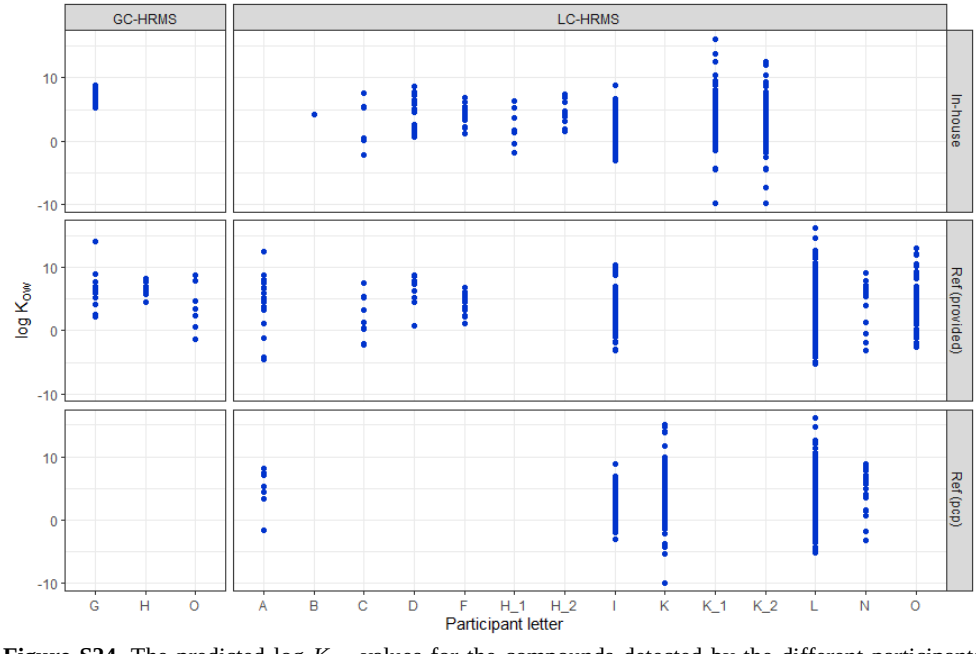


Figure S24. The predicted log K_{OW} values for the compounds detected by the different participants through suspect screening of the fish samples from Teltow Canal. The data is divided into sample preparation method (In-house, Ref (provided) and Ref (pcp)) and method of analysis (LC-HRMS and GC-HRMS). Reported compounds that were ambiguously identified (not containing a name/SMILES/other identifier, or containing several for the same m/z) were excluded from this figure since no single log K_{OW} could be calculated.

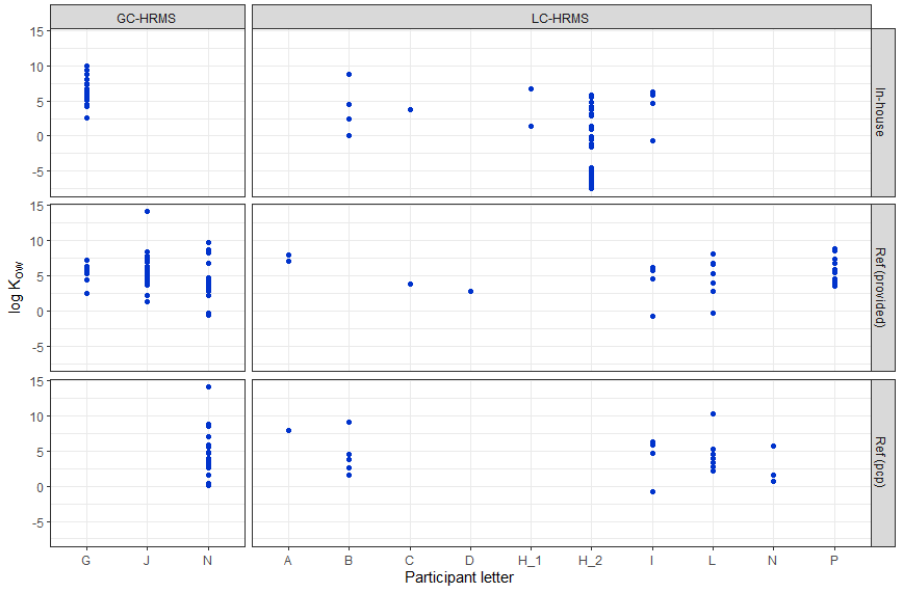


Figure S25. The predicted log K_{OW} values for the compounds detected by the different participants through non-target screening of the fish samples from Teltow Canal. The data is divided into sample preparation method (In-house, Ref (provided) and Ref (pcp)) and method of analysis (LC-HRMS and GC-HRMS). Reported compounds that were ambiguously identified (not containing a name/SMILES/other identifier, or containing several for the same m/z) were excluded from this figure since no single log K_{OW} could be calculated.

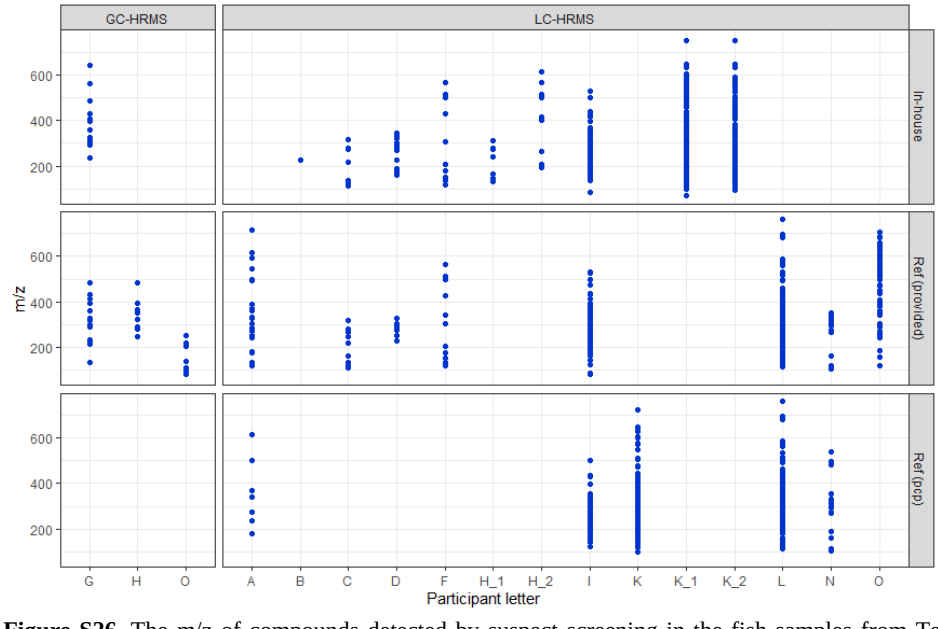


Figure S26. The m/z of compounds detected by suspect screening in the fish samples from Teltow Canal. Reported compounds that were ambiguously identified (not containing a name/SMILES/other identifier, or containing several for the same m/z) were excluded from this figure.

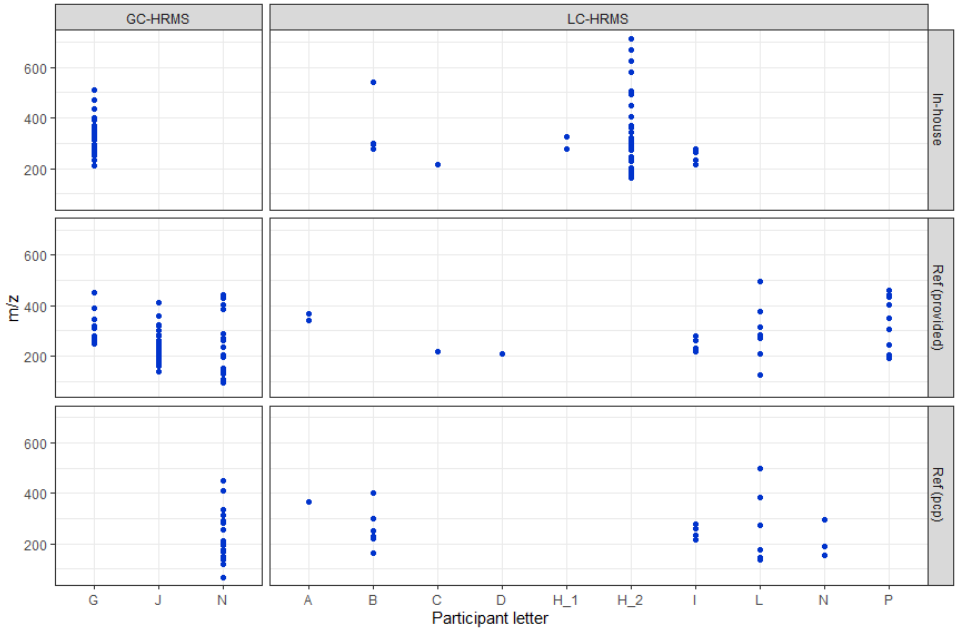


Figure S27. The m/z of compounds detected by non-target screening in the fish samples from Teltow Canal. Reported compounds that were ambiguously identified (not containing a name/SMILES/other identifier, or containing several for the same m/z) were excluded from this figure.

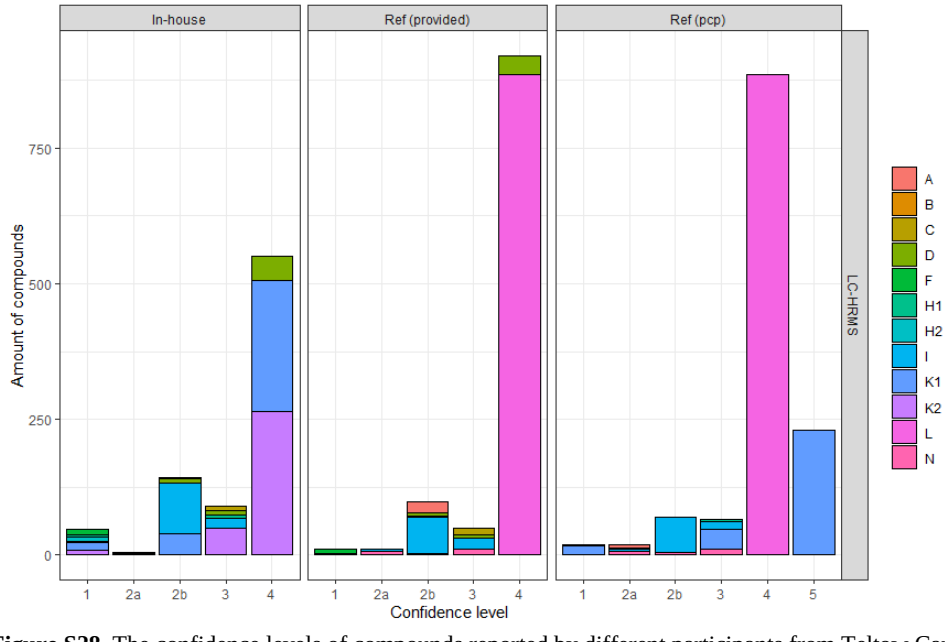


Figure S28. The confidence levels of compounds reported by different participants from Teltow Canal using suspect screening and LC-HRMS. The numbers are however approximate since not all participants reported confidence levels of their features.

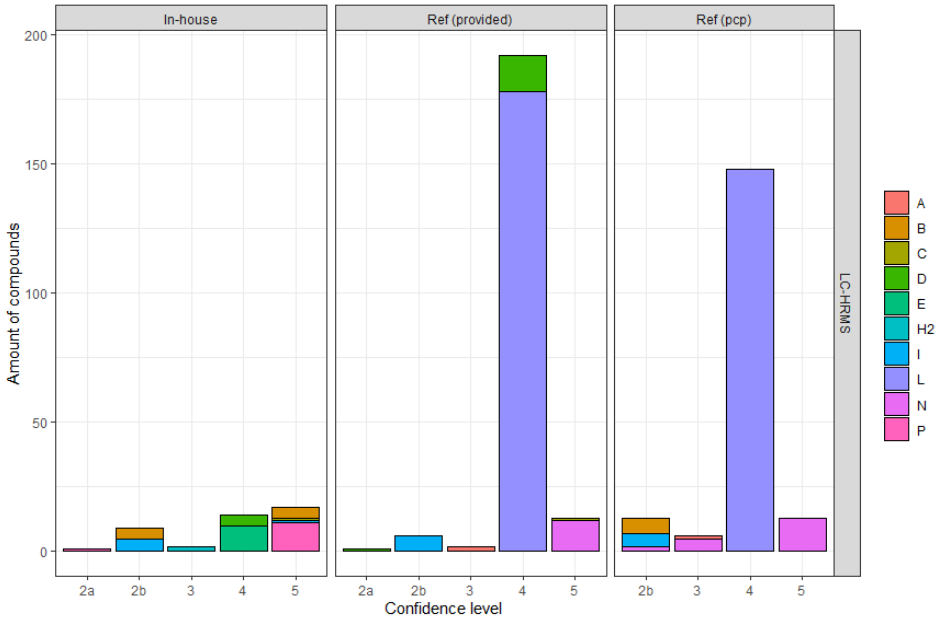


Figure S29. The confidence levels of compounds reported by different participants from Teltow Canal using non-target screening and LC-HRMS. The numbers are however approximate since not all participants reported confidence levels of their features. It is also worth mentioning that not all workflows included the reporting of confidence level 4 and 5.

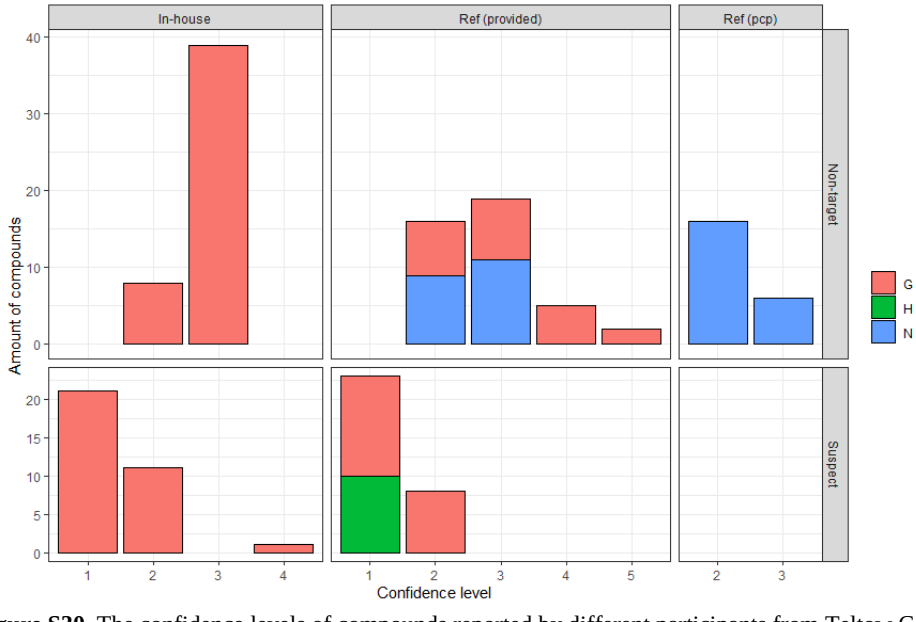


Figure S30. The confidence levels of compounds reported by different participants from Teltow Canal using GC-HRMS and suspect or non-target screening. The numbers are however approximate since not all participants reported confidence levels of their features.

9. Detection of compounds from samples of Lake Stechlin

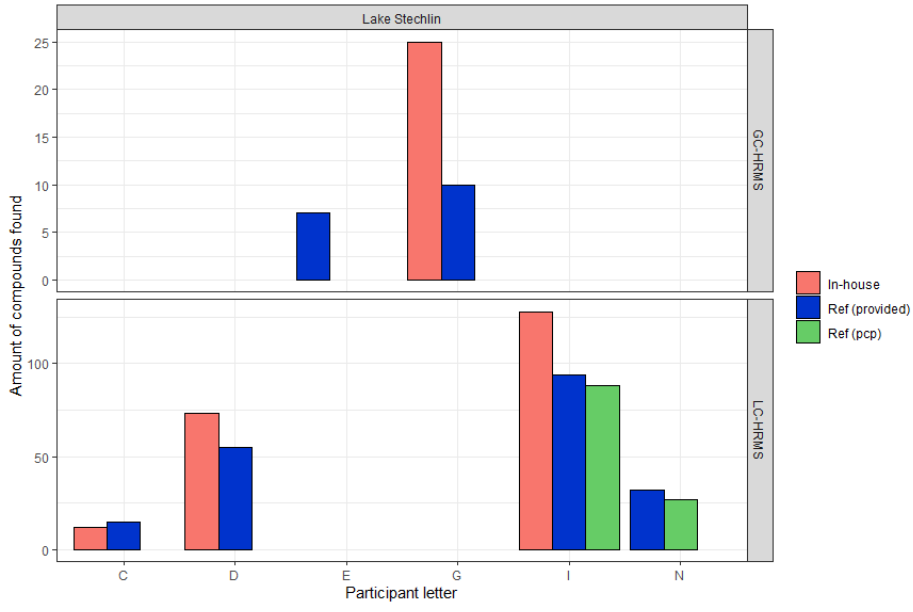


Figure S31. The amount of compounds found through suspect screening in fish from Lake Stechlin by the different participants. The data is divided into sample preparation method (sample prepared through participant's in-house = red, provided sample prepared through reference method = blue, sample prepared through reference method by the participant = green), and method of analysis.

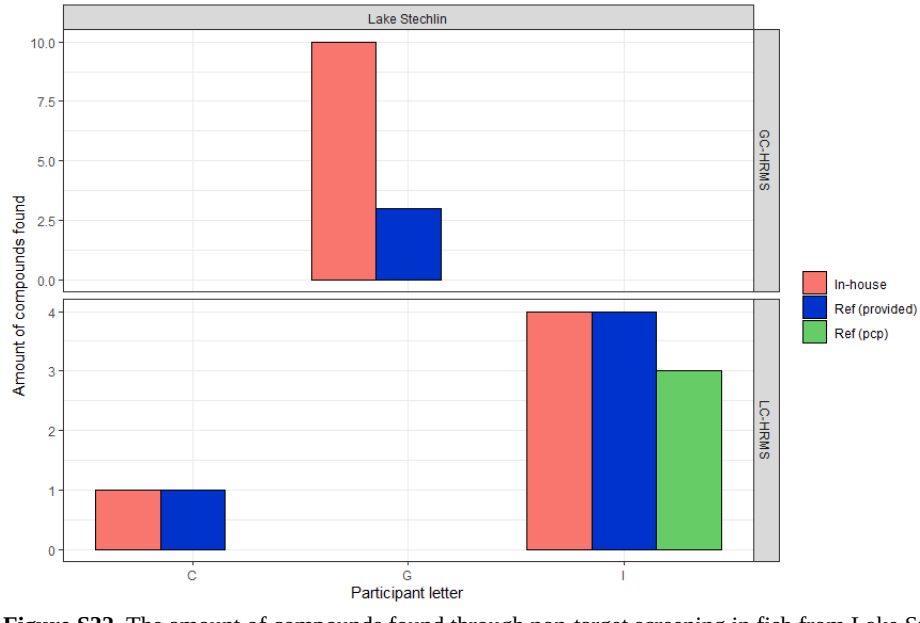


Figure S32. The amount of compounds found through non-target screening in fish from Lake Stechlin by the different participants. The data is divided into sample preparation method (sample prepared through participant's in-house = red, provided sample prepared through reference method = blue, sample prepared through reference method by the participant = green, and method of analysis.

Table S10. The ranges of log K_{OW}'s and m/z detected by the different methods for identification, analysis and sample preparation of fish samples from Lake Stechlin. The number of identified compounds refer to unique chemicals within the specified group, not counting reports of m/z with a molecular formula as sole identifier.

Method of identification	Method of analysis	Method of sample preparation	Min log K_{OW}	Max log K_{OW}	Min m/z	Max m/z	# Identified compounds
Suspect	LC-HRMS	Ref (provided)	-3.16	11.81	82.04	734.56	145
Suspect	LC-HRMS	In-house	-3.07	10.4	82.04	714.51	153
Suspect	LC-HRMS	Ref (pcp)	-3.16	8.84	104.11	714.51	115
Suspect	GC-HRMS	Ref (provided)	2.31	14.12	194.23	485.71	17
Suspect	GC-HRMS	In-house	5.87	8.91	235.01	643.53	25
Suspect	GC-HRMS	Ref (pcp)	-	-	-	-	0
Non-target	LC-HRMS	Ref (provided)	-0.73	6.27	216.15	279.26	5
Non-target	LC-HRMS	In-house	3.78	6.27	216.15	279.26	4
Non-target	LC-HRMS	Ref (pcp)	4.64	6.27	216.15	279.26	3
Non-target	GC-HRMS	Ref (provided)	3.78	7.30	216.15	311.04	3
Non-target	GC-HRMS	In-house	3.78	7.30	216.15	345.00	5
Non-target	GC-HRMS	Ref (pcp)	-	-	-	-	0

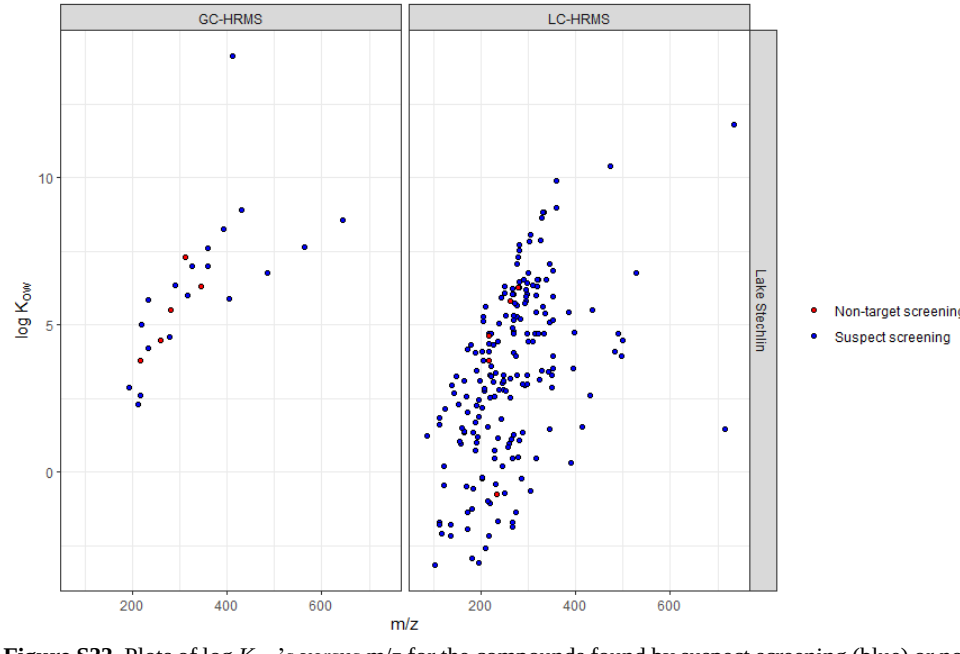


Figure S33. Plots of log K_{OW}'s versus m/z for the compounds found by suspect screening (blue) or non-target screening (red) in fish from Lake Stechlin. The data is divided into method of analysis, but all three sample preparation methods (In-house, Ref (provided) and Ref (pcp)) are included. Reported compounds that were ambiguously identified (not containing a name/SMILES/other identifier, or containing several for the same m/z) were excluded from this figure since no single log K_{OW} could be calculated.

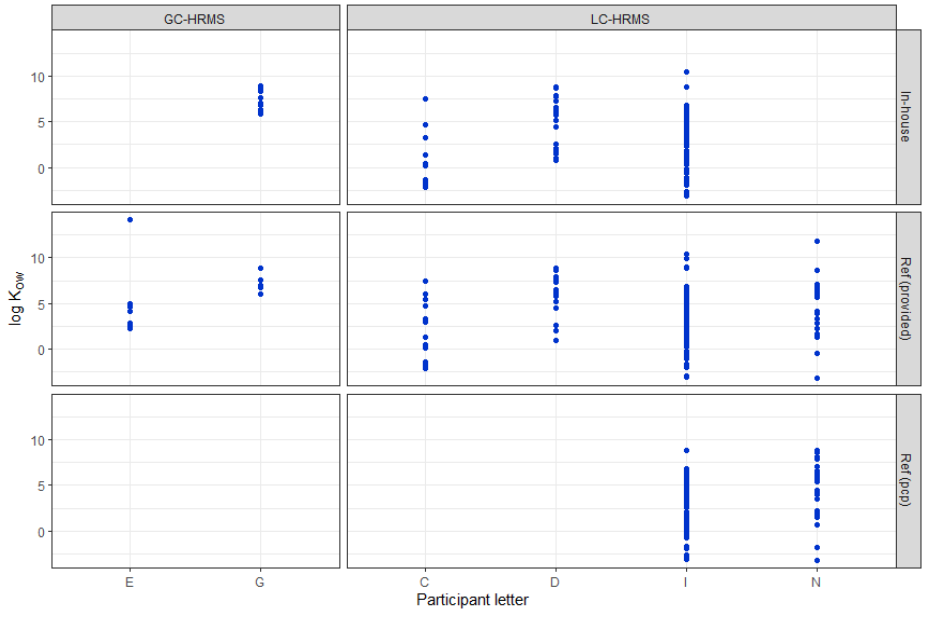


Figure S34. The predicted log K_{OW} values for the compounds detected by suspect screening in the fish samples from Lake Stechlin. Reported compounds that were ambiguously identified (not containing a name/SMILES/other identifier, or containing several for the same m/z) were excluded from this figure since no single log K_{OW} could be calculated.

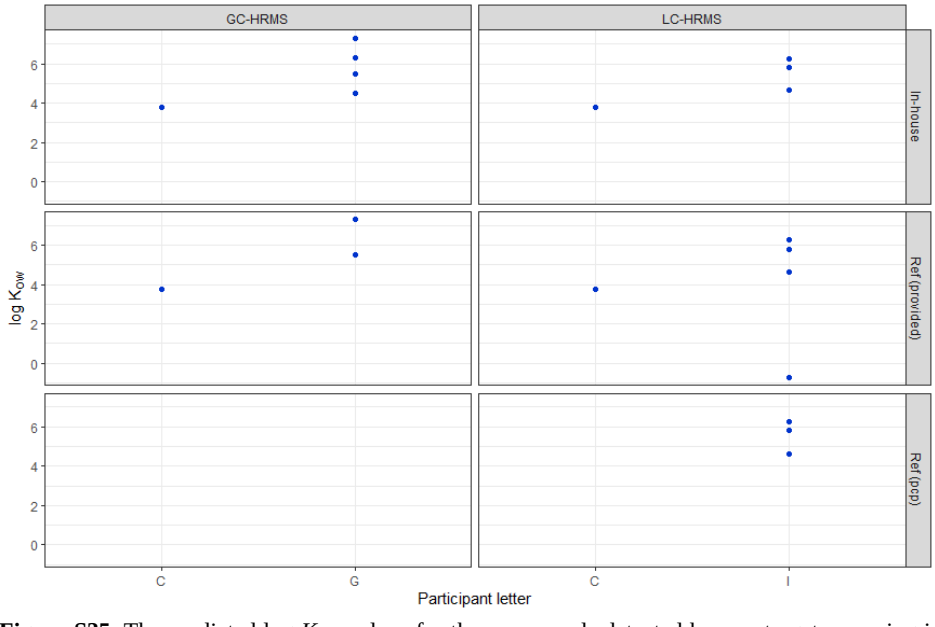


Figure S35. The predicted log K_{OW} values for the compounds detected by non-target screening in the fish samples from Lake Stechlin. Reported compounds that were ambiguously identified (not containing a name/SMILES/other identifier, or containing several for the same m/z) were excluded from this figure since no single log K_{OW} could be calculated.

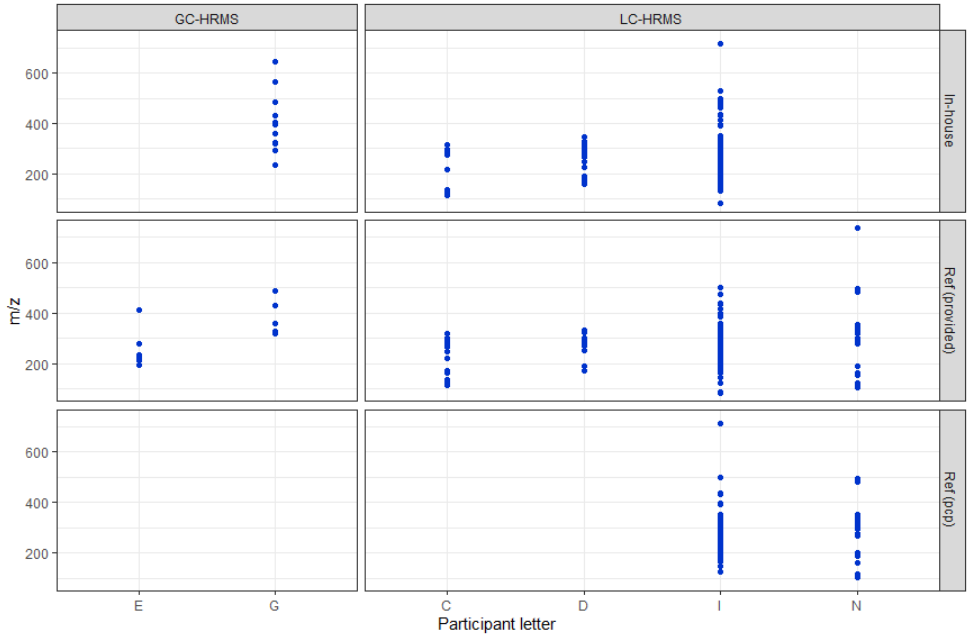


Figure S36. The m/z of compounds detected by suspect screening in the fish samples from Lake Stechlin. Reported compounds that were ambiguously identified (not containing a name/SMILES/other identifier, or containing several for the same m/z) were excluded from this figure.

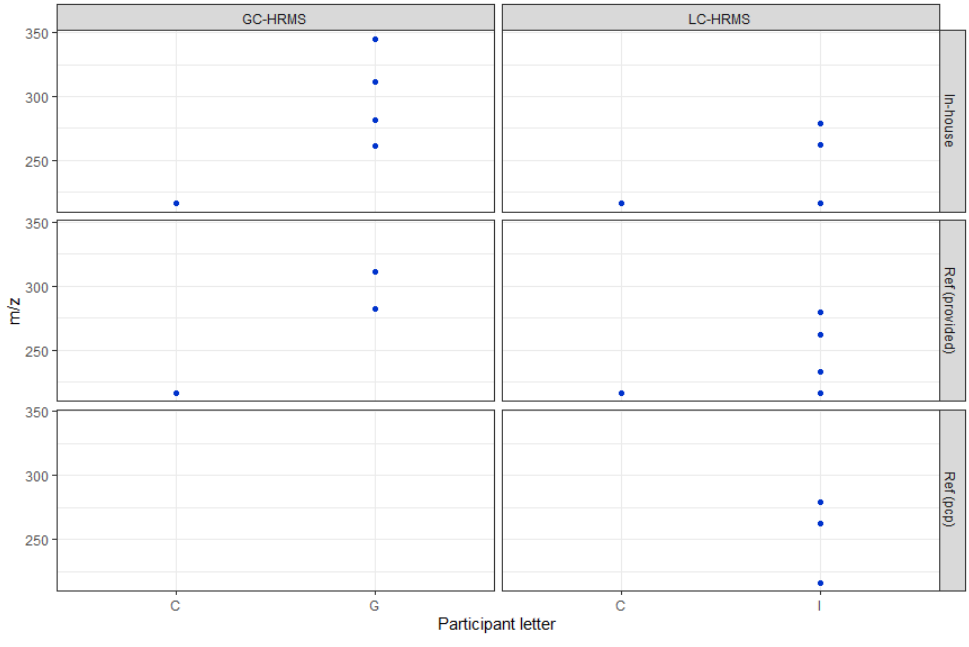


Figure S37. The m/z of compounds detected by non-target screening in the fish samples from Lake Stechlin. Reported compounds that were ambiguously identified (not containing a name/SMILES/other identifier, or containing several for the same m/z) were excluded from this figure.

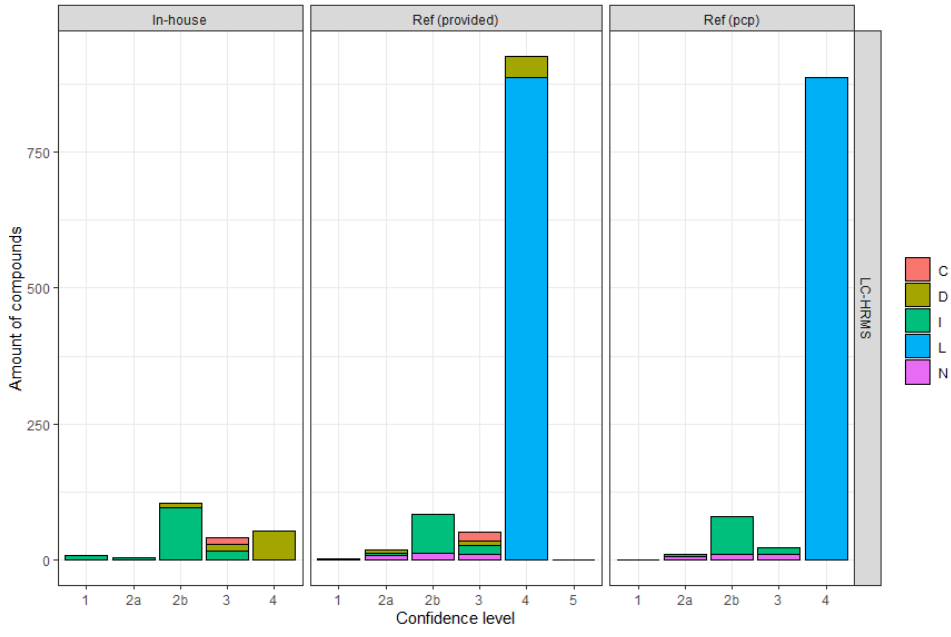


Figure S38. The confidence levels of compounds reported by different participants from Lake Stechlin using suspect screening and LC-HRMS. The numbers are however approximate since not all participants reported confidence levels of their features.

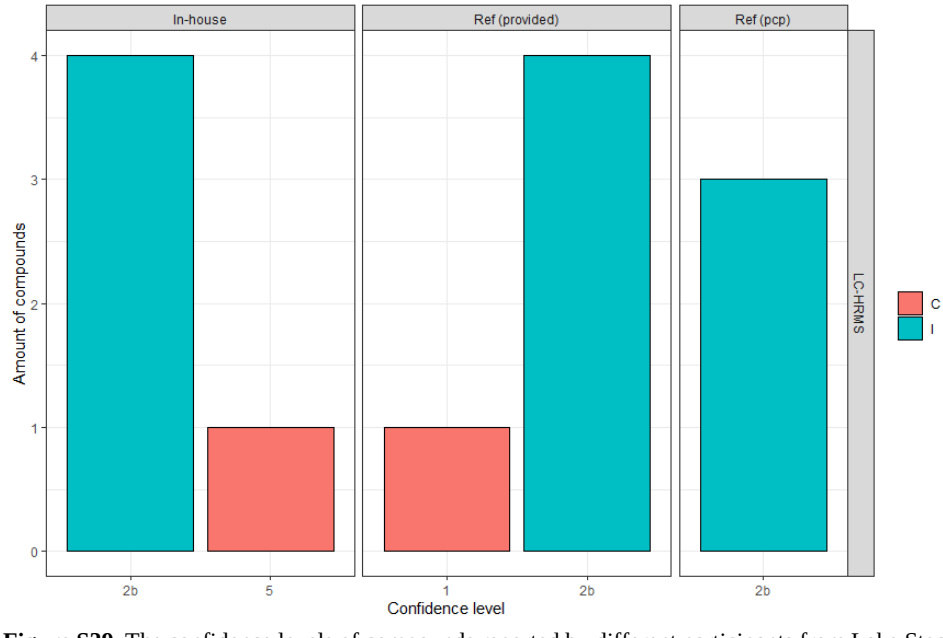


Figure S39. The confidence levels of compounds reported by different participants from Lake Stechlin using non-target screening and LC-HRMS. The numbers are however approximate since not all participants reported confidence levels of their features.

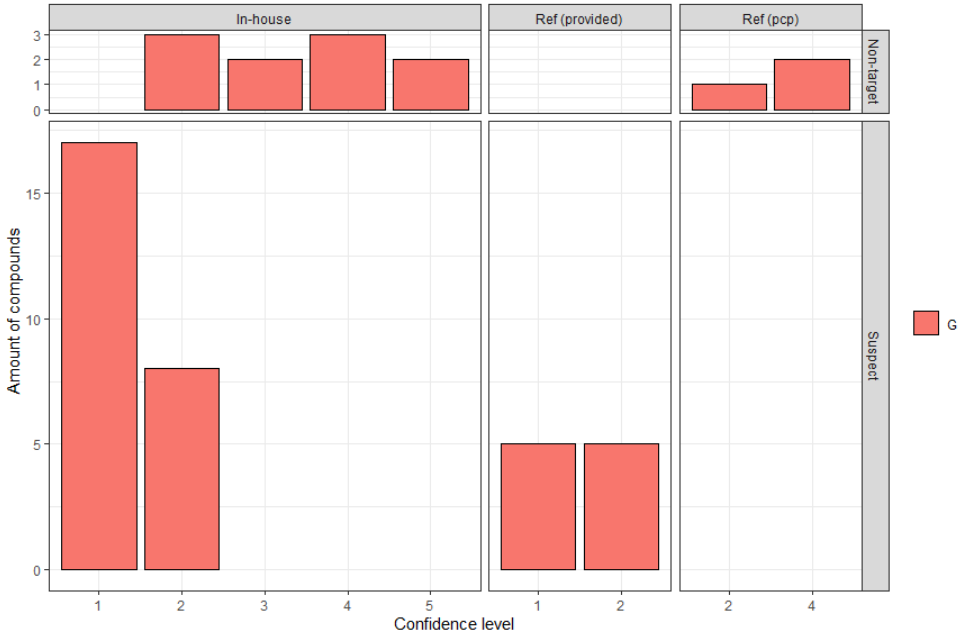


Figure S40. The confidence levels of compounds reported by the participant, identified from Lake Stechlin using GC-HRMS and suspect or non-target screening. The numbers are however approximate since not all participants reported confidence levels of their features.

References

1. Chaker J., Gilles E., Léger T., Jégou B. and David A. From Metabolomics to HRMS-Based Exposomics: Adapting Peak Picking and Developing Scoring for MS1 Suspect Screening. Anal. Chem. 2021, 93, 1792-1800.

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Doctoral Thesis No. 2025:73

The aquatic environment contains tens of thousands of pollutants from varying anthropogenic sources. To simplify the identification process, this thesis applies effect-directed analysis (EDA) to prioritise compounds with potential toxic relevance. A method for EDA was developed and applied to various matrices, including wastewater treatment plant effluent, urban snow, bottled water and tap water. An additional strategy involved assessing bioavailable water pollutants through fish tissue analysis, providing a focused lens for identifying environmentally relevant contaminants.

Sofia Lindblad received her doctoral education at the Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences. She holds a BSc in Chemistry, an MSc in Organic Chemistry and an MSc in Environmental Toxicology from Uppsala University.

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