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Differences in the internal PFAS patterns of herbivores, omnivores and carnivores - lessons learned from target screening and the total oxidizable precursor assay

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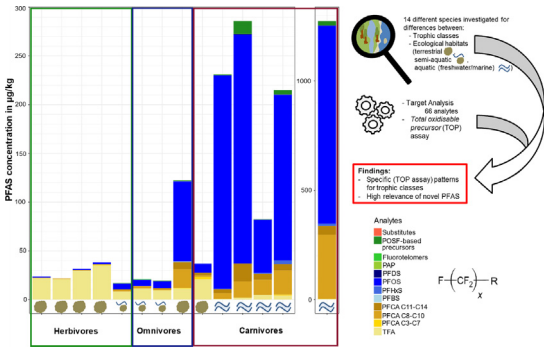
HIGHLIGHTS

- PFAS in several specimen types were analysed by target analysis and TOP assay.
- PFAS concentrations and patterns varied strongly between trophic classes.
- The ecological habitat also affected the internal PFAS contamination.
- Consistent patterns of formed PFCAs in the TOP assay for trophic classes.
- No differences in the PFCA formation potential and pattern between liver and musculature.

GRAPHICAL ABSTRACT



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ABSTRACT

Per- and polyfluorinated alkyl substances (PFAS) are a group of anthropogenic chemicals, which are not (fully) biodegradable and accumulate in different environmental compartments worldwide. A comprehensive, quantitative analysis – consisting of target analysis (66 different analytes, including e. g. ultrashort-chain perfluorinated carboxylic acids (PFCAs), precursor compounds and novel substitutes) and the *Total Oxidisable Precursor* (TOP) assay (including trifluoroacetic acid (TFA)) – were conducted to analyse the PFAS concentrations and patterns in 12 mammalian and two bird species from different areas of Germany and Denmark. The PFAS contamination was investigated in dependance of the trophic class (herbivores, omnivores, carnivores), ecological habitat (terrestrial, (semi-) aquatic) and body tissue (liver, musculature). PFAS concentrations were highest in carnivores, followed by omnivores and herbivores, with ΣPFAS concentration ranging from 1274 µg/kg (Eurasian otter liver) to 22 µg/kg (roe deer liver). TFA dominated in the herbivorous species, whereas perfluorooctanesulfonic acid (PFOS) and the long-chain PFCAs covered the majority of the PFAS contamination in carnivorous species. Besides trophic class, ecological habitat also affected the PFAS levels in the different species, with terrestrial herbivores and omnivores showing higher PFAS concentration than their aquatic counterparts, whereas for carnivores this relationship was reversed. The TOP assay analysis indicated similar trends, with the PFCA formation pattern differing significantly between the trophic classes. TFA was formed predominantly in herbivorous and omnivorous species, whereas in carnivorous species a broad spectrum of PFCAs (chain-

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length C2–C14) was formed. Musculature tissue of six species exhibited significantly lower PFAS concentrations than the respective liver tissue, but with similar PFAS patterns. The comprehensive approach applied in the present study showed, that primarily the trophic class is decisive for the PFAS concentration, as herbivores, omnivores and carnivores clearly differed in their PFAS concentrations and patterns. Additionally, the TOP assay gave novel insights in the PFCA formation potential in biota samples.

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are anthropogenic, highly fluorinated aliphatic compounds, differing in their carbon chain length and functional groups (Buck et al., 2011). Following the *Organisation for Economic Co-operation and Development* (OECD) definition, PFAS contain at least one perfluorinated methyl group (—CF_3) or methylene group (—CF_2—) (OECD, 2021). Based on this definition, the PFAS group covers more than six million individual substances (PubChem, 2022). Their chemical structure and the extremely strong and stable C—F bond render PFAS chemicals with unique properties (high thermal/chemical stability, dirt-/water-/fat-repellence), having led to a broad range of industrial applications since the 1950s (Buck et al., 2011). Due to the strength of the C—F bond, PFAS resist biodegradation, photooxidation and hydrolysis (Sznajder-Katarzyńska et al., 2019). PFAS have been found in all environmental compartments worldwide and with some compounds being subject to (long-range) atmospheric transport, PFAS have been reported even in remote environments such as the Arctic and Antarctica (Houde et al., 2006; Lee and Mabury, 2014; Kotthoff et al., 2020; Cousins et al., 2022; Guckert et al., 2022). Certain PFAS have toxic properties and can biomagnify in food webs, posing a toxicological risk for wildlife and humans (Giesy and Kannan, 2001; Lau et al., 2004; Müller et al., 2011).

As mainly long-chain perfluoroalkyl acids (PFAAs) have been marketed and these were proven to be more bioaccumulative than their short-chain analogues, past regulatory activities specifically focused on perfluoroalkyl sulfonic acids (PFSAs, number of carbon chain-length (n_C) ≥ 6), perfluoroalkyl carboxylic acids (PFCAs, n_C ≥ 8) and their corresponding anions (Buck et al., 2011). Perfluorooctanesulfonic acid (PFOS), perfluorooctanoic acid (PFOA), their derivatives, salts and related compounds were included in the international *Stockholm Convention on Persistent and Organic Pollutants* (POPs) to prohibit their use (Stockholm Convention). Further PFAS such as perfluorohexanesulfonic acid (PFHxS), its salts and related compounds as well as the PFCAs (C9–C14) are being considered for inclusion in the Stockholm Convention. In the European Union PFOS/PFOA and their derivatives have already been restricted under the EU's POPs regulation (Commission of the European Communities, 2020).

In response to the regulatory activities, manufacturers substituted regulated PFAAs with novel PFAS like perfluoroalkyl ether acids (e. g. 4,8-dioxa-3*H*-perfluorononanoic acid (DONA), hexafluoropropylene oxide dimer acid (HFPO-DA)) or short chain PFAS (Ateia et al., 2019; Munoz et al., 2019; Zhang et al., 2019). However, information about fate, transport, exposure, toxicity and bioaccumulation of these PFAS in the environment are scarce (Wang et al., 2017; Ateia et al., 2019). Certain alternative PFAS were already detected in biotic and abiotic matrices and their potential for persistence, mobility and potential for long range transport was proven (Munoz et al., 2019). Furthermore, short chain PFAAs accumulate in plants and agricultural crops, exhibiting an additional concern for the human and ecosystem health (Lesmeister et al., 2021).

Food is the main exposure pathway of PFAS for mammalian and bird species (Giesy and Kannan, 2001; Falk et al., 2012) Previous studies of PFAS in wildlife mainly investigated species that are in contact with each other (bioaccumulation along one food chain) and/or originate from the same ecosystem (e. g. terrestrial, marine, limnic), rather than comparing different environmental compartments. (Kannan et al., 2005; Müller et al., 2011; Chen et al., 2021; Huang et al., 2022). Furthermore, these studies primarily focused on legacy PFAS (PFSAs and PFCAs) and selected polyfuorinated precursor compounds, covering only a fraction of those PFAS which can be captured by target analysis. Therefore, bioaccumulation of especially long-chain PFAAs along specific food chains has been reported, while information about novel PFAS (e. g. ultrashort-chain PFCAs, substitutes) and comparison of different ecosystems is lacking (Kannan et al., 2005; Müller et al., 2011; Huang et al., 2022).

In this study liver samples of 14 different mammalian and bird wildlife species collected from 2015 to 2020 in Germany and Denmark were analysed for their PFAS pattern. This was done to learn to which extent the internal PFAS concentration is influenced by the trophic class of the species in the respective food chain (herbivores, omnivores and carnivores) and their habitat (terrestrial and (semi-) aquatic). It was investigated whether the trophic class of the species shows specific PFAS patterns/PFAA formation potentials and if the results for the trophic class differ between the ecological habitats. Additionally, the extent of – in addition to the legacy PFAS – novel PFAS (e. g. ultrashort-chain PFCAs, substitute compounds) and a wide range of precursor compounds accumulating in the different trophic classes and ecological habitats of the species was explored.

For this purpose, a very broad range of 66 PFAS were analysed and the *Total Oxidisable Precursors* (TOP) assay was applied. The TOP assay offers novel insights into the PFCA formation potential in biota samples by transforming PFAA-precursor compounds into measurable PFCAs (Houtz and Sedlak, 2012). To complement the interspecies comparison, musculature tissue from selected species was analysed to determine if the patterns observed by target analysis and TOP assay were liver specific.

2. Materials and methods

2.1. Sampling and sample treatment

The sampling design of the present study aimed at a general comparison of animals from different trophic classes and from different ecosystem types and environmental compartments. Instead of a representative sampling strategy, only a small number of samples was examined for each species and its habitat. Therefore, PFAS profiles likely contribute more to understanding PFAS accumulation patterns in and between food webs than providing detailed information on individual species studied and how they relate to each other. This is especially true when different species were compared within the same environmental ecosystem, since, for example, local differences in PFAS levels in terrestrial systems are known depending on the type of land use (Rupp et al., 2023) Additionally, it must be pointed out, that the different species were partly sampled in different years/regions and come from different age groups.

Liver tissue (n = 60) from 14 different species was collected by different German authorities and research institutes (Table SI01). The sampling was carried out by the different institutions under the legal regulations and necessary permits. The herbivorous species collected were: European hare (*Lepus europaeus*, n = 1, pooled sample), roe deer (*Capreolus capreolus*, n = 10, pooled samples), red deer (*Cervus elaphus*, n = 3, individual samples), chamois (*Rupicapra rupicapra*, n = 3, individual samples), European beaver (*Castor fiber*, n = 4, pooled samples); the omnivorous species were: nutria (*Myocastor nutrias*, n = 4, individual samples), common eider duck (*Somateria mollissima*, n = 1, pooled sample), wild boar (*Sus scrofa*, n = 11, pooled samples, data first reported in (Rupp et al., 2023)); and the carnivorous species consisted of European wildcat (*Felis silvestris*, n = 9, individual samples), Eurasian otter (*Lutra lutra*, n = 2, pooled samples), Great cormorant (*Phalacrocorax carbo*, n = 8, pooled and individual samples), harbour porpoise (*Phocoena phocoena*, n = 2, pooled samples), harbour seal (*Phoca vitulina*, n = 1, pooled sample), and grey seal

(*Halichoerus grypus*, $n = 1$, pooled sample). Additionally, for Eurasian otter ($n = 2$, pooled samples), harbour porpoise (n = 2, pooled samples), harbour seal (n = 1, pooled sample) and grey seal (n = 1, pooled sample), musculature tissue was also analysed. The sampling was conducted between 2015 and 2020 in different parts of Germany (all species except for common eider duck) and Denmark (common eider duck), from sites without known specific PFAS contamination history. An overview of the samples investigated, including information on sampling location, sex, age and sample processing is provided in Table SI01.

All samples were stored at −18 °C. In general, pooling of the samples for each species was performed when at least three individual samples from the same origin, gender and age were available. No pooling was performed for nutria, wildcat, chamois and red deer. For musculature tissue, at least one set of pooled material was available. All samples were homogenised by using a rotor stator disperser (ULTRA-TURRAX T25 from IKA Labortechnik, Staufen/Germany, equipped with the tool "S 25 N – 18 G – ST"). Sample aliquots of approximately 10 g were filled into pressure lock backs until analysis.

Sampling and sample processing of roe deer livers from the German Environmental Specimen Bank (German ESB) is described in a respective guideline (Tarricone et al., 2018). In brief, livers of a desired number of 14 one-year old individuals per sampling area were cryomilled to 200 μm and stored above liquid nitrogen.

2.2. Chemical analysis

All 66 tissue samples (60 liver and 6 musculature) were analysed applying target analysis and the TOP assay analysis. Analysis was performed in two different laboratories (group of analytes A and B) and based on liquid chromatography tandem mass spectrometry (RP-LC-MS/MS) as well as ion chromatography quadrupole time of flight mass spectrometry (IC-QTOF).

2.2.1. Reagents and standards

The reagents used for analysis were high purity grade as described in SI A.1. In total, 66 different analytes were included, of which 42 PFAS were analysed quantitatively and 24 PFAS qualitatively, based on their theoretical multiple reaction monitoring (MRM) transitions. If detected, the qualitatively measured PFAS were not included in data used for tables/figures/calculations. For quantification, 73 reference standards (including mass-labelled standards) were used. The full list of target compounds and internal standards as well as the compound classes, suppliers and used acronyms are listed in Table SI 02. In summary, the target list included four PFSAs (n_C = 4, 6, 8 and 10), 13 PFCAs (n_C = 2–14), six substitute compounds (two chlorinated perfluoroalkyl ether sulfonic acids and perfluoroalkyl mono- and di-ether carboxylic acids as well as one fluorotelomer sulfonamide amine oxide and fluorotelomer sulfonamidopropyl betaine) and 19 precursors compounds consisting of seven different classes: fluorotelomer phosphate mono- and diesters (monoPAP/diPAP), perfluorooctane sulfonamido phosphate diester (diSAmPAP), fluorotelomer sulfonic acids (FTSAs), alkylated and non-alkylated perfluorooctane sulfonamides (FOSAs), alkylated and non-alkylated perfluorooctane sulfonamidoacetic acids (FOSAAs), alkylated and non-alkylated perfluorooctane sulfonamidoethanols (FOSEs).

2.2.2. Analysis

The samples were analysed in two different laboratories, with different analytical methods to match the different requirements of the analytes. The distribution of the PFAS between the two laboratories (group A and group B) is provided in Table SI 02. Analysis of group A analytes and the TOP assay analysis were conducted in the same laboratory. The protocols of solid-liquid extraction, clean-up and instrumental analysis are presented in SI A.2.

In short, for samples analysed in laboratory A, 0.5 g of sample was extracted twice with acetonitrile/water (9/1, *v*/v). The combined extract was stored overnight at −18 °C for phase separation. The acetonitrile phase was removed, evaporated and the residues were re-extracted twice with acetonitrile, followed by evaporation to dryness and reconstitution in methanol/water 8/2, *v*/v.

For TOP assay analysis the extract was divided into two aliquots before being stored overnight at −18 °C. One aliquot for the oxidation process and the other aliquot for reference (without oxidation) to determine the formation potential of PFCAs from precursors. The reference aliquot was treated like the target analysis samples. The aliquot for oxidation was spiked with aqueous potassium persulfate solution and incubated for 20 h at 85 °C. Afterwards, the sample was evaporated to dryness and subject to the previously described clean-up with acetonitrile.

For samples analysed in laboratory B, 1 g of sample was extracted twice with acetonitrile. Clean-up was performed using graphitized carbon and glacial acetic acid. After centrifugation, the supernatant was mixed with aqueous ammonium solution, stored overnight at −18 °C and centrifuged. The extracts were filtered and used for analysis.

Instrumental analysis was performed via IC-QTOF and three methods of RP-LC-MSMS with minor variations SI A.2. The mass spectrometric conditions for each analyte such as cone voltage, collision energy and mass transitions are given in Table SI 03.

2.3. Quality control and statistical analysis

2.3.1. Quality assurance

Quality assurance was performed according to (Rupp et al., 2023). In short, the isotope dilution approach was used for quantification. For analytes without corresponding mass-labelled standard, a structurally related internal standard was used. Table SI 03 offers an overview of the used mass-labelled standards for each analyte. To evaluate instrumental drifts, external calibration curves were analysed prior and after each analytical sequence. Furthermore, instrumental performance and carryover was checked repeatedly by running instrumental blanks and calibration standards.

Procedural blanks were included in the complete workflow to determine blank values. In case of blank signals, the average signal area detected in the blanks was subtracted from the sample data in the batch.

Additionally, present blank values were included in the limit of quantification (LOQ) determination (LOQ ≥ tenfold standard deviation of procedural blanks). In absence of blank contamination, the LOQ was determined by signal-to-noise ratio (S/N ≥ 10). Due to the variation of matrix effects within one type of matrix, also values below the typical LOQ were reported if the signal fulfilled both conditions.

Method performance (i. e. precision and accuracy) was determined by triplicate spike/recovery experiments. Liver tissue of bream (*Abramis brama*) from the German ESB was used as proxy for the liver samples analysed in this study. For group A analytes musculature tissue of bream was also validated SI A.3. Validation results of target and TOP assay analysis are provided in the supporting information (Tables SI05 and SI06).

Oxidation of precursor compounds in the TOP assay analysis was ensured by spiking separate samples of each matrix with precursor material prior oxidation. The absence of the spiked precursor material after the TOP assay, was defined as prerequisite for sufficient oxidation conditions of the assay. Additionally, oxidation was controlled visually, as in biota samples a clear and colorless liquid was present after oxidation. However, as there is still a low probability of incomplete oxidation, the formation potential has to be regarded as a minimum.

For quality control of selected PFAS, the certified JRC pike-perch musculature reference material IRMM 427, was analysed with every batch of samples. The differences to the measurement results were calculated and compared to the combined expanded uncertainty of measurement and reference value (Dabrio Ramos et al., 2015) Table SI 04. The results for all PFAS in the reference material were found to be unbiased – with the exception of PFDoDA, where the analysis resulted in systematically lower levels (63 % of the reference value). The lower findings of PFDoDA indicate that the actual values for PFDoDA in the samples are presumably higher than the determined values.

2.3.2. Statistical data analysis

Due to the large number of analytes with individual LOQs and varying LOQs depending on the species, values < LOQ were treated as zero. Samples were analysed once for each method. Calculated concentrations for each species refer to wet weight (ww) and are given as arithmetic mean (except for samples with $n = 1$) if not stated differently. As PFAS are generally found at high concentrations in liver tissue, as compared to other tissue types, the interspecies comparison is solely based on liver samples.

The PFCA formation potential in the TOP assay was calculated as the difference between the PFCA concentrations in the oxidised extract and the reference extract from the TOP assay (ΔTOP). The ΔTOP results are provided in the supporting information (SI B).

Statistical analysis (test for normal distribution (Shapiro Wilk Test) and significance (*t*-Test, ANOVA)) as well as the figures were performed with the software R. Significance was tested at confidence level $\alpha = 0.05$ for all data. Statistical analysis was only conducted for sample numbers $n \geq 3$. Samples of different sex and age were treated equally, as the sampling size was not sufficient for statistical analysis. Results of statistical analysis have to be interpreted with caution, as individual and pooled samples were compared. For comparison of the PFAS patterns, the data for the principal component analysis (PCA) was transformed to molar concentrations and later normalised to the sum of all concentrations.

3. Results and discussion

3.1. Terrestrial species

The mean ΣPFAS concentration in the terrestrial liver species analysed followed the order wild boar > wildcat > hare > red deer > chamois > roe deer (Fig. 1, Table SI07). In herbivores, PFCAs dominated the PFAS pattern, especially the ultrashort-chain PFCA TFA which accounted for more than >90 % of the total PFAS load (Fig. 2). In addition to TFA, PFCAs with chain-length C8–C14 were detected, with individual PFCA concentrations < 0.4 μg/kg. Among PFSAs, only PFOS was detected in terrestrial herbivores (max. 1.9 μg/kg in hare). In roe deer, PFOS was not detected. However, this was the only species without PFOS findings in this study.

Similar to the PFAS pattern in herbivores, PFCAs, in particular the ultrashort-chain PFCAs were also the dominant group of PFAS in wildcat, the only terrestrial carnivore in this study (TFA 21 μg/kg, PFPrA 2.2 μg/kg). However, wildcats had comparatively higher concentrations of C7–C14 PFCAs (max. 1 μg/kg PFDA and PFTrDA) and PFOS (9.4 μg/kg). In both, herbivores and wildcat only few polyfluorinated compounds were detected in concentrations ≤ 0.04 μg/kg − i. e. diSAmPAP and EtFOSAA in herbivores and 6:2 diPAP, 8:2 FTSA as well as qualitatively FBSA in wildcat.

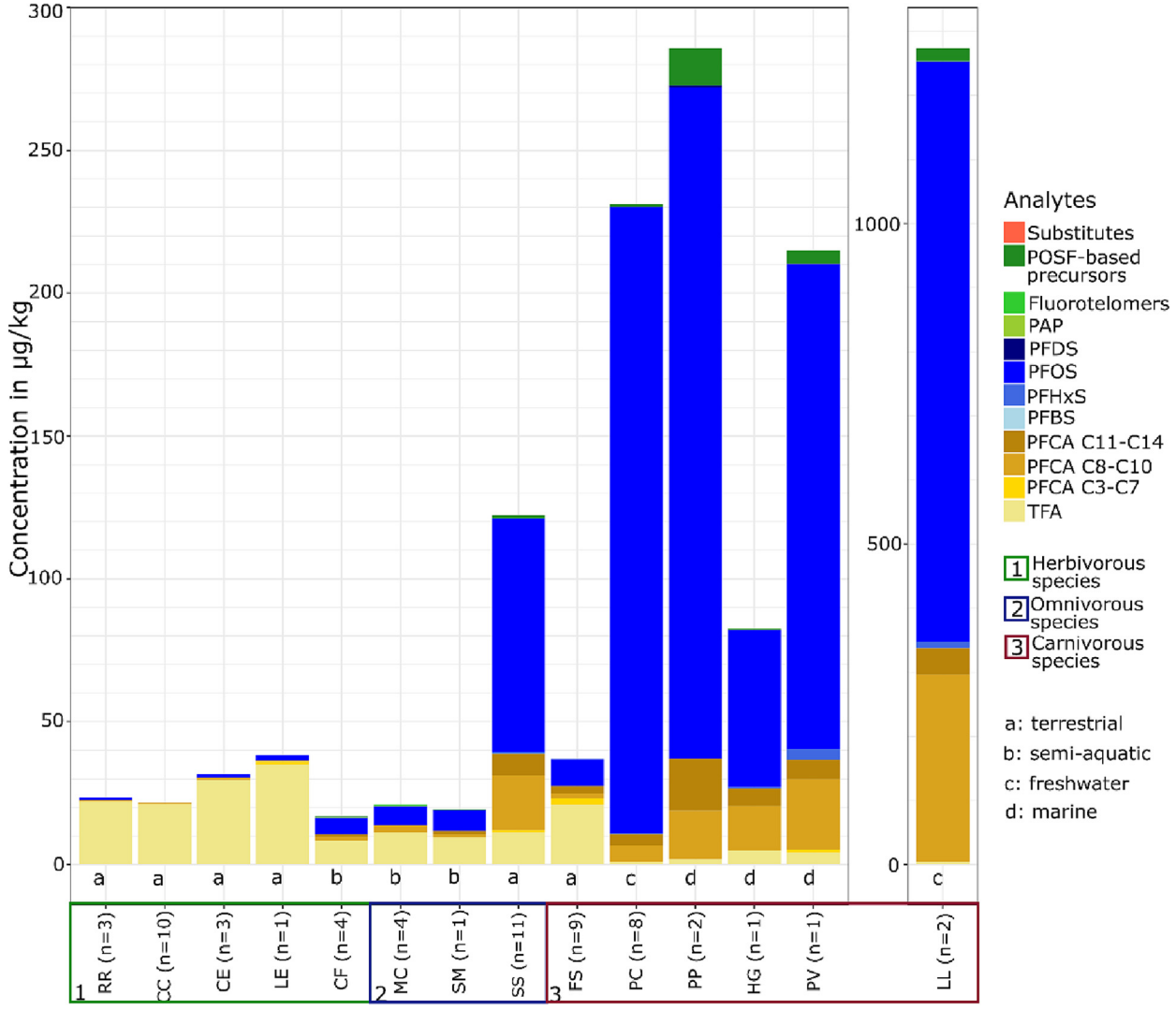

Fig. 1. Total PFAS concentrations in livers from different species determined by target analysis. n represents the number of samples analysed. Except for red deer, chamois, nutria and wildcat (each individual samples), the samples are pooled and consist of at least 3 individuals. For the cormorant, individual as well as pooled samples were analysed. Used abbreviations: red deer (CE), roe deer (CC), chamois (RR), hare (LE), beaver (CF), nutria (MC), common eider duck (SM), wild boar (SS, from (Rupp et al., 2023)), wildcat (FS), otter (LL), cormorant (PC), harbour porpoise (PP), grey seal (HG), harbour seal (PV).

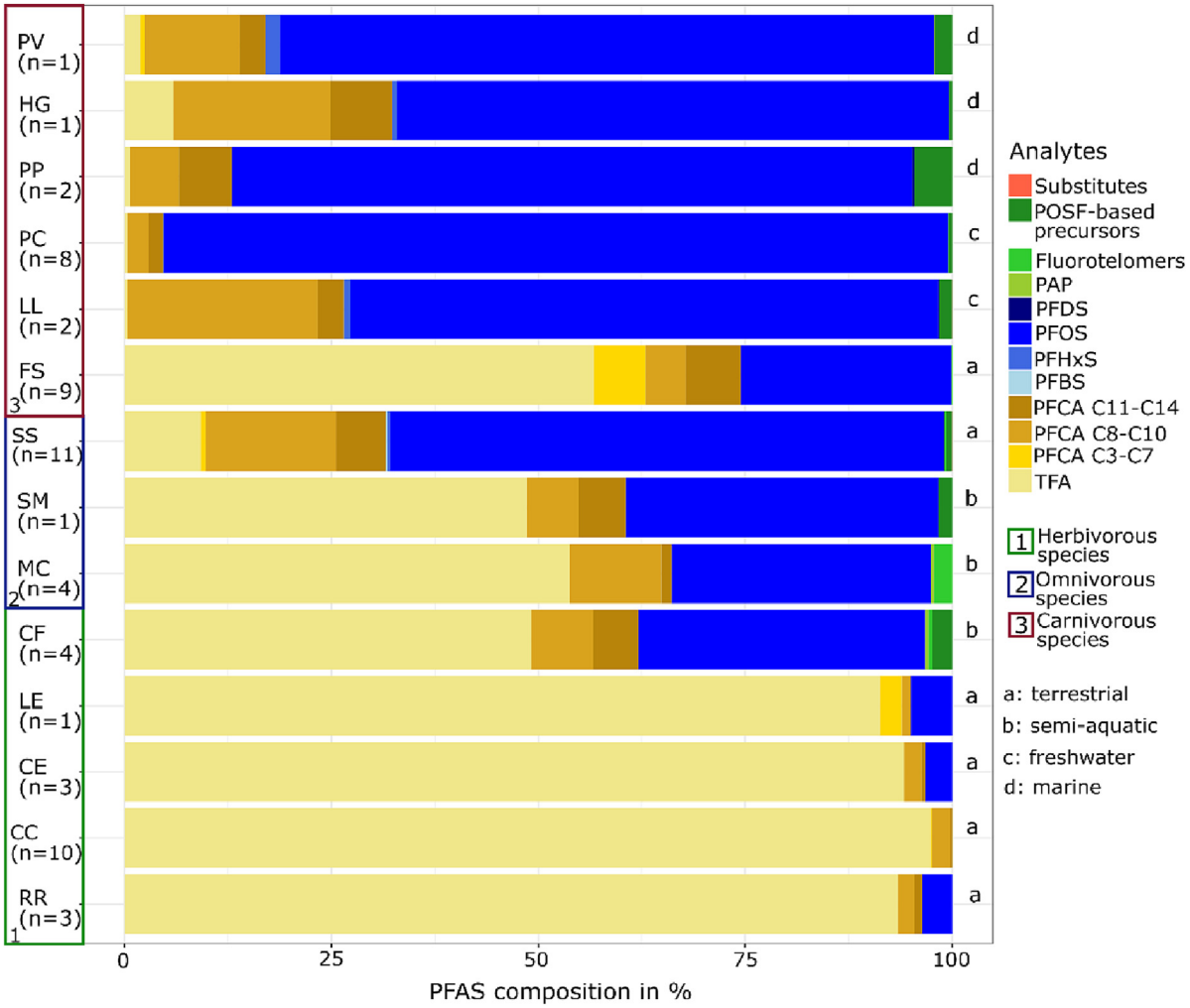

Fig. 2. Differences in the PFAS composition in livers from different species determined by target analysis. n represents the number of samples analysed. Except for red deer, chamois, nutria and wildcat (each individual samples), the samples are pooled and consist of at least 3 individuals. For the cormorant, individual as well as pooled samples were analysed. Used abbreviations: red deer (CE), roe deer (CC), chamois (RR), hare (LE), beaver (CF), nutria (MC), common eider duck (SM), wild boar (SS, from (Rupp et al., 2023)), wildcat (FS), otter (LL), cormorant (PC), harbour porpoise (PP), grey seal (HG), harbour seal (PV).

Contrary to the terrestrial herbivores and wildcat, in wild boar PFOS was the dominant PFAS (82 μg/kg), followed by the PFCAs TFA and PFNA (both 11 μg/kg, Fig. 1). Furthermore, the PFSAs PFBS, PFHxS, PFDS, and the PFCAs with chain-lengths C4 and C7–C14 were detected. In addition to the PFAAs, several polyfluorinated compounds (10:2 diPAP, diSAmPAP, 6:2 and 8:2 FTSA, Me- and EtFOSAA, EtFOSE and FBSA) were detected in wild boar with a maximum concentration of 5.9 μg/kg for EtFOSE. Wild boar was the only terrestrial species in which PFAS substitutes (6:2 Cl-PFESA, 6:2 FTNO) were identified (first reported in Rupp et al., 2023).

The high TFA concentrations in herbivorous species are consistent with recent TFA results in terrestrial German ecosystems (Freeling et al., 2020, 2022). In general, TFA is not expected to accumulate in animal tissue because it is hydrophilic and rapidly eliminated (Holoday, 1977; Frank et al., 2002). Therefore, the TFA is assumed to mainly reflect the level of TFA content of the current diet and local habitat at the time of sampling. Recently, significant correlations were reported between TFA in locusts and in plants on which they feed, collected from the same farmland in China (Lan et al., 2020). Nevertheless, it can be expected that due to its persistence, TFA will remain in the environment which leads to a continuous and long-lasting exposure Atmospheric transformation and deposition of halogenated refrigerants are discussed as sources of TFA, as well as pesticides that form TFA during biotic and abiotic transformation (Behringer et al., 2021; Seiber and Cahill, 2022).

PFOS and long-chain PFCAs were present at significantly higher concentrations in wildcat compared to terrestrial herbivores ($p < 0.05$, Table SI08), likely due to the exclusively carnivorous diet of wildcat (Lozano et al., 2006) and the accumulation of longer chained PFAAs in food webs (Lozano et al., 2006; Kelly et al., 2009). Nevertheless, overall PFCA levels in wildcat were low, with a high TFA contribution to ΣPFAS (57 %). This could be explained by consuming small herbivorous rodents or insects as the main diet in a short food chain with low bioaccumulation potential (Lozano et al., 2006; Shukla et al., 2021).

The omnivorous species wild boar exhibited the highest PFAS contamination of the terrestrial species analysed. Its opportunistic feeding behaviour, including e. g. plants, insects, and small rodents provides a wide range of different PFAS sources (Cuevas et al., 2010). Due to its digging and rooting behaviour (Kowalczyk et al., 2018), wild boar is in close contact with soil as well as organisms present in the soil (e. g. earthworms) and therefore particularly exposed to atmospheric deposition of PFAS, as soils and organisms in the soil are a major repository for PFAS (Rankin et al., 2016; Kowalczyk et al., 2018; Parolini et al., 2022; Sörengård et al., 2022).

The ΣPFAS findings in the present study for the herbivorous species exceed previous reports for livers of terrestrial herbivores (roe deer and chamois; mean 1.6–10.1 μg/kg) (Falk et al., 2012; Riebe et al., 2016; Falk et al., 2019; Kotthoff et al., 2020). This is primarily due to the inclusion of TFA in the present study, as it was not considered in the cited studies. After

subtracting TFA concentrations from ΣPFAS (mean 0.6–3.3 μg/kg), the results of the present study are slightly lower than in the previous studies. The concentrations of ΣPFAS and PFOS determined in the omnivorous wild boar are consistent with previously reported data (Brambilla et al., 2017; Kowalczyk et al., 2018).

3.2. Semi-aquatic herbivores and omnivores

Despite beaver and nutria being sampled inland and the common eider duck in coastal areas, the profiles and patterns in livers of these three species were similar, with mean ΣPFAS concentrations of 17 to 21 μg/kg (Fig. 1). Major contributions to the ΣPFAS concentrations were determined for TFA (8.4–11.3 μg/kg) and PFOS (5.9–7.3 μg/kg). In addition, long-chain PFCAs C8–C14 were determined.

While beaver and nutria are predominantly herbivorous, the common eider duck is mainly carnivorous (Laursen and Møller, 2022). Smaller differences might be accounted for by the different diet or migration behaviour of the common eider duck, whereby the common eider reflects the PFAS contamination of different areas (Laursen et al., 2019). Larger differences are not expected as the common eider duck mainly feeds on biota of low trophic classes e. g. bivalves (Laursen and Møller, 2022). Beaver and nutria exhibited multiple findings of polyfluorinated compounds (e. g. 10:2 diPAP, diSAmPAP, FTSAs, FBSA) whereas in common eider duck only FBSA, FHxSA and FOSA were detected. As the beaver and nutria were both sampled in urban catchments, the higher detection frequency of polyfluorinated substances of the ΣPFAS (Fig. 2) might derive from urban contamination (Chen et al., 2019; Lan et al., 2020). The levels of PFOS in beaver and common eider duck are consistent with data reported in the literature (6.6 μg/kg, respectively 7.7 μg/kg) (Falandysz et al., 2007; Kelly et al., 2009).

3.3. Freshwater and marine carnivores

3.3.1. Profiles in liver samples

Mean ΣPFAS concentrations in the livers of freshwater (otter, cormorant) and marine fish-feeding top predators followed the order otter > harbour porpoise > cormorant > harbour seal > grey seal (Fig. 1, Table SI07). For all those species the predominant PFAS was PFOS (67–95 %, Fig. 2), followed by PFNA and PFDA. In otter, the PFSAs PFBS, PFHxS and PFDS were also detected, while no PFSAs other than PFHxS were found in the other species (except for harbour porpoise). The long-chain PFCAs C8–C14 were detected in species from both ecosystems. In the species from marine ecosystem, diSAmPAP, 8:2 FTSA, FOSA as well as the qualitatively measured FBSA and FSHxA, were the only polyfluorinated compounds determined. The pattern of polyfluorinated compounds in otter and cormorant was more diverse (e. g. 10:2 diPAP, diSAmPAP, FTSAs, FASAs, FASAAs). Besides, multiple substitute compounds (6:2 Cl-PFESA, 8:2 Cl-PFESA, 6:2 FTNO) were also detected at low concentrations in the aquatic freshwater species.

The otter results are consistent with previously reported high concentrations of ΣPFAS and PFOS concentrations for otter in Northern Europe (Roos et al., 2013; Androulakakis et al., 2022). The high level of ΣPFAS is associated with more frequent detections of polyfluorinated compounds and substitutes. This could be explained by higher PFAS emissions in freshwater systems compared to coastal and marine systems (Androulakakis et al., 2022).

The cormorant accounted for the highest percentage of PFOS in the total PFAS load (mean 95 %) compared to the other piscivorous species. However, a strong spread in the ΣPFAS and PFOS concentration could be observed for the eight cormorant samples (29–640 μg/kg, Table SI06), which is likely attributable to the sampling site, as there seemed to be no correlation with sex or age. Nevertheless, the results for PFOS in cormorant liver are in agreement with piscivorous birds reported in the early 2000s (Kannan et al., 2002; Houde et al., 2006) – despite the fact that PFOS and PFOA concentrations in Western Europe tend to decrease since then (Falk et al., 2019; Kotthoff et al., 2020).

The marine species share a similar PFAS profile, with the harbour porpoise and harbour seal having higher ΣPFAS concentrations compared to the grey seal. This discrepancy is likely to be explained by the different sampling regions (North Sea for harbour porpoise/seal, and Baltic sea for the grey seal), as all three marine species share the same ecological niche and feeding behaviour. As the species enter adjacent estuaries in search for food, they might be stronger exposed to anthropogenic influences, which could result in the high levels of contamination (Carter et al., 2001; Taupp, 2022). In general – and despite targeting more analytes in the present study – the ΣPFAS results for the marine species are lower or at the lower limit compared to data in the literature from previous years (Kannan et al., 2002; van de Vijver et al., 2003; van de Vijver et al., 2007; Ahrens et al., 2009; Galatius et al., 2013; Androulakakis et al., 2022), which reflects the decreasing environmental concentrations of legacy PFAS.

3.3.2. Tissue distribution

PFAS are known to preferentially bioaccumulate in liver tissue (Müller et al., 2011; Greaves et al., 2012). To complement the interspecies comparison in liver, PFAS profiles were also determined in musculature tissue for the piscivorous species.

Indeed, concentrations of ΣPFAS in liver were significantly higher than in musculature tissue (5-fold (grey seal) to 28-fold (otter), Fig. SI02), but in both tissue types PFOS was the dominant PFAS (Fig. SI03). The relative amount of PFOS and long-chain PFCAs ($n_C \geq 8$) did not differ significantly, while the relative concentration of short-chain PFCAs ($n_C < 8$) was significantly higher in musculature than in liver tissue. In general, the relative concentration of precursors in musculature was also higher than in liver tissue.

In contrast to liver tissue, in the musculature tissue, the differences in the total PFAS concentrations between the species were minor. The results for the PFAS trends in liver and musculature tissue are consistent with data reported in harbour seals, polar bears and fish (Ahrens et al., 2009; Greaves et al., 2012; Kowalczyk et al., 2020; Chen et al., 2021). However, data on the accumulation of short-chain PFAS in different animal body tissues is scarce, as previous studies mainly focus on long-chain PFAS, lacking information on the differences in tissue distribution of short-chain PFAS.

3.4. Interspecies comparison

PFAS concentrations in the investigated species decreased in the order freshwater carnivore > marine carnivore > terrestrial omnivore > terrestrial carnivore > terrestrial herbivore > semi-aquatic omnivore/herbivore. Although, it needs to be kept in mind that animals were not always from the same region, year and not of the same sex and age.

A principal component analysis (PCA) was performed to obtain unbiased insight into the differences in the PFAS patterns of the liver samples of the different species (Fig. 3). The principal components (PC) 1 and 2 explain 61 % of the total variance in the data. PC2 clearly separates the terrestrial herbivores from aquatic carnivores. A unique distribution pattern can be seen between the carnivorous wildcat and terrestrial herbivores rather than a clear separation of the wild cat. In general, terrestrial herbivores cluster identically and are strongly affected by high TFA and low PFOS concentrations resulting in high scores of PC2. Similar clustering can be seen for the omnivorous common eider duck and nutria and herbivorous beaver. The clustering is also influenced by TFA, but to a smaller extent than in the terrestrial herbivores.

Most of all, clustering of beaver and nutria is affected by polyfluorinated compounds. Clustering of wild boar is driven by PC2, being influenced by PFOS and TFA. Due to the high PFOS content, wild boar clearly separates from the other clusters of terrestrial species. The piscivorous species are mainly affected by PFOS and the long-chain PFCAs and therefore group differently from the herbivorous species but overlap with wild boar due to PFOS. However, separation of wild boar and piscivorous species is achieved when PC3 is considered. PC3 explains 6.4 % of the total variance, so that 67.4 % of the total variance is explained by the first three PC. While wild

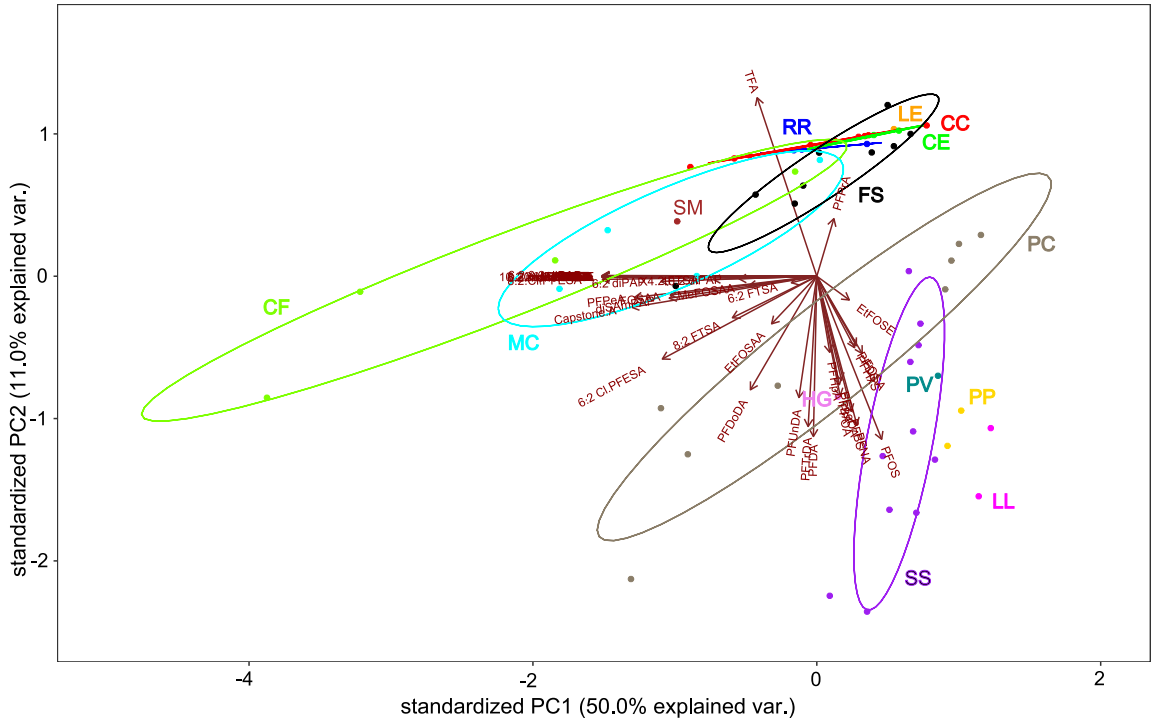

Fig. 3. Principal component analysis showing the PFAS pattern in the livers analysed by target analysis. Illustrated are the first two principal components (PC), explaining 61 % of the variance embedded within the data. Ellipses show 68 % confidence intervals for the respective sample groups. The loadings of each analyte for PC1 and PC2 are listed in table SI10. Used abbreviations: red deer (CE, $n = 3$), roe deer (CC, $n = 10$), chamois (RR, n = 3), hare (LE, n = 1), beaver (CF, $n = 4$), nutria (MC, n = 4), common eider duck (SM, n = 1), wild boar (SS, $n = 11$, from (Rupp et al., 2023)), wildcat (FS, $n = 9$), otter (LL, $n = 2$), cormorant (PC, $n = 8$), harbour porpoise (PP, n = 2), grey seal (HG, n = 1), harbour seal (PV, n = 1). n represents the number of samples analysed. Except for red deer, chamois, nutria and wildcat (each individual samples), the samples are pooled and consist of at least 3 individuals. For the cormorant, individual as well as pooled samples were analysed.

boar clustering is mainly affected by PFBS, PFBA, PFOA and the polyfluorinated EtFOSE via PC3, piscivores are influenced by the long-chain PFUnDA, PFDS, PFOS and especially FOSA.

The large differences in the PFAS pattern and concentrations between the carnivorous terrestrial (wildcat) and all aquatic species might be explained by the differences in trophic classes and the ecological habitat. In general, food chains are longer in aquatic environments than in terrestrial ecosystems, resulting in aquatic prey having higher PFAS levels (Chase, 2000; Eriksson et al., 2016). Furthermore, species-specific physiological processes (e. g. absorption, excretion, distribution, conversion rate) and prey pattern also affect the PFAS burden. For example, research on the faeces of domestic cats showed high excretion rates for long-chain PFCAs ($n_C \geq 8$) (Ma et al., 2020), which could explain the atypical PFCA pattern in wildcat compared to the other carnivorous species.

3.5. TOP assay analysis

Clear trends in the concentrations and patterns of the PFAS analysed in dependence of the trophic class and/or habitat of the different species were found in this study. These relationships were further studied by TOP assay to determine the formation potential for PFCAs from partially unknown precursor compounds. Due to the aggressive conditions in the process, the TOP assay only forms PFCAs and does not simulate the biotransformation processes in the environment, in which also PFSAs and intermediate products may be formed (Houtz and Sedlak, 2012; Casson and Chiang, 2018). However, the TOP assay gives a good estimate for both PFSA and PFCA precursors in the environment. The PFCA formation potential is expressed as organic fluorine, for which the organic fluorine content of each analyte was calculated with the respective PFAS concentrations.

3.5.1. Interspecies comparison of the PFCA formation potential and pattern

The formation potential in liver tissue ranged from <0.01 μg/kg (common eider duck, roe deer, hare) to 13.2 μg/kg organic fluorine (grey seal, Table 1). Relative to the ΣPFCA concentration measured by target analysis, the increase in ΣPFCA concentrations after TOP assay ranged from 1 % (wildcat) to 74 % (grey seal).

While the TOP assay analysis showed no significant differences in the PFCA formation potential for either the trophic class or the ecological habitat of the analysed species, it exhibited different patterns of PFCAs for herbivores, omnivores and carnivores. In terrestrial herbivores and nutria TFA accounted for >99 % of the total PFCAs formed (Fig. 4). In contrast, in beaver and the omnivorous wild boar TFA accounted for 75 %, and 90 %, respectively. The percentage of TFA in the total formation potential was much lower for carnivores, with a maximum of 27 % determined in otters. In carnivorous species, the pattern of PFCAs formed is broad, covering all the analysed PFCAs. Their patterns differed between species, with PFUnDA and PFDoDA dominating in wildcat and otter, and PFHxA, PFHpA and PFOA dominating in grey seal. While the PFCA pattern of wildcat from target analysis resembled that of the herbivores, the pattern of PFCAs formed by the TOP assay resembled that of a carnivore.

The low PFCA formation potential of all liver samples agrees with the low concentration of known precursors determined in target analysis (Table SI07). Both findings may reflect in vivo transformation of precursors in the metabolically active liver (Rand and Mabury, 2014; Chen et al., 2015; Liu et al., 2020). The data of body tissues (Sections 3.3.2 and 3.5.2) points in the same direction.

The TFA formation potential in herbivores and omnivores possibly derives from fluorinated compounds containing only isolated CF_3-groups, which are released upon oxidation, such as agrochemicals (Kaczyński et al., 2021; Seiber and Cahill, 2022). The low findings of polyfluorinated PFAS by target analysis support this thesis (Table SI07). Additionally for the semi-aquatic beaver and nutria, which have been sampled in close proximity to urban catchment, fluorinated compounds in wastewater might also account for the TFA formation potential (Scheurer et al., 2017).

For the carnivorous species, due to variability of PFCAs formed (C2–C14), the organic fluorine is likely to result from precursor compounds

Table 1
Organic fluorine (OF) concentrations detected as ΣPFCAs in μg/kg. n represents the number of samples analysed. Except for red deer, chamois, nutria and wildcat (each individual samples), the samples are pooled and consist of at least 3 individuals. For the cormorant, individual as well as pooled samples were analysed. Used abbreviations: red deer (CE), roe deer (CC), chamois (RR), hare (LE), beaver (CF), nutria (MC), common eider duck (SM), wild boar (SS, from (Rupp et al., 2023)), wildcat (FS), otter (LL), cormorant (PC), harbour porpoise (PP), grey seal (HG), harbour seal (PV).

Species	Increase in ΣPFCAs from TOP assay in μg/kg OF	ΣPFCAs from target analysis in μg/kg OF	ΣPFCAs after TOP assay relative to the ΣPFCA concentration from target analysis in %
Liver			
RR (n = 3[a])	3.0	11.5	126
CE (n = 3[a])	3.8	15.4	125
CC (n = 10)	<0.01	12.6	–
LE (n = 1)	<0.01	18.4	–
CF (n = 4)	0.7[b]	5.8	112
MC (*n* = 4[a])	0.6	7.5	108
SM (n = 1)	<0.01	6.3	–
SS (n = 11)	7.1[b]	25.9	127
FS (n = 9[a])	0.2	14.8	101
LL (n = 2)	6	235.7	103
PC (n = 8)	3.7[b]	8.5	143
PP (*n* = 2)	5.8	25.7	122
HG (n = 1)	13.2	17.7	174
PV (*n* = 1)	1.1	24.7	104
Musculature (F)			
LLF (n = 2)	3.1	5.8	154
PPF (n = 2)	8.1	3.2	350
HGF (n = 1)	1.2	3.0	138
PVF (n = 1)	1.3	6.0	122

[a] Individual samples.
[b] Outliers identified via Shapiro-Wilk-Test excluded.

with fluorinated alkyl chains, which were not included in target analysis, such as perfluorinated phosphinic acids (PFPiAs). PFPiAs were found in different prey fish and could, among other unknown precursors compounds, account for the organic fluorine formation in cormorant and grey seal (Chen et al., 2021).

For certain carnivorous species (otter, harbour porpoise and harbour seal) the concentration of precursor compounds determined by target analysis exceeded the formation potential determined by the TOP assay (Table SI07). This could either be due to: i) non-detectable/unknown/not extractable PFAS/oxidation products (e. g. perfluoromethoxypropionic acid (PFMOPrA)) (Zhang et al., 2019; Göckener et al., 2022), ii) poor correction by internal standard (IS) which is only added after the oxidation step, iii) loss of precursor compounds by the TOP assay, e. g. volatilisation (i. e. FOSA/FOSE) (Del Vento et al., 2012) or iv) depending on the precursor compound, loss of organic fluorine due to oxidative mineralisation of precursor compounds (Janda et al., 2019).

Altogether, the broad spectrum of PFCAs released by the TOP assay in carnivores indicates the presence of different precursor compounds and outlines the bioaccumulation potential of precursor compounds in the food web. According to the different patterns of the formed PFCAs, this bioaccumulation potential differs between herbivores, carnivore and omnivores.

3.5.2. Tissue specific PFCA formation potential and pattern

In liver and musculature tissue of piscivorous predators, the PFCA formation potential in musculature and liver is similar (Table 1). Both, the absolute and the relative increase in organic fluorine between musculature and liver were insignificant. However, due to the lower PFCA concentrations determined in musculature by target analysis, the relative increases appear higher. Especially striking was the high formation potential in musculature of harbour porpoise (350 %), which fits the high percentage (19 %) of perfluorinated compounds seen in the PFAS pattern (Fig. SI03). The largest discrepancies between the PFCA formation potential in liver and musculature were observed for the grey seal (tenfold higher in liver tissue). The formation potential is likely to derive from unknown precursor compounds.

Regarding the pattern of formed PFCAs, only minor differences between liver and musculature tissue were observed (Fig. 4). In musculature tissue, the long-chain PFCAs had a higher formation potential compared to the short-chain PFCAs (68 % vs. 32 %). For liver tissue, the ratio between short-chain PFCAs and long-chain ($n_C \geq 8$) PFCAs was equal (50 % each). However, between musculature and liver tissue, differences in the formation potential of short-/long-chain PFCAs were not significant.

Significant differences, though, were observed for the amount of explainable organic fluorine (polyfluorinated compounds determined by the target analysis) between liver and musculature tissue. Musculature tissue shows significantly higher ratios of unidentified precursor compounds (Table SI09), which might be due to a lower metabolic activity in musculature tissue.

4. Conclusions

In a comprehensive, quantitative analysis, the PFAS concentrations and patterns of 66 PFAS were investigated in 14 different mammalian and avian species including herbivores, omnivores and carnivores from different ecological habitats (terrestrial, semi-aquatic, marine) and in different body tissues (liver and musculature). This study confirms a ubiquitous presence of PFAS in wildlife.

In general, PFAS concentrations in liver tissue decreased in the order semi-aquatic carnivore > marine carnivore > terrestrial omnivore > terrestrial carnivore > terrestrial herbivore > semi-aquatic omnivore/herbivore, due to PFAS enrichment in longer food chains. PFAS patterns differed significantly, with TFA dominating in (predominantly) herbivorous species, whereas in carnivores PFOS, and to a lesser extent long-chain PFCAs ($n_C \geq 8$) dominated. Novel substitute compounds were detected only sporadically (wild boar, otter, cormorant) and at low concentrations. The major contribution of TFA to the total PFAS contamination in herbivores highlights the importance of including TFA in future biota screening studies.

TFA was also the dominant PFCA formed in the liver of herbivores in the TOP assay, whereas in carnivores, the PFCAs C2–C14 were formed. It appears important to extend the target analysis and TOP assay analyte spectrum with respect to additional precursor compounds (e. g. PFPiAs and phosphonic acids) and transformation compounds (e. g. PFMOPrA) in future studies.

For the first time, the PFCA formation potential and patterns in different body tissues was investigated, which neither differed significantly for the absolute formation potential, nor the pattern of formed PFCAs, between liver and musculature. However, as the samples sizes for musculature tissues were comparatively small, further research in regards to the formation potential in different body tissues is necessary.

CRediT authorship contribution statement

Marc Guckert: Investigation, Formal analysis, Visualization, Writing – original draft. **Jana Rupp:** Investigation, Formal analysis, Writing – review & editing. **Gudrun Nürenberg:** Investigation, Writing – review & editing. **Karsten Nödler:** Supervision, Writing – review & editing. **Jan Koschorreck:** Supervision, Resources, Writing – review & editing. **Urs Berger:** Funding acquisition, Supervision, Writing – review & editing. **Wiebke Drost:** Supervision, Resources, Writing – review & editing. **Ursula Siebert:** Resources, Writing – review & editing. **Gudrun Wibbelt:** Resources, Writing – review & editing. **Thorsten Reemtsma:** Supervision, Writing – review & editing.

Data availability

All data used are provided in the supporting information.

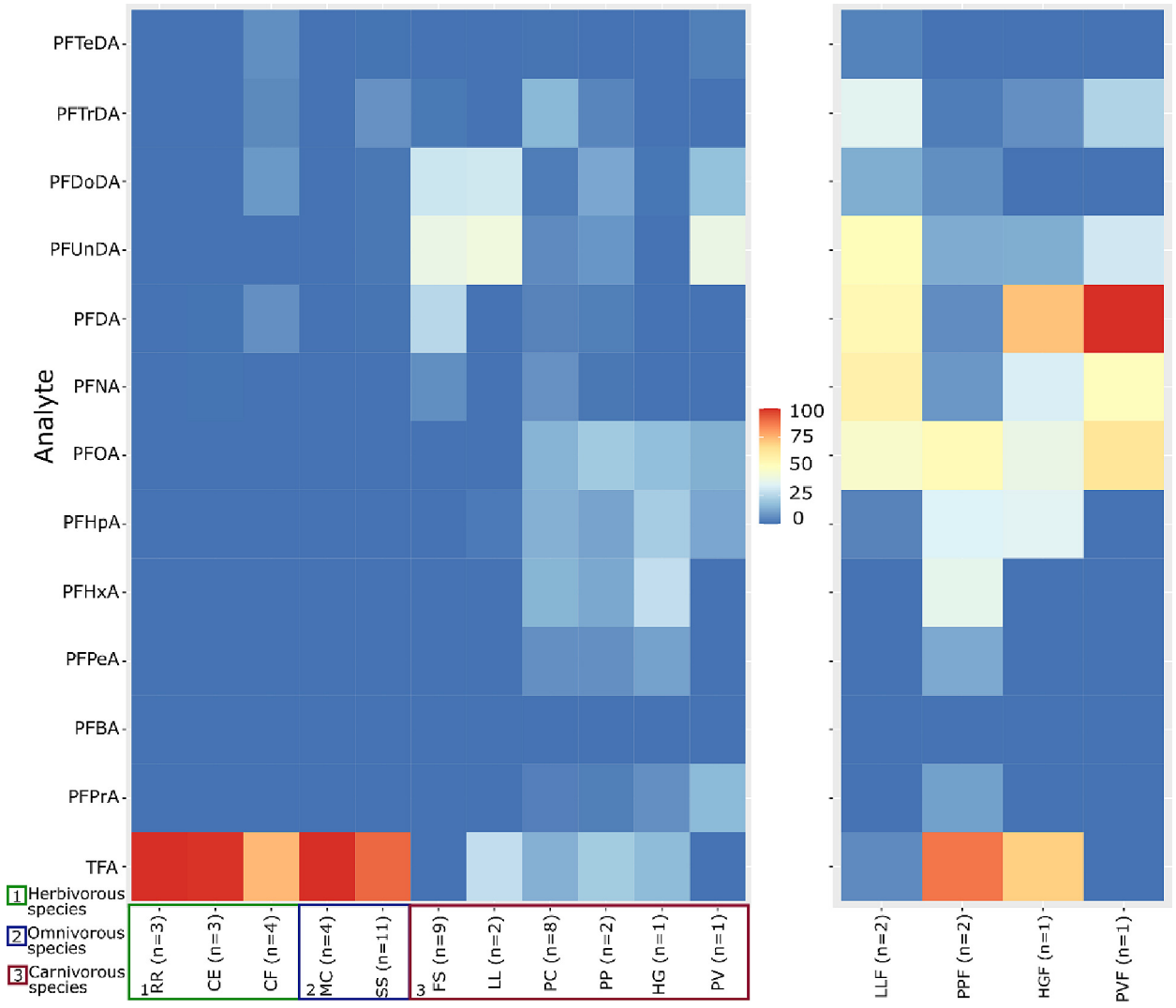

Fig. 4. Heatmap showing the pattern of PFCAs formed upon TOP assay analysis. Species with <0.01 μg/kg PFCA formation potential are excluded. n represents the number of samples analysed. Except for red deer, chamois, nutria and wildcat (each individual samples), the samples are pooled and consist of at least 3 individuals. For the cormorant, individual as well as pooled samples were analysed. Left: difference between livers (L) from different species; right: PFCA formation potential in organs other than liver (musculature (F)) for certain species. Used abbreviations: red deer (CE), roe deer (CC), chamois (RR), hare (LE), beaver (CF), nutria (MC), common eider duck (SM), wild boar (SS, from (Rupp et al., 2023)), wildcat (FS), otter (LL), cormorant (PC), harbour porpoise (PP), grey seal (HG), harbour seal (PV).

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.162361.

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