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

Andreas Androulakis<sup>1</sup>, Nikiforos Alygizakis<sup>1,2</sup>, Georgios Gkotsis<sup>1</sup>, Maria-Christina Nika<sup>1</sup>, Varvara Nikolopoulou<sup>1</sup>, Erasmia Bizani<sup>1</sup>, Elizabeth Chadwick<sup>4</sup>, Alessandra Cincinelli<sup>5</sup>, Daniela Claßen<sup>3</sup>, Sara Danielsson<sup>6</sup>, Rene W.R.J. Dekker<sup>7</sup>, Guy Duke<sup>8</sup>, Natalia Glowacka<sup>2</sup>, Hugh A.H. Jansman<sup>9</sup>, Oliver Krone<sup>10</sup>, Tania Martellini<sup>5</sup>, Paola Movalli<sup>7</sup>, Sara Persson<sup>6</sup>, Anna Roos<sup>6</sup>, Emily O'Rourke<sup>4</sup>, Ursula Siebert<sup>11</sup>, Gabriele Treu<sup>3</sup>, Lee Anthony Walker<sup>12</sup>, Jaroslav Slobodnik<sup>2</sup> and Nikolaos S. Thomaidis<sup>1\*</sup>

<sup>1</sup>National and Kapodistrian University of Athens, Panepistimiopolis Zografou, 15771 Athens, Greece

<sup>2</sup>Environmental Institute, Okružná 784/42, 97241 Koš, Slovak Republic

<sup>3</sup>German Environment Agency, 06844 Dessau, Germany

<sup>4</sup>Cardiff University, Biomedical Science Building, Museum Avenue, Cardiff, CF10 3AX, UK

<sup>5</sup>Department of Chemistry "Ugo Schiff", University of Florence, 50019 Sesto Fiorentino, Italy

<sup>6</sup>Naturhistoriska riksmuseet, Box 50007, 104 05 Stockholm, Sweden

<sup>7</sup>Naturalis Biodiversity Center, 2333 RA Leiden, The Netherlands

<sup>8</sup>Environmental Change Institute, University of Oxford, 3 South Parks Rd, Oxford OX1 3QY, United Kingdom

<sup>9</sup>Wageningen Environmental Research, 6700 AA Wageningen, The Netherlands

<sup>10</sup>Leibniz Institute for Zoo and Wildlife Research, Department of Wildlife Diseases, Alfred-Kowalke-Strasse 17, 10315 Berlin, Germany

<sup>11</sup>Institute for Terrestrial and Aquatic Wildlife Research, University of Veterinary Medicine Hannover, 25761 Buesum, Germany

<sup>12</sup>UK Centre for Ecology and Hydrology, Lancaster, LA1 4AP, United Kingdom

\*Corresponding author

Nikolaos S. Thomaidis

Laboratory of Analytical Chemistry, Department of Chemistry, National and Kapodistrian University of Athens, Panepistimiopolis Zografou, 15771 Athens, Greece

E-mail: [ntho@chem.uoa.gr](mailto:ntho@chem.uoa.gr)

Phone: +30 210 727 4317

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37 **Abstract**

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

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

68 **Keywords**

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

70

## 71 1. Introduction

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

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

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

94 kidneys, and bile secretion and not in fat tissues (**Jones et al., 2003; Perez et al., 2013**). Additionally, PFAS  
95 levels have been reported to be very high in human liver cells (**Domingo et al., 2012; Fliedner et al.,**  
96 **2020**).

97 Currently, perfluorooctanesulfonate (PFOS) and its salts are listed under Annex B of the Stockholm  
98 Convention for Persistent Organic Pollutants (**UNEP, 2009**), while perfluorooctanoic acid (PFOA), its salts  
99 and PFOA-related compounds were added to Annex A in 2019. Perfluorohexane sulfonate (PFHxS) has  
100 been proposed for inclusion (**UNEP, 2018**). The phase-out of the legacy compounds and their replacement  
101 with structurally similar PFAS has been the most common industry policy in the last decades (**Wang et al.,**  
102 **2013; Wang et al., 2017**). This poses a great environmental danger, since most emerging PFAS also show  
103 high toxicity, yet are to this day not routinely monitored or part of any regulatory guideline (**Cao et al.,**  
104 **2019**). Up to this day there are nearly 5000 PFAS that are broadly used in several industrial and commercial  
105 applications (**Buck et al., 2011**).

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

112 Several analytical regimes have been developed for the determination of PFAS in various matrices,  
113 including sediments, ground- and freshwater (**Joerss et al., 2019; Simmonet-Laprade et al., 2019**), fish  
114 and other aquatic organisms (**Babut et al., 2017; Liu et al., 2017; Fair et al., 2019**), birds (**Munoz et al.,**  
115 **2017; Lopez-Antia et al., 2019; Russell et al., 2019**) and mammals (**Boisvert et al., 2019; Cui et al., 2019;**  
116 **Gui et al., 2019**). Solid phase extraction (SPE) and liquid–liquid extraction (LLE) are the main techniques

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

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

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

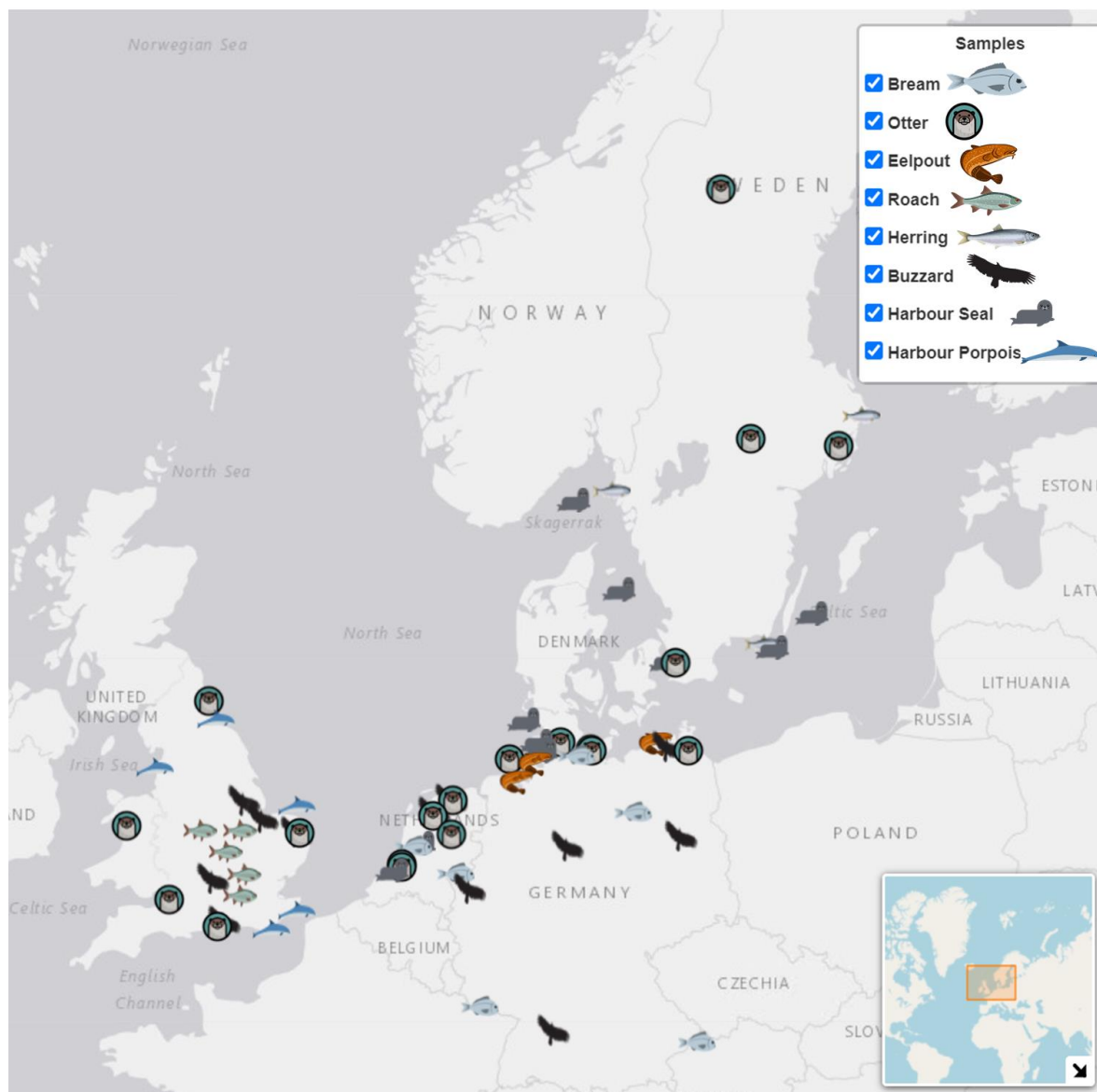
## 141 2. Material and Methods

### 142 2.1 Study area and sampling strategy

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



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



167  
168 **Figure 1.** Sample collection sites and their spatial distribution. Interactive version of the map is available  
169 in the following link: [https://norman-data.eu/LIFE\\_APEX\\_PFA5\\_Tier1/](https://norman-data.eu/LIFE_APEX_PFA5_Tier1/)

170

## 171 2.2 Chemicals and reagents

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

178

## 179 2.3 Extraction of samples

180 All LIFE APEX samples collected from ESBs, NHMs and other scientific collections were sent to NKUA for  
181 their pre-treatment. The documentation and condition of the delivered samples were thoroughly  
182 checked, and unique sample codes were given to the samples. For the calculation of the % water content  
183 of the samples, empty petri-dishes with the respective code of each sample were weighed. This was  
184 followed by the segmentation of the samples and their placement into petri-dishes in an isolated room.  
185 The petri-dishes including the wet samples were then weighed. All samples were kept refrigerated (-80°C)  
186 for at least 5 hours, as a pre-treatment step before lyophilization. Afterwards, the samples' freeze-drying  
187 (-55°C, 0.05 mbar, Capacity: 5 kg/24h, Telstar Lyoquest Freeze Dryer) in accordance with the standardized  
188 operational procedure (SOP) for the lyophilization took place, followed by the weighting of the petri-  
189 dishes including the freeze-dried samples. Accordingly, the % water content was calculated. The weights  
190 and % water content, as well as any other freeze-drying relevant information were registered in a specific  
191 file. The homogenization of each sample using pestle and mortar or multi in an isolated room was then  
192 performed. Between homogenizations all lab instruments were cleaned using milli-Q water and acetone.  
193 All freeze-dried samples were then stored (-80°C) in amber glass vials. Accelerated Solvent Extraction

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

198

## 199 **2.4 Instrumental Analysis**

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

205

## 206 **2.5 Quality assurance and quality control**

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

216 method was assessed with recovery experiments in muscle and liver samples. Extraction recoveries for  
217 target analytes were determined (n=5) at one concentration level (100 ng/g ww). Recoveries were  
218 determined by comparing the concentrations obtained after the whole sample preparation with the initial  
219 spiking levels. Satisfactory recoveries 80<recovery<120% were achieved for the majority of the substances  
220 for both matrices (**Table S5**). To ensure a correct quantification, method precision was determined as  
221 relative standard deviation (%RSD) from the recovery experiments, processed with the described method.  
222 Precision limit <20% RSD was met for all analytes indicating the good precision of the method developed.  
223 Regarding sensitivity, limit of detection (LODs, lowest analyte concentration with S/N ratio of 3) and limit  
224 of quantification (LOQs, concentration with S/N ratio of 10 and imprecision lower than 20%) were  
225 estimated. Finally, matrix effect was evaluated as the percentage of suppression or enhancement. Matrix  
226 suppression was observed for 41 and 43 compounds for liver and muscle matrix respectively. The  
227 identification and confirmation criteria for the analysis of the target substances was based on the  
228 Commission Decision 2002/657/EC. To confirm the presence of the compounds, the retention time of the  
229 compounds (2.5 % of tolerance) and relationship between the two transitions (difference of less than 20  
230 %) were used. The detected PFAS were quantified using isotopic dilution (**Table S3** in supplementary  
231 material). If IS standards were not available, then standard addition method was used. All quantitative  
232 results were expressed in ng/g wet weight (ww). In order to express the detected PFAS concentration in  
233 ng/g ww, the moisture content (%) of the liver and muscle tissues were considered. Especially for PFOS,  
234 samples were diluted 5 times for the quantitation, since it was initially out of the linear range. PFAS with  
235 values between LOD and LOQ were replaced by LOQ/2 (**European Commission, 2009**). Method detection  
236 limits (MDLs), method quantification limits (MQLs), linearity curves and retention times for target PFAS  
237 can be found in Table S4, while the recoveries for all analytes spiked into liver and muscle samples are  
238 displayed in Table S5.

239

240  
241  
242  
243

### 244 3. Results and Discussion

#### 245 3.1 PFAS occurrence in the samples

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

257

258 **Table 1.** Mean  $\Sigma$ PFAS concentration and range (ng/g ww) among the tissues of different species in this  
259 study. N (pooled) values represent the number of samples analyzed for each species.

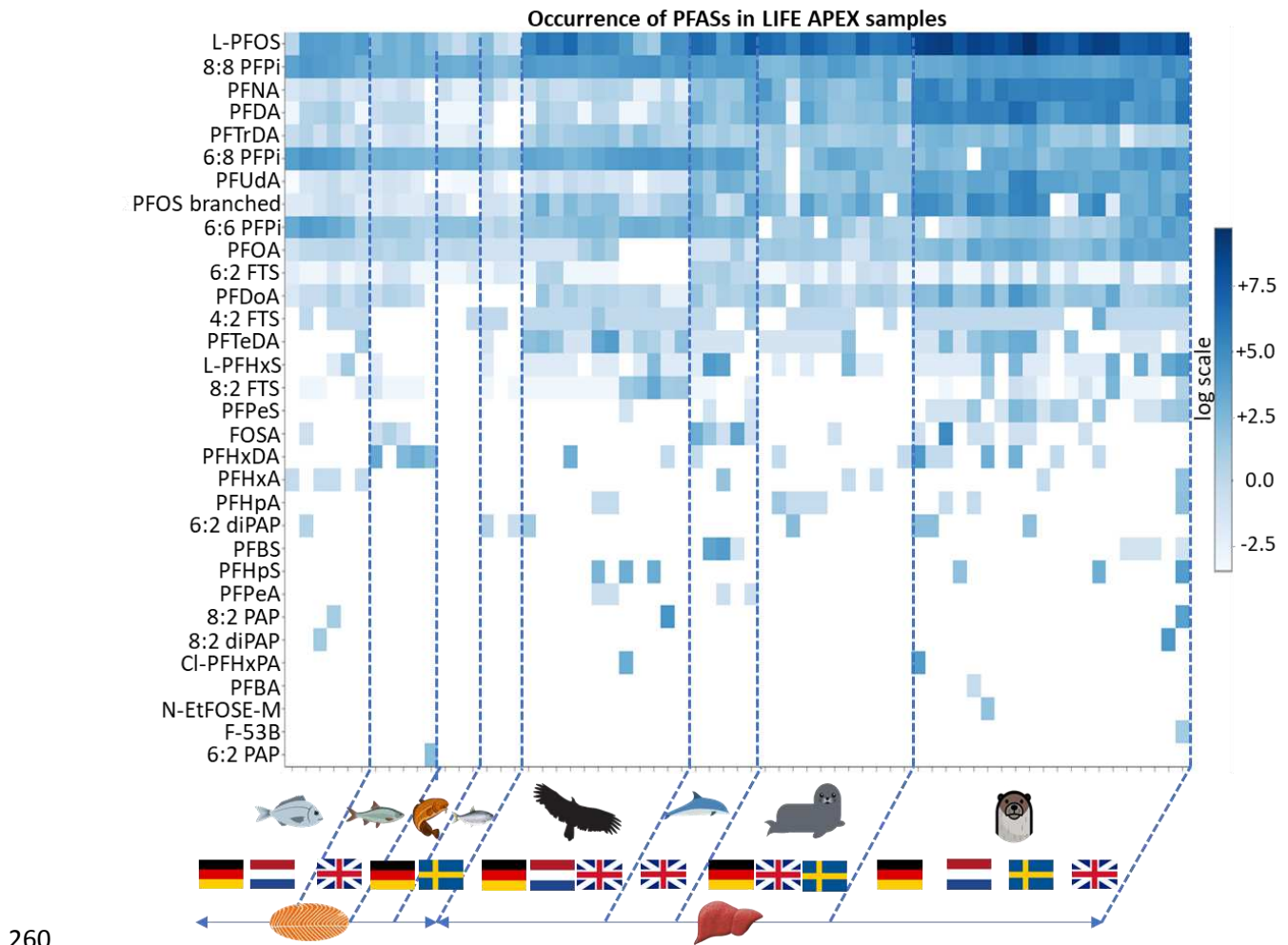
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Species	Tissue	n (pooled)	$\Sigma$ PFAS (ng/g ww)	Concentration range (ng/g ww)	Habitat
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Eelpout	Muscle	3	57	46-66	Marine
Herring	Muscle	3	25	16-39	Marine
Bream	Muscle	6	190	100-325	Freshwater
Roach	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



260

261 **Figure 2.** Heatmap representing the occurrence of PFAS in the LIFE APEX samples. The concentration levels  
 262 are given in  $\text{ng g}^{-1}$  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  $<\text{MDL}$  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. PFOA, 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

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

277

### 278 **3.2 Prey samples**

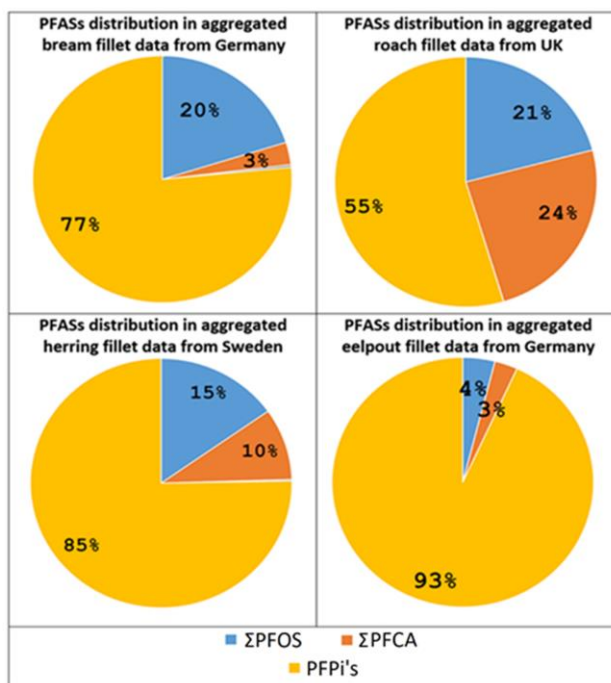
279 As regards the muscle samples of the four edible fish species examined in this research, the average  
280  $\Sigma$ PFAS<sub>bream</sub> (190 ng/g ww) was the highest among the four prey species, followed by  $\Sigma$ PFAS<sub>roach</sub> (77 ng/g  
281 ww),  $\Sigma$ PFAS<sub>eelpout</sub> (57 ng/g ww) and  $\Sigma$ PFAS<sub>herring</sub> (25 ng/g ww). Since no outliers were identified among the  
282 individual measurements the average and median concentrations coincide across all investigated AP&P  
283 species. The PFAS profile of all edible fish analyzed in the framework of this study is predominantly  
284 characterized by the presence of PFPI's, with the exception of the pooled bream sample from the  
285 Netherlands, that was collected in the province of South Holland. For this sample, 63% of  $\Sigma$ PFAS was PFOS,  
286 20% 8:8 PFPI, 8% 6:8 PFPI, and 18% C8-C14 PFCAs. For all other fish samples in this study PFPI's dominated  
287 the respective PFAS ratios, reflecting the fact that these compounds are increasingly used as PFOS  
288 alternatives in surfactants and pesticide ingredients. The predominant analogues were, again, 6:8 PFPI  
289 and 8:8 PFPI.  $\Sigma$ PFPI's was 77% of the total PFAS yield for bream specimens from Germany, 93% for eelpout  
290 from the same country, 55% for roach collected in the river network of UK, and 75% for the herring  
291 specimens collected along the Swedish coast in the Baltic. PFHxA was detected at an average  
292 concentration of 0.7 ng/g ww in the five pooled samples from Germany.  $\Sigma$ PFCAs (C8-C14) accounted for



293 3-10% for bream and eelpout from Germany and herring from Sweden. Yet carboxylic acids in pooled  
 294 roach fillets from the UK were at higher levels than  $\Sigma$ PFOS, with an average concentration of 20 ng/g ww  
 295 (24% of  $\Sigma$ PFAS for these samples; **Figure 3**).

296  
 297 PFOS was 20% of the total PFAS yield for bream from Germany, 4% for eelpout from Germany, 21% for  
 298 roach from the UK, and 15% for herring from Sweden, respectively. The low PFAS levels in eelpout samples  
 299 were comparable to those found in similar studies (**Couderc et al., 2015; Giari et al., 2015**). In general,  
 300 the quantitative results for the fish samples from Germany are comparable with the PFAS profiling for  
 301 bream and eelpout matrices in a recent study by Kotthoff et al (**Kotthoff et al., 2020**).

302



303  
 304 **Figure 3.** Relative contribution (%) of  $\Sigma$ PFOS,  $\Sigma$ PFCA and PFPI's to  $\Sigma$ PFAS concentrations in the muscle  
 305 tissues of the different fish species. Bream: n = 5, Roach: n = 5, Herring: n = 3, Eelpout: n = 3.

306

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

316

### 317 **3.3 Apex predator samples**

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

#### 321 **Eurasian otter (freshwater top predator)**

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

329 yield in the 20 otter samples of our study. For otters, which is the only specie that was sampled in all  
330 involved counties within this study, 98% of ΣPFOS was linear PFOS (L-PFOS) and 2% was branched PFOS.  
331 The remaining 10-20% of the PFAS cocktail corresponds mainly to long-chain PFCAs (C8-C13), with PFTeDA  
332 (C14) appearing the least abundant. Nevertheless, an important 8% of PFPI's detected in the otter samples  
333 from the UK is not to be neglected and suggests a slightly alternative chemicals' exposure of these animals.  
334

### 335 **Harbour and grey seal and harbour porpoise (marine apex predators)**

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

353 Along the same line, the 5 pooled liver tissues of harbour porpoises collected from the shores of the UK  
354 were the second most contaminated samples. The PFAS pattern showed a remarkable similarity to the  
355 PFAS profile outlined for the otters from the UK. The composition of PFAS was the following: 79%  $\Sigma$ PFOS,  
356 13% PFPI's and FTSA's, 4% PFCAs (C8-C16), 2% PFBS, and 2% PFHxS. Ultralong-chain PFHxDA was detected  
357 in a recent (2019) specimen from the Blackpool coastal area at a concentration of 0.90 ng/g ww. PFTeDA  
358 was detected in 4 out of 5 pooled harbour porpoise samples in this study at a consistent concentration of  
359 < 0.5 ng/g ww. The high levels of PFOS are in good agreement with the results of another study by Van de  
360 Vijver *et al.* (**Van de Vijver et al., 2004**). Harbour porpoises from Northern Europe were found to be heavily  
361 contaminated with PFOS and to a lesser extent with perfluorocarboxylates.

362 Despite the fact that the average  $\Sigma$ PFAS concentration of the aggregated otter samples is approximately  
363 6 times higher than the respective harbour porpoise samples in this study, the PFAS profile for both  
364 species is very similar. The aforementioned marine mammals live and hunt for prey in river estuaries and  
365 marine and brackish water ecosystems along the coast, while otters are inland water predators.  
366 Therefore, it can be concluded that both these taxa are recipients of the same array of PFAS due to their  
367 exposure to the same aquatic continuum. The specific dolphin species is exclusively located near harbours  
368 and sites of anthropogenic activity, where POPs are washed off through river system discharges (**Booth**  
369 **et al., 2013**). Otters are inhabitants of the upper part of the same network. Although, patterns of harbour  
370 porpoise from the UK are similar to seals patterns from the Netherlands, Germany and Sweden, the  
371 reason why the seals are less burdened than the analyzed porpoises in this study should be further  
372 investigated.

### 373 **Common buzzard (terrestrial apex predator)**

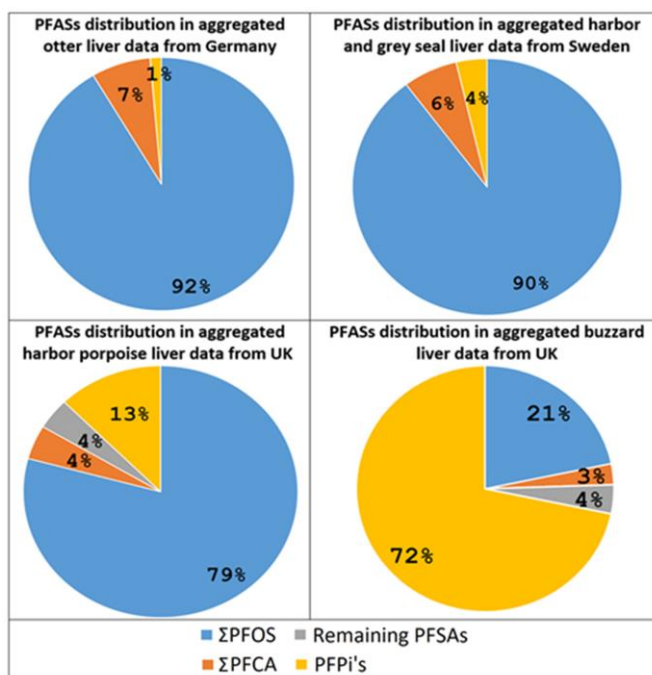
374 Common buzzards were found to be the least contaminated, yet most variable of the apex predator  
375 species studied in terms of PFAS profiling within the frameworks of this study. The latter is probably due

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

382 For German buzzard samples, PFOS was the most abundant PFAS, accounting for 80% of the total  
383 concentration levels. 3% of  $\Sigma$ PFAS was attributed to C8-C16 PFCAs. PFHxDA was detected in a pooled  
384 sample from the agroforestry area of Mecklenburg-Vorpommern at a concentration of 22 ng/g ww. The  
385 remaining 17% of  $\Sigma$ PFAS for this population accounted for PFPi's, with 6:8 PFPi and 8:8 PFPi being the  
386 predominant congeners, as in the case of seals from the Netherlands and harbour porpoises from the UK.  
387 For the Dutch samples as well, more than 50% of the total PFAS yield was  $\Sigma$ PFOS. This percentage is a lot  
388 lower than in the German specimens. Higher percentages of PFPi's (30%), C7-C14 PFCAs (17%), and 2% of  
389 PFHpS were observed in the Dutch avian predators, while higher levels of PFTeDA (50 ng/g ww, on  
390 average) and traces of PFPeA, PFHpA, and PFHxS (< 1ng/g ww) were noted. British birds of prey were the  
391 only predator specimens in this study for which PFOS was not the predominant compound in the total  
392 PFAS burden. The most abundant was 8:8 PFPi (41%), followed by 6:8 PFPi (24%),  $\Sigma$ PFOS (21%), 6:6 PFPi  
393 (5%), and 8:2 FTS (2%). The percentages of C9-C16 PFCAs and  $\Sigma$ PFASs except PFOS were 3% and 4% of the  
394 total PFAS amount quantified in the UK buzzard samples, respectively. PFHxDA was detected in a pooled  
395 buzzard sample at a concentration of 0.9 ng/g ww, while just fairly low PFOA levels were documented (0.4  
396 - 6 ng/g ww). The distribution of PFAS for selected predators is shown in **Figure 4**.

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

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



406  
 407 **Figure 4.** Relative contribution (%) of ΣPFOS, ΣPFCA, PFPI's, and PFASs excluding PFOS to ΣPFAS  
 408 concentrations in the liver tissues of the selected apex predator species. Otters: n = 5, Seals: n = 5, Harbour  
 409 Porpoises: n = 5, Common Buzzards: n = 5.

410  
 411 **3.4 PFAS patterns**

412 Throughout this research, major differences in the PFAS patterns between apex predators and their prey  
 413 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

438 PFPI's have been reportedly used as defoaming components in pesticide formulations, as well as leveling  
439 and wetting agents in industrial and commercial applications (**De Silva et al., 2012**), it should be noted  
440 that it is not known whether PFPI's containing pesticides or other PFPI related products were applied in  
441 any of this project's sampling locations. In general, the use of PFPI's in pesticide formulations further  
442 complicate characterization of wastewater sources from agricultural sources. On the basis of the presence  
443 of PFPI's in fish and apex predators, we recommend further research to determine the effect of these  
444 substances. While the contribution of PFPI's to the PFAS burden in all samples, determined on the basis  
445 of comparison to PFCAs and PFSAs, was dominant, PFAPAs were consistently below detection limits. De  
446 Silva *et al.* observed the same PFPI's:PFAPAs ratio in the framework of their recent study on  
447 perfluoroalkylphosphinic acids levels in northern pike, double-crested cormorants, and bottlenose  
448 dolphins (**De Silva et al., 2016**). Additionally, we identified microquantities of PFBA, PFPeA, PFHpA,  
449 PFHxDA, PFBS, and PFPeS only in AP livers but not in prey muscle tissues. On the contrary, PFOA had a  
450 100% FoA in the prey specimens, yet was below LOD in several predator samples. It could be supposed  
451 that the differences in the PFAS between apex predators and prey could be a result of the metabolism  
452 and following biotransformation PFAS undergo across the food web. Precursor metabolism and  
453 biotransformation processes are complex fields of research that have not yet been fully investigated. The  
454 ratio precursor:analyte:metabolite is dynamic and depends on a number of factors, the combination of  
455 which may alter the chemicals' mix from taxon to taxon or even at the individual level. Foraging habits,  
456 dwelling area/foraging location, migration behavior, sex, age and size strongly influence the PFAS  
457 concentrations across a wildlife population. However, sex and body length of the fish species does not  
458 influence the bioaccumulation of PFAS, according to previous studies, suggesting that the size of fish does  
459 not affect PFAS levels (**Ye et al., 2008; Quinete et al., 2009**).



#### 460 **4. Conclusions**

461 The present study presents insights into the frequency of occurrence and concentrations of PFAS in  
462 Eurasian otters, grey and harbour seals, harbour porpoises and common buzzards as well as four fish  
463 species (bream, roach, herring and eelpout) collected from 61 sampling sites in Germany, the Netherlands,  
464 Sweden and the United Kingdom. The analysis of 65 liver and muscle tissues for 56 PFAS shows that all  
465 analysed specimens were primarily contaminated with PFOS, while the three PFPI's included in this study  
466 exhibited FoA 100%. Additionally, our findings demonstrate that C9 to C13 PFCAs generally occur at high  
467 concentrations in apex predator livers despite phase-outs and increasing regulation of these compounds  
468 together with C8-based PFAS. The negligible detection of C4-C7 PFCAs in all AP tissues may indicate that  
469 the top predators in this study were not exposed to short-chain PFCAs via their prey or may suggest a low  
470 bioaccumulation potential of these compounds. PFAS concentrations were one to four orders of  
471 magnitude higher in predator liver tissues than in fish muscle. Apart from the difference in the PFAS  
472 metabolism in livers and muscles, the significant difference in total body size between predators and prey  
473 has to be taken into consideration when comparing total PFAS levels. All the above points to a widespread  
474 PFAS contamination in otters, seals, harbour porpoises and, to a lesser degree, common buzzards. While  
475 the PFAS contamination in fish muscles was lower than in predator livers, it was still considerably high.  
476 PFAS relative contribution varied among different species, due to the different binding affinity of PFAS for  
477 proteins and fats that are tissue- and organism-specific. Furthermore, the results show an association  
478 between the PFAS concentrations in apex predators and the geographical origin of the specimens. Despite  
479 the fact that the sixty-one sampling areas of this study were diverse, in terms of terrain, climate as well  
480 coordinates, a basic correlation between the geographical origin of the samples and the type as well as  
481 levels of PFAS in them was observed. This has to be factored in together with the type of matrix and its  
482 lipid/protein content, when drawing conclusions about what species were most contaminated and why.  
483 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.

504

505

506 **Conflict of interest**

507 The authors declare no conflict of interest.

508

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