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DEPARTMENT OF CHEMISTRY

DOCTORAL THESIS

"Development of methods for the determination of new per- and polyfluoroalkyl substances in environmental samples and assessment of their environmental risk"

ANDREAS ANDROULAKAKIS

M.Sc. CHEMIST

ATHENS, 31 MAY 2022

ΕΘΝΙΚΟ ΚΑΙ ΚΑΠΟΔΙΣΤΡΙΑΚΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΑΘΗΝΩΝ

ΣΧΟΛΗ ΘΕΤΙΚΩΝ ΕΠΙΣΤΗΜΩΝ

ΤΜΗΜΑ ΧΗΜΕΙΑΣ

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ABSTRACT

Per- and polyfluoroalkyl substances (PFASs) are a class of persistent organic pollutants (POPs). They are widely used in industrial and consumer applications and are known for their persistence, long-distance migration and toxicity. Various PFAS have been manufactured and distributed over the years at global scale. Decades of relevant research on these emerging contaminants has revealed that PFAS are bio-accumulative and possibly carcinogenic to animals as well as humans. Following regulations and public concern about their impact on ecosystems and uncertain environmental fate, many legacy PFAS have been banned and the industrial production has switched to alternative fluoroalkyl substances. Recently, novel PFAS classes have been identified in numerous environmental matrices. The high variety of legacy and emerging PFAS across the ecosystems is alarming and calls for an efficient monitoring strategy for the quantitative determination of known substances as well as the elucidation and discovery of new compounds. This is crucial for PFAS management and risk assessment in the environment and merits the attention of regulators.

The objective of the thesis was to develop novel workflows employing state-of-the-art target and suspect screening tools and apply them on samples obtained from important European ecosystems such as the Danube River Basin (DRB), the Black Sea (BlS), the North Sea (NS), and the Baltic Sea (BS).

In this context, an introduction on PFAS, workflows and techniques for their identification is presented in Chapter 1. Various instrumental analysis techniques and screening approaches are presented, explored, and compared on basis of their efficiency and applicability, together with sampling, pre-treatment and extraction methods in air, water, abiotic solid matrices and biological matrices. The scope of the thesis is elaborated in Chapter 2. Chapter 3 outlines the development of an LC/MS-MS methodology for the determination of 29 target PFAS in trace quantities. This method was applied on water samples from Austria. Chapter 4 describes the multiresidue and highly sensitive target screening workflow utilizing liquid chromatography tandem mass spectrometry (LC/MS-MS) capable to quantify compounds at the picogram range. The fully validated method was applied to 65 recent specimens of a terrestrial apex predator (Common buzzard), freshwater and marine apex predators (Eurasian otter, harbour porpoise, grey seal, harbour seal) and their potential prey (bream, roach, herring, eelpout) from northern Europe (United Kingdom, Germany, the Netherlands and Sweden). 56 compounds from 14 classes were measured. In Chapter 5, the occurrence of 4,777 PFAS in the Danube river basin (DRB) was investigated by target and suspect screening. Target screening involved the investigation of PFAS with reference standards, as described in the LC/MS-MS method covered in the previous chapter. Suspect screening covered 4,777 PFAS retrieved from the NORMAN

Substance Database, including all individual PFAS lists submitted to the NORMAN network. PFAS having a risk score above 1 in at least one matrix were prioritized.

SUBJECT AREA

Analytical Chemistry

KEYWORDS

Emerging contaminants, PFAS, high-resolution mass spectrometry (HRMS), liquid chromatography tandem mass spectrometry (LC-MS/MS), wide-scope suspect screening, ecotoxicological risk assessment

ΠΕΡΙΛΗΨΗ

Οι πολυφθοριωμένες ενώσεις (PFAS) αποτελούν μια κατηγορία εμμενόντων οργανικών ρύπων (POPs). Είναι ευρέως χρησιμοποιούμενες σε πληθώρα βιομηχανικών και οικιακών εφαρμογών, ενώ διακρίνονται για τη μακρά διατήρησή τους στο περιβάλλον, την ικανότητά τους να διασπείρονται σε μεγάλες αποστάσεις από τα σημεία εκπομπής τους, καθώς και για τον ισχυρά τοξικό τους χαρακτήρα. Χιλιάδες πολυφθοριωμένες ουσίες έχουν συντεθεί και χρησιμοποιηθεί συστηματικά τα τελευταία χρόνια, σε παγκόσμια κλίμακα. Έρευνες των τελευταίων δεκαετιών πάνω στους συγκεκριμένους ρύπους έχουν καταδείξει πως οι πολυφθοριωμένες ουσίες είναι βιοσυσσωρευόμενες και πιθανώς καρκινογόνες για τα ζώα και τους ανθρώπους. Εφαρμόζοντας σχετικές νομοθεσίες και λόγω της αβεβαιότητας που χαρακτηρίζει τις ακριβείς επιπτώσεις τους στα οικοσυστήματα, πολλές εξ αυτών έχουν παύσει να παράγονται. Απεναντίας, νέες πολυφθοριωμένες ενώσεις παράγονται διαρκώς τα τελευταία χρόνια και χρησιμοποιούνται για την αντικατάσταση των ήδη απαγορευμένων, αρχικών ομολόγων τους. Πρόσφατα, πλειάδα νέων πολυφθοριωμένων ουσιών έχουν ανιχνευθεί σε διαφορετικά περιβαλλοντικά υποστρώματα. Ο μεγάλος αριθμός παλαιότερων αλλά και νεότερων πολυφθοριωμένων ρύπων στο περιβάλλον είναι πολύ ανησυχητικός και καθιστά επιτακτική την ανάγκη πλαισίου ιχνηλάτησης, παρακολούθησης και καταγραφής τους, με απώτερο σκοπό τον ποσοτικό προσδιορισμό των ήδη γνωστών ενώσεων και την ταυτοποίηση υπόπτων ή άγνωστων πολυφθοριωμένων ρύπων σε περιβαλλοντικά δείγματα. Αυτό είναι κρίσιμο για τη διαχείριση των εν λόγω ουσιών και την αποτίμηση του περιβαλλοντικού τους κινδύνου.

Κύριος στόχος της παρούσας διατριβής είναι η ανάπτυξη εργαλείων ολοκληρωμένης χημικής ανάλυσης των πολυφθοριωμένων ενώσεων και η εφαρμογή τους σε σημαντικά ευρωπαϊκά οικοσυστήματα όπως η λεκάνη απορροής του Δούναβη, η Μαύρη Θάλασσα, η Βόρειος Θάλασσα και Βαλτική.

Στα πλαίσια του σκοπού αυτού, στο Κεφάλαιο 1 εισάγονται οι πολυφθοριωμένοι ρύποι και οι τεχνικές ταυτοποίησής τους, ακολουθούμενοι από λεπτομερή περιγραφή των στόχων της διατριβής στο Κεφάλαιο 2. Στο Κεφάλαιο 3 περιγράφεται η ανάπτυξη μεθόδου για τον προσδιορισμό 29 πολυφθοριωμένων ουσιών σε υδατικά δείγματα από την Αυστρία. Στο Κεφάλαιο 4 περιγράφεται μια πλήρης πορεία στοχευμένης ανάλυσης για τον ποσοτικό προσδιορισμό 56 πολυφθοριωμένων ουσιών από 14 κλάσεις σε 65 δείγματα ιστών θηρευτών και της λείας τους από την Κεντρική και Βόρειο Ευρώπη. Για τον σκοπό αυτό αναπτύχθηκε και επικυρώθηκε πλήρως τεχνική υγρής χρωματογραφίας συζευγμένη με φασματομετρία μαζών χαμηλής διακριτικής ικανότητας (LC/MS-MS).

Στο Κεφάλαιο 5 ερευνάται η ύπαρξη 4,777 πολυφθοριωμένων ουσιών στη λεκάνη απορροής του Δούναβη με στοχευμένη και ύποπτη σάρωση. Η στοχευμένη σάρωση συμπεριέλαβε 56

πρότυπες ουσίες, καθώς και το αναλυτικό πρωτόκολλο που περιγράφεται στο προηγούμενο κεφάλαιο. Η ύποπτη σάρωση έκανε χρήση 4,777 καταχωρημένων ουσιών στη βάση δεδομένων του δικτύου NORMAN, η οποία περιλαμβάνει όλες τς λίστες καταγραφής πολυφθοριωμένων ουσιών που έχουν κατατεθεί στον οργανισμό. Η πορεία αυτή χρησιμοποιήθηκε για την ανίχνευση νέων πολυφθοριωμένων ουσιών σε πλειάδα διαφορετικών δειγμάτων προερχόμενων από τη λεκάνη απορροής του Δούναβη με σκοπό την προτεραιοποιήσή τους.

ΘΕΜΑΤΙΚΗ ΠΕΡΙΟΧΗ

Αναλυτική Χημεία

ΛΕΞΕΙΣ ΚΛΕΙΔΙΑ

Αναδυόμενοι ρύποι προτεραιότητας, πολυφθοριωμένες ενώσεις, φασματομετρία μαζών υψηλής διακριτικής ικανότητας, LC-MS/MS, ευρεία ύποπτη σάρωση, αποτίμηση περιβαλλοντικού κινδύνου

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Finally, I would like to thank my partner, my parents, grandparents, and sister for their continuous support and their encouragement throughout my PhD and my life in general.

to my family

PREFACE

All the experimental parts of this thesis were conceived and performed at the Laboratory of Analytical Chemistry, Department of Chemistry of the National and Kapodistrian University of Athens, Greece, under the supervision of Dr. Nikolaos Thomaidis.

The data processing work for the suspect screening case study was conducted between the Laboratory of Analytical Chemistry, Department of Chemistry of the National and Kapodistrian University of Athens (Greece) and the laboratory of Environmental Institute (Slovakia).

An extended Electronic Supplementary Material of 30 pages, consisting of 7 Sections (including 1 Figure and 10 Tables) is also available along with this thesis at http://users.uoa.gr/~androulakakis/PhD/.

The first experimental part of this doctoral thesis (Chapter 2) has been financed by the European Union through the LIFE APEX project (LIFE17 ENV/SK/000355, https://lifeapex.eu/)

Samples presented in Chapter 3 were collected in cooperation with TU Vienna.

The study presented in Chapter 5 was implemented under the Joint Danube Survey Program (http://www.danubesurvey.org) and was funded by the European Union.

Samples for this case study were collected in cooperation with International Commission for the Protection of the Danube River (ICPDR).

Last but not least, I would like to express my gratitude to The Praxis Network within The Foundation for Research and Technology - Hellas (FORTH) for supporting me throughout the last part of my PhD studies.

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CHAPTER 1: Poly- and perfluoroalkyl substances (PFAS): screening methods for their environmental analysis

This chapter has been submitted for publication as a review paper in Environmental Science: Advances

1.1. Introduction

Per- and poly-fluoroalkyl substances (PFAS) are a group of highly fluorinated aliphatic chemicals that have been broadly used in several industrial and household applications since the 1940s, due to their high stability, and water/lipid resistance **[1]**. PFAS are identified as emerging contaminants **[2]** and are persistent, bio-accumulative and possibly carcinogenic to animals as well as humans **[3]**. The increased half-lives of these chemicals in both wildlife and humans render them to extremely hazardous for the environment **[4]**. Therefore, the monitoring of PFAS is essential to promote environmental regulation and to assess their environmental fate. However, because of their unique physicochemical properties PFAS tend to accumulate at trace levels in various environmental matrices, which hinders the analytical procedure for their identification and quantification. Several analytical regimes have been developed for the determination of PFAS in a various matrices, including sediments, ground- and freshwater **[5, 6]**, fish and other aquatic organisms **[7-9]**, birds **[10-12]** and mammals **[13-15]**.

Solid phase extraction (SPE) and liquid–liquid extraction (LLE) are the techniques that have been applied in the extraction, purification and pre-concentration of PFAS in environmental samples in the recent years **[16-18]**. The phase-out of the legacy compounds and their replacement with

structurally similar PFAS has been the most common industry policy in the last decades **[19, 20]**. This fact poses a great environmental danger, since most emerging PFAS also show high toxicity, yet are to this day not routinely monitored or part of any regulatory guideline **[21]**. Additionally, many PFAS undergo transformation in wastewater treatment plants as well as metabolic alteration in humans and livestock **(Table 1)**. Up to this day there are nearly 5000 PFAS that are broadly used in several industrial and commercial applications **[22]**. Currently, targeted methodologies only cover a small fraction of the existing PFAS, while the vast majority of the PFAS precursors remains unknown **[23]**. Establishing analytical approaches that employ untargeted screening to elucidate and, ultimately, identify unknown PFAS is a necessary step toward the understanding of the full scale environmental risk and hazardous potential of these contaminants. Up to this day, liquid chromatography (LC) coupled with mass spectrometric (MS) or tandem mass spectrometric (MS/MS) detection is the golden standard for targeted PFAS determination **[24-26]**; Profiting from the latest improvements in the field of high resolution mass spectrometry (HRMS), novel PFAS have been continuously identified in a variety of environmental matrices **[27]**. Recently, time-of-flight mass spectrometry (TOF-MS), Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) and orbitrap instrumentations have been utilized for structure proposal of unknown PFAS molecules from more than twenty established classes **[28]** and contribute to the developing field of emerging PFAS discovery via suspect and non-target screening **[29]**.

In the following sections the most commonly used instrumental analysis approaches and extraction methods per matrix type, literature reported limits of detection and quantification, as well as sampling techniques are presented.

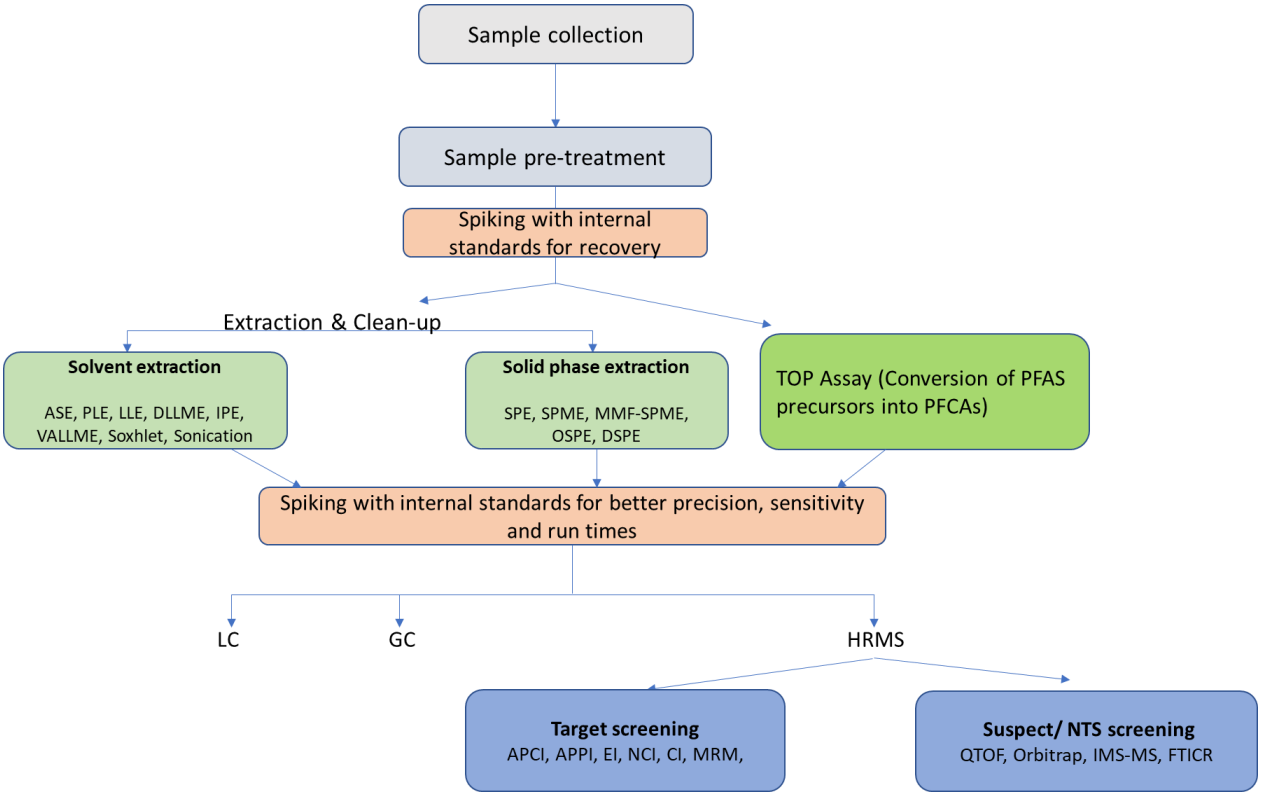


Figure 1. *Graphic illustration of PFAS analyses regimes.*

1.2. Air samples

Ionic and zwitterionic perfluoroalkyl acids (PFAAs) as well as neutral fluorotelomer alcohols (FTOHs) are the two most abundant classes in air particles. Both of these PFAS families are considered to be highly volatile and therefore find their way to airborne matter. Perfluoroalkane sulphonamido ethanols (FASEs) and perfluoroalkane sulphonamides (FASAs) have been also identified in air samples. Collected PFAS are measured by gas chromatography-mass spectrometry (GC-MS) or HPLC-MS/MS.

1.2.1. Collection and storage

Both active and passive sampling methods are utilized in the collection of PFAS in air samples **[30]**. Sorbent-impregnated polyurethane foam (SIP) disks and simple polyurethane foam (PUF) disks are the most common tools in the airborne PFAS sampling, thanks to their easy handling and consistent efficiency **[31]**. Simplistic passive air samplers have been allegedly used at site locations where active sampling was not possible **[32]**. For instance, SIP foams have been frequently used as a passive air sampler for air PFAS monitoring **[33]** due to their easy handling and sustainability. In the last decade, high volume PUF samplers equipped with quartz-fibre filters

(QFFs)/ glass-fibre filters (GFFs) and Amberlite® XAD®-2 resin were designed for the acquisition and storage of big particle masses containing volatile FTOHs **[34]**. Conventional low volume active air samplers are equipped with solid phase extraction (SPE) cartridges to enable the collection of PFAS classes with diverse physicochemical properties and volatilities. All purpose, strongly hydrophilic, reversed-phase, water-wettable polymers, such as OASIS HLB (Waters Inc., Milford, MA, USA), or hydroxylated polystyrene-divinylbenzene copolymers, such as and ISOLUTE ENV+ (Biotage AB, Uppsala, Sweden) have been traditionally used for neutral PFAS sampling **[35]**. A two-layer SPE set-up consisting of higher carbon (HC)-C18 and weak anion exchange (WAX) material was recently employed for the sampling of various PFAS classes **[36]**. Typically, the sampling volume for air particle is in the range of 2 to 200 m^3 for indoor spaces and 300 to 2,500 m^3 for outdoor area **[30, 36]**. To detect airborne PFAS in the ppb range, field blanks are also collected during the sampling campaigns, while eliminating background contamination during all stages prior to analysis, including washing, storage and transport and pre-treatment of samplers, is crucial too. Quality control (QC) is an integral element of PFAS analysis in air samples. All samplers are individually placed in polypropylene (PP) containers or wrapped with aluminum foil and stored at -20°C up to the sample extraction stage in the laboratory.

1.2.2. Extraction, clean-up and concentration

In the recent years, mainly SPE cartridges are used for indoor air monitoring, due to their easy and standardizable handling as well as low solvent volumes, especially for the monitoring of polyfluoroalkyl phosphate diesters (diPAPs) **[37]**. In most of the methods, SPE cartridges are precleaned with methanol or ethyl acetate, then dried with nitrogen before use **[35]**. For XAD and PUF methods, preliminary Soxhlet extraction is performed with organic solvents such as methanol and dichloromethane **[38]**. 1:1 mixtures of acetone and petroleum ether have also been employed for the extraction of analytes from SIP discs **[39]**. These disks are generally saturated with XAD-like resins, then stored in solvent-rinsed glass jars until used **[32]**. On the other hand, GFFs and QFFs are individually wrapped with aluminum foil, then baked for 18-24 h at 450°C for the removal of contaminants **[40]**. Additional clean-up utilizing the graphitised carbon sorbent ENVI-Carb (Supelco, Bellefonte, PA, USA) has been used in cases of highly contaminated indoor samples **[41]**.

1.2.3. Instrumental analysis and measurement results

Neutral PFAS are usually determined by GC-MS coupled with electron ionisation (EI) or, in fewer cases, with chemical ionisation (CI) in selected ion monitoring (SIM) mode **[32, 42]**. The golden standard for ionic PFAS detection involves analysis by HPLC-MS/MS with electrospray ionisation (ESI) **[37]**. In both cases negative ionization (ESI-) is preferred. For GC, a WAX column can be

employed to improve analyte separation **[35, 41]**. Ionic PFAS are adequately separated by a C18 column with an aqueous and methanol/acetonitrile mobile phase buffered with 5 mM ammonium acetate. As Gremmel et al. stated, in negative ESI, FTOHs, FASEs and FOSEs form acetate adducts ($[M + Ac]^-$), which are only stable at lower temperatures **[43]**. Therefore, the addition of ammonium acetate to the mobile phase is a common practice, as part of the latest analytical regimes. Particularly for the short-chain (C2-C4) perfluorinated carboxylic acids (PFCAs), Tian et al. suggested the use of an ion exchange column (Shodex RSpak JJ-50 2D; Showa Denko America, Inc., New York, NY, USA) compatible with HPLC **[37]**. According to their results, the ion-exchange resin beads improved the recovery of these substances. Historically, PFAS have been significantly less monitored in air samples than they have been monitored in aqueous and biological samples. Mainly, there are records about airborne PFAS from Central and Northern Europe, Northern America and Antarctica. Overall, perfluoro sulfonic acids (PFSAs) concentrations in outdoor air samples have shown increasing trends in the last decade, unlike FTOHs, FASAs, FASEs and PFCAs, whose levels have been stable for the same period **[44]**. Due to their volatile property, FOTHs comprise the class that has been more thoroughly investigated. Rauert et al. reported that FTOHs were detected at fairly high concentrations during the 2009-2015 Global Atmospheric Passive Sampling (GAPS) survey, ranging from <0.4 to 21 pg/m^3 on the glacial terain, and 40 to 238 pg/m^3 in industrial and urban areas **[44]**. In the framework of another study conducted at the northernmost continuously inhabited place in the world, Alert (Qikiqtaaluk Region, Nunavut, Canada) between 2006 and 2014, FTOHs, FASAs and FASEs were detected at concentrations < 0.17-30 pg/m^3, <0.014-0.82 pg/m^3 and <0.10-4.8 pg/m^3, respectively **[45]**. As for indoor spaces, PFCAs and PFSAs have been detected primarily in households, while FASA/FASE levels were higher in more visited places, such as hotels. In their relevant study from 2018 Yao et al. reported that the yields of these substances were in range of nondetectable (ND) up to almost 2,500 pg/m^3 **[36]**. FTOH numbers, on the other hand, were alarmingly higher, ranging from 246 to 62,100 pg/m^3, as presented in the same case survey.

1.2.4. Discussion

In general, passive samplers coupled to SIP disks are primarily used for the monitoring of outdoor air masses, while SPE cartridges are employed for dust monitoring in closed spaces. Sampling and extraction methodologies for air samples are imporoved for zwitterionic, cationic, and anionic compounds, yet there is space for imporivement regarding emerging PFAS. Nevertheless, the main challenge when monitoring PFAS in air matrices lies in the fact that there is no standardised methodology. Assessing the technical difficulties regarding the collection of indoor as well as outdoor air particles will greatly help towards the development of a universally applicable and standardized sampling protocol. Last but not least, multiresidue analytical methods for anionic and neutral PFAS in particles remain limited. To characterise the environmental fate and long range transport of PFAS, new analytical methods are needed for the less studied air matrices.

1.3. Aqueous matrices

Almost every established PFAS class has been historically detected in several parts the aquatic continuum, including drinking water, surface water, groundwater, wastewater, snowmelt, landfill leachate, brakish as well as sea water **[46-48]**. The Geological Survey of the United States (USGS, https://www.usgs.gov) published in 2006 a detailed description of the different aqueous samples sources, types as well as all available sampling techniques for various aqueous samples up to that point **[49].**

1.3.1. Collection and storage

This report comprises the basis of today's syllabus for sampling, transporting and storing different water samples. Sampling of drinking water, surface water, and wastewater samples is fairly easy and is performed utilizing pre-cleaned buckets or stainless steel grab samplers. As sampling technology advances through the last years various autosamplers are available in the market, for instance Liquiport 2010 CSP44 by Endress and Hauser AG, Reinach, Switzerland **[50]**. Lake and open ocean water samples are collected by more sophisticated circuits, such as a conductivity-temperature-depth meter (CTD) system equipped with narrow, cylindrical, long plastic tubes (commercially available as „Niskin-X bottles") capped at both ends **[2]**. The collecting depth for the samples is specifically delineated in the sampling protocol of each study. As for groundwater sample collection, digging trenches through the soil is the most popular technique. Usually, two samples are collected from the upper aquifer, while at least three samples are collected from the lower aquifer Samples are collected utilizing simple vacuum tubes **[48]**. Typically, the sampling volume ranges between 0.1 and 2 litres, highly depending on the water sample type. Afterwards, samples are transfered to a pre-washed high-density polyethylene (HDPE) [51], or polypropylene (PP) container **[52]**, while in some cases even plain glass bottles may be applicable **[53]**. They remain stored at either 4°C (simple refrigeration) or -20°C (deep freeze) until further analysis **[54]**. Field blanks are HPLC grade water or distilled water, unless otherwise noted in the analytical protocol **[51]**.

1.3.2. Extraction, clean-up and concentration

SPE cartridges have been predominantly used for water samples enrichment and clean-up, as well as improved sensitivity of the analytical procedure **[52, 53, 55]**. HLB series or Strata-X cartridges (Phenomenex, Torrance, CA, USA) are widely employed for targeted multi-class PFAS analysis **[56-58]**. Methanol is frequently used as the elution solvent. For heavily contaminated samples, such as wastewater and sludge matrices, an additional clean-up step with ENVI-Carb

can be applied after SPE clean-up **[51]**. Oasis WAX (Waters, Inc.) and Strata X-AW (Phenomenex) are also used for high through-put PFAS analysis of water samples **[53, 59]**. Some additional techniques that have been applied more sporadically, yet with satisfying recoveries for the extraction of analytes from aqueous matrices are multiple monolithic fibre solid-phase microextraction (MMF-SPME), liquid-liquid extraction (LLE) the and dispersive liquid-liquid microextraction (DLLME). MMF-SPME utilizing a monolith-based adsorbent that generates anion-exchange as well as pfuorophillic interactions with PFCAs was evaluated for the precize detection of long-chain PFCAs in environmental water and milk samples in the pg/L range **[60]**. LLE and its primare ecological application, DLLME, are often applied for the clean-up of PFAS burdened aqueous samples. Recent DLLME approaches **[57, 61]** utilise less extraction solvent, yet achieving sufficient recoveries (80.6% - 121% for house water, river water and urine samples) and relatively low detection limits (0.6 - 8.7 ng/L for water and urine samples). Vortex-assisted liquid-liquid microextraction (VALLME), another recent LLE extension, employs a vortex mixer instead of dispersive solvent, a simpler alternative to the two different solvent systems employed in traditional DLLME methods. This technique was applied for legacy PFSAs and perfluoroalkane sulfonamides (FASAs) analyses in seawater by LC- LTQ-Orbitrap HRMS **[62]**, as well as PFOS determination in tap, river and well water samples **[63]**. A direct injection (DI) approach followed by liquid chromatography tandem mass spectrometry (DI-LC-MS/MS) for targeted PFAS analysis of various water samples including drinking water, ground water, river water, lake water and wastewater has been reported with satisfactory sensitivity has been recently reported **[50]**.

1.3.3. Instrumental analysis and measurement results

The golden standard for instrumental analysis of PFAS in aquatic matrices, especially for target screening, remains HPLC-MS/MS **[52, 54, 59, 60, 64]**. Nevertheless, in some cases HRMS such as orbitrap or time-of-flight (TOF)-MS are employed for quantitative and qualitative analyses **[58, 62]**. MS is generally operated in ESI-negative mode due to the fact that most legacy and emerging PFAS are anionic. Although neutral PFAS such as FASAs, FASEs and FTOHs can still be ionized using ESI, atmospheric pressure chemical ionisation (APCI) and atmospheric pressure photoionisation (APPI) have also been tested **[55]**. Mean concentrations of seven long-chain PFCAs (C6-C12) and two PFSAs (C4 and C6) in Australian wastewater treatment plant (WWTP) influents and effluents were found to be in the range of 0.3 to 25 ng/L **[51]**. The effluent concentration levels were particularly alarming. Elevated levels of iconic PFAS, namely PFOS and PFOA have been repeatedly reported in studies about PFAS monitoring in Asian bodies. An important study involving water samples collected in 2016 from 65 rivers and 34 coastal drain outlets around the Bohai Sea, China **[65]** indicated high PFOS and PFOA levels as well as increasing trends in the concentrations of these two chemicals. Various tap water samples were analysed for the determination of 14 legacy target compounds, including four PFSAs (C4C10) and 10 PFCAs (C5-

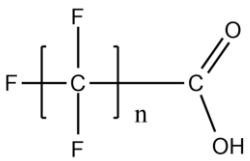
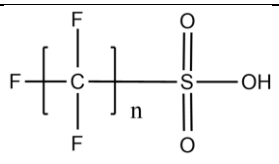
C14), in South Korea in 2017 **[66]**. With reported concentrations up to 189.6 ng/L, this particular case study raised serious concerns about the presence of PFAS in households, hospitals and other human foundations. The investigation of global concentrations, patterns and trends of novel PFAS in aqueous samples, including cyclic PFSAs, perfluoroalkyl phosphonic acids (PFPAs), perfluoroether sulphonic acids (PFESAs) and perfluoroether carboxylic acids (PFECAs) has been a very important topic in the relative literature. Well-known as well as novel PFAS, including 6:2 chlorinated polyfluorinated ether sulphonic acid (6:2 Cl-PFESA, commercially known as F-53B) and 6:2 FTSA, were detected in ground water samples collected from 13 non-industrialised cities in Jiangsu Province, China, were 2.7 - 556 ng/L **[53]**. Hexafluoropropylene oxide dimer acid (HFPO-DA, commercially known as GenX), hexafluoropropylene oxide trimer acid (HFPO-TA) and 6:2 Cl-PFESA, have been widely detected in developed as well as developing countries, such as China, the United States, the United Kingdom, Sweden, Germany, the Netherlands and South Korea. This validates last decade‘s hypothesis that the PFOS and PFOA alternatives‘ levels will eventually be comparable to the original PFAS concentrations in Asia, Europe, and America. The surprisingly high frequency of appearance (FoA) of F-53B (>95%) in several Chinese water bodies has raised the concerns of regulators over the past decade **[67]**. The screening of Short-chain PFCAs is still a developing field of research. In one of the few available publications on that subject, it was reported that (C2-C8) PFCAs were detected in the range of 0.056 mg/ L (PFPrA) to 2.2 mg/L (TFA) from aquifer water collected at polluted sites in the state of Baden-Wuerttemberg, Germany **[56]**. PFOA and TFA were the most abundant analytes. Nevertheless, it has to be noted that some researchers constrict the term „Short-chain PFCAs“ to PFCAs whose molecule’s carbon chain strictly consists of 2 to 5 carbon atoms.

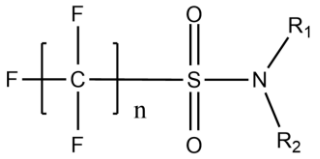
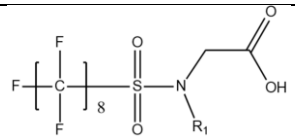
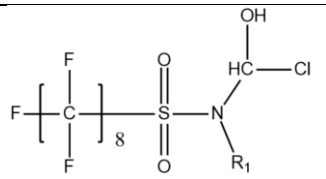
1.3.4. Discussion

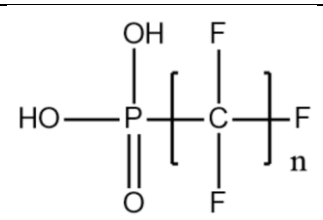
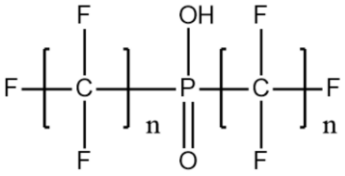
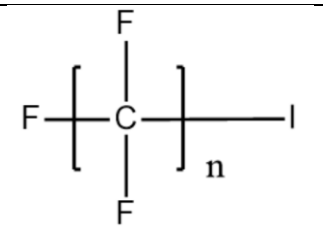
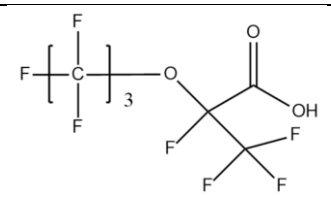
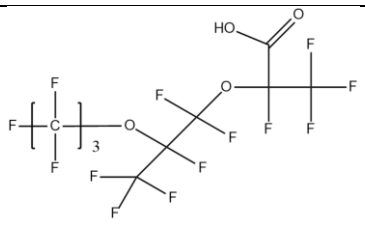
The most notable development for PFAS analysis in aqueous matrices during in the recent years can be observed in the field of extraction and clean-up techniques. More specifically, the specialization of LLE towards the development of DLLME, VALLE as well as micro-SPE significantly decreased the required sample volume and the amount of extraction solvent needed **[58, 62]**, enabling for greener applications of established techniques. Multiresidue and simultaneous analytical methods have been developed and validated for the analysis of a wide range of emerging PFAS, such as 6:2 Cl-PFESA, HFPO-DA and HFPO-TA, together with legacy PFAS. Various technical difficulties remain though, namely the method optimization for all target analytes. This is a multifaceted process and hence still very challenging. Additionally, Short-chain PFAS have been proven to be significantly more affected by matrix effects that cause ionization and signal suppression, resulting in higher LODs **[56]**. Since Short-chain PFAS (C6 or shorter) have been progressively manufactured and used at the global scale as alternatives for long-chain homologues, yet are more volatile and diffusive, more research is in order to determine their

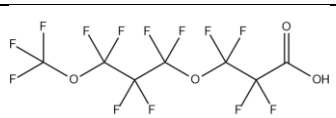
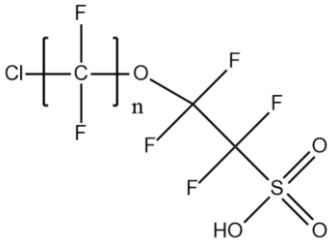
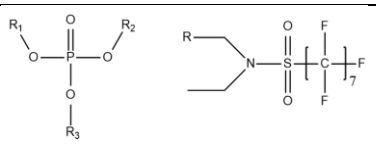
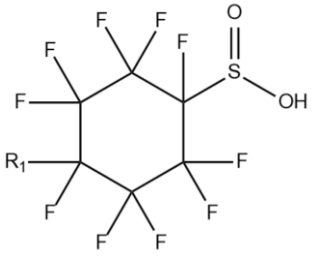
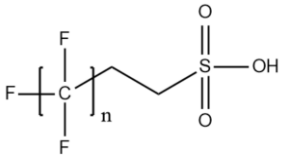
environmental fate and toxicology is a necessity. Valid evidence shows that short-chain PFAS are more likely to accumulate in wastewater treatment facilities **[68]**. Therefore, developing robust and multiresidue analytical methods is essential for grasping the chemical properties of both original and novel PFAS in aqueous matrices.

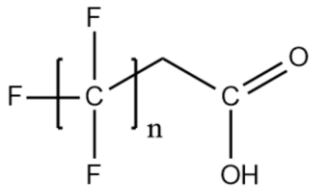
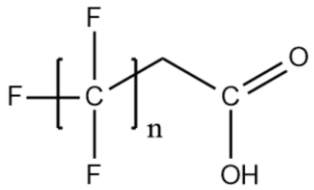
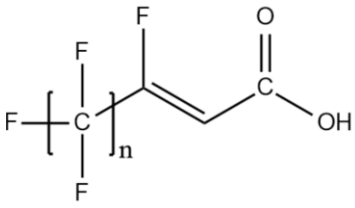
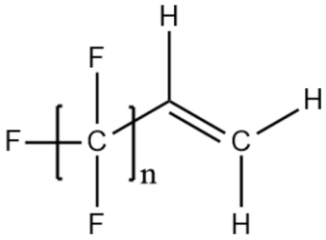
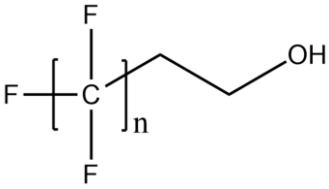
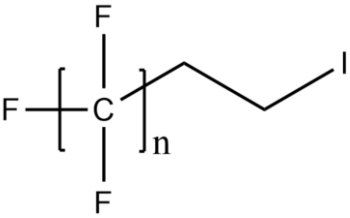
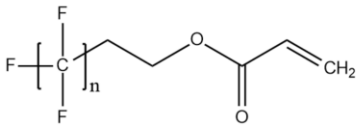
Table 1. *List of poly- and perfluoroalkyl substances (PFAS) and their structure.*

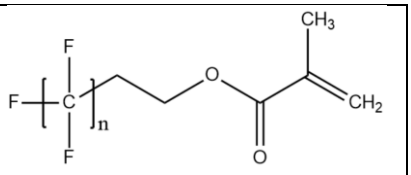
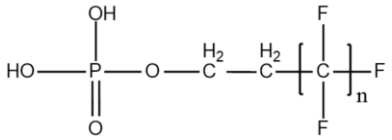
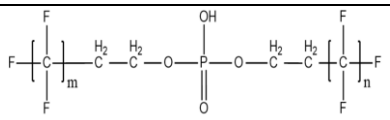
Group	Compound Name	Acronym	Structure
Perfluoroalkyl carboxylic acids (PFCAs)	Trifluoroacetic acid (n = 2)	TFA	
	Perfluoropropanoic acid (n = 3)	PFPrA	
	Perfluorobutanoic acid (n = 4)	PFBA	
	Perfluoropentanoic acid (n = 5)	PFPeA	
	Perfluorohexanoic acid (n = 6)	PFHxA	
	Perfluoroheptanoic acid (n = 7)	PFHpA	
	Perfluorooctanoic acid (n = 8)	PFOA	
	Perfluorononanoic acid (n = 9)	PFNA	
	Perfluorodecanoic acid (n = 10)	PFDA	
	Perfluoroundecanoic acid (n = 11)	PFUnDA	
	Perfluorododecanoic acid (n = 12)	PFDoDA	
	Perfluorotridecanoic acid (n = 13)	PFTrDA	
	Perfluorotetradecanoic acid (n = 14)	PFTeDA	
	Perfluorohexadecanoic acid (n = 16)	PFHxDA	
	Perfluorooctadecanoic acid (n = 18)	PFODA	
Perfluoroalkyl sulphonic acids (PFSAs)	Perfluorobutane sulphonic acid (n = 4)	PFBS	
	Perfluoropentane sulphonic acid (n = 5)	PFPeS	

	Perfluorohexane sulphonic acid (n = 6)	PFHxS	
	Perfluoroheptane sulphonic acid (n = 7)	PFHpS	
	Perfluorooctane sulphonic acid (n = 8)	PFOS	
	Perfluorononane sulphonic acid (n = 9)	PFNS	
	Perfluorodecane sulphonic acid (n = 10)	PFDS	
	Perfluorododecane sulphonic acid (n = 12)	PFDoDS	
Perfluoroalkane sulphonamides (FASAs)	Perfluorooctane sulphonamide (n = 8, R1 = H, R2 = H)	FOSA	
	N-Methyl fluorobutane sulphonamide (n = 4, R1 = H, R2 = H)	MeFBSA	
	N-Methyl fluorooctane sulphonamide (n = 8, R1 = CH3, R2 = H)	MeFOSA	
	N-Ethyl fluorooctane sulphonamide (n = 8, R1 = C2H5, R2 = H)	EtFOSA	
N-Alkyl perfluoroalkane sulphonamido acetic acids (FASAAs)	Perfluorooctane sulphonamidoacetic acid (R1 = H)	FOSAA	
	N-Methyl fluorooctane sulphonamido acetic acid (R1 = CH3)	MeFOSAA	
	N-Ethyl fluorooctane sulphonamido acetic acid (R1 = C2H5)	EtFOSAA	
N-Alkyl perfluoroalkane sulphonamido ethanols (FASEs)	2-(N-Methyl fluorooctane sulphonamido)-ethanol (R1 = CH3)	MeFOSE	
	2-(N-Ethyl fluorooctane sulphonamido)-ethanol (R1 = C2H5)	EtFOSE	

Perfluoroalkyl phosphonic acids (PFPAs)	Perfluorohexane phosphonic acid (n = 6)	PFHxPA	
	Perfluorooctane phosphonic acid (n = 8)	PFOPA	
	Perfluorodecane phosphonic acid (n = 10)	PFDPA	
Perfluoroalkyl phosphinic acids (PFPiAs)	6:6 Perfluoroalkyl phosphinic acid (m = 6, n = 6)	6:6 PFPiA	
	6:8 Perfluoroalkyl phosphinic acid (m = 6, n = 8)	6:8 PFPiA	
	8:8 Perfluoroalkyl phosphinic acid (m = 8, n = 8)	8:8 PFPiA	
Perfluoroalkyl iodides (PFAIs)	Perfluorohexyl iodide (n = 6)	PFHxI	
	Perfluorooctyl iodide (n = 8)	PFOI	
	Perfluorodecyl iodide (n = 10)	PFDI	
Perfluoroether carboxylic acids (PFECAs)	Hexafluoropropylene oxide dimer acid	HFPO-DA (trade name: GenX)	
	Hexafluoropropylene oxide trimer acid	HFPO-TA	

	4,8-Dioxa-3H-perfluorononanoic acid	ADONA	
Perfluoroether sulphonic acids (PFESAs)	6:2 Chlorinated polyfluorinated ether sulphonic acid (n = 6)	6:2 Cl-PFESA (trade name: F-53B)	
	8:2 Chlorinated polyfluorinated ether sulphonic acid (n = 8)	8:2 Cl-PFESA	
	10:2 Chlorinated polyfluorinated ether sulphonic acid (n = 10)	10:2 Cl-PFESA	
Perfluorooctane sulphonamido ethanol-based phosphate esters (SAmPAPs)	Phosphate diester of N-ethylperfluorooctane sulphonamido ethanol (R1 = R, R2 = R, R3 = H)	SAmPAP diester	
	Phosphate triester of N-ethylperfluorooctane sulphonamido ethanol (R1 = R, R2 = R, R3 = R)	SAmPAP triester	
Cyclic perfluoroalkyl sulphonic acids (cyclic PFSAs)	Perfluoromethylcyclohexane sulphonic acids (R1 = CH3)	PFMeCHS	
	Perfluoroethylcyclohexane sulphonic acids (R1 = C2H5)	PFECHS	
Fluorotelomer sulphonic acids (FTSAs)	n:2 Fluorotelomer sulphonic acids (n = 4, 6, 8, 10)	n:2 FTSA	

Fluorotelomer carboxylic acids (FTCAs)	n:2 Fluorotelomer carboxylic acids (n = 6, 8, 10)	n:2 FTCA	
	n:3 Fluorotelomer carboxylic acids (n = 5, 7)	n:3 FTCA	
Fluorotelomer unsaturated carboxylic acids (FTUCAs)	n:2 Fluorotelomer unsaturated carboxylic acids (n = 6, 8, 10)	n:2 FTUCA	
Fluorotelomer olefins (FTOs)	n:2 Fluorotelomer olefins (n = 6, 8, 10)	n:2 FTO	
Fluorotelomer alcohols (FTOHs)	n:2 Fluorotelomer alcohols (n = 4, 6, 8, 10, 12)	n:2 FTOH	
Fluorotelomer iodides (FTIs)	n:2 Fluorotelomer iodides (n = 4, 6, 8)	n:2 FTI	
Fluorotelomer acrylates (FTACs)	n:2 Fluorotelomer acrylates (n = 4, 6, 8, 10, 12)	n:2 FTAC	

Fluorotelomer methacrylates (FTMACs)	n:2 Fluorotelomer methacrylates (n = 6, 8)	n:2 FTMAC	
Polyfluoroalkyl phosphate monoesters (monoPAPs)	n:2 Polyfluoroalkyl phosphate monoesters (n = 4, 6, 8, 10)	n:2 monoPAP	
Polyfluoroalkyl phosphate diesters (diPAPs)	n:2 Polyfluoroalkyl phosphate diesters (m = n = 4, 6, 8, 10)	n:2 diPAP	
	4:2/n:2 Polyfluoroalkyl phosphate diesters (m = 4, n = 4, 6)	4:2/n:2 diPAP	
	6:2/n:2 Polyfluoroalkyl phosphate diesters (m = 6, n = 6, 8, 10, 12, 14)	6:2/n:2 diPAP	
	8:2/n:2 Polyfluoroalkyl phosphate diesters (m = 8, n = 8, 10, 12)	8:2/n:2 diPAP	
	10:2/10:2 Polyfluoroalkyl phosphate diesters (m = 10, n = 10)	10:2/10:2 diPAP	

1.4. Abiotic solid matrices

The extended use of aqueous film-forming foam (AFFF) on firefighting training grounds has been the primare source of PFAS contamination of land areas **[69]**. PFAS research in abiotic solid matrices mainly focuses on the quantitative identification of compounds in various soil matrices **[69, 70]**, while constantly aiming towards the optimization of extraction and clean-up methods for emerging PFAS **[71]**. On the other hand PFAS monitoring in dust is a novel field of research, therefore methods for dust analysis call for further exploration. Finally, earth core and borehole samples have been investigated to estimate time series and temporal trends of legacy and emerging PFAS **[72]**.

1.4.1. Collection and storage

Abiotic solid samples include dust particles, soil, and sediments. Dust samples are generally collected utilizing simple household tools, such as a vacuum cleaner bag or a precleaned bristle brush **[73]**. As soil cores are regarded, the prescribed sampling depth is playing a determining

role. Surface soil sampling is carried out by using a stainless-steel trowel or a shovel precleared with methanol **[74]**. This applies to the top 15 cm of the soil. For a given area, each soil sample generally consists of a series of sub-samples. This is done for representative sampling purposes. Typically, up to five sub-samples are collected from the center of the given site up to 100 m^2 and 1 soil core is extracted from each of the four corners of the area **[75]**. Common tools for surface sediment sampling include a Van Veen grab sampler and Ponar grab sampler, while MC-400 Multi-corer samplers are used for the collection of more complex core samples **[76]**. Such samplers may capture the sedimentation process of several decades in samples that cosist of various bed layers. Core samples are routinely dissected into parts with 0.5-2 cm of thickness each, using a stainless-steel knife before further treatment **[77]**. In some cases, earth core samples can be collected using a diver with an acryl tube **[78]**. In general, after sampling, solid samples are transpoirted to the laboratory wrapped in aluminum foil. Pre-treatment includes drying and calculation of the water content, sieving, homogenization, and storing in PP containers refrigerated at 4°C or frozen at -20°C until analysis **[79]**. Solid comercial products such as textile, fabrics and food packaging parts are bought from local retailers. Non-specific, generic and unbiased selection of these consumable samples is a necessity for the reproduction of real case scenarios **[80]**. As a rule of the thumb, textile or food packaging components are cut in pieces with an acreage of up to 100 cm^2 each, and stored in PP bottles until analyte extraction and analysis **[81]**.

1.4.2. Extraction, clean-up and concentration

With the exception of a promising extraction method developed by Wang et al in 2018 **[82]**, no worth-mentioning advancements in pre-treatment approaches can be seen in the extraction field for abiotic matrices. According to the aforementioned study, solvent extraction proved to achieve better analyte recoveries for the target compounds than SPE. Especialy for dust samples, methods tend to be more sophisticated and involve sequences of extraction steps as well as fractionating with several solvents **[73, 83]**, due to the fact that these matrices are routinely analysed for both volatile and non-volatile PFAS; Generally, pre-treatment methods consist of Soxhlet extraction, PLE, and supported liquid extraction (SLE), followed by additional clean-up procedures for heavily contaminated matrices, i.e. sludge samples, using graphite carbon materials such as ENVI-Carb, SLE or IPE **[31, 84]**. The most commonly used combination is SLE followed by ENVI-Carb or an SPE cartridge (e.g., OASIS WAX, OASIS HLB or C18) under neutral or basic conditions. Arguably, the main focus of generic pre-treatment processes for sediments and soils should be newly discovered and suspect cationic and zwitterionic PFAS that are characterized by increased hydrophobicity **[71, 78]**.

1.4.3. Instrumental analysis and measurement results

For ionic PFAS, the instrumentation used as the golden standard for target screening is HPLC-ESI(-)-MS/MS, as in the case of aqueous matrices analysis. LC conditions are generally similar to those applied for air and aqueous matrices. A few studies essentially employed Orbitrap-MS **[79]** or TOF-MS **[85]**. Nevertheless, it was recently reported that an alkyl-perfluorinated C8 column (Epic FO LB, ES Industries, Inc., West Berlin, NJ, USA) achieved better chromatographic separation of PFAS isomers than a RP C18 column **[86]**. This has to be further tested in upcoming studies. GC-MS is the predominant method for volatile PFAS analysis in solids. Studies from the last 15 years have been steadily reporting PFOS and PFOA terrestrial sediment concentrations in the range of ND to 623 ng/g dry weight (dw) and ND to 16 ng/g dw, respectively **[87, 88]**. This demonstrates the vast difference in sediment contamination by PFOS and the less of an environmental threat PFOA, on an average. Marine sediment core samples collected in developing Asian counties, such as China and Korea, displayed similar trends, in terms of PFOS/PFOA ratios and abundance **[72]**. It should be noted, that surface sediment layers were characterized by significantly higher ΣPFAS values compared to borehole samples, which was also the case for core sediment samples from Great Lakes region of North America **[89]**. diPAPs and PFPiAs were detected in the upper most sediment layer from the same region during monitoring campaigns between 2006 and 2009. In the framework of per- and polyfluorinated chemicals long-term monitoring, emerging PFAS, such as 6:2 Cl-PFESA and its analogues, and 6:2 fluorotelomer sulphamide alkylbetaines (FTABs), have also been detected in abiotic solid matrices and other parts of the aquatic continuum Rarely traced perfluorooctane sulphonamido ethanol-based phosphate (SAm- PAP) diester and triester were also detected in freshwater sediments from Lake Tai, Yangtze Delta area, China. **[27]**. As the occrence of PFAS in dust samples is regarded, both levels and profile of ΣPFAS depend on the location as well as anthropogenic activity at the various collection sites. Samples collected from hotels, hospitals and public buildings are predominatly characterized by higher levels of FTOHs, while samples from households contain much fewer FTOHs. In the latter samples short-chain PFCAs are the most abundant PFAS class **[36]**. Last but not least, PAPs and, especially, diPAPs are regularly detected in dust samples in noteble concentrations, according to the literature **[73, 90]**.

1.4.4. Discussion

Time series analyses as well as numerical simulations point out towards the fact that sediments, soils and sludges are arguably the most important environmental sink for PFAS, followed by surface water bodies and open seas **[91]**. A fair amount of emerging PFAS homologues have been detected in recent studies **[71, 92]**. These substances tend to exhibit strong adsorption to solid matrices **[93]**. Dust is one of the most important means of human exposure to PFAS, since it can easily coalesce with indoor particles, calling for a robust and versatile method for PFAS deterination in trace concentrations. Overall, the urge for the continuous development of

techniques for the extraction of legacy as well as newly discovered PFAS classes, for both volatile as well as non-volatile compounds, is a priority.

1.5. Biological samples

The main focal point of PFAS screening in biological samples has been the development of efficient extraction and clean-up techniques, since biological samples consist of complex matrices **[57, 84]**. IPE and alkaline digestion followed by LLE have been the main pre-treatment methods **[94]**. Eluates are commonly subjected to an additional clean-up stage with an SPE cartridge containing HLB, WAX or ENVI-Carb resin. In the recent years, Accelerated Solvent Extraction (ASE) has been applied for the extraction of analytes from complex biological matrices due to its green profile and technical advantages, compared to traditional extraction methods **[95]** Multiple studies have investigated the occurrence of established as well as new PFAS and their isomers from most of the known classes. Special focus has been given on homologues from the PFECAs and PFESAs **[65, 96]**, perfluoroethylcyclohexane sulphonate (PFECHS) **[97],** PFPAs and perfluorophosphinates (PFPiAs) **[98]**, polyfluoroalkyl phosphate monoesters (monoPAPs) and diPAPs **[99, 100]** families. Additionally, rare cationic and zwitterionic compounds were recently identified in various fish tissues **[101]**. In the prism of green chemistry, several eco-friendly techniques, including focused ultrasound solid-liquid extraction (FUSLE) and turbulent flow chromatography (TFC) are assessed **[57]**. Overall, plasma, serum and breast milk are the most thoroughly studied biological matrices so far **[84]**, but many recent studies are focusing on urine, hair and nail for human biomonitoring **[102, 103]**, as well as wildlife to help improve chemicals management in the environment **[8, 12, 13]**.

1.5.1. Collection and storage

Biological samples cover a variety of complex matrices such as tissues of mammals, birds, fish, seafood, invertebrates, vegetables, eggs, as well as biological fluids such as urine, blood, plasma etc **[7, 10, 104].** Birds, fish and invertebrate samples are commonly captured using nest traps **[105]**, gill nets and bottom trawls **[65]**. They can also be purchased from local markets **[106]**. After sampling, organs and tissues, including brain, liver, heart, gonads, adipose and muscle tissue, among others, are carefully transported to the analytical laboratory. Afterwards, they get homogenized with precleaned stainless-steel tools **[107]**. Bird egg samples are processed by separating the yolk from the albumen and homogenizing the two parts **[108]**. Human milk, urine and blood samples are collected and stored in polypropylene bags and tubes **[104, 109]**. After sampling, most biological samples are stored in polypropylene containers and remain frozen at -20°C or hyper freezed at -80 °C until analysis.

1.5.2. Extraction, clean-up and concentration

The most widely used methods for analyte extraction from biota and human matrices are SPE, SLE, LLE, and IPE; alkaline digestion and acetonitrile protein precipitation have been also employed for the clean-up of biological matrices **[16-18, 103]**. A recent study by Androulakakis et al. involved generic pre-treatment methods and the utilization of Accelerated Solvent Extraction (ASE) for the extraction of PFAS from various top predator livers as well as fish muscle tissues that were collected in Northern Europe **[95]**. The advantages of ASE in comparison to conventional extraction techniques lie in the faster extraction of analytes, the more efficient contact between analytes and solvents, and the smaller consumption of solvents. All these methods result in cleaner extracts using a greener approach. Furthermore, additional clean-up at -30°C for 2 h after analyte extraction by SPE was proven to be effective for the removal of lipid components, which are responsible for signal suspension or enhancement during complex matrices analysis **[110]**. Finally, the most modern trend to report in the field of human biomonitoring, is the reduction and/or simplification of pre-treatment steps and the usage of online SPE systems.

1.5.3. Instrumental analysis and measurement results

The mainstream analytical technique for PFAS analysis of biological samples is HPLC-MS/MS. To a smaller extent HPLC instrumentations coupled to orbitrap or time-of-flight (TOF)-MS are used for biota analysis. Last decade‘s most prominent advantage in the field of PFAS analysis in wildlife and human matrices is the application of online SPE or dualcolumn systems coupled with HPLC-MS/MS **[99]**. Temporal trends and time series analyses of human serum and blood samples for the biomonitoring of PFOS and PFOA in the Northern hemisphere clearly depict reduced levels and a low frequency of appearance of these chemicals, although their occurence in the environment remains steadily high **[111]**. The overall increasing trend of PFAS levels in human and wildlife samples from China, especially close to sites where PFAS are used or produced, constitutes one of the biggest environmental challenges at the global scale and a major concern for regulators **[65]**. Next to the established and in most countries banned legacy PFAS, novel PFAS have been detected in diverse biological samples. The rising levels of these compounds next to their increasing detection rate is the aftereffect of the phasing out of original PFAS and the industrial shift towards alternatives **[100, 101]**. Recently, 6:2 Cl-PFESA was detected for the first time in the liver of a eurasian otter from the East Anglia region, UK, at a concentration of 3.3 ng/g ww, in the framework of a study of assessing PFAS occurence patterns in top predators and their prey from Northern Europe **[95]** Both 6:2 and 8:2 Cl-PFESA as well as PFOS had bioaccumulated and been biomagnified in the marine ecosystem of the Bohai Sea on the east coast of Mainland

China, as demonstrated in the findings of a recent research by Chen et al. 78 6:2 Cl-PFESA and, to a lesser extent, HFPO-TA were detected in various tissues from black spotted frogs near a PFAS mass production and manufacturing unit in China **[96]**, validating once again the response of PFAS concentrations in the environment to the production rate of fluorochemicals in the area. PFPAs and PFPiAs are two groups of emerging PFAS that have raised the concerns of scientists, regulators and policy makers over the recent years, due to their increasing FoA and proven toxicity, bioaccumulation potential, and mobility. 141 samples of dolphins, fish as well as hunting birds collected between 2004 and 2011 in aquatic and terrestrial ecosystems of North America were analysed for PFPAs and PFPiAs **[98]**. PFPiAs were detected in all animals at fair levels, while most PFPAs were found below LOD. In the field of human biomonitoring, the vast majorit of PFAS related studies that were published since 2000 cover the determination of legacy and emerging PFAS in blood and serum of adults and infants. Fewer studies focus on organs, breast milk, urine, hair and nail samples **[104, 112, 113]**. Nevertheless, it has been scientiffically established that nail matrix acts as the best indicator for human exposure to PFAS, with PFOS tested as case study compound **[114]**. In another recent study, 6:2 Cl-PFESA was detected in most of the analyzed urine, hair and nail samples that were derived from two populations with different exposure rates and conditions **[115]**.

1.5.4. Discussion

Method development is a priority for the assessment of the exposure of wildlife and humans to legacy and emerging PFAS through robust and multiresidue analysis of these chemicals. Especially in Asian countries where most fluoropolymer production takes place, and thousands of precursors, metabolites and biotransformation products of legacy PFAS are in the environment, it is crucial to measure organisms‘ exposure to PFAS alternatives such as PFESAs, PFECAs and PFPiAs through biomonitoring and chemicals management. The recent discovery of hundreds of novel PFAS belonging to more than 10 different classes in biota, including mammals, birds, fish and mussels from around the globe, by non-target mass spectrometry strategies is alarming for regulators and warrants for the urge of revolutionary techniques in the field of untargeted PFAS analysis in biological samples. A recent study involving the NTS screening of pooled fish liver samples from China **[101]** pointed out the fact that the analytical advancements in the field of PFAS analysis cannot cope with the rapid industrial production of PFAS alternatives. The development of ethical and non-invasive sample collection techniques, more efficient clean-up methods, and improved ionisation techniques for MS analysis and, are essentially needed. Although, numerous publications on targeted analysis of wildlife and humans for legacy and novel PFAS determination by LC-MS/MS have been reported since 2015 **[9, 11, 15]**, the wide variety of physicochemical properties of PFAS hindered the development of a sufficient pretreatment method that eliminates or at least significantly reduces matrix effect complex

tissues and other biological in samples. Isotope dilution **[100, 116]** and matrix-matched calibration curves have been important tools towards better quantitation and overall analytical performance **[99]**. In order to lessen matrix effects, the volume of the initial sample's volume and the amount of the injected extract in the instrument sequence need to be reduced. This is not always feasible, though. Yet, the biggest challenge in targeted PFAS analysis remains the fact that less than 80 reference standards are currently available, while matrices that are representative of all possible biological samples are also not available. The ratio of commercially available reference standards to known PFAS does not exceed 0.015%, up to this day **[117]**. Last but not least, the loss of sensitivity caused by ionisation suppression due to ESI approaches adds to non-detection, which is often unrealistic. pre-clean-up lyophilization, sample freezing after SPE extraction **[99]**, graphite carbon (e.g., ENVI-Carb) clean-up **[100]** and/or addition of 1-methyl piperazine to the LC-MS/MS mobile phase **[99]** could possibly reduce the lipid content of the final extract, but none of the above can act as full remedy for complex and fatty matrices. As mentioned above, the high production rhythm of the unmonitored alternatives, whose physicochemical properties, toxicology, long-range transport effect and bioaccumulation potential are unknown, calls for sophisticated, robust and efficient risk assessmet of suspect and newly discovered compounds and their environmental threat.

1.6. Untargeted analyses

1.6.1. Suspect screening

Highly sensitive and specific analytical methods have contributed greatly to targeted PFAS determination and quantification in environmental samples. However, the ramping number of legacy and emerging PFAS in the society, currently, warrants for HRMS techniques that aim for the discovery of unknown or suspect compounds in the environment, without the necessity of reference standards. The timely discovery of novel, yet possibly hazardous PFAS contributes to early warning strategies via their risk assessment and the monitoring of their early life-cycle, prohibiting – if necessary- the global distribution of these compounds. Some of the most aknowledged and reliable databases for PFAS suspect screening include SFISHFLUORO on the NORMAN Suspect List Exchange **[100]**, as well as EPAPFASINV, PFASKEMI and PFASOECD on the U.S. Environmental Protection Agency (EPA) CompTox Chemistry Dashboard **[35]**. However, it has to be noted that at least 20% of the ~5000 PFAS listed in these databases has no molecular formula and/or structural information. Suspect screening can be performed against databases containing either exact mass and isotope patterns **[118-120]**, generated molecular formulas, MS/MS data retrieved from literature and open source databases (e.g. Massbank **[50, 103]**, MZCloud **[102]**), or in-silico MS/MS predictions (e.g. MetFrag **[50]** and CFM-ID **[50]**). Typically, it is applicable during the step of full-scan acquisition, where *m/z* ratios are acquired over defined

mass ranges with a specified scanning frequency and/or resolving power (the ability of the instrument to separate two adjacent peaks, RP).

Suspect screening can be further utilized for the proposal of per- and polyfluorinated molecular formulas, taking advantage of HRMS instruments‘ mass accuracy (≤ 5 ppm, or even <1 ppm, depending on its calibration efficiency) **[51, 52, 59, 121, 122]**. Lastly, suspect screening can be performed in the phase of MS/MS structural characterization by isolating a specific parent ion, fragmenting it by collision-induced dissociation (CID) or electron-capture dissociation **[92]**, and collecting the product ions in full-scan mode for complete MS/MS spectra attainment. The rapid scan of numerous samples and the possibility of retrospective screening of stored samples **[123, 124]**, is a key advancement of this untargeted HRMS strategy over conventional MS/MS techniques.

1.6.2. Non-target screening

For over a decade, nontarget HRMS methodologies act as powerful tools for the discovery of numerous PFAS that are endlessly manufactured and discharged into the ecosystems at the global scale **[27, 67]**. Just recently, more than 950 unknown PFAS, disregarding possible branched isomers, were detected in various environmental media **[67]**. The proposed structures of the aforementioned analytes contributes greatly to next-level PFAS research and the risk assessment of these substances and their side-products **[29, 57]**. The main focus of formula elucidation and structure proposal of unknown PFAS via NTS methodologies has found applications in AFFF and surfactant samples. Several novel PFAS in screened AFFF fractions were identified using fast atom bombardment (FAB)-MS and quadrupole TOF-MS **[125, 126]**. Additional and so-far-unkown PFAS from various classes including anions, zwitterions, cations and neutral species were also identified in AFFF samples **[127]**, and fluorocarbon surfactants **[128]**. More case studies involve the implimentation of NTS pathways on freshwater **[85]**, drinking water **[129]** and wastewater matrices **[130]**. Additionally, airborne particulate matter **[131]**, human blood samples **[132]**, and various fish tissues **[101]** were screened for the detection of unmonitored emerging PFAS. Four new PFAS classes consisting of more than 165 homologues, in total, were also reported in pooled fish samples collected downstream from a fluorochemical industry, in 2018 **[101]**.

An unconventional NTS study alternated between scan events with high and low collision energies (CE) for the screening of parent ions as probable PFAs precursors by TOF-MS **[128]**. This study led to the discovery of 5 anionic, 30 cationic, 15 zwitterionic and 40 neutral PFAS, raising the number of newly established fluorosubstances in the last five years by another 90. This type of scanning needs to be further tested together with in-source fragmentation flagging scans for anionic PFAS **[101]**. Both of these scanning strategies could be powerful tools in the developing field of PFAS NTS screening. Multidimensional analysis techniques such as GC × GC or LC × LC

coupled to TOF-MS that have been developed for non-target analysis of organic contaminants in dust samples **[133]**, could be potentially applicable in the development of PFAS NTS strategies for environmental matrices. So far, the main pre-treatment methodologies for target analysis have been applied in NTS strategies too. The most widely used clean-up and extraction approaches that have been adopted as part of NTS pathways are SPE **[101]**, SLE **[132]** (also coupled to activated carbon filtration **[85, 131]**), as well as filtration/dilution combinations **[128]**. Nevertheless, the main fault of these methods lies in the fact that they have only been assessed for anionic compounds, that have comprised the majority of target analytes for decades. Thus, the loss of cationic, zwitterionic and neutral PFAS with different physicochemical properties during NTS is inevitable [85, 131]. The above was demonstrated in the framework of a study analysing firefighting foams in soils. It was found that analyte recoveries of cationic and zwitterionic PFAS to be out of the 70-170% range when using standardized pre-treatment techniques optimised for anionic PFAS applied **[71]**. Therefore, the urge to carefully select should be one of the main focal points in the improvement of future NTS strategies within PFAS research. The employment of generic, non-intrussive pre-treatment methods, together with extraction procedures that are able to capture diverse PFAS, while simultainously reducing matrix effects and signal interferences are a priority in future PFAS research **[131]**.

Overall, in-depth non-target workflows have good chances of discovering potential PFAS transformation products, intermediates, manufacturing impurities, as well as PFAS not listed in available suspect databases, yet several elucidation schemes have led to identifications that remain tentative. It is a necessity that, in frequent cases, NTS must be followed by authentic chemical standard synthesis for utter confirmation, further targeted and quantitative monitoring by highly sensitive MS/MS instrumentations, as well as toxicology studies, towards a holistic risk assessment of newly discovered substances. NTS is closely connected with chemical prioritization schemes. Robust, effective yet cost-benefit chemical prioritization schemes are indefeasible aspects of modern chemicals monitoring. When finally deciding what PFAS ought to be prioritized, scientists, regulators and policy makers greatly affect the big-scale synthesis of fluorochemicals and their environmental footprint. Therefore, the current monitoring degree, toxicological testing, detection frequency and possible increasing trends in environmental samples for the watched substances should be taken into consideration **[110]**.

1.6.3. TOPA and TOF for PFAS and precursors analysis

A vast and constantly increasing number of PFAS homologues, including legacy compounds and their precursors currently exist, often in just trace concentrations, in environmental matrices. The fact that the majority of these precursors are unknown, due to commercial unavailability of their standards for quantification creates the biggest analytical challenge in the field of PFAS analysis. For that, and in order to mitigate leaks in the environmental fate of precursors, as a

result of their possible biodegradation as intermediates, new methods have been developed to detect fluoro-substances. The most well-known methods include total oxidisable precursor (TOP) assays **[134]**, fluorine-19 nuclear magnetic resonance (19F NMR) spectroscopy **[135]**, inductively coupled plasma-mass spectrometry (ICP-MS/MS) **[136]**, and X-ray photoelectron spectroscopy (XPS) **[137]**. Methods for total fluorine (TF) analysis have also been developed for the discovery of PFAS precursors in complex environmental matrices. These methods include total organic fluorine (TOF) analysis, particle-induced gamma ray emission (PIGE) spectroscopy and combustion ion chromatography (CIC). After PFAS are extracted from the matrices of interest by SPE, TOF yield can be measured by PIGE **[138]**. PIGE is a very fast technique and effective for quantitative analysis, but the requirement of gamma radiation for nuclear activation makes it costly and rarerly applied. CIC involves the adsorption of both PFAS and their precursors on an activated carbon matrix (ACM). The ACM gets then combusted and the released fluorine from every fluorinated substance is measured. Through that procedure, the control of the mass balance is possible, yet no individual components can be traced **[139]**. Extractable organic fluorine (EOF) assay using CIC has been used for the analysis of water matrices, sediments and various biological samples since it was first reported by Miyake et al., in 2007 **[140, 141]**. Adsorbable organic fluorine (AOF) assay, utilises activated carbon adsorbent, and also employs CIC but to the authors‘ knowledge there has not been a direct comparison between EOF and AOF aproaches so far. The TOP assay, originally developed by Houtz and Sedlak in 2012 **[134]**, is perhaps the most promising and therefore most widely used non-destructive and non-specific method to report. It achieves the best detection selectivity from the available non-specific methods, but only for precursors that can be oxidised to certain perfluoroalkyl carboxylic and sulfonic acids (PFAAs). This is done by comparing a given matrix prior and after oxidation by hydroxyl radicals. This method can be facilitated by utilizing a simple LC-MS/MS system, yet is highly precise and can effectively target possible precursors of specific PFAAs. recently, TOP assay has been applied to various water samples to evaluate the tradeoff between selectivity and inclusivity in PFAS analyses **[142]**. Additionally, it was implimented in the analysis of effluent wastewater samples for the assessment of the environmental fate of per- and polyfluoroalkyl Ether Acids **[143]**. Ultrashort-chain perfluoroalkyl carboxylic acids were included in the TOP assay protocol in 2019 **[56]**, while zwitterionic, cationic, and anionic PFAS were integrated into the TOP assay groundwater protocol the same year **[144]**. TOF, on the other hand, was applied to detect organic and inorganic fluoride from seawater and blood samples **[140, 141]**. Extraction was performed by SPE and IPE with methyl tert-butyl ether (MTBE) and hexane as solvents. CIC was then applied for the determination of PFAS and precursors in both sample types.

CHAPTER 2: SCOPE AND OBJECTIVES

2.1. The analytical problem

Despite their partial banning, thousands of PFAS enter daily the environment due to anthropogenic activities, generating complex cocktails in various consistencies, which may potentially harm the ecosystem and human health. Moreover, TPs, precursors, and alternatives enter the aquatic environment constantly during the mechanical, biological and chemical processes that take place in the industry, finding their way to both wildlife and humans. Fluorinated pollutants enter the ecosystems and have potential carcinogenic and toxic effects on living organisms (e.g. algae, crustaceans and fish, birds, mammals). Many PFAS end up also in humans either through the trophic chain, industrial products such as firefighting foams and packaging or even directly through air and water.

To tackle this problem and protect the environment, EU water legislation (e.g. WFD **[145]**, EQS directive **[146]**), is oriented towards specific monitoring programmes for PFAS, with the aim to measure concentration levels of specific legacy pollutants, while at the same time exploring the existence of emerging and unknown compounds by establishing extended suspect lists of thousands of PFFAS. Despite the efforts of the policy makers, the legislation is limited on monitoring of just a few established PFAS, and overlooks the risk derived from the occurrence of thousands of unknown precursors, alternatives, intermediates and TPs.

Recent developments in advanced analytical instrumentation, especially in the field of HRMS have given the analytical scientists the opportunity to broaden their horizons. LC-HRMS has proven to be a powerful tool in the hands of researchers to detect and reveal the identity of many unknown PFAS in the environment. The high specificity of hybrid mass spectrometers such as QTOF instruments enabling suspect screening regimes, combined with the extraordinary

selectivity and sensitivity of LC-ESI-MS/MS methods via thorough target screening analyses, enables the holistic analysis of very complex matrices.

The main limitation in the environmental analysis of PFAS lies primarily in the vast number of newly composed alternatives, as well as the thousands precursors, intermediates and metabolites of known compounds. Reference standards as well as analytical methods exist for just of the known fluorinated pollutants, as they are reported in the most recently updated suspect lists **[147]**.

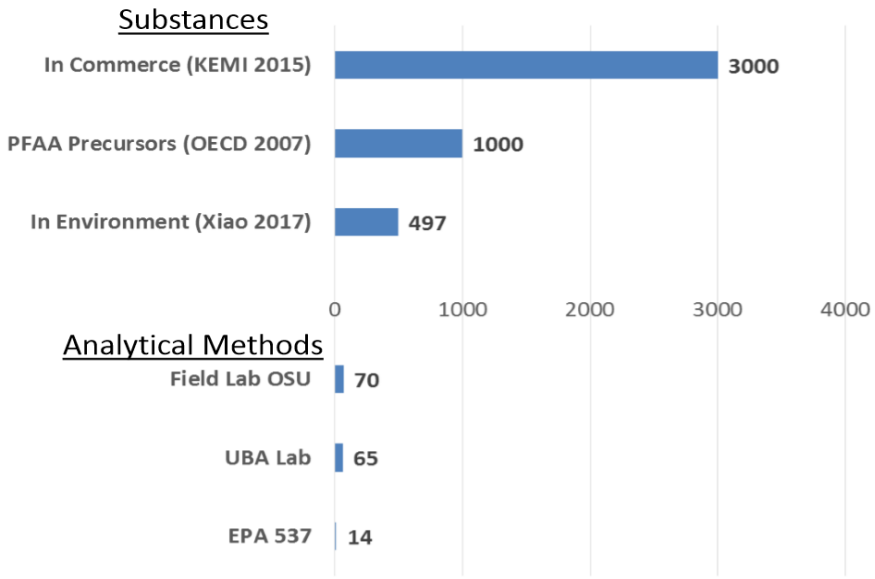


Figure 2. *Emerging poly- and perfluoroalkyl substances in the aquatic environment: A review of current literature. Source: Xiao, F. (2017). Water Research, Volume 124, 1 November 2017, Pages 482-495.*

Although this highlights the urge for sound untargeted screening methods in the field of PFAS environmental analysis, the generated data from advanced HRMS instrumentations is not possible to be fully interpreted and taken advantage of yet. Up to this day HRMS vendors use in-house data formats and have built their acquisition tools based on them, which hinders further development of some promising research schemes.

Consequently, universal data formats as well as harmonized acquisition software in suspect and non-target screening when combined with newly developed targeted analytical techniques, such as TOPA and TOF assays, could solve the environmental problems in PFAS research faced by the scientific community and the policy makers.

In the context of this thesis, two screening approaches are presented, namely a highly sensitive liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS)

method that was applied in iconic biota samples from various ecosystems across Europe and a suspect screening method that has been developed in cooperation with the Environmental Institute of Slovak Republic, which covered 4,777 PFAS retrieved from the NORMAN Substance Database, employing LC-HRMS chromatograms obtained from the Digital Sample Freezing Platform (DSFP).

Emphasis will be given on how to establish a complete monitoring technique for PFAS that combines the strengths of both target screening by highly sensitive, low resolution mass spectrometry and suspect screening by retrospectively utilizing hundreds of electronically stored chromatograms, in order to holistically assess the risk posed by legacy and emerging PFAS in the environment.

2.2. Research objectives and scope

The objective of this thesis was to develop novel methodologies for the investigation of the occurrence of PFAS in the environment and apply them in European ecosystems. To achieve the objective, advanced analytical instrumentation and cutting-edge software tools were developed and applied on the collected samples. The thesis is organized in three case studies, each one described in the following three chapters.

Chapter 3 describes the development of an LC/MS-MS methodology for the determination of 29 target PFAS in trace quantities. This method was applied on lake water samples from Austria and accounts for the initial attempt for the development of a target screening approach for legacy and emerging PFAS in aqueous samples.

Chapter 4 describes the extended multiresidue and highly sensitive target screening workflow utilizing liquid chromatography tandem mass spectrometry (LC/MS-MS) capable to quantify compounds at the picogram range. The fully validated method was applied to 65 recent specimens of a terrestrial apex predator (Common buzzard), freshwater and marine apex predators (Eurasian otter, harbour porpoise, grey seal, harbour seal) and their potential prey (bream, roach, herring, eelpout) from northern Europe (United Kingdom, Germany, the Netherlands and Sweden). 56 compounds from 14 classes were measured.

Chapter 5 presents the findings of a suspect screening campaign. The occurrence of 4,777 PFAS in the Danube river basin (DRB) was investigated by target and suspect screening. Target screening involved the investigation of PFAS with reference standards, as described in the LC/MS-MS method covered in the previous chapter. Suspect screening covered 4,777 PFAS retrieved from the NORMAN Substance Database, including all individual PFAS lists submitted to the NORMAN network. PFAS having a risk score above 1 in at least one matrix were prioritized.

Chapter 3: Determination of 29 PFAS in aqueous samples from Austria by LC-MS/MS

3.1. Introduction

PFAS compose a vast class of chemicals that includes perfluoroalkyl acids (PFAAs) and more specifically perfluoroalkyl carboxylic acids (PFCAs) and perfluoroalkyl sulfonic acids (PFSAs) **[148]**. PFAS are persistent, bio-accumulative and possibly carcinogenic to animals as well as humans **[3]**. Since the 1940s, they have been broadly used in several applications due to their particular physicochemical properties **[149]**. They have been extensively used in foam mixtures for fire-extinguishing purposes and surfactants **[150, 151]**. Additionally, these versatile substances have been used in leather as well as textile treatment processes **[152]**. PFAS end up in the aquatic ecosystems primarily through industrial wastewater **[153]** . Short-chain PFAS display increased mobility in sediment and water layers, which classifies them as exceptionally hazardous for the environment, yet up to this day these substances have not been adequately monitored **[154]**.

Currently, perfluorooctanesulfonate (PFOS) and its salts are listed under Annex B of the Stockholm Convention for Persistent Organic Pollutants **[155]**, while perfluorooctanoic acid (PFOA), its salts and PFOA-related compounds were added to Annex A in 2019. Perfluorohexane sulfonate (PFHxS) has been proposed for inclusion **[156]**. The phase-out of the legacy compounds and their replacement with structurally similar PFAS has been the most common industry policy in the last decades **[19, 20]**. This poses a great environmental danger, since most emerging PFAS also show high toxicity, yet are to this day not routinely monitored or part of any regulatory guideline **[21]**. Up to this day there are nearly 5000 PFAS that are broadly used in several industrial and commercial applications **[22]**.

Several analytical regimes have been developed for the determination of PFAS in aquatic matrices, including sediments, ground- and freshwater **[5, 6]**. Solid phase extraction (SPE) and liquid–liquid extraction (LLE) are the main techniques that have been applied in the extraction, purification and pre-concentration of PFAS in environmental samples in the recent years **[16-18]**. Liquid chromatography (LC) coupled with mass (MS) or tandem mass spectrometric (MS/MS) detection is the golden standard for the determination of PFAS **[24-26]**; for some PFAS limits of detection at the picogram range can easily be achieved **[157, 158]**.

The objective of the present study was to develop a robust, quick and efficient LC-MS/MS method in order to assess the PFAS presence in water bodies that are of importance to humans and livestock. We particularly thrived to determine the contamination by 29 legacy and emerging PFAS of lake samples in Austria, as part of the Joint Danube Survey monitoring program.

3.2. Materials and methods

3.2.1 study area and selected samples

8 surface water samples were carefully selected for the testing of the developed method. The study covered lake samples from Austria that were received in September 2019 from TU Vienna, in the framework of the project Joint Danube Survey (https://www.icpdr.org/main/activities-projects/joint-danube-survey). 4 additional samples, including procedural blanks and calibration samples, were used for quality control purposes.

3.2.2 Chemicals and reagents

All target compounds and ISs were purchased from Wellington Laboratories (Guelph, Ontario, Canada) (≥98%). The target PFCAs were perfluorobutanoic acid (PFBA; C4), perfluoropentanoic acid (PFPeA; C5), perfluorohexanoic acid (PFHxA; C6), perfluoroheptanoic acid (PFHpA; C7), perfluorooctanoic acid (PFOA; C8), perfluorononanoic acid (PFNA; C9), perfluorodecanoic acid (PFDA; C10), perfluoroundecanoic acid (PFUdA; C11), perfluorododecanoic acid (PFDoA; C12), perfluorotridecanoic acid (PFTrDA; C13), perfluorotetradecanoic acid (PFTeDA; C14), PFHxDA (Perfluorohexadecainoic acid; C16), and Perfluorooctadecanoic acid (PFODA; C18). The target PFSAs were potassium perfluorobutanesulfonate (PFBS; C4), sodium perfluoropentanesulfonate (PFPeS; C5), sodium perfluorohexanesulfonate (PFHxS; C6), sodium perfluoroheptanesulfonate (PFHpS; C7), sodium perfluorooctanesulfonate (PFOS; C8), sodium perfluorononanesulfonate (PFNS; C9), and sodium perfluorodecanesulfonate (PFDeS; C10). The target FASAs were perfluorooctane sulfonamide (PFOSA), N-methylperfluorooctane sulfonamide (N-MeFOSA), and N-ethylperfluorooctane sulfonamide (N-EtFOSA). The target FTSAs were 4:2 fluorotelomer

sulfonic acid (4:2 FTS), 6:2 fluorotelomer sulfonic acid (6:2 FTS), and 8:2 fluorotelomer sulfonic acid (8:2 FTS). The target list of analytes in this study also included the perfluoroether carboxylic acids (PFECAs) hexafluoropropylene oxide dimer acid (HFPO-DA; trade name Gen-X) and 4,8-dioxa-3H-perfluorononanoic acid (trade name ADONA), as well as the perfluoroether sulphonic acid (PFESA) 6:2 chlorinated perfluoroether sulfonic acid (6:2 Cl-PFESA; trade name F-53B). Labelled Perfluoro-n-[$^{13}C_4$]butanoic acid ($^{13}C_4$-PFBA), Perfluoro-n-[$^{13}C_5$]pentanoic acid ($^{13}C_5$-PFPeA), Perfluoro-n-[1,2,3,4,6-$^{13}C_5$]hexanoic acid ($^{13}C_5$-PFHxA), Perfluoro-n-[1,2,3,4-$^{13}C_4$]heptanoic acid ($^{13}C_4$-PFHpA), Perfluoro-n-[$^{13}C_8$]octanoic acid ($^{13}C_8$-PFOA), Perfluoro-n-[$^{13}C_9$]nonanoic acid ($^{13}C_9$-PFNA), Perfluoro-n-[1,2,3,4,5,6-$^{13}C_6$]decanoic acid ($^{13}C_6$-PFDA), Perfluoro-n-[1,2,3,4,5,6,7-$^{13}C_7$]undecanoic acid ($^{13}C_7$-PFUdA), Perfluoro-n-[1,2-$^{13}C_2$]dodecanoic acid ($^{13}C_2$-PFDoA), Perfluoro-n-[1,2-$^{13}C_2$]tetradecanoic acid ($^{13}C_2$-PFTeDA), Sodium perfluoro-1-[2,3,4-$^{13}C_3$]butanesulfonate ($^{13}C_3$-PFBS), Sodium perfluoro-1-[1,2,3-$^{13}C_3$]hexanesulfonate ($^{13}C_3$-PFHxS), Sodium perfluoro-1-[$^{13}C_8$]octanesulfonate ($^{13}C_8$-PFOS), Perfluoro-1-[$^{13}C_8$]octanesulfonamide ($^{13}C_8$-PFOSA), Sodium 1H,1H,2H,2H-perfluoro-1-[1,2-$^{13}C_2$]hexanesulfonate ($^{13}C_2$-4:2FTS), Sodium 1H,1H,2H,2H-perfluoro-1-[1,2-$^{13}C_2$]octanesulfonate ($^{13}C_2$-6:2FTS), Sodium 1H,1H,2H,2H-perfluoro-1-[1,2-$^{13}C_2$]decanesulfonate ($^{13}C_2$-8:2FTS), N-methyl-d_3-perfluoro-1-octanesulfonamidoacetic acid (d_3-N-MeFOSAA), and N-ethyl-d_5-perfluoro-1-octanesulfonamidoacetic acid (d_5-N-EtFOSAA) were used as internal standards. In order to acquire the m/z (Da) of the precursor ion for the 29 target PFAS and the 32 internal standards, as well as the optimum MS/MS parameters for the product ions, including m/z (Da), collision energy (eV) and tube lens values (V), 1 µg/mL solutions in MeOH of each target compound and IS were directly infused into the triple quadrupole mass analyzer in low collision energy mode. The compound catalogue, including the abbreviation, compound class, and optimized LC-MS/MS parameters for the analytes in this case study, can be found in **Table 2**, while the CAS numbers and the internal standards (IS) used as surrogates for quantification purposes can be found in **Table 3**. Acetonitrile (ACN) and Methanol (MeOH) of LC-MS grade were purchased from Merck (Darmstadt, Germany), Oasis-HLB disks were purchased from Labicom (Olomouc, Czechia), RC syringe filters (4 mm diameter, 0.2 µm pore size) from Phenomenex (USA) and formic acid (FA) 99% was obtained from Sigma-Aldrich, Fluka (Buchs, Switzerland). Distilled water was provided by a Milli-Q apparatus (Millipore Direct-Q UV, Bedford, MA, USA). Ammonium acetate (p.a., purity 99.0% or greater) purchased from Sigma-Aldrich (Buchs, Switzerland) was used for the eluents in the HPLC– MS/MS method. All standard stock solutions were prepared in MeOH and stored in the dark at 4 °C. Mixtures of target analytes standard solutions were prepared in MeOH at final concentrations of 25, 50 and 100 ng mL^{-1} and used for spiking. Eppendorf tubes (Sarstedt, Nümbrecht, Germany) were used during sample preparation.

***Table 2**. Optimized tandem MS parameters for the compounds measured in this study.*

Compound class	Compound name	Abbreviation	Precursor Ion (Da)	Product Ion (Da)	Collision energy (eV)	Tube lens (V)
Perfluoroalkyl carboxylic acids (PFCAs)	Perfluorobutanoic acid	PFBA	213	169	6	59
	Perfluoropentanoic acid	PFPeA	263	219	6	59
	Perfluorohexanoic acid	PFHxA	313	269 (119[b])	9 (22[b])	50
	Perfuoroheptanoic acid	PFHpA	363	319 (169[b])	11 (18[b])	50
	Perfuorooctanoic acid	PFOA	413	369 (169[b])	11 (18[b])	37
	Perfuorononanoic acid	PFNA	463	419 (169[b])	11(18[b])	50
	Perfuorodecanoic acid	PFDA	513	469 (169[b])	13 (18[b])	50
	Perfuoroundecanoic acid	PFUdA	563	519 (169[b])	11 (16[b])	50
	Perfluorododecanoic acid	PFDoA	613	569 (169[b])	13 (22[b])	50
	Perfluorotridecanoic acid	PFTrDA	663	619 (169[b])	13 (34[b])	60
	Perfluorotetradecanoic acid	PFTeDA	713	669 (419[b])	13 (380[b])	70
	Perfluorohexadecanoic acid	PFHxDA	813	768	16	75
	Perfluorooctadecanoic acid	PFODA	869	269 (468[b])	28 (23[b])	80
Perfluoroalkyl sulphonic acids (PFSAs)	Potassium perfluorobutanesulfonate[a]	PFBS	299	99 (80[b])	44 (36[b])	50
	Sodium perfluoropentanesulfonate[a]	PFPeS	349	80 (99[b])	40 (36[b])	50
	Sodium perfluorohexanesulfonate[a]	PFHxS	399	80 (99[b])	48 (44[b])	50

	Sodium perfuoroheptanesulfonate[a]	PFHpS	449	80 (99[b])	50 (46[b])	50
	Sodium perfluorooctanesulfonate and branched isomers[a]	PFOS	499	80 (99[b])	53 (43[b])	104
	Sodium perfluorononanesulfonate[a]	PFNS	549	80 (99[b])	76 (48[b])	50
	Sodium perfluorodecanesulfonate[a]	PFDS	599	80 (99[b])	60 (60[b])	50
Perfluorooctane sulfonamides (FOSAs)	Perfluorooctane sulfonamide[a]	PFOSA	498	78	10	50
	N-Methylperfluorooctane sulfonamide[a]	N-MeFOSA	512	169 (219[b])	28 (28[b])	112
	N-Ethylperfluorooctane sulfonamide[a]	N-EtFOSA	526	169 (219[b])	32 (28[b])	103
Fluorotelomer sulphonic acids (FTSAs)	1H,1H,2H,2H-perfluorohexanesulfonate[a]	4:2 FTS	327	307 (81[b])	16 (44[b])	95
	1H,1H,2H,2H-perfluorooctane sulfonate[a]	6:2 FTS	427	407 (81[b])	28 (44[b])	95
	1H,1H,2H,2H-perfluorodecane sulfonate[a]	8:2 FTS	527	507 (80[b])	32 (52[b])	95
Perfluoroether carboxylic acids (PFECAs)	4,8-dioxa-3H-perfluorononanoic acid	ADONA	377	251 (85[b])	12 (36[b])	50
	hexafluoropropylene oxide dimer acid	Gen-X	329	285 (185[b])	5 (24[b])	50
Perfluoroether sulphonic acids (PFESAs)	6:2 chlorinated perfluoroether sulfonic acid[a]	F-53B	531	351 (99[b])	26 (38[b])	50
Internal Standards	Perfluoro-n-[$^{13}C_4$]butanoic acid	$^{13}C_4$-PFBA	217	172	6	59
	Perfluoro-n-[$^{13}C_5$]pentanoic acid	$^{13}C_5$-PFPeA	268	223	6	59
	Perfluoro-n-[1,2,3,4,6-$^{13}C_5$]hexanoic acid	$^{13}C_5$-PFHxA	318	273	9	50
	Perfluoro-n-[1,2,3,4-$^{13}C_4$]heptanoic acid	$^{13}C_4$-PFHpA	367	322	11	50
	Perfluoro-n-[$^{13}C_8$]octanoic acid	$^{13}C_8$-PFOA	421	376	11	37
	Perfluoro-n-[$^{13}C_9$]nonanoic acid	$^{13}C_9$-PFNA	472	427	11	50
	Perfluoro-n-[1,2,3,4,5,6-$^{13}C_6$]decanoic acid	$^{13}C_6$-PFDA	519	474	13	50

Perfluoro-n-[1,2,3,4,5,6,7-$^{13}C_7$]undecanoic acid	$^{13}C_7$-PFUdA	570	525	11	50
Perfluoro-n-[1,2-$^{13}C_2$]dodecanoic acid	$^{13}C_2$-PFDoA	615	570	13	50
Perfluoro-n-[1,2-$^{13}C_2$]tetradecanoic acid	$^{13}C_2$-PFTeDA	715	670	13	70
Sodium perfluoro-1-[2,3,4-$^{13}C_3$]butanesulfonate	$^{13}C_3$-PFBS	302	99	44	50
Sodium perfluoro-1-[1,2,3-$^{13}C_3$]hexanesulfonate	$^{13}C_3$-PFHxS	402	99	48	50
Sodium perfluoro-1-[$^{13}C_8$]octanesulfonate	$^{13}C_8$-PFOS	507	99	53	104
Perfluoro-1-[$^{13}C_8$]octanesulfonamide	$^{13}C_8$-PFOSA	506	78	78	10
Sodium 1H,1H,2H,2H-perfluoro-1-[1,2-$^{13}C_2$]hexanesulfonate	$^{13}C_2$-4:2FTS	329	81	16	80
Sodium 1H,1H,2H,2H-perfluoro-1-[1,2-$^{13}C_2$]octanesulfonate	$^{13}C_2$-6:2FTS	429	81	28	80
Sodium 1H,1H,2H,2H-perfluoro-1-[1,2-$^{13}C_2$]decanesulfonate	$^{13}C_2$-8:2FTS	529	81	32	80
N-methyl-d_3-perfluoro-1-octanesulfonamidoacetic acid	d_3-N-MeFOSAA	573	419	28	112
N-ethyl-d_5-perfluoro-1-octanesulfonamidoacetic acid	d_5-N-EtFOSAA)	589	419	32	103

[a] pseudo-MRM mode, [b] confirmation ion

3.2.3. Extraction of samples

Sample extraction was carried out using the protocol described by Alygizakis et al. **[159]**. In short, samples were cleaned up and preconcentrated 4000 times on Atlantic HLB-M Disk using HORIZON SPE-DEX 4790 (USA) with 47 mm disk holder according to the extraction program described in Section S1-3 (SI). Extracts were evaporated using gentle stream of nitrogen and reconstituted with 500 µL of 50:50 methanol:water for analysis. Before instrumental analysis

extracts were filtered through RC syringe filters of 4 mm diameter and 0.2 μm pore size (Phenomenex, USA).

During the sample preparation it was noted that the physicochemical properties (i.e. solubility/lipophilicity and acidity) of the molecules greatly vary depending on the chain length and the acidic group present in the PFAS molecule. This was in good agreement with the relative literature **[18]**. Furthermore, special care was taken during sample manipulation, treatment and analysis, since there is an actual risk of contamination during the whole analytical process, owing to the presence of fluorinated polymers in commonly used laboratory materials and equipment. More specifically, all labware, weighing and dissection tools were prescreened and rinsed with methanol before use in order to reduce the contamination likelihood.

***Table 3**. List of PFAS, their CAS number and the respective internal standard (IS) used for quantification.*

Compound	CAS	Respective IS used for quantification
PFBA	456-22-4	13C4-PFBA
PFPeA	5989-64-0	13C5-PFPeA
PFHxA	307-24-4	13C5-PFHxA
PFHpA	375-85-9	13C4-PFHpA
PFOA	335-93-3	13C8-PFOA
PFNA	444-03-1	13C9-PFNA
PFDeA	335-76-2	13C6-PFDA
PFUnA	2058-94-8	13C7-PFUdA
PFDoA	307-55-1	13C2-PFDoA
PFTrDA	72629-94-8	13C2-PFTeDA
PFTeDA	376-06-7	13C2-PFTeDA
PFHxDA	67905-19-5	13C2-PFTeDA
PFODA	16517-11-6	13C2-PFTeDA
PFBS	375-73-5	13C3-PFBS
PFPeS	2706-91-4	13C3-PFHxS
PFHxS	355-46-4	13C3-PFHxS
PFHps	375-92-8	13C8-PFOS
PFOS	2795-39-3	13C8-PFOS
PFNS	68259-12-1	13C8-PFOS
PFDeS	335-77-3	13C8-PFOS
PFOSA	754-91-6	13C8-PFOSA
N-MeFOSA	31506-32-8	d3-N-MeFOSAA
N-EtFOSA	4151-50-2	d5-N-EtFOSAA)
4:2 FTS	757124-72-4	13C2-4:2FTS
6:2 FTS	27619-97-2	13C2-6:2FTS
8:2 FTS	39108-34-4	13C2-8:2FTS

ADONA	51460-26-5	13C8-PFOA
GenX	62037-80-3	13C8-PFOA
F-53B	73606-19-6	13C8-PFOS

3.2.4. Instrumental Analysis

All measurements were performed using a UHPLC Thermo Accela pump incorporating a column thermostat, a degasser, and an autosampler (San Jose, CA, U.S.). The selected mass spectrometric system was a Thermo TSQ Quantum Access triple quadrupole mass analyzer. Chromatographic separation was performed using an XTerra MS C18 (100 mm × 2.1 mm, 3.5 µm) column from Waters and the column temperature was set at 25 °C; Phenomenex C18 guard columns (4.0 mm × 2.0 mm, 5 µm) were used during the entire experimental procedure. The operating parameters of ESI, sheath gas, auxiliary gas, capillary temperature, and spray voltage were based on the method developed by Arvaniti et al.**[68]**. The electrospray ionization voltage was applied at -2.5 kV. The sheath gas (N_2) flow rate was set at 60 A.U. (Arbitrary Units), the auxiliary gas (N_2) flow rate was set at 20 A.U., the ion transfer capillary temperature was set at 200 °C, and the collision pressure was set at 1.5 mTorr. Chromatographic analyses were carried out using a gradient elution program with 5 mM ammonium acetate aqueous solution (solvent A) and MeOH (solvent B) as a binary mobile phase mixture at a flow rate of 100 mL/min. The gradient elution started with 20% (v/v) MeOH and increased linearly to 75% MeOH in 1.5 min, and then to 100% MeOH in 10.0 min which was held for 5.0 min (until 15.0 min), reverted to 20% MeOH and re-equilibrated for 5.0 min (from 15.0 to 20.0 min) at 20% MeOH (total run time of 20 min). Multiple Reaction Monitoring (MRM) was applied for all PFCAs and the emerging PFECAs ADONA and Gen-X, while for all remaining PFAS a pseudo-MRM approach was selected. When following the pseudo-MRM approach, the triple-quad is still operated in MRM mode, with collision gas on and an optimized collision energy, as selected from the method validation experiment. The difference from the traditional MRM is the fact that the precursor and product ions are set to the same value. The final in-vial composition of all samples and standard solutions was in MeOH/5 mM ammonium acetate (50 : 50, % v/v), and was injected into the column with full-loop injection (10 µL). Data were acquired with the Xcalibur 4.3.0 software package (Thermo Scientific).

3.2.5. Quality assurance and quality control

The identification and confirmation criteria for the analysis of the 29 analytes in this study was based on the Commission Decision 2002/657/EC. To confirm the presence of the compounds, the retention time of the compounds (2.5 % of tolerance) and relationship between the two transitions (difference of less than 20 %) were used. The detected PFAS were quantified using isotopic dilution. If IS standards were not available, then standard addition method was used.

The calibration curves obtained for the investigated compounds at 7 levels (0.1 ng/L, 1 ng/L, 10 ng/L, 25 ng/L, 50 ng/L, 75 ng/L and 100 ng/L) were linear with $r^2 > 0.98$ in all cases. Accuracy of the method was assessed with recovery experiments in surface water samples. Extraction recoveries for target analytes were determined (n=5) at two concentration levels, 10.0 and 100.0 ng L^{-1}. Most analytes showed recovery efficiency between 80 and 110%.

To ensure a correct quantification, method precision was determined as relative standard deviation (%RSD) from the recovery experiments, processed with the described method (method repeatability). Precision limit <15% RSD was met for all analytes, indicating the good precision of the method.

Regarding sensitivity, method limit of detection (MDLs, lowest analyte concentration with S/N ratio of 3) and method limit of quantification (MQLs, concentration with S/N ratio of 10 and imprecision lower than 20%) were estimated in surface water. MLODs and MLOQs were calculated from the recovery experiments at the lowest concentration spiked.

Matrix effect (ME) was evaluated and the results are expressed as percentage of suppression or enhancement. ME was calculated at one concentration level (100.0 ng L^{-1}) according to the equation:

$$ME = \left(\frac{SM - AC}{STD} - 1\right) * 100$$

where SM refers to the area of the peak in the spiked matrix at the final step of reconstruction, AC is the average peak area in the sample and STD stands for the peak area in a methanol standard solution spiked at the same concentration. Matrix suppression was observed for 21 compounds, while 8 compounds showed signal enhancement due to the matrix. Results are summarized in **Tables 4 and 5**.

Table 4. *MDLs, MQLs, linearity curves and retention times for target PFAS.*

Compounds	MDL (ng/L)	MQL (ng/L)	Equation for standard addition calibration curve y=ax+b	R^2	Rt (min)
4:2 FTS	0.64	1.89	y = 16718x + 217.83	R^2 = 0.993	10.13
6:2 FTS	0.63	1.89	y = 27626x + 2E+06	R^2 = 0.990	12.46
8:2 FTS	1.80	4.90	y = 19659x + 7517.5	R^2 = 0.990	14.07
ADONA	0.13	0.32	y = 236082x + 34031	R^2 = 0.996	11.56
GenX	0.16	0.32	y = 17198x + 958.19	R^2 = 0.98	10.60
F53B	0.14	0.32	y = 150544x + 7357.1	R^2 = 0.995	13.73

PFBA	0.55	1.56	y = 26762x - 3750.5	R^2 = 0.990	5.89
PFPeA	0.67	1.06	y = 43120x + 387.89	R^2 = 0.998	8.67
PFHxA	0.25	0.61	y = 47932x + 13108	R^2 = 0.991	10.23
PFHpA	0.25	0.73	y = 91898x + 66748	R^2 = 0.995	11.46
PFOA	0.25	0.54	y = 111209x + 49570	R^2 = 0.992	12.48
PFNA	0.25	0.54	y = 180461x + 52083	R^2 = 0.995	13.37
PFDA	0.25	0.54	y = 88190x + 6939.4	R^2 = 0.997	14.08
PFUnA	0.03	0.09	y = 151570x + 32553	R^2 = 0.996	14.43
PFDoA	0.44	1.10	y = 168940x + 33467	R^2 = 0.997	14.80
PFTrDA	0.23	0.50	y = 194357x + 90250	R^2 = 0.993	15.07
PFTeDA	0.40	1.10	y = 85524x + 123586	R^2 = 0.992	15.41
PFHxDA	0.59	1.80	y = 669.49x + 307.32	R^2 = 0.96	16.33
PFODA	0.66	2.17	y = 55802x + 8498.3	R^2 = 0.992	16.50
PFBS	1.30	3.62	y = 3984.4x - 1501.7	R^2 = 0.995	9.04
PFPeS	0.56	1.43	y = 4040.9x - 1437.1	R^2 = 0.990	10.42
PFHxS	0.85	2.83	y = 2960.5x - 804.59	R^2 = 0.994	11.55
PFHpS	1.26	4.95	y = 3609.2x - 3172.3	R^2 = 0.9995	12.50
L-PFOS	0.33	1.00	y = 2644.8x - 821.66	R^2 = 0.96	13.37
monosubstituted PFOS	0.10	0.30	y = 2849.8x - 311.60	R^2 = 0.997	13.07
PFNS	0.53	1.44	y = 1808.5x - 6202.8	R^2 = 0.998	14.06
PFDS	1.33	2.84	y = 3401.5x - 8710.7	R^2 = 0.98	14.42
PFOSA	0.32	1.13	y = 2723.3x - 955.93	R^2 = 0.995	14.32
N-MeFOSA	1.25	3.79	y = 2640x - 1505.3	R^2 = 0.990	14.29
N-EtFOSA	1.25	3.79	y = 87194x + 56711	R^2 = 0.995	11.50

Table 5. *The recoveries (mean ± SD%) of target PFAS spiked into surface water samples.*

Compounds	**Recoveries surface water (%) (n = 5)**	**%RSD for method (n=5)**	**%RSD instrumental (n=5)**	**Matrix effect surface water (%)**
4:2 FTS	82 ± 7	10	6	9
6:2 FTS	96 ± 10	10	6	15
8:2 FTS	100 ± 4	9	6	7

ADONA	94 ± 12	13	3	46
GenX	97 ±13	12	5	33
F53B	106 ± 5	14	2	28
PFBA	98 ± 5	17	5	-15
PFPeA	89 ± 9	12	7	-14
PFHxA	95 ± 8	7	7	-13
PFHpA	101 ± 3	10	6	-20
PFOA	99 ± 11	3	7	-7
PFNA	100 ± 4	3	1	-39
PFDA	109 ± 6	14	2	-13
PFUnA	90± 5	5	2	-70
PFDoA	96 ± 5	19	6	-60
PFTrDA	102 ± 14	16	6	-15
PFTeDA	95 ± 14	13	6	-13
PFHxDA	114 ± 5	12	3	19
PFODA	84 ± 3	12	3	29
PFBS	105 ± 4	14	10	-7
PFPeS	91 ± 10	13	6	-19
PFHxS	99 ± 10	14	8	-38
PFHpS	107 ± 12	14	4	-18
L-PFOS	102 ± 5	11	5	-75
monosubstituted PFOS	97 ± 4	10	4	-70
PFNS	87 ± 8	22	4	-85
PFDS	107 ± 6	15	4	-34
PFOSA	85 ± 5	17	7	-13
N-MeFOSA	107 ± 11	12	10	-2
N-EtFOSA	82 ± 13	14	4	-30

3.3. Results & Discussion

C6-C9 PFCAs were detected in all samples. In detail, PFHxA (1.0-1.3 ng L^{-1}), PFHpA (0.8-1.4 ng L^{-1}), PFOA (10.7-16.7 ng L^{-1}), and PFNA (0.5-0.7 ng L^{-1}) were present at each sampling station. Overall, PFOA was the chemical found at the highest concentration range, with a median concentration at 13 ng L^{-1}. PFTeDA was also detected in one sample at a concentration of 0.4 ng L^{-1}.

It is worth to mention that ADONA was also omnipresent, however, at low ng L^{-1} concentration levels (0.4-0.6 ng L^{-1}). According to relative literature the PFOA alternatives, GenX and ADONA, are probably more likely to be detected in water where fluoropolymers are manufactured. For this reason, these two compounds are less likely to be detected where chemicals containing PFAS are actually used **[160]**. This hypothesis needs to be further investigated for the case of these lake samples.

Although PFOS was detected in only one sampling location, the measured concentration was clearly above its regulatory EQS limit value of 0.65 ng L^{-1} **[161]**. Several independent studies have already reported PFOS above its EQS value; e.g. concentrations up to 26 ng L^{-1} in the JDS3 **[162]** and 35 ng L^{-1} in surface water from Austria **[163]**. The present study showed generally lower concentrations of PFOA, despite its high FoA, comparing to previous reports (e.g. 37 ng L^{-1} in the JDS3 samples **[162]** and 19 ng L^{-1} in Austria **[163]**).

All remaining PFAS were below MDL, with the exceptions of PFHxS and PFPeS that were detected in one and three samples, respectively. PFHxS was found at a concentration of 3.9 ng L^{-1}, while the median concentration for PFPeS was 1.7 ng L^{-1}.

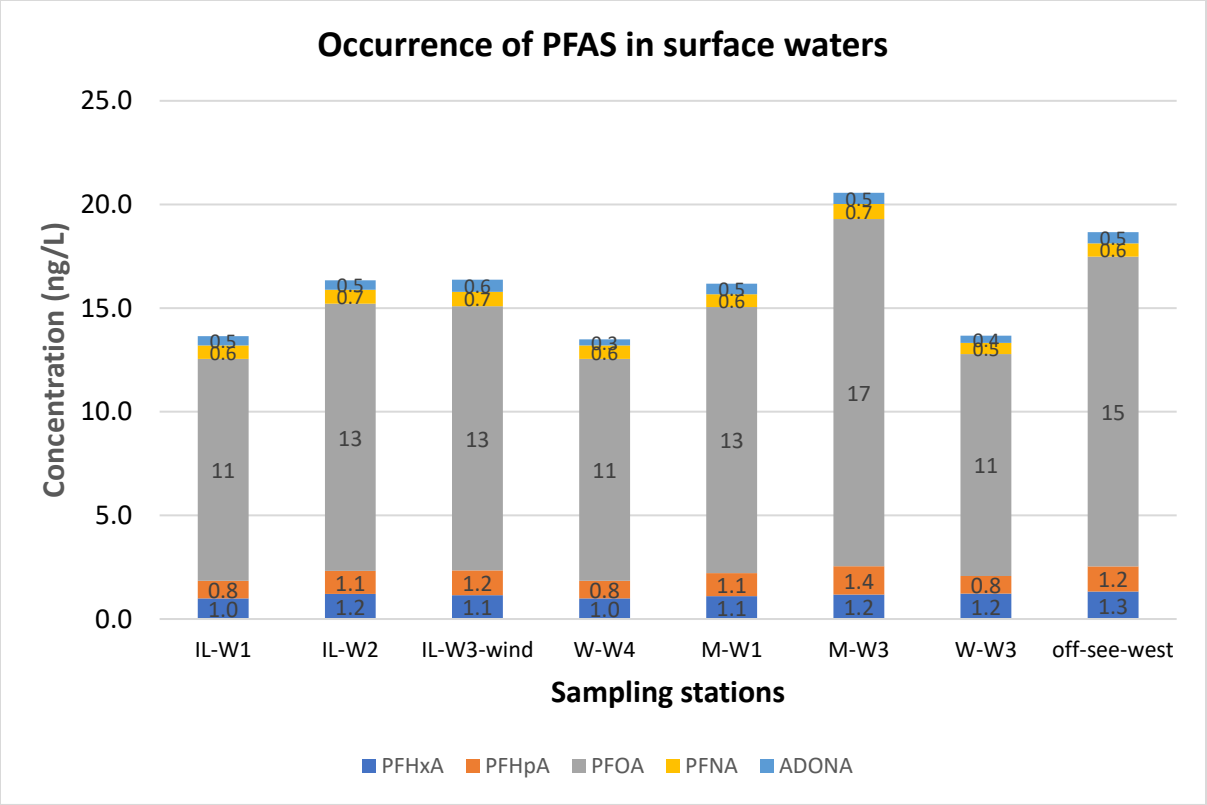


***Figure 3**. Occurrence of PFAS in lake samples from Austria.*

The findings of the present case study pose a good reminder that perfluorinated substances may pose a serious threat to the ecosystem, as shown by the case of PFOS **[162, 164]** even when detected at trace concentration levels.

Chapter 4: Determination of 56 per- and polyfluoroalkyl substances in top predators and their prey from Northern Europe by LC-MS/MS

This case study has been published in Chemosphere, Volume 287, Part 2, January 2022, 131775

4.1. Introduction

PFAS are recognized endocrine disrupting chemicals, and animal studies have suggested multiple pathways of impact that include disruption of reproductive hormones and impaired signaling of thyroid hormones **[153, 165]**. The enormous number of homologues, metabolites and precursors of all known PFAS classes (>4000 variations according to OECD records) and the knowledge gap regarding their environmental fate and hazardous potential makes them a subject of continuous concern **[166]**. The increased half-lives of PFAS in both wildlife and humans render them extremely hazardous for the environment **[4]**. Biomonitoring of PFAS in living organisms is an evolving field of research. Legacy PFAS have been detected in human blood cells **[104, 109]**, breast milk **[113]** seminal plasma **[112]**, and umbilical cord blood **[167]**. Unlike the majority of persistent organic pollutants (POPs), they tend to accumulate in the kidneys, and bile secretion

and not in fat tissues **[168, 169]**. Additionally, PFAS levels have been reported to be very high in human liver cells **[170, 171]**.

Additionally, many PFAS undergo transformation in wastewater treatment plants as well as metabolic alteration in humans and livestock. This creates the urge for PFAS precursors, metabolites, intermediate – and final products to be incorporated in targeted analytical methodologies together with the parent analytes **[172-174]** . In order to limit the environmental as well as health-related risks from the manufacture and use of PFAS, a restriction proposal is being elaborated under REACH in 2021.

Competent analytical techniques have been developed for the determination of PFAS in fish and other aquatic organisms **[7-9]**, birds **[10-12]** and mammals **[13-15]**.

To the best of our knowledge, despite the high number of available analytical methodologies for the determination of PFAS in the environment, few studies have reported the simultaneous determination of multi-class PFAS in contemporaneously collected samples from differing trophic levels within an ecosystem. Environmental Specimen Banks (ESBs), scientific collections (SCs) and Natural History Museums (NHMs) have contributed to water management, chemicals' monitoring, and regulation. Systematic and opportunistic sampling campaigns have been conducted for decades, collecting various tissues from apex predators and their prey (AP&P). Sample collections are guided by standardized protocols and operate under well-controlled conditions to allow for chemicals investigations. The EU funded LIFE Apex project **(LIFE17 ENV/SK/000355, 2018-2022, www.lifeapex.eu)** was initiated to bring together sample collections and analytical laboratories with the objective to apply generic sample preparation and instrumental methods for the generation of contaminant data for apex predators and their prey in support of chemicals management **[175, 176]**.

The objective of the present study was to investigate the PFAS exposure among varying trophic levels including apex predators and fish species, that are also widely consumed by humans. We specifically aimed to expand the LC-MS/MS methodology as described in *Chapter 3* by doubling the target analytes and determine the exposure to established and newer PFSA/PFCA contaminants and several PFSA precursors in livers of common buzzards, Eurasian otters, harbour and grey seals and harbour porpoises and muscle tissues of their major prey species, from several regions across Germany, Sweden, the Netherlands and the United Kingdom.

4.2 Materials and Methods

4.2.1 Study area and sampling strategy

Within the framework of LIFE APEX, 65 samples of common buzzards, Eurasian otters, harbour and grey seals and harbour porpoises and several fish species from various ecosystems across central and northern Europe were retrieved from ESBs, SCs and NHMs (**Table S4-1** in Electronic Supplementary Information) and screened for 56 legacy and emerging PFAS from 14 classes. All apex predator samples in this study were liver tissues, while only fillet (muscle tissue) was extracted from the fish species for the PFAS target screening. This was done according to the project's strategic plan, which received approval by the EU. More specifically, the rationale was primarily ethical. Additionally, there were certain limitations concerning the sample availability from the specimen providers, namely it would have involved excessive fish sampling for the collection of enough pooled liver quantity to be compared with the predator liver samples in terms of PFAS contamination. On the other hand, as the predator screening is regarded, we aimed to analyze liver tissues since it is there where PFAS are primarily accumulated and metabolized. Sampling was carried out by two environmental specimen banks (German and Swedish ESBs), five research collections (UK Centre for Ecology & Hydrology, Cardiff University, University of Veterinary Medicine Hannover, Leibniz Institute for Zoo and Wildlife Research and Wageningen University & Research) and one natural history museum (Naturalis Biodiversity Center) over a 4 year period between 2015 and 2018 in Central and Northern Europe. 65 pooled samples of muscle and liver tissue were, obtained from 61 different locations across Germany, the Netherlands, Sweden and the United Kingdom (**Figure 4**). The 8 species collected were the following: Bream (*Abramis brama*), Roach (*Rutilus rutilus*), Herring (*Clupea harengus*), Eelpout (*Zoarces viviparus*), Harbour porpoise (*Phocoena phocoena)*, Eurasian otter (*Lutra lutra*), Harbour seal (*Phoca vitulina*), Grey seal (*Halichoerus grypus*), and Common buzzard *(Buteo buteo*). All samples were processed at the collectors' facilities and, subsequently, frozen at -20 °C or -80 °C, shipped to and stored at -80 °C at the National and Kapodistrian University of Athens (NKUA) or at the Laboratory of Analytical Chemistry of University of Athens (Greece). Muscle and liver tissue samples were kept frozen and thereafter freeze-dried before analyses. Sampling was conducted under EU research licenses/permits.

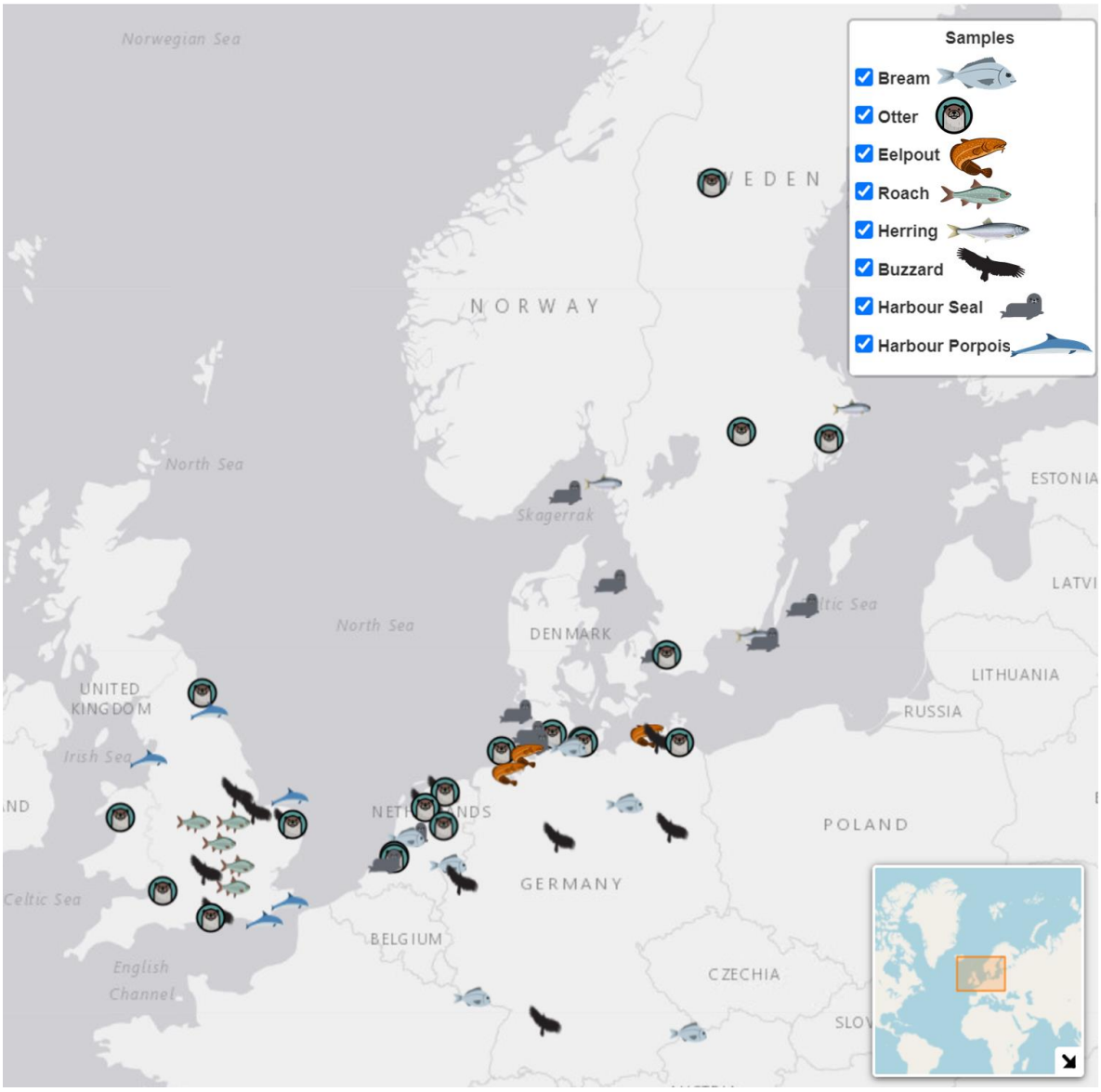


Figure 4.*Sample collection sites and their spatial distribution. Interactive version of the map is available in the following link: https://norman-data.eu/LIFE_APEX_PFAS_Tier1/.*

4.2.2 Chemicals and reagents

The full list of target compounds, internal standards, and consumables that were used in this study can be found in section 2 of the supplementary information. In summary the target list included 13 PFCAs (C3-C14, C16 and C18; Cn refers to the carbon chain-length of the molecule), 7 PFSAs, 3 FASAs, 4 PFAPAs, 3 PFPi's, 5 FTOHs, 2 PAPs, 2 diPAPs, 6 FTAS, 3 FTUAs, 2 FASEs, 3 FTSAs, 2 PFECAs and 1 Cl-PFESA. The compound catalogue, including their abbreviation, compound class, and optimized LC-MS/MS parameters, can be found in **Table S4-2**.

4.2.3 Extraction of samples

All LIFE APEX samples collected from ESBs, NHMs and other scientific collections were sent to NKUA for their pre-treatment. The documentation and condition of the delivered samples were thoroughly checked, and unique sample codes were given to the samples. For the calculation of the % water content of the samples, empty petri-dishes with the respective code of each sample were weighed. This was followed by the segmentation of the samples and their placement into petri-dishes in an isolated room. The petri-dishes including the wet samples were then weighed. All samples were kept refrigerated (-80°C) for at least 5 hours, as a pre-treatment step before lyophilization. Afterwards, the samples' freeze-drying (-55°C, 0.05 mbar, Capacity: 5 kg/24h, Telstar Lyoquest Freeze Dryer) in accordance with the standardized operational procedure (SOP) for the lyophilization took place, followed by the weighting of the petri-dishes including the freeze-dried samples. Accordingly, the % water content was calculated. The weights and % water content, as well as any other freeze-drying relevant information were registered in a specific file. The homogenization of each sample using pestle and mortar or multi in an isolated room was then performed. Between homogenizations all lab instruments were cleaned using milli-Q water and acetone. All freeze-dried samples were then stored (-80°C) in amber glass vials. Accelerated Solvent Extraction (ASE) was used for the extraction of the analytes from the biota matrices, followed by a clean-up step using SPE (in-house mixed mode cartridges, see below). More details about the extraction protocol that was followed in this study can be found in the **Electronic Supplementary Information**. After the injections in the LC-ESI-MS/MS the vials with the remaining extracts were stored in the freezer (-80°C).

4.2.4 Instrumental Analysis

All measurements were performed using a UHPLC Thermo Accela pump incorporating a column thermostat, a degasser, and an autosampler (San Jose, CA, U.S.). The selected mass spectrometric system was a Thermo TSQ Quantum Access triple quadrupole mass analyzer. Details regarding the instrumentation and the chromatographic separation of the target PFAS can be found in the **Electronic Supplementary Information** section. The MS/MS parameters for PFAS analysis are presented in **Table S4-2**.

4.2.5 Quality assurance and quality control

To reduce possible contamination, all labware, weighing and dissection tools were prescreened and rinsed with methanol before use, as in the case of all analyses described in the previous chapter. Additionally, the use of adequate isotope labeled ISs (added prior to extraction) can to some extent compensate for variable recovery and matrix effects among samples. Prior to daily use, we flushed the LC column with elution solvents [MeOH/5 mM ammonium formate (70 : 30, % v/v)] before initiating a sequence. The analytical method was evaluated under the optimized

conditions in terms of linearity, sensitivity, accuracy, repeatability and matrix effects. **Table S4-4a** and **Table S4-4b** summarize the method performance parameters. Seven-point calibration curves were generated using linear regression analysis. The linearity was qualified by linear correlation coefficient, R^2. The reference standard calibration curves obtained for the SRM transitions were linear with $R^2 > 0.95$ in all cases. Accuracy of the method was assessed with recovery experiments in muscle and liver samples. Extraction recoveries for target analytes were determined (n=5) at one concentration level (100 ng/g ww). Recoveries were determined by comparing the concentrations obtained after the whole sample preparation with the initial spiking levels. Satisfactory recoveries 80<recovery<120% were achieved for the majority of the substances for both matrices (**Table S4-4b**). To ensure a correct quantification, method precision was determined as relative standard deviation (%RSD) from the recovery experiments, processed with the described method. Precision limit <20% RSD was met for all analytes indicating the good precision of the method developed. Regarding sensitivity, limit of detection (MDLs, lowest analyte concentration with S/N ratio of 3) and limit of quantification (MQLs, concentration with S/N ratio of 10 and imprecision lower than 20%) were estimated. Finally, matrix effect was evaluated as the percentage of suppression or enhancement. Matrix suppression was observed for 41 and 43 compounds for liver and muscle matrix respectively. The identification and confirmation criteria for the analysis of the target substances was based on the Commission Decision 2002/657/EC. To confirm the presence of the compounds, the retention time of the compounds (2.5 % of tolerance) and relationship between the two transitions (difference of less than 20 %) were used. The detected PFAS were quantified using isotopic dilution (**Table S4-5** in Electronic Supplementary Information). If IS standards were not available, then standard addition method was used. All quantitative results were expressed in ng/g wet weight (ww). In order to express the detected PFAS concentration in ng/g ww, the moisture content (%) of the liver and muscle tissues were considered. Especially for PFOS, samples were diluted 5 times for the quantitation, since it was initially out of the linear range. PFAS with values between LOD and LOQ were replaced by LOQ/2 **[177]**. Method detection limits (MDLs), method quantification limits (MQLs), linearity for the standard addition calibration curves and retention times for target PFAS can be found in **Table S4-4a**, while the recoveries for all analytes spiked into liver and muscle samples are displayed in **Table S4-4b.**

4.3 Results and Discussion

4.3.1 PFAS occurrence in the samples

The quantitative determination of PFAS in complex biological matrices such as muscle or liver samples is a very detailed process that requires accuracy and precision. Despite the knowledge that has been made in the field over the last decades, there are still gaps and uncertainties. As

mentioned in the relevant literature, both negative as well as positive systematic errors may occur at several steps of an analytical scheme. This includes analyte losses and sample contamination, respectively. Moreover, biases may also take place during sampling and storage. Last but not least, matrix effects may affect important analytical parameters, such as instrumental response and measurement reproducibility, while recovery losses are likely to happen at any stage of a multi-step sample preparation and clean-up process. Bearing all the above in mind, the mean ΣPFAS concentrations and ranges (ng/g ww) in the tissues among AP&P species were calculated and are presented in **Table 6**. The individual concentration levels for the target substances in the samples are presented in **Figure 5**, sorted by the frequency of appearance (FoA).

***Table 6**. Mean ΣPFAS concentration and range (ng/g ww) among the tissues of different species in this study. N (pooled) values represent the number of samples analyzed for each species.*

Species	Tissue	n (pooled)	ΣPFAS (ng/g ww)	Concentration range (ng/g ww)	Habitat
Eelpout	Muscle	3	57	46-66	Marine
Herring	Muscle	3	25	16-39	Marine
Bream	Muscle	6	190	100-325	Freshwater
Roach	Muscle	5	77	56-100	Freshwater
Eurasian otter	Liver	20	6321	1942-20236	Freshwater
Harbour/Grey seal	Liver	11	803	244-1517	Marine
Harbour porpoise	Liver	5	1079	357-2692	Marine
Common buzzard	Liver	12	426	217-1092	Terrestrial

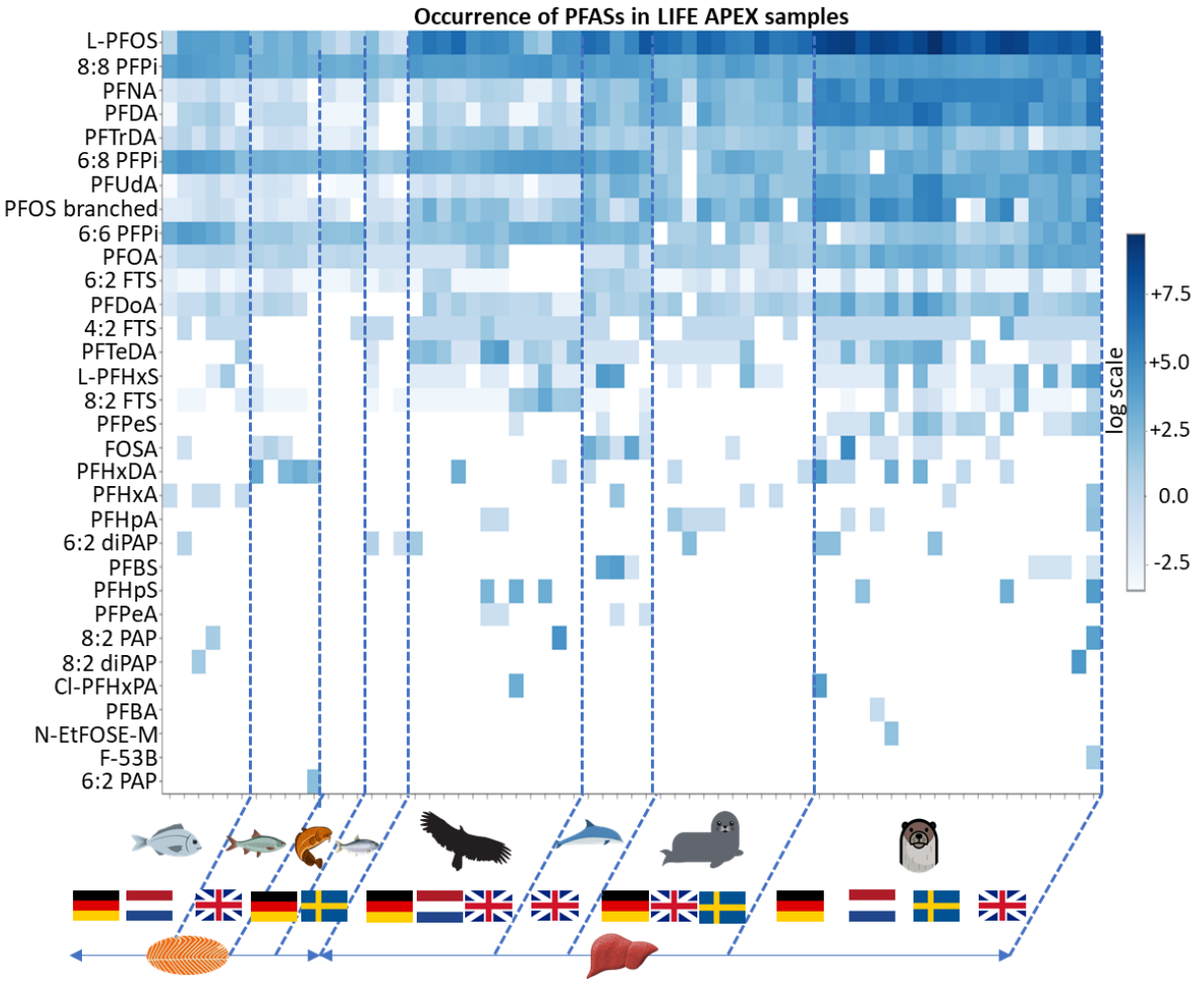


***Figure 5**.Heatmap representing the occurrence of PFAS in the LIFE APEX samples. The concentration levels are given in ng g-1 wet weight in logarithmic scale. The analytes are sorted based on their frequency of appearance (FoA) in the samples. . Clear white colour represents values <MDL for the respective analyte.*

PFOS, 6:6 PFPi, 6:8 PFPi and 8:8 PFPi were detected in all AP&P tissues. C9-C13 PFCAs were detected at noteworthy concentrations in the examined predator liver tissues, and in fairly high levels in the fish muscle tissues. PFODA, PFNS, PFDS, N-MeFOSA, N-EtFOSA, N-MeFOSE, GenX, ADONA as well as all FTOHs, FTASs, FTUAs, and PFAPAs were not detected in any sample. Exception was Cl-PFHxPA, which was detected in two apex samples (a pooled otter sample from Germany and a pooled buzzard sample from UK). ΣPFAS in AP&P tissues ranged from 16 to 20,200 ng/g ww, with the latter being detected in an individual Eurasian otter sample from the Dutch province Overijssel. The highest ΣPFAS concentration in fish muscle was found in a pooled bream sample from Danube Jochenstein (325 ng/ g ww), while the most contaminated taxon overall was Eurasian otter (average ΣPFAS concentration of 6300 ng/g ww). The only positive detection of

the Chinese PFOS alternative F-53B in this study was for an otter sample from the East Anglia region in the UK at a concentration of 3.3 ng/g ww. To the best of our knowledge this is the first time this emerging Cl-PFESA has been detected in Eurasian otters.

4.3.2 Prey samples

As regards the muscle samples of the four edible fish species examined in this research, the average $\Sigma PFAS_{bream}$ (190 ng/g ww) was the highest among the four prey species, followed by $\Sigma PFAS_{roach}$ (77 ng/g ww), $\Sigma PFAS_{eelpout}$ (57 ng/g ww) and $\Sigma PFAS_{herring}$ (25 ng/g ww). Since no outliers were identified among the individual measurements the average and median concentrations coincide across all investigated AP&P species. The PFAS profile of all edible fish analyzed in the framework of this study is predominantly characterized by the presence of PFPi's, with the exception of the pooled bream sample from the Netherlands, that was collected in the province of South Holland. For this sample, 63% of ΣPFAS was PFOS, 20% 8:8 PFPi, 8% 6:8 PFPi, and 18% C8-C14 PFCAs. For all other fish samples in this study PFPi's dominated the respective PFAS ratios, reflecting the fact that these compounds are increasingly used as PFOS alternatives in surfactants and pesticide ingredients. The predominant analogues were, again, 6:8 PFPi and 8:8 PFPi. ΣPFPi's was 77% of the total PFAS yield for bream specimens from Germany, 93% for eelpout from the same country, 55% for roach collected in the river network of UK, and 75% for the herring specimens collected along the Swedish coast in the Baltic. PFHxA was detected at an average concentration of 0.7 ng/g ww in the five pooled samples from Germany. ΣPFCAs (C8-C14) accounted for 3-10% for bream and eelpout from Germany and herring from Sweden. Yet carboxylic acids in pooled roach fillets from the UK were at higher levels than ΣPFOS, with an average concentration of 20 ng/g ww (24% of ΣPFAS for these samples; **Figure 6**).

PFOS was 20% of the total PFAS yield for bream from Germany, 4% for eelpout from Germany, 21% for roach from the UK, and 15% for herring from Sweden, respectively. The low PFAS levels in eelpout samples were comparable to those found in similar studies **[178, 179]**. In general, the quantitative results for the fish samples from Germany are comparable with the PFAS profiling for bream and eelpout matrices in a recent study by Kotthoff et al **[180]**.

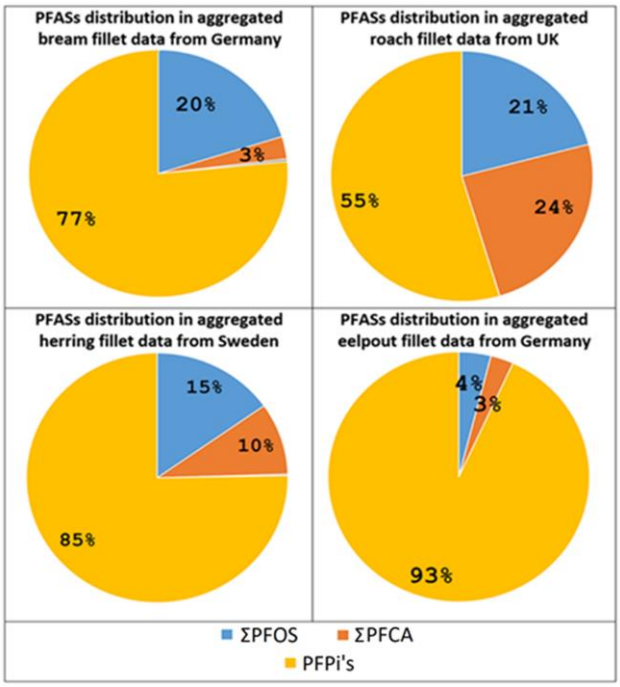


Figure 6*.Relative contribution (%) of ΣPFOS, ΣPFCA and PFPi's to ΣPFAS concentrations in the muscle tissues of the different fish species. Bream: n = 5, Roach: n = 5, Herring: n = 3, Eelpout: n = 3.*

We found that freshwater fish was notably more contaminated than coastal/marine fish (**Table S4-5** in Electronic Supplementary Information). This suggests that fish that live in brackish or open sea ecosystems are less exposed to PFAS and other man-made chemicals than those living in freshwater ecosystems. River and lake fish may be more highly exposed to emissions from anthropogenic activities such as industry and tourism **[181, 182]**. The environmental fate of PFAS follows either sorption to the soil and leakage to the groundwater fluxes and aquifers or discharged through the surface water system to deltas and, eventually, the open sea. For this reason, fish that live in a pristine environment are less exposed to chemicals' contamination, including PFAS, PCBs, DDTs **[183, 184]**.

4.3.3 Apex predator samples

PFAS preferably bind to serum proteins and are typically high in well-vascularized organs, notably in liver tissue as the main organ of albumin synthesis **[170]**. We found overall ΣPFAS levels in apex predator livers up to 4 orders of magnitude higher than the respective values in prey muscle tissues.

Eurasian otter (freshwater top predator)

It has been frequently emphasized in recent studies on dietary intakes of otters as well as other campaigns for the assessment of chemicals management for aquatic mammals and other wildlife, that otters suffer a significant contamination of emerging contaminants **[185]**. Evidence to date suggests that terrestrial foods contribute very little to the nutritional ecology of Eurasian otters, that are mostly piscivorous **[186]**. Representing a large proportion of its diet, fish are responsible for the passing of a large amount of PFAS and other POPs to the metabolism of otters **[187]**. It is worth mentioning that linear and branched isomers of PFOS account for more than 80% of the ΣPFAS yield in the 20 otter samples of our study. For otters, which is the only specie that was sampled in all involved counties within this study, 98% of ΣPFOS was linear PFOS (L-PFOS) and 2% was branched PFOS. The remaining 10-20% of the PFAS cocktail corresponds mainly to long-chain PFCAs (C8-C13), with PFTeDA (C14) appearing the least abundant. Nevertheless, an important 8% of PFPi's detected in the otter samples from the UK is not to be neglected and suggests a slightly alternative chemicals' exposure of these animals.

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

The same is valid for the case of the total of 11 seal samples analyzed within this campaign. Although the total amount of PFAS detected in seal livers is on average 8 times lower than the ΣPFAS quantified in the otters' livers due to the relation marine - freshwater predators, the chemicals palette is similar for both aquatic predators. More specifically, for harbour and grey seals collected from German and Swedish coasts ΣPFOS accounts for 90% of the ΣPFAS burden. In the case of the individual harbour seal samples collected in the Netherlands, 23% of the ΣPFAS corresponds to PFPi's, 1% to FTSAs, and less than 1% to PFOSA traces. This indicates the localized occurrence of PFOS alternatives. The predominant congeners were 6:8 PFPi and 8:8 PFPi. 6% of the seals' PFAS profile from the Netherlands is linked to the identification of PFCAs (C8-C13) and just 1% corresponds to PFHxS. The remaining and still very high percentage (69% of ΣPFAS) is to be attributed to ΣPFOS. The results of our study are in good agreement with the findings of Van de Vijver *et al.* on increasing PFAS concentrations in otters and ringed seals from Sweden **[188]**, reporting that otters have historically been exposed to an order of magnitude higher PFAS contamination compared to seals from adjacent or neighboring areas. Changes in the diet of harbour and grey seal may also affect the level and pattern of PFAS, but also the seasonal changes in the diet of their fish prey will determine the accumulation of pollutants in these marine mammals. Overall, harbour seals have been shown to respond to varying prey availability and distribution by exhibiting high flexibility in their movement ecology and diet.

Along the same line, the 5 pooled liver tissues of harbour porpoises collected from the shores of the UK were the second most contaminated samples. The PFAS pattern showed a remarkable similarity to the PFAS profile outlined for the otters from the UK. The composition of PFAS was

the following: 79% ΣPFOS, 13% PFPi's and FTSAs, 4% PFCAs (C8-C16), 2% PFBS, and 2% PFHxS. Ultralong-chain PFHxDA was detected in a recent (2019) specimen from the Blackpool coastal area at a concentration of 0.90 ng/g ww. PFTeDA was detected in 4 out of 5 pooled harbour porpoise samples in this study at a consistent concentration of < 0.5 ng/g ww. The high levels of PFOS are in good agreement with the results of another study by Van de Vijver *et al.* **[189]**. Harbour porpoises from Northern Europe were found to be heavily contaminated with PFOS and to a lesser extent with perfluorocarboxylates.

Despite the fact that the average ΣPFAS concentration of the aggregated otter samples is approximately 6 times higher than the respective harbour porpoise samples in this study, the PFAS profile for both species is very similar. The afforementioned marine mammals live and hunt for prey in river estuaries and marine and brackish water ecosystems along the coast, while otters are inland water predators. Therefore, it can be concluded that both these taxa are recipients of the same array of PFAS due to their exposure to the same aquatic continuum. The specific dolphin species is exclusively located near harbours and sites of anthropogenic activity, where POPs are washed off through river system discharges **[190].** Otters are inhabitants of the upper part of the same network. Although, patterns of harbour porpoise from the UK are similar to seals patterns from the Netherlands, Germany and Sweden, the reason why the seals are less burdened than the analyzed porpoises in this study should be further investigated.

Common buzzard (terrestrial apex predator)

Common buzzards were found to be the least contaminated, yet most variable of the apex predator species studied in terms of PFAS profiling within the frameworks of this study. The latter is probably due to seasonal changes in the diet of common buzzards and birds of prey in general, resulting of fluctuations in the level and pattern of PFAS. Common buzzards have been shown to respond to varying prey availability and distribution by exhibiting high flexibility in their spatial and temporal movement ecology and diet **[191].** Yet, the fact that no prey species of common buzzards (rodents, rabbits etc.) were included in this study is a limiting factor in drawing robust conclusions for the occurrence of PFAS in buzzards.

For German buzzard samples, PFOS was the most abundant PFAS, accounting for 80% of the total concentration levels. 3% of ΣPFAS was attributed to C8-C16 PFCAs. PFHxDA was detected in a pooled sample from the agroforestry area of Mecklenburg-Vorpommern at a concentration of 22 ng/g ww. The remaining 17% of ΣPFAS for this population accounted for PFPi's, with 6:8 PFPi and 8:8 PFPi being the predominant congeners, as in the case of seals from the Netherlands and harbour porpoises from the UK. For the Dutch samples as well, more than 50% of the total PFAS yield was ΣPFOS. This percentage is a lot lower than in the German specimens. Higher percentages of PFPi's (30%), C7-C14 PFCAs (17%), and 2% of PFHpS were observed in the Dutch avian predators, while higher levels of PFTeDA (50 ng/g ww, on average) and traces of PFPeA,

PFHpA, and PFHxS (< 1ng/g ww) were noted. British birds of prey were the only predator specimens in this study for which PFOS was not the predominant compound in the total PFAS burden. The most abundant was 8:8 PFPi (41%), followed by 6:8 PFPi (24%), ΣPFOS (21%), 6:6 PFPi (5%), and 8:2 FTS (2%). The percentages of C9-C16 PFCAs and ΣPFSAs except PFOS were 3% and 4% of the total PFAS amount quantified in the UK buzzard samples, respectively. PFHxDA was detected in a pooled buzzard sample at a concentration of 0.9 ng/g ww, while just fairly low PFOA levels were documented (0.4 - 6 ng/g ww). The distribution of PFAS for selected predators is shown in **Figure 7**.

This versatility regarding the PFAS profile of the only terrestrial predator species in this study could be linked to the wide range of their foraging areas and diet composition **[192, 193]**. The fact that common buzzards were found to be the least contaminated among the studied apex predator species, strengthens the hypothesis that the environmental fate of PFAS , is to end up in the aquatic environment, also due to their high water solubility, thus rendering terrestrial predators less subject to contamination. However, it is worthful to mention that terrestrial contamination may respond more slowly to restrictions in the use of POPs. For example, polybrominated diphenyl ethers (PBDEs) declined in gannet eggs **[108]** but no significant decline in sparrowhawk livers was observed **[194].**

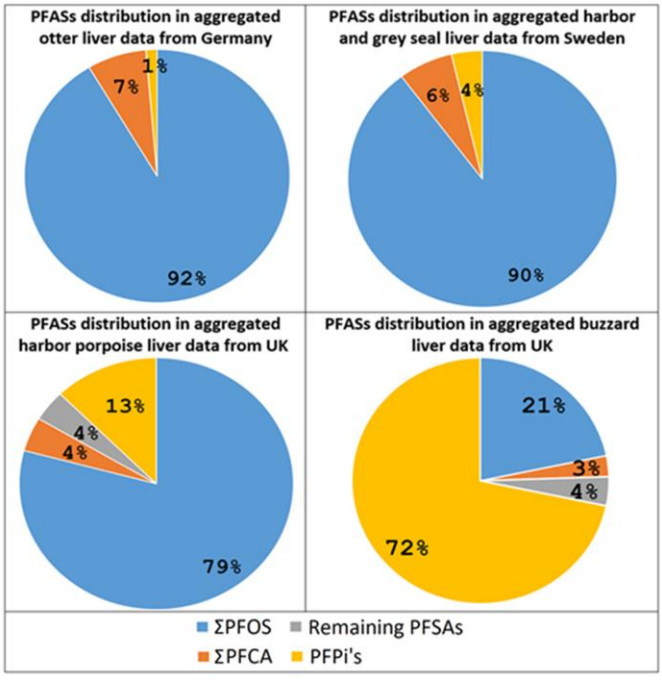


Figure 7.*Relative contribution (%) of ΣPFOS, ΣPFCA, PFPi's, and PFSAs excluding PFOS to ΣPFAS concentrations in the liver tissues of the selected apex predator species. Otters: n = 5, Seals: n = 5, Harbour Porpoises: n = 5, Common Buzzards: n = 5.*

4.3.4 PFAS patterns

Throughout this research, major differences in the PFAS patterns between apex predators and their prey was observed. More specifically, a noteworthy aberration in the PFOS levels was spotted. PFOS was proved to be prone to bioaccumulation, since it was detected in fairly low concentrations in the prey samples but in high concentrations in the predator specimens. The vast differences in the PFOS and other PFAS' levels between prey and predators can partly be attributed to the different tissues used. Zafeiraki et al. **[106]** report the following trend of ascending PFAS concentrations in the tissues of analyzed sharks from the Mediterranean for which all 5 organs were available: gonads > heart > liver ≈ gills > muscle. For completeness purposes, a liver-to-liver comparison between AP and P should be further investigated. We would also like to highlight that an average contribution of 0.02% of branched-PFOS to ΣPFOS was also observed in all samples in this study. These findings suggest that environmental and/or physiological processes, such as sediment – water partitioning, transformation, and bioaccumulation, discriminate between linear and branched isomers, based on different physicochemical properties between isomers. The slightly higher water solubility of branched-PFOS isomers compared to linear-PFOS **[195]** raises the overall toxicity of ΣPFOS. Finally, our results are in agreement with relevant studies showing accumulation of linear PFOS, yet no significant accumulation of the branched isomers in living organisms **[196]**.

The 100% detection frequency of PFPi's, could be attributed to the high persistence and long-range transport potential of this emerging and relatively under-studied PFAS class **[197]**. Like other PFAS, PFPi's are also surfactants possessing a hydrophobic and lipophobic perfluoroalkyl tail connected to a polar anionic headgroup. They are proteophilic and accumulate in protein-rich tissues, such as liver **[198]**. PFPi's are similar to PFOS in terms of chemical structure, containing a perfluorinated carbon tail attached to a phosphinate through a carbon-phosphorus bond **[88]**, therefore they are expected to have similar physicochemical properties, bioaccumulation potential, and even higher acute toxicity than PFOS. The latter hypothesis is based on the fact that PFPi's usually have longer carbon chain length (≥12 C atoms) than PFOS. It has been verified that PFAS with longer carbon chain length are significantly more toxic than the shorter ones **[199]**. Although PFPi's have been reportedly used as defoaming components in pesticide formulations, as well as leveling and wetting agents in industrial and commercial applications **[200]**, it should be noted that it is not known whether PFPi's containing pesticides or other PFPi related products were applied in any of this project's sampling locations. In general, the use of PFPi's in pesticide formulations further complicate characterization of wastewater

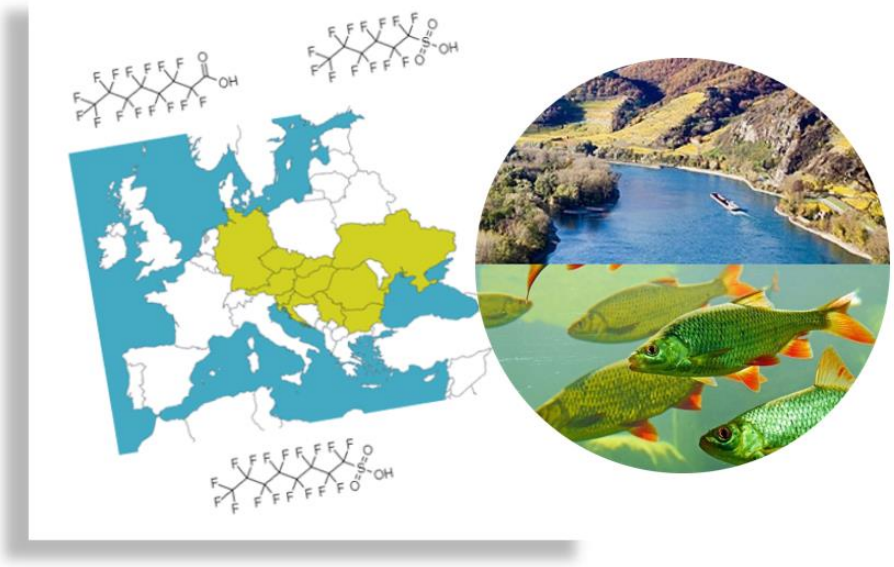
sources from agricultural sources. On the basis of the presence of PFPi's in fish and apex predators, we recommend further research to determine the effect of these substances. While the contribution of PFPi's to the PFAS burden in all samples, determined on the basis of comparison to PFCAs and PFSAs, was dominant, PFAPAs were consistently below detection limits. De Silva *et al.* observed the same PFPi's:PFAPAs ratio in the framework of their recent study on perfluoroalkylphosphinic acids levels in northern pike, double-crested cormorants, and bottlenose dolphins **[98]**. Additionally, we identified microquantities of PFBA, PFPeA, PFHpA, PFHxDA, PFBS, and PFPeS only in AP livers but not in prey muscle tissues. On the contrary, PFOA had a 100% FoA in the prey specimens, yet was below LOD in several predator samples. It could be supposed that the differences in the PFAS between apex predators and prey could be a result of the metabolism and following biotransformation PFAS undergo across the food web. Precursor metabolism and biotransformation processes are complex fields of research that have not yet been fully investigated. The ratio precursor:analyte:metabolite is dynamic and depends on a number of factors, the combination of which may alter the chemicals' mix from taxon to taxon or even at the individual level. Foraging habits, dwelling area/foraging location, migration behavior, sex, age and size strongly influence the PFAS concentrations across a wildlife population. However, sex and body length of the fish species does not influence the bioaccumulation of PFAS, according to previous studies, suggesting that the size of fish does not affect PFAS levels **[201, 202]**.

4.4. Conclusions

The present study presents insights into the frequency of occurrence and concentrations of PFAS in Eurasian otters, grey and harbour seals, harbour porpoises and common buzzards as well as four fish species (bream, roach, herring and eelpout) collected from 61 sampling sites in Germany, the Netherlands, Sweden and the United Kingdom. The analysis of 65 liver and muscle tissues for 56 PFAS shows that all analysed specimens were primarily contaminated with PFOS, while the three PFPi's included in this study exhibited FoA 100%. Additionally, our findings demonstrate that C9 to C13 PFCAs generally occur at high concentrations in apex predator livers despite phase-outs and increasing regulation of these compounds together with C8-based PFAS. The negligible detection of C4-C7 PFCAs in all AP tissues may indicate that the top predators in this study were not exposed to short-chain PFCAs via their prey or may suggest a low bioaccumulation potential of these compounds. PFAS concentrations were one to four orders of magnitude higher in predator liver tissues than in fish muscle. Apart from the difference in the PFAS metabolism in livers and muscles, the significant difference in total body size between predators and prey has to be taken into consideration when comparing total PFAS levels. All the above points to a widespread PFAS contamination in otters, seals, harbour porpoises and, to a lesser degree, common buzzards. While the PFAS contamination in fish muscles was lower than

in predator livers, it was still considerably high. PFAS relative contribution varied among different species, due to the different binding affinity of PFAS for proteins and fats that are tissue- and organism-specific. Furthermore, the results show an association between the PFAS concentrations in apex predators and the geographical origin of the specimens. Despite the fact that the sixty-one sampling areas of this study were diverse, in terms of terrain, climate as well coordinates, a basic correlation between the geographical origin of the samples and the type as well as levels of PFAS in them was observed. This has to be factored in together with the type of matrix and its lipid/protein content, when drawing conclusions about what species were most contaminated and why. Focusing on the interaction extent between humans and wildlife, it was clear that otters and seals, which inhabit freshwater or marine ecosystems often affected by intense anthropogenic activity, are more exposed to contamination by PFAS and other POPs than buzzards whose diet derives from terrestrial food webs. More research is needed to further deepen our knowledge on the environmental fate of PFAS and their accumulation in AP&P.

Chapter 5: Target and suspect screening of 4,777 PFAS in wastewater, river water, ground water and biota samples in the Danube River Basin



This case study has been submitted for publication in Journal of Hazardous Materials

5.1 Introduction

Being the second longest river in Europe **[203]**, the Danube serves as the major source of drinking water for approximately 20 million people living in its catchment area **[148]**. The Danube river also acts as the key ecosystem of the region **[204]** and it is therefore essential to ensure its water quality. Extensive human activities, including large scale industrial production, contribute to releasing numerous contaminants of emerging concern (CECs) into the Danube river **[205]**. Many of these CECs may pose a threat to human health and ecosystems **[206]**.

The CECs include novel and so far unknown PFAS **[207, 208]**. PFAS is a large group of chemicals. They include substances with diverse chemical structures and thus physicochemical properties resulting in different environmental fate. Long-chain PFAS are well-known for being bioaccumulative and persistent in the environment, perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) pose potential reproductive risk to animals and humans which could last for decades **[209]**. The two compounds were found to be the dominant PFAS in the DRB in previous studies **[162, 210]**. However, considering studies from other parts of Europe, it could have been expected that there are more PFAS to be revealed. The identification, risk

assessment and prioritization of PFAS pose a challenge for chemicals management in the Danube region. A comprehensive list of PFAS candidates and advanced analytical approaches are required for performing such a study. Currently, only PFOS (and its derivatives) is listed as a priority substance in the Environmental Quality Standards Directive (2013/39/EU). An environmental quality standard (EQS) of 9.1 µg kg^{-1} wet weight in biota (fish) is recommended for the respective regulatory monitoring programmes **[211, 212]**. Currently, the EQS for PFAS is under discussion and additional perfluoroalkyl acids (PFAAs) have been suggested for monitoring **[213, 214]**. Given the large number of PFAS and the aquatic studies often limited to a small number of known compounds, further environmental studies may reveal other PFAS that require regulatory monitoring and follow up measures to protect the aquatic environment of the DRB **[111]**.

So far, majority of the investigations targeting PFAS in the DRB were performed solely with "traditional" target screening **[162].** Although bioassays as well as sum parameters such as the total oxidizable precursor (TOP) assay or extractable organic fluorine (EOF) assay already exist to determine the total PFAS content in environmental samples, further analysis is needed to determine the identity of individual PFAS **[210]**. These "total organofluorine" methods provide the total occurrence profile of all fluorinated compounds (including PFAS), while liquid and gas chromatography techniques coupled with high resolution mass spectrometry (LC-HRMS; GC-HRMS) could serve as complementary methods which provide comprehensive characterization of the PFAS occurrence profile **[143]**. Suspect screening has proven to be a powerful tool to reveal novel CECs in environmental samples in addition to traditional target screening **[215-217],** and it allows for partially closing the gap in detection of ever growing list of PFAS **[218, 219]**. The prerequisite for large-scale suspect screening include advanced HRMS techniques **[220]**, harmonized analytical procedures with big data processing tools **[221]**, and sharing information via chemical databases **[222]**. The NORMAN Database System (https://www.norman-network.com/nds/) contains all support information and software tools, which are required for hosting and processing of data obtained by wide-scope target and suspect screening of PFAS in the environment. The NORMAN Digital Sample Freezing Platform (DSFP) **[123]** archives GC- and LC-HRMS chromatograms from a wide range of environmental samples across Europe and allows for simultaneous retrospective screening of tens of thousands of CECs, including PFAS, in each sample **[123]**.

The aims of the study were (1) to screen for PFAS in the DRB with special focus on revealing the presence of novel PFAS in wastewater, river water, groundwater, sediment, and biota samples; (2) to investigate distribution of detected PFAS in each matrix; and (3) perform environmental risk assessment and prioritize detected PFAS to characterise the potential threat of PFAS pollution in the DRB.

5.2 Materials and methods

5.2.1 Investigated samples

The study covered 95 environmental samples including 22 wastewater (11 influent and 11 effluent, from municipal wastewater treatment plants), 51 river water, 7 groundwater, 11 biota and 4 sediment samples, all obtained within the Joint Danube Survey 4 (JDS4) organised by the International Commission for the Protection of the Danube River (ICPDR) in June and July 2019. Six additional samples (procedural blanks) were used for quality control purposes.

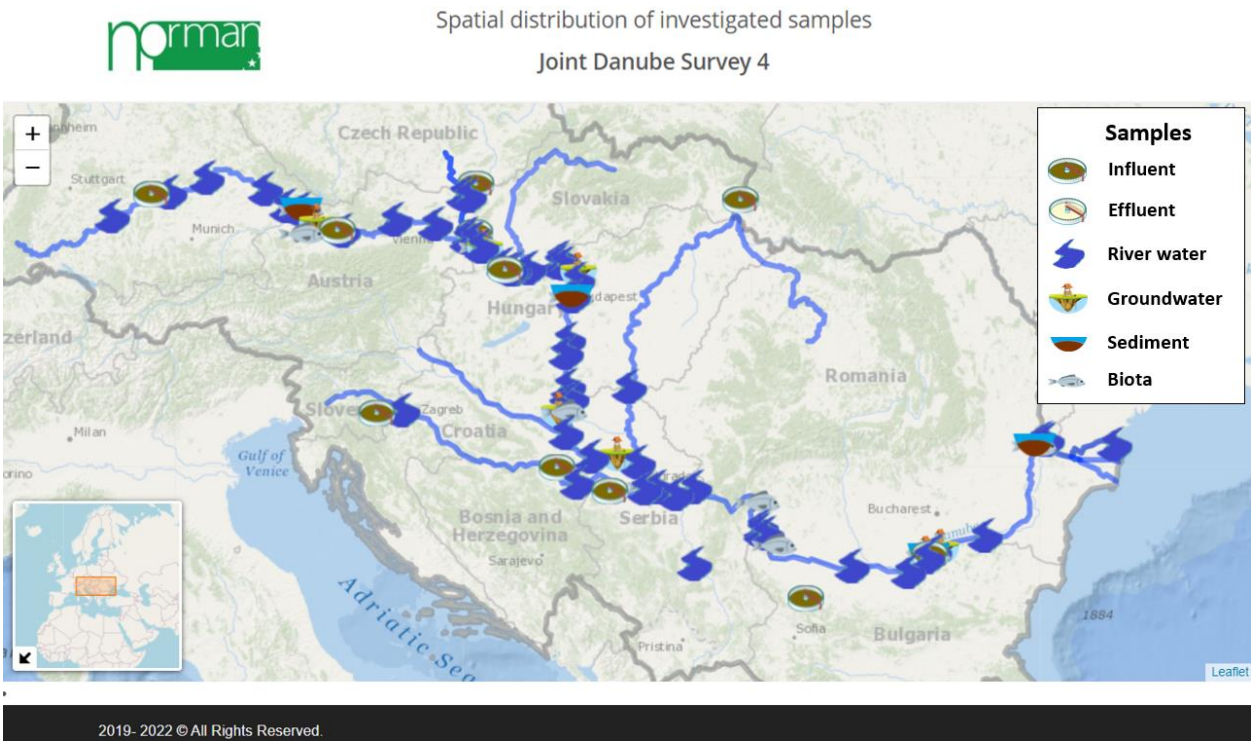


Figure 8*.Spatial distribution of the investigated samples collected in context of the JDS4. Online interactive map is available at https://norman-data.eu/JDS4_Samples.*

5.2.2 Sample preparation and instrumental analysis

All water samples (groundwater, river water, influent and effluent wastewater) underwent solid phase extraction (SPE) with HORIZON SPE-DEX 4790 device (USA). Following SPE, samples were concentrated with an automated extraction program, with the use of Atlantic HLB-M Disk equipped with 47 mm disk holder **[223]**. HLB cartridges showed lower background levels and higher recoveries for PFAS (especially for short chain PFAS such as 1,1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10-henicosafluoro-12-iodoheptadecane, perfluorobutanesulfonic acid (PFBS)) when compared with cartridges such as Sep-pak tC18 **[224]**.

The present sample treatment program provides extraction for a broad range of organic substances with the effort to retain as many targeted analytes as possible. Nonetheless, some substances could have been lost during the sample preparation process in our study, especially for short chain PFAS as there could be difference in retention owing to their shorter hydrophobic chains. The strategies to address such analytical gaps could be found elsewhere **[225]**. The limit of detection, recovery, repeatability, and matrix effect of the extraction program could be found elsewhere **[223]**. The volume of sample for influent wastewater, effluent wastewater, river water, and groundwater was 200 mL, 2 L, 4 L, and 4L, respectively.

Sediment samples were processed using a validated protocol **[226]**: freeze-dried sediment samples (0.2 g each) were spiked with the corresponding surrogates, and kept in contact overnight. Samples were then extracted with 2 mL methanol–Milli-Q water (50:50; v/v), at pH 2.5 with 0.1% EDTA and 0.5% formic acid) by 1 min of vortex-mixing, followed by 50°C ultrasonic extraction for 15 min. Extracted samples were centrifuged at 4000 rpm for 10 min and the supernatant was collected in glass tubes. The procedure was repeated three times and, in total, 6 mL supernatant was collected.

Biota extraction was performed following an optimized multiresidue method **[227]**. Briefly, 1 g of each properly homogenized sample was spiked with target compounds and internal standards (IS). After 10 – 15 min of rest, 2 mL of Milli-Q water containing 0.1% formic acid and 0.1% EDTA was added to the samples, followed by 2 mL of methanol and 2 mL of acetonitrile to enhance the extraction recovery **[228]**. After vortex-mixing for 30 sec, samples underwent ultrasonic-assisted extraction at 60°C for 20 min, followed by centrifugation at 4000 rpm for 10 min. Supernatant was transferred to new polypropylene tubes and kept at 23°C for 12 h. After another round of centrifugation, supernatant was defatted using 5 mL of hexane by vortex-mixing for 1 min, followed by centrifugation at 4000 rpm for 10 min. Hexane layer was removed and extracts were collected.

The extracted samples were evaporated to dryness under a gentle stream of nitrogen at temperature below 40°C, reconstituted in methanol and water (50:50; v/v) and filtered with a 0.2 µm RC filter. The total volume of the extract was approximately 500 µL in all cases. The extract was split in two vials with 250 µL inserts.

One extract was analysed by a fully-validated targeted LC-MS/MS method **[229].** Isotopic dilution was used for quantification of targeted PFAS. The target list can be found in **Table S5-1** in the supplementary material. More details about the chromatographic conditions and the instrumental setup can be found elsewhere **[229]**.

The second extract was analysed by ultra-high performance electrospray ionization quadrupole time of flight (UHPLC-ESI-QTOF) method. The LC system was a Dionex UltiMate 3000 RSLC (Thermo Fisher Scientific, USA), which was coupled to a Maxis Impact QTOF (Bruker, Germany). Information about the instrumental conditions can be found elsewhere **[230]**. The data was used for suspect screening of PFAS after exporting the files to mzML using Bruker CompassXport

3.0.9.2. (Bruker Daltonics, Germany). The files were uploaded to DSFP that was used to perform retrospective suspect screening **[123]**.

5.2.3 Quality assurance and quality control

Sampling was conducted within the Joint Danube Survey 4 (JDS4) organised by the ICPDR. Logistic planning during the JDS4 sampling campaign and locations of the sampling sites are thoroughly described in the JDS4 Scientific Report **[231]**. During the transport, the samples were kept at 4°C to avoid degradation and were processed immediately upon arrival to the laboratory. All samples were spiked with internal standards at 50 ng L^{-1} level, to ensure that the extraction protocol and instrumental analysis performed as expected. For each matrix, an artificial sample acting as procedural blank was used to avoid reporting false positives. The electrospray ionization (ion transfer tube and skimmer cone) for both instruments were cleaned before analyzing the extracts following the cleaning protocol: (i) Milli-Q water and isopropanol (70:30) in a sonication device (at temperature of 50^{o}C) for 15 minutes, (ii) Milli-Q water (15 minutes, 50^{o}C) and (iii) methanol (15 minutes, 50^{o}C).

The mass spectrometers were calibrated before the analysis and were set to operational mode for 1 hour. The chromatographic system was running with the initial gradient conditions for 1 hour. Low resolution target screening identifications passed two multiple-reaction monitoring criteria as requested by the Commission Decision 2002/657/EC **[232]**. Recovery ranged from 42 to 144%, while satisfactory recoveries (between 80 to 120%) were observed for the targeted analytes in all matrices studied. All suspect HRMS detections passed the following identification criteria: i) mass accuracy <2 mDa, ii) RT prediction ±20 % if within the applicability domain **[233]**, iii) presence of at least two qualifier fragment ions, except substances that do not yield more fragments because of their chemical structure and iv) compliant isotopic fit in case isotopic peaks were available. All suspect identifications were verified manually to reduce the possibility of reporting false positives.

5.2.4 Suspect screening of PFAS

The chemical structures of 4,777 PFAS were retrieved from the NORMAN Substance and Suspect List Exchange Database modules of the NORMAN Database System **[234]**. This covered all individual PFAS lists that have been submitted to the NORMAN network until July 2021. The list of the 4,777 screened substances has been submitted to the NORMAN Suspect List Exchange Database **[234]** and is available as list S89: PRORISKPFAS.

MS fragmentation prediction was established with the CMF-ID software **[235]** based on the chemical structures of the retrieved PFAS. Retention Time Index (RTI) prediction was established with the special tool 'Development and Prediction of Retention Time Indices for LC-HRMS' (version 2.5.0; University of Athens, Greece) **[236]** based on the chemical structures of the retrieved PFAS. The MS fragmentation and RTI predictions of the studied PFAS were compared

against the LC-HRMS chromatograms collected from the DRB in an automated manner after uploading the data to DSFP **[123]**.

For suspect screening, a detection entails a match in *m/z* of below 2 mDa, plausible retention time (± 20% of the predicted RTI) and match of at least two MS/MS fragments. **Figure 9** is a typical challenging example of PFAS detection in LC-HRMS chromatogram recorded in data-independent acquisition mode. The signal was detected in four surface water and one groundwater samples from Hungary. The observed MS/MS fragments with *m/z* 284.9779, 168.9894 and 118.9926 corresponding to fragments $C_5F_{11}O^-$, $C_3F_7^-$ and $C_2F_5^-$ respectively, which clearly indicate the presence of a PFAS compound. When performing suspect screening of thousands of compounds, it is crucial to group compounds with the same molecular formula that yield signals of the same intensity. In this specific example (group 762, **Figure 9b**), there were six candidate substances in the suspect list that comply with the experimental evidence. Here, one should be aware, that many commercial PFAS mixtures are chemically translated to a single MS-ready compound **[237]**. For example, PFAS with the CAS Nos. 13252-13-6, 62037-80-3, 67963-75-1, 67118-55-2, 165951-17, 165951-18-8 result in one MS-ready compound. Examples of chemical structures that fit to the same experimental data are presented in **Figure 9c**. To reveal which one of the six suspected PFAS is present in the sample, one has to use additional evidence (e.g., ion mobility MS mobilogram) and ultimately purchase or synthesize the reference standard. In this study, we considered such case as the detection (by suspect screening) of the candidate PFAS with the lowest PNEC, and proceeded with the ecotoxicological risk assessment accordingly.

The same methodology was applied for all the identifications to get the highest quality results possible. Detection limit of suspect screening was 1.00 ng L^{-1} for groundwater samples; 1.25 ng L^{-1} for river water samples; 2.00 ng L^{-1} for wastewater samples; 1.5 ng g^{-1} wet weight for biota samples; and 5.0 ng g^{-1} for sediment samples. To enable environmental risk assessment, semi-quantification of the detected PFAS was performed using the standard addition calibration curve of the structurally most similar compound in the target list **[238]**.

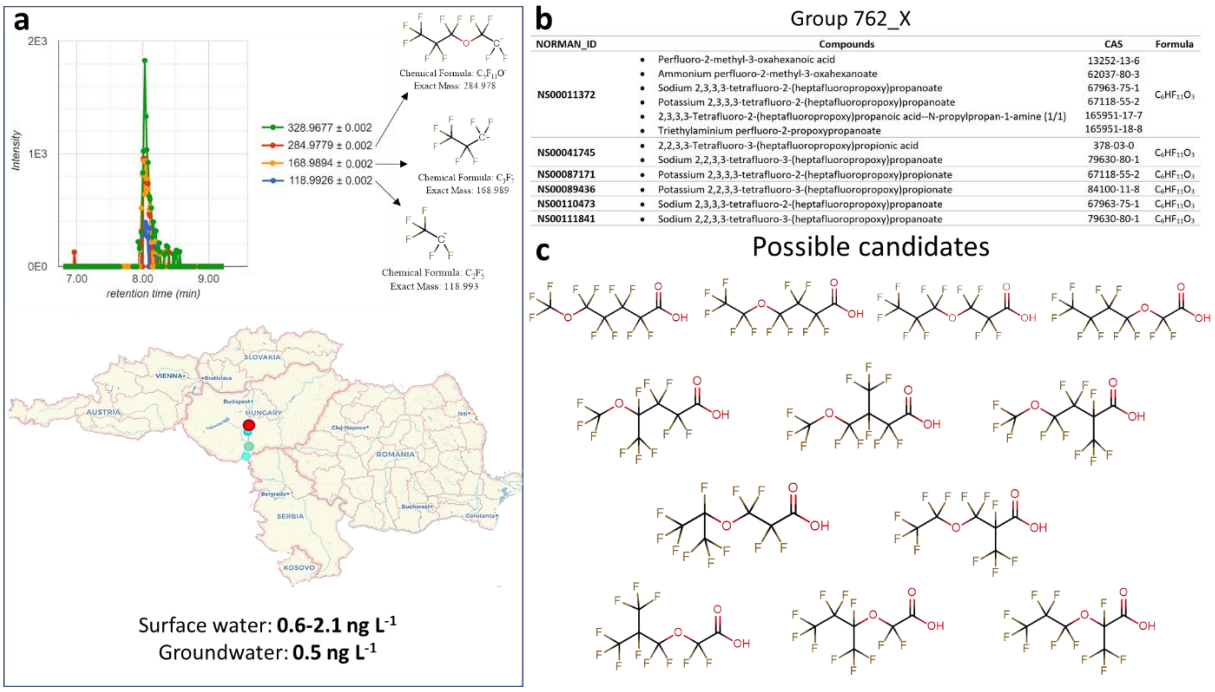


Figure 9*. A typical example of suspect screening of PFAS in the river water samples (JDS4-26, 27, 28, 29) and groundwater sample (Surany region). 9a shows the available experimental evidence, the spatial distribution of the detected signal and an estimation of the concentration of the suspected compound. 9b provides the list of suspected compounds that match with the available experimental evidence (the one with the lowest PNEC was selected for ecotoxicological risk assessment). 9c provides additional possible candidates from a non-target screening perspective.*

5.2.5 Risk assessment of identified PFAS

Ecotoxicity threshold values (predicted no effect concentrations; PNECs) for the 4,777 analysed PFAS were retrieved from the NORMAN Ecotoxicology database (https://www.norman-network.com/nds/ecotox/). PNECs were selected using the following order of credibility: (a) legislative thresholds (EQS values); (b) experimental PNEC values from reference laboratories; (c) *in silico* predicted PNEC (if no experimental PNEC was available) **[239]**. The list of PNECs of detected PFAS per matrix (wastewater, river water, groundwater, and biota) are provided in **Table S5-2A** in the supplementary material. For risk assessment of effluent wastewater, a conversion of the individual concentrations to freshwater concentrations with a factor of 5 was adopted (optionally 2 or 10 **[240]**).

Frequency of Appearance (FoA, on a scale of 0 to 1), Frequency of PNEC Exceedance (FoE, 0 - 1) and Extent of PNEC Exceedance (EoE, 0 - 1) were calculated for the detected PFAS in the 95 JDS4 samples **[241]**. FoA of PFAS shows the percentage of sites where the substance was detected;

FoE represents the percentage of sites with PNEC exceedance. EoE was established by computing the score for exceedance of the environmental threshold value, which involves dividing the 95th percentile of the measured environmental concentrations at each site (MEC_{95}) by the lowest PNEC of the chemical derived for the studied matrix. The translation from the score for exceedance of environmental threshold value (MEC_{95}/lowest PNEC ratio) to EoE is shown in **Table S5-2C** in the supplementary material.

The concentration range of the detected PFAS and its median in each matrix are shown in **Table S5-2B** in the supplementary material. Risk score was assigned to each of the identified PFAS as the sum of FoA, FoE and EoE, which ranges from 0 to 3 per substance per matrix.

5.2.6 PNEC analysis – grouping of PFAS by functional group

The 4,777 studied PFAS were assigned to groups according to their functional groups similarity, in accordance with the Ecological Structure Activity Relationships (ECOSAR) Predictive Model **[242, 243]**. The prediction of PNEC for substances lacking experimental data was derived using state-of-the-art models with defined applicability domain **[42]**. Nevertheless, the manual verification of predicted PNECs is important. Unfortunately, actual empirical data to develop PNECs in a manner that would be consistent with the development of PNECs for REACH dossiers exists for a limited number PFAS substances. Ranges of PNECs of these PFAS groups comprising at least 30 PFAS were compared. The aim was to find out whether PFAS in these groups could be assigned 'an average' or estimated characteristic PNEC value for the whole group to be used in further risk assessment.

5.3 Results and discussion

5.3.1 Occurrence of PFAS

In total, 82 PFAS were detected by target and/or suspects screening. Five were observed only in influent wastewater samples but not in effluent wastewater samples (2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10-nonadecafluorodecyl 2-methylprop-2-enoate (CAS RN: 23069-32-1, also found in one groundwater sample), 2-ethyl-4-(1,1,1,2,3,3,3-heptafluoropropan-2-yl)-3-(2-methylpropane-1-sulfinyl)benzoic acid (CAS RN: 1355554-99-2), ethenyl 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctyl carbonate (CAS RN: 96383-57-2, also found in one sediment sample), 1,1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,12,12,13,13-heptacosafluoro-15-iodopentadecane (CAS RN: 146983-96-2), and ethyl 4,4,5,5,6,6,7,7,8,8,9,9,9-tridecafluoro-2-iodononanoate (CAS RN: 165281-74-3)), which suggests that they have been successfully removed during the wastewater treatment. Nevertheless, all five compounds possess high logKow values (>5) which indicate high potential to bio-concentrate in living organisms. These substances are potentially of regulatory interest and the sources need to be

investigated. Further monitoring efforts in other European river basins may be required to gather enough evidence about the substances.

The study involved only four sediment samples which was insufficient to generate results representative of the occurrence of PFAS in the entire basin. Therefore, risk assessment and prioritization were not performed for the sediment samples. Nevertheless, four PFAS were detected in these sediment samples, including ((perfluorodecyl)methyl)oxirane (CAS_RN: 38565-54-7, in three samples), 3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,12,12,12-henicosafluorododecanal (CAS_RN: 864551-38-2, in three samples), 2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,11-henicosafluoroundecanal (CAS_RN: 63967-42-0, in two samples), and ethenyl 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctyl carbonate (CAS_RN: 96383-57-2, in one sample). Some of them were also detected in samples of wastewater and/or groundwater, indicating these potential contamination source.

Figure 10 shows the occurrence of PFAS in river water, groundwater, effluent wastewater, and biota samples (detected by target and/or suspect screening). The majority of studied PFAS were detected in river water, effluent wastewater and groundwater samples: over 30 PFAS per matrix with some overlaps. PFOS was the only PFAS that was detected in all four matrices studied. Out of the eight PFAS detected in biota samples, six were not detected in the other matrices studied (PFOS in all four matrices, perfluorodecanoic acid (CAS_RN: 335-76-2) in biota and effluent wastewater). 22 PFAS were detected in river water but not in effluent wastewater, which indicates that industrial emissions or alternative contamination sources should be sought for.

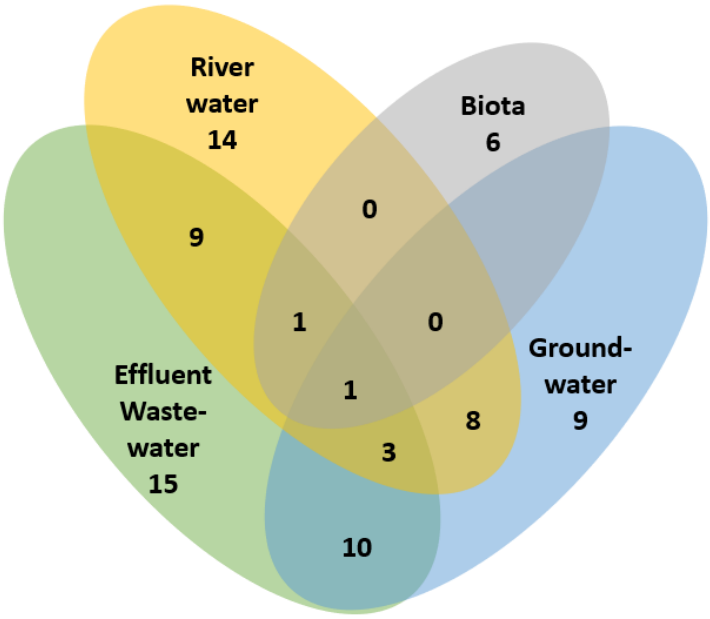


Figure 10*. Venn diagram showing occurrence of identified PFAS (covering both target and suspect screening) in 80 JDS4 river water, effluent wastewater, groundwater and biota samples; results from influent wastewater and sediment samples were not included (see text above).*

The analysed JDS4 samples were collected from Germany, Austria, Czech Republic, Slovakia, Hungary, Slovenia, Croatia, Serbia, Romania, Bulgaria and Ukraine. The heat map of the presence of PFAS by country and matrix is shown in **Figure 11**, including 10 PFAS identified by target screening (indicated by '*' in **Figure 11**) and 72 additional PFAS determined by suspect screening.

Results suggested that the highest number of PFAS were present in river water. A gradual decline in number of detected PFAS was observed from upstream countries (Germany, Austria, etc.) to downstream countries (Ukraine, Bulgaria, etc.). The study results were consistent with the previous study on JDS4 samples aiming at the identification of selected PFAS (PFOS (CAS_RN: 1763-23-1), PFOA (CAS_RN: 335-67-1), perfluorohexanoic acid (PFHxA; CAS_RN: 307-24-4), etc.) [244].

Figure 11 shows that six PFAS (1,1,1,2,2,3,3,4,4,5,5,6,6-tridecafluoroundecane (CAS_RN: 1287702-48-0), 6:2 fluorotelomer methacrylate (CAS_RN: 2144-53-8), 4,4,5,5,6,6,7,7,8,8,9,9,9-tridecafluorononyl prop-2-enoate (CAS_RN: 216389-85-4), (heptadecafluorooctyl)phosphonic acid--4-methylaniline (1/1) (CAS_RN: 1263361-03-0), PFOPA – (heptadecafluorooctyl)phosphonic acid--4-methylaniline (1/1) (CAS_RN: 40143-78-0), and perfluoroundecanoic acid (CAS_RN: 2058-94-8)) were detected in biota samples, whereas they were not detected in wastewater, river water, groundwater or sediment samples. Such PFAS may be the product of metabolism in the fish. These substances were not detected in river water, which indicates their presumably high hydrophobicity.

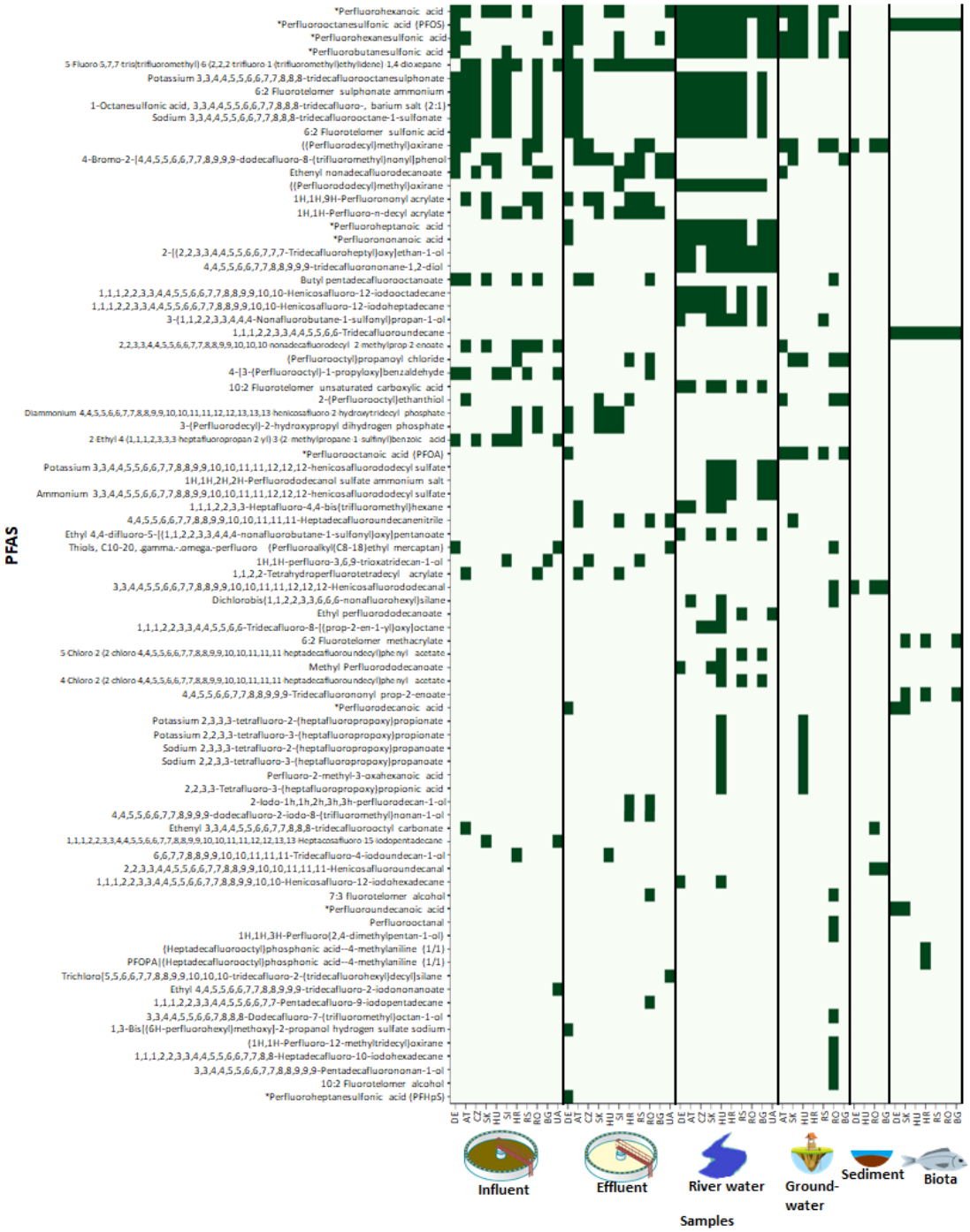


***Figure 11**. Heat map of PFAS occurrence organized by matrix (influent wastewater, effluent wastewater, river water, groundwater, sediment and biota samples) and by country (Germany DE, Austria AT, Czech Republic CZ, Slovakia SK, Hungary HU, Croatia HR, Serbia RS, Romania RO, Bulgaria BG, Ukraine UA) arranged in the order of total frequency of appearance (from high to low). CAS RN and InChiKey of the detected PFAS are available at **Table S5-2A** in the supplementary material.*

5.3.2 Target and suspect screening of PFAS

In total, 82 PFAS were identified. Ten PFAS were detected by target screening in samples of influent and effluent wastewater, river water, groundwater and biota. Suspect screening of PFAS in JDS4 samples resulted in detection of 73 PFAS which belong to a wide range of chemical classes (including carboxylic acids, sulfonic acids, alcohols, etc), of which perfluorohexanesulfonic acid (PFHxS) was detected by both target and suspect screening analytical approaches. In conclusion, suspect screening allowed for detection of 72 additional PFAS that were missed by target screening.

It should be noted that the ten PFAS determined by target screening were all detected also by suspect screening, but there was insufficient evidence for identification of nine of them (did not pass through minimum identification requirement criteria) as there were less than two matches of their MS fragments. A possible reason for such observation is that the lower limit of detection (LOD) of triple quadrupole MS used in target screening was lower than that of HRMS utilized for suspect screening. For example, LOD of suspect screening (using the present LC-HRMS method) in river water and biota samples was 1.25 ng L^{-1} and 1.5 ng g^{-1} w.w., respectively; while LOD of target screening (using the present highly sensitive LC-MS/MS method) in river water and biota samples was below 0.02 ng L^{-1} and 0.5 ng g^{-1} w.w., respectively for most of the targeted analytes. Moreover, concentrations of the ten PFAS identified by target screening were compared with the semi-quantitative concentrations obtained by suspect screening methodology. The values were in the same order of magnitude for majority of the PFAS in all matrices. PFAS concentrations measured by target screening were higher than PFAS concentrations estimated by suspect screening for 221 out of the 224 detections (10 PFAS in total, detected 224 times), by 2.2 times higher on average. The finding is suggestive of a correction factor of 3.2 for the estimated PFAS concentration from suspect screening. A larger scope study for comparing estimated PFAS concentration from target and suspect screening would give a more robust correction factor. Considering that the detected concentrations are in the trace analysis range (low-µg L^{-1} and ng L^{-1}) such differences might be considered acceptable for screening purposes and risk assessment. Target compounds could nonetheless be excluded from suspect screening approach as a result. The study demonstrates that qualitative and semi-quantitative analysis by suspect screening provides complementary results in the PFAS screening.

Previous study on Danube River water (Germany) revealed the presence of numerous unknow PFAS by direct total oxidizable precursor (dTOP) assay, total PFAS concentration detected by dTOP was more 10 times higher than the total PFAS concentration of 41 targeted substances (among the targeted and suspected compounds of the present study) **[245]**. This is consistent with the results of the present study that suspect screening reveals the presence of many PFAS that could be missed by target screening.

Obviously, target screening yielded identification and quantification of PFAS with higher confidence compared to suspect screening alone. Nevertheless, the study has proven that

suspect screening is a significant addition to the “traditional” target screening, both in terms of the number of PFAS identified and number of samples in which PFAS were detected.

5.3.3 Risk assessment and prioritization

Risk score was established for the detected PFAS by comparing the detected concentration level (from target screening and semi-quantification of suspect screening) to the PNEC in each of the studied matrices. **Figure 13** shows radial plots of risk scores of the detected PFAS in (a) wastewater, (b) river water, (c) groundwater, and (d) biota.

PFOS, the only PFAS regulated under the WFD **[246]**, received risk score of over 2.9 (out of the maximum 3) in both river water and biota, which was the highest among the detected PFAS. The high risk score of PFOS in biota suggests its environmental significance. Moreover, PFOS was the highest ranking PFAS as the risk score indicated a concern for all four matrices studied (risk score of 3 in biota, 2.9 in river water, 1.4 in groundwater, and 1.2 in wastewater). Despite being listed under Annex B of the Stockholm Convention on Persistent Organic Pollutants since May 2009 **[247]**, PFOS is still commonly found in commercial products including fire-fighting foams and surfactants **[248]**.

Risk scores indicated a potential concern for other PFAS, including PFHxS (river water: 1.9; groundwater: 1.6, perfluorohexanoic acid (PFHxA) (wastewater: 1.00; groundwater: 1.63), and PFOA (river water: 2.2; groundwater: 1.6). On top of the aforementioned three PFAS and PFOS, 14 additional PFAS ((perfluorododecyl)methyl)oxirane, 1,1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10-henicosafluoro-12-iodooctadecane, PFBS, ((perfluorodecyl)methyl)oxirane, butyl pentadecafluorooctanoate, (perfluorooctyl)propanoyl chloride, 2-(perfluorooctyl)ethanthiol, 5-fluoro-5,7,7-tris(trifluoromethyl)-6-(2,2,2-trifluoro-1-(trifluoromethyl)ethylidene)-1,4-dioxepane, 4-bromo-2-[4,4,5,5,6,6,7,7,8,9,9,9-dodecafluoro-8-(trifluoromethyl)nonyl]phenol, 1H,1H,9H-perfluorononyl acrylate, 1H,1H-perfluoro-n-decyl acrylate, ethenyl nonadecafluorodecanoate, and 1,1,1,2,2,3,3,4,4,5,5,6,6-tridecafluoroundecane) were ranked as potential threat using target and suspect screening. They were detected in the studied samples and ranked in each of the matrices as shown in **Figure 13**. Target screening alone would not reveal the importance of the occurrence of these PFAS compounds in the environment.

The detection of 82 different PFAS in samples collected across the DRB (as shown in **Figure 10**) indicates a potentially on-going, large-scale occurrence of PFAS in Europe. Systematic monitoring of PFAS, with particular attention to those suspected to cause adverse effects to ecosystem and human health, and their cessation from the environment is obviously required. This is consistent with the Chemicals Strategy for Sustainability by the European Commission, where phasing out the use of PFAS is one of the key actions listed **[249]**. Initial steps of such comprehensive study have been taken by the NORMAN network **[219, 250]**. Data management techniques are available for a large scale retrospective screening of PFAS across Europe in an automated manner

[123, 251]. Such study would facilitate the development of future regulatory monitoring of PFAS in Europe, and outline its impact to various stakeholders, including chemical manufacturers, communities and consumers **[252, 253]**.

The groundwater EQSs and/or threshold values of some PFAS were proposed **[254-256]** - 3 μg L^{-1} for PFBS, 1 μg L^{-1} for PFHxA, and 0.1 μg L^{-1} for PFOA. Each of the three PFAS was detected by target screening in five out of the seven groundwater samples in the presented study, 10 out of 15 determined concentrations of these three compounds were above the aforementioned EQSs and/or threshold values. A limit value of 0.1 μg L^{-1} was introduced for the sum of 20 PFAS (PFBA, PFPA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoDA, PFTrDA, PFBS, PFPS, PFHxS, PFHpS, PFOS, PFNS, PFDS, perfluoroundecane sulfonic acid, perfluorododecane sulfonic acid, perfluorotridecane sulfonic acid) in drinking water **[257]**, of which some of them were detected in groundwater in this study. For the assessment of other PFAS, the proposed threshold value for individual PFAS in the EU Drinking Water Directive 2018 recast (0.1 μg L^{-1}) **[258]** was considered. The 31 detected PFAS in groundwater samples were detected at concentrations above 0.1 μg L^{-1}. The results indicate the presence of PFAS in the groundwater of the DRB at threatening levels.

The screened for 4,777 PFAS were assigned into 132 groups according to the functional groups present in the compound, using the ECOSAR compound classification model. Out of these, 29 groups contained 30 or more PFAS each; the PNEC range per group are included in **Figure 12**. The 29 groups (amines, alcohols, sulfonamides, carboxylic acids, ketones, epoxides, amides, ethers, phosphates, acid halides, sulfoxides, esters, sulfonic acids, aldehydes, benzoic acids, anilines, phosphodiesters, nitro/nitroso compounds, thioethers, methacrylates, aliphatic chains or rings, acrylate, phenols, sulfonate esters, sulfones or sulfonyl halides, benzyl halides, carbamate esters, alkoxy silanes, silanes) showed no significant difference in the PNEC ranges. Nine groups (amines, alcohols, sulfonamides, carboxylic acids, amides, ethers, esters, sulfonic acids, aliphatic chains or rings which account for majority of the PFAS analyzed), contained more than 300 PFAS (some PFAS belong to more than one group depending on their chemical structure). The PNEC range of each group spanned over five orders of magnitude or more. The above discussed individual PFAS groups can be found in the NORMAN Substance Database under ‘Use Category’.

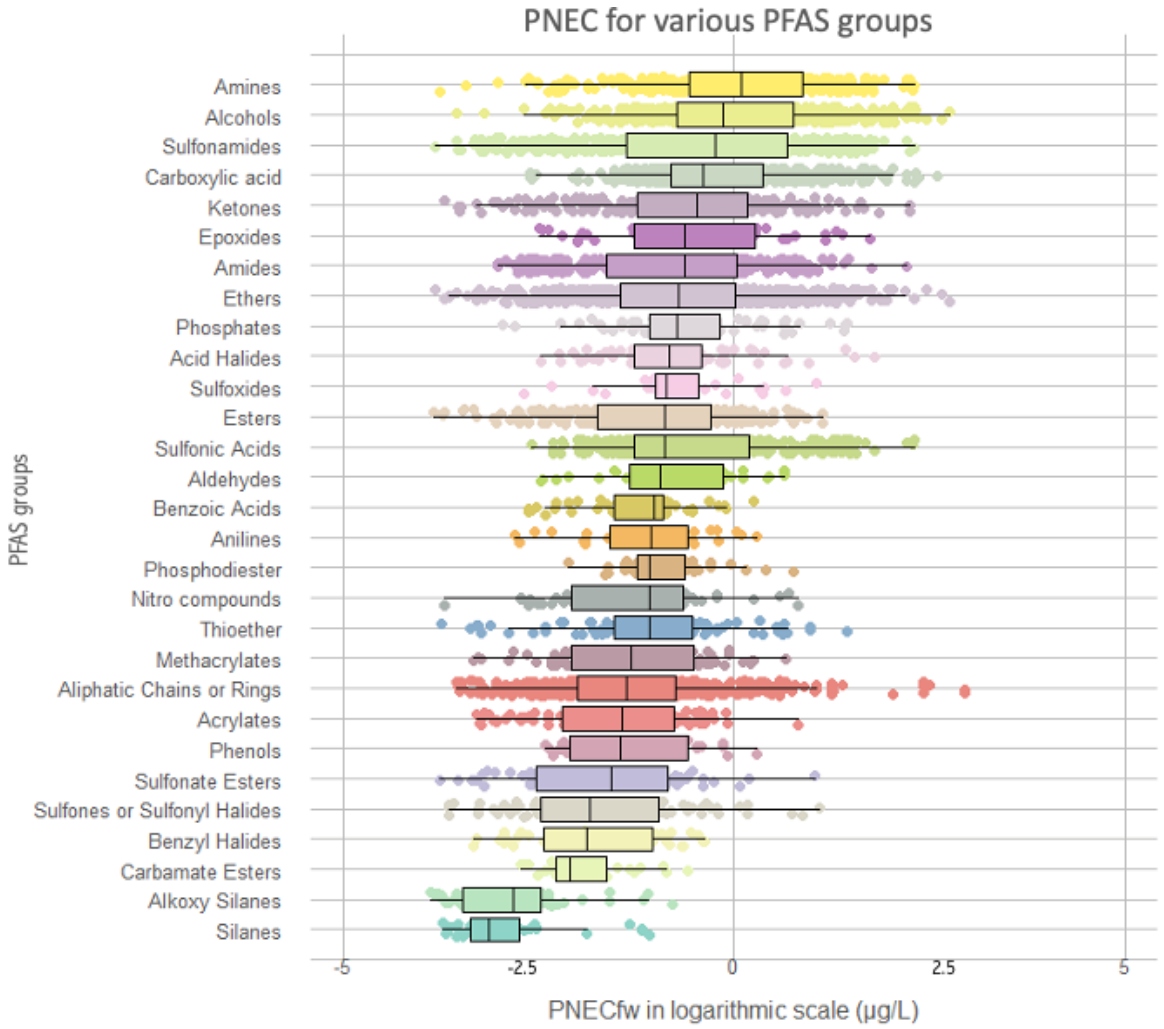


Figure 12. PNEC for various PFAS groups (with at least 30 PFAS each).

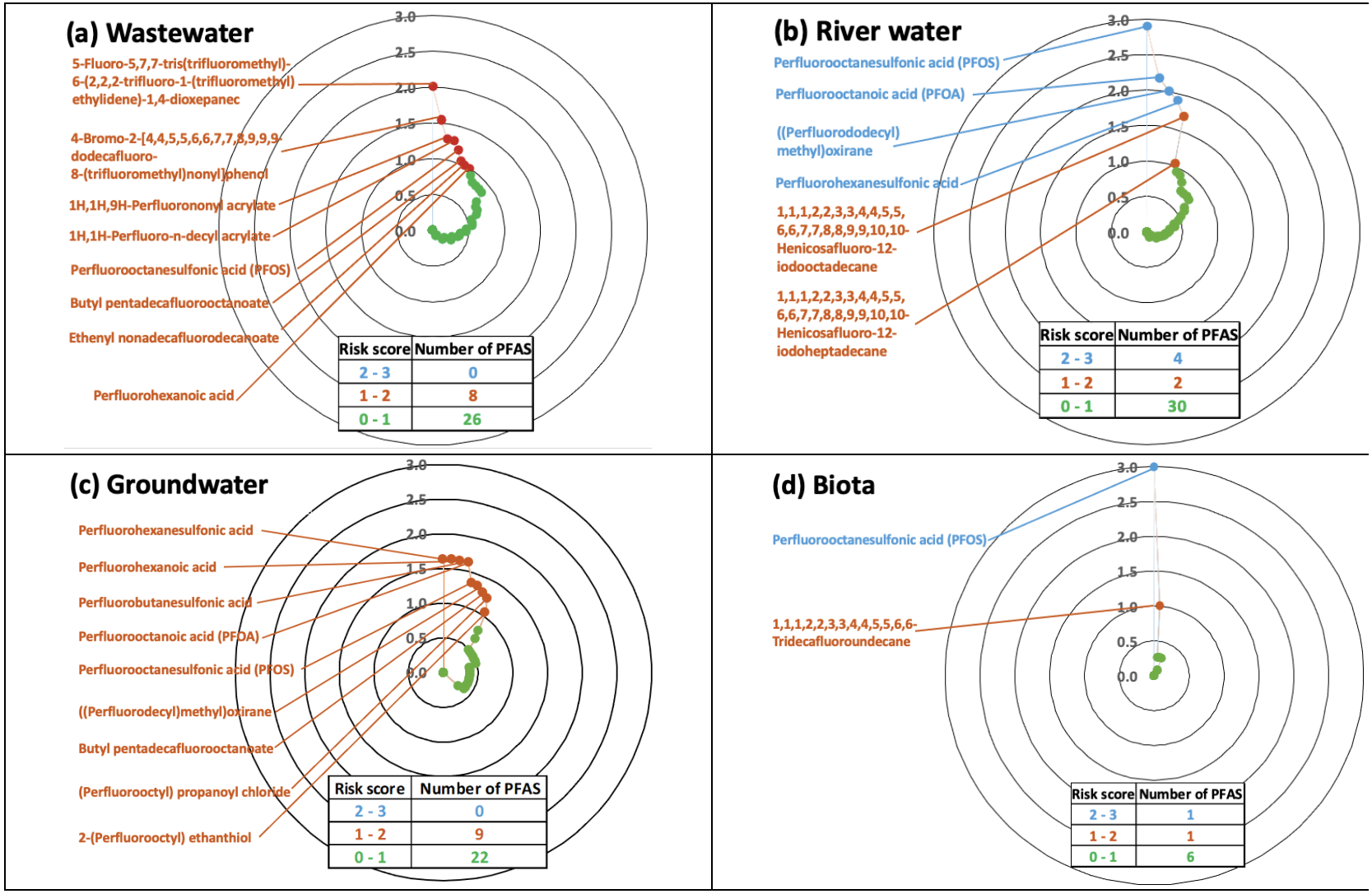


Figure 13. *Radial plots of risk scores of detected PFAS in (a) wastewater, (b) river water, (c) groundwater, and (d) biota. Some of the PNEC values were obtained using in silico tools, risk scores derived from such values involve predictive nature.*

*Risk Score (0 to 3) = Frequency of Appearance (0 to 1) + Samples with PNEC exceedance (0 to 1) + Extent of PNEC exceedance (0 to 1)

5.4 Conclusions

Target and suspect screening of 4,777 PFAS in 95 surface river water, wastewater, groundwater, biota and sediment samples collected within the JDS4 (11 countries) resulted in detection of 82 PFAS, of which 72 were detected only by suspect screening. PFOS was the only compound detected in all water and biota matrices studied. The detected PFAS were prioritized based on the total risk score representing the sum of Frequency of Appearance (FoA; 0-1), Extent of PNEC Exceedance (EoE; 0-1) and Frequency of PNEC Exceedance (FoE; 0-1). 18 PFAS were ranked with the risk score above '1' in at least one of the four matrices. Using this methodology, suspect screening led to revealing 13 PFAS of potential environmental concern, which not detected by target screening. The proposed groundwater EQSs for PFBS, PFHxA and PFOA have been frequently exceeded. All 31 PFAS detected in groundwater samples were detected at concentrations above the proposed threshold value for individual PFAS level. These results indicate the presence of PFAS in the groundwater of the DRB at threatening levels.

The screened for 4,777 PFAS were assigned into 132 groups according to the functional groups present in the compounds, using the respective ECOSAR functionality. Sub-groups of PFAS showed no significant difference in the PNEC ranges.

The findings of the study revealed the presence of PFAS at threatening levels in various environmental compartments in a big part of Europe covered by the DRB. Except PFOS, none of the PFAS with high risk score are monitored under the current legislative framework. There is an obvious need to monitor and assess the risk of PFAS in Europe in a more comprehensive approach in terms of environmental matrices and ever-increasing number of PFAS produced by industry. The NORMAN network has already conducted initial steps in this direction. The screening approach utilized in this study can be applied retrospectively to all environmental samples archived in DSFP (currently more than 2,800) for a large scale detection of PFAS across Europe, utilizing the automated function for suspect screening. The data management techniques required are already available. The outcomes of such study would shed light on the design of future regulatory monitoring of PFAS and ultimately contribute to support phasing out PFAS in Europe.

CHAPTER 6: Conclusions

PFAS are a general class of man-made chemicals that have been used for almost 70 years, but only since 2001 have some of these been identified as global contaminants. Widespread environmental contamination of legacy long-chain poly- and per-fluoroalkyl substances has triggered chemical regulatory action and a global transitioning to alternative PFAS. More than 5000 PFAS are recognized on various lists, but few have been monitored despite ample evidence of unidentified organic fluorine in human and environmental samples. Over the last decade, PFAS research has shifted from original compound classes such as perfluoroalkyl sulphonic acids (PFSAs) and perfluoroalkyl carboxylic acids (PFCAs) toward new fluorinated compounds possessing one or more perfluoroalkyl (-CnF2n-) moieties. At the same time untargeted and unspecific workflows have led to the discovery of potential PFAS transformation products, intermediates, manufacturing impurities, as well as PFAS that are not listed in available suspect databases **[166]**.

In the context of this thesis, an extensive literature review on the most recent analytical method development for PFAS in air, water, abiotic solid matrices and biological matrices was conducted. In the discursive *Chapter 1* of this booklet various instrumental analysis techniques and screening approaches were presented, explored, and compared on basis of their efficiency and applicability, together with sampling, pre-treatment and extraction methods. Additionally, all promising non-target and non-specific approaches of the last decade up to 2021 were addressed as the key-element in future PFAS analysis.

Chapter 3 described the development of a robust, quick and efficient LC/MS-MS methodology for the determination of 28 target PFAS in trace quantities. This multiresidue method was validated on lake water samples from Austria and its QA/QC elements were found to be first-rate.

The aforementioned method was later on extended to 56 target analytes in order to meet the standards of established reference LC-MS/MS methodologies (Field Lab OSU, UBA Lab, EPA 537).

In *Chapter 4* the fully validated LC-MS/MS method was applied to complex biological matrices. 65 recent specimens of a terrestrial apex predator (Common buzzard), freshwater and marine apex predators (Eurasian otter, harbour porpoise, grey seal, harbour seal) and their potential prey (bream, roach, herring, eelpout) from northern Europe (United Kingdom, Germany, the Netherlands and Sweden) were analyzed for the presence of legacy and emerging PFAS, employing a highly sensitive liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) method. 56 compounds from 14 classes were measured; 13 perfluoroalkyl carboxylic acids (PFCAs), 7 perfluoroalkyl sulphonic acids (PFSAs), 3 perfluorooctane sulfonamides (FOSAs), 4 perfluoroalkylphosphonic acids (PFAPAs), 3 perfluoroalkylphosphinic acids (PFPi's), 5 telomer alcohols (FTOHs), 2 mono-substituted

polyfluorinated phosphate esters (PAPs), 2 di-substituted polyfluorinated phosphate esters (diPAPs), 6 saturated fluorotelomer acids (FTAS), 3 unsaturated fluorotelomer acids (FTUAs), 2 N-Alkyl perfluorooctane sulfonamidoethanols (FOSEs), 3 fluorotelomer sulphonic acids (FTSAs), 2 perfluoroether carboxylic acids (PFECAs) and 1 chlorinated perfluoroether sulphonic acid (Cl-PFESA). All samples were lyophilized before analysis, in order to enhance extraction efficiency, improve the precision and achieve lower detection limits. The analytes were extracted from the dry matrices through generic methods of extraction, using an accelerated solvent extraction (ASE), followed by clean-up through solid phase extraction (SPE). Method detection limits and method quantification limits ranged from 0.02 to 1.25 ng/g wet weight (ww) and from 0.05 to 3.79 ng/g (ww), respectively. Recovery ranged from 40 to 137 %. Method precision ranged from 3 to 20 %RSD. The sum of PFAS concentration in apex predators livers ranged from 0.2 to 20.2 μg/g (ww), whereas in the fish species muscle tissues it ranged from 16 to 325 ng/g (ww). All analysed specimens were primarily contaminated with PFOS, while the three PFPi's included in this study exhibited frequency of appearance (FoA) 100%. C9 to C13 PFCAs were found at high concentrations in apex predator livers, while the overall PFAS levels in fish fillets also exceeded ecotoxicological thresholds. The findings of our study showed a clear association between the PFAS concentrations in apex predators and the geographical origin of the specimens, with samples that were collected in urban and agricultural zones being highly contaminated compared to samples from pristine or semi-pristine areas. The high variety of PFAS and the different PFAS composition in the apex predators and their prey (AP&P) samples was found to be alarming and strengthened the importance of PFAS monitoring across the food chain.

In *Chapter 5* the occurrence of 4,777 PFAS was investigated in the Danube river basin (DRB) by target and suspect screening. Target screening involved the investigation of PFAS with reference standards utilizing the LC-ESI-MS/MS method as described in the previous chapter. Suspect screening covered 4,777 PFAS retrieved from the NORMAN Substance Database, including all individual PFAS lists submitted to the NORMAN network. Mass spectrometry fragmentation and retention time index predictions of the retrieved PFAS were established for PFAS screening in LC-HRMS chromatograms obtained from DRB samples using Digital Sample Freezing Platform (DSFP). 82 PFAS were detected in the 95 environmental samples from wastewater, river water, groundwater, sediment and biota. Suspect screening detected 72 PFAS that were missed by target screening. Prediction of no effect concentration was performed for ecotoxicological risk assessment, which involved the derivation of risk scores as the sum of frequency of appearance (FoA), frequency of PNEC exceedance (FoE) and extent of PNEC exceedance (EoE). 18 PFAS were prioritized having a risk score above 1 in at least one matrix. The detection of PFAS across Danube indicated a potentially large-scale migration of PFAS in Europe, which requires systematic monitoring by regulatory bodies.

The extended literature review as presented in the *Chapter 1* of the present thesis as well as *Chapters 4 and 5* have been published in sound scientific journals.

Overall, it is well agreed upon that PFAS are the most persistent synthetic chemicals to date and they hardly degrade in the natural environment, hence referred to as “forever chemicals”. Despite strict legislation about their monitoring and environmental fate, the cycle of thousands of PFAS remains unmapped and, therefore, undisrupted (**Figure 14**).

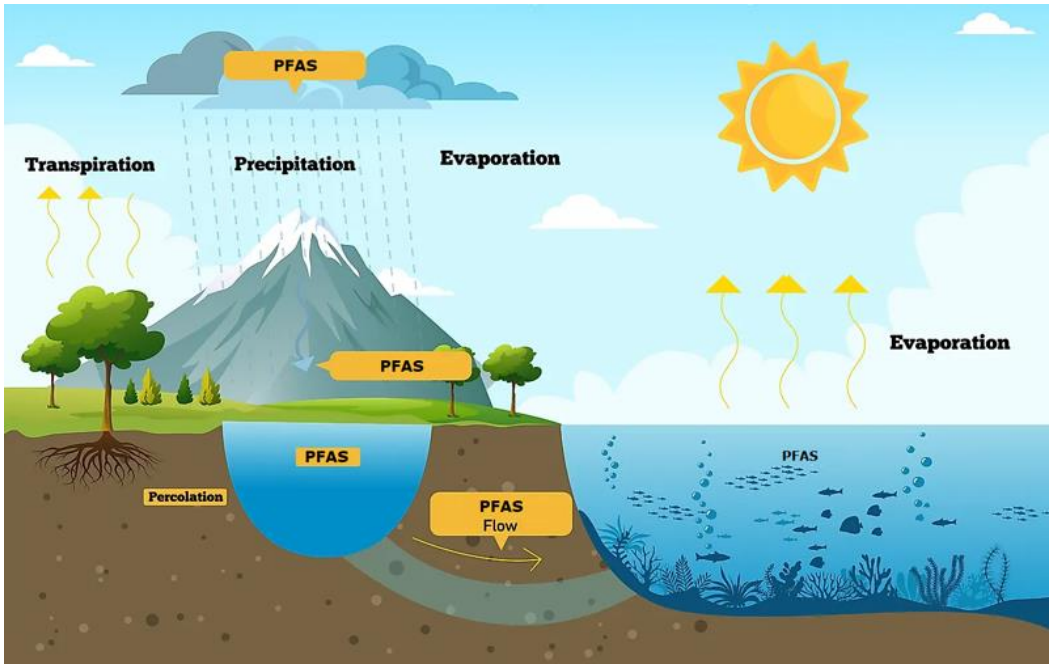


***Figure 14**. The PFAS Cycle, source: Michigan.gov/PFASResponse.*

Further pushing the analytical limits of LC-MS/MS methods for well-established PFAS to the trace spectrum together with the discovery of emerging homologues before they are global contaminants via suspect and non-target screening schemes could mitigate future contamination if strategic techniques can be developed to prioritize some of these substances for synthesis and confirmation, further monitoring, source elucidation and hazard characterization.

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