Determination of 56 per- and polyfluoroalkyl substances in top predators and their prey from Northern Europe by LC-MS/MS

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Abstract

Per- and polyfluoroalkyl substances (PFAS) are a group of emerging substances that have proved to be persistent and highly bioaccumulative. They are broadly used in various applications and are known for their long-distance migration and toxicity. In this study, 65 recent specimens of a terrestrial apex predator (Common buzzard), freshwater and marine apex predators (Eurasian otter, harbour porpoise, grey seal, harbour seal) and their potential prey (bream, roach, herring, eelpout) from northern Europe (United Kingdom, Germany, the Netherlands and Sweden) were analyzed for the presence of legacy and emerging PFAS, employing a highly sensitive liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) method. 56 compounds from 14 classes were measured; 13 perfluoroalkyl carboxylic acids (PFCAs), 7 perfluoroalkyl sulphonic acids (PFSAs), 3 perfluorooctane sulfonamides (FOSAs), 4 perfluoroalkylphosphonic acids (PFAPAs), 3 perfluoroalkylphosphinic acids (PFPi's), 5 telomer alcohols (FTOHs), 2 mono-substituted polyfluorinated phosphate esters (PAPs), 2 di-substituted polyfluorinated phosphate esters (diPAPs), 6 saturated fluorotelomer acids (FTAS), 3 unsaturated fluorotelomer acids (FTUAs), 2 N-Alkyl perfluorooctane sulfonamidoethanols (FOSEs), 3 fluorotelomer sulphonic acids (FTSAs), 2 perfluoroether carboxylic acids (PFECAs) and 1 chlorinated perfluoroether sulphonic acid (Cl-PFESA). All samples were lyophilized before analysis, in order to enhance extraction efficiency, improve the precision and achieve lower detection limits. The analytes were extracted from the dry matrices through generic methods of extraction, using an accelerated solvent extraction (ASE), followed by clean-up through solid phase extraction (SPE). Method detection limits and method quantification limits ranged from 0.02 to 1.25 ng/g wet weight (ww) and from 0.05 to 3.79 ng/g (ww), respectively. Recovery ranged from 40 to 137 %. Method precision ranged from 3 to 20 %RSD. The sum of PFAS concentration in apex predators livers ranged from 0.2 to 20.2 µg/g (ww), whereas in the fish species muscle tissues it ranged from 16 to 325 ng/g (ww). All analysed specimens were primarily contaminated with PFOS, while the three PFPi's included in this study exhibited frequency of appearance

(FoA) 100%. C9 to C13 PFCAs were found at high concentrations in apex predator livers, while the overall PFAS levels in fish fillets also exceeded ecotoxicological thresholds. The findings of our study show a clear association between the PFAS concentrations in apex predators and the geographical origin of the specimens, with samples that were collected in urban and agricultural zones being highly contaminated compared to samples from pristine or semi-pristine areas. The high variety of PFAS and the different PFAS composition in the apex predators and their prey (AP&P) samples is alarming and strengthens the importance of PFAS monitoring across the food chain.

Keywords

PFAS, LC-MS/MS, buzzard, otter, harbour porpoise, harbour seal, grey seal

1. Introduction

Per- and poly-fluoroalkyl substances (PFAS) compose a vast class of chemicals that includes perfluoroalkyl acids (PFAAs) and more specifically perfluoroalkyl carboxylic acids (PFCAs) and perfluoroalkyl sulfonic acids (PFSAs) **(EFSA, 2011)**. PFAS are persistent, bio-accumulative and possibly carcinogenic to animals as well as humans **(Ahrens, 2011)**. Since the 1940s, they have been broadly used in several applications due to their particular physicochemical properties **(Prevedouros et al., 2006)**. They have been extensively used in foam mixtures for fire-extinguishing purposes and surfactants **(De Voogt and Saez, 2006; Richardson, 2008)**. Additionally, these versatile substances have been used in leather as well as textile treatment processes **(Villagrasa et al., 2006)**. PFAS end up in the aquatic ecosystems primarily through industrial wastewater **(Rappazzo et al., 2017)** . Short-chain PFAS display increased mobility in sediment and water layers, which classifies them as exceptionally hazardous for the environment, yet up to this day these substances have not been adequately monitored **(Brendel et al., 2018)**.

PFAS are recognized endocrine disrupting chemicals, and animal studies have suggested multiple pathways of impact that include disruption of reproductive hormones and impaired signaling of thyroid hormones **(Rappazzo et al., 2017; Gardener et al., 2021)**. The enormous number of homologues, metabolites and precursors of all known PFAS classes (>4000 variations according to OECD records) and the knowledge gap regarding their environmental fate and hazardous potential makes them a subject of continuous concern **(Nakayama et al., 2019)**. The increased half-lives of PFAS in both wildlife and humans render them extremely hazardous for the environment **(Zhang et al., 2013)**.

Biomonitoring of per- and polyfluoroalkyl substances in living organisms is an evolving field of research. Legacy PFAS have been detected in human blood cells **(Lau et al., 2007; Goralczyk et al., 2015)**, breast milk **(Motas Guzman et al., 2016)** seminal plasma **(Guruge et al., 2005)**, and umbilical cord blood **(Inoue et al., 2004)**. Unlike the majority of persistent organic pollutants (POPs), they tend to accumulate in the

kidneys, and bile secretion and not in fat tissues **(Jones et al., 2003; Perez et al., 2013)**. Additionally, PFAS levels have been reported to be very high in human liver cells **(Domingo et al., 2012; Fliedner et al., 2020)**.

Currently, perfluorooctanesulfonate (PFOS) and its salts are listed under Annex B of the Stockholm Convention for Persistent Organic Pollutants **(UNEP, 2009)**, while perfluorooctanoic acid (PFOA), its salts and PFOA-related compounds were added to Annex A in 2019. Perfluorohexane sulfonate (PFHxS) has been proposed for inclusion **(UNEP, 2018)**. The phase-out of the legacy compounds and their replacement with structurally similar PFAS has been the most common industry policy in the last decades **(Wang et al., 2013; Wang et al., 2017)**. This poses a great environmental danger, since most emerging PFAS also show high toxicity, yet are to this day not routinely monitored or part of any regulatory guideline **(Cao et al., 2019)**. Up to this day there are nearly 5000 PFAS that are broadly used in several industrial and commercial applications **(Buck et al., 2011)**.

Additionally, many PFAS undergo transformation in wastewater treatment plants as well as metabolic alteration in humans and livestock. This creates the urge for PFAS precursors, metabolites, intermediate – and final products to be incorporated in targeted analytical methodologies together with the parent analytes **(Lee et al., 2010; Wang et al., 2011; Zhao et al., 2013)** . In order to limit the environmental as well as health-related risks from the manufacture and use of PFAS, a restriction proposal is being elaborated under REACH in 2021.

Several analytical regimes have been developed for the determination of PFAS in various matrices, including sediments, ground- and freshwater **(Joerss et al., 2019; Simmonet-Laprade et al., 2019)**, fish and other aquatic organisms **(Babut et al., 2017; Liu et al., 2017; Fair et al., 2019)**, birds **(Munoz et al., 2017; Lopez-Antia et al., 2019; Russell et al., 2019)** and mammals **(Boisvert et al., 2019; Cui et al., 2019; Gui et al., 2019)**. Solid phase extraction (SPE) and liquid–liquid extraction (LLE) are the main techniques

that have been applied in the extraction, purification and pre-concentration of PFAS in environmental samples in the recent years **(Powley et al., 2005; Wolf and Reagen, 2011; Groffen et al., 2019)**. Liquid chromatography (LC) coupled with mass (MS) or tandem mass spectrometric (MS/MS) detection is the golden standard for the determination of PFAS **(Weremiuk et al., 2006; Fernandez-Sanjuan et al., 2010; Llorca et al., 2011)**; for some PFAS limits of detection at the picogram range can easily be achieved **(Gosetti et al., 2010; Zhao et al., 2011)**.

To the best of our knowledge, despite the high number of available analytical methodologies for the determination of PFAS in the environment, few studies have reported the simultaneous determination of multi-class PFAS in contemporaneously collected samples from differing trophic levels within an ecosystem. Environmental Specimen Banks (ESBs), scientific collections (SCs) and Natural History Museums (NHMs) have contributed to water management, chemicals' monitoring, and regulation. Systematic and opportunistic sampling campaigns have been conducted for decades, collecting various tissues from apex predators and their prey (AP&P). Sample collections are guided by standardized protocols and operate under well-controlled conditions to allow for chemicals investigations. The EU funded LIFE Apex project **(LIFE17 ENV/SK/000355, 2018-2022, www.lifeapex.eu)** was initiated to bring together sample collections and analytical laboratories with the objective to apply generic sample preparation and instrumental methods for the generation of contaminant data for apex predators and their prey in support of chemicals management **(Movalli et al., 2019; Badry et al., 2020)**.

The objective of the present study was to investigate the PFAS exposure among varying trophic levels including apex predators and fish species, that are also widely consumed by humans. We specifically aimed to determine the exposure to established and newer PFSA/PFCA contaminants and several PFSA precursors in livers of common buzzards, Eurasian otters, harbour and grey seals and harbour porpoises and muscle tissues of their major prey species, from several regions across Germany, Sweden, the Netherlands and the United Kingdom.

2. Material and Methods

2.1 Study area and sampling strategy

Within the framework of LIFE APEX, 65 samples of common buzzards, Eurasian otters, harbour and grey seals and harbour porpoises and several fish species from various ecosystems across central and northern Europe were retrieved from ESBs, SCs and NHMs (**Table S1** in supplementary information) and screened for 56 legacy and emerging PFAS from 14 classes. All apex predator samples in this study were liver tissues, while only fillet (muscle tissue) was extracted from the fish species for the PFAS target screening. This was done according to the project's strategic plan, which received approval by the EU. More specifically, the rationale was primarily ethical. Additionally, there were certain limitations concerning the sample availability from the specimen providers, namely it would have involved excessive fish sampling for the collection of enough pooled liver quantity to be compared with the predator liver samples in terms of PFAS contamination. On the other hand, as the predator screening is regarded, we aimed to analyze liver tissues since it is there where PFAS are primarily accumulated and metabolized. Sampling was carried out by two environmental specimen banks (German and Swedish ESBs), five research collections (UK Centre for Ecology & Hydrology, Cardiff University, University of Veterinary Medicine Hannover, Leibniz Institute for Zoo and Wildlife Research and Wageningen University & Research) and one natural history museum (Naturalis Biodiversity Center) over a 4 year period between 2015 and 2018 in Central and Northern Europe. 65 pooled samples of muscle and liver tissue were, obtained from 61 different locations across Germany, the Netherlands, Sweden and the United Kingdom (**Fig. 1**). The 8 species collected were the following: Bream (*Abramis brama*), Roach (*Rutilus rutilus*), Herring (*Clupea harengus*), Eelpout (*Zoarces viviparus*), Harbour porpoise (*Phocoena phocoena)*, Eurasian otter (*Lutra lutra*), Harbour seal (*Phoca vitulina*), Grey seal (*Halichoerus grypus*), and Common buzzard *(Buteo buteo*). All samples were processed at the collectors' facilities and, subsequently, frozen at -20 °C or -80 °C, shipped to and stored at -80 °C at

the National and Kapodistrian University of Athens (NKUA) or at the Laboratory of Analytical Chemistry of University of Athens (Greece). Muscle and liver tissue samples were kept frozen and thereafter freeze-dried before analyses. Sampling was conducted under EU research licenses/permits.

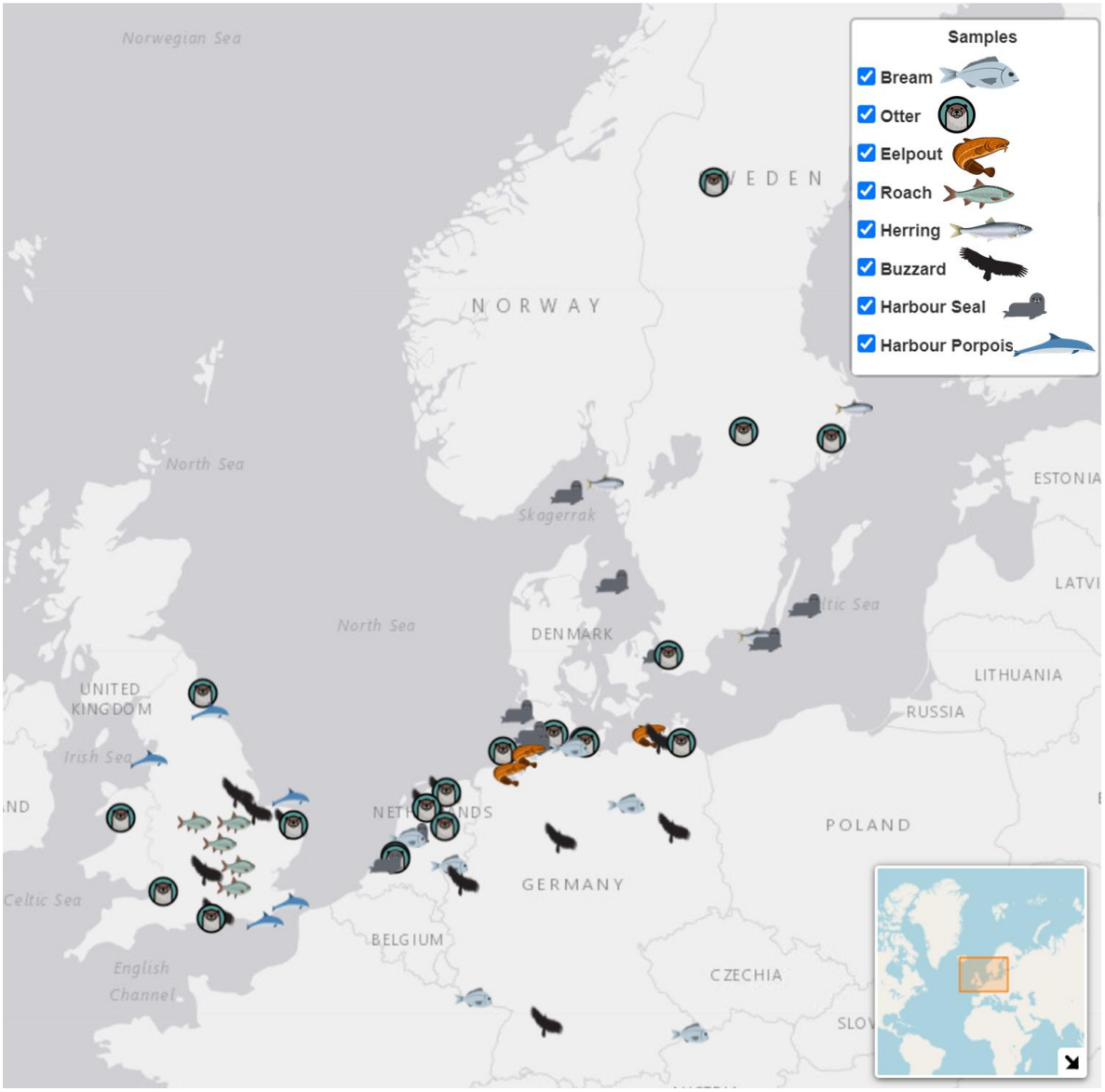


Figure 1. Sample collection sites and their spatial distribution. Interactive version of the map is available in the following link: https://norman-data.eu/LIFE_APEX_PFAS_Tier1/

2.2 Chemicals and reagents

The full list of target compounds, internal standards, and consumables that were used in this study can be found in section 2 of the supplementary information. In summary the target list included 13 PFCAs (C3-C14, C16 and C18; Cn refers to the carbon chain-length of the molecule), 7 PFSAs, 3 FASAs, 4 PFAPAs, 3 PFPi's, 5 FTOHs, 2 PAPs, 2 diPAPs, 6 FTAS, 3 FTUAs, 2 FASEs, 3 FTSAs, 2 PFECAs and 1 Cl-PFESA. The compound catalogue, including their abbreviation, compound class, and optimized LC-MS/MS parameters, can be found in **Table S2**.

2.3 Extraction of samples

All LIFE APEX samples collected from ESBs, NHMs and other scientific collections were sent to NKUA for their pre-treatment. The documentation and condition of the delivered samples were thoroughly checked, and unique sample codes were given to the samples. For the calculation of the % water content of the samples, empty petri-dishes with the respective code of each sample were weighed. This was followed by the segmentation of the samples and their placement into petri-dishes in an isolated room. The petri-dishes including the wet samples were then weighed. All samples were kept refrigerated (-80°C) for at least 5 hours, as a pre-treatment step before lyophilization. Afterwards, the samples' freeze-drying (-55°C, 0.05 mbar, Capacity: 5 kg/24h, Telstar Lyoquest Freeze Dryer) in accordance with the standardized operational procedure (SOP) for the lyophilization took place, followed by the weighting of the petri-dishes including the freeze-dried samples. Accordingly, the % water content was calculated. The weights and % water content, as well as any other freeze-drying relevant information were registered in a specific file. The homogenization of each sample using pestle and mortar or multi in an isolated room was then performed. Between homogenizations all lab instruments were cleaned using milli-Q water and acetone. All freeze-dried samples were then stored (-80°C) in amber glass vials. Accelerated Solvent Extraction

(ASE) was used for the extraction of the analytes from the biota matrices, followed by a clean-up step using SPE (in-house mixed mode cartridges, see below). More details about the extraction protocol that was followed in this study can be found in the **Supporting Information**. After the injections in the LC-ESI-MS/MS the vials with the remaining extracts were stored in the freezer (-80°C).

2.4 Instrumental Analysis

All measurements were performed using a UHPLC Thermo Accela pump incorporating a column thermostat, a degasser, and an autosampler (San Jose, CA, U.S.). The selected mass spectrometric system was a Thermo TSQ Quantum Access triple quadrupole mass analyzer. Details regarding the instrumentation and the chromatographic separation of the target PFAS can be found in the **Supporting Information** section. The MS/MS parameters for PFAS analysis are presented in **Table S2**.

2.5 Quality assurance and quality control

To reduce possible contamination, all labware, weighing and dissection tools were prescreened and rinsed with methanol before use. Additionally, the use of adequate isotope labeled ISs (added prior to extraction) can to some extent compensate for variable recovery and matrix effects among samples. Prior to daily use, we flushed the LC column with elution solvents [MeOH/5 mM ammonium formate (70 : 30, % v/v)] before initiating a sequence. The analytical method was evaluated under the optimized conditions in terms of linearity, sensitivity, accuracy, repeatability and matrix effects. **Table S4** and **Table S5** summarize the method performance parameters. Seven-point calibration curves were generated using linear regression analysis. The linearity was qualified by linear correlation coefficient, R^2. The reference standard calibration curves obtained for the SRM transitions were linear with $R^2 > 0.95$ in all cases. Accuracy of the

method was assessed with recovery experiments in muscle and liver samples. Extraction recoveries for target analytes were determined (n=5) at one concentration level (100 ng/g ww). Recoveries were determined by comparing the concentrations obtained after the whole sample preparation with the initial spiking levels. Satisfactory recoveries 80<recovery<120% were achieved for the majority of the substances for both matrices (**Table S5**). To ensure a correct quantification, method precision was determined as relative standard deviation (%RSD) from the recovery experiments, processed with the described method. Precision limit <20% RSD was met for all analytes indicating the good precision of the method developed. Regarding sensitivity, limit of detection (LODs, lowest analyte concentration with S/N ratio of 3) and limit of quantification (LOQs, concentration with S/N ratio of 10 and imprecision lower than 20%) were estimated. Finally, matrix effect was evaluated as the percentage of suppression or enhancement. Matrix suppression was observed for 41 and 43 compounds for liver and muscle matrix respectively. The identification and confirmation criteria for the analysis of the target substances was based on the Commission Decision 2002/657/EC. To confirm the presence of the compounds, the retention time of the compounds (2.5 % of tolerance) and relationship between the two transitions (difference of less than 20 %) were used. The detected PFAS were quantified using isotopic dilution (**Table S3** in supplementary material). If IS standards were not available, then standard addition method was used. All quantitative results were expressed in ng/g wet weight (ww). In order to express the detected PFAS concentration in ng/g ww, the moisture content (%) of the liver and muscle tissues were considered. Especially for PFOS, samples were diluted 5 times for the quantitation, since it was initially out of the linear range. PFAS with values between LOD and LOQ were replaced by LOQ/2 **(European Commission, 2009)**. Method detection limits (MDLs), method quantification limits (MQLs), linearity curves and retention times for target PFAS can be found in Table S4, while the recoveries for all analytes spiked into liver and muscle samples are displayed in Table S5.

3. Results and Discussion

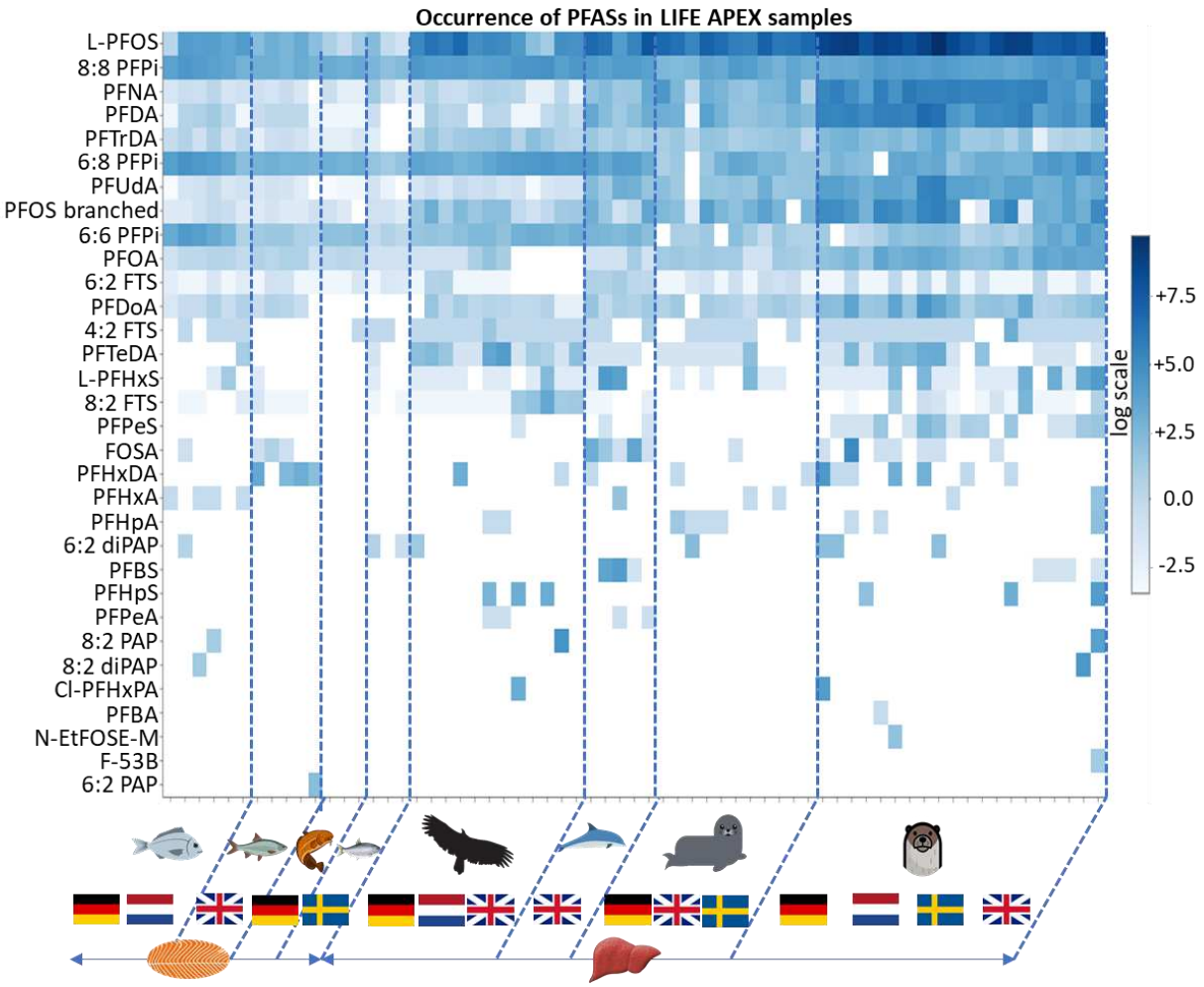
3.1 PFAS occurrence in the samples

The quantitative determination of PFAS in complex biological matrices such as muscle or liver samples is a very detailed process that requires accuracy and precision. Despite the knowledge that has been made in the field over the last decades, there are still gaps and uncertainties. As mentioned in the relevant literature, both negative as well as positive systematic errors may occur at several steps of an analytical scheme. This includes analyte losses and sample contamination, respectively. Moreover, biases may also take place during sampling and storage. Last but not least, matrix effects may affect important analytical parameters, such as instrumental response and measurement reproducibility, while recovery losses are likely to happen at any stage of a multi-step sample preparation and clean-up process. Bearing all the above in mind, the mean ΣPFAS concentrations and ranges (ng/g ww) in the tissues among AP&P species were calculated and are presented in **Table 1**. The individual concentration levels for the target substances in the samples are presented in **Figure 2**, sorted by the frequency of appearance (FoA).

Table 1. Mean ΣPFAS concentration and range (ng/g ww) among the tissues of different species in this study. N (pooled) values represent the number of samples analyzed for each species.

Species	Tissue	n (pooled)	ΣPFAS (ng/g ww)	Concentration range (ng/g ww)	Habitat

Eelpout	Muscle	3	57	46-66	Marine
Herring	Muscle	3	25	16-39	Marine
Bream	Muscle	6	190	100-325	Freshwater
Roach	Muscle	5	77	56-100	Freshwater
Eurasian otter	Liver	20	6321	1942-20236	Freshwater
Harbour/Grey seal	Liver	11	803	244-1517	Marine
Harbour porpoise	Liver	5	1079	357-2692	Marine
Common buzzard	Liver	12	426	217-1092	Terrestrial



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261 **Figure 2**. Heatmap representing the occurrence of PFAS in the LIFE APEX samples. The concentration levels

262 are given in ng g^{-1} wet weight in logarithmic scale. The analytes are sorted based on their frequency of

263 appearance (FoA) in the samples. Clear white colour represents values <MDL for the respective analyte.

264

265 PFOS, 6:6 PFPi, 6:8 PFPi and 8:8 PFPi were detected in all AP&P tissues. C9-C13 PFCAs were detected at

266 noteworthy concentrations in the examined predator liver tissues, and in fairly high levels in the fish

267 muscle tissues. PFODA, PFNS, PFDS, N-MeFOSA, N-EtFOSA, N-MeFOSE, GenX, ADONA as well as all FTOHs,

268 FTASs, FTUAs, and PFAPAs were not detected in any sample. Exception was Cl-PFHxPA, which was

269 detected in two apex samples (a pooled otter sample from Germany and a pooled buzzard sample from

UK). ΣPFAS in AP&P tissues ranged from 16 to 20,200 ng/g ww, with the latter being detected in an individual Eurasian otter sample from the Dutch province Overijssel. The highest ΣPFAS concentration in fish muscle was found in a pooled bream sample from Danube Jochenstein (325 ng/ g ww), while the most contaminated taxon overall was Eurasian otter (average ΣPFAS concentration of 6300 ng/g ww). The only positive detection of the Chinese PFOS alternative F-53B in this study was for an otter sample from the East Anglia region in the UK at a concentration of 3.3 ng/g ww. To the best of our knowledge this is the first time this emerging Cl-PFESA has been detected in Eurasian otters.

3.2 Prey samples

As regards the muscle samples of the four edible fish species examined in this research, the average $\Sigma PFAS_{bream}$ (190 ng/g ww) was the highest among the four prey species, followed by $\Sigma PFAS_{roach}$ (77 ng/g ww), $\Sigma PFAS_{eelpout}$ (57 ng/g ww) and $\Sigma PFAS_{herring}$ (25 ng/g ww). Since no outliers were identified among the individual measurements the average and median concentrations coincide across all investigated AP&P species. The PFAS profile of all edible fish analyzed in the framework of this study is predominantly characterized by the presence of PFPi's, with the exception of the pooled bream sample from the Netherlands, that was collected in the province of South Holland. For this sample, 63% of ΣPFAS was PFOS, 20% 8:8 PFPi, 8% 6:8 PFPi, and 18% C8-C14 PFCAs. For all other fish samples in this study PFPi's dominated the respective PFAS ratios, reflecting the fact that these compounds are increasingly used as PFOS alternatives in surfactants and pesticide ingredients. The predominant analogues were, again, 6:8 PFPi and 8:8 PFPi. ΣPFPi's was 77% of the total PFAS yield for bream specimens from Germany, 93% for eelpout from the same country, 55% for roach collected in the river network of UK, and 75% for the herring specimens collected along the Swedish coast in the Baltic. PFHxA was detected at an average concentration of 0.7 ng/g ww in the five pooled samples from Germany. ΣPFCAs (C8-C14) accounted for

3-10% for bream and eelpout from Germany and herring from Sweden. Yet carboxylic acids in pooled roach fillets from the UK were at higher levels than ΣPFOS, with an average concentration of 20 ng/g ww (24% of ΣPFAS for these samples; **Figure 3**).

PFOS was 20% of the total PFAS yield for bream from Germany, 4% for eelpout from Germany, 21% for roach from the UK, and 15% for herring from Sweden, respectively. The low PFAS levels in eelpout samples were comparable to those found in similar studies **(Couderc et al., 2015; Giari et al., 2015)**. In general, the quantitative results for the fish samples from Germany are comparable with the PFAS profiling for bream and eelpout matrices in a recent study by Kotthoff et al **(Kotthoff et al., 2020)**.

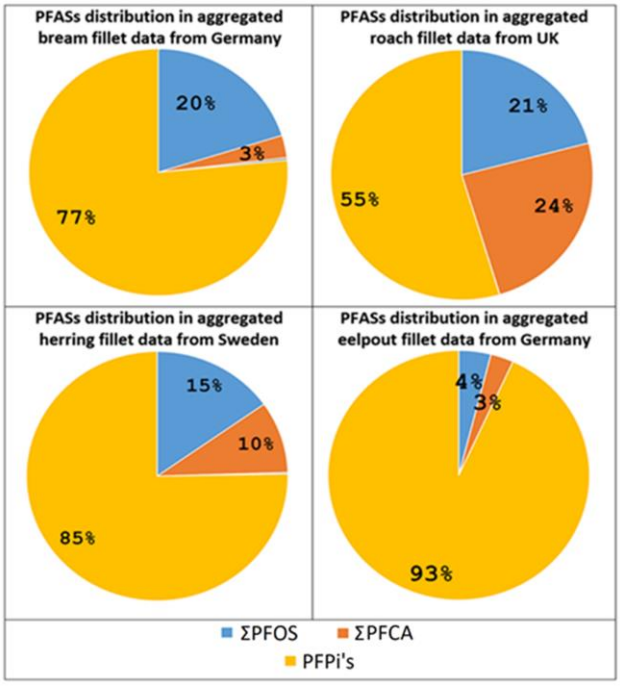

Figure 3. Relative contribution (%) of ΣPFOS, ΣPFCA and PFPi's to ΣPFAS concentrations in the muscle tissues of the different fish species. Bream: n = 5, Roach: n = 5, Herring: n = 3, Eelpout: n = 3.

We found that freshwater fish was notably more contaminated than coastal/marine fish (**Table S4** in supplementary information). This suggests that fish that live in brackish or open sea ecosystems are less exposed to PFAS and other man-made chemicals than those living in freshwater ecosystems. River and lake fish may be more highly exposed to emissions from anthropogenic activities such as industry and tourism **(Denys et al., 2014; Cerveny et al., 2016)**. The environmental fate of PFAS follows either sorption to the soil and leakage to the groundwater fluxes and aquifers or discharged through the surface water system to deltas and, eventually, the open sea. For this reason, fish that live in a pristine environment are less exposed to chemicals' contamination, including PFAS, PCBs, DDTs **(Faxneld et al., 2014; Mazzoni et al., 2020)**.

3.3 Apex predator samples

PFAS preferably bind to serum proteins and are typically high in well-vascularized organs, notably in liver tissue as the main organ of albumin synthesis **(Fliedner et al., 2020)**. We found overall ΣPFAS levels in apex predator livers up to 4 orders of magnitude higher than the respective values in prey muscle tissues.

Eurasian otter (freshwater top predator)

It has been frequently emphasized in recent studies on dietary intakes of otters as well as other campaigns for the assessment of chemicals management for aquatic mammals and other wildlife, that otters suffer a significant contamination of emerging contaminants **(Krawczyk et al., 2016)**. Evidence to date suggests that terrestrial foods contribute very little to the nutritional ecology of Eurasian otters, that are mostly piscivorous **(Lyach and Čech, 2017)**. Representing a large proportion of its diet, fish are responsible for the passing of a large amount of PFAS and other POPs to the metabolism of otters **(Roos et al., 2013)**. It is worth mentioning that linear and branched isomers of PFOS account for more than 80% of the ΣPFAS

yield in the 20 otter samples of our study. For otters, which is the only specie that was sampled in all involved counties within this study, 98% of ΣPFOS was linear PFOS (L-PFOS) and 2% was branched PFOS. The remaining 10-20% of the PFAS cocktail corresponds mainly to long-chain PFCAs (C8-C13), with PFTeDA (C14) appearing the least abundant. Nevertheless, an important 8% of PFPi's detected in the otter samples from the UK is not to be neglected and suggests a slightly alternative chemicals' exposure of these animals.

Harbour and grey seal and harbour porpoise (marine apex predators)

The same is valid for the case of the total of 11 seal samples analyzed within this campaign. Although the total amount of PFAS detected in seal livers is on average 8 times lower than the ΣPFAS quantified in the otters' livers due to the relation marine - freshwater predators, the chemicals palette is similar for both aquatic predators. More specifically, for harbour and grey seals collected from German and Swedish coasts ΣPFOS accounts for 90% of the ΣPFAS burden. In the case of the individual harbour seal samples collected in the Netherlands, 23% of the ΣPFAS corresponds to PFPi's, 1% to FTSAs, and less than 1% to PFOSA traces. This indicates the localized occurrence of PFOS alternatives. The predominant congeners were 6:8 PFPi and 8:8 PFPi. 6% of the seals' PFAS profile from the Netherlands is linked to the identification of PFCAs (C8-C13) and just 1% corresponds to PFHxS. The remaining and still very high percentage (69% of ΣPFAS) is to be attributed to ΣPFOS. The results of our study are in good agreement with the findings of Van de Vijver *et al.* on increasing PFAS concentrations in otters and ringed seals from Sweden **(Van de Vijver et al., 2005)**, reporting that otters have historically been exposed to an order of magnitude higher PFAS contamination compared to seals from adjacent or neighboring areas. Changes in the diet of harbour and grey seal may also affect the level and pattern of PFAS, but also the seasonal changes in the diet of their fish prey will determine the accumulation of pollutants in these marine mammals. Overall, harbour seals have been shown to respond to varying prey availability and distribution by exhibiting high flexibility in their movement ecology and diet.

Along the same line, the 5 pooled liver tissues of harbour porpoises collected from the shores of the UK were the second most contaminated samples. The PFAS pattern showed a remarkable similarity to the PFAS profile outlined for the otters from the UK. The composition of PFAS was the following: 79% ΣPFOS, 13% PFPi's and FTSAs, 4% PFCAs (C8-C16), 2% PFBS, and 2% PFHxS. Ultralong-chain PFHxDA was detected in a recent (2019) specimen from the Blackpool coastal area at a concentration of 0.90 ng/g ww. PFTeDA was detected in 4 out of 5 pooled harbour porpoise samples in this study at a consistent concentration of < 0.5 ng/g ww. The high levels of PFOS are in good agreement with the results of another study by Van de Vijver *et al.* **(Van de Vijver et al., 2004)**. Harbour porpoises from Northern Europe were found to be heavily contaminated with PFOS and to a lesser extent with perfluorocarboxylates.

Despite the fact that the average ΣPFAS concentration of the aggregated otter samples is approximately 6 times higher than the respective harbour porpoise samples in this study, the PFAS profile for both species is very similar. The afforementioned marine mammals live and hunt for prey in river estuaries and marine and brackish water ecosystems along the coast, while otters are inland water predators. Therefore, it can be concluded that both these taxa are recipients of the same array of PFAS due to their exposure to the same aquatic continuum. The specific dolphin species is exclusively located near harbours and sites of anthropogenic activity, where POPs are washed off through river system discharges **(Booth et al., 2013).** Otters are inhabitants of the upper part of the same network. Although, patterns of harbour porpoise from the UK are similar to seals patterns from the Netherlands, Germany and Sweden, the reason why the seals are less burdened than the analyzed porpoises in this study should be further investigated.

Common buzzard (terrestrial apex predator)

Common buzzards were found to be the least contaminated, yet most variable of the apex predator species studied in terms of PFAS profiling within the frameworks of this study. The latter is probably due

to seasonal changes in the diet of common buzzards and birds of prey in general, resulting of fluctuations in the level and pattern of PFAS. Common buzzards have been shown to respond to varying prey availability and distribution by exhibiting high flexibility in their spatial and temporal movement ecology and diet **(Kappers et al., 2017).** Yet, the fact that no prey species of common buzzards (rodents, rabbits etc.) were included in this study is a limiting factor in drawing robust conclusions for the occurrence of PFAS in buzzards.

For German buzzard samples, PFOS was the most abundant PFAS, accounting for 80% of the total concentration levels. 3% of ΣPFAS was attributed to C8-C16 PFCAs. PFHxDA was detected in a pooled sample from the agroforestry area of Mecklenburg-Vorpommern at a concentration of 22 ng/g ww. The remaining 17% of ΣPFAS for this population accounted for PFPi's, with 6:8 PFPi and 8:8 PFPi being the predominant congeners, as in the case of seals from the Netherlands and harbour porpoises from the UK. For the Dutch samples as well, more than 50% of the total PFAS yield was ΣPFOS. This percentage is a lot lower than in the German specimens. Higher percentages of PFPi's (30%), C7-C14 PFCAs (17%), and 2% of PFHpS were observed in the Dutch avian predators, while higher levels of PFTeDA (50 ng/g ww, on average) and traces of PFPeA, PFHpA, and PFHxS (< 1ng/g ww) were noted. British birds of prey were the only predator specimens in this study for which PFOS was not the predominant compound in the total PFAS burden. The most abundant was 8:8 PFPi (41%), followed by 6:8 PFPi (24%), ΣPFOS (21%), 6:6 PFPi (5%), and 8:2 FTS (2%). The percentages of C9-C16 PFCAs and ΣPFSAs except PFOS were 3% and 4% of the total PFAS amount quantified in the UK buzzard samples, respectively. PFHxDA was detected in a pooled buzzard sample at a concentration of 0.9 ng/g ww, while just fairly low PFOA levels were documented (0.4 - 6 ng/g ww). The distribution of PFAS for selected predators is shown in **Figure 4**.

This versatility regarding the PFAS profile of the only terrestrial predator species in this study could be linked to the wide range of their foraging areas and diet composition **(Kruger, 2002; Butet et al., 2010)**. The fact that common buzzards were found to be the least contaminated among the studied apex

predator species, strengthens the hypothesis that the environmental fate of PFAS , is to end up in the aquatic environment, also due to their high water solubility, thus rendering terrestrial predators less subject to contamination. However, it is worthful to mention that terrestrial contamination may respond more slowly to restrictions in the use of POPs. For example, polybrominated diphenyl ethers (PBDEs) declined in gannet eggs **(Crosse et al., 2012)** but no significant decline in sparrowhawk livers was observed **(Crosse et al., 2013).**

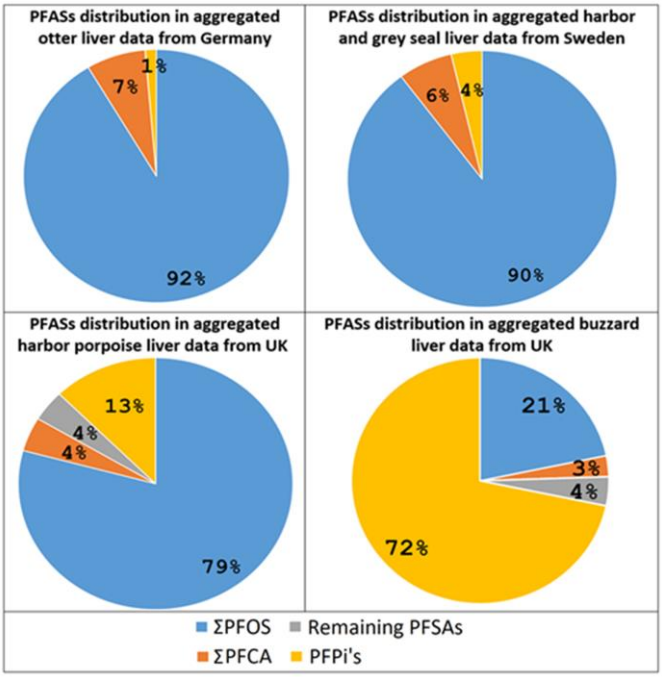

Figure 4. Relative contribution (%) of ΣPFOS, ΣPFCA, PFPi's, and PFSAs excluding PFOS to ΣPFAS concentrations in the liver tissues of the selected apex predator species. Otters: n = 5, Seals: n = 5, Harbour Porpoises: n = 5, Common Buzzards: n = 5.

3.4 PFAS patterns

Throughout this research, major differences in the PFAS patterns between apex predators and their prey was observed. More specifically, a noteworthy aberration in the PFOS levels was spotted. PFOS was

414 proved to be prone to bioaccumulation, since it was detected in fairly low concentrations in the prey
415 samples but in high concentrations in the predator specimens. The vast differences in the PFOS and other
416 PFAS' levels between prey and predators can partly be attributed to the different tissues used. Zafeiraki
417 et al. **(Zafeiraki et al., 2019)** report the following trend of ascending PFAS concentrations in the tissues of
418 analyzed sharks from the Mediterranean for which all 5 organs were available: gonads > heart > liver ≈
419 gills > muscle. For completeness purposes, a liver-to-liver comparison between AP and P should be further
420 investigated. We would also like to highlight that an average contribution of 0.02% of branched-PFOS to
421 ΣPFOS was also observed in all samples in this study. These findings suggest that environmental and/or
422 physiological processes, such as sediment – water partitioning, transformation, and bioaccumulation,
423 discriminate between linear and branched isomers, based on different physicochemical properties
424 between isomers. The slightly higher water solubility of branched-PFOS isomers compared to linear-PFOS
425 **(Sharpe et al., 2010)** raises the overall toxicity of ΣPFOS. Finally, our results are in agreement with relevant
426 studies showing accumulation of linear PFOS, yet no significant accumulation of the branched isomers in
427 living organisms **(Greaves and Letcher, 2013)**.

428 The 100% detection frequency of PFPi's, could be attributed to the high persistence and long-range
429 transport potential of this emerging and relatively under-studied PFAS class **(Wang et al., 2016)**. Like other
430 PFAS, PFPi's are also surfactants possessing a hydrophobic and lipophobic perfluoroalkyl tail connected to
431 a polar anionic headgroup. They are proteophilic and accumulate in protein-rich tissues, such as liver
432 **(Rand and Mabury, 2014)**. PFPi's are similar to PFOS in terms of chemical structure, containing a
433 perfluorinated carbon tail attached to a phosphinate through a carbon-phosphorus bond **(Lee and**
434 **Mabury, 2017)**, therefore they are expected to have similar physicochemical properties, bioaccumulation
435 potential, and even higher acute toxicity than PFOS. The latter hypothesis is based on the fact that PFPi's
436 usually have longer carbon chain length (≥12 C atoms) than PFOS. It has been verified that PFAS with
437 longer carbon chain length are significantly more toxic than the shorter ones **(Kudo et al., 2006)**. Although

PFPi's have been reportedly used as defoaming components in pesticide formulations, as well as leveling and wetting agents in industrial and commercial applications **(De Silva et al., 2012)**, it should be noted that it is not known whether PFPi's containing pesticides or other PFPi related products were applied in any of this project's sampling locations. In general, the use of PFPi's in pesticide formulations further complicate characterization of wastewater sources from agricultural sources. On the basis of the presence of PFPi's in fish and apex predators, we recommend further research to determine the effect of these substances. While the contribution of PFPi's to the PFAS burden in all samples, determined on the basis of comparison to PFCAs and PFSAs, was dominant, PFAPAs were consistently below detection limits. De Silva *et al.* observed the same PFPi's:PFAPAs ratio in the framework of their recent study on perfluoroalkylphosphinic acids levels in northern pike, double-crested cormorants, and bottlenose dolphins **(De Silva et al., 2016)**. Additionally, we identified microquantities of PFBA, PFPeA, PFHpA, PFHxDA, PFBS, and PFPeS only in AP livers but not in prey muscle tissues. On the contrary, PFOA had a 100% FoA in the prey specimens, yet was below LOD in several predator samples. It could be supposed that the differences in the PFAS between apex predators and prey could be a result of the metabolism and following biotransformation PFAS undergo across the food web. Precursor metabolism and biotransformation processes are complex fields of research that have not yet been fully investigated. The ratio precursor:analyte:metabolite is dynamic and depends on a number of factors, the combination of which may alter the chemicals' mix from taxon to taxon or even at the individual level. Foraging habits, dwelling area/foraging location, migration behavior, sex, age and size strongly influence the PFAS concentrations across a wildlife population. However, sex and body length of the fish species does not influence the bioaccumulation of PFAS, according to previous studies, suggesting that the size of fish does not affect PFAS levels **(Ye et al., 2008; Quinete et al., 2009)**.

4. Conclusions

The present study presents insights into the frequency of occurrence and concentrations of PFAS in Eurasian otters, grey and harbour seals, harbour porpoises and common buzzards as well as four fish species (bream, roach, herring and eelpout) collected from 61 sampling sites in Germany, the Netherlands, Sweden and the United Kingdom. The analysis of 65 liver and muscle tissues for 56 PFAS shows that all analysed specimens were primarily contaminated with PFOS, while the three PFPi's included in this study exhibited FoA 100%. Additionally, our findings demonstrate that C9 to C13 PFCAs generally occur at high concentrations in apex predator livers despite phase-outs and increasing regulation of these compounds together with C8-based PFAS. The negligible detection of C4-C7 PFCAs in all AP tissues may indicate that the top predators in this study were not exposed to short-chain PFCAs via their prey or may suggest a low bioaccumulation potential of these compounds. PFAS concentrations were one to four orders of magnitude higher in predator liver tissues than in fish muscle. Apart from the difference in the PFAS metabolism in livers and muscles, the significant difference in total body size between predators and prey has to be taken into consideration when comparing total PFAS levels. All the above points to a widespread PFAS contamination in otters, seals, harbour porpoises and, to a lesser degree, common buzzards. While the PFAS contamination in fish muscles was lower than in predator livers, it was still considerably high. PFAS relative contribution varied among different species, due to the different binding affinity of PFAS for proteins and fats that are tissue- and organism-specific. Furthermore, the results show an association between the PFAS concentrations in apex predators and the geographical origin of the specimens. Despite the fact that the sixty-one sampling areas of this study were diverse, in terms of terrain, climate as well coordinates, a basic correlation between the geographical origin of the samples and the type as well as levels of PFAS in them was observed. This has to be factored in together with the type of matrix and its lipid/protein content, when drawing conclusions about what species were most contaminated and why. Focusing on the interaction extent between humans and wildlife, it was clear that otters and seals, which

484 inhabit freshwater or marine ecosystems often affected by intense anthropogenic activity, are more
485 exposed to contamination by PFAS and other POPs than buzzards whose diet derives from terrestrial food
486 webs. More research is needed to further deepen our knowledge on the environmental fate of PFAS and
487 their accumulation in AP&P.
488

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500

501 **Disclaimer**

502 The content of this article reflects only the authors' views and the Research Executive Agency is not
503 responsible for any use that may be made of the information it contains.
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Conflict of interest

The authors declare no conflict of interest.

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