Quantification of target per- and polyfluoroalkyl substances (PFASs) and extractable organic fluorine (EOF) in crayfish from Lake Vättern, Sweden.

Independent Project for the Degree of Bachelor in Chemistry, HT19, KE015G

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Abstract

Per- and polyfluorinated substances (PFASs) are a family of persistent organic pollutants in which the aliphatic carbon chains have been fluorinated, either completely or partially. One subclass of PFASs are perfluoroalkyl acids (PFAAs), in which perfluoroalkyl carboxylic acids (PFCAs) and perfluoroalkyl sulfonic acids (PFSAs) are two prominent groups. PFASs are toxic to the environment, as well as humans, and are found to have a developmental, neurological and immunological toxicity. They are also found to be possibly carcinogenic. In this study, the quantity of target PFASs was determined in 11 samples of hepatopancreas in crayfish from different locations in Lake Vättern, Sweden, using liquid chromatography coupled to tandem mass spectrometry. The hepatopancreas is the crayfish detoxification organ and therefore, it is also where persistent organic pollutants are possibly accumulated. Extractable organic fluorine concentration was also determined, using combustion ion chromatography to account for other fluorinated compounds not included in the target analysis of 31 PFASs. The amount of target PFASs was compared to the extractable organic fluorine concentration, giving an idea of how much of the extractable organic fluorine that consists of identified PFASs in relation to unidentified organic fluorine.

The measured concentrations of target PFASs were compared to measured concentrations in a previous study (2008) of PFASs in the hepatopancreas of crayfish from two different locations in Lake Vättern. Concentrations of the measured target PFASs were also related to if the sampling sites for each pooled sample was located close to a known point source, Karlsborg military airport. A sum of the average of PFHxS, PFOS, PFOA and PFNA for all sample sites were compared to a combined tolerable weekly intake (TWI) for these four compounds, established by the European Food Safety Authority (EFSA). As well as the intake of PFOS and PFOA compared individually against their TWI.

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1. Introduction

1.1 Background

1.1.1 Per- and polyfluoroalkyl substances, PFASs

Per- and polyfluoroalkyl substances, PFASs, are a family of anthropogenic chemicals with certain beneficial properties, resulting in them having many application areas for industrial and consumer purposes. The properties that make PFASs desirable for consumer purposes are their hydrophobic and oleophobic traits, which are useful for preventing e.g. stains. PFASs also have surface-tension lowering properties [1]. These compounds have a high stability, both chemically and biologically, due to the strong carbon-fluorine chemical bonds [2]. Application areas of PFASs are diverse and they can be found as one of the main components in aqueous film forming foams (AFFF). The AFFF are frequently used for training activities involving fire-fighting at sites such as military bases and airports [1]. Other application areas include textile, leather, apparel, oil production, pesticides etc. [3].

Aliphatic carbon chains that are completely fluorinated are termed perfluoroalkyl substances, whilst polyfluoroalkyl substances are the term for the substances that have an incomplete replacement of hydrogen atoms by fluorine [1]. There are different compound subclasses of PFASs, such as perfluoroalkyl acids (PFAAs) which includes perfluoroalkyl carboxylic acids (PFCAs) and perfluoroalkyl sulfonic acids (PFSAs) [3]. Long-chain PFCAs are defined by the carbon chain having eight or more perfluorinated carbons (i.e. ≤ 7 perfluorinated carbons). Long-chain PFSAs are defined by the carbon chain having six or more carbons that are perfluorinated (i.e. ≤ 6 perfluorinated carbons) [4]. Precursors of these compounds are also acknowledged in the long-chain PFASs category. Two of the most renowned long-chain PFASs are perfluorooctanoic acid (PFOA) and perfluorooctanesulfonate (PFOS) [3]. Studies have shown that long-chain PFSAs and PFCAs are highly environmentally persistent and bioaccumulative, especially the compounds with chain lengths longer than eight perfluorocarbons [2]. The general rule for bioaccumulation of PFASs is that it increases with the length of the perfluoroalkyl chain, as well as with the trophic level of the organism [5].

Precursors of PFASs are compounds that, when degraded, transforms into another PFAS, e.g. in soil and sediment the biodegradation of 6:2 fluorotelomer alcohol (6:2 FTOH) results in short-chain PFCAs (C_5, C_6, C_4) [6]. Fluorotelomer sulfonic acids (FTSAs) are also a class of precursors for PFCAs and they are intermediates in the transformation of e.g. fluorotelomer mercaptoalkylamid sulfonates (FTSASs). These FTSASs can be found in new AFFFs. There are other fluorotelomer intermediates, such as saturated and unsaturated fluorotelomer carboxylic acids (FTCAs and FTUCAs respectively). These intermediates are a part of a common degradation pathway, which is a mutual pathway for several PFASs. The FTCAs/FTUCAs have no known consumer or industrial purposes, so the presence of these compounds can be seen as an indication of an active degradation of fluorotelomer-based precursors [7].

1.1.2 Health effects of PFASs

There are multiple ways that humans are exposed to PFASs, these exposure pathways include consumption of contaminated water and seafood (and other types of food), as well as exposure by inhalation of contaminated indoor air [1]. The exposure of PFASs to humans by seafood is the main focus in this project. According to the European Food Safety Authority (EFSA), 86% of the dietary exposure of PFASs comes from seafood [8]. Concentrations of PFASs in seafood are higher at locations that are closer to the site of contamination. A probable correlation between PFOA exposure and the development of high cholesterol, thyroid disease, pregnancy induced hypertension, ulcerative colitis, as well as kidney and testicular cancer has been established [1]. PFOA is classified as "possibly carcinogenic to humans" by the International Agency for Research on Cancer (IARC). Research has also shown that there is a statistically significant association between PFAS exposure and suppression in the response of antibodies to vaccinations in that of children, adolescents and adults [1]. Many of the PFASs are found to be toxic to both the environment and humans, with adverse health effects for humans including developmental toxicity, neurotoxicity, immunotoxicity, as well as the possible carcinogenicity [7].

1.1.3 Tolerable weekly intake (TWI) and regulations

The European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain (CONTAM) estimated a human half-life of approximately 5 years for PFOS and 2-4 years for PFOA. A tolerable weekly intake (TWI) for PFOS has been established to 13 ng/kg body weight (bw) per week, whilst the TWI for PFOA is 6 ng/kg bw per week [8]. A combined tolerable weekly intake (TWI) for PFHxS, PFOS, PFOA and PFNA has been established to 8 ng/kg body weight (bw) per week [9]. PFOS and PFOA are restricted under Annex A of the Stockholm Convention on persistent organic pollutants (POPs). This convention states that the production and use of PFOS, PFOA and their precursors is to be eliminated [10]. PFOS is also restricted under an EU regulation, EU POPs Regulation (EU, 2019). PFOA, as well as its precursors, are also regulated on an EU level under the regulation (EU, 2006) called Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). The REACH list of Substances of Very High Concern (SVHCs) has multiple other PFASs listed on it [11]. The restrictions are few when looking at the total number of listed PFASs, which is 4730 PFASs according to the Organization for Economic Co-operation and Development, OECD [12].

1.1.4 Crayfish

Crayfish are a type of seasonal seafood caught (in streams and lakes) and eaten in Sweden, as well as other Nordic countries, during late summer. They are omnivores that eat, amongst other things, insect larvae, clams, shells, hard roe, fish and cadaver. The parts of the crayfish that is most commonly consumed is the large tail muscle, a special digestive gland called the hepatopancreas and hard roe (when present). Fat composition is higher in the hepatopancreas than in that of the tail muscle tissue. Hepatopancreas is an organ that functions as both a pancreatic gland and liver in crayfish, which entails that the hepatopancreas' role is that of a

detoxification organ. Because of the detoxification function of the hepatopancreas, persistent organic pollutants may accumulate in this organ. The hard roe has a higher fat composition than that of the tail muscles. In caught crayfish, hard roe is not always present, so this tissue would not be ideal for establishing pollutant concentrations [13].

1.1.5 Lake Vättern

Lake Vättern is the main source of drinking water to that of approximately 280 000 people and is also Sweden's second largest freshwater lake. Since the inflow of water is low in relation to the total volume of water in the lake, these circumstances result in an average residence time for water of approximately 60 years. Aquatic environments can be exposed to PFASs, as well their precursors, through point sources e.g. sites where AFFF has been used for fire-fighting activities. A military airport located in Karlsborg near Lake Vättern has used AFFF for fire-fighting drills at sites in the area. At one of the sites, leakage into the aquatic environment occurred which was confirmed through measurements of surface water in connection to the site [14].

1.1.6 Extractable organic fluorine (EOF)

Measuring extractable organic fluorine (EOF) is a mass-balance analysis that gives an indication of how much of the extractable organic fluorine that consists of target PFASs (known measured compounds). It also gives an indication of the amount of unknown/unquantifiable organic fluorine present [15]. Organic fluorine, OF, refers to the compounds that have one or more carbon-fluorine bonds, there is also inorganic fluorine which includes fluoride ions, salt and metal complexes. For organic fluorine analysis there are two fractions, the extractable organic fluorine fraction and the unquantifiable organic fluorine fraction. The latter fraction consists of an unidentified organic fluorine part and an organic fluorine part that is identified, but not quantified [16].

1.1.7 Previous findings of PFASs in crayfish

A previous study of PFASs in crayfish from Motala and Aspa bruk, Lake Vättern showed a sum value for six PFCAs to be around 22 μg/kg in Motala and 24 μg/kg in Aspa Bruk. The sum value for three PFSAs was found to be 10 μg/kg in Motala, 16 μg/kg in Aspa Bruk and the value for perfluorooctancesulfonamide (PFOSA) was found to be less than 0.10 μg/kg at both locations in Lake Vättern. In total, there were 13 locations and between 6-20 individual crayfish per location included in this study from 2008 [13].

1.2 Aim

The aim of this project is to quantify target PFASs in crayfish from Lake Vättern, as well as measure the extractable organic fluorine. Aiming to evaluate how much the target analytes contribute to the extractable organic fluorine and how much of the extractable organic fluorine that consists of unidentified organic fluorine. Further, concentrations of PFASs in

crayfish caught at different locations in Lake Vättern will be evaluated. Finally, the human exposure to PFASs from crayfish, because of the adverse health effects associated with high PFAS exposure, will also be estimated.

2. Experimental

2.1 Sample pooling

In this study, crayfish were caught at 11 different locations by civilians during the autumn of 2019, with between 2-5 individual crayfish caught per location. In Table 1 below, the project number, sample name, number of crayfish caught at respective locations, the date at which the crayfish were caught, as well as the location of the caught crayfish is shown. The locations of the caught crayfish have also been approximated on a map (Figure 1) on which MH-19-016:001 is represented by the number 1, MH-19-016:002 is represented by number 2 on the map, continuing in the same manner for all compounds.

Table 1. Sample information on crayfish samples collected in Lake Vättern (9 locations), Lake Unden (sample 3) and Munksjön (sample 2) from year 2019, analyzed for per-and polyfluoroalkyl substances.

Name	Name in report	Number of crayfish	Date of catch	Location
MH-19-016:001	1	3		Vättern
MH-19-016:002	2	3		Lake Unden
MH-19-016:003	3	4	190814	Undens, Karlsborgs
MH-19-016:004	4	3	190810	Flisen
MH-19-016:005	5	2		Motala
MH-19-016:006	6	3	190920	Norrviken, Olshammar
MH-19-016:007	7	3	190907	Hämtviken, north of Västanvik
MH-19-016:008	8	3	190821	Vadstena
MH-19-016:009	9	3	190825	Nässja
MH-19-016:010	10	2	190827	Stenbrottsudden, Vänneviken
MH-19-016:011	11	5	190916	North of Stora Aspön

There were 11 different sampling sites from Lake Vättern, yielding different numbers of crayfish (Table 1). The target organ for analysis was the hepatopancreas, located in the abdominal cavity of the crayfish. Hepatopancreas was the target organ since that is where the persistent organic pollutants are expected to be found, due to the organ’s detoxification

function [13]. The crayfish were cut open using a pair of surgical scissors and the material was collected using pliers as well as a spatula. The tissue inside the claws and the bottom part of the crayfish was removed and saved in 50 mL PP-tubes. The crayfish were in different states of decomposition and the material from crayfish Sample 6-11 was gathered two days after the thawing process took place. Whilst the material from crayfish Sample 1-5 was gathered one day after the thawing process took place. Everything that could possibly be conceived as the hepatopancreas was gathered in a pre-cleaned PP-tube, then the "hepatopancreas" from the crayfish caught at the same location were combined in the same tube. The amount of material used for each pooled sample ranged from 0.1067g to 1.567g for the target analysis fraction and 0.4551g to 3.069g for the extractable organic fluorine analysis. Variation in the sample amounts can be explained by the number of crayfish available from each location and the sizes of the crayfish. Approximately one third of the collected sample was transferred to a PP-tube meant for target PFAS analysis and approximately two thirds remained in the original tube aimed for extractable organic fluorine (EOF) analysis (since the combustion ion chromatography instrument has lower sensitivity, more sample is needed). The sample was homogenized using a spatula or a scalpel. The homogenization was performed inside the PP-tubes. Sample weights can be seen in Table 7 in the Appendix.

The tools for collecting the sample material (hepatopancreas) were cleaned in between the locations, not in between individual crayfish. The cleaning in between was done by rinsing with methanol (HPLC grade), washing with tap water and detergent and then rinsing with methanol again. The samples were kept in the freezer (-20ºC) before extraction.

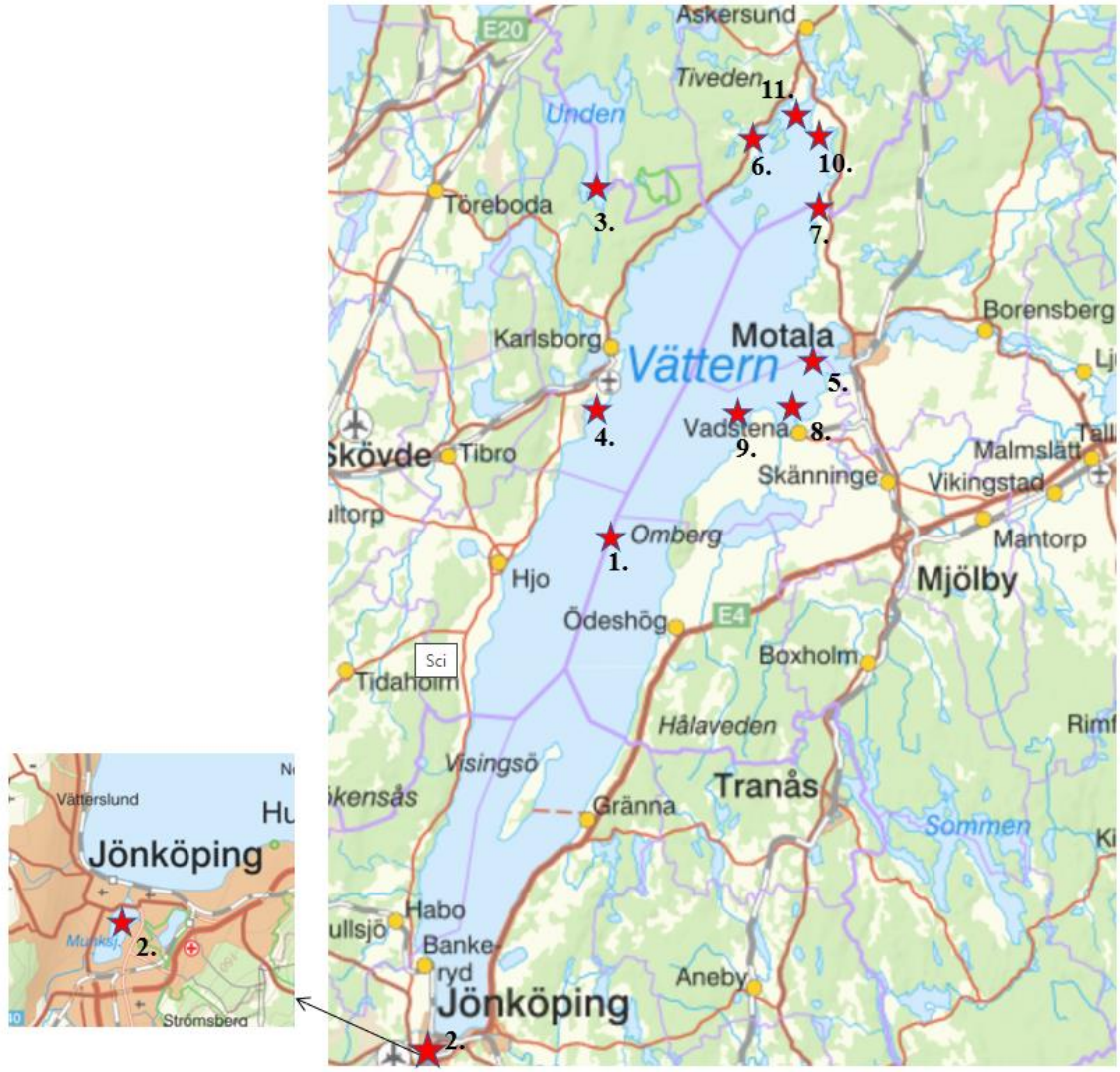

Figure 1. Approximate sampling sites of crayfish from Lake Vättern, Lake Munksjön and Lake Unden analyzed for fluorinated organic compounds.

2.2 Chemicals and Materials

2.2.1 Chemicals

The chemicals used in this project was tetrabutylammonium bisulfate (≥99.0), from Sigma-Aldrich (Switzerland), ammonium acetate (≥99.0%), from Sigma-Aldrich (Germany), MilliQ-water (18.2 MΩ), methanol (HPLC-grade, ≥99.8%), from Fisher scientific (Trinidad and Tobago), methanol (LC-MS-grade, ≥99.9%), ammonia solution (analytical reagent grade, 25%), from Fisher Scientific, tert-Butyl methyl ether MTBE (HPLC grade, ≥99.8%) from Sigma-Aldrich, sulfuric acid 95-97% (reagent grade) from Scharlab (Spain) and acetic acid (PhEur) from SAFC (Arklow, Ireland).

2.2.2 Materials

The material used in this project was Oasis Weak Anion Exchange (WAX) 6cc Cartridge 150mg 30 µm solid-phase extraction columns from Waters Corporation (Milford, USA), solid-phase extraction manifold, solid-phase extraction equipment, automatic pipettes, automatic syringe (eVol), vacuum assembly, RapidVap from LABCONCO, polypropylene tubes 15 mL (PP-tubes), centrifuge from Sigma, vortex, ultrasonication bath, scale, syringes (3 mL, 6 mL), Acrodisc Syringe Filters 13 mm/0.2 µm, flat bottomed LC-vials , total recovery LC-vials, maximum recover LC-vials, all from Waters (USA), Pasteur pipettes and pH-paper.

2.2.3 Standards

Standards were used to quantify the target PFAS analytes. One such standard that was used is a carbon 13 labelled internal standard that is affected by the sample matrix in the same manner as the target compounds. The internal standard is also used to account for any loss of analytes during the sample preparation process and this is accounted for using a recovery standard (RS). The ratio between the internal standard and recovery standard are used to calculate the recovery, i.e. how much of the sample that was in the extract versus how much was in the sample from the beginning. There was also an addition of native standard (Calibration standard) in each batch standard.

Table 2. Labelled, ^{13}C, per- and polyfluoroalkyl substances included in the internal standard (IS) and recovery standard (RS) used for the analysis of PFAS in crayfish samples.

Internal standard		**Recovery standard**	
Chemical name	Acronym	Chemical name	Acronym
perfluorobutanoate	PFBA	perfluorobutanoate	PFBA
perfluoropentanoate	PFPeA	perfluoropentanoate	PFPeA
Perfluorohexanoate	PFHxA	perfluorohexanoate	PFHxA
perfluoroheptanoate	PFHpA	perfluorooctanoate	PFOA
perfluorooctanoate	PFOA	perfluorononanoate	PFNA
perfluorononanoate	PFNA	perfluorodecanoate	PFDA
perfluorodecanoate	PFDA	perfluoroundecanoate	PFUnDA
perfluoroundecanoate	PFUnDA	perfluorohexane sulfonate	PFHxS
perfluorododecanoate	PFDoDA	perfluorooctane sulfonate	PFOS
perfluorohexadecanoate	PFHxDA	4:2 fluorotelomer sulfonate	4:2 FTSA
perfluorotetradecanoate	PFTeDA		
perfluorobutane sulfonate	PFBS		
perfluorohexane sulfonate	PFHxS		
perfluorooctane sulfonate	PFOS		
6:2 fluorotelomer sulfonate	6:2 FTSA		
8:2 fluorotelomer sulfonate	8:2 FTSA		
perfluorooctanesulfonamide	PFOSA		

Table 3. Native per- and polyfluoroalkyl substances included in the native standards (CS) and internal standard (used as native standard) used in the batch standards.

Native standard	
Chemical name	Acronym
perfluorobutanoate	PFBA
perfluoropentanoate	PFPeA
perfluorohexanoate	PFHxA
perfluoroheptanoate	PFHpA
perfluorooctanoate	PFOA
perfluorononanoate	PFNA
perfluorodecanoate	PFDA
perfluoroundecanoate	PFUnDA
perfluorododecanoate	PFDoDA
perfluorotridecanoate	PFTrDA
perfluorotetradecanoate	PFTeDA
perfluorohexadecanoate	PFHxDA
perfluorooctadecanoate	PFOcDA
linear perfluorobutane sulfonate	L-PFBS
linear perfluoropentane sulfonate	L-PFPeS
linear perfluorohexane sulfonate	L-PFHxS
linear perfluoroheptane sulfonate	L-PFHpS
linear perfluorooctane sulfonate	L-PFOS
linear perfluorononane sulfonate	L-PFNS
linear perfluorodecane sulfonate	L-PFDS
linear perfluorododecane sulfonate	L-PFDoDS
4:2 fluorotelomer sulfonate	4:2 FTSA
6:2 fluorotelomer sulfonate	6:2 FTSA
8:2 fluorotelomer sulfonate	8:2 FTSA
perfluorooctane sulfonamide	PFOSA
perfluorobutane sulfonamide	FBSA
N-Methyl perfluorobutane sulfonamide	MeFBSA
perfluorohexane sulfonamide	PFHxSA
N-Methyl perfluorohexane sulfonamide	MePFHxSA
6:2 fluorotelomer unsaturated carboxylic acid	$^{13}C_2$-6:2 FTUCA
10:2 fluorotelomer unsaturated carboxylic acid	$^{13}C_2$-10:2 FTUCA

2.3 Extraction method

The pooled samples aimed for target PFAS analysis (fraction A) were spiked with 2000 pg internal standard before the start of the ion-pair extraction. Batch standards were prepared for respective batch, spiking with internal standard and native standard. Thereafter, the procedure shown in Figure 2 was performed.

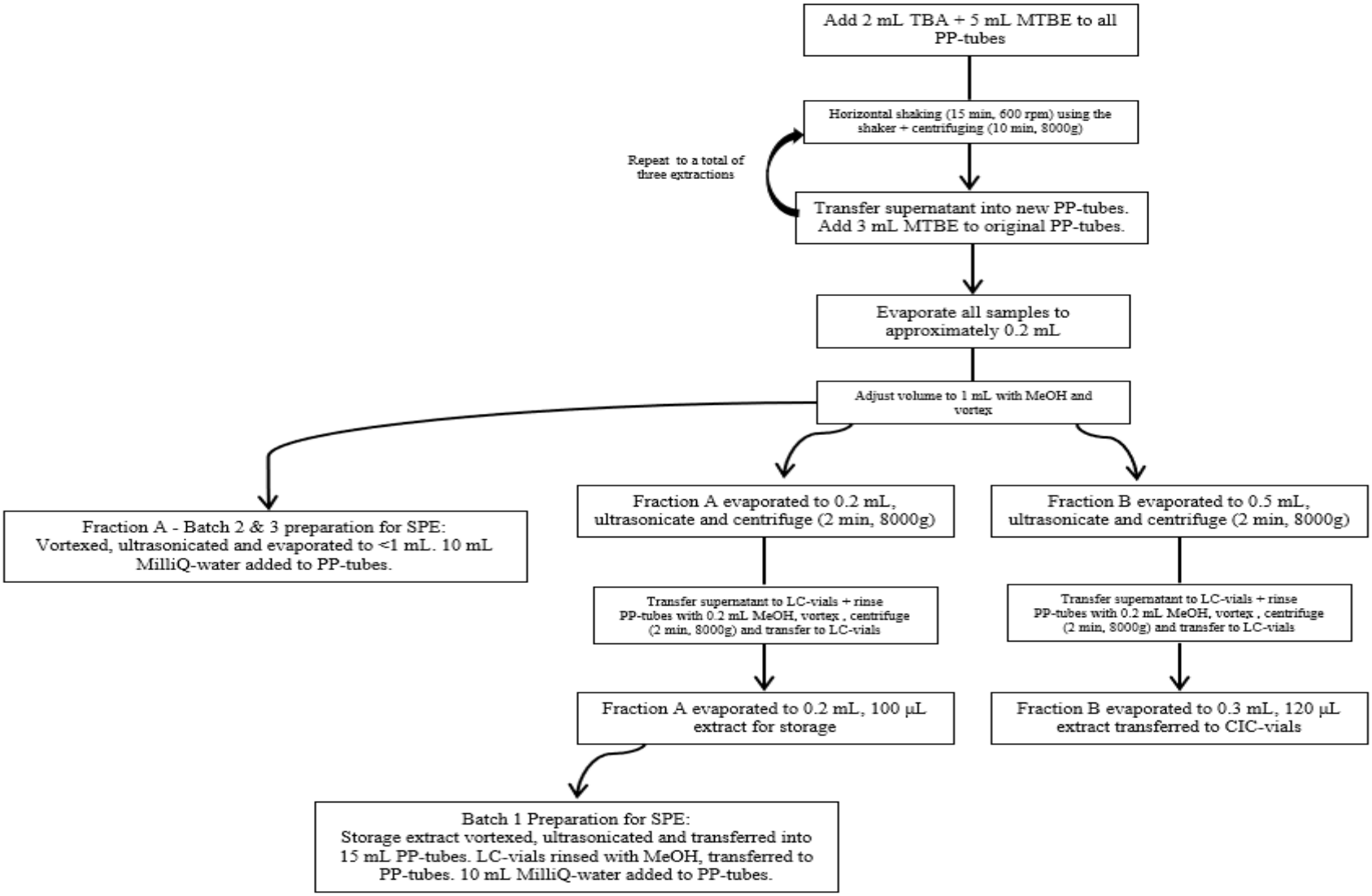

Figure 2. Ion-pair extraction procedure for extraction of PFASs from crayfish samples from Swedish lakes.

The ion pair reagent TBA is used to separate the inorganic fluorine from organic-fluorine, resulting in the PFASs residing in the organic phase of the liquid partition and the inorganic fluorine in the aqueous phase [16]. A total of three MTBE cycles was performed, in which the organic phase was transferred into a new PP-tube. This was followed by evaporation and then switching of the solvent from MTBE to methanol. Batch 1 underwent a complete ion pair extraction before being prepared for the clean-up procedure, whilst batch 2 and 3 were prepared for clean-up after the solvent switch (seen in Figure 2). A more in-depth description of the extraction can be found under Additional Information in the Appendix. After the ion-pair extraction, a solid-phase extraction was done as a clean-up procedure in the manner shown in Figure 3.

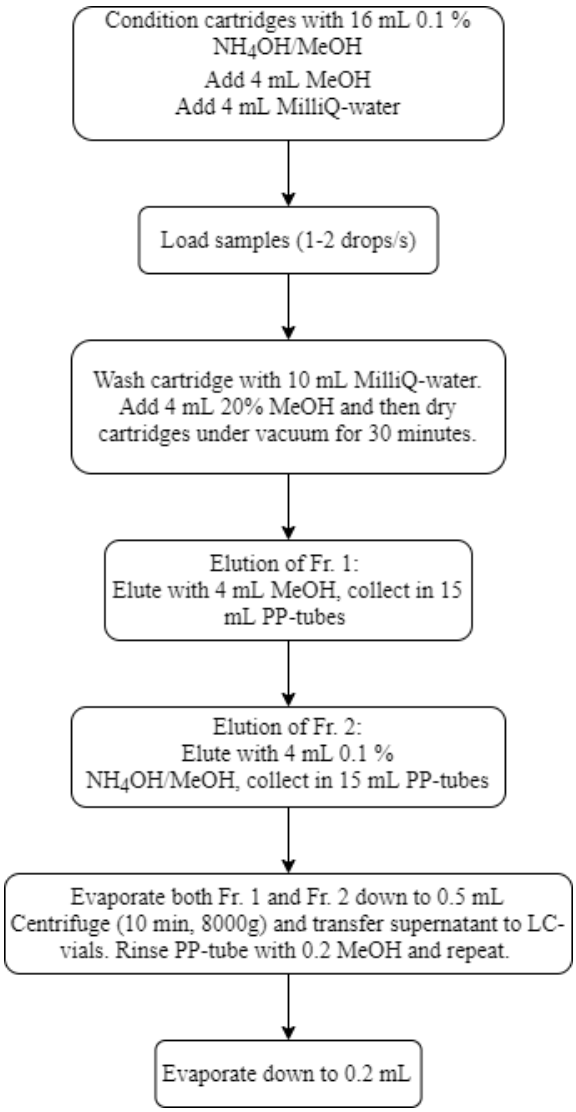

Figure 3. The solid-phase extraction, clean-up procedure that was used for all fraction A (target PFAS) samples.

The sample extracts were diluted in MilliQ-water before the solid-phase extraction. A clean-up procedure was done because the samples had a very dirty matrix. Solid-phase extraction was done by first conditioning the cartridges, then loading the samples onto the cartridges, thereafter the cartridges were washed and the elution was carried out in two fractions (seen in

Figure 3). In fraction 1, the neutral compounds are eluted and in fraction 2 the anionic compounds are eluted. For a more in-depth description of the clean-up procedure, see Additional Information in the Appendix.

After the solid-phase extraction clean-up procedure, the extracts were spiked with 2000 pg recovery standard before transferring 100 µL into new LC-vials. To the new LC-vials, 150 µL of the aqueous phase was added. The samples were then frozen overnight (-21 ºC) and thereafter centrifuged (6000g for 20 minutes). If the supernatant was visually transparent, 100 µL of the supernatant was transferred into a new LC-vial for analysis. For three samples (QCA2, QCA3 and A:002), the supernatant was not visually transparent and the whole sample was filtered using a three mL syringe and 0.2 µm filters into a new LC-vial. For the extracts of fraction 2, all samples except for the blanks (BA1, BA2 and BA3) needed to be filtered. The filtering was done in the same manner as that of fraction 1.

2.3.1 Fraction B – Preparation for Extractable Organic Fluorine analysis

The samples from batch one was prepared for extractable organic-fluorine analysis by diluting them 1:1, which was achieved by adding 120 µL methanol to the 120 µL extract in the combustion ion chromatography vials. Thereafter, the samples were vortexed, ultrasonicated and centrifuged (6000g for five minutes). Then 120 µL of the 1:1 dilution was transferred into new combustion ion chromatography vials. As for fraction B batch 2 & 3, these were prepared to a dilution of 1:1 by adding methanol to one mL. Before transferring 120 µL of the extracts into combustion ion chromatography vials, the samples were vortexed, ultrasonicated and centrifuged (6000g for five minutes).

2.4 Instrumental analysis

2.4.1 Target analysis

The separation and quantification of the individual target PFASs was performed using an UPLC-MS/MS system (Xevo TQ-S, Waters Corporation, Milford, USA) with electronspray ionization (ESI) as ion source, negative mode (Zspray, Waters). The column used for analysis was Acquity UPLC BEH C18 1.7 µm (2.1x100mm Column). Separation of target compounds was achieved by applying a gradient from 99% of mobile phase A (2mM NH_4Ac in 70:30 MilliQ:methanol) to 100% mobile phase B (2 mM in NH_4Ac in MeOH).

2.4.2 Extractable organic fluorine analysis

The extractable organic fluorine was determined using a combustion ion chromatograph (CIC) that measures the concentration of fluoride ions, F^-. In the CIC, the extract is combusted in a furnace (Combustion Module, AnalytikJena) at a temperature between 1000-1050 ºC. The combustion step converts organic fluorine into hydrogen fluoride, HF, that is then dissolved in MilliQ-water (920 Absorber Module, Metrohm). An ion exchange column (Metrosep A Supp 5-150/4.0, Metrohm) separates the anions so that the F^- concentration can be determined (930 Compact IC Flex, Metrohm).

2.5 Quality control/Quality assurance

The extraction method was tested using a fish sample (MH-17-013:35) before extraction of the crayfish samples. This fish sample was then used as quality control (QC) sample, one QC per batch. For additional information on how the quality control sample was used as test of the method, see Additional information in the Appendix. Below, the previously measured values of the fish sample used as QC (theoretical concentration) and the average concentration of the target analytes in the fish samples (measured concentration) can be seen. Table 4 also shows the relative standard deviation (RSD) of the measured concentration.

Table 4. The measured concentration in the fish samples of perch from Finland used as reference material to the crayfish. QC is the previously measured concentration the PFASs, average QC is the mean concentration of PFASs in the fish samples measured in this study and relative standard deviation shows the variability of the values from this study.

	Previous QC (ng/g)	Average QC Samples (ng/g)	RSD (%)
L-PFHxS	0.28	0.418	13.8
PFHpS	0.28	0.406	11.5
PFNA	4.80	5.74	9.57
L-PFOS	34.0	52.2	0.787
PFDA	3.09	3.83	7.18
PFUnDA	10.9	12.8	8.37
PFDoDA	0.908	1.87	13.2
PFTrDA	5.59	1.78	7.43
PFOA	<0.25	0.529	40.4
PFTDA	0.560	0.404	N/A
PFHxDA	<0.7	<1.031	N/A
PFOcDA	<0.7	<0.017**	N/A
PFDoDS	<0.04	<0.098**	N/A
PFNS	<0.04	<0.015**	N/A
PFDS	<0.04	<0.040	N/A
PFBS	<0.04	<0.144	N/A
PFBA	<0.2	<0.704	N/A
PFPeA	<0.04	<0.082	N/A
PFHxA	<1.7	<0.022	N/A
PFHpA	<0.04	<0.030	N/A
6:2 FTSA	<0.04	<0.014	N/A
PFPeS	<0.04	<0.011**	N/A
8_2_FTSA	<0.04	<0.133**	N/A
6_2FTUCA293	n.q.	<0.026	N/A
10_2_FTUCA493	n.q.	<0.032**	N/A
4:2 FTSA	<0.04	<0.090**	N/A

**Lowest point of calibration was used for limit of quantification of the respective compounds.
N/A refers to the values not being available.

As a reference material to the crayfish, a fish sample (MH-17-013:35) of perch from Finland with a previously measured concentration was used. As can be seen in Table 4, the average concentration for the fish sample measured in this project was higher than that of the previously measured concentration. The measured concentrations of PFOA has a variability of approximately 40% (seen in Table 4 and in Table 15) giving these values a higher uncertainty because of the high relative standard deviation of the QC and the blanks. In comparison, the relative standard deviation values for the other compounds shown in Table 4 range from 0.787 to 13.8 indicating a good repeatability. The reproducibility is somewhat poor when looking at the values measured for PFOS and PFTrDA in the previous study compared to the values of respective compound in this study. For PFOS, the measured concentration in this study was much higher, whilst for PFTrDA it was much lower than in the previous study. The differences in the measured concentrations could be due to differences in the method of extraction and clean-up between the previous study and this study.

To account for loss of analyte and matrix effects, an internal standard (IS) was added to fraction A, which was aimed for target analysis. The internal standard recovery was then used in the quantification of the different PFASs.

Procedural blanks, one for each batch, were extracted and treated in the same manner as the samples, with the purpose to control for any contamination that might be introduced during the procedure. One batch standard per batch was analyzed on the instrument before and after respective batch to control for the stability of the instrument. Before and after every batch standard, an instrumental blank consisting of methanol was run to monitor possible contamination from the system itself.

For the CIC analysis, approximately every sixth injection was a combustion blank with the purpose to check the stability of the instrument during the run. Another purpose of the combustion blank is to remove any carry-over fluorine contamination. A batch standard with known concentration (200 ng F/ mL) was also used to control for the stability of the instrument. The batch standard was run before, in-between and after the samples.

2.6 Data handling

MassLynx V.2 and Quantify MFC Application (TargetLynx XS) was used for the quantification of the target PFASs. In Targetlynx there were certain criteria that determined whether the compound was quantified, or not. First criteria were a signal-to-noise ratio (S/N) greater than 10 for the compound for it to be quantifiable. Second condition was the 1º Ratio Flag, that marks quantifiable samples as “NO” depending on if the quantitative and the qualitative transitions differs too much in retention time (RT).

For the analysis of the extractable organic fluorine data, the quantity of target PFASs was converted to the fluorine equivalents (ng F/g) for the different sample sites (Sample 1-11) using the following formula (Formula 1).

$$C_F = N_F \cdot \frac{MW_F}{MW_{PFAS}} \cdot C_{PFAS} \quad (1)$$

The fluorine equivalent concentration (ng F/g) is represented by C_F, N_F represents the number of fluorine in the respective target PFASs, MW_F represents the molecular weight of fluorine, molecular weight of respective PFASs is represented by MW_{PFAS} and C_{PFAS} represents the concentration of the respective target PFASs acquired from the LC-MS/MS analysis [17]. Then the resulting equivalents were related to the extractable organic fluorine quantity of the respective sample sites (seen in Figure 6 and 7).

3. Result and discussion

Concentrations of target PFASs and extractable organic-fluorine was determined in 9 pooled samples of the "hepatopancreas" in crayfish from various locations around Lake Vättern, one sample from Lake Unden and one sample from Lake Munksjön. Both unidentified organic-fluorine and target PFASs could be measured in all samples.

3.1 Target PFAS analysis

The sum of PFASs in the samples range from 18.9-59 (ng/g). In total 31 target PFASs were analyzed in this study, of which three PFSAs (PFHxS, PFHpS and PFOS) and seven PFCAs (PFOA, PFNA, PFDA, PFUnDA, PFDoDA, PFTrDA and PFTeDA) could be found in the majority of the samples.

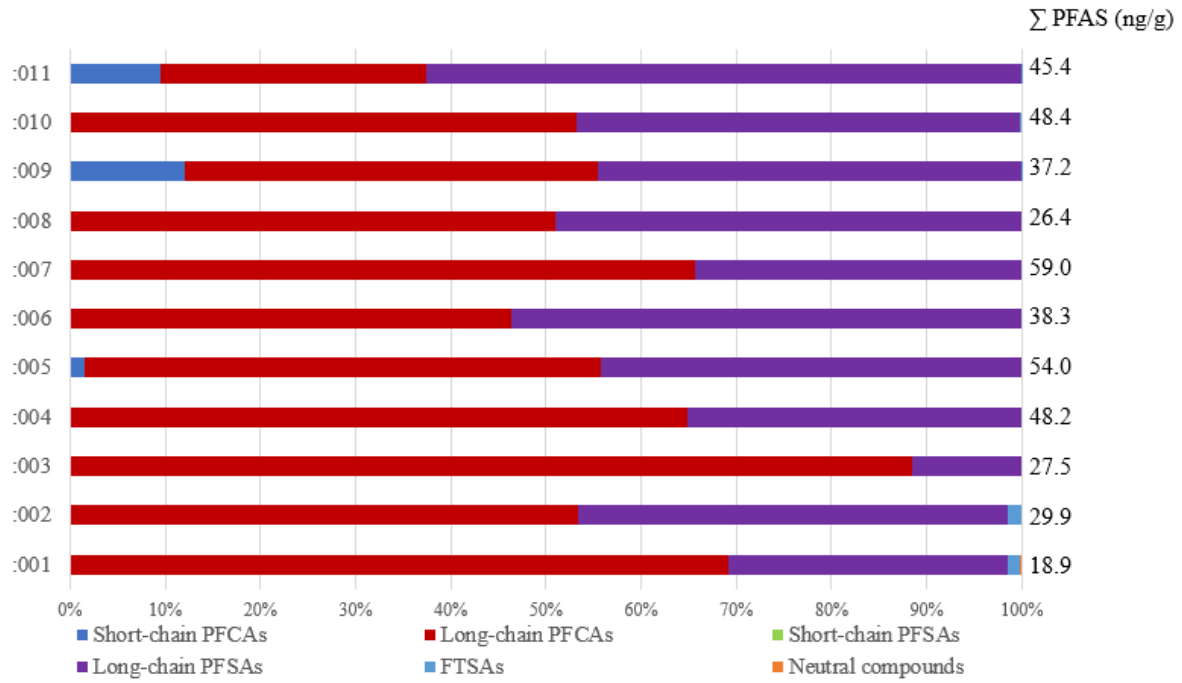

Figure 4. Normalization of the different groups of target PFASs (i.e. short-chain PFCAs, short-chain PFSAs, long-chain PFSAs, long-chain PFCAs and FTSAs) in crayfish samples from Sweden.

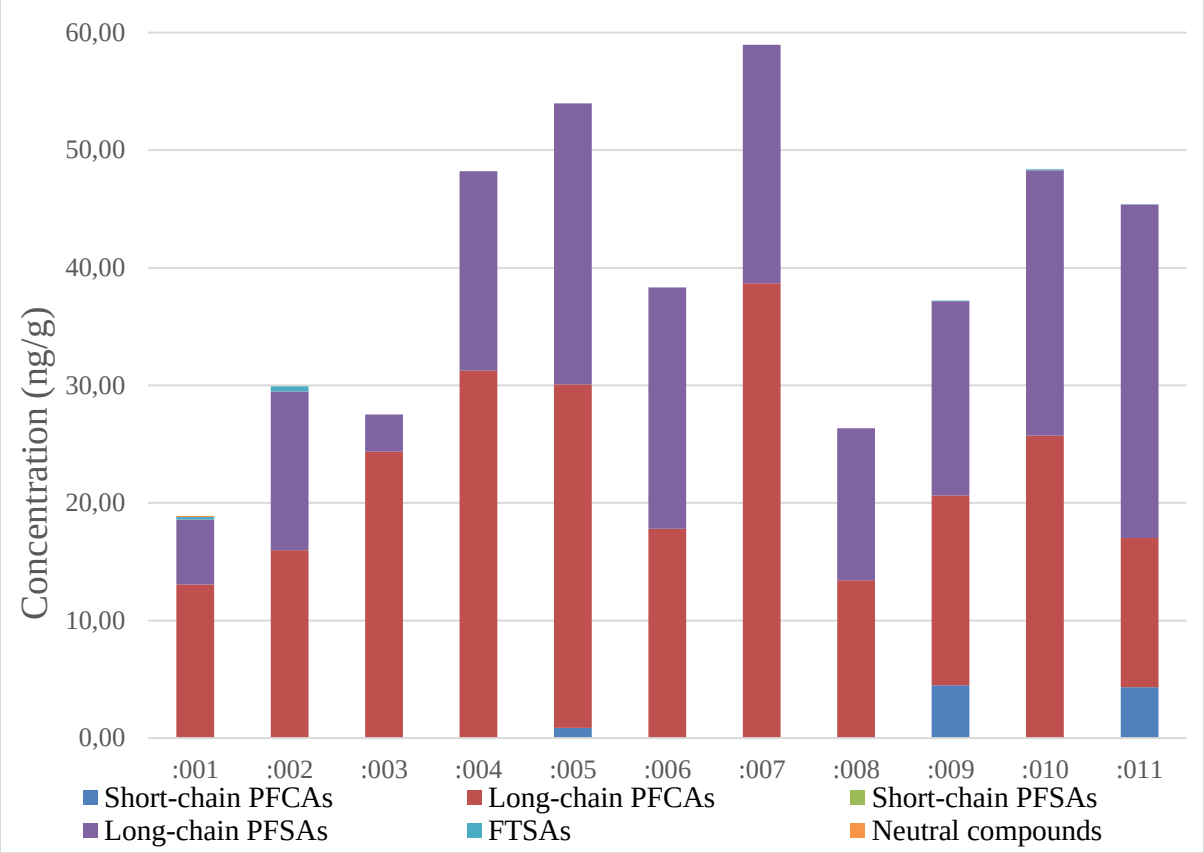

Figure 5. Total concentration (ng/g) in relation to the concentration of the groups of target PFASs (i.e. short-chain PFCAs, short-chain PFSAs, long-chain PFSAs, long-chain PFCAs and FTSAs) and the total amount of target PFASs present in crayfish samples.

The anionic compounds, such as PFCAs and PFSAs, were eluted in the second fraction and the concentration of these compounds was determined from the data gathered in MassLynx. Procedural blanks were used for the calculation of limit of quantification (LOQ). For compounds marked with "**" (seen in Table 9 and Table 11 in Appendix) the lowest point of calibration for that compound was used for LOQ. The semi-quantifiable compounds are marked with a "*" (seen in Table 9 in the Appendix) and were still included in the sum calculation of the different subclasses of PFASs.

Locations of the sampling sites for the samples are illustrated in Figure 1. Sample 4, closest to Karlsborg and the military airport, had a measured concentration of 48.2 ng/g. The highest concentrations were measured in Sample 5 and 7, 54 and 59 ng/g respectively. These sampling sites are located on the opposite side of Lake Vättern from Karlsborg and the military airport, with Sample 7 further away than 5. Sample 8 had a concentration of 26.4 ng/g which is lower than the concentration of Sample 2, 29.9 ng/g. The location of Sample 8 is close to the airport and was sampled next to Sample 5. Sample 2 is the sample located furthest away from the military airport of all the samples, in Lake Munksjön. Sample 3 has a concentration of 27.5 ng/g, and was sampled in Lake Unden that is part of the inflow system to Lake Vättern and has no known sources of PFASs. This leads to the conclusion that the contamination in this Sample 3 could come from atmospheric sources. The concentration of

One known point source is Karlsborg military airport so looking at the sum value of all PFASs for each location, the general expectation is that the concentration is highest closest to Karlsborg. Based on this, the concentration in Sample 4 is thus conceivably expected to be the highest, but it is not. The highest measured concentrations are from Sample 5 and 7, further away from the point source than Sample 4. Sample 2, from Lake Munksjön is located close to a big city which could likely be expected to contribute to an increased PFAS contamination. The measured concentration of Sample 8 is lower than that of Sample 2, despite being located closer to Karlsborg than Sample 2 as well as being located next to one of the sampling locations with highest measured concentration (Sample 5). Sampling site for Sample 1 is not specified other than "Vättern", this makes it hard to determine if there is any connection between the distance from Karlsborg's airport and the concentration in the sample.

Based on these results, there seems to be no connection between the distance from the airport and the measured concentration at the different sampling sites. This could be because of the fact that external forces, such as wind and currents, haven't been accounted for and that these forces have a bigger impact on where the PFASs concentrate than distance. It could also be because the age of the crayfish is unknown, which could have an impact on the measured concentrations since the older crayfish would have more time to accumulate contaminants. Further, it could also be explained by their food source, since their diet consists of fish and insects, among other things, that have the ability to move over a larger area than the crayfish and can therefore move through areas with varying contamination of PFASs. A crayfish located in an area that has low contamination could eat a fish that is highly contaminated increasing the level of contamination in that area. If the Also, the assortment of crayfish per location is too small to come to a conclusion about correlation between location and concentration. Had there been bigger differences between the measured concentrations at the different locations, the conclusion would have been that further studies were to be made. As this is not the case, there is no conclusion about the locations to be made.

In biota samples such as crayfish, high concentrations of long-chain PFCAs and PFSAs are expected to be found. By looking at Figures 4 and 5, it can be concluded that long-chain PFCAs and PFSAs are the subclasses of PFASs that are the most abundant in the samples.

When looking at the sum value of PFCAs, the results range from 13.1 (ng/g) to 38.7 (ng/g) between the different locations (found in Table 9. in the Appendix). The previously measured value for PFCAs hepatopancreas of crayfish from Lake Vättern was measured to 22 ng/g and 24 ng/g, in the study from 2008. Concentrations, for both locations in Lake Vättern, (22 and 24 ng/g) are located in the range of the measured concentrations (13.1 to 38.7 ng/g) in this study. The sum value for PFSAs in this study range from 3.16 to 28.4 ng/g, with the sum value for PFSAs in the previous study at 10 ng/g and 16 ng/g. Those sum values for PFSAs (10 and 16 ng/g) also falls within the range of measured concentrations of PFSAs in this study (3.16-28.4 ng/g). The value for PFOSA in this study is below the limit of quantification, which corresponds to the results of <0.10 ng/g in the previous study. The differences between this study and the previous study from 2008, is that there were only two

different locations in Lake Vättern in the previous study compared to the nine different locations in this study. In this study, the assortment of crayfish from each location was small (between 2-5 individuals per location), whilst in the previous study they had between 6-20 individuals caught per location. Despite the small assortment of crayfish in this study, the results are corroborated by the measured concentrations in the study from 2008 being similar to the measured concentrations of PFCAs and PFSAs in this study. This result also shows that, despite regulations put on both PFOS and PFOA [9], [10] the concentrations of PFCAs and PFSAs are approximately the same in crayfish from 2008 and crayfish from 2019.

Table 5. Average amounts of PFHxS, PFOS, PFOA, PFNA and sum PFASs from all sample sites.

	Average amount (ng/g)
PFHxS	1.02
PFOS	15.4
PFOA	5.32
PFNA	3.59
∑PFASs	39.4

The sum of the average concentrations of PFHxS, PFOS, PFOA and PFNA is 25.3 ng/g (based on the values in Table 5). A combined tolerable weekly intake (TWI) for these four PFASs is set to 8 ng/kg body weight (bw) per week. To put this into perspective, two examples will be used for comparison to the TWI of which one is a worst-case scenario where the edible material is estimated to 0.5 kg crayfish. The person eating it has a bodyweight of 75 kg and the sum of the average measured concentration for PFHxS, PFOS, PFOA and PFNA is 25300 ng/kg (25.3 ng/g). Then the following approximation can be done.

$$\frac{(0.5\ \text{kg} \cdot 25300\ \text{ng/kg})}{75\ \text{kg}} = 169\ \ ng/kg\ \text{bw}$$

In the worst-case scenario, a person is thus exposed to 169 ng/kg bw PFHxS, PFOS, PFOA and PFNA per serving crayfish. To estimate a less severe case, the weight of the edible material is estimated to 0.1 kg and all other factors are kept the same. This results in exposure of 33 ng/kg bw PFHxS, PFOS, PFOA and PFNA per serving crayfish. To put this into perspective, an estimation of how often crayfish from Lake Vättern can be eaten was calculated by dividing 33 ng/kg – and 169 ng/kg bw PFHxS, PFOS, PFOA and PFNA with the tolerable weekly intake for these four compounds. In the less severe case, eating crayfish more than every four weeks would be exceeding the TWI. As for the worst-case scenario, eating crayfish more than every 21 weeks would be exceeding the TWI.

Taking the individual TWI for PFOS and PFOA into account, the average amount of PFOS measured was approximately 15400 ng/kg (15.4 ng/g) and 5300 ng/kg (5.32 ng/g) for PFOA. Thus, resulting in a worst-case exposure scenario of 103 ng/kg bw PFOS per serving and 35.3 ng/kg bw PFOA per serving crayfish from Lake Vättern. In the less severe case, the exposure of PFOS is estimated to 20.5 ng/kg bw PFOS per serving and 7.07 ng/kg bw PFOA per

serving crayfish. In the less severe case, eating crayfish from Lake Vättern more than every two weeks would be exceeding the TWI for PFOS and more than every week (eating crayfish multiple times in a week) would exceed the TWI for PFOA. As for the worst-case scenario, eating crayfish from Lake Vättern more than every eight weeks would exceed the TWI for PFOS and more than every six weeks would exceed the TWI for PFOA.

This estimation does not take into account other exposure sources of PFHxS, PFOS, PFOA and PFNA or that these four compounds only make up 64.2 percent of the sum value of all PFASs analyzed in this study (calculated based on the sum PFASs value and the sum of the average concentrations of PFHxS, PFOS, PFOA and PFNA from Table 5). These values are only in the “hepatopancreas” of the crayfish, so the total exposure of these four PFASs from crayfish is still unknown. Thus, the weight of the edible material used in the estimations are composed of other parts of the crayfish than the hepatopancreas, these parts of the crayfish may or may not be consumed more often than the hepatopancreas. But the concentration in the hepatopancreas is assumed to represent the worst-case scenario and the highest concentration of PFASs in crayfish.

3.2 Extractable organic fluorine (EOF) analysis

In the extractable organic fluorine analysis, unidentified organofluorine compounds were found in all samples analyzed. The unidentified fraction varied between more than a 100 percent identified compounds (giving a negative unidentified value) and 93 percent, when using the blank corrected data (seen in Figure 6). When looking at the data without blank correction the unidentified fraction varied between 14.4 and 93.1 percent (seen in Figure 7).

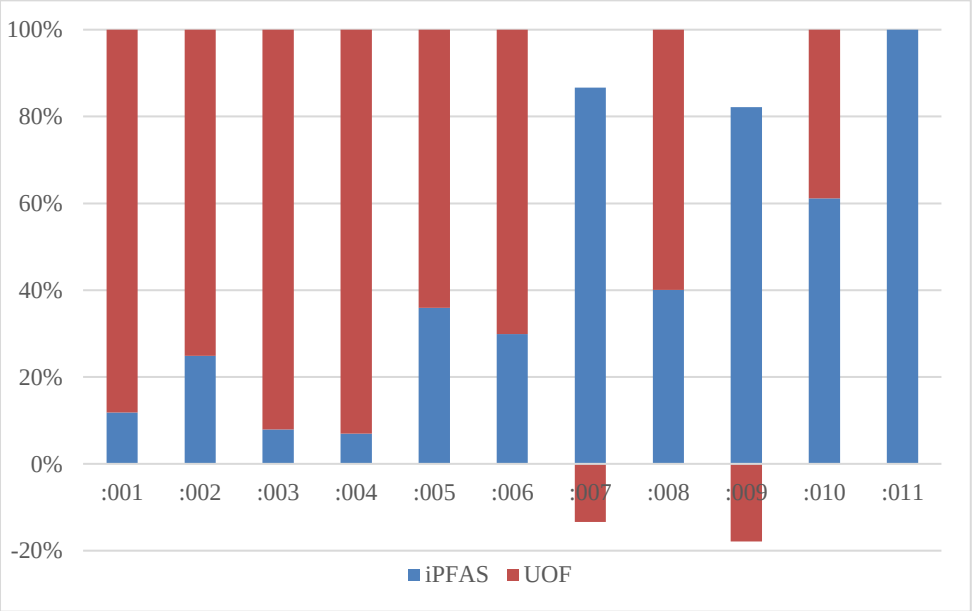

Figure 6. The percentage of identified target PFASs (iPFAS) relative to the percentage of unknown organic fluorine (OEF) normalized to 100%, with procedural blank correction.

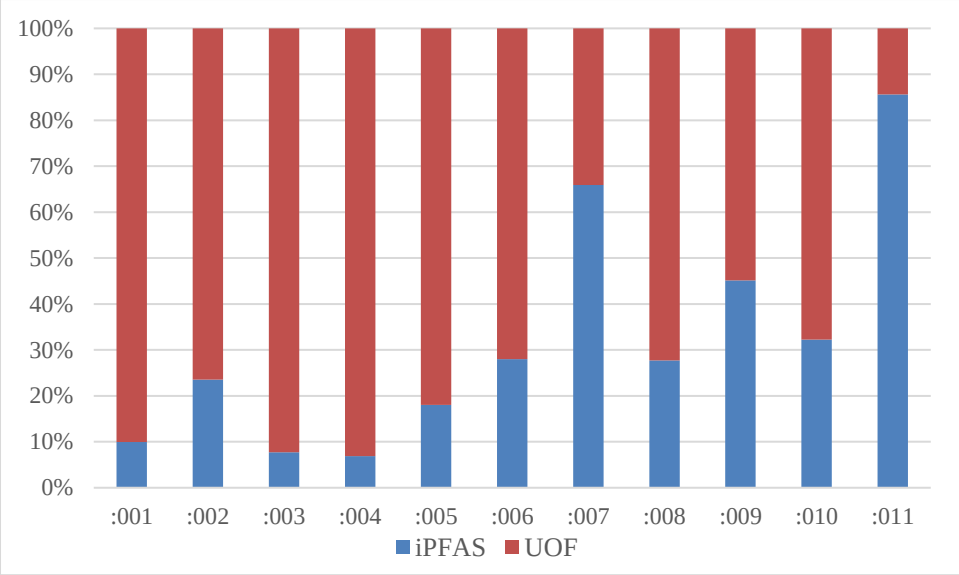

Figure 7. The percentage of identified target PFASs (iPFAS) relative to the percentage of unknown organic fluorine (OEF) normalized to 100%, without procedural blank correction.

Concentrations of target PFASs that were below limit of quantification were not included in the calculation of the equivalent concentration, but would still be included under the unidentified organic fluorine. Since the fraction of unidentified organic fluorine consists of both unidentified organic fluorine as well as target PFASs over limit of detection but below limit of quantification. This should have no greater impact than a small overestimation of the unidentified fraction of the extractable organic fluorine, since concentrations below limit of quantification are very low.

Table 6. The calculated mean, standard deviation (STD) and relative standard deviation (RSD) of the fluoride anion peak area for the procedural blanks and the quality control samples from combustion ion chromatography analysis.

Mean area of blanks	0.091
STD area blanks	0.039
RSD blanks (%):	42.2
Mean area of QC	0.094
STD area QC	0.040
RSD QC (%):	42.2

Based on the relative standard deviation for the fluoride area in the blanks and QC samples, the concentration in the samples would also have a variability of 42 percent. This can be confirmed when comparing Figure 6 and 7, since the values of identified PFASs in Sample 7 and 9 are a 100 percent identified in Figure 6. Whilst in Figure 7, the composition of Sample 7 is 65.9 percent and in Sample 9 it is 45.2 percent of the unidentified organic fluorine. The varying blank values, could be explained by the method of extraction. Since the partitioning of the organic and inorganic phase in ion-pair extraction are difficult to distinguish between, some of the inorganic phase (containing the inorganic fluorine) could have been transferred with the organic phase. Inorganic fluorine contamination could therefore explain the high blank values. For Sample 11, the concentration of target PFASs was more than ten times

higher than the extractable organic fluorine concentration which resulted in a normalized value of 1125 percent of identified PFASs and -1025% unidentified organic fluorine (seen in Table 12 in the Appendix). Therefore, the unidentified organic fluorine value for Sample 11 was identified as an outlier and excluded from the result. The sum concentration of target PFAS in Sample 11 was similar to that of the other samples, making this value more credible and therefore it is still reported.

Because of the high variability in the procedural blanks and the QC samples, the unidentified organic fluorine values in the samples are uncertain and only an approximation of the actual amount of unidentified organic fluorine. Given this, a more plausible result would be that the unidentified organic fluorine fraction is larger than that of the identified PFASs since it is highly unlikely that all organic fluorine is identified. The values in Figure 7 are not blank corrected, which would lead to an overestimation of organic fluorine. Whilst the blank corrected values in Figure 6, underestimates the unidentified organic fluorine fraction because of the high blank values caused by a possible contamination of inorganic fluorine. In conclusion, it is plausible that the true value of unidentified organic fluorine is somewhere in between the blank corrected values and the values without correction.

4. Conclusion

Target PFASs and unidentified organic fluorine was found in all (11 samples) crayfish samples that were analyzed. The sum of measured concentrations for long-chain PFCAs ranged from 13.1 to 38.7 ng/g, concentrations for short-chain PFCAs ranged from 0.86 to 4.49 ng/g and for long-chain PFSAs the concentrations ranged between 3.16-28.4 ng/g. For FTSAs, the sum concentrations ranged from 0.034 to 0.450 ng/g. The only concentration measured for any of the neutral compounds was FBSA found in Sample 1, with a concentration of 0.05 ng/g. The results from a previous study (from 2008) of concentrations of PFCAs and PFSAs in the hepatopancreas of crayfish, was in the range of concentrations measured in this study corroborating the result.

The true value of the unidentified organic fluorine is uncertain, but the amount of unidentified organic fluorine is concluded to be higher than that of the target PFASs. This indicates a production and use of organic fluorine from other unidentified PFASs or other fluorinated compounds that are not classified as PFASs, such as certain fluorinated pesticides and pharmaceuticals. The uncertainty of the true value for unidentified organic fluorine comes from high blank concentrations, resulting in varying amounts of unidentified organic fluorine depending on if the samples were blank corrected or not.

For the target PFAS analysis, no apparent connection between the concentration in the different samples and their distance from the military airport could be found. This could be due to external factors, such as the age of the crayfish, wind and currents, not being accounted for. The sum of PFASs from all locations ranged from 18.9 ng/g in Sample 1 to 59 ng/g in Sample 7. It was also concluded that in this study long-chain PFSAs and PFCAs have bioaccumulated to a larger extent than their short-chain homologues.

Comparing a combined tolerable weekly intake for PFHxS, PFOS, PFOA and PFNA to the sum of the average of these four compounds lead to the conclusion that in the worst-case scenario, eating crayfish more than every 21 weeks would be exceeding the TWI of 8 ng/kg bw per week. In the less severe estimation, eating crayfish from Lake Vättern more than every four weeks would be exceeding the TWI. Comparing the TWI individually for PFOS and PFOA to the average amount measured of the respective compounds, lead to the conclusion that in the less severe case eating crayfish from Lake Vättern more than every two weeks would exceed the TWI for PFOS and eating crayfish multiple times in a week would exceed the TWI set for PFOA. In the worst-case scenario, eating crayfish more than every eight weeks would exceed the TWI for PFOS and more than every six weeks would exceed the TWI for PFOA. The total exposure of PFASs from crayfish is still unknown, since it was only the hepatopancreas that was analyzed in this study. Thus, the weight of the edible material used in the estimations are composed of other parts of the crayfish than the hepatopancreas, these parts of the crayfish may or may not be consumed more often than the hepatopancreas. But the concentration in the hepatopancreas is assumed to represent the worst-case scenario and the highest concentration of PFASs, since the tail muscle and other tissues are assumed to have lower concentrations of PFASs.

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6. Appendix

Table 7. The weights of the pooled samples after dividing them into the target analysis fraction (A fraction) and the extractable organofluorine fraction (B fraction).

Sample name	Weight target analysis (g)	Weight EOF analysis (g)
MH-19-016:001	0.6829	1.2582
MH-19-016:002	1.086	2.9642
MH-19-016:003	1.4296	2.827
MH-19-016:004	0.3474	1.1277
MH-19-016:005	0.1067	0.4551
MH-19-016:006	0.7072	1.4152
MH-19-016:007	0.6371	1.9823
MH-19-016:008	1.1194	2.244
MH-19-016:009	1.393	2.4218
MH-19-016:010	0.7648	1.7367
MH-19-016:011	1.5674	3.0685

Difference in size, as well as the number of crayfish at each location, results in the varying sample amounts being extracted. The varying crayfish sample amounts can be seen in Table 7.

Table 8. Shows the weights of MilliQ-water in the blanks from the different batches (Blank-A1, BA1, is for fraction A in batch one, whilst BB1 is the blank for fraction B in batch 1). It also shows the weight of the reference samples (QCA1, QCB1, QCA2, QCB2, QCA3 and QCB3).

Sample name	**Weight (g)**
BA1	1.0021
BB1	1.932
QCA1	0.2698
QCB1	0.2686
BA2	1.0024
BB2	2.0209
QCA2	0.2583
QCB2	0.3019
BA3	1.02
BB3	2.0141
QCA3	0.2749
QCB3	0.3293

The blank weights were divided in a similar manner as that of the samples, the blanks aimed for LC analysis were filled with approximately one mL MilliQ-water and the ones aimed for CIC analysis were filled with two mL (to simulate the one third and two thirds relationship of the samples), as can be seen in Table 8. Despite the varying weights of the samples, blanks and QC samples, the solvent amount in the MTBE extraction was not adjusted based on amount, it was kept the same for all samples.

6.1 Additional information

Solutions prepared

TBA (tetra-n-butyl ammonium hydrogen sulfate) 0.5 M solution (20.68 g of tetrabutylammonium bisulfate Lot # BCCB8729 in 121 mL MilliQ water).

Aqueous phase was prepared (0.030832 of ammonium acetate Lot# BCBV88695 to 200 mL)

The solution of 0.1 % NH_4OH/MeOH was prepared by adding 1.6 mL ammonium solution (25%) Lot# 1846504 to 400 mL MeOH.

For the 0.01 % NH_4OH/MilliQ-water solution, it was prepared by adding 0.04 mL ammonium solution to 100 mL MilliQ-water.

The 20 % methanol solution was prepared by adding 20 mL MeOH to 80 mL MilliQ-water.

Ammonium acetate buffer (pH 4) was prepared by first making a solution of ammonium acetate (0.025 mM), adding 60 mL water to 0.1185 g ammonium acetate. Then, 349.5 mL of MilliQ-water was weighed on a scale and added to 0.5 mL acetic acid creating an acetic acid solution. Thereafter, 50 mL of the 0.025 mM ammonium acetate solution was mixed with the acetic acid solution to a total volume of 250 mL. The pH of the solution was controlled using pH-paper. Acetic acid solution, as well as, concentrated acetic acid was added until the color corresponded with that of pH 4.

Test of the method (reference material):

Before the analysis of the sample crayfish, a test of the method was performed using muscle samples from fish. The15 mL PP-tubes were cleaned using methanol (HPLC grade). Approximately 0.25 g of fish sample (MH-17-013:35) were added to each of the PP-tubes and thereafter 10 µL of IS was added to all PP-tubes in fraction A. Two mL of tetra-n-butyl hydrogen sulfate (TBA) solution was added to each of the tubes, followed by the addition of five mL methyl tert-butyl methyl ether (MTBE, HPLC grade) in each of the tubes. The tubes were then placed horizontally on a shaker for 15 minutes at 600 rpm and thereafter the tubes were placed in the centrifuge at 8000 g for ten minutes. This separates the organic solvent phase (in which the PFASs, if present, are dissolved) from the aqueous phase and gives a supernatant of organic solvent phase. The supernatant was then removed using a pasteur pipette and transferred to a new PP-tube. In the aqueous phase the inorganic fluorine would be dissolved, making it important not to accidentally pipette any of the aqueous phase into the new tubes. Since the combustion ion chromatography (CIC) method cannot distinguish the origin of the fluorine. Thereafter, three mL of MTBE was added to the original PP-tubes and the process of shaking and centrifuging was repeated in the same manner as previously. The supernatant was removed in the same manner as previously as well, and the same process was repeated once more after that. This results in three cycles of MTBE extraction.

Using the RapidVap (30-40ºC, 400 mBar), the samples were evaporated down to 0.2 mL in the PP-tubes. Thereafter, the volume was adjusted to one mL with methanol using Pasteur pipettes. The samples were then evaporated down to 0.5 mL, vortexed and centrifuged for two minutes at 8000 g. The supernatant was transferred to flat bottomed LC-vials. A volume of 200 µL methanol was added into the PP-tubes, these were then vortexed and centrifuged in the same manner as previously. The supernatant was then transferred to the corresponding LC-vials and the volume was evaporated down to 250 µL (250 µL MeOH in a new vial was used as reference to be able to determine the volume of the samples). The B fraction was transferred to the corresponding vials for CIC analysis, 120 µL of each sample and weighed. Aqueous phase was added to fraction A in the LC vials, 375 µL was added to each of the vials, as well as 10 µL RS. Fraction A was centrifuged, first at 6000 g for 1 min (to see if the vials break), then for 10 min at 6000 g. The samples were cloudy and to achieve clear extracts possible to inject on the LC-system, the samples were put in the freezer (-20ºC) overnight and then centrifuged at 6000 g for 10 minutes. Thereafter, the samples were filtered using a filter and syringe, pre-cleaned with methanol.

Extraction - Batch 1 method 1

The samples (A:001, A:002, A:003, A:004, B:001, B:002, B:003 and B:004) were thawed and four 15 mL PP-tubes were cleaned using methanol (HPLC-grade). The blanks were prepared by adding one mL MilliQ-water to one PP-tube (Blank-A1, BA1) and two mL MilliQ-water to another (Blank-B1, BB1). The reference samples (QCA1, QCB1) were weighed to approximately 0.25 g, exact weights can be found in Table 8. Thereafter, 2000 pg of internal standard (IS) was added to the 40% and 80% batch standards, as well as the samples of fraction A. The batch standards were then spiked with 5 µL native standard (CS-mix 205, 493 and IS-mix 215).

Two mL of tetra-n-butyl hydrogen sulfate (TBA) solution was added to each of the tubes, followed by the addition of five mL methyl tert-butyl methyl ether (MTBE) HPLC grade in each of the tubes. The tubes were then placed horizontally on a shaker for 15 minutes at 600 rpm and thereafter the tubes were placed in the centrifuge at 8000 g for ten minutes. This resulted in a supernatant of organic solvent phase in which the PFASs, if present, resided. The organic solvent phase was transferred into a new 15 mL PP-tube. Thereafter, three mL MTBE solvent was added to each of the original 15 mL PP-tubes and the shaking as well as the centrifuging cycle was repeated. Another MTBE solvent extraction was performed as described above, resulting in a total of three cycles. The supernatant of each of the MTBE extraction cycles were combined in the new 15 mL PP-tubes to a total volume of approximately 10 mL.

Using the RapidVap (40 ºC, ≤400 mBar the pressure was lowered to speed up the evaporation), all samples were evaporated down to approximately 0.2 mL and the volume was adjusted to 1 mL using an automatic pipette. Thereafter, the samples were vortexed and the fraction A samples were evaporated down to 0.2 mL. The fraction B samples were evaporated down to 0.5 mL and both fractions were ultrasonicated for ten minutes following the evaporation step. All samples were then centrifuged for two minutes at 8000 g and

transferred into LC-vials. 0.2 mL methanol was added to each of the PP-tubes, these were then vortexed and centrifuged (2 minutes 8000 g). The supernatant was then transferred into the corresponding LC-vial to a total volume of approximately 0.4 mL (fraction A) and approximately 0.7 mL (fraction B). Thereafter, fraction A was evaporated down to 0.2 mL and fraction B was evaporated down to 0.3 mL inside the LC-vials using the RapidVap (60 ºC, ≤400 mBar), as well as two LC-vials containing 0.2 mL and 0.3 mL respectively, as a reference of volume for the evaporation. The vials were then vortexed, ultrasonicated for five minutes and then centrifuged for ten minutes at 6000 g. For the extractable organofluorine analysis, 120 µL of the extract was transferred from the LC-vial to a CIC-vial and the remaining 180 µL was stored in the LC-vials. The samples aimed for target analysis were split into 40% MeOH sample, 80% MeOH sample and the remainder was left for storage. To the 40% MeOH samples, 50 µL extract, 2.5 µL RS and 72.5 µL aqueous phase was added. To the 80% MeOH samples, 50 µL extract, 2,5 µL RS, 25 µL aqueous phase and 47.5 µL MeOH was added. Approximately,100 µL extract remained as storage in the LC-vials.

These samples were still oily and became cloudy when the aqueous phase was added, making them not injectable on the LC. The samples were therefore centrifuged for ten minutes and then frozen overnight in -20 ºC. In the morning, the sample vials were ultrasonicated for ten minutes and then centrifuged again. The samples were still cloudy, which resulted in them being filtered using a 0.22 µm filter and three mL syringe into new maximum recovery vials. After filtration and centrifugation (10 min, 8000g), the samples were injectable on the LC.

Batch 1- Preparation for SPE cleanup

The storage extract of fraction A for batch 1 was vortexed and ultrasonicated before it was transferred into a 15 mL PP-tube. Then the vials were rinsed using methanol which was then transferred to the corresponding PP-tube. Thereafter, ten mL MilliQ-grade water was added to each of the PP-tubes.

Extraction - Batch 2 & 3

The samples were extracted using three cycles of MTBE extraction, in the same manner as previously. Thereafter, one mL of methanol was added to each of the PP-tubes and then the samples were vortexed and ultrasonicated for five minutes. The samples of fraction A were thereafter evaporated down to < 1 mL and the fraction B samples were evaporated down to approximately 0.5 mL. Then ten mL MilliQ-grade water was added to the fraction A samples.

SPE Cleanup – Fraction A from Batch 1, 2 & 3

The cartridges (6 cc, 150 mg) were conditioned by adding 16 mL of the 0.1 % ammonium hydroxide solution in methanol to the cartridges and letting it pass through. This was done using a 5 mL automatic pipette and sections of four mL added four times. The second conditioning step was done by passing four mL methanol through the cartridges. Thereafter, four mL of MilliQ-water was passed through the cartridges. The samples were then loaded onto the cartridges (1-2 drops/s). Then the PP-tubes were rinsed with 0.5 mL methanol and transferred to the corresponding PP-tubes while there was approximately five mL sample still

in the cartridges. Washing of the cartridges was done by passing ten mL MilliQ-water through the cartridge, then four mL 20% methanol was passed through and then the cartridges were dried under vacuum for 30 minutes. The cartridges were covered with aluminum foil in between addition of the different solutions, to prevent contamination.

Fraction 1 was eluted with four mL methanol and collected in 15 mL PP-tubes. Thereafter, fraction 2 was eluted with four mL 0.1 % ammonium hydroxide in methanol and the fraction was collected in 15 mL PP-tubes. The samples of both fraction one and two were evaporated down to approximately 0.5 mL in the PP-tubes. Thereafter, the extracts were centrifuged before the supernatant was transferred into new LC-vials. Then the PP-tubes were rinsed by adding 0.2 mL methanol, followed by centrifugation and then the supernatant was transferred into the corresponding LC-vials. The extracts were evaporated down to 0.2 mL in the LC-vials, using a LC-vial with 0.2 mL methanol as volume reference.

6.2 Tables

Table 9. Shows the concentration of anionic compounds eluted in fraction 2 of the solid-phase extraction.

Fraction 2 (ng/g)	**A001**	**A002**	**A003**	**A004**	**A005**	**A006**	**A007**	**A008**	**A009**	**A010**	**A011**
PFBA	<0.700	<0.700	<0.700	<0.700	0.862	<0.700	<0.700	<0.700	<0.700	<0.700	<0.700
PFPeA	<0.104	<0.104	<0.104	<0.104	<0.104	<0.104	<0.104	<0.104	<0.104	<0.104	<0.104
PFBS	<0.144	<0.144	<0.144	<0.144	<0.144	<0.144	<0.144	<0.144	<0.144	<0.144	<0.144
PFHxA	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	4.49	<0.006	4.32
PFHpA	0.160	<0.009	<0.009	<0.009	0.521	<0.009	<0.009	<0.009	<0.009	<0.009	<0.009
PFHxS	0.557	0.534	0.176	0.847	1.44	0.717	2.78	0.663	1.20	1.40	0.883
PFHpS	<0.006	<0.006	<0.006	<0.006	<0.006	0.424	0.421	0.258	0.592	0.363	1.13
PFOA	4.64	1.16	1.01	7.66	8.33	1.18	15.0	3.38	5.59	8.09	2.53
PFNA	1.61	0.70	1.11	6.51	5.82	2.70	5.61	3.10	4.54	5.66	2.17
PFOS99	4.96	12.8	2.98	16.1	22.4	19.4	17.1	12.0	14.7	20.8	26.4
PFDA	1.07	1.91	1.95	3.59	2.67	1.91	4.04	2.76	2.38	2.58	2.25
PFUnDA	1.35	1.73	6.00	4.80	3.63	3.36	5.91	4.19	3.63	2.73	5.72
PFDS	<0.040	0.113	<0.040	<0.040	<0.040	<0.040	<0.040	<0.040	<0.040	<0.040	<0.040
PFDoDA	0.981	3.94	3.03	2.21	1.77	2.11	2.74	<0.035	<0.035	1.35	<0.035
PFTrDA	2.33	2.69	8.71	5.23	5.02	5.16	3.27	<0.014	<0.014	4.42	<0.014
PFTeDA	0.917*	3.86*	2.56	1.25*	1.46	1.38	2.14*	<0.066	<0.066	0.907	<0.066
PFHxDA	<0.623	<0.623	<0.623	<0.623	<0.623	<0.623	<0.623	<0.623	<0.623	<0.623	<0.623
6_2_FTSA	0.239	0.450*	<0.016	<0.016	<0.016	<0.016	<0.016	<0.016	0.034	0.086	0.035
6_2FTUCA293	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026	<0.026
6_2FTUCA243	<0.009**	<0.009**	<0.009**	<0.009**	<0.009**	<0.009**	<0.009**	<0.009**	<0.009**	<0.009**	<0.009**
10_2_FTUCA493	<0.032**	<0.032**	<0.032**	<0.032**	<0.032**	<0.032**	<0.032**	<0.032**	<0.032**	<0.032**	<0.032**
10_2_FTUCA243	<0.032**	<0.032**	<0.032**	<0.032**	<0.032**	<0.032**	<0.032**	<0.032**	<0.032**	<0.032**	<0.032**
PFPeS	<0.011**	<0.011**	<0.011**	<0.011**	<0.011**	<0.011**	<0.011**	<0.011**	<0.011**	<0.011**	<0.011**
PFDoDS	<0.098**	<0.098**	<0.098**	<0.098**	<0.098**	<0.098**	<0.098**	<0.098**	<0.098**	<0.098**	<0.098**
PFNS	<0.015**	<0.015**	<0.015**	<0.015**	<0.015**	<0.015**	<0.015**	<0.015**	<0.015**	<0.015**	<0.015**
4_2_FTSA	<0.090**	<0.090**	<0.090**	<0.090**	<0.090**	<0.090**	<0.090**	<0.090**	<0.090**	<0.090**	<0.090**
8_2_FTSA	<0.133**	<0.133**	<0.133**	<0.133**	<0.133**	<0.133**	<0.133**	<0.133**	<0.133**	<0.133**	<0.133**
PFOcDA	<0.017**	<0.017**	<0.017**	<0.017**	<0.017**	<0.017**	<0.017**	<0.017**	<0.017**	<0.017**	<0.017**

*Semi-quantifiable compounds.

**Lowest point of calibration was used for LOQ of the respective compounds.

Table 10. Concentration of the different subclasses of PFASs.

Concentration (ng/g)	**:001**	**:002**	**:003**	**:004**	**:005**	**:006**	**:007**	**:008**	**:009**	**:010**	**:011**
Short-chain PFCAs	0.00	0.00	0.00	0.00	0.86	0.00	0.00	0.00	4.49	0.00	4.32
Long-chain PFCAs	13.1	16.0	24.4	31.2	29.2	17.8	38.7	13.4	16.1	25.7	12.7
Short-chain PFSAs	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Long-chain PFSAs	5.52	13.5	3.16	17.0	23.9	20.5	20.3	12.9	16.5	22.6	28.4
FTSAs	0.239	0.450	0.000	0.000	0.000	0.000	0.000	0.000	0.034	0.086	0.035
Neutral compounds	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
∑PFASs (ng/g)	18.9	29.9	27.5	48.2	54.0	38.3	59.0	26.4	37.2	48.4	45.4

Table 11. Concentration of neutral compounds eluted in fraction 1 of solid-phase extraction.

Fraction 1 (ng/g)	**A001**	**A002**	**A003**	**A004**	**A005**	**A006**	**A010**	**A011**
FBSA	0.05	<0.041	<0.041	<0.041	<0.041	<0.041	<0.041	<0.041
MeFBSA	<0.005**	<0.005**	<0.005**	<0.005**	<0.005**	<0.005**	<0.005**	<0.005**
PFHxSA	<0.101**	<0.101**	<0.101**	<0.101**	<0.101**	<0.101**	<0.101**	<0.101**
MePFHxSA	<0.020**	<0.020**	<0.020**	<0.020**	<0.020**	<0.020**	<0.020**	<0.020**
PFOSA	<0.052**	<0.052**	<0.052**	<0.052**	<0.052**	<0.052**	<0.052**	<0.052**

**Lowest point of calibration was used for LOQ of the respective compounds.

Table 12. Concentration of target PFASs in relation to values of extractable organic fluorine, with procedural blank correction. The percentage of identified PFAS (iPFAS) in relations to unidentified organofluorine (UOF) is also shown.

With blank corr.	Target PFAS (ng F/g)	EOF analysis (ng F/g)	iPFAS	UOF
:001	3.63	30.7	12%	88%
:002	6.43	25.8	25%	75%
:003	5.96	75.2	8%	92%
:004	10.6	151	7%	93%
:005	28.5	79.2	36%	64%
:006	17.4	58.3	30%	70%
:007	26.2	22.1	118%	-18%
:008	10.0	24.9	40%	60%
:009	19.2	15.1	128%	-28%
:010	24.7	40.5	61%	39%
:011	18.9	1.68	1125%	-1025%
QC1	22.3	21.2	105%	-5%
QC2	51.0	129	39%	61%
QC3	49.8	-122	-41%	141%

Table 13. Concentration of target PFASs in relation to values of extractable organic fluorine, without procedural blank correction. The percentage of identified PFAS (iPFAS) in relations to unidentified organofluorine (UOF) is also shown.

Without blank corr.	Target PFAS (ng F/g)	EOF analysis (ng F/g)	iPFAS	UOF
:001	3.81	38.2	10.0%	90.0%
:002	6.83	29.0	23.5%	76.5%
:003	6.08	78.6	7.74%	92.3%
:004	11.0	160	6.88%	93.1%
:005	29.4	163	18.1%	81.9%
:006	23.9	85.2	28.0%	72.0%
:007	27.2	41.3	65.9%	34.1%
:008	11.6	41.9	27.7%	72.3%
:009	20.4	45.2	45.2%	54.8%
:010	26.6	82.5	32.2%	67.8%
:011	21.8	25.4	85.6%	14.4%
QC1	22.3	56.4	39.6%	60.4%
QC2	51.0	255	20.0%	80.0%
QC3	49.8	99.7	50.0%	50.0%

Table 14. The concentration of ng F/g in the procedural blanks.

	C (ng F/g)
Blank 1	4.89
Blank 2	18.8
Blank 3	36.2

Table 15. The average of the blank concentration and standard deviation for the PFASs, used to calculate limit of detection, limit of quantification and the relative standard deviation.

Concentration (ng/g)	Average blank concentration	Standard deviation	LOD	LOQ	Relative standard deviation (%)
PFBA	0.088	0.061	0.271	0.700	70.0
PFPeA	0.008	0.010	0.037	0.104	125.5
FBSA	0.004	0.004	0.015	0.041	82.5
PFBS	0.020	0.008	0.045	0.103	40.3
MeFBSA	0	0	0	0	0
PFHxA	0.003	0.0003	0.004	0.006	8.47
4:2 FTSA	0	0	0	0	0
PFHpA	0.005	0.0004	0.006	0.009	9.80
PFPeS	0	0	0	0	0
PFHxS	0.009	0.004	0.021	0.047	40.1
PFHpS	0.0008	0.001	0.003	0.006	68.4
PFOA	0.071	0.032	0.168	0.393	45.3
PFNA	0.004	0.002	0.011	0.028	53.2
PFOSA	0	0	0	0	0
PFOS99	0.021	0.014	0.064	0.164	68.8
PFDA	0.004	0.001	0.007	0.013	18.9
PFUnDA	0.011	0.005	0.025	0.059	44.5
PFNS	0	0	0	0	0
PFDS	0.004	0.004	0.015	0.040	81.0
PFDoDA	0.013	0.002	0.020	0.035	16.3
PFTrDA	0.004	0.001	0.007	0.014	27.7
PFDoDS	0	0	0	0	0
PFTDA	0.014	0.005	0.030	0.066	36.3
PFHxDA	0.151	0.047	0.293	0.623	31.2
PFOcDA	0	0	0	0	0
6:2 FTSA	0.002	0.001	0.006	0.016	95.2
6:2 FTUCA293	0	0	0	0	0
6:2 FTUCA243	0	0	0	0	0
PFHxSA	0	0	0	0	0
MePFHxSA	0	0	0	0	0
10:2 FTUCA493	0	0	0	0	0
10:2 FTUCA243	0	0	0	0	0
8:2 FTSA	0	0	0	0	0