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Journal Pre-proofs

Full length article

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Journal Pre-proofs

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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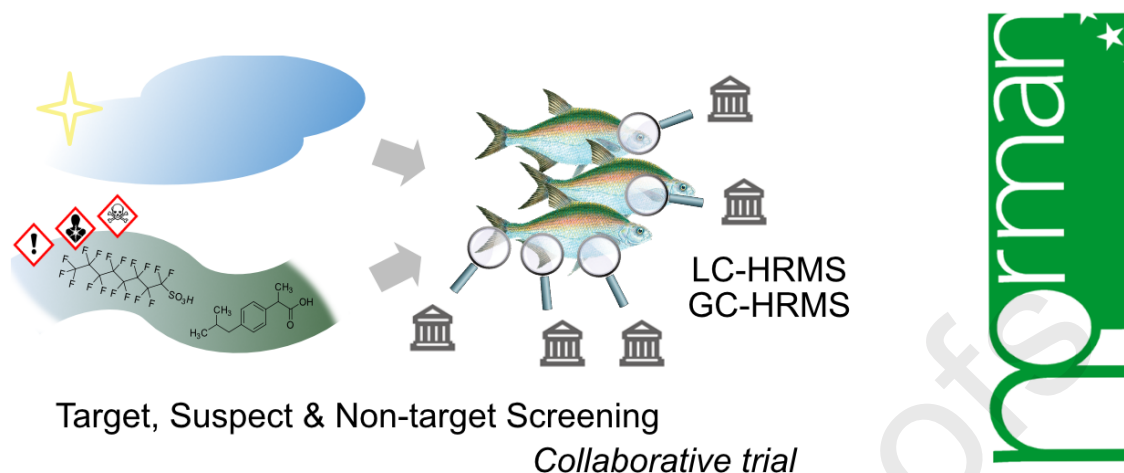
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Graphical abstract



Highlights

- Collaborative trial of 16 laboratories on suspect and non-target screening (NTS) in fish
- Comparison of test extracts by in-house and reference methods (GC-HRMS, LC-HRMS)
- Reproducibility between sample preparation methods was not significantly different
- On average, 41% (LC-HRMS) and 37% (GC-HRMS) of spiked compounds were identified

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 ranging from -9.9 to 16 and mass-to-charge ratio (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.

Keywords:

Suspect and non-target analysis; biota; LC-HRMS; GC-HRMS; collaborative trial; exposome

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 interests 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) (Hollender et al., 2017, Anna et al., 2016, Hogenboom et al., 2009, Chen et al., 2022) 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., 2015a, 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 efficiently removing 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 (Caballero-Casero et al., 2021b).

The NORMAN network started as a European research project in 2005, 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., 2021a). 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 to determine the range of detectable chemicals and to provide future recommendations for harmonization.

2. Materials and methods

2.1. Samples and experimental design

Bream (*Abramis brama*) samples from the reference site Lake Stechlin (longitude 13.0278N, latitude 53.1514E) and the more polluted site Teltow Canal (longitude 13.1900N, 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 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., 2022a), 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 ($c = 50 \text{ ng mL}^{-1}$, equivalent tissue concentration 300 ng g^{-1} dry weight (dw) for each compound) and with 19 compounds for analysis by GC-HRMS ($c = 75 \text{ ng mL}^{-1}$, 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., 2015b) and representing 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 participants’ own in-house methods for sample preparation and/or preparing their own extracts following 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). As mentioned briefly above, 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 following 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.

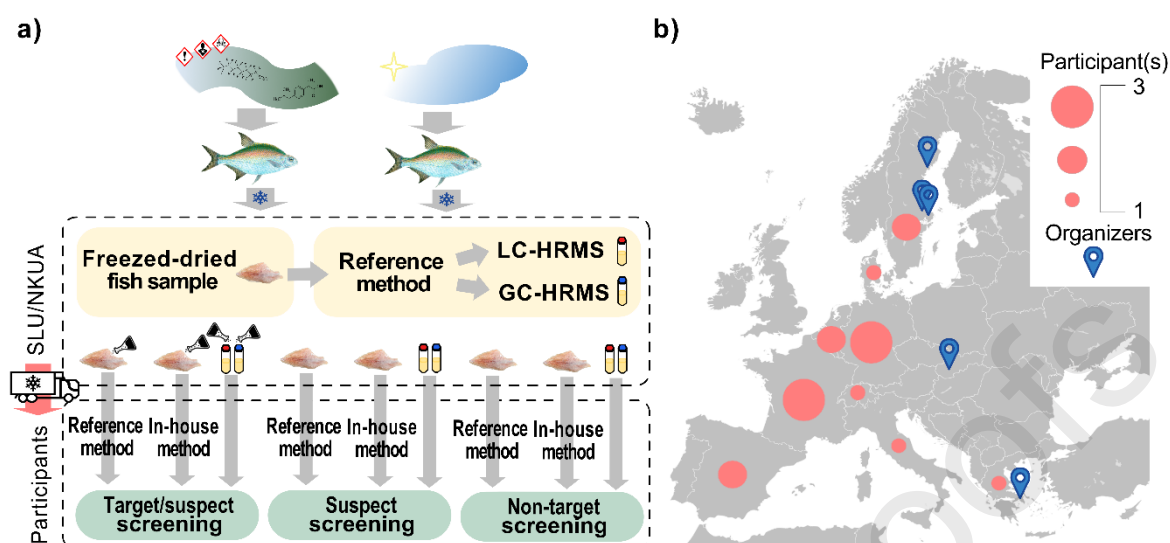


Fig. 1. Experimental design of the collaborative trial and participant map. a) Design of the study. Fish samples and reference extracts prepared by the reference methods were sent to the participants for LC-HRMS and GC-HRMS analysis. The participants analysed the extracts provided and/or prepared their 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 was analysed using target/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.

In addition to the fish samples and reference extracts, two mixtures of the reference standards used for preparing the spiked samples were provided to the participants. Retention time 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 retention times 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^{-1} for LC-HRMS analysis and 1 mg L^{-1} 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. 1b). 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 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.

2.3. Reference methods for sample preparation

Preparation of extracts for LC-HRMS analysis (Fig. 2a) 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 same reference method for their sample preparation. The extracts for GC-HRMS analysis (Fig. 2b) were prepared at NKUA, Greece, following an existing protocol (Badry et al., 2022b) 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 participants and the method used is given in Table 1.

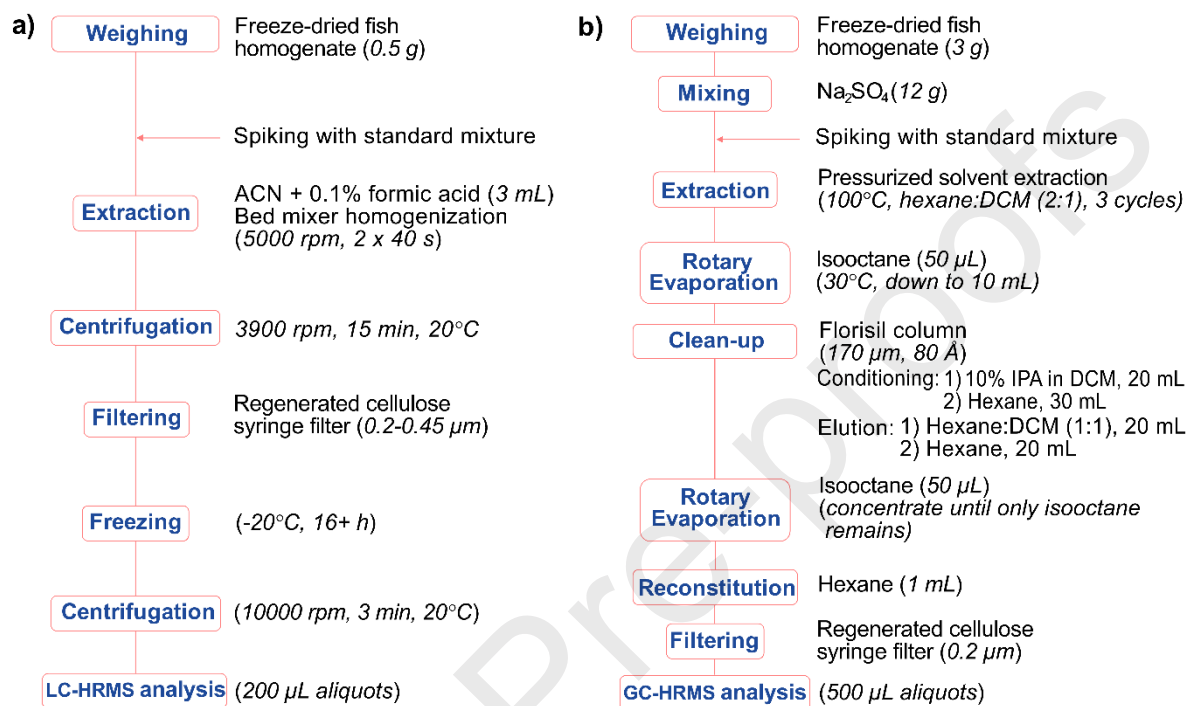


Fig. 2. Reference methods used in sample preparation for analysis by a) LC-HRMS and b) GC-HRMS. ACN: acetonitrile. DCM: dichloromethane. IPA: isopropyl alcohol.

2.4. 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).

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
				ABCDEFGHIJKLMNO

		Ref (provided)	x	x	x	x	x	x	x				x	x	x	x	x	
	LC-HRMS	Ref (pcp)	x											x			x	
		In-house		x	x	x	x		x	x			2x		x		x	
Lake Stechlin (spiked)	Target/suspect screening	Ref (provided)							x	x							x	x
	GC-HRMS	Ref (pcp)																x
		In-house							x									
		Ref (provided)	x	x	x	x							x		x		x	
	LC-HRMS	Ref (pcp)	x	x									x	x	x		x	
		In-house		x	x	x	x						2xx		2x			
	Suspect screening	Ref (provided)							x	x	x							x
	GC-HRMS	Ref (pcp)																
		In-house							x									
Teltow		Ref (provided)	x	x	x								x		x		x	x
	LC-HRMS	Ref (pcp)	x	x									x		x		x	
		In-house		x	x	x	x						2xx					
	Non-target screening	Ref (provided)							x				x					x
	GC-HRMS	Ref (pcp)																x
		In-house							x									

2.5. 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. retention time (RT), m/z intensity, MS/MS data, type of workflow, proposed compound identity, molecular formula, CAS, SMILES and identification confidence level (Schymanski et al., 2014)). For the spiked samples, target or suspect analysis was used in screening for the known compounds, while only suspect analysis, using participants' own suspect lists, was used for the screening of the unknown spiked compounds (see section 3.1). Furthermore, suspect screening with the participants' own suspect lists as well as non-target screening, was performed of non-spiked fish samples from Teltow Canal and Lake Stechlin. Additional 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.

2.6. Data analysis

For comparison of the overall methods (sample preparation, analysis workflow etc.) used by 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 obtained using the reference method, 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).

In suspect and non-target screening, 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, predicted $\log K_{OW}$ and molecular mass of the detected and undetected compounds were assessed to aid comparison 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.

3. Results and discussion

3.1. Known and unknown spiked compounds

Samples from Lake Stechlin had been spiked with 32 and 19 compounds for LC- and GC-HRMS analysis, respectively, of which five compounds each had been disclosed to the participants. This part of the study had the purpose to assess the frequency of correct identifications, while sample preparation and data analysis methods varied. The percentages of known (5 for LC-HRMS and GC-HRMS, respectively, of known identity) 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 analysed, 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 0-185 for the LC-HRMS analyses (although ≤ 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, some participants reported compounds that were not added during spiking. Such

findings were not reproducible and could be due to inter-laboratory variability and are more 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 have 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±17% for in-house method, 28±17% for provided extracts, 36±12% for reference method). A similar assessment of the GC-HRMS results was impeded by the low number of participants (14% for in-house method (1 participant), 29±17% for provided extracts (4 participants), 21% for 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 to 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 obtained 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 participants (Tables S2-S3 in SD1) were fairly similar, so data processing seemed to be the most important source of variation (Figures S1-S19 in SD1). In general, participants followed data processing work flows 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 of the identified compounds by the participants. Furthermore, if the unknown spiked compounds were not included in the participants' suspect library then the participant was not able to identify them. This highlights the importance of the smart selection of suspect libraries. However, 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.

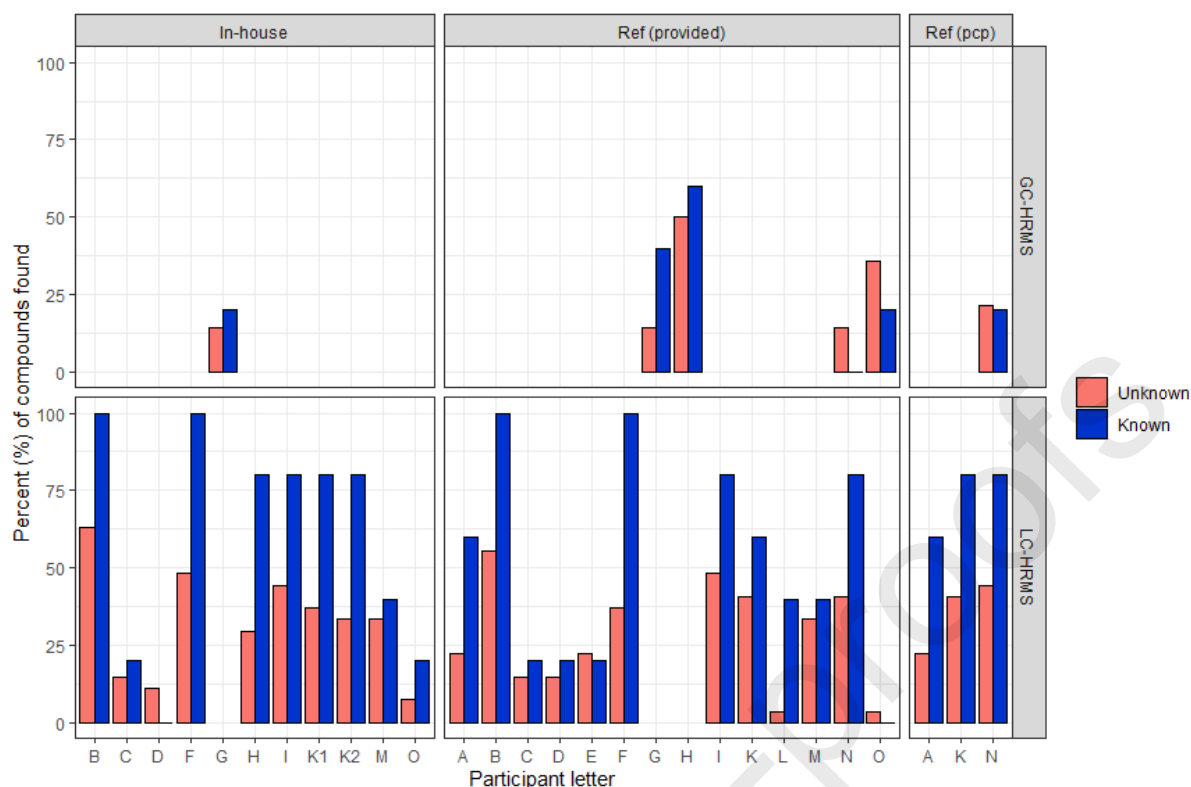


Fig. 3. Detected spiked compounds. Percentage of spiked compounds correctly identified by the different participants ($n = 16$, codes A-P) for unknown spiked compounds (red bars; identity not disclosed, LC-HRMS 100% = 27; GC-HRMS 100% = 14) and known spiked compounds (blue bars; identity revealed to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

The participants who identified the 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 $\log K_{OW}$ (-2.5-2.7) 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), fenciclonil 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 used separation methods since, as stated before, 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.

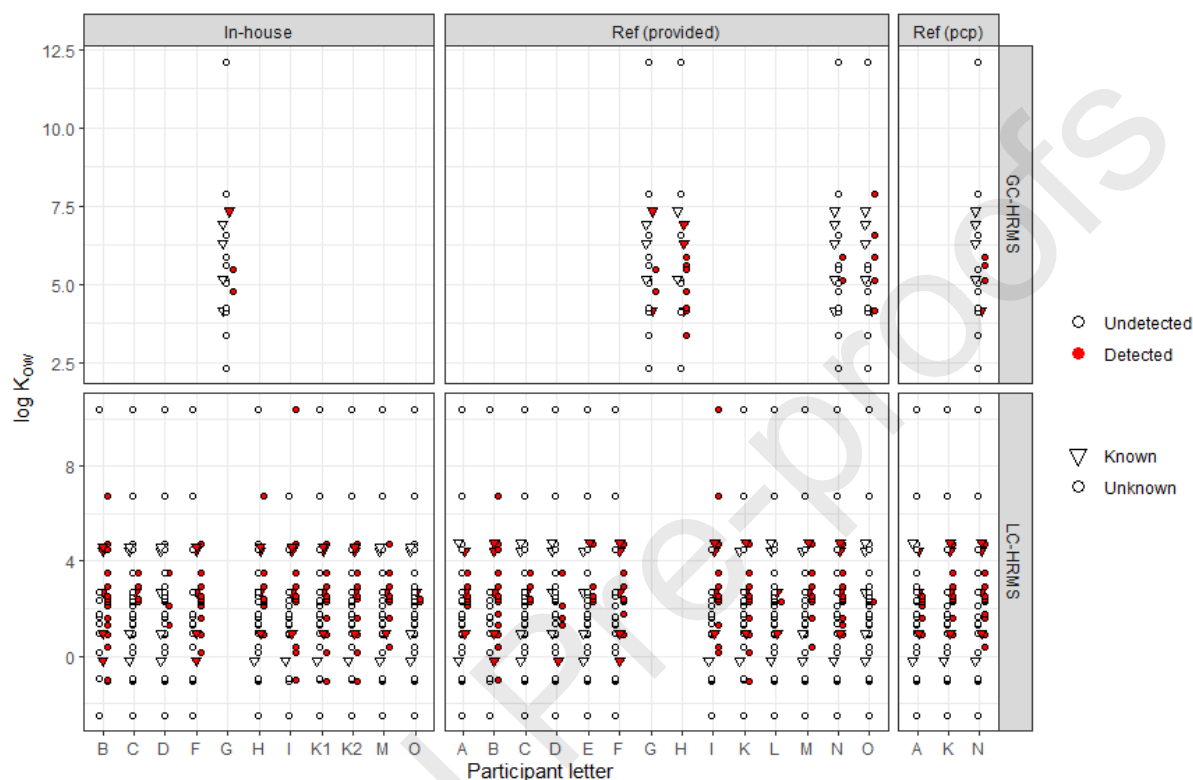


Fig. 4. Predicted $\log K_{OW}$ values of spiked compounds. $\log K_{OW}$ values of spiked compounds predicted by participants ($n = 16$, code A-P) using EPI Suite 4.0, along with indication of positive (red) or negative (white) detection. Unknown compounds (spiked compounds whose identity was not disclosed)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

3.2. 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 suggest that most laboratories performed at a similar level. The high average number of features reported by LC-HRMS participants, in comparison to the median number of features, can be explained by the high number of identified unique features by a few participants. Suspect screening with LC-HRMS analysis performed on fish samples from Teltow Canal led to 16 features being 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 overlapping features, at least one of the participants identified that compound at confidence level 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 confidence level 1 and 2 (although these are underestimates, since not all participants reported confidence levels of features identified). 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 a range of $\log K_{OW}$ values of -9.9 to 16 (Fig. S24 in SD1) and m/z 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 GC-HRMS results ($p < 0.05$), but not for LC-HRMS results ($p > 0.05$) in suspect and non-target screening of fish tissue 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 from which the fish was obtained. The number of features detected by the different participants using suspect screening in fish samples from Teltow Canal showed high variation for both GC-HRMS and LC-HRMS (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 be explained mainly by different suspect lists and data processing and 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), but harmonized data processing is often difficult because often vendor software is used and these programs are largely “black boxes”.

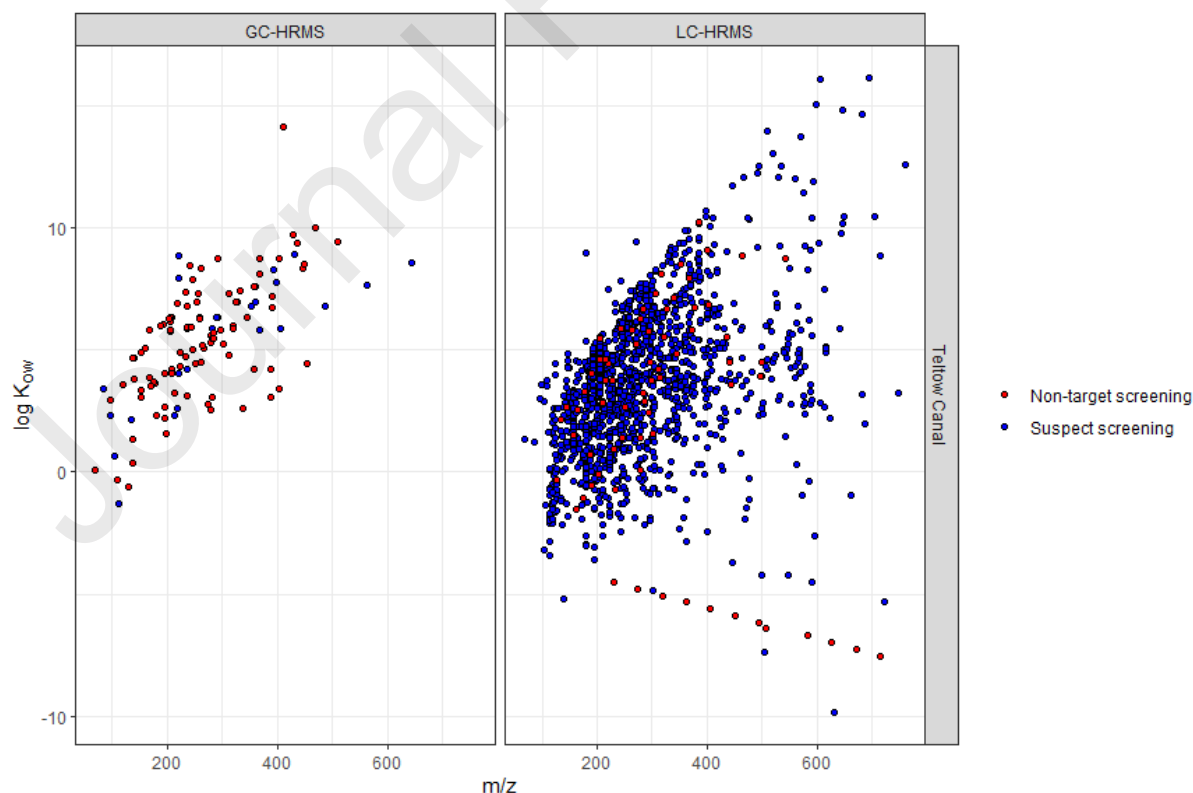


Fig. 5. Range of detected features. m/z values versus $\log K_{OW}$ values for the features found by all participants by suspect screening (blue) or non-target screening (red) in fish samples from Teltow Canal using (left) GC-HRMS

analysis and (right) LC-HRMS analysis) and all three sample preparation methods (reference extract provided, extract prepared through repeating the reference protocol, or extract prepared through the participant's in-house protocol). Reported features that were ambiguously identified (not containing a name/SMILES/other identifier, or containing several for the same m/z) are excluded from the diagram since no single log K_{OW} value could be calculated.

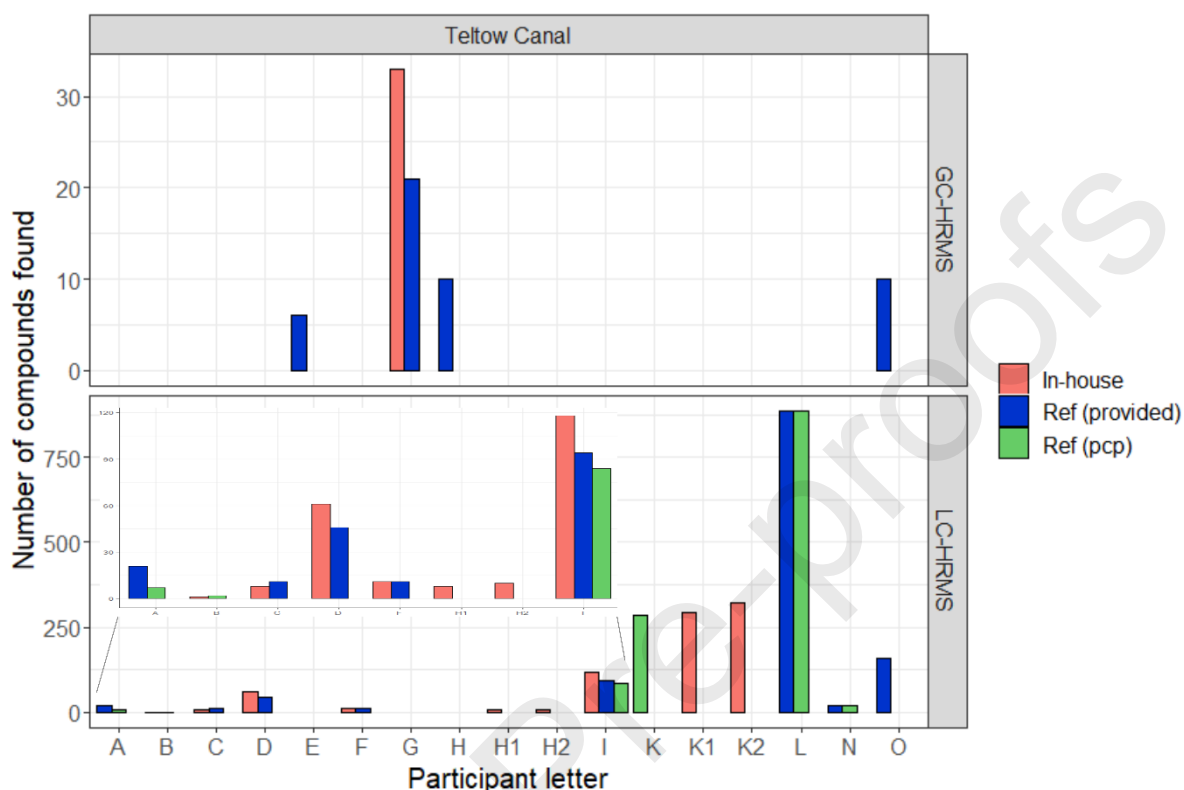


Fig. 6. Compounds detected by suspect screening. Number of compounds (at all confidence levels) in fish samples from Teltow Canal reported by participants ($n = 16$, code A-P) using the suspect screening approach, for different sample preparation methods (reference extract provided = blue, extract prepared through the reference method by the participant = green, extract prepared through 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 allocated sequential numbers following the laboratory code letter (e.g. K1, K2).

3.3. 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. Thus, non-target screening was only applied to samples from Teltow Canal. The participants were asked to highlight the ten compounds with the highest intensity. 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 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 after extraction with both the reference and in-house method.

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 (\geq 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 made (Aalizadeh et al., 2021).

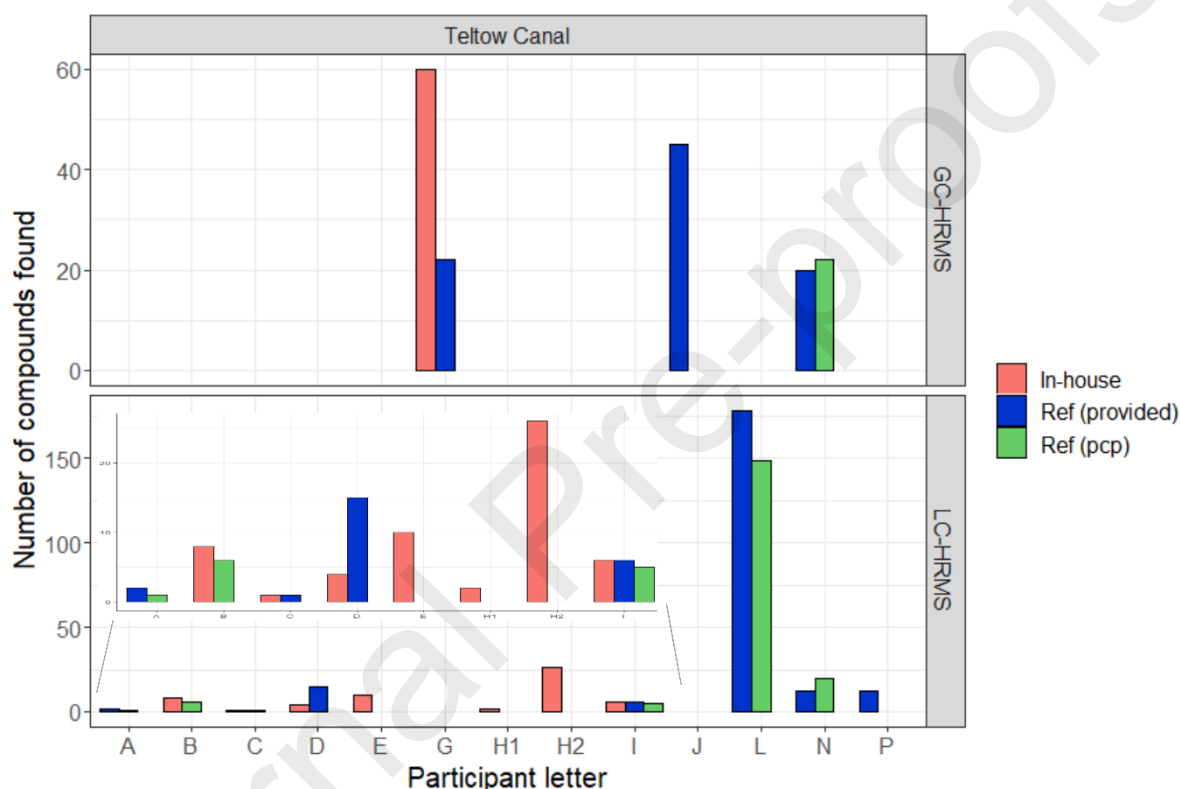


Fig. 7. Compounds detected by non-target screening. Number of compounds reported (on all confidence levels) in fish samples from Teltow Canal by participants ($n = 16$, code A-P) when using the non-target screening approach, for different sample preparation methods (reference extract provided = blue, extract prepared through the reference method by the participant = green, extract prepared through 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 allocated sequential numbers following the laboratory code letter (e.g. H1, H2).

4. 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 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 being 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 (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 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 procedures and pipelines 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 confidence level (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 Munsch:** 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**

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Declaration of Competing Interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data to this article can be found online at [link].

Supplementary data 1 at [link].

Supplementary data 2 at [link].

Supplementary data 3 at [link].

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Declaration of Competing Interest

The authors declare no competing interests.

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