Fluorine Mass Balance and Suspect Screening in Marine Mammals from the Northern Hemisphere

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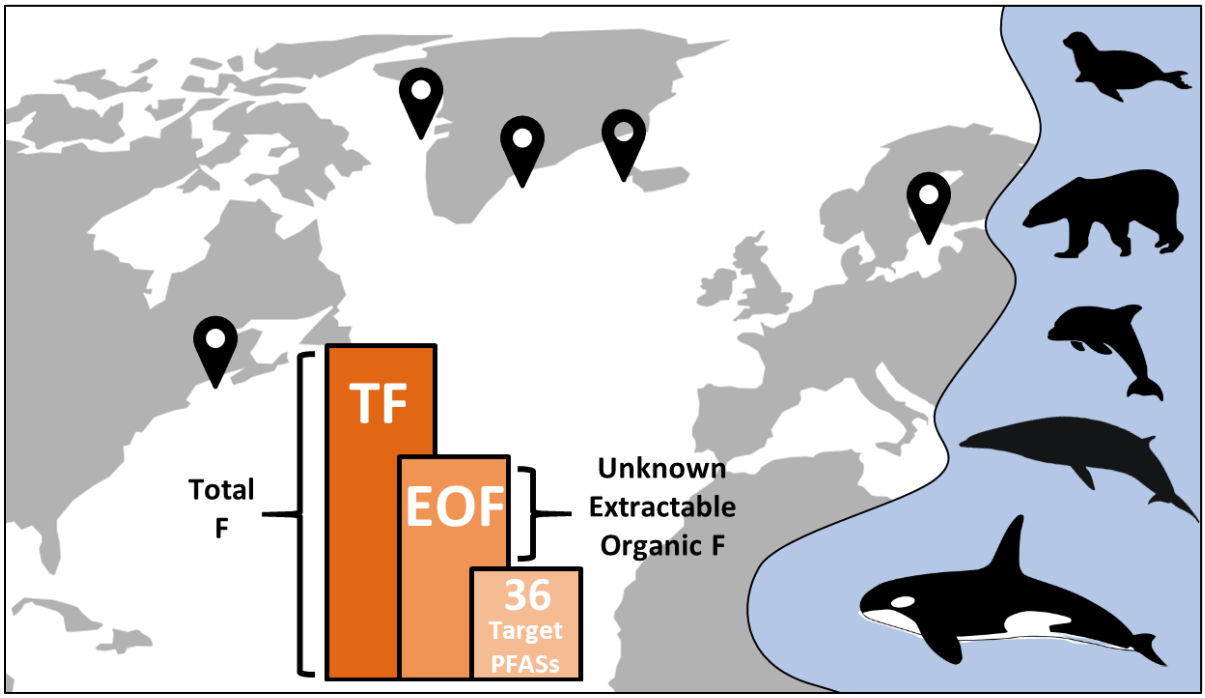
1 **TOC ART**

2 
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ABSTRACT

There is increasing evidence that the ~20 routinely monitored per- and polyfluoroalkyl substances (PFASs) account for only a fraction of extractable organofluorine (EOF) occurring in the environment. To assess whether PFAS exposure is being underestimated in marine mammals from the Northern Hemisphere, we performed a fluorine mass balance on liver tissues from 11 different species using a combination of targeted PFAS analysis, EOF and total fluorine determination, and suspect screening. Samples were obtained from the east coast United States (US), west and east coast of Greenland, Iceland, and Sweden from 2000-2017. Of the 36 target PFASs, perfluorooctane sulfonate (PFOS) dominated in all but one Icelandic and three US samples, where the 7:3 fluorotelomer carboxylic acid (7:3 FTCA) was prevalent. This is the first report of 7:3 FTCA in polar bears (~1000 ng/g, ww) and cetaceans (<6-190 ng/g, ww). In 18 out of 25 samples, EOF was not significantly greater than fluorine concentrations derived from sum target PFASs. For the remaining 7 samples (mostly from the US east coast), 30-75% of the EOF was unidentified. Suspect screening revealed an additional 33 PFASs (not included in the targeted analysis) bringing the total to 59 detected PFASs from 12 different classes. Overall, these results highlight the importance of a multi-platform approach for accurately characterizing PFAS exposure in marine mammals.

INTRODUCTION

Per- and polyfluoroalkyl substances (PFASs) are a diverse class of chemicals used throughout society.[1,2] Perfluoroalkyl chains possess a wide range of unique properties, including extreme stability and combined oil/water repellency. These attributes have led to the use of PFASs in a broad range of products, including fire-fighting foams, textiles, non-stick cookware, food wrapping paper, paints, cosmetics, in addition to many other industrial applications.[3,4] The most well-studied PFASs are the perfluoroalkyl acids (PFAAs), in particular the perfluoroalkyl carboxylic acids (PFCAs), such as perfluorooctanoic acid (PFOA), and the perfluoroalkyl sulfonic acids (PFSAs), such as perfluorooctanoic sulfonic acid (PFOS). PFSAs and PFCAs are suggested to be the final breakdown products of most PFASs.[5]

The bioaccumulation potential of PFASs is strongly correlated with perfluoroalkyl chain length; structures containing ≥8 fluorinated carbons for PFCAs and ≥6 fluorinated carbons for PFSAs are considered bioaccumulative.[6–8] PFAAs are present in the blood of humans and wildlife globally, including remote polar regions.[9–11] Unlike classical persistent organic pollutants (e.g. polychlorinated biphenyls), PFASs accumulate primarily in protein-rich tissues such as liver and blood.[12] PFASs have been linked to various toxicological effects, e.g. reproductive deficits,[13,14] immunotoxicity,[15,16] thyroid hormone disruption,[17–19] and disturbance of lipid metabolism.[20] Due to their persistent, bioaccumulative and toxic properties as their widespread distribution, PFASs have received global attention over the last few decades leading to several regulatory initiatives.[21–23] However, development and manufacturing of alternative PFASs (which are largely uncharacterized in terms of risks) remain ongoing, despite numerous examples of their environmental occurence.[24,25] and therefore hard to detect or often overseen in analyses of environmental samples and wildlife tissue samples.

Recent research by the Organization for Economic Co-operation and Development (OECD) identified 4730 CAS numbers related to PFASs.[2] However, since only a small fraction (<20) of these substances are routinely monitored, PFAS exposure may be underestimated. Indeed, the large quantities of unidentified extractable organofluorine (EOF) in environmental samples (56-100%),[26–29] cosmetics (68-100%),[30] aqueous film forming foam (AFFF; ~50%),[31] human blood (15-67%),[32] and wildlife (68-90%)[33,34] are cause for considerable concern. Moreover, recent investigations using non-target and suspect-screening analytical workflows have uncovered an unprecedented number of novel PFAS structures, some of which may account for this unidentified organofluorine.[25,35–39] However, since standards are unavailable for most of these compounds, the importance of their contribution to overall PFAS exposure remains unclear.

As top predators, marine mammals are vulnerable to persistent and bioaccumulative substances and are among the highest exposed organisms on the planet. Recent investigations in polar bear serum identified 35 additional PFASs that were not included in targeted analyses.[40] This included cyclic or unsaturated PFSAs, ether PFSAs, unsaturated ether-, cyclic ether- or carbonyl PFSAs, and x:2 chlorinated perfluoroalkyl ether sulfonates. The present study builds upon the work of Liu et al.[40] by combining suspect screening with organofluorine mass balance to comprehensively assess PFAS exposure in eleven different marine mammal species from different locations within the Northern Hemisphere (Table S1). To the best of our knowledge, this is the first time organofluorine mass balance combined with suspect screening has been conducted in marine mammals.

MATERIALS AND METHODS

Sample Collection

Marine mammal liver samples included in this study originated from five different locations within the Northern Hemisphere (Table S1). A full list of samples, including information on species (including Latin names), year, age, sex, sampling location, weight, and length are provided in Table S1. A brief overview is provided here. Species from the US Atlantic coast included grey seal, harbor seal, harbor porpoise, and pygmy sperm whale; samples were obtained between the years 2000 and 2012 from stranded animals. Samples from Sweden were collected between 2011 and 2016 from by-caught animals (seals), animals shot during domestic hunting (seals), or from stranded animals (harbor porpoise). Grey and harbor seals as well as harbor porpoise were collected from the south, while ringed seals were collected from the northern Baltic. Samples from Greenland included harp and ringed seals, harbor porpoise, white beaked dolphin, killer whale, humpback whale, minke whale (fetus), and polar bear (including a mother and cub) were collected with help from local Inuit subsistence hunters from 2000-2016. Targeted PFAS data for ringed seal (2012), polar bears (2012), and killer whales (2013) from East Greenland were previously reported in Gebbink et al.[41] but were re-analyzed in the present work. Icelandic seal samples were derived from animals that were by-caught in 2009 and 2010 and included grey, harbor and harp seal. CITES numbers for export and import permissions are provided in the supporting information (SI, Table S2 and S3). Liver tissues were shipped in individual PP-tubes on dry ice, after which they were stored at −20 °C until analysis. The present study was originally designed so that every sample would include a pool of liver tissue from multiple animals, with mixed sexes and ages. However, this was not possible for some species due to low sample availability, and therefore some samples consist of liver tissue from only one animal, while pooled samples consisted of liver tissues from 2-10 animals.

Chemicals and Reagents

Native and isotopically-labelled PFAS standards included in the targeted analysis were purchased from Wellington Labs (Guelph, Canada). Structures and abbreviations of individual PFASs are provided in Table S3. A total of 36 PFASs were targeted in the present work, including 14 perfluoroalkyl carboxylic acids (PFCAs; $C_{4\text{-}16}$, C_{18}), 8 perfluoroalkyl sulfonic acids (PFSAs; $C_{4\text{-}11}$), perfluorooctane sulfonamide (FOSA), 3 perfluoroalkane sulfonamidoacetic acids (FOSAA, MeFOSAA, EtFOSAA), 2 chlorinated polyfluorinated ether sulfonates (Cl-PFESAs; 9Cl-PF3ONS, 11Cl-PF3OUdS), ADONA, HFPO-DA, 3 fluorotelomer sulfonates (4:2, 6:2, and 8:2 FTSAs), and 3 fluorotelomer carboxylic acids (3:3, 5:3, and 7:3 FTCAs). Linear (L) and branched (br) isomers were determined separately for some substances (see Table S5). For some target analytes an analogous internal standard (IS) was lacking and these were therefore semi-quantified (see Table S5).

Overview of Fluorine Mass Balance Approach

The experimental approach for assessing fluorine mass balance is depicted in Figure S1, and was performed as follows. Three portions of tissue were removed of homogenates of a single liver or pooled sample. The first portion was fortified with an internal standard mix, extracted as described in the next paragraph, and analyzed using both UPLC-MS/MS (targeted analysis) and UPLC-Orbitrap-MS (suspect screening). The second portion was extracted using the same methods but without addition of internal standard, and the resulting extract was analyzed for EOF by combustion ion chromatography (CIC). For comparability to targeted PFAS concentrations, EOF concentrations were recovery-corrected based on the results of a spike-recovery experiment (see QC section). The third portion of tissue was combusted directly on the CIC for determination of total fluorine (TF). Approximately 25% of the samples were run in triplicate. Assuming that all liver tissues display similar instrumental variation, the highest relative standard deviation (RSD)

for each analyte was used to estimate standard deviations for all other samples (i.e. those not run as replicates).

Sample Preparation

Liver samples were stored in 13 ml polypropylene (PP) tubes at -20 ℃ prior to analysis. Sub-sampling was done using a stainless-steel knife of which the blades were pre-cleaned with methanol. For targeted analysis, approximately 0.5 g of liver homogenate was thawed at room temperature and internal standard (IS) solution was added prior to extraction using the procedure described by Powley et al.[42] (detailed description is provided in the SI). The final extract was fortified with recovery standards (RSs; $^{13}C_8$-PFOA and $^{13}C_8$-PFOS) and 500 µl of 4mM NH_4OAc (aq) and then stored at -20 ℃ until analysis. The extraction procedure for EOF analysis was the same as for target PFAS analysis, with the exception that standards and buffer were not included, and the final extracts were concentrated to ~200 µl under a stream of nitrogen. For TF analysis, 100 mg neat liver was analyzed directly, with no fortification of standards.

Instrumental Analysis and quality control

Targeted analysis

Targeted analysis was carried out on an Acquity UPLC (Waters) coupled to a triple quadrupole mass spectrometer (Xevo TQS, Waters), equipped with a BEH (Ethylene Bridged Hybrid) C_{18} column (1.7 µm, 50 × 2.1 mm, Waters), based on a previously described method.[43] The gradient program is specified in Table S5. MS source conditions are provided in the SI. Quantification was performed using MassLynx 4.1 (Waters), via a 9-point calibration curve ranging from 0.008 to 150 ng/ml (linear, 1/x weighting). Precursor and product ions are presented in Table S6. Analytes lacking an analogous labeled standard were quantified using the IS with the closest retention time

and the data quality was defined as semi-quantitative (semiQ). Branched isomers were quantified using the calibration curve of the linear isomer. Limits of quantification (LOQs) are presented in Table S6.

To determine method accuracy and precision, spike/recovery experiments were performed using homogenized seal liver. Seal liver samples (0.5 g) spiked with 10 ng native standard mix showed very good recoveries for most compounds (73-130%; Figure S2). The exceptions were PFHxDA, PFOcDA, 4:2 FTSA, and 8:2 FTSA, which showed very high recoveries (278%, 397%, 212%, and 227%, respectively), while HFPO-DA, 3:3 FTCA, 5:3 FTCA, and 7:3 FTCA showed very low recoveries (22%, 34%, 55%, and 53%, respectively). These deviating recoveries are likely due to matrix effects, which were not accounted for because of the absence of an exactly matching isotopically-labeled internal standard (see detailed discussion in the SI and Figure S2). NIST certified reference material 1957 (CRM 1957) was used for external method validation, and results were generally in good agreement with certified values (see Table S8). Finally, each batch of samples was processed together with three method blanks and control seal liver tissue (spiked and unspiked), and between every 8-10 instrumental injections a standard was included to monitor instrumental drift.

Total- and extractable organofluorine analysis

Measurements of TF and EOF were carried out using CIC (Thermo-Mitsubishi) using previously described methods.[30,44] A detailed description is provided in the SI and the IC gradient program is provided in Table S9. Quantification was performed using a standard calibration curve prepared at 0.05 to 100 µg F/ml (R^2>0.98). For EOF measurements the mean fluoride concentration in the method blanks was subtracted from all samples. For TF analysis, instrumental (boat) blank fluoride

concentrations were subtracted. The method quantification limit (LOQ) was defined as the mean concentration plus three times the standard deviation of the method blanks.

Spike/recovery experiments with NaF and PFOS over a range of concentrations revealed that inorganic fluorine was removed efficiently by the extraction procedure, as intended, even at the highest fortification level of 2000 ng F (Figure S3). In contrast, fluorine concentrations increased linearly (R^2>0.99) with increasing fortification of PFOS. A comparison of the measured concentration of PFOS using CIC to the amount fortified revealed an average recovery of 69% ± 2% (± standard deviation), which is excellent considering that no internal standard is used for this procedure. This value was used for recovery-correction of all EOF concentrations.

For comparison of sum PFAS concentrations to EOF and TF, concentrations of target PFASs were converted to their corresponding concentration in fluorine equivalents (C_{F_PFAS}) according to eqn 1**Error! Reference source not found.**:

(1) $$\boldsymbol{C_{F\_PFAS} = C_{PFAS} \cdot n_F \cdot A_F / MW_{PFAS}}$$

where C_{PFAS} is the concentration of the target compound, n_F is the number of fluorine atoms in the target compound, A_F is the atomic weight of fluorine (g/mol), and MW_{PFAS} is the molecular weight of the target compound. The sum of known extractable fluorine concentration (ΣC_{F_PFAS}) was calculated by summing the fluorine concentrations from all individual PFASs. Values <LOQ were set to 0 for calculating ΣC_{F_PFAS}. EOF concentrations (C_{F_EOF}) were corrected using the average PFOS recovery, obtained from spike/recovery experiments. Correction for analyte-specific recoveries would presumably give more accurate results, but this is impossible for unknown PFASs or PFASs lacking standards which contribute to the EOF. Another option is to extract the samples without using ISs, split the final extract and analyze this in both target and total fluorine

analysis, adding IS to the fraction for targeted analysis only.[45] Although this approach leads to inaccuracies in the targeted data (since these data would be uncorrected for procedural losses), an additional extraction for targeted analysis with ISs could be included, assuming sufficient sample availability. Overall, correcting the EOF data using PFOS recoveries is reasonable in this case given that a) PFOS is the predominant PFAS in most samples, b) PFOS recoveries are generally representative of recoveries for most perfluoroalkyl acids, and c) targeted results were not compromised using this approach.

Statistical comparisons of ΣC_{F_PFAS} and C_{F_EOF} were done with 1-tailed T-tests with unequal variances, assuming that ΣC_{F_PFAS} can only be less than or equal to the C_{F_EOF} concentrations. In cases where the C_{F_EOF} appeared to be lower than ΣC_{F_PFAS}, the fluorine balance was considered closed.

Suspect screening

Suspect screening was carried out using a Dionex Ultimate 3000 liquid chromatograph coupled to a Q Exactive HF Orbitrap (Thermo Scientific), based on a previously described method.[46] Instrumental parameters are provided in the SI. The instrument was run in negative ion, full scan (200-1200 m/z) data dependent acquisition (DDA) MS/MS mode based on an inclusion list derived from a combination of online databases (abbreviated here as EPA,[47] KemI,[48] OECD,[49] and Trier[50]), literature,[38,40,51–54] and features identified from PFAS homologue series mining (details below) during pre-screening experiments. The resolution was set to 120 000 (15 000 for MS/MS) and the automatic gain control (AGC) was set to 3e6. Other instrumental parameters are presented in Table S10. Data processing was carried out using Xcalibur 3.1 and Compound Discoverer 3.1® (Thermo Scientific). The workflow included peak retention time alignment, peak integration (using a mass

tolerance of 5 ppm, a minimum signal to noise (S/N) ratio of 30 and a minimum peak intensity of 1e6), grouping and gap-filling (peak integration at S/N = 10 for peaks detected at S/N = 30 in at least one sample). Blank subtraction was carried out by removing all peaks with areas less than 3 times the average peak area in the method blank.

A total of 17973 features remained following data pre-processing. These features were subjected to homologue series mining using the R-package 'nontarget'[55] which was used to screen exact masses for homologue series differing by -CF_2- (49.9 Da) and -C_2F_4- (99.9 Da) fragments, which are characteristic for PFASs. Each homologue series was then checked manually in the extracted ion chromatogram (EIC) for good peak shapes and an increasing retention time with mass-to-charge. At this point, in-source fragments were removed by comparing retention times, exact mass, and MS/MS spectra (if available). The resulting list of exact masses and their MS/MS spectra were annotated through comparison to databases and/or literature. In one case, MS/MS spectra were predicted using the *in silico* fragmentation predictor MetFrag.[56] Confidence levels (CLs) were assigned according to Schymanski et al.[57] (see SI for details).

RESULTS AND DISCUSSION

Overview of PFAS concentrations in marine mammals

Of the 36 target PFASs analyzed in the present work, 20 were quantifiable in one or more samples: PFHpA, PFOA (L), PFNA, PFDA, PFUnDA, PFDoDA, PFTrDA, PFTeDA, PFPeDA, PFHxDA, PFBS, PFHxS (L+Br), PFHpS, PFOS (L+Br), PFDS (L+Br), FOSA (L+Br), 9Cl-PF3OUdS, 5:3 FTCA, 7:3 FTCA, and 6:2 FTSA. Peaks were also observable for FOSAA (L), MeFOSAA (L), EtFOSAA (L), and 11Cl-PF3OUdS, but concentrations were always <LOQ. PFBA, PFPeA, PFHxA, PFOA (Br), PFOcDA, PFPeS, PFNS, PFUnDS, FOSAA (Br), MeFOSAA (Br),

EtFOSAA (Br), ADONA, HFPO-DA, 3:3 FTCA, 4:2 FTSA, and 8:2 FTSA were all below quantification limits in all samples. Both concentrations and PFAS profiles varied widely among species, sampling location, and sampling year (Figure 1). The highest sum PFAS concentrations (i.e. Σ_{36}PFAS) among all species were observed in polar bears (3600-4000 ng/g), which were an order of magnitude higher than most other marine mammals (Figure 1). As apex predators, polar bears are among the most chemically contaminated species on the planet.[58] The three most predominant compounds in polar bears were PFOS, 7:3 FTCA and PFNA, which made up 45-51%, 23-28% and 9-13% of the Σ_{36}PFAS, respectively. 7:3 FTCA has not been reported in polar bears before and it is therefore particularly surprising that this compound makes up such a large fraction of the total PFAS concentration. Σ_{36}PFAS profiles were very similar between all polar bears, Σ_{36}PFAS concentrations were only slightly higher for the female polar bear compared to her cub, which is concerning due to health risks associated with chemical exposure at this early developmental stage.

In cetacean liver samples, the highest Σ_{36}PFAS concentrations were observed in killer whales from East Greenland (614 ± 49 ng/g, ww), while in seals the highest Σ_{36}PFAS concentrations were detected in harbor seals (640 ± 51 ng/g, ww) and ringed seals (536 ± 43 ng/g, ww) from Sweden. PFOS dominated the Σ_{36}PFAS fraction in samples from all locations, except for samples from the US Atlantic coast, where 7:3 FTCA was dominant. For harbor seal and harbor porpoise from the US Atlantic coast, 7:3 FTCA accounted for up to 64 and 71% of Σ_{36}PFAS concentrations, respectively, which may indicate that these animals were located in closer proximity to the source(s) of 7:3 FTCA. Seals from Iceland contained low Σ_{36}PFAS levels compared to the other samples, i.e. 23, 43, and 67 ng/g for grey seal, harp seal, and harbor seal, respectively.

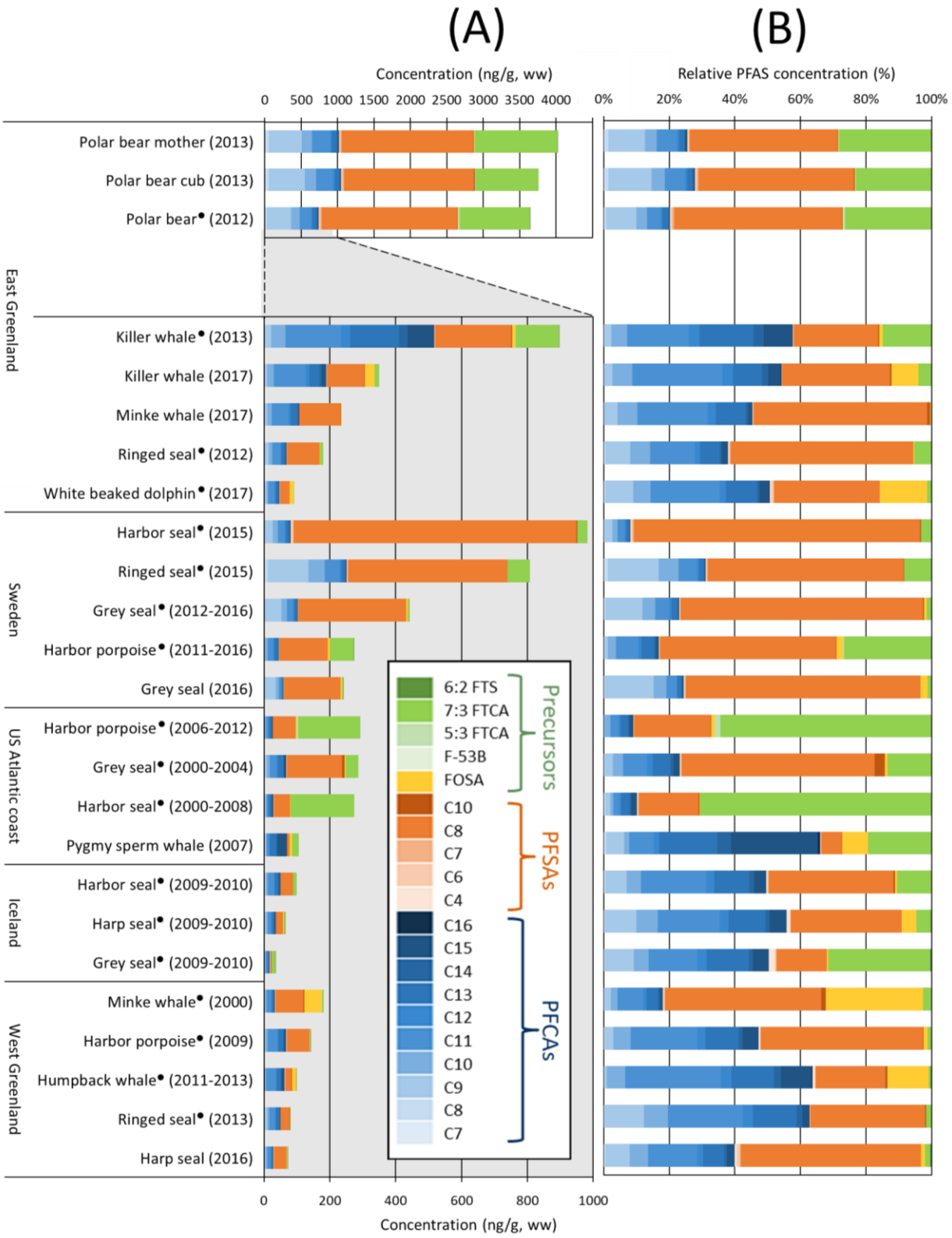

Figure 1. (A) Sum of targeted PFASs (note the separate concentration axis for polar bears) and (B) normalized concentrations for marine mammals sorted according to their sampling location. • = pooled samples (*n*=2-10). Detailed sample information is available in Table S2.

Inter-species and geographical differences in PFCA distribution

The distribution of PFCA homologues is shown in Figure 2. Among all samples, a characteristic odd/even chain length pattern was observed, wherein the concentration of a given odd chain-length PFCA in most cases exceeds the concentration of its adjacent even chain-length homologues (i.e. PFNA exceeds PFOA and PFDA, PFUnDA exceeds PFDA and PFDoDA, etc). This pattern has been widely reported in the literature,[41,59–61] and is suggested to occur due to atmospheric oxidation of fluorotelomer alcohols (FTOHs) to corresponding even- and odd-chain length PFCAs, followed by preferential bioaccumulation of the odd (i.e. longer) chain-length homologue.[62] Despite this consistent pattern, the overall distribution of PFCA homologues was remarkably different among species. Species-specific metabolism may explain these differences.[63] For example, the dominant PFCA in polar bears from East Greenland was PFNA (C9), while PFUnDA (C11) was dominant in cetaceans (except for the pygmy sperm whale) from Greenland, the US, and Sweden. In comparison, the dominant PFCA in pygmy sperm whale was PFPeDA (C15; 28.0 ng/g, ww). The unique profile in pygmy sperm (n=1) whale was not explainable by differences in sampling year amongst cetaceans. While C15 has not been quantified in pygmy sperm whales before, long-chain PFCAs (specifically PFTrDA (C13)) were previously reported to make up a large fraction of the total PFAS concentration in pygmy sperm whales.[64,65] Diet may partly explain this unique pattern, since pygmy sperm whales were one of the few species investigated here (in addition to white-beaked dolphin) that feed offshore on small fish, squid, octopus, and other invertebrates.[66] However, we cannot be sure that the pattern is representative for the species, since the liver of only one pygmy sperm whale was analyzed. For seals, the PFCA distribution varied among sampling locations, suggesting geographical differences in exposure source (Figure 2). In seals from Sweden (both Baltic Sea and west-coast Skagerrak/Kattegat straits) the most prevalent PFCA homologue

was PFNA (C9), whereas for seals from the Atlantic Ocean (i.e. US, Greenland, Iceland), PFUnDA (C11) represented the highest fraction. These differences (which were not explainable by differences in sampling year), point to a common source of exposure in seals from the US, Greenland, and Iceland that is unique relative to that of the Baltic Sea and Skagerrak/Kattegat straits.

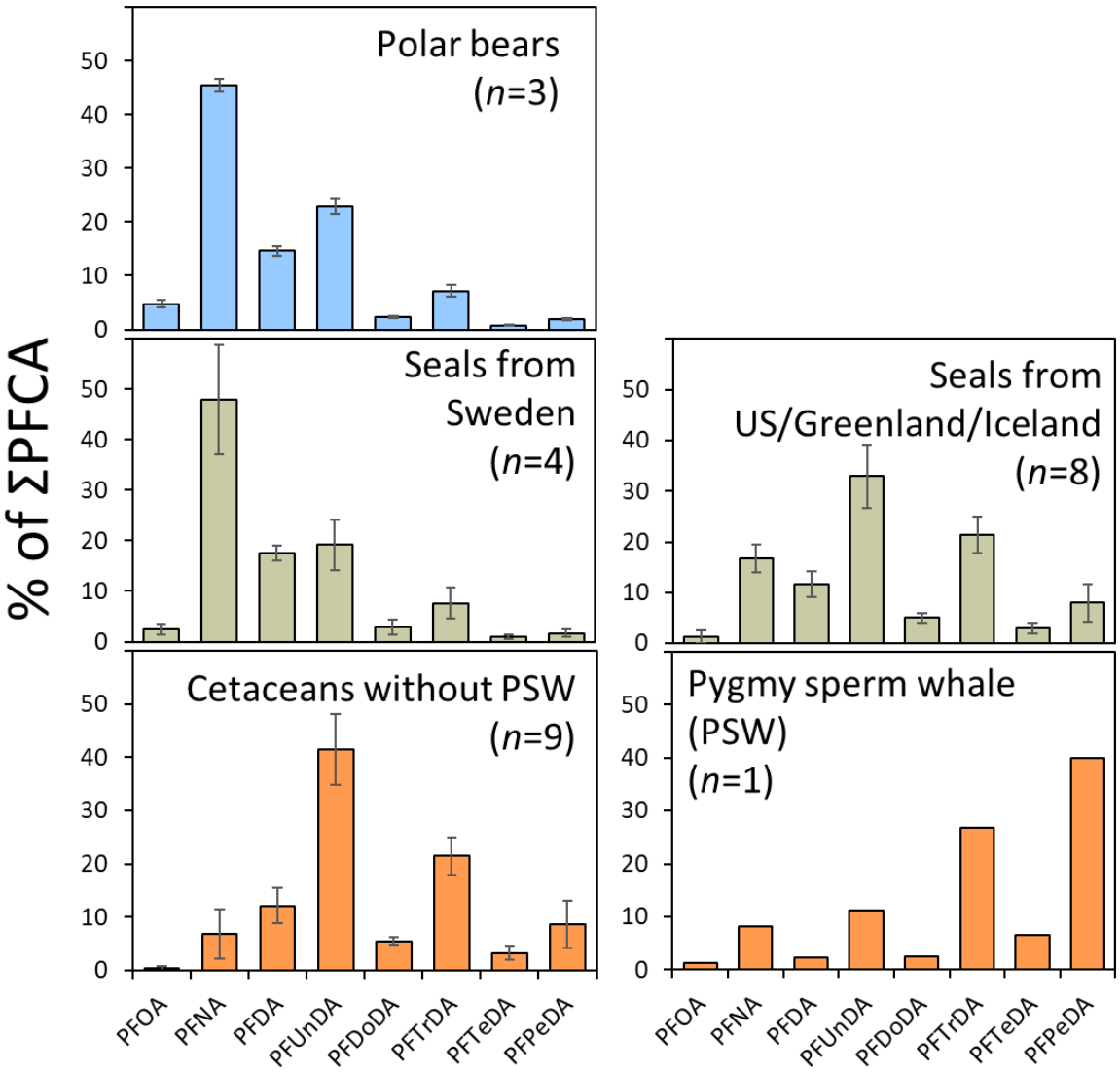

Figure 2. Average percent contribution of PFCAs (C8-C15) to ΣPFCA concentrations (error bars represent standard deviation) in polar bears, seals (grouped by locations with similar patterns), and cetaceans (Pygmy sperm whale and other cetaceans from Sweden/US/Greenland).

Inter-species differences in FOSA concentrations

FOSA:PFOS ratios were generally much higher for cetaceans (0.01-1.28; average 0.33), compared to other marine mammals (0-0.14; average 0.02). The exception was for harbor porpoises, which contained consistently lower FOSA:PFOS ratios (0.02-0.04; average 0.03). Previous studies have observed similar results, with Galatius et al.[67] hypothesizing that smaller cetacean species (i.e. harbor porpoises) might have a higher capacity for transformation. FOSA is the most commonly observed PFOS precursor in wildlife. While FOSA usually occurs at lower concentrations than PFOS, a review of the current literature (see Figure S4) revealed that FOSA:PFOS ratios are higher in cetaceans (0.2-1.0) compared to other marine mammals (ratio <0.005; Figure S4).[68–71] This unique pattern is attributed to a phylogenetic difference in the ability of cetacean species to transform FOSA to PFOS.[67]

Elevated concentrations of 7:3 FTCA

The 7:3 FTCA was the second most prevalent PFAS (next to PFOS), and is reported here for the first time in cetaceans and polar bears. FTCAs are not used in consumer products or industrial applications,[72] but may form from biodegradation of fluorotelomer alcohols.[73] 7:3 FTCA has been observed previously in biological samples such as birds (16.2 ng/g, ww in water birds and 0.01-0.84 ng/g, dw in eagle-owl feathers),[74,75] fish (0.07-0.21 ng/g, ww),[75] human whole blood (from technicians working with ski wax; 3.9 ng/ml)[76] and breast milk (<42 pg/ml),[43] and seals (0.5-2.5 ng/g, ww).[77] However, concentrations are typically much lower than those observed in the present study (e.g. polar bear mother: 1131 ng/g, ww and harbor seal: 192 ng/g, ww). Suspect screening also revealed the presence of other X:3 FTCA homologues (see section on non-targeted and

299 suspect screening). The origin of FTCAs in marine mammals remains unclear and requires further
300 investigation.

301 **Fluorine mass balance**

302 An overview of the fluorine mass balance including the sum target PFAS (ΣC_{F_PFAS}), EOF
303 (C_{F_EOF}), and TF (C_{F_TF}) concentrations is presented in Figure 3. A total of seven out of 25 samples
304 displayed significantly (i.e. $p<0.05$ or $p<0.1$) higher C_{F_EOF} compared to ΣC_{F_PFAS} concentrations
305 (Figure 3A). This included the pooled polar bear sample from East Greenland from 2012 (32%
306 unidentified EOF); pooled East Greenland killer whale from 2013 (35% unidentified EOF); pooled
307 ringed seal from Sweden from 2015 (45% unidentified EOF); and finally, the pooled harbor
308 porpoise, pooled grey seal, pooled harbor seal, and the pygmy sperm whale (all sampled 2000-
309 2012) from the US Atlantic coast (30-75% unidentified EOF). These results show that exposure
310 of these species to organofluorine is indeed underestimated in some cases. Animals sampled from
311 the US Atlantic coast contained the largest fraction of unidentified EOF, which may indicate that
312 these animals are closer to the source(s) of unidentified organofluorine. Notable, however, is the
313 fact that the US samples also tended to be older than those sampled at other sites. C_{F_EOF} and
314 ΣC_{F_PFAS} concentrations were not significantly different in 9 of the samples, indicating a closed
315 EOF mass balance. Another 9 samples displayed slightly lower C_{F_EOF} than their respective
316 ΣC_{F_PFAS} concentrations, likely caused by under-reporting of C_{F_EOF} due to recovery-correction
317 using PFOS (see methods section). While we considered the EOF mass balance to be closed for
318 these samples, the source of this under-reporting requires further investigation. TF concentrations
319 were consistently higher than EOF and target PFASs for all samples, which may be attributed to
320 the presence of inorganic and/or- non-extractable organic fluorine in the tissues. Overall,
321 percentage of unknown TF ranged from 10-93% (average 58%).

Sum target PFAS concentrations, EOF and TF were natural log (ln)-linearly correlated with one another (Figure 4; $p<0.001$; R^2 0.58-0.77), which can be expected since the organofluorine mass balance was closed or nearly closed in most samples. The unidentified fraction of the EOF could consist of novel PFASs, metabolites and/or transformation products of PFASs. Fluorinated pharmaceuticals and/or pesticides may also accumulate in marine mammals,[78] but given their low percentage of fluorine (i.e. these substances typically only contain a few fluorine atoms), they are not expected to make a significant contribution to EOF or TF concentrations unless they are present in very high abundance. Trifluoroacetic acid (TFA) was also considered since it occurs naturally in sea water at high concentrations (up to 17-190 ng/L in the Northern Atlantic[79]) and is ubiquitous throughout the entire aquatic environment.[79] However, this was ultimately ruled out since TFA is non-bioaccumulative and therefore not expected to occur in marine mammals.[80]

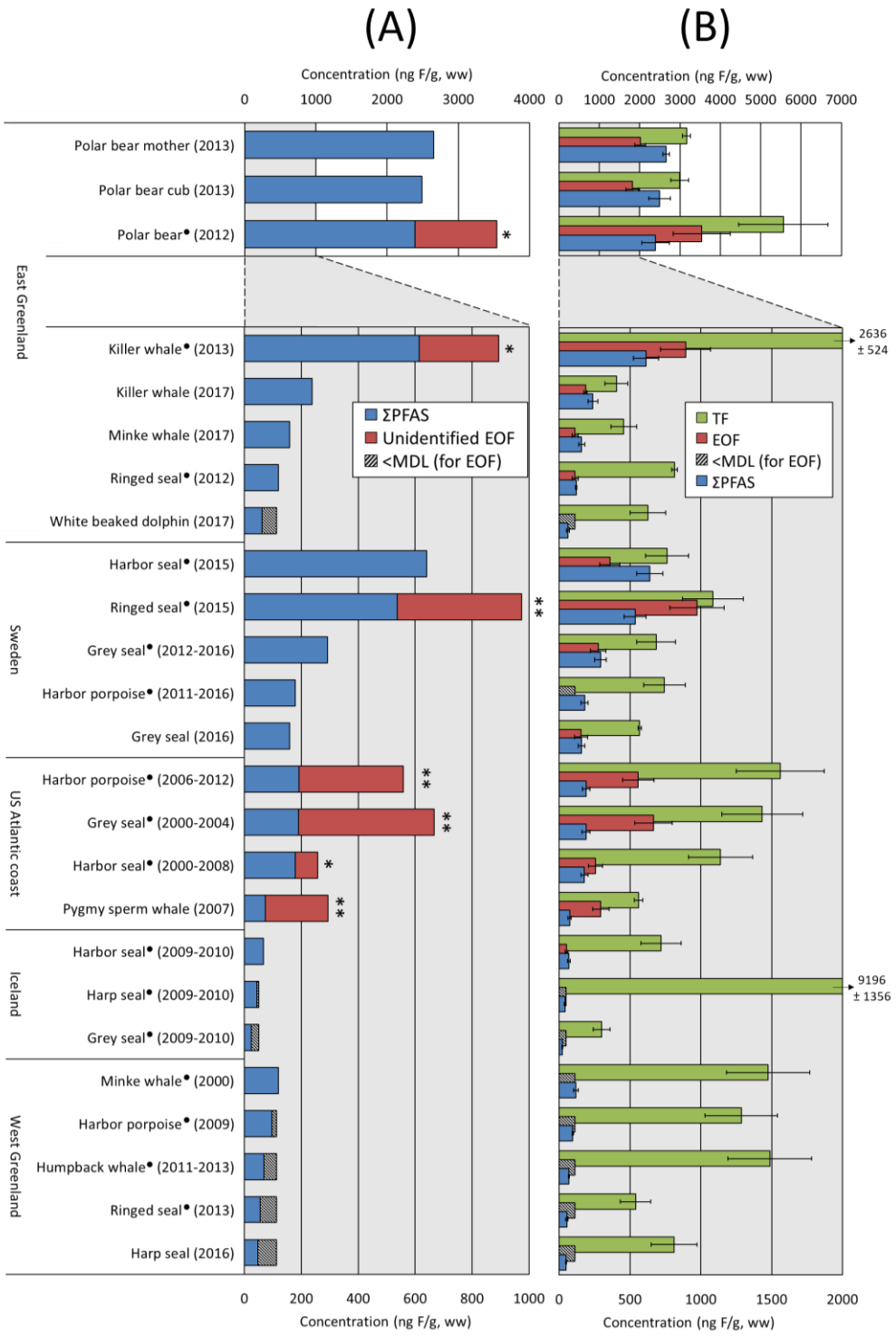

Figure 3. (A) Sum target PFAS and unidentified extractable organofluorine (EOF) concentrations in ng F/g, ww. Significantly higher EOF concentrations are denoted by asterisks (*p<0.1; **p<0.05, 1-sided T-test, unequal variance). (B) Concentrations of target PFASs, EOF, and total fluorine (TF) in ng F/g, ww. Error bars indicate the standard deviation. Note the separate concentration axis for polar bears. • = pooled samples (*n*=2-10). Detailed sample information is available in Table S2.

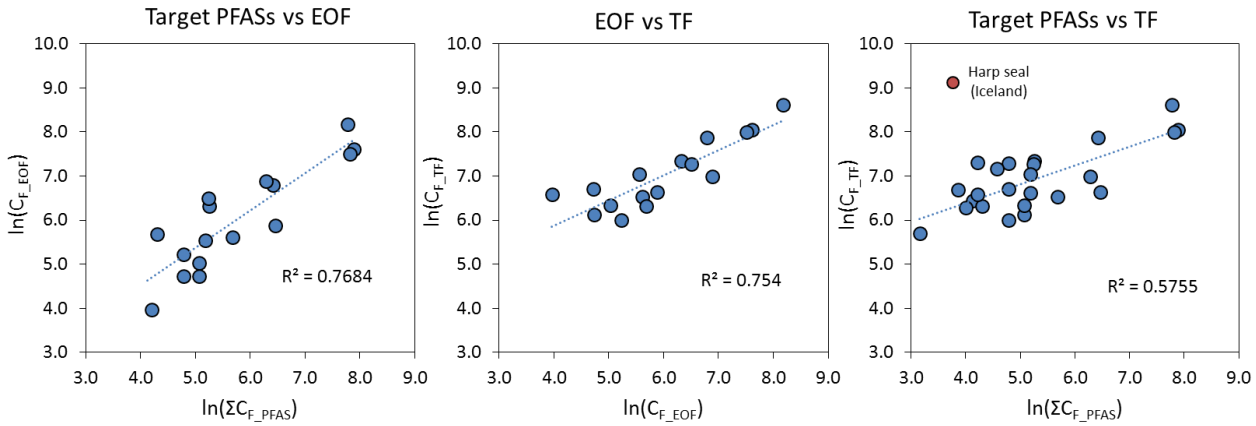

Figure 4. Natural log (ln)-linear correlations between sum target PFAS, EOF and TF concentrations. Data <LOQ were excluded. P-values were < 0.001 in all cases.

PFAS suspect screening

Figure 5 summarizes the PFASs that were identified via suspect screening along with the relative abundance of each suspect in individual samples. Classes 1-7 (PFCAs, PFSAs, FTCAs, FTSAs, FASAs, FASAAs, an Cl-PFESAs) were present in our target list, but additional homologues from some of these classes were identified through homologue series mining. Classes 8-11 (PFECAs, d/c PFSAs, ether PFSAs, and enol-ether/cyclic ether or carbonyl PFSAs) were identified by matching exact masses (and MS/MS fragments when available) to those in literature. Finally, class 12 was flagged through homologue series mining; thereafter we attempted structural elucidation through database matching and comparison of MS/MS spectra to *in silco* fragmentation predictions.

Among the FTCAs, 5 additional homologues were detected that were not present in our target list (i.e. 6:3 and 8:3 – 11:3 FTCAs; < 2 ppm mass error). These substances displayed a similar fragmentation pattern to target FTCAs; thus a high degree of confidence (CL=2a) is ascribed to

their assignment, despite an absence of standards (Figure S5). 5:3, 6:3, and 7:3 FTCAs showed highest abundancies in polar bears, while 8:3-11:3 FTCAs showed highest abundancy in harbor porpoise and ringed seals from the US, when comparing peak areas to other samples. All three samples from the US contained significant quantities of unidentified EOF. We posit that quantification of the full suite of FTCA homologues may account for a large portion of the missing EOF in these samples.

10:2 and 12:2 FTSAs (class 4) and C4-C7 FASAs (class 5) were not included in our target list and were identified through a combination of homologue series mining and by comparing their MS/MS fragments to those homologues for which standards were available (i.e. 6:2 and 8:2 FTSAs, and FOSA; see Figures S6-7). Notably, the peak area of 10:2 FTSA was elevated in all polar bear samples and the US harbor seal sample compared to other samples, suggesting that this target may contribute to the missing EOF observed in these samples. Among FASA homologues, perfluorobutane sulfonamide (FBSA) is particularly notable as this substance is a degradation product of a wide range of substances derived from perfluorobutanesulfonyl fluoride, which replaced PFOS-precursors in the early 2000s.[81] FBSA was present mainly in cetaceans and in all animals from Sweden. FBSA has previously been reported in several fish species in Canada and The Netherlands[82] and one study even reported FBSA in polar bear liver at concentrations of 0.4 ng/g ww.[83]

Perfluoroalkyl ether carboxylates (PFECAs; class 8, C8-11) were identified by matching the exact mass of multiple homologues to those reported previously in water,[84,85] and particulate matter.[52] While C3-C8[84] and C10-C15[52] PFECAs have been reported previously, to the best of our knowledge this is the first report of C9 PFECA homologue in the environment. Similarly, a homologue series of double bond or cyclic PFSAs (d/c PFSAs; class 9, C8-C10) were identified

by first matching the parent mass and MS/MS spectrum for perfluoroethylcyclohexanesulfonate (PFECHS; C8; Figure S10) to those reported previously in polar bear serum.[40] Notably, PFECHS was prevalent in both ringed seals and harbor seals from Sweden relative to other samples, the former of which was found to have a significant quantity of missing EOF.

MS/MS data was not available for either C6-C9 ether-PFSAs (class 10) and C7-C9 enol-ether/cyclic-ether/carbonyl PFSAs (class 11) due to low peak intensities. Therefore, tentative identification (i.e. CL=3-4) was carried out by matching the exact mass of the precursor ions to those reported previously in polar bear serum.[40] For class 11, peaks for the C10 homologue eluted both at retention time 5.03 and 5.55 suggesting a mixture of structures (e.g. both an enol ethers and a cyclic ether).

Finally, one of the compounds of the "unknown" class (class 12; $C_nF_{2n+1}H_{10}$-C_5SO_4N) was originally matched with a methyl ester structure listed in both the OECD and KemI lists (CAS# 87988-69-0; mass error = 0.456 ppm). However, methyl esters are generally non-detectable by ESI-MS so this structure was ruled out.[86] Alternatively, this substance may be an isomer or in-source fragment of a neutral compound. This feature displayed the highest peak areas in the harbor porpoise and pygmy sperm whale from the US (which had a large fraction of unidentified EOF). Ultimately, confirming the identity of this substance and quantifying it is necessary to assess how much it contributed to the unidentified EOF fraction.

Overall, an additional 33 PFASs were identified through our suspect screening workflow, which were not included in the targeted analysis, bringing the total number of substances detected at a CL of 1-4 to 59 substances from 12 different PFAS classes (not including isomers). We note that the highest peak areas for suspects were not always in samples containing significant unknown

EOF. This should not be surprising, considering that EOF measurements are based on fluorine equivalents, rather than molecular weight-based concentrations, and because the contribution to EOF from a few dominant substances (e.g. PFOS) may dwarf that of some important novel PFAS. Thus, while EOF remains an important tool for prioritizing samples for closer scrutiny; suspect screening (and ultimately quantification) of novel PFASs is clearly needed to obtain a complete picture of PFAS exposure in wildlife.

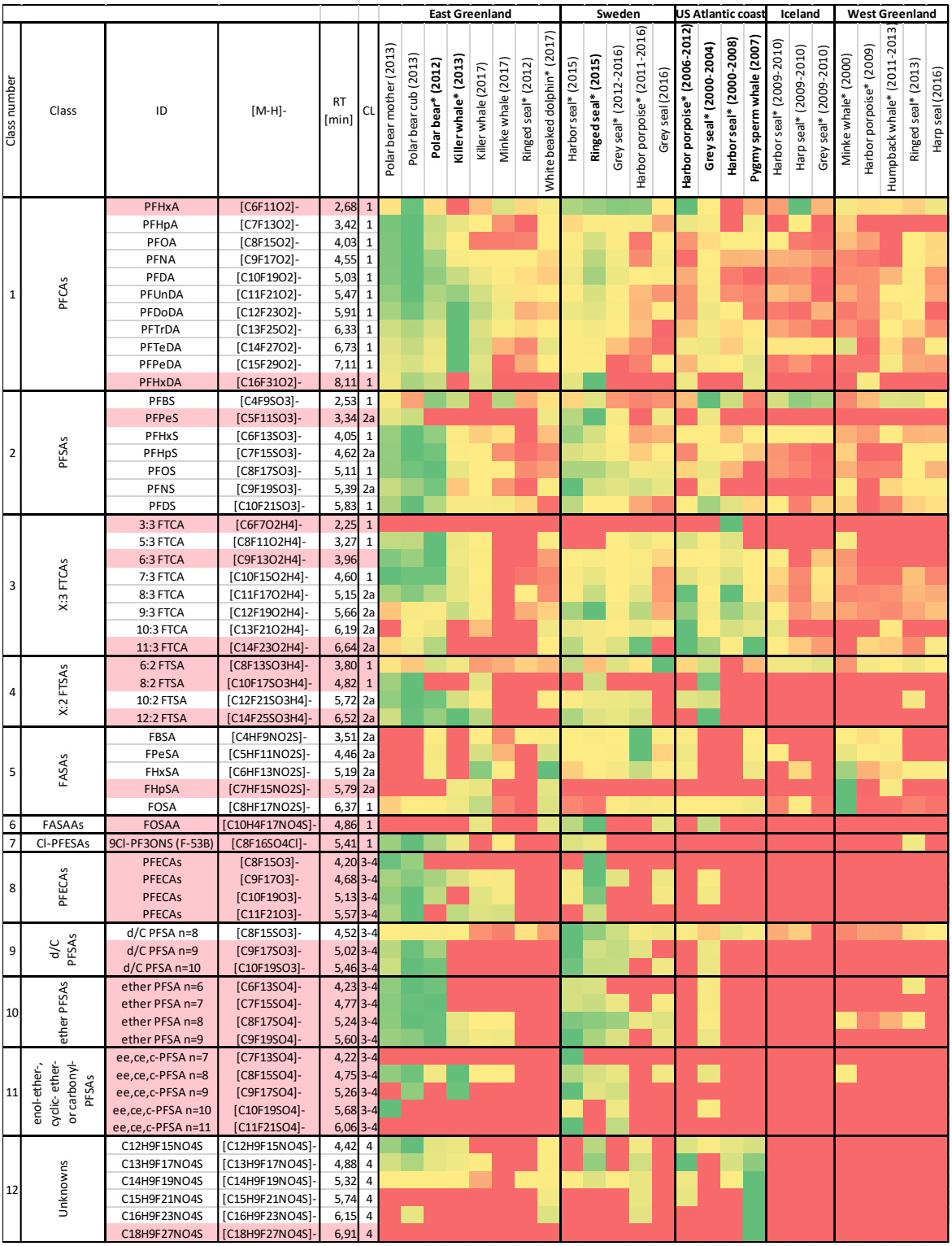

Figure 5. Heatmap showing relative abundance of PFASs identified by suspect screening across all samples. Data are normalized row-wise based on the maximum response observed across all samples for a given substance. Green indicates high abundance, red indicates low abundance. Pink shading indicates suspects identified by manual inspection of the data. Bold font indicates samples where a significant gap in EOF was identified.

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SUPPORTING INFORMATION

Further information on chemicals and reagents, sample preparation, instrumental analysis, along with results of spike/recovery experiments (targeted and CIC analysis), literature review of FOSA:PFOS ratios, EICs for suspects, detailed sampling information, CITES permits, target PFASs, LC mobile phase gradient, MS and RTs for target PFASs, LOQs, NIST results, eluent programs for CIC analysis, HRMS parameters.

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