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# Screening of organic pollutants in mollusc and fish samples from the Portuguese coast by combining liquid and gas chromatography with high resolution mass spectrometry

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ABSTRACT

In recent years, the presence of organic pollutants (OPs) in biota samples, especially in species which are intended for human consumption, has generated major concern. Thus, legislation on presence and levels has been created and/or updated in order to achieve safe food products for consumers. During the years 2020 and 2022 in the frame of the project "Contaminant levels in species of commercial interest captured in the Portuguese Maritime Area - CEIC", different species of fish and molluscs were collected in 4 main areas of the Portuguese coast. Samples were processed by matrix solid-phase dispersion (MSPD) and injected into gas and liquid chromatographic systems coupled to high resolution mass spectrometry analysers. A total of 82 OPs were identified using liquid chromatography (LC), most being pharmaceuticals, 56 OPs were identified by gas chromatography (GC) using a high-resolution mass spectra library. In addition, 38 chlorine/bromine-containing compounds were tentatively identified using the low resolution NIST 2017 database, most of which are used as industrial chemicals. Cluster analysis of samples revealed grouping according to the type of analysed tissue, sampling area and campaign. For the *per-* and polyfluoroalkyl substances (PFAS) detected, a quantitative methodology was applied to evaluate concentration levels. Several fish muscle and bivalve mollusc samples exhibited PFAS concentrations that exceeded the permitted levels established by current regulations, with perfluorooctanoic acid (PFOA) showing particularly elevated levels in the analysed samples. In conclusion, the study highlights the widespread occurrence of organic pollutants, particularly PFAS, in commercial marine species along the Portuguese coast, underscoring the urgent need for continued monitoring.

## 1. Introduction

The presence of persistent organic pollutants (POPs) has been documented in several aquatic environments because of the indiscriminate synthesis and widespread usage in the last decades. This group of pollutants is of special concern due to their bioaccumulation capability, their potential transfer to the food chain and the impact on several wildlife species of the marine environment and seafood consumers health (Beduk et al., 2022). Although international regulations have minimized the primary emissions of some POPs into the environment, they are still present both in source and remote regions (Lunder Halvorsen et al., 2023) and the monitoring in the environment is crucial to identify the main sources and to control the emissions of these compounds. Current legislation controls the release of some POPs, listed in the Stockholm Convention of 2009 (Part A of Annex I to Regulation (EC) No 850/2004), into the environment. Also, the maximum levels of some

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POPs families, such as PAHs and PCBs levels in molluscs and other seafoods are controlled through the Regulation (EU) 2023/915 (EC, 2023).

Contaminants of emerging concern (CEC) is a terminology that has been used to refer to a very extensive group of chemicals that includes organic pollutants (OPs) with growing concern due to their potential adverse effects on ecosystems and human health. Some CECs, such as *per*- and polyfluoroalkyl substances (PFAS), have had restrictions placed on their use and allowable levels in the environment (EESC, 2023) and recently were also added to (EU) 2023/915 regulations restricting their presence in seafood and molluscs (EC, 2023). However, there is still a lack of comprehensive regulation and monitoring data for most CECs (Dulio et al., 2018).

Several authors reported the presence of OPs in environmental samples of fish tissue (Manjarrés-López et al., 2023; Rebryk et al., 2023), mussels (Meng et al., 2022) and clams (Castro et al., 2022; Manjarrés-López et al., 2023; Meng et al., 2022; Rebryk et al., 2023). In this context, marine biota is usually used as an indicator of pollution status in the marine environment, especially for bioaccumulative and persistent chemicals. The extraction methods most frequently used for the identification of OPs from marine biota are pressurized liquid extraction (Matsukami et al., 2016), microwave assisted extraction (Ma et al., 2013), solid-liquid extraction by shaking (Green et al., 2008) and accelerated solvent extraction (Kim et al., 2011). However, after extraction, a clean-up step is usually needed to remove co-extracted lipids and other interfering substances. In this sense, matrix solid-phase dispersion (MSPD) is a promising alternative for sample preparation, since it incorporates extraction and clean-up into a single step, reducing solvent consumption, cost and eliminating the need for dedicated instrumentation (Barker, 2000). Methods for MSPD using acidic silica and octadecylsilane ($C_{18}$) have been used to effectively remove nonpolar interferences in mollusc and fish tissue matrices prior to the determination of several CECs classes (Ros et al., 2016; Villaverde-de-Sáa et al., 2013).

For instrumental analysis, gas chromatography (GC) (Castro et al., 2022; Lee et al., 2019; Rebryk et al., 2023) and liquid chromatography (LC) coupled to mass spectrometry (MS) (Gago-Ferrero et al., 2015; Salgueiro-González et al., 2019; Wilson et al., 2021) are the most popular techniques for the identification of OPs in marine biota screening studies.

Thus, the aim of this work was to design and apply a screening strategy to detect CECs and POPs, covering a wide range of physicochemical properties, in fish and clam samples from the Portuguese coast. To reach this objective, the combination of different MSPD-based protocols and determination by liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) and gas chromatography coupled to high resolution mass spectrometry (GC-HRMS) was used. Additionally, a dedicated methodology for the quantification of a specific subgroup of identified PFAS was applied to evaluate the compliance with the requirements of recent legislation on those compounds.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Ultrapure deionized water (18.2 MΩ/cm) was supplied by a RephiLe Bioscience Genie equipment (Acton, MA, USA). Methanol (MeOH) and acetonitrile (ACN), both LC-MS grade, were purchased from Merck (Darmstadt, Germany). Ethyl acetate (AcOEt) GC–MS grade, sulfuric acid ($H_2SO_4$) 95–98 % and glacial acetic acid (HAc) were provided by Panreac (Castellar del Vallès, Spain). Ammonia 7 N in methanol was purchased from Acros Organics (Antwerp, Belgium) and 25 % ammonium hydroxide (aqueous solution) LC-MS quality was purchased from Scharlab (Barcelona, Spain). Ammonium fluoride with a purity ≥99.99 % was from Merck. The silylation reagent, *N*-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), and the C7–C40 saturated alkanes standard solution were provided by Sigma-Aldrich (St. Louis, MO, USA). Florisil (60–100 mesh) and primary-secondary amine–bonded silica (PSA) were provided by Supelco (Bellefonte, PA, USA). Silica gel 60 (0.040–0.063 mm) was provided by Merck. Diatomeas earth was provided by VWR Chemicals (Barcelona, Spain) and $Na_2SO_4$ by ITW Reagents (Milano, Italy).

An internal standard (IS) solution of 1 mg/L (hereinafter mix IS A) was prepared in methanol. Among those substances, 20 were used in LC and 5 were used in GC for signal intensities correction after each chromatographic analysis when using each of the instrumental methodologies (Table S1, supplementary material).

A standard mixture of 2 mg/L containing 7 PFCAs (perfluoroalkyl carboxylic acids) and 2 PFSAs (perfluorinated sulfonic acids) in methanol and an IS mixture (hereinafter mix IS B) of 2 mg/L of 9 isotopically labelled PFCAs and PFSAs in methanol (Table S2, supplementary material) were purchased from Wellington Laboratories (Ontario, Canada).

All analytical solutions were stored at −20 °C.

### 2.2. Samples

Liver and muscle of fish samples of *Trachurus trachurus* (atlantic horse mackerel, coded as HOM), *Solea solea* (dover sole, coded as LING), *Microchirus azevia* (azevia, coded as AZ), *Microchirus variegatus* (thickback sole, coded as AZR), *Trisopterus luscus* (pouting, coded as BIB) and the whole body of clam samples of *Spisula solida* (thick trough shell, coded as SSOL) were collected in one sampling campaign in September 2020 and, additionally, in June 2022 another sample campaign of SSOL was collected in different areas of the Portuguese coast (Fig. 1) within the project "Contaminant levels in species of commercial interest captured in the Portuguese Maritime Area - CEIC" by the authors from the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) and the Portuguese Institute for Sea and Atmosphere (IPMA). The samples were collected in four regions of Portugal: North (N), Center (C), Lisbon (L) and Algarve (A) as detailed in Table S3 (supplementary material). The north part is the most industrialized area of the country with an urban area of petrochemical industry near the sampling points. The center of Portugal presents also an important industry activity (mainly paper industry) and an agricultural activity of cereals, olives, and vineyards. The Lisbon region is the main financial and economic area of Portugal, an important tourist attraction and a significant area of electronic industry. The Algarve (south part of Portugal) is the most popular tourist destination in Portugal specially in the summer period (EURES, 2022; IEFP, 2022; INE, 2022).

After the collection, the samples were lyophilized during 48 h at −60 °C in a Scanvac Cool Safe equipment (LaboGene, Lillerød, Denmark) and sent to USC laboratories for analysis where pooled samples were prepared according to the information given in Table S3.

### 2.3. Sample preparation for screening

Freeze-dried samples were submitted to two different MSPD protocols to detect analytes with a wider range of properties (Fig. 2). In method A, based on Villaverde de Saá et al. (Villaverde-de-Sáa et al., 2013), 0.5 g of freeze-dried sample (0.2 g in case of liver samples) spiked with the internal standards (mix IS A) were mixed in a glass mortar with 0.5 g of PSA to achieve complete homogenization. A 10 mL polypropylene syringe was prepared by filling in (from bottom to top) 0.5 g of silica, 1.75 g of acidified silica (10 % (*w*/w) $H_2SO_4$), 1.75 g of florisil (deactivated with 5 % (w/w) $H_2O$) and the homogenized mixture sample. Analytes were eluted with 10 mL of dichloromethane. The eluate was concentrated to dryness under a nitrogen stream and reconstituted in 100 μL of ethyl acetate.

In method B, based on Castro et al. (Castro et al., 2020), 0.5 g of freeze-dried sample (0.2 g in case of liver samples) spiked with the internal standards (mix IS A) were mixed in a glass mortar with 1.2 g of activated silica. A 10 mL polypropylene syringe was prepared by filling

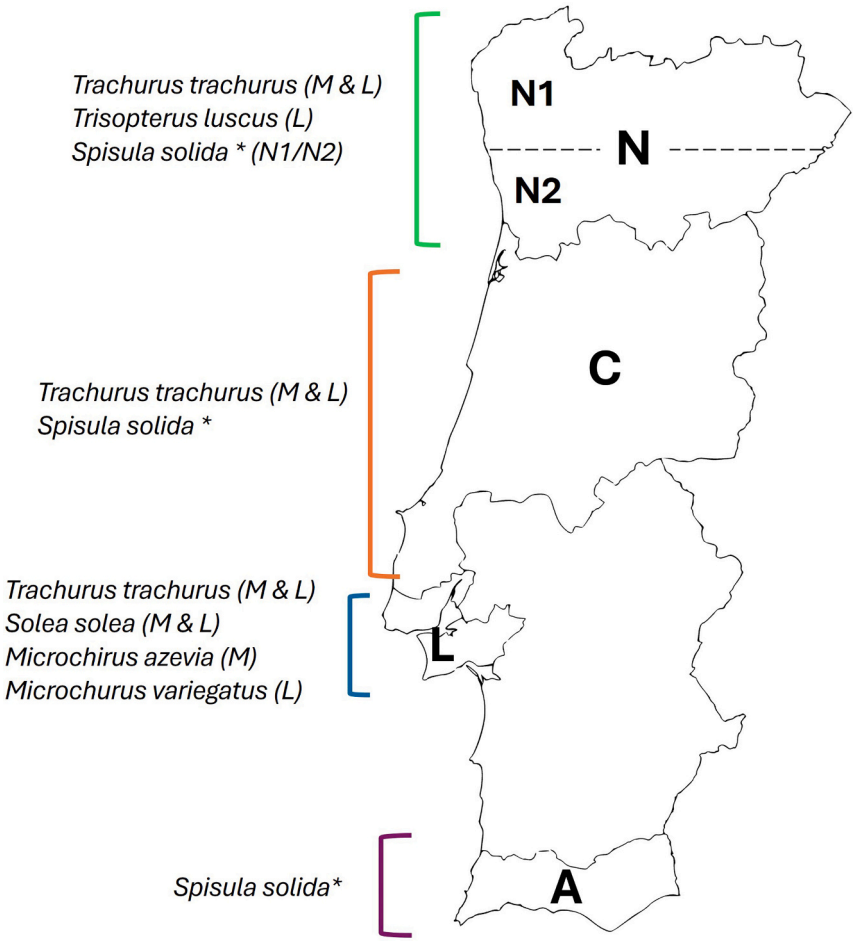


**Fig. 1.** Sampling zones, species and analysed tissue (M: muscle, L: liver). *For *Spisula solida* 2 sampling campaigns were performed and the whole body was processed. Specific description and codes can be found in Supplementary Material Table S3.

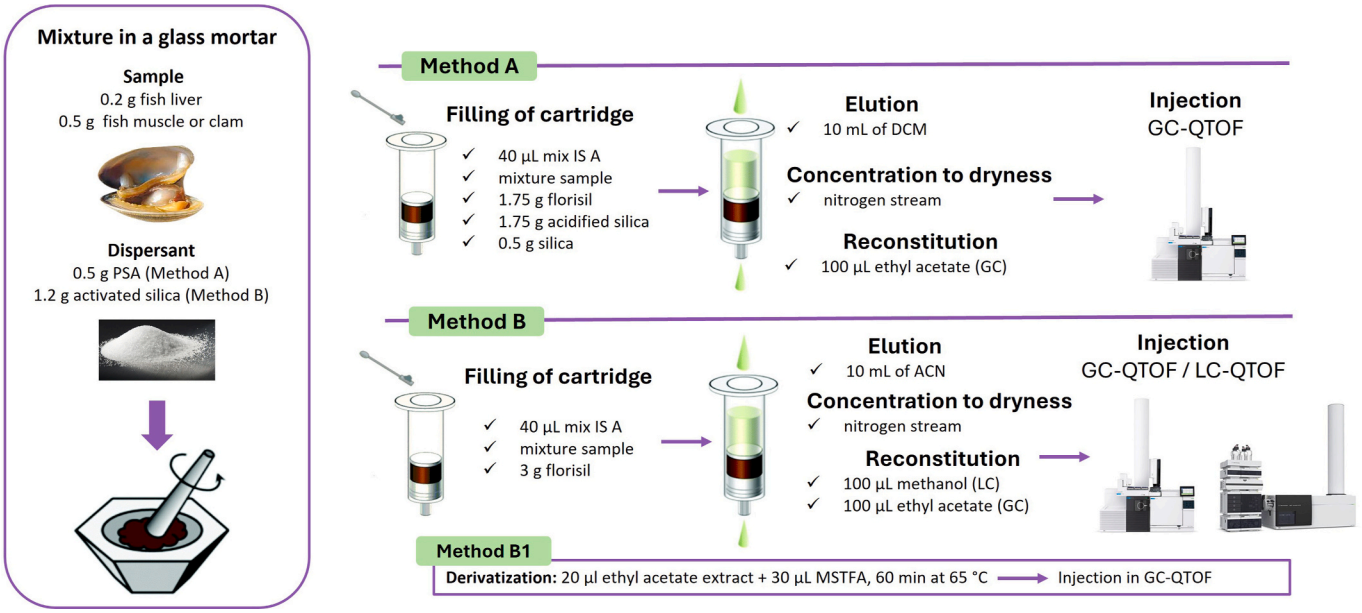


**Fig. 2.** Scheme of the three analytical methods applied for the sample preparation.

in (from bottom to top) 3 g of florisil (deactivated with 5 % (w/w) $H_20$), a polyethylene frit and the homogenized mixture sample. Analytes were eluted with 10 mL of acetonitrile. The eluate was concentrated to dryness under a nitrogen stream and reconstituted in 100 µL of ethyl acetate (for gas chromatography injection) or reconstituted in 100 µL of methanol (for liquid chromatography injection). An aliquot of 20 µL of

each extract reconstituted in ethyl acetate was derivatized with 30 μL of the silylation reagent MSTFA by heating in an oven for 60 min at 65 °C (method B1).

To reduce contamination problems, for both methods, florisil and silica were previously washed in a pressurized liquid extraction (PLE) system with acetonitrile and ethyl acetate at 60 °C. Then, the sorbents were dried in an oven for 24 h at 120 °C. Procedural blanks of both MSPD methods were performed together with each batch of samples.

### 2.4. Liquid chromatography-quadrupole-time of flight (LC-QTOF) analysis

Methanol extracts obtained by method B were analysed using an Agilent Technologies (Santa Clara, CA, USA) liquid chromatograph coupled to quadrupole time-of-flight (LC-QTOF) system. The LC instrument comprised a degasser, a binary pump, a thermostated column compartment and an autosampler. The mass spectrometer was a QTOF (Agilent 6550 QTOF) furnished with an ESI JetStream interface.

Chromatographic separation was carried out with a ZORBAX Eclipse Plus C18 column (2.1 × 50 mm; particle size 1.8 μm) supplied by Agilent Technologies. Two microliters of the sample extract were injected in positive and negative modes. As mobile phases, ultrapure water (5 mM ammonium acetate) (A) and methanol (5 mM ammonium acetate) (B) were used at a flow rate of 0.3 mL/min and the column temperature was fixed at 35 °C. The gradient elution started with 2 % B, increasing to 100 % B in 22 min, held for 4 min. Subsequently, it returned to initial conditions (2 % B) and held for 4 min for column back-conditioning.

The Agilent MassHunter Workstation software B.10.00 was used for instrument control and data acquisition. Data acquisition was performed by data dependent analysis (DDA) with an auto MS/MS method using three consecutive injections per sample excluding the precursor ions selected for tandem mass spectrometry (MS/MS) fragmentation in the previous injections (iterative acquisition). For these iterative MS/MS experiments the mass exclusion tolerance was fixed at 20 ppm for mass error and at 0.2 min for retention time.

The collision energies used for the fragmentation in auto MS/MS mode were 10, 20 and 40 V. The acquisition frequency and the scan range in single-stage MS and MS/MS were 3 and 6 spectra per second, and 60–1100 *m/z* and 30–1000 *m/z*, respectively. The QTOF mass spectrometer was operated at 2 GHz and continuously recalibrated using a reference solution through a secondary ESI nebulizer according to manufacturer's instructions.

### 2.5. Gas chromatography-quadrupole-time of flight (GC-QTOF) analysis

Samples extracts obtained by method A, B and B1 were analysed with a 7890A gas chromatograph (GC) coupled to a 7200 QTOF mass spectrometer from Agilent Technologies. The system was controlled by MassHunter Acquisition B.07.06 software (Agilent).

GC analysis was performed on a HP-5MS capillary column (30 m × 250 μm i.d., 0.25 μm film thickness) supplied by Agilent Technologies. High purity helium (99.9999 %, Nippon Gases, Madrid, Spain) was used as carrier gas at a constant flow rate of 1 mL/min. Transfer line, MS source and MS quad temperatures were set at 280 °C, 150 °C and 230 °C, respectively. The oven temperature program was as follows: 50 °C (held for 1 min), then ramped to 290 °C at 10 °C/min (held for 15 min). The total runtime was 40 min and the solvent delay was set at 3.5 min (5 min of solvent delay for the derivatized samples). One microliter of extract was injected at 280 °C in splitless mode during 1 min using a 10 μL syringe. The injector was equipped with an Agilent ultra-inert liner containing glass wool.

The QTOF mass spectrometer was operated at 2 GHz, the electron ionization (EI) source voltage was 70 eV with an emission current of 5 μA. Data were acquired in the mass range of 40 to 1000 *m/z*, at a frequency of 5 spectra per second. The QTOF mass axis was automatically recalibrated every 3 injections by infusion of a commercial solution of perfluorotributylamine in the EI source.

### 2.6. LC-QTOF suspect screening workflow

The scheme of the screening workflow for LC-QTOF is presented in Fig. 3. The suspect screening workflow was performed using a HRMS library of 3227 chemical products (e.g., pharmaceuticals, pesticides, drugs and industrial chemicals). This library was created combining four commercial libraries supplied by Agilent (ForTox PCDL, Water PCDL, Pesticides PCDL and VetDrugs PCDL) and a non-commercial MS/MS library developed at the USC (Castro et al., 2021).

For the identification of compounds an automatic search was performed using the algorithm "Find by Auto MS/MS", which extracts features and compares their MS and MS/MS spectra against the library. Thus, the software generates a list with the suspect compounds identified in the sample and calculates a normalized score parameter (0–100) combining mass deviation, isotope abundance and spacing between ions. As screening criteria, a minimum peak area of 1000 counts, maximum mass error (±5 ppm) and score higher than 80 were set as cut-off values. Also, MS/MS spectra were compared at the different collision energies with the recorded in library, at least two product ions in the MS/MS spectrum should match the recorded in library. Moreover, a manual verification was performed filtering by peak shape. Finally, when standards were available in the laboratory, they were injected into the system to unequivocally confirm the compound based on both retention time and MS/MS spectrum matching. Identification levels were used to denote confidence in identification based on the proposal made by Schymansky et al. (Schymanski et al., 2015).

### 2.7. GC-QTOF suspect screening workflow

The suspect screening workflow for GC-QTOF data analysis (Fig. 3) was performed using the deconvolution algorithm "SureMass". For the spectra comparison, two HRMS libraries were used: a commercial Agilent Pesticides library of over 800 compounds (modified to include retention index, RIs) and an in-house library developed in the laboratory with 356 experimental spectra, which includes 116 trimethylsilyl derivatives spectra (Castro et al., 2022). Moreover, the low-resolution NIST (The National Institute of Standards and Technology) 2017 library was used to cover a wider number of compounds.

Compound identification using HRMS libraries, with a pure weight factor (PWF) of 0.7, was performed, as described in Castro et al. (Castro et al., 2022), using a retention index difference (Delta RI) less than 20 s, a maximum mass error of 50 ppm, and a match factor higher than 70. Thus, the compounds that satisfied these criteria were selected as tentatively confirmed (level 2a). When the identification was performed using the NIST 17 low-resolution library, a filter focusing on the presence of chlorine and bromine heteroatoms in the molecule's formula prediction and with a minimum mass score of 80 was applied to those tentatively identified chemicals. Using this low-resolution library, the identification of the compounds was assigned as level 3 due to the lack of retention indexes and low-resolution nature of the library spectra.

### 2.8. Data treatment of the LC-QTOF and GC-QTOF results

For the analysis and interpretation of the LC-QTOF and GC-QTOF results, only those compounds with signals at least 3 times higher than the signal obtained in the blanks were considered. For this comparison of signals, the peak area ratio of the analytes and the corresponding IS were considered, the corresponding IS were assigned by structural similarity and retention time with the analyte. For this purpose, the software Agilent MassHunter Quantitative Analysis B10 was used.

In addition, using the web tool ClustVis (https://biit.cs.ut.ee/clustvis; Metsalu and Vilo, 2015), a statistical treatment was carried out and provided a cluster heat map using the Manhattan distance by the average method.

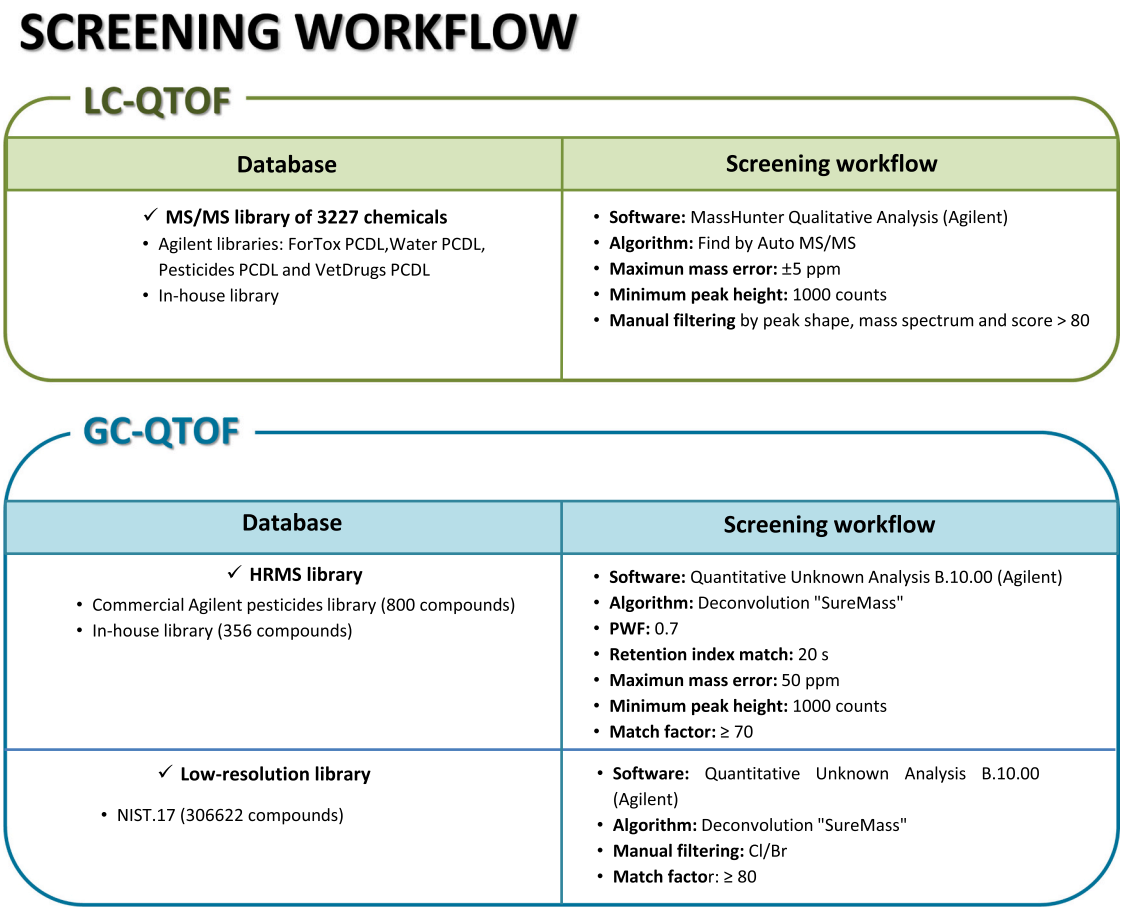


**Fig. 3.** Scheme of the screening workflow applied for the LC-QTOF and GC-QTOF.

### *2.9. Quantification of PFAS congeners by ultra(high)-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) analysis*

Freeze-dried samples were submitted to a sample preparation based on a MSPD protocol developed by Villaverde-de-Sáa et al. (Villaverde-De-Sáa et al., 2012). Briefly, in this protocol 0.5 g of freeze-dried sample spiked with the internal standards (mix IS B) was mixed with 0.2 g of diatomaceous earth in a glass mortar. A 10 mL polypropylene syringe was filled (from bottom to top) with 1 g of $Na_2SO_4$, 4 g of silica gel and the mixture of 0.5 g of freeze-dried sample with 0.2 g of diatomaceous earth. Analytes were eluted with 20 mL of acetonitrile. The eluate was concentrated under a nitrogen stream to a volume of 500 µL acetonitrile.

Acetonitrile extracts were analysed using a Waters Acquity UPLC® H class system (Milford, MA, USA) coupled to a XEVO TQD triple quadrupole (QqQ) mass spectrometer for the quantification of a mixture of 9 PFAS identified in the screening protocol and the legislated ones following the methodology proposed by Montes et al. (Montes et al., 2020). The chromatographic separation was performed at 40 °C on an Acclaim™ mixed-mode WAX-1 column (50 × 3 mm I.D., particle size 3 µm) from Thermo (Waltham, MA, USA) and the determination was performed by MS/MS in Selected Reaction Monitoring (SRM) mode acquiring two precursor/product ion transitions per analyte (except for PFBA, for which one transition was acquired) and one transition per IS. Table S4 (supplementary material) compiles SRM transitions (Qn), optimal collision energies (CE), cone voltages (CV) and retention times (RT) for each analyte.

The software MassLynx v4.1 and TargetLynx v4.1 (Waters) were used for control of the UHPLC-MS/MS system and data treatment, respectively.

As the average percentages of humidity in the species analysed in this work (calculated as the difference in weight after the freeze-drying process, relative to the initial weight and multiplied by 100) were 81 % in clam samples and 79 % in fish muscle, the concentrations were transformed to wet weight to easily compare the results with the limits stablished in the Regulation (EU) 2023/915.

#### *2.9.1. Validation of the UHPLC-MS/MS methodology*

The UHPLC-QqQ method for the quantification of 22 PFAS has been validated previously (Montes et al., 2020). For quality assurance and quality control (QA/QC) the following parameters were evaluated for the 9 selected PFAS: linear range, determination coefficient ($R^2$), instrumental method precision and instrumental quantification limits (IQL). Briefly, the IQLs, calculated for a signal to noise ratio of 10, ranged from 0.05 ng/mL to 0.5 ng/mL, the linear range spanned the IQLs to 100 ng/mL. Precision in terms of relative standard deviation (% RSD) values for 5 replicates of standards injected at 1 ng/mL were below 14 % and at 100 ng/g were below 13 % for the intra-day precision studies (Table S5, see supplementary material).

Also, the combination of the MSPD protocol (Villaverde-De-Sáa et al., 2012) and the UHPLC-QqQ method was evaluated for those 9 PFAS in terms of trueness, precision and method quantification limits (MQLs). Trueness and precision were assessed by recovery studies performed in mussel samples spiked with the standards (25 ng/g) (Table S5, see supplementary material). IS-corrected percentages of recovery (%R) for triplicate analyses of mussel samples comprised between 66 % and 132 %, with %RSD comprised between 1 % and 25 %. The MQLs calculated using the IQLs and the percentages of recovery of the mussel samples ranged from 0.002 ng/g to 0.07 ng/g for the clam samples, fish muscle and fish liver (Table S5, see supplementary material).

## 3. Results and discussion

### 3.1. Compounds identified by the MSPD-LC-QTOF screening workflow

A total of 82 compounds (see supplementary material, Table S6) were identified in at least one of the 18 analysed samples (7 clam samples and 11 fish tissue samples) using the Method B MSPD protocol followed by identification by LC-QTOF. From them, 29 were unequivocally identified (confirmation level 1) and the remaining compounds were set to confirmation level 2a, since a match between the experimental MS/MS spectra and the spectra recorded in the library was found, but the analytical standard was not available for confirmation. Fig. S1 (see supplementary material) shows an example of MS/MS spectrum comparison for an identified compound at level 1, metalaxyl, detected in a clam sample (SSOL_N1_A). Metalaxyl (DF – Detection Frequency - 67 %) is a fungicide widely used in agriculture which was also extensively reported as surface water pollutant (Fernández-García et al., 2024).

Fig. S2 presents the number of compounds detected according to their use by MSPD-LC-QTOF, the 82 identified compounds can be classified in 5 groups, pharmaceuticals (35), industrial chemicals (30), pesticides (10), cosmetics (5) and food additives (2). Table 1 summarizes the top ten compounds more frequently identified using this screening workflow among the 18 analysed samples, the number of samples in which they were detected, the detection frequency, the confirmation level and the usual uses of each compound. The highest detection frequency (DF) was found for acetanilide (100 % DF) followed by 4-hydroxybenzoic acid and 4-nitrophenol (83 % DF) being all within the industrial chemicals category. Both, 4-hydroxybenzoic acid and 4-nitrophenol are industrial chemicals frequently reported in literature as water pollutants (Castro et al., 2022.; Tisler et al., 2022). However, acetanilide, used for dye production and rubber vulcanization, was only found in tissues from fish exposed to stormwater run-off (Du et al., 2017).

### 3.2. Compounds identified by the MSPD-GC-QTOF screening workflow

#### 3.2.1. Identification based on HRMS libraries

A total of 56 compounds (see supplementary material Table S7) were identified in at least one of the 18 samples using the high-resolution spectral libraries. From them, 48 were identified by library spectra and retention index matching (confirmation level 1). The remaining compounds were classified as confirmation level 2a since a match between the experimental MS/MS spectra and the recorded in library was found. As an example, Fig. S3 (see supplementary material) depicts the chromatogram and spectra of 2,6-diisopropylnapthalene identified in the liver of a pout fish sample (BIB_LIVER N) processed with the MSPD method A. This compound presented a DF of 39 % in all the analysed samples and is used in the food industry to prevent sprouting of stored vegetables. The comparison between the sample spectrum (upper part of Fig. S3b) and the spectral library spectrum (lower part of Fig. S3b) showed a coincidence factor of 94.1 and a retention index difference (Delta RI) of −10, which allowed its identification.

As shown in Fig. S2, using this methodology, most of the identified pollutants were industrial chemicals or (by)products (44) whereas pharmaceuticals were predominant for the LC-based workflow. Table 2 shows the top ten compounds more frequently identified by MSPD (methods A, B and B1) and GC-QTOF, the number of samples in which they were detected, the DF, the confirmation level and some usual uses of each compound. The species with the highest DF in the samples were the polycyclic aromatic hydrocarbons (PAH) pyrene and fluoranthene with detection frequencies of 93 % and 78 %, respectively. Also, 2,2-bis (4-chlorophenyl)-1,1-dichloroethylene (4,4'-DDE), and 2,3′,4,4′,5-pentachlorobiphenyl (PCB 118) presented high DF. Several authors have reported high DF for PAHs and PCBs in mollusc samples (Rodil et al., 2019). The presence of these compounds in clam and fish samples could be attributed to their presence in release from petrochemical industry and other industrial processes and products. Regarding the rest of identified compounds, 7 were cosmetics, 3 pesticides, 1 food additive and 1 pharmaceutical. The most frequently detected compound from these later families was 2-ethylhexyl salicylate (a UV filter) with a detection frequency of 83 %.

#### 3.2.2. Identification based on low-resolution spectral library (NIST 17)

A total of 38 compounds were tentatively identified using MSPD (methods A, B and B1) and GC-QTOF with the NIST 17 screening approach (confirmation level 3). These analytes can be found in supplementary material (Table S8). As an example, Fig. S4 (see supplementary material) shows the chromatogram and spectra of 2,3,6-trichloro-1,1'-Biphenyl (PCB 24) identified in one of the clam samples (SSOL_N2_A) processed by method B. This compound, which presented a DF of 39 % belongs to the group of polychlorinated biphenyls (PCB) that were widely used in industry as dielectric and hydraulic fluids, as oil modifiers, as heat exchange fluid and in vacuum pumps. The experimental spectrum (upper part of Fig. S4b) was compared with the library spectra with a coincidence factor of 90.4.

As already mentioned in Section 2.7, this search was limited to Cl/Br containing chemicals and includes, among others, PCBs, chlorinated and brominated phenols, etc. These types of halogenated compounds have been previously identified in environmental samples using GC–MS (Badea et al., 2020; Cariou et al., 2016; Simonnet-Laprade et al., 2022).

### 3.3. Cluster analysis of the compounds identified in clam samples

A cluster heat-map (Fig. 4) was built with the results obtained from the clam samples, as normalized relative area (see Section 2.8). This

**Table 1**
Top ten most frequently detected compounds by MSPD-LC-QTOF.

| Compound | CAS | No. Clam samples detections ($n$ = 7) | No. Fish tissue sample detections ($n$ = 11) | % Detection | ID Level | Uses |
|---|---|---|---|---|---|---|
| Acetanilide | 103-84-4 | 7 | 11 | 100 | 2a | Industrial chemical |
| 4-Hydroxybenzoic acid | 99-96-7 | 4 | 11 | 83 | 1 | Industrial chemical and cosmetic |
| 4-Nitrophenol | 100-02-7 | 7 | 8 | 83 | 1 | Industrial chemical |
| Perfluoro-n-decanoic acid | 335-76-2 | 4 | 8 | 67 | 1 | Surfactant |
| Norethisterone | 51-98-9 | 6 | 5 | 61 | 2a | Drug |
| 4-Chlorophenol | 106-48-9 | 2 | 8 | 56 | 2a | Industrial chemical |
| Metalaxyl | 57837-19-1 | 4 | 6 | 56 | 1 | Pesticide |
| p-Phenetidine | 156-43-4 | 7 | 3 | 56 | 2a | Industrial chemical |
| Vigabatrin | 60643-86-9 | 5 | 4 | 50 | 2a | Drug |
| p-(Aminomethyl)benzoic acid | 56-91-7 | 4 | 4 | 44 | 2a | Industrial chemical |

**Table 2**
Top ten most frequently detected compounds by MSPD-GC-QTOF.

| Compound | CAS | No. Clam samples detections (n = 7) | No. Fish samples detections (n = 11) | % Detection | ID Level | Uses |
|---|---|---|---|---|---|---|
| Pyrene | 129-00-0 | 7 | 10 | 94 | 1 | Industrial chemical |
| 2-Ethylhexyl salicylate | 118-60-5 | 7 | 8 | 83 | 1 | Cosmetic |
| 2,2-Bis(4-chlorophenyl)-1,1-dichloroethylene | 72-55-9 | 4 | 10 | 78 | 1 | Industrial chemical |
| 2,3',4,4',5-Pentachlorobiphenyl | 31508-00-6 | 4 | 10 | 78 | 1 | Industrial chemical |
| Fluoranthene | 206-44-0 | 6 | 8 | 78 | 2a | Industrial chemical |
| Naphthalene | 91-20-3 | 3 | 9 | 67 | 1 | Industrial chemical |
| 1,1′-Biphenyl | 92-52-4 | 6 | 5 | 61 | 1 | Industrial chemical |
| 2,3,3',4,4',5-Hexachlorobiphenyl | 38380-08-4 | 6 | 5 | 61 | 2a | Industrial chemical |
| Diphenyl ether | 101-84-8 | 3 | 7 | 56 | 1 | Industrial chemical |
| Phenanthrene | 85-01-8 | 5 | 5 | 56 | 1 | Industrial chemical |

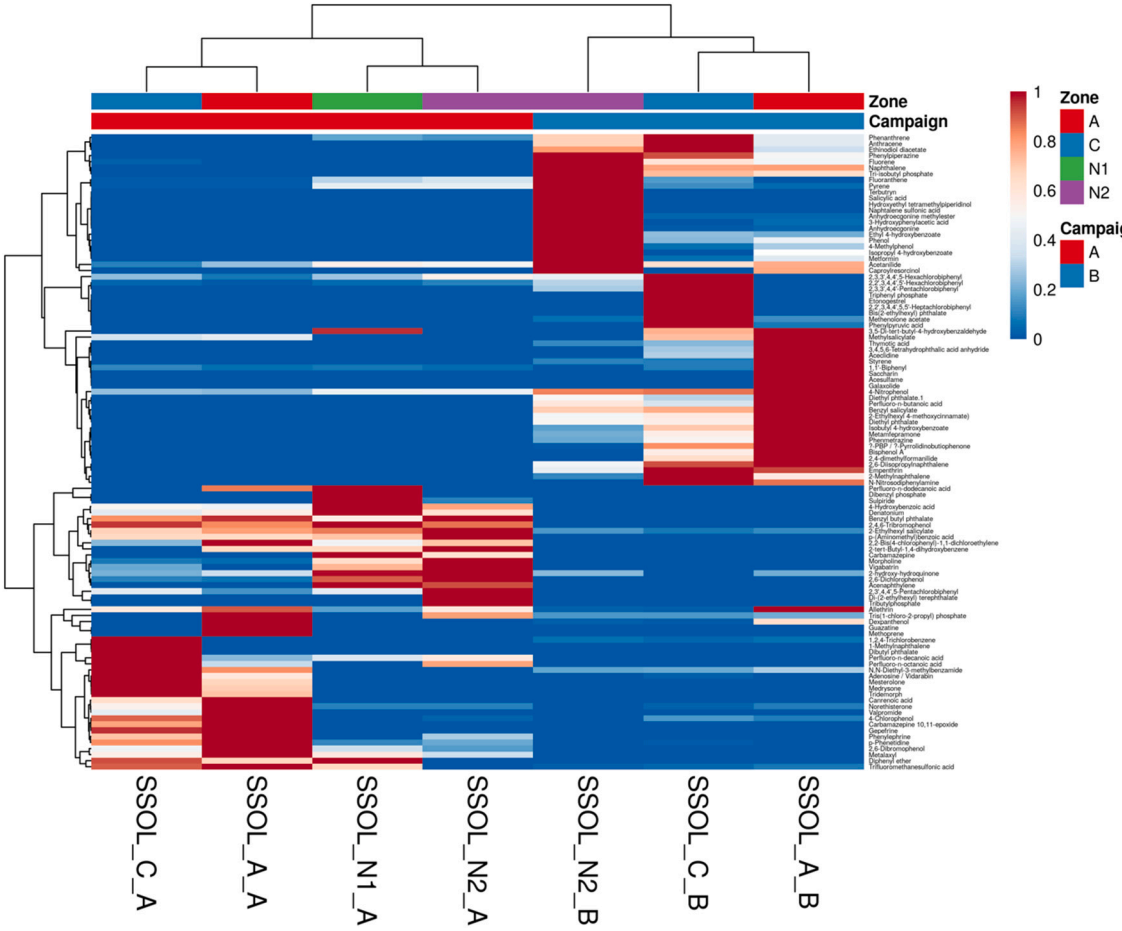


**Fig. 4.** Cluster analysis of clam samples. Location: A: Algarve; C: center; N: north. Campaign: A: September 2020, B: June 2022. Created with https://biit.cs.ut.ee/clustvis (Metsalu and Vilo, 2015). Information regarding sample coding can be found in Table S3. Raw data accessible in https://doi.org/10.5281/zenodo.14621070.

figure shows the identified compounds (rows) in the 7 clam samples (columns). The colour indicates the relative abundance of a given compound. Clam samples are separated in six categories according to the location (A, C, N1, N2) and the two sampling campaigns (A and B). A clear clustering of samples from the two different campaigns is observed. In campaign A the clams were out of the reproductive period whereas in campaign B they were mature, which could be one of the explanations for the differences in clustering between sampling campaigns. Also, in campaign A, samples from the zones A (Algarve, south of Portugal) and C (center) are clustered together containing pesticides such as metalaxyl

and pharmaceuticals (such as mesterolone and valpromide) being different from those from the N zone (north) containing mostly industrial chemicals (such as 4-hydroxybenzoic acid and 2,6-dichlorophenol). These results reflect the differences between the most industrialized area of the country (northern region) and the C area and specially A region which is more associated with agriculture and tourism sectors.

Campaign B also shows different identified compounds based on the sampling locations. Compounds identified in samples from the N (north) area continue to be mainly related to industrial activity, e.g. the PAHs pyrene and phenanthrene, whereas in zone A (Algarve) pharmaceuticals and cosmetics are still predominant (e.g. benzyl salicylate). The profile in the zone C (center) in this sampling campaign is more heterogeneous compared with campaign A, with a lower predominance of pharmaceuticals and a higher presence of industrial chemicals, such as 2,3,3′,4,4′,5-hexachlorobiphenyl (PCB-157).

Although some differences between the campaigns and the location of the samples were observed, most of the compounds identified in the clam samples are industrial chemicals. Among others, pyrene, phenanthrene, tris(1-chloro-2-propyl) phosphate and PFDoA were also previously reported in clam samples collected in the neighbouring coast of Spain (Castro et al., 2022; Rodil et al., 2019).

### 3.4. Cluster analysis of the compounds identified in the fish tissue samples

The results obtained from the fish tissue samples were analysed by clustering as detailed in Section 2.8.

As shown in Fig. 5, the compounds identified in the fish samples are divided into two clear subgroups according to the type of tissue (muscle and liver). In the muscle, all the identified pharmaceuticals were detected in the L (Lisbon) area, which is more impacted by the tourism. However, in the N and C areas (more industrialized areas) there are a higher normalized intensity of industrial compounds such as 2,6-dichlorophenol, pyrene and tris (1-chloro-2-propyl) phosphate, among others.

On the other hand, in the liver samples, although industrial chemicals are predominant in all the areas, there are differences between the compounds identified in the north of Portugal (N) being predominant compounds like styrene, diisobutyl phthalate or 4-nitrophenol and the compounds identified in the center (C) and Lisbon zone (L) where PFAS (e.g., PFOA, PFNA, PFBA and PFOS) are the most abundant pollutants. The presence of PFAS mostly in the center (C) and Lisbon (L) zones points to the proximity of an urban area of electronic industry (Glüge et al., 2020).

### 3.5. Correlation analysis of the compounds identified in the clam samples and fish tissue samples

Considering the fish and clam samples together, there are notable similarities in the compounds identified across the different sampling areas. In the northern region (N1, N2, and N), most compounds identified were industrial reagents. This finding aligns with the fact that this is the most industrialized area of Portugal, heavily influenced by the petrochemical industry. In contrast, in the center (C) and the Algarve (A) zones there are a higher prevalence of pesticides and pharmaceuticals, reflecting the significant influence of agriculture and tourism in these areas. Meanwhile, in the Lisbon region (L), industrial reagents were predominantly identified, with most being PFAS compounds, likely due to the proximity of the electronic industry. Since in campaign B only

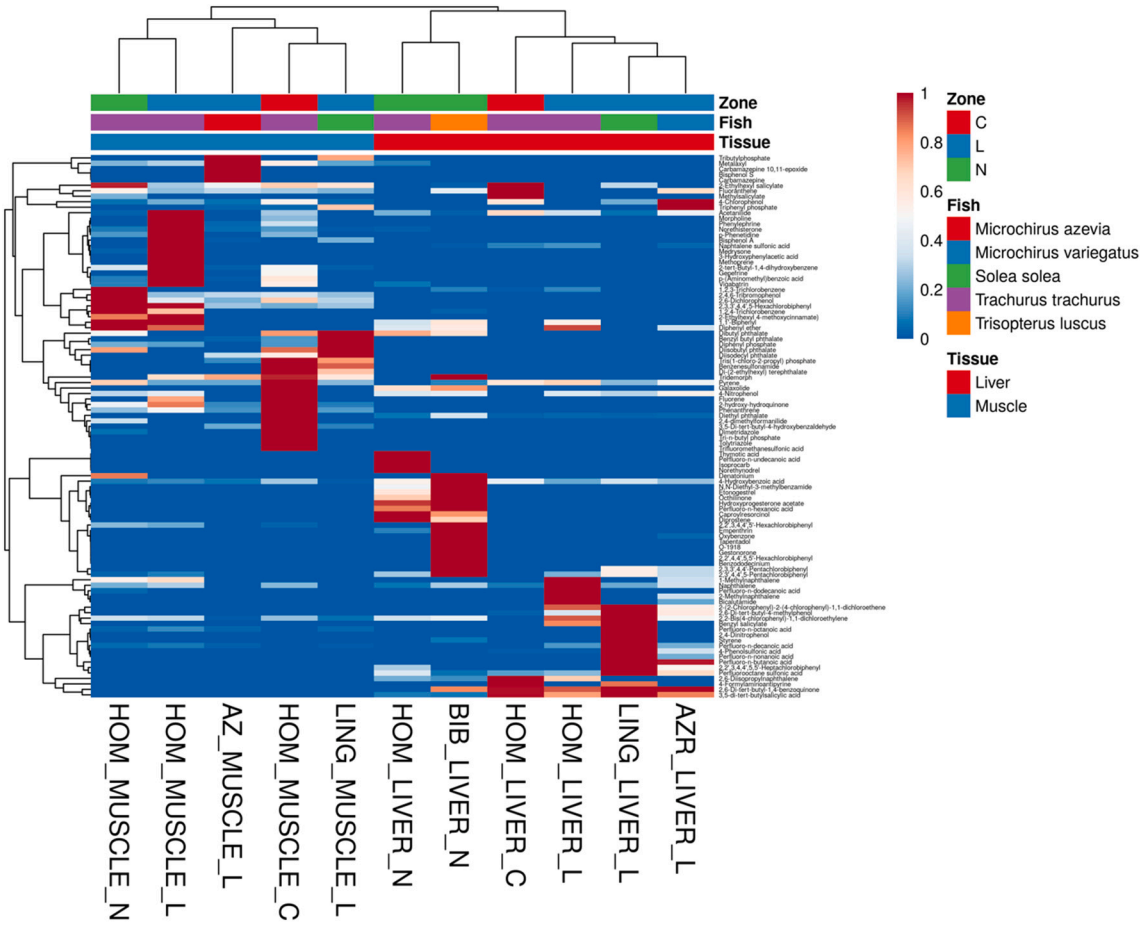


**Fig. 5.** Cluster analysis of fish tissues. Location: C: center; L: Lisbon; N: north. Campaign: September 2020. Created with https://biit.cs.ut.ee/clustvis (Metsalu and Vilo, 2015). Information regarding sample coding can be found in Table S3. Raw data accessible in https://doi.org/10.5281/zenodo.14621070.

clam samples were analysed, any correlation with the fish samples cannot be established.

### 3.6. Quantification of PFAS in clam and fish tissue samples

According to the recent Regulation (EU) 2023/915 (EC, 2023), some of the PFAS detected in these screening campaigns must be quantified in foodstuff samples, including bivalve molluscs and fish meat. Considering that PFAS presented a high DF in all the species specially in those samples collected in center and Lisbon areas, a quantitative methodology based on MSPD and UHPLC-MS/MS (Section 2.9) was adapted and applied to accurately quantify the identified PFAS and the legislated ones. This quantitative methodology was only applied to the 10 fish samples and 7 clam samples where PFAS were originally detected. During the analysis process, three procedural blanks were performed together with each sample batch and blank concentrations were subtracted from the samples' ones. As shown in Table 3, all the investigated PFAS were found in at least one sample at concentrations ranging from LOQ to 5.8 ng/g wet weight (ww). All the PFAS identified in the previous screening were detected using the quantitative method in addition to PFHxS that was not originally identified through the screening methodology but could finally be quantified using this target approach.

In terms of concentration, PFOA and PFOS were the analytes detected at higher levels in the samples (up to 5.3 ng/g ww). The presence of higher concentrations of PFOA and PFOS, long chain PFAS, can be attributed to their acknowledged capacity to bioaccumulate in fish samples. In fact, levels in liver (the highest fat content matrix) were higher than those found in molluscs and fish muscle. To date, very few studies have focused on the analysis of PFAS in mollusc, fish muscle and fish liver samples and in agreement with present results, PFOA and PFOS were the predominant PFAS detected (Villaverde-De-Sáa et al., 2012).

The permitted levels of PFAS set in the Regulation (EU) 2023/915 are 5 ng/g ww as sum of PFOS, PFOA, PFNA and PFHxS in bivalve mollusc and 2 ng/g ww in fish muscle except for *Trachurus trachurus* (HOM) with a permitted levels slightly higher (8 ng/g ww) (Table S9). However, if the meat is intended for infant feed the permitted level in muscle of all fish species is set at 2 ng/g.

Focusing on clam samples, the levels found in the analysed samples (as sum of regulated congeners) are lower than the regulated ones, except for sample SSOL_C_B. However, when analysing the regulated congeners separately, 5 out of 7 samples presented levels of PFOA up to five times higher than the allowed (0.7 ng/g ww). Thus, PFOA appears as the major congener in clam samples with concentrations higher than reported in previous studies in this Atlantic European region (Villaverde-De-Sáa et al., 2012), which reinforces the concern about the presence of this substance in marine environments.

Regarding fish muscle, the permitted levels are higher for *Trachurus trachurus* (HOM) than for the other analysed species. For that reason, none of the HOM muscle samples surpassed the limit (8 ng/g ww) whereas for both *Solea solea* (LING) and *Microchirus azevia* (AZ) muscle samples the concentrations found were higher than allowed (2 ng/g ww). Again, when analysing the results for the congeners individually all muscle samples presented higher levels than permitted for at least one congener (Table 3 and Table S9).

As shown in Fig. 6, PFOA was identified in 15 out of 17 analysed samples with concentration levels above the allowed limit for 5 out of 7 clam samples (0.7 ng/g ww) and for LING and AZ muscle meat (0.2 ng/g ww). Also, the concentrations for 1 out of 3 HOM muscle samples were higher than allowed (1.0 ng/g ww). Thus, PFOA concentrations exceeded the regulated levels in most of the samples reaching concentrations up to 5.2 ng/g ww in the case of the liver of *Microchirus variegatus* (AZR), although the legislation does not establish a threshold for the case of liver.

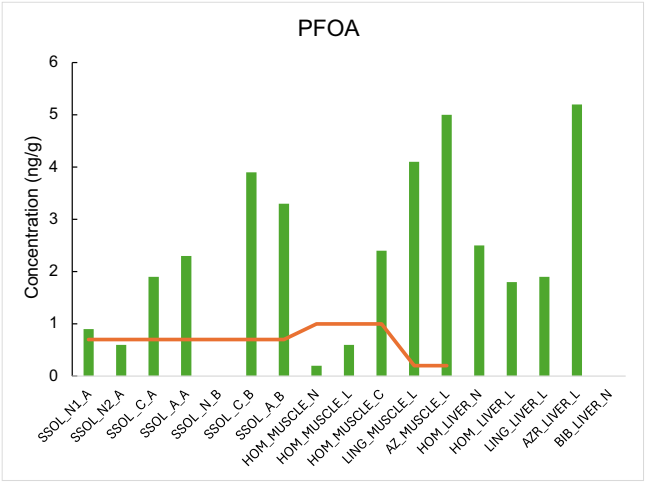


**Fig. 6.** Concentration (ng/g ww) of PFOA in the 17 analysed samples (green columns) and the maximum concentration level permitted by legislation 1.0 ng/g ww for HOM muscle, 0.2 ng/g ww for other fish muscle and 0.7 ng/g ww for bivalve molluscs (orange line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 3**
Concentration (ng/g) ww of 9 PFAS in the biota samples ($n = 2$ replicates).

| Sample | PFBA | PFHxA | PFOA[a] | PFNA[a] | PFDeA | PFUnA | PFDoA | PFHxS[a] | PFOS[a] | ΣPFAS[a] |
|---|---|---|---|---|---|---|---|---|---|---|
| HOM_MUSCLE_N | 0.5 | 0.1 | 0.2 | – | 0.4 | 0.4 | 0.3 | 0.3 | 0.7 | 1.2 |
| HOM_MUSCLE_L | 0.2 | 0.1 | 0.6 | 0.3 | 0.7 | – | – | 0.7 | 0.7 | 2.3 |
| HOM_MUSCLE_C | 2.1 | 0.3 | 2.4 | 0.7 | 0.7 | 0.7 | 0.8 | 0.7 | 0.8 | 4.5 |
| HOM_LIVER_N | 1.7 | 1.6 | 2.5 | – | 2.2 | 2.5 | 2.0 | 1.6 | 3.2 | 7.2 |
| HOM_ LIVER_L | 1.5 | 1.4 | 1.8 | – | 3.7 | – | – | 1.6 | 2.6 | 6.0 |
| LING_MUSCLE_L | 3.1 | 2.2 | 4.1 | 0.8 | 0.4 | – | – | 0.3 | 0.9 | 6.0 |
| LING_LIVER_L | 1.5 | – | 1.9 | 5.0 | 2.0 | – | 1.7 | 1.6 | 4.2 | 12.7 |
| AZ_MUSCLE_L | 5.8 | 2.9 | 5.0 | – | – | – | – | 0.7 | – | 5.6 |
| AZR_LIVER_L | 2.1 | 1.2 | 5.2 | 3.4 | – | – | – | 1.6 | 5.3 | 15.5 |
| BIB_LIVER_N | 1.1 | 1.4 | – | – | – | – | – | – | 1.9 | 9.0 |
| SSOL_N1_A | 1.1 | 0.6 | 0.9 | 0.6 | – | – | – | – | 0.6 | 2.1 |
| SSOL_N2_A | 0.6 | 0.2 | 0.7 | – | 0.3 | – | 0.6 | 0.6 | 0.6 | 1.8 |
| SSOL_C_A | 0.6 | 0.2 | 1.9 | – | 0.3 | – | – | 0.6 | 0.6 | 3.1 |
| SSOL_A_A | 0.6 | 0.2 | 2.3 | 0.6 | 0.3 | – | 0.6 | 0.6 | 0.6 | 4.1 |
| SSOL_N2_B | <MQL | – | – | – | – | – | – | 0.6 | 0.6 | 1.2 |
| SSOL_C_B | 2.3 | 1.5 | 3.9 | – | – | – | – | 0.6 | 0.7 | 5.2 |
| SSOL_A_B | 2.9 | 1.4 | 3.3 | – | – | – | – | 0.6 | – | 4.0 |

[a] PFAS included in the European regulation (EU) 2023/915 [5] (EC, 2023). MQL: method quantification limit.

## 4. Conclusions

When dealing with the OPs screening in biota samples, a combination of different sample preparation and determination strategies is essential to cover a variety of compounds with different physicochemical characteristics. In this work, different MSPD protocols previously applied in mollusc samples were used, together with a combination of suspect screening workflows using GC-QTOF and LC-QTOF analysis and different spectral libraries allowing the tentative identification of 176 OPs, where 77 of them were unequivocally identified by reference standards. Whereas pharmaceuticals were primarily detected using LC-QTOF analysis, GC-QTOF identification was more successful for industrial chemicals, being 2-ethylhexyl salicylate, fluoranthene, acetanilide or 4-nitrophenol some of the OPs more frequently detected. The cluster analysis allowed the grouping of OPs by fish tissue and interestingly, a distinct profile was observed for liver and muscle, independent of the fish species. Regarding clam analysis, samples collected during different campaigns clustered separately, suggesting a maturation-based and location-based influence. Using the screening LC-QTOF workflow, 9 PFAS were identified (only one sample was free of contamination from this OPs class), and this fact, together with the recent changes in foodstuff European regulation 2023/915 (where maximum allowed levels for four PFAS were included) promoted the need for the application of a quantitative MSPD-LC-MS/MS methodology to 17 samples. The levels of the regulated PFAS were higher than those allowed by the regulation in some fish muscle samples and bivalve mollusc. The results of this study emphasize the need of additional monitoring and analysis of OPs in fish and mollusc samples. This is essential to implement new regulatory frameworks aimed at reducing the concentrations of these compounds and, consequently, minimizing both environmental and human exposure to these substances.

## CRediT authorship contribution statement

**Sandra Méndez:** Writing – original draft, Methodology, Investigation. **Rosa Montes:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Conceptualization. **Joana Raimundo:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Daylin López-Castillo:** Investigation. **Javier López-Vázquez:** Investigation. **Miguel Caetano:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Clara Lopes:** Resources. **Cátia Figueiredo:** Resources. **Marlene Pinheiro:** Investigation. **Nélson Alves:** Investigation. **Ricardo Capela:** Investigation. **Susana Barros:** Investigation. **Hugo Morais:** Investigation. **Teresa Neuparth:** Writing – review & editing, Resources, Conceptualization. **Miguel.M. Santos:** Writing – review & editing, Funding acquisition, Conceptualization. **José Benito Quintana:** Writing – review & editing, Supervision, Resources, Conceptualization. **Rosario Rodil:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Rosario Rodil reports financial support was provided by Spanish Scientific Research Agency (Agencia Estatal de Investigación). Rosa Montes reports financial support was provided by Government of Galicia (Xunta de Galicia). Joana Raimundo reports financial support was provided by Portuguese Institute for the Sea and Atmosphere. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2025.118598.

## Data availability

Data contained in this research article can be found in Zenodo repository (DOI:10.5281/zenodo.14621070).

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