Characterizing the organohalogen iceberg

Kyra Miranda Spaan

Characterizing the organohalogen iceberg

Kyra Miranda Spaan

Academic dissertation for the Degree of Doctor of Philosophy in Environmental Sciences at Stockholm University to be publicly defended on Friday 13 December 2024 at 09.30 in De Geersalen, Geovetenskapens hus, Svante Arrhenius väg 14 and online via Zoom, public link is available at the department website.

Abstract
Organohalogen compounds (OHCs) represent a diverse group of organic substances containing fluorine, chlorine, bromine and/or iodine, many of which are well-known for their environmental persistence, bioaccumulation and toxicity. Despite regulations and bans on several problematic OHCs, new compounds continue to emerge as replacements, challenging existing analytical techniques. The concept of the OHC "iceberg" is that we only measure a fraction ("the tip") of all OHCs in a sample. This thesis aims to quantify the size of the OHC iceberg and apply state-of-the-art analytical techniques to identify the part we cannot see. To achieve this, extractable organohalogen (EOX; where X = F, Cl, or Br) mass balance methods were developed and applied, using a combination of combustion ion chromatography (CIC) and target analyses. Subsequently, high resolution mass spectrometry (HRMS)-based suspect and nontarget screening approaches were applied to further characterize the unknown fractions of EOX.

The lack of standardization for extractable organofluorine (EOF) mass balance methods has raised concerns about data reproducibility. In Paper I, an interlaboratory comparison was conducted to assess the fluorine mass balance method across three laboratories, using both water and sludge samples. The EOF-CIC method demonstrated promising accuracy and robustness, over a wide range of concentrations (60 to 2500 ng/L F). Paper II presents the first multi-halogen mass balance in wastewater treatment plant (WWTP) sludge, a useful approach to prioritize samples for follow-up investigation. Total halogen (TX) and EOX were determined in municipal sewage sludge as well as in standard reference materials (SRMs). Chlorinated paraffins (CPs) made up ~92% of extractable organochlorine (EOCl), while brominated flame retardants accounted for ~54% of extractable organobromine (EOBr) and per- and polyfluoroalkyl substances (PFAS) accounted for only 2% of the EOF. Additionally, unidentified EOF in non-polar CP extracts suggest the existence of organofluorine(s) with chemical properties unlike those of conventional PFAS. In Paper III the unknown fraction of EOF in WWTP sludge was further investigated, focusing on fluoropharmaceuticals and -pesticides. HRMS-based suspect screening was applied and sixteen pharmaceutical substances (including transformation products [TPs]), one pesticide and thirteen conventional PFAS were confirmed at confidence levels 1-4. Although the newly detected organofluorine compounds contained few fluorine atoms, their high concentrations resulted in significant contributions to the EOF. The known EOF fraction increased from 2% to 27% identified, of which ~22% was accounted for by fluoropharmaceuticals. In Paper IV, sludge and SRM extracts from Paper II containing unidentified EOCl and EOBr were reanalyzed using HRMS with ion mobility (IM) separation. Out of 17,982 peaks, 3,890 were prioritized using isotope patterns, collision cross section (CCS) values, and mass defect filters, resulting in the detection of 54 legacy OHCs and 30 unknown OHCs, of which 11 were tentatively identified.

Keywords: *Organohalogen compounds, combustion ion chromatography, mass balance, sewage treatment plant sludge, suspect screening, nontarget screening.*

Stockholm
University

Department of Environmental Science

Stockholm University, 106 91 Stockholm

CHARACTERIZING THE ORGANOHALOGEN ICEBERG

Kyra Miranda Spaan

Characterizing the organohalogen iceberg

Kyra Miranda Spaan

Table of contents

Abstract

Organohalogen compounds (OHCs) represent a diverse group of organic substances containing fluorine, chlorine, bromine and/or iodine, many of which are well-known for their environmental persistence, bioaccumulation and toxicity. Despite regulations and bans on several problematic OHCs, new compounds continue to emerge as replacements, challenging existing analytical techniques. The concept of the OHC "iceberg" is that we only measure a fraction ("the tip") of all OHCs in a sample. This thesis aims to quantify the size of the OHC iceberg and apply state-of-the-art analytical techniques to identify the part we cannot see. To achieve this, extractable organohalogen (EOX; where X = F, Cl, or Br) mass balance methods were developed and applied, using a combination of combustion ion chromatography (CIC) and target analyses. Subsequently, high resolution mass spectrometry (HRMS)-based suspect and nontarget screening approaches were applied to further characterize the unknown fractions of EOX.

The lack of standardization for extractable organofluorine (EOF) mass balance methods has raised concerns about data reproducibility. In **Paper I**, an interlaboratory comparison was conducted to assess the fluorine mass balance method across three laboratories, using both water and sludge samples. The EOF-CIC method demonstrated promising accuracy and robustness, over a wide range of concentrations (60 to 2500 ng/L F). **Paper II** presents the first multi-halogen mass balance in wastewater treatment plant (WWTP) sludge, a useful approach to prioritize samples for follow-up investigation. Total halogen (TX) and EOX were determined in municipal sewage sludge as well as in standard reference materials (SRMs). Chlorinated paraffins (CPs) made up ~92% of extractable organochlorine (EOCl), while brominated flame retardants accounted for ~54% of extractable organobromine (EOBr) and per- and polyfluoroalkyl substances (PFAS) accounted for only 2% of the EOF. Additionally, unidentified EOF in non-polar CP extracts suggest the existence of organofluorine(s) with chemical properties unlike those of conventional PFAS. In **Paper III** the unknown fraction of EOF in WWTP sludge was further investigated, focusing on fluoropharmaceuticals and -pesticides. HRMS-based suspect screening was applied and sixteen pharmaceutical substances (including transformation products [TPs]), one pesticide and thirteen conventional PFAS were confirmed at confidence levels 1-4. Although the newly detected organofluorine compounds contained few fluorine atoms, their high concentrations resulted in significant contributions to the EOF. The known EOF fraction increased from 2% to 27% identified, of which ~22% was accounted for by fluoropharmaceuticals. In **Paper IV**, sludge and SRM extracts from Paper II containing unidentified EOCl and EOBr were reanalyzed using HRMS with ion mobility (IM) separation. Out of 17,982 peaks, 3,890 were prioritized using isotope patterns, collision cross section (CCS) values, and mass defect filters, resulting in the detection of 54 legacy OHCs and 30 unknown OHCs, of which 11 were tentatively identified.

Sammanfattning

Halogenerade organiska ämnen (OHCs) är en mångsidig grupp av organiska ämnen som innehåller fluor, klor, brom och/eller jod, varav många är kända för att vara långlivade, bioackumulerande och toxiska. Trots reglering och förbud mot flera problematiska OHCs ersätts dessa ofta med nya ämnen, vilket utmanar befintliga analysmetoder. Konceptet med metaforen OHC "isberget" är att endast en bråkdel ("toppen") mäts av de totala OHCs som finns i ett prov. Denna avhandling syftar till att kvantifiera den totala storleken på det så kallade isberget och att tillämpa moderna analytiska tekniker för att identifiera den osynliga delen av OHC-isberget. För att uppnå detta utvecklades och tillämpades massbalansmetoder för extraherbara organohalogener (EOX; där X = F, Cl eller Br) med förbränningsjonkromatografi (CIC) i kombination med riktade (target) analyser. Därefter tillämpades högupplöst masspektrometri (HRMS)-baserade metoder för misstänkt och icke-riktad screening för att ytterligare karakterisera de okända fraktionerna av EOX.

Avsaknaden av standardiserade metoder för massbalans av extraherbar organofluor (EOF) har lett till att reproducerbarheten av data blivit ifrågasatt. I **Publikation I** genomfördes en interlaboratoriestudie för att jämföra fluor-massbalansmetoden, med både vatten- och slamprov. EOF-CIC-metoden visade lovande noggrannhet och robusthet i ett brett koncentrationsspann (60 till 2500 ng/L F). **Publikation II** presenterar den första multi-halogenmassbalansen i slam från ett avloppsreningsverk (WWTP), en användbar strategi för att prioritera prover för uppföljande undersökningar. Den totala mängden halogena ämnen (TX) och EOX mättes i kommunalt avloppsslam samt i standardreferensmaterial (SRMs). Klorparaffiner (CPs) utgjorde ungefär 92% av extraherbar organoklor (EOCl), medan bromerade flamskyddsmedel stod för ungefär 54% av extraherbar organobrom (EOBr), och per- and polyfluorerade ämnen (PFAS) utgjorde endast 2% av EOF. Dessutom tyder oidentifierad EOF i icke-polära CP-extrakt på existensen av organofluor med kemiska egenskaper som skiljer sig från konventionell PFAS. I **Publikation III** undersöktes den okända fraktionen av EOF i WWTP-slam vidare, med fokus på fluorerade läkemedel och pesticider. HRMS-baserad misstänkt screening tillämpades där 16 läkemedel (inklusive transformationsprodukter [TPs]), 1 pesticid och 13 konventionella PFAS bekräftades på konfidentsnivåerna 1-4. Även om de nyupptäckta organofluorföreningarna har få fluoratomer resulterade deras höga koncentrationer i ett betydande bidrag till EOF. Den kända EOF-fraktionen ökade från 2% till 27%, varav 22% var kopplad till fluorerade läkemedel. I **Publikation IV** omanalyserades slam- och SRM-extrakt från Publikation II med oidentifierade EOCl och EOBr med HRMS och jonmobilitetsseparation (IM). Av 17 982 toppar prioriterades 3 890 med hjälp av isotopmönster, collision cross section (CCS)-värden och massdefektfilter, vilket resulterade i detektion av 54 kända ämnen med riktad analys och 30 okända halogena ämnen, varav 11 kunde preliminärt identifieras.

Samenvatting

Organische halogeenverbindingen (OHC's) zijn een diverse groep van stoffen die koolstof en waterstof bevatten, en waar één of meerdere waterstofatomen zijn vervangen door fluor, chloor, broom en/of jood. Vele van deze OHCs zijn persistent, bioaccumulerend en toxisch. Door regelgeving en verboden duiken nieuwe verbindingen op ter vervanging van problematische OHC's. Deze alternatieven vormen een uitdaging voor bestaande analytische technieken. Het concept van de OHC 'ijsberg' verwijst naar het feit dat we meestal slechts een fractie ('de top') van de totale OHC's in een monster meten. Dit proefschrift heeft als doel de totale omvang van de zogenoemde OHC ijsberg te kwantificeren en moderne analytische technieken toe te passen om zowel de verbindingen in de top van de ijsberg als in de verborgen lagen te identificeren. Om dit te bereiken, werd een methode ontwikkeld met verbrandingsionchromatografie (CIC) voor massabalansmetingen van extraheerbaar organisch halogeen (EOX; waar X = F, Cl of Br) en deze werd toegepast in combinatie met doelstofanalyses. Vervolgens werden op basis van hoge-resolutie massaspectrometrie (HRMS) *suspect* en generieke (*nontarget*) screeningsmethodes toegepast om de onbekende fracties van de EOX verder te karakteriseren.

In **Publicatie I** werd een interlaboratoriumonderzoek uitgevoerd om de fluor-massabalansmethode in drie laboratoria te vergelijken, in zowel water- als slibmonsters. De EOF-CIC-methode toonde veelbelovende nauwkeurigheid en robuustheid. **Publicatie II** presenteert de eerste multi-halogeen-massabalans in slib van een afvalwaterzuiveringsinstallatie (WWTP), een veelbelovende aanpak om monsters te prioriteren voor vervolgonderzoek. Totale halogeen (TX) en EOX werden bepaald in gemeentelijk rioolslib evenals in standaardreferentiematerialen (SRM's). Chloorparaffines (CP's) maakten ongeveer 92% uit van de EOCl terwijl gebromeerde vlamvertragers goed waren voor ongeveer 54% van de EOBr, en PFAS slechts 2% van de EOF uitmaakten. Bovendien suggereert het ongeïdentificeerde EOF in apolaire CP-extracten het bestaan van organisch fluor met eigenschappen die verschillen met die van conventionele PFAS. In **Publicatie III** werd de onbekende fractie van EOF in slib van de WWTP verder onderzocht, met focus op fluoromedicijnen en -pesticiden. HRMS-gebaseerde suspect screening werd toegepast en zestien farmaceutische stoffen (inclusief transformatieproducten [TP's]), één pesticide en dertien conventionele PFAS werden bevestigd op vertrouwensniveaus 1-4. Hoewel deze farmaceutische stoffen en pesticiden weinig fluoratomen bevatten, resulteerden hun hoge concentraties in een significante bijdrage aan de EOF. De bekende EOF-fractie steeg van 2% naar 27%, waarvan 22% werd toegeschreven aan gefluoreerde farmaceutische stoffen. In **Publicatie IV** werden slib- en SRM-extracten uit **Publicatie II** met onbekend EOCl en EOBr opnieuw geanalyseerd met HRMS met ionmobiliteitsscheiding (IM). Van de 17.982 pieken werden er 3.890 geprioriteerd met behulp van isotopische patronen, collision cross section (CCS)-waarden en massadefectfilters, wat resulteerde in de identificatie van 54 bekende en 30 onbekende halogeenverbindingen, waarvan er 11 konden worden geïdentificeerd op vertrouwensniveaus 4-5.

List of publications

The following papers are included in this thesis and referred to in the text by their Roman numerals.

I   Anna Kärrman, Leo Yeung, <u>Kyra Spaan</u>, Frank-Thomas Lange, Minh Nguyen, Merle Plassmann, Cynthia de Wit, Marco Scheurer, Raed Awad, Jonathan Benskin. **Can determination of extractable organofluorine (EOF) be standardized? First interlaboratory comparisons of EOF and fluorine mass balance in sludge and water matrices**. *Environmental Science: Processes & Impacts 2021,* 23(10), 1458-1465.

II <u>Kyra Spaan</u>, Bo Yuan, Merle Plassmann, Jonathan Benskin, Cynthia de Wit. **Characterizing the Organohalogen Iceberg: Extractable, multi-halogen mass balance determination in municipal wastewater treatment plant sludge**. *Environmental Science and Technology 2023, 57(25), 9309-9320.*

III <u>Kyra Spaan</u>*, Fredric Seilitz*, Merle Plassmann, Cynthia de Wit, Jonathan Benskin. **Pharmaceuticals account for a significant proportion of the extractable organic fluorine in municipal wastewater treatment plant sludge.** *Environmental Science and Technology Letters 2023,* 10(4), 328-336.

IV <u>Kyra Spaan</u>, Xiaodi Shi, Cynthia de Wit, Jonathan Benskin. **Nontarget screening of organochlorine and organobromine compounds in municipal wastewater treatment plant sludge using gas chromatography - atmospheric pressure chemical ionization - ion mobility - mass spectrometry.** *Manuscript.*

*Shared first authorship

Contribution to papers

I   Participated in study design; performed extractions, instrumental analysis and data processing for SU; participated in writing the manuscript.

II Designed and coordinated the study; performed sampling, extractions, and instrumental analyses (except CP analysis); developed CIC method; carried out data analysis (except CP analysis); took a lead role in writing the manuscript.

III Participated in study design, instrumental analysis and data processing; took a lead role in writing the manuscript.

IV Participated in study design, instrumental analysis and data processing; took a lead role in writing the manuscript.

Abbreviations

APCI	Atmospheric pressure chemical ionization
BFRs	Brominated flame retardants
CCS	Collision cross section
CIC	Combustion ion chromatography
CL	Confidence level
CI-OPEs	Chlorinated organophosphate esters
CPs	Chlorinated paraffins
EOBr	Extractable organobromine
EOCl	Extractable organochlorine
EOF	Extractable organofluorine
EOX	Extractable organohalogen
GC	Gas chromatography
HFRs	Halogenated flame retardants
HRMS	High resolution mass spectrometry
IM	Ion mobility
LC	Liquid chromatography
LRMS	Low resolution mass spectrometry
MD	Mass defect
OHCs	Organohalogen compounds
PBDEs	Polybrominated diphenyl ethers
PFAS	Per- and polyfluoroalkyl substances
POPs	Persistent organic pollutants
SRM	Standard reference material
TP	Transformation product
TX	Total halogen
WWTP	Waste water treatment plant

Introduction

Chemicals are all around us, both good ones and bad ones. While chemicals have improved our quality of life in many areas, their spread around the globe can have harmful impacts on both humans and the environment. We already know about many bad chemicals, which we can measure and regulate. However, as the French philosopher René Descartes stated in the 17th century: "We do not describe the world we see, we see the world we can describe." This applies to chemicals as well: we usually only measure what we know. Yet, advanced measurement techniques now allow us - analytical chemists - to look for the unknown. In this thesis different techniques were used to estimate the so-called "chemical iceberg" focusing on halogenated chemicals (which are often the bad ones). The aim was to determine the total size of the "iceberg" and the fraction of known halogenated chemicals (the "tip of the iceberg") in sludge samples from a wastewater treatment plant in Stockholm. Thereafter state-of-the-art techniques were applied to characterize those that were unidentified (the unseen part of the "iceberg"). Chemicals found in sludge mirror their use in society and should be monitored as the sludge may be released back into the environment as fertilizer and could pose long-term risks to both ecosystems and human health.

Organohalogen compounds

Organohalogen compounds (OHCs) encompass a broad class of both natural and anthropogenic chemicals that contain halogen atoms (fluorine, chlorine, bromine, and iodine) covalently bound to carbon atoms. Man-made OHCs are widely used for various industrial, agricultural and medical purposes, finding applications as flame retardants, plasticizers, biocides, surfactants, coolants and lubricants. Currently, more than 30,000 individual OHCs are registered in inventories worldwide (Wang *et al.*, 2020).

Despite their utility, the same properties that make OHCs useful - such as their persistence - have been linked to environmental and health concerns. Many OHCs are environmentally persistent, bioaccumulative and toxic, which has led to numerous regulations and global phase-out initiatives. The UN Stockholm Convention on Persistent Organic Pollutants (POPs), for example, aims to eliminate and/or restrict the production and use of a number of

chemicals (UNEP, 2023). The majority listed in the UN Stockholm Convention are halogenated, highlighting their problematic nature. Well-known classes of highly halogenated pollutants include per- and polyfluoroalkyl substances (PFAS), chlorinated paraffins (CPs) and brominated flame retardants (BFRs), such as polybrominated diphenyl ethers (PBDEs; Figure 1).

Regulated chemicals are often replaced by new substances with similar or even worse properties, a process known as "regrettable substitution". PBDEs and hexabromocyclododecane (HBCDD), for example, have been replaced by numerous emerging flame retardants, while perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) have been replaced by short chain PFAS and polyfluoroalkyl substances. Although many prominent pollutants have been regulated, a large number remain unknown and unmonitored due to their confidentiality or ambiguously described identity (UNEP, 2018; Wang *et al.*, 2020). Furthermore, some compounds are transformed after release into the environment by, for example, photolysis, hydrolysis, and/or metabolism and form transformation products (TPs) with properties that might differ from their parent compounds. This situation creates a constant and arduous task to develop and validate analytical methods that can detect and quantify novel OHCs, in order to enable risk assessment, monitoring and regulation.
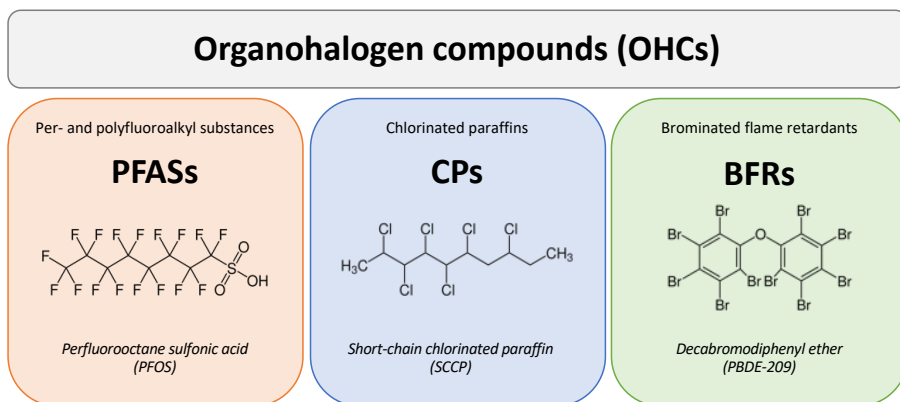

Figure 1. Three groups of highly halogenated chemicals with an example compound structure for each group.

Halogen mass balance approach

Capturing the large number and diversity of OHCs in a single targeted method is challenging and currently not possible, leading to concerns that exposure to OHCs may be underestimated. For this reason, there is growing interest in so-called "organohalogen mass balance" experiments, which seek to quantify the fraction of unidentified organohalogen in samples.

The halogen mass balance approach is used to understand the distribution of different forms of halogens (Figure 2). Total halogen (TX) represents all halogenated species in a sample, including both organic and inorganic substances. Extractable organohalogen (EOX) is the fraction that can be extracted from the sample, which is designed to remove inorganic halogens (IX) but may also inadvertently remove some organohalogens (i.e. non-extractable organohalogen; NEOX). The known portion of EOX is associated with specific target OHCs.

Due to the large number of OHCs and their tendency to be environmentally persistent, there is a growing demand for regulation on a class-wide, rather than individual substance basis. This is especially relevant for PFAS, which are estimated to encompass more than 7 million substances under the revised definition of PFAS: any chemical containing at least one perfluorinated methyl ($-CF_3$) group or a perfluorinated methylene ($-CF_2-$) group (Schymanski *et al.*, 2023). In 2023, a universal PFAS restriction was submitted to the European Chemicals Agency (ECHA) by five EU member states (Germany, Denmark, Netherlands, Norway, and Sweden). The EU revised Drinking Water Directive has recently adopted a group approach for PFAS ('PFAS-Total') that can be either used as a complement or replacement for the 0.10 µg/L threshold for the sum of 20 commonly measured PFAS ($\sum PFAS20$) including 10 perfluoroalkyl carboxylic acids (PFCAs; C_{4-13}) and 10 perfluoroalkyl sulfonic acids (PFSAs; C_{4-13}) (European Union, 2020). Since no single analytical method can capture all PFAS, the 'PFAS-Total' approach, with a current threshold concentration of 0.50 µg/L, has three recommended methods that can all provide useful results and proxy measurements, however none of the methods are standardized. The methods included are the total oxidizable precursor (TOP) assay, combustion ion chromatography after extraction of organofluorine (EOF-CIC), and liquid-chromatography - high resolution mass spectrometry (LC-HRMS) (European Union, 2024).
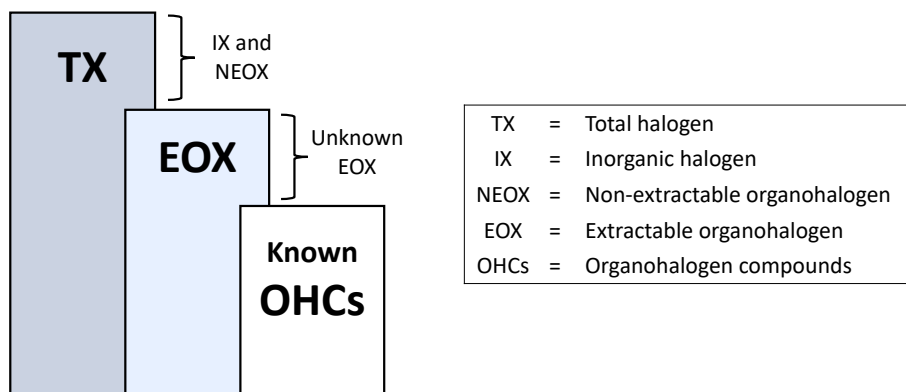

Figure 2. Halogen mass balance concept.

Previous organohalogen mass balance studies

The earliest organohalogen mass balance experiments were performed during the late 1990s and 2000s, when instrumental neutron activation analysis (INAA) was used to determine extractable organochlorine (EOCl), extractable organobromine (EOBr) and extractable organo-iodine (EOI) across a wide range of environmental samples, including biota (Kiceniuk *et al.*, 1997; Kannan *et al.*, 1999; Kawano *et al.*, 2003, 2007, 2008; Wan *et al.*, 2010), incinerator ashes (Matsui *et al.*, 2003), air (Xu *et al.*, 2005), and pine needles (Xu *et al.*, 2003). Targets measured in these studies included polychlorinated biphenyls (PCBs), dichlorodiphenyl trichloroethanes (DDTs), hexachlorocyclohexanes (HCHs), polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans (PCDD/Fs), and PBDEs. All of these studies revealed a significant portion of EOX that remained unidentified.

In comparison, fluorine mass balance experiments were first introduced in 2007 when targeted PFAS analyses were paired with extractable organofluorine (EOF) determined by combustion ion chromatography (CIC) in seawater (Miyake *et al.*, 2007). The fluorine mass balance approach has since then been applied to many other matrices, including biological samples (Yeung *et al.*, 2009; Kärrman *et al.*, 2019; Koch *et al.*, 2019; Kaiser *et al.*, 2020; Miaz *et al.*, 2020; Schultes *et al.*, 2020; Spaan *et al.*, 2020) consumer products (Schultes *et al.*, 2018, 2019) and sewage sludge (Yeung *et al*., 2017; Aro *et al*., 2021).

Searching the unknown: high resolution mass spectrometry

Advances in analytical chemistry and computing have greatly expanded the possibilities for molecular structure elucidation, thereby revolutionizing analysis of complex environmental samples (Hollender *et al.*, 2017). High resolution mass spectrometry (HRMS) instruments have high mass accuracy (± 0.001 Da), and allow resolution of peaks with very small mass differences (e.g., resolution >200,000 at *m/z* 200). HRMS has enabled suspect and nontarget screening, making it possible to screen for a broad range of compounds in a single run, without the need of authentic standards, which might be too expensive, restricted, or not commercially available (Hollender *et al.*, 2017).

Nontarget screening can be broadly defined as the characterization of the chemical composition of any given sample without the use of *a priori* knowledge regarding the chemical content in the sample (Place *et al.*, 2021). Suspect screening is an example of nontarget analysis and aims to identify

known chemicals by matching experimental data with predefined suspect lists. Due to the sheer quantity of data produced in a nontarget experiment, the features of interest must be prioritized. This can be accomplished in a number of ways, for example by exact mass matches with a library database, homologue series search, and characteristic isotope pattern search (Hollender *et al.*, 2023). Fragmentation data (MS^2) can give additional information about the identity of a feature, acting like a fingerprint. The environmental chemistry community often uses a five-level framework to describe the confidence level (CL) in the identification of chemical features by HRMS (Schymanski *et al.*, 2014) where CL1 entails the highest confidence with a confirmed structure achieved using a reference standard. CL2 suggests a probable structure based on library matches or diagnostic evidence. CL3 are tentative candidates where evidence exists for a possible structure. For CL4 an unequivocal molecular formula can be assigned, and for CL5 there is an exact mass of interest, but information to assign a formula is lacking.

Sample of interest: waste water treatment plant sludge

Waste water treatment plants (WWTPs) are a mirror of chemical use in society. Many chemicals from commercial and household sources end up in wastewater and urban runoff, entering WWTPs. There are three main pathways for release of chemicals from WWTPs into the environment: via volatilization into the atmosphere, via effluent, and via sludge disposal. Sewage sludge has historically been an unwanted by-product from wastewater treatment. It can, however, be an important resource, as it contains nutrients, such as phosphorus (which is a finite resource), nitrogen, micronutrients and organic matter. On average, ~50% of sludge in Europe is used in agriculture, ~20% is incinerated and the rest is used for land reclamation (around mines for example) or disposed in landfills (EurEau, 2021). EU member states utilize sewage sludge very differently, depending on local needs, priorities and opportunities. For example, in the Netherlands and Switzerland incineration is the only permitted option, while the Baltic region shows a high share of sewage sludge use in agriculture (Sichler *et al.*, 2022). However, due to the presence of contaminants, including heavy metals, pharmaceuticals and organic pollutants, there is an ongoing debate about the use of sludge (or biosolids – as it is called after treatment) in agriculture and meeting regulatory requirements (Holmgren *et al.*, 2020).

In Sweden, every year about 200,000 tonnes of sludge are produced, of which ~50% of the treated sludge is reused on farmland (Revaq, 2022). The application of sewage sludge as fertilizer can result in soil contamination and absorption by organisms like earthworms, posing a risk of bioaccumulation in

terrestrial food webs, as was observed with PBDEs (Sellström *et al.*, 2005). Application of sewage sludge on agricultural land is regulated by the EU sludge directive from nearly 40 years ago, which focuses mainly on heavy metals (86/278/EEC), as well as by Swedish regulations: SNFS 1994:2 and § 20 of SFS 1998:944. Revaq is a voluntary sludge quality assurance certification system for WWTPs in Sweden and has been established by the Swedish Water & Wastewater Association (Svenskt Vatten) in co-operation with the Federation of Swedish Farmers (LRF), the Swedish Food Federation and the Swedish EPA. About 5 million people (~50% of the Swedish population) are connected to Revaq-certified WWTPs. Revaq aims to ensure that sludge is safe to be used in agriculture and requires the WWTPs to be committed to source control work, and to reducing the concentrations of heavy metals, trace elements, and pathogens. It also includes a phase-out list for chemicals in upstream industries, and will include measurements of ∑PFAS4 and ∑PFAS22 (Revaq, 2025).

There are currently no thresholds for organic pollutants in sewage sludge in Sweden. However, the Swedish EPA has proposed thresholds for the year 2030 for decabromodiphenyl ether (PBDE-209), CPs, ∑PCB7 and PFOS of 0.5, 2, 0.04, and 0.02 mg/kg dw, respectively and 10 ng/kg dw for PCDD/Fs (as toxic equivalents [TEQ]) (Naturvårdsverket, 2013). WWTPs are a major filter for chemicals from the technosphere to the environment. It is therefore of great importance to ensure that harmful substances are identified and prevented from entering the wastewater system so that sludge can be reused in a sustainable manner. Characterization of sewage sludge is also valuable as it can be potentially used as a proxy for human exposure and could be applied as an early warning system to prevent bioaccumulation of hazardous chemicals (Gil-Solsona *et al.*, 2021).

The few OHC mass balance data available for sludge have focused on PFAS and organofluorine, and showed that a significant proportion of EOF is unidentified (Yeung *et al.*, 2017; Aro *et al.*, 2021). To the best of our knowledge EOCl or EOBr mass balance studies have not been previously carried out in sludge, nor have there been any OHC mass balance studies involving emerging brominated or chlorinated contaminants.

Hypotheses and objectives

The overarching hypothesis in this dissertation is that the presence of OHCs is underestimated when relying solely on targeted analytical methods. To address this, the main objectives were to:

a) Analyze samples for the presence of known compounds by targeting highly halogenated compounds such as PFAS, CPs, and halogenated flame retardants.

b) Develop a multi-halogen CIC method and estimate the total size of the organohalogen iceberg.

c) Quantify the fraction of unknown organohalogen in the samples.

d) Characterize the "unseen" part of the organohalogen iceberg, by applying suspect and nontarget screening using HRMS.

Paper I aimed to compare the EOF-based mass balance approach between three different laboratories in sludge and water matrices, intending to standardize determination of EOF and fluorine mass balance. We assessed accuracy and reproducibility, evaluated suitability for regulatory applications and identified further improvements for the method.

Paper II aimed to present a CIC method for multihalogen detection, determine EOF, EOCl and EOBr concentrations and compare the results with target OHC concentrations as well as quantify the unknown percentage of EOX in sewage sludge and standard reference materials.

Paper III aimed to identify unknown fluorinated substances in sewage sludge samples, using liquid chromatography - HRMS suspect screening and focusing our search on fluoropharmaceuticals and -pesticides.

Paper IV aimed to apply nontarget screening with a halogen prioritization workflow by a gas chromatograph - ion mobility - HRMS and identify unknown chlorinated and brominated species in sewage sludge and standard reference material.

Methods

Samples and sample information

In **Paper I**, we analyzed various sludge and water matrices, providing the first interlaboratory comparison of EOF globally. The samples included two ultrapure water samples fortified with a PFAS-mix at high and low levels, two groundwater samples known to have high and low contamination levels, respectively, a pooled effluent sample, and a pooled sewage sludge sample. To compare instrumental performance directly, a pooled groundwater extract was distributed among the labs for direct analysis.

For **Papers II-IV**, sewage sludge samples were collected at the Henriksdal WWTP in Stockholm (870,000 PE) in November 2019. The WWTP has two inlets consisting mainly of domestic and commercial wastewater plus urban runoff. The plant has an average sewage treatment capacity of 273,000 m^3/day (SVOA, 2019) and the average residence time of the sludge is 19 days. The major treatment steps include mechanical, chemical and biological treatment. The sludge is digested both aerobically and anaerobically, after which it is dewatered. The collected sludge is the final product after treatment. After sampling, the sludge samples were freeze-dried and homogenized using a mortar and pestle, after which the sample was stored in the freezer until analysis.

Water content (67%) was determined by weighing the sample before and after freeze-drying. Organic matter (64%) was determined by loss on ignition, heating the sample at 105 ^{o}C for 12 h followed by 550 ^{o}C for 2 h. Organic carbon (39%) was estimated using a conversion factor of 0.6 times the organic matter (van Leeuwen and Vermeire, 2007).

Sample extractions

Multiple extraction methods were applied throughout this thesis targeting these three main OHC analyte groups; PFAS, CPs and halogenated flame retardants (HFRs). An overview on the different types of samples, extraction methods and instrumental analyses is presented in Figures 3 and 4.

The PFAS extraction procedures for the aqueous and sludge matrices in **Paper I** were based on established methods with small modifications, described below. Aqueous samples were filtered through glass fiber filters and were subsequently extracted by solid phase extraction (SPE) method using weak anion exchange (WAX) adopted from Miyake *et al.*, (2007). The cartridges were conditioned with 0.1% ammonium hydroxide in methanol, followed by methanol and subsequently milliQ water. The aqueous samples were adjusted to a pH of 4 using glacial acetic acid and were then loaded onto the cartridge. Washing was done by rinsing with 0.01% ammonium hydroxide in milliQ water, followed by just milliQ water, ammonium acetate buffer at pH 4, and finally 20% methanol, all of which were discarded. The cartridges were centrifuged and elution was done with 0.1% ammonium hydroxide in methanol. Sludge samples were fortified with methanol and placed in an ultrasonic bath and were extracted two times. The extract was then reduced and subjected to ENVI-Carb clean-up based on an established method (Powley *et al.*, 2005).

The final extracts of the aqueous and sludge samples were split. One portion was fortified with buffer and an internal standard mix and stored for target analysis, while the second portion was set aside for EOF analysis using CIC. The reason we spiked internal standards after extraction instead of prior to it, is that CIC measures the total fluorine in a sample and standards would contribute to the fluorine signal. Although we do not account for losses during the extraction method, it allows us to directly compare EOF concentrations derived with CIC with target PFAS concentrations.

For **Paper II**, three extraction methods were applied on sludge from the Henriksdal WWTP, based on established methods specifically targeting PFAS, CPs, and HFRs, respectively (Figure 4).

The PFAS extraction was the same as in **Paper I** and based on a method described by (Powley *et al.*, 2005), as described above.

The CP extraction was based on a previously developed method using accelerated solvent extraction (ASE) (Yuan *et al.*, 2010). Briefly, ASE cells were packed with freeze-dried sludge and filled with diatomaceous earth to nearly full. The cell was extracted with *n*-hexane:acetone (1:1). Thereafter lipids were removed using concentrated sulfuric acid. Elemental sulfur was removed with activated copper, and the final clean-up step involved a multilayer SPE cartridge. The final extract was stored for analysis.

The HFR extractions were based on an earlier study with some small modifications (Nylund *et al.*, 1992) and fractionation was applied according to a clean-up method developed by Sahlström *et al.*, (2012). The freeze-dried sludge was extracted with acetone and rotated and centrifuged. The solid

residue was extracted again with acetone:*n*-hexane (3:1) mixture. The liquid phases were combined in a separatory funnel with buffer solution. After gentle rocking, the organic phase was re-extracted with *n*-hexane:diethyl ether (9:1) and the solvent was exchanged to isooctane. The extract was added onto an SPE column for fractionation. Fraction I (FrI) was eluted with *n*-hexane and included the nonpolar analytes PBDEs, decabromodiphenyl ethane (DBDPE), and emerging BFRs. Fraction II (FrII) included 2-ethylhexyl 2,3,4,5-tetrabromobenzoate (EH-TBB), 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE), and bis(2-ethylhexyl) tetrabromophthalate (BEH-TEBP) and was eluted with *n*-hexane:diethyl ether (9:1). Fraction III (FrIII) was eluted with ethyl acetate and split into FrIIIa and FrIIIb, which contained tetrabromobisphenol A (TBBPA), α- β-, and γ- 1,2,5,6,9,10-hexabromocyclododecane (α-, β-, and γ-HBCDD) and FrIIIb contained chlorinated organophosphate esters (Cl-OPEs).

In **Paper III**, the same PFAS extracts as described above were used for suspect screening. The CP and HFR extracts were reanalyzed using nontarget screening in **Paper IV**.
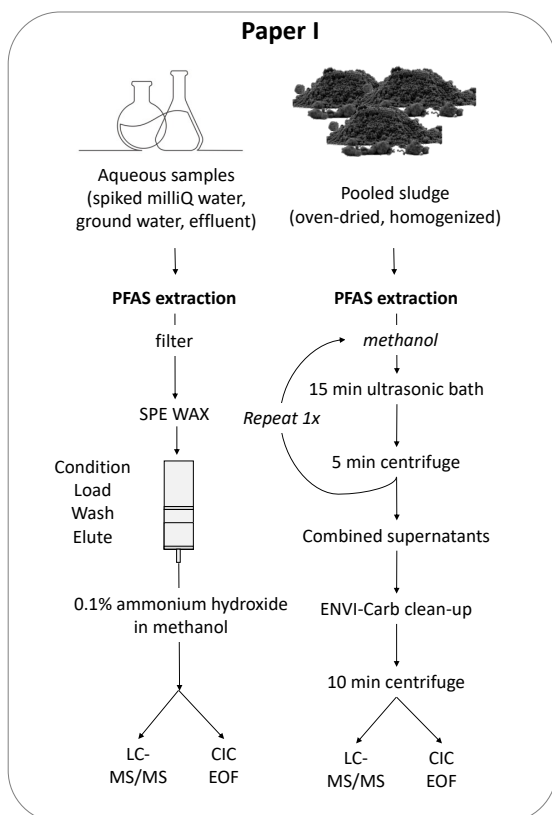

*Figure 3. Summary overview of the different types of samples, extraction methods, and instrumental analyses applied in **Paper I**.*
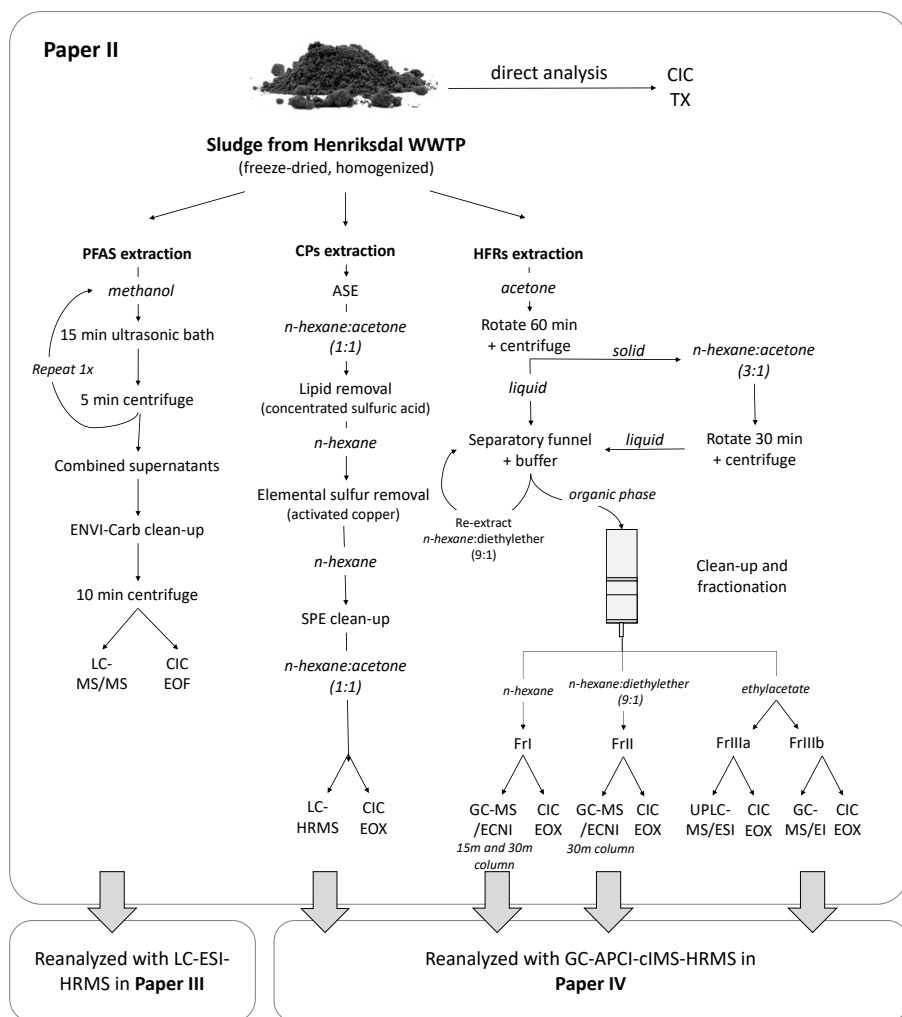
*Figure 4. Summary overview of the different extraction methods, and instrumental analyses used in **Papers II-IV**.*

Instrumental analyses

Analyses of TX and EOX were done using CIC. Target analyses were performed by both LC and gas chromatography (GC) instruments in conjunction with both low resolution mass spectrometry (LRMS) and HRMS (Figure 4). Further details on each instrumental method are briefly discussed below.

Combustion ion chromatography

The CIC method is a main pillar in this thesis, and is applied to conduct halogen measurements. The instrument consists of four main components: i) the autosampler, ii) the combustion oven, iii) the absorption tube, and iv) the ion chromatograph (Figure 5). Samples (neat sludge or extracts) were placed into a ceramic boat, and combusted at ~1000 °C. At this high temperature all substances are mineralized and organohalogens are converted into inorganic halides after which they are absorbed in a water-filled absorption unit. This solution is then injected onto an ion chromatograph where the halide ions are separated and measured via an ion conductivity meter.
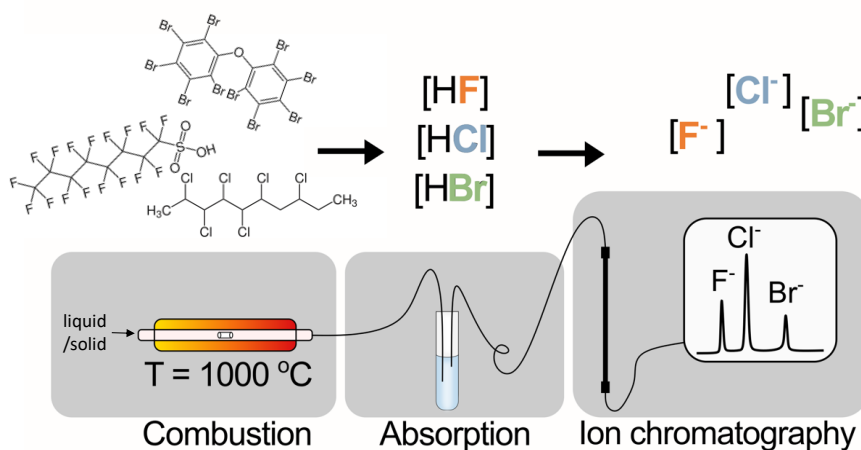

Figure 5. Schematic drawing of the combustion ion chromatograph (CIC).

PFAS analysis

For PFAS determination in **Paper I and II**, instrumental analysis was carried out by ultra-performance liquid chromatography – tandem mass spectrometry (UPLC-MS/MS) and included 16 target PFAS ("PFAS-16"), consisting of 11 perfluoroalkyl carboxylic acids (PFCAs; $C_{4-14,16}$), 4 perfluoroalkyl sulfonic acids (PFSAs; $C_{4,6,8,10}$) and perfluorooctane sulfonamide (FOSA).

CPs analysis

Targeted analysis of very short-, short-, medium-, and long-chain CPs (vSCCPs, SCCPs, MCCPs, LCCPs) was carried out in **Paper II** by UPLC - atmospheric pressure chemical ionization - HRMS (UPLC-APCI-HRMS) using a QExactive HF Orbitrap (Thermo Fisher Scientific) in full scan (m/z 250-2,000) mode with a resolution of 120,000 full width at half maximum (FWHM). A total of 342 CP homologues (expressed as C_nCl_m, n = 6-33 and m = 1-16) were measured. Quantification was carried out based on a C_nCl_m-profile

reconstruction method (Bogdal *et al.*, 2015). Sixteen commercial mixtures were used for quantification. The chlorine weight was calculated based on the semi-quantitative determination of the degree of chlorination obtained by the instrument.

HFRs analysis

In **Paper II**, FrI and FrII from the HFR extracts were analyzed on a GC mass spectrometer (GC-MS) equipped with an electron capture negative ionization (ECNI) source. Since highly brominated PBDEs are prone to thermal degradation (Hites, 2004), the octaBDEs, nonaBDEs and decaBDEs were analyzed using a short column (15 m), while the remaining BDEs were analyzed on a long column (30 m). FrIIIa was run on a LC-MS. FrIIIb analysis was carried out on a GC-MS with electron ionization (GC-MS/EI).

Halogen mass balance

In order to compare target analyses with EOF and EOX concentrations in **Paper I** and **Paper II**, respectively, the target compound concentrations were converted to halogen equivalents (C_{X_OHC}; i.e., mass of halogen per mass of sample [ng X/g]). This was calculated according to the following equation:

Equation 1: $C_{X\_OHC} = C_{OHC} * n_X * A_X / MW_{OHC}$

Where C_{OHC} is the concentration of the target OHC (ng/g), n_X is the number of halogens in the molecule, A_X is the atomic mass of the halogen (g/mol), and MW_{OHC} is the molecular weight of the OHC (g/mol).

Nontarget and suspect screening

In **Paper III**, suspect screening was applied to the sludge extracts from PFAS analysis. Extracts were analyzed on a high resolution mass spectrometer (Orbitrap) coupled to an LC. Full scan data were acquired in both positive and negative mode. The data were preprocessed in Compound Discoverer v3.3, by low-intensity peak removal, retention time alignment, feature grouping, and blank subtraction. Exact masses were compared with a compiled suspect list, which included 340 fluoropharmaceuticals (Inoue *et al.*, 2020), 424 fluoro-agrochemicals (Ogawa *et al.*, 2020), and 498 conventional PFAS from previous literature. All exact mass hits with the suspect list were added to an inclusion list in order to collect fragmentation spectra (MS^2). Tentative identification was achieved by comparing acquired MS^2 spectra to the mzCloud database, as well as the in silico MS^2 prediction tools MetFrag and SIRIUS4 CSI:FingerID. For confirmation and quantification of suspects,

samples were reanalyzed together with procured authentic standards. For confirmed substances, a list of potential transformation products (TPs) was predicted by BioTransformer v3.0 and MS^2 spectra were acquired for those TPs that were present in the extract using an inclusion list. Authentic standards or a structurally similar compound were used for (semi)quantification.

In **Paper IV**, CP and HFR extracts from Paper II were reanalyzed on a GC - APCI coupled to an ion mobility - HRMS (GC-APCI-IM-HRMS) according to a previous method (Shi *et al.*, 2024). IM provides an additional dimension to the data: a collision cross section (CCS) value, which depends on the ion's size, shape and charge. Polyhalogenated compounds occupy a specific region of chemical space defined by *m/z* and CCS (MacNeil *et al.*, 2022). Halogenated compounds tend to have shorter drift times, and consequently lower CCS values, which could be explained by their more condensed molecular conformation compared to non-halogenated compounds with similar *m/z* (Mullin *et al.*, 2020). The raw data were preprocessed in Progenesis QI (v3.0, Waters Corporation). Nontarget screening was carried out by prioritizing features using three filters that all indicate the presence of a halogen: i) isotope patterns - identified using a *HaloSelect* script (Zhang *et al.*, 2019); ii) CCS screening - by setting a filter selecting CCS values < 0.2 *m/z* + 100 Å^2 and CCS values < 250 Å^2, a significant number of polyhalogenated compounds is captured (MacNeil *et al.*, 2022); and iii) mass defect (MD) - features with low or negative mass defect are more likely to contain halogens (Dolios *et al.*, 2019).

Features matching two or more of the prioritization filters were further analyzed by checking the extracted ion chromatograms in the raw data for good peak shape and isotopic pattern. Possible molecular formulae were calculated using the *cheminfo.org* website (Cheminfo.org, 2024) by inserting the observed monoisotopic mass and considering both molecular ions and protonated ions ([M]$^{+\bullet}$ and [M+H]$^+$), constrained by the number of halogen atoms after inspecting isotopic patterns. In addition to molecular formulae search, a simultaneous search in the PubChem database was done.

Results and discussion

Interlaboratory comparison of EOF and fluorine mass balance (Paper I)

Fluorine mass balance studies are increasing in interest as targeted analytical methods, measuring only a limited number of compounds, do not capture the total burden of PFAS and other organofluorines in a sample. Many studies have reported large fractions of unknown EOF in various matrices. However, there has been little effort to standardize fluorine mass balance methods using the same instrument and extraction method, which is necessary to ensure good reproducibility of data.

In **Paper I** we presented the first interlaboratory study comparing EOF and fluorine mass balance measurements. This study involved a comparison of fluorine mass balance measurements in sludge and various aqueous matrices across three laboratory participants and we assessed the potential for standardizing EOF methods.

Accuracy was determined between the laboratories by repeated measurements of EOF in water samples spiked with known concentrations, and ranged from 85-101% for low-level spikes and 76-109% for high-level spikes. The measurements covered a wide range of concentrations from ~60 to ~2,500 ng/L F and showed good reproducibility between labs. The revised EU Drinking Water Directive proposed a 'PFAS Total' measurement, requiring measurement uncertainties of <50% (European Union, 2020), which was the case for all samples. Although procedural blanks were relatively high for two laboratories (151 and 124 ng/L F) compared to the third laboratory (13 ng/L F), reporting limits were still well within the range for regulatory purposes, as the requirements for the revised Drinking Water Directive limit are proposed at 500 ng/L 'PFAS Total'. Efforts to reduce the background levels in procedural blanks are needed.

EOF measurements are highly dependent on the extraction method that is applied (Kaiser *et al.*, 2020). For example, (ultra)short-chain PFAS (such as trifluoroacetic acid) are often not captured using common PFAS extraction methods, as was the case in this study. Therefore, a separate extraction method may be necessary to get a more comprehensive picture of the total organofluorine content.

Overall, this first interlaboratory study between the participants demonstrated that EOF methods using CIC can be standardized. Despite the small participant number and poor extraction efficiency for short-chain PFAS, the method showed promising accuracy, robustness and reporting limits.

Multihalogen mass balance (Paper II)

The first organohalogen mass balance studies were conducted during the mid-1990s and measured EOCl, EOBr and EOI using instrumental neutron activation analysis (INAA) (Loganathan *et al.*, 1995; Kiceniuk *et al.*, 1997; Kannan *et al.*, 1999). In recent years fluorine mass balance studies have become increasingly more common and have been applied to many different types of samples (Shelor *et al.*, 2024). However, there still remains a significant gap in our knowledge on how much target compounds contribute to the total OHC burden.

In **Paper II**, a multihalogen mass balance method was developed using CIC, where we extended our existing EOF-CIC method to include, in addition to fluorine, also chlorine and bromine measurements. This method was applied to municipal WWTP sludge and standard reference materials (SRMs). TX determination, performed by directly combusting the sludge without any prior extraction, was highest for TCl (205 ± 27 µg/g dw), then TF (106 ± 16 µg/g dw), and finally TBr (7 ± 1 µg/g dw). EOX concentrations were an order of magnitude lower. Three separate extracts were analyzed each focusing on a specific OHC class: EOF/PFAS, EOCl/CPs, and EOCl+EOBr/HFRs (Figure 6).

The fluorine mass balance showed that of the measured EOF (304 ± 116 ng F/g) in sludge only 2% was characterized by target PFAS, of which PFOS was the most prevalent target at 7 ± 1 ng/g dw. In the NIST domestic sludge SRM 2781, the EOF concentration was measured at 3,590 ± 311 ng F/g dw, of which 6% was identified by target PFAS. The chlorine mass balance was calculated for two extracts, one for CPs and one for HFRs. The CPs explained nearly all of the EOCl (92%) in the CP extracts, while the Cl-HFRs only explained <1% of the EOCl in the HFR extracts. The CP extraction was also applied to indoor dust SRM 2585 and revealed 106% of the 17,000 ± 1,600 ng Cl/g dw EOCl was characterized by CPs. The extract for the bromine mass balance was separated into three fractions. The first and least polar fraction was characterized 63% by PBDEs. The second, slightly more polar, fraction was identified 19% by BFRs. For the third fraction EOBr measurements were below the detection limit.

This first multihalogen mass balance assessment in municipal WWTP sludge offers a new approach to prioritization of samples for follow-up

investigation. In the examined sludge, CPs accounted for the majority of EOCl in the CP extract. PFAS and HFRs only partially explained the EOF and EOCl/EOBr in the extracts and these were therefore of interest to further investigate in Papers III and IV (Figure 6).

Fluorine suspect screening (Paper III)

Following the results from Paper II, only 2% of the EOF was identified by target PFAS in municipal wastewater treatment plant sludge. Therefore, **Paper III** focused on investigating the unidentified fraction of the EOF by reanalyzing the extracts and applying HRMS suspect screening, hypothesizing that pharmaceuticals and pesticides could be important contributors to the unknown EOF in wastewater treatment plant sludge.

Suspect screening revealed the presence of eight fluoropharmaceuticals, one fluoropesticide and eight pharmaceutical TPs at confidence levels of 1-4. Suspects were (semi-)quantified with either an authentic standard, a structurally similar standard, or with a parent compound standard. The highest fluorine contributions to EOF were determined for a TP of ticagrelor (155 ± 43 ng/g dw; 4.0%), ezetimibe (127 ± 2 ng/g dw; 3.9%), and bicalutamide (60 ± 26 ng/g dw; 3.5%). Conventional PFAS accounted for 7% of the EOF, of which the highest contribution was for 5:3 FTCA (12 ng/g dw; 2.4%) followed by PFOS (7.2 ng/g dw; 1.1%).

This study revealed for the first time that fluoropharmaceuticals and their TPs significantly contributed to the EOF, increasing the percentage of known EOF from 2% to 27%. This result shows that even though fluoro-pharmaceuticals and pesticides typically contain only one or a few fluorine atoms, at high concentrations they can still contribute significantly to the fluorine mass balance. Pharmaceuticals are not necessarily persistent, but their widespread use can lead to elevated concentrations in sludge. Although this study characterized a larger fraction of the EOF in sludge, 73% remains unidentified. Further scrutinization to close the fluorine mass balance could include GC-based analyses for detection of neutral and more hydrophobic fluorinated compounds.

Nontarget organohalogen screening (Paper IV)

In **Paper IV**, the EOCl and EOBr fractions of sewage sludge, which were only partially characterized in Paper II were further investigated using nontarget screening by GC-APCI-IM-HRMS. A prioritization workflow was used involving three filters: isotope pattern, CCS, and MD. Since halogenated

compounds occupy a specific region of chemical space defined by *m/z* and CCS, they can be prioritized for further investigation (MacNeil *et al.*, 2022). Isotope patterns were selected using a *HaloSelect* script (Zhang *et al.*, 2019), and a MD filter was applied as negative MDs are indicative for the presence of chlorine and/or bromine atoms.

The prioritization workflow was evaluated using halogenated target compounds including PBDEs, PCBs, and Cl-OPEs, where 52 out of 54 (96%) of the standards matched all three prioritization criteria, demonstrating high confidence in the approach. From a total of 17,982 detected peaks, 3,890 were prioritized, and those matching ≥2 prioritizations were further assessed by examining extracted ion chromatograms for peak shape and isotopic pattern. The latter helped determine the number of chlorines and/or bromines present in a suspect compound.

Thirty unknown halogenated compounds were selected, and tentative molecular formulas were calculated using exact monoisotopic mass, constrained by the number of halogens based on the isotope patterns. Tentative identifications were then searched for in the PubChem database and MS^2 fragments (if available) were used to increase confidence. Eleven compounds were tentatively identified at CL 4, including the pesticides chloroxynil, 3,4-dichloroaniline, trichloroaniline, bromocyclen, chlorinated cyclodienes chlordane and its impurity, chlordene and a pharmaceutical metabolite, sertraline ketone.

This study shows the great advantage IM-derived CCS values give for halogen-specific screening using HRMS. The combination of isotope pattern search together with the CCS threshold values and MD filters to prioritize chlorinated and brominated compounds allowed prioritization of OHCs in a large dataset. Although none of the prioritizations provides unequivocal evidence of a chlorinated or brominated compound itself, their combined application improves confidence in flagging OHCs. Future studies will focus on confirming the tentatively identified unknowns and to (semi-)quantify these substances in order to determine their overall contribution to the organochlorine and organobromine mass balance.
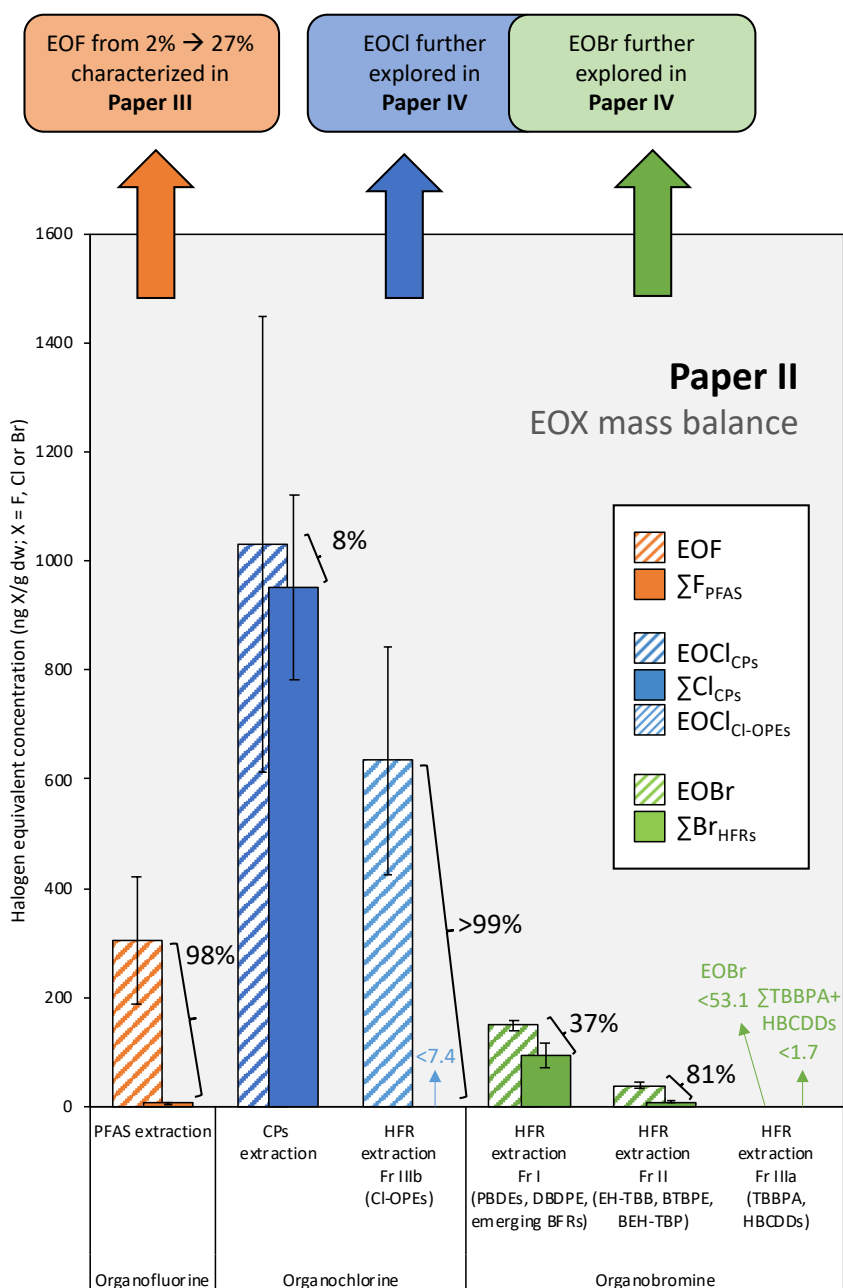
Figure 6. Extractable organofluorine, -chlorine and -bromine mass balances in sewage sludge from Paper II (n=3, error bars indication standard deviation of replicate extractions). EOX concentrations from CIC are compared to the halogen equivalent concentrations form target analyses. Percentages show the size of the gap between identified and unidentified EOX. EOF was further characterized in Paper III, and EOCl and EOBr were further explored in Paper IV.

Conclusions and future perspectives

The overarching hypothesis that the presence of OHCs in WWTP sludge is underestimated, when only using targeted methods, was confirmed. In this thesis, an interlaboratory EOF study was conducted, WWTP sludge was analyzed for the presence of known compounds, and a multihalogen CIC method was developed and applied to measure the size of the so-called organohalogen iceberg. Moreover, the fraction of unknown organohalogens in sludge was quantified and the "unseen" part of the organohalogen iceberg was further characterized using suspect and nontarget screening.

The interlaboratory comparison in **Paper I** showed that CIC-based fluorine mass balance studies can be standardized. Although the number of participants was small, this initial study shows promising results for standardization of EOF and fluorine mass balance studies using CIC. Accuracy was good and measured values were consistent between the laboratories, indicating good reproducibility. However, more studies with larger numbers of participants and inclusion of SRMs are needed to improve confidence and better understanding of the limitations of the method.

The multihalogen mass balance study in **Paper II** presented the possibility of measuring EOF, EOCl, and EOBr simultaneously using CIC. Comparison of these measurements with target analyses revealed that CPs explain the majority of EOCl, while PFAS and BFRs only explain a smaller part of EOF and EOBr (2% and 54%), respectively. Extraction methods applied were specifically designed to capture our target analytes. In order to get a more comprehensive picture, a more generic extraction method involving limited sample processing would be preferable to reduce the risk of losing compounds during sample preparation. However, this will also mean that certain matrix interferences might hinder detection as the sensitivity is decreased (Travis *et al.*, 2021).

In **Paper III**, pharmaceuticals were found to contribute significantly to the organofluorine mass balance. Sixteen fluoropharmaceuticals and one fluoropesticide were detected using suspect screening by high resolution mass spectrometry. These compounds made up ~22% of the EOF, while conventional PFAS only accounted for ~5%. Nevertheless, the majority of EOF (73%) remains unidentified, which raises concerns as there is no information on the persistence and potential toxicological properties of the

unknown organofluorine compounds. Speculatively, the unidentified EOF could consist of neutral PFAS – such as perfluoroalkyl sulfonamide copolymers, fluorotelomer alcohols, silicones and olefins. GC-based analyses could be applied to include neutral or more hydrophobic fluorinated substances.

Paper IV explored the unidentified fractions of EOCl and EOBr in sludge extracts from Paper II. A nontarget prioritization workflow for halogenated compounds in large datasets was investigated, by analyzing data gathered using a GC-APCI-IM-HRMS analysis. CCS values derived via ion mobility separation were used to prioritize halogenated species, in combination with isotope patterns and mass defects. Thirty unknown halogenated compounds were selected in the sewage sludge extracts and SRM, of which eleven were tentatively identified. However, only qualitative analysis was carried out and their concentrations and contribution to the organochlorine and organobromine mass balance remain unclear.

Overall, achieving a comprehensive coverage of all contaminants in a sample is challenging, due to the diversity of chemical properties, detection limits, and limitations of analytical techniques. There are always trade-offs to consider when choosing a method. Therefore, a combination of analytical technologies is the key to cover a variety of chemicals with different chemical properties. Mulithalogen mass balance studies offer a novel approach for prioritizing samples for follow-up analysis. HRMS techniques extend the spectrum of compounds that can be detected and identified, which provides a more comprehensive picture. This thesis improves the knowledge on pollution of OHCs in WWTP sludge samples by application of multiple techniques to uncover a variety of chemicals and estimate the total burden of OHCs in WWTP samples. Although these nontarget and multihalogen mass balance studies provide a more comprehensive picture of contamination, some chemicals may remain outside the spotlight – or the unseen part of the OHC "iceberg", due to analytical limitations.

Acknowledgements

References

Aro, R., Carlsson, P., Vogelsang, C., Kärrman, A. and Yeung, L.W. (2021) 'Fluorine mass balance analysis of selected environmental samples from Norway', *Chemosphere*, 283. Available at: https://doi.org/10.1016/j.chemosphere.2021.131200.

Aro, R., Eriksson, U., Kärrman, A., Chen, F., Wang, T. and Yeung, L.W.Y. (2021) 'Fluorine Mass Balance Analysis of Effluent and Sludge from Nordic Countries', *ACS ES&T Water*, 1(9), pp. 2087–2096. Available at: https://doi.org/10.1021/acsestwater.1c00168.

Bogdal, C., Alsberg, T., Diefenbacher, P.S., Macleod, M. and Berger, U. (2015) 'Fast quantification of chlorinated paraffins in environmental samples by direct injection high-resolution mass spectrometry with pattern deconvolution', *Analytical Chemistry*, 87(5), pp. 2852–2860. Available at: https://doi.org/10.1021/ac504444d.

Cheminfo.org (2024) *Molecular formula from monoisotopic mass and PubChem*. Available at: https://www.cheminfo.org/Spectra/Mass/MF_from_monoisotopic_mass_and_pubchem/index.html (Accessed: 20 September 2024).

Dolios, G., Patel, D., Arora, M. and Andra, S.S. (2019) 'Mass defect filtering for suspect screening of halogenated environmental chemicals: A case study of chlorinated organophosphate flame retardants', *Rapid Communications in Mass Spectrometry*, 33(5), pp. 503–519. Available at: https://doi.org/10.1002/rcm.8370.

EurEau (2021) *Waste water treatment - sludge management*, *Briefing note*. Available at: https://www.eureau.org/resources/briefing-notes/5629-briefing-note-on-sludge-management/file.

European Union (2020) *Directive (EU) 2020/2184, EU (revised) Drinking Water Directive.*, *Official Journal of the European Communities*. Available at: https://eur-lex.europa.eu/eli/dir/2020/2184/oj.

European Union (2024) 'Technical guidelines regarding methods of analysis for monitoring of per- and polyfluoroalkyl substances (PFAS) in water intended for human consumption', *Official Journal of the European Union*, C/2024/491.

Gil-Solsona, R., Nika, M.-C., Bustamante, M., Villanueva, C.M., Foraster, M., Cosin-Tomás, M., Alygizakis, N., Gómez-Roig, M.D., Llurba-Olive, E., Sunyer, J., Thomaidis, N.S., Dadvand, P. and Gago-Ferrero, P. (2021) 'The Potential of Sewage Sludge to Predict and Evaluate the Human Chemical Exposome', *Environmental Science & Technology Letters*, 8(12), pp. 1077–1084. Available at: https://doi.org/10.1021/acs.estlett.1c00848.

Hites, R.A. (2004) 'Polybrominated Diphenyl Ethers in the Environment and in People: A Meta-Analysis of Concentrations', *Environmental Science and Technology*, 38(4), pp. 945–956. Available at: https://doi.org/10.1021/es035082g.

Hollender, J., Schymanski, E.L., Singer, H.P. and Ferguson, P.L. (2017) 'Nontarget Screening with High Resolution Mass Spectrometry in the Environment: Ready to Go?', *Environmental Science and Technology*, 51(20), pp. 11505–11512. Available at: https://doi.org/10.1021/acs.est.7b02184.

Hollender, J., Schymanski, E.L., Ahrens, L., Alygizakis, N., Béen, F., Bijlsma, L., Brunner, A.M., Celma, A., Fildier, A., Fu, Q., Rauert, C., Rostkowski, P., Samanipour, S., Schulze, B., Schulze, T., Singh, R.R., Slobodnik, J., … Krauss, M. (2023) 'NORMAN guidance on suspect and non-target screening in environmental monitoring', *Environmental Sciences Europe*, 35(75). Available at: https://doi.org/10.1186/s12302-023-00779-4.

Holmgren, G., Larsson, F.K., Johansson, M.H. and Lindblad Hammar, I. (2020) *Hållbar slamhantering*. Stockholm: Statens Offentliga Utredningar.

Inoue, M., Sumii, Y. and Shibata, N. (2020) 'Contribution of Organofluorine Compounds to Pharmaceuticals', *ACS Omega*, 5(19), pp. 10633–10640. Available at: https://doi.org/10.1021/acsomega.0c00830.

Kaiser, A.M., Aro, R., Kärrman, A., Weiss, S., Hartmann, C., Uhl, M., Forsthuber, M., Gundacker, C. and Yeung, L.W.Y. (2020) 'Comparison of extraction methods for per- and polyfluoroalkyl substances (PFAS) in human serum and placenta samples—insights into extractable organic fluorine (EOF)', *Analytical and Bioanalytical Chemistry*, pp. 865–876. Available at: https://doi.org/10.1007/s00216-020-03041-5.

Kannan, K., Kawano, M., Kashima, Y., Matsui, M. and Giesy, J.P. (1999) 'Extractable organohalogens (EOX) in sediment and biota collected at an estuarine marsh near a former chloralkali facility', *Environmental Science and Technology*, 33(7), pp. 1004–1008. Available at: https://doi.org/10.1021/es9811142.

Kärrman, A., Wang, T., Kallenborn, R., Langseter, A.M., Grønhovd, S.M., Ræder, E.M., Lyche, J.L., Yeung, L., Chen, F., Eriksson, U., Aro, R. and Fredriksson, F. (2019) *PFASs in the Nordic environment*.

Kawano, M., Falandysz, J. and Morita, M. (2008) 'Instrumental neutron activation analysis of extractable organohalogens in marine mammal, harbour porpoise (Phocoena phocoena) and its feed, Atlantic herring (Clupea harengus), from the Baltic Sea', *Journal of Radioanalytical and Nuclear Chemistry*, 278(2), pp. 263–266. Available at: https://doi.org/10.1007/s10967-008-0401-8.

Kawano, M., Falandysz, J. and Wakimoto, T. (2003) 'Instrumental neutron activation analysis of extractable organohalogens (EOX) in Antarctic marine organisms', *Journal of Radioanalytical and Nuclear Chemistry*, 255(2), pp. 235–237. Available at: https://doi.org/10.1023/A:1022563611414.

Kawano, M., Falandysz, J. and Wakimoto, T. (2007) 'Instrumental neutron activation analysis of extractable organohalogens in the Antarctic Weddell seal (Leptonychotes weddelli)', *Journal of Radioanalytical and Nuclear Chemistry*, 272(3), pp. 501–504. Available at: https://doi.org/10.1007/s10967-007-0611-5.

Kiceniuk, J.W., Holzbecher, J. and Chatt, A. (1997) 'Extractable organohalogens in tissues of beluga whales from the Canadian Arctic and the St. Lawrence Estuary', *Environmental Pollution*, 97(3), pp. 205–211. Available at: https://doi.org/10.1016/S0269-7491(97)00100-0.

Koch, A., Kärrman, A., Yeung, L.W.Y., Jonsson, M., Ahrens, L. and Wang, T. (2019) 'Point source characterization of per- and polyfluoroalkyl substances (PFASs) and extractable organofluorine (EOF) in freshwater and aquatic invertebrates', *Environmental Science: Processes & Impacts*, 21(11), pp. 1887–1898. Available at: https://doi.org/10.1039/C9EM00281B.

van Leeuwen, C.J. and Vermeire, T.G. (2007) *Risk Assessment of Chemicals: An Introduction*, *Chemicke Listy*. Dordrecht, The Netherlands: Springer. Available at: https://doi.org/10.2166/9781789061987_0025.

Loganathan, B.G., Kannan, K., Watanabe, I., Kawano, M., Irvine, K., Kumar, S. and Sikka, H.C. (1995) 'Isomer-Specific Determination and Toxic Evaluation of Polychlorinated Biphenyls, Polychlorinated/brominated Dibenzo-p-dioxins and

Dibenzoftirans, Polybrominated Biphenyl Ethers, and Extractable Organic Halogen in Carp from the Buffalo River, New York', *Environmental Science and Technology*, 29(7), pp. 1832–1838. Available at: https://doi.org/10.1021/es00007a021.

MacNeil, A., Li, X., Amiri, R., Muir, D.C.G., Simpson, A., Simpson, M.J., Dorman, F.L. and Jobst, K.J. (2022) 'Gas Chromatography-(Cyclic) Ion Mobility Mass Spectrometry: A Novel Platform for the Discovery of Unknown Per-/Polyfluoroalkyl Substances', *Analytical Chemistry*, 94(31), pp. 11096–11103. Available at: https://doi.org/10.1021/acs.analchem.2c02325.

Matsui, M., Kashima, Y., Kawano, M., Matsuda, M., Ambe, K., Wakimoto, T. and Doi, R. (2003) 'Dioxin-like potencies and extractable organohalogens (EOX) in medical, municipal and domestic waste incinerator ashes in Japan', *Chemosphere*, 53(8), pp. 971–980. Available at: https://doi.org/10.1016/S0045-6535(03)00587-3.

Miaz, L.T., Plassmann, M.M., Gyllenhammar, I., Bignert, A., Sandblom, O., Lignell, S., Glynn, A. and Benskin, J.P. (2020) 'Temporal trends of suspect-and target-per/polyfluoroalkyl substances (PFAS), extractable organic fluorine (EOF) and total fluorine (TF) in pooled serum from first-time mothers in Uppsala, Sweden, 1996-2017', *Environmental Science: Processes and Impacts*, 22(4), pp. 1071–1083. Available at: https://doi.org/10.1039/c9em00502a.

Miyake, Y., Yamashita, N., Rostkowski, P., So, M.K., Taniyasu, S., Lam, P.K.S. and Kannan, K. (2007) 'Determination of trace levels of total fluorine in water using combustion ion chromatography for fluorine: A mass balance approach to determine individual perfluorinated chemicals in water', *Journal of Chromatography A*, 1143(1–2), pp. 98–104. Available at: https://doi.org/10.1016/j.chroma.2006.12.071.

Mullin, L., Jobst, K., DiLorenzo, R.A., Plumb, R., Reiner, E.J., Yeung, L.W.Y. and Jogsten, I.E. (2020) 'Liquid chromatography-ion mobility-high resolution mass spectrometry for analysis of pollutants in indoor dust: Identification and predictive capabilities', *Analytica Chimica Acta*, 1125, pp. 29–40. Available at: https://doi.org/10.1016/j.aca.2020.05.052.

Naturvårdsverket (2013) *Hållbar återföring av fosfor*. Rapport 6580. Available at: http://www.naturvardsverket.se/Documents/publikationer6400/978-91-620-6580-5.pdf?pid=9620.

Nylund, K., Asplund, L., Jansson, B., Jonsson, P., Litzen, K. and Sellström, U. (1992) 'Analysis of some polyhalogenated organic pollutants in sediment and sewage sludg', *Chemosphere*, 19(12), pp. 463–466. Available at: https://doi.org/10.20595/jjbf.19.0_3.

Ogawa, Y., Tokunaga, E., Kobayashi, O., Hirai, K. and Shibata, N. (2020) 'Current Contributions of Organofluorine Compounds to the Agrochemical Industry', *iScience*, 23(9), p. 101467. Available at: https://doi.org/10.1016/j.isci.2020.101467.

Place, B.J., Ulrich, E.M., Challis, J.K., Chao, A., Du, B., Favela, K., Feng, Y.L., Fisher, C.M., Gardinali, P., Hood, A., Knolhoff, A.M., McEachran, A.D., Nason, S.L., Newton, S.R., Ng, B., Nuñez, J., Peter, K.T., … Williams, A.J. (2021) 'An Introduction to the Benchmarking and Publications for Non-Targeted Analysis Working Group', *Analytical Chemistry*, 93(49), pp. 16289–16296. Available at: https://doi.org/10.1021/acs.analchem.1c02660.

Powley, C.R., George, S.W., Ryan, T.W. and Buck, R.C. (2005) 'Matrix effect-free analytical methods for determination of perfluorinated carboxylic acids in environmental matrixes', *Analytical Chemistry*, 77(19), pp. 6353–6358. Available at: https://doi.org/10.1021/ac0508090.

Revaq (2022) *Revaq Årsrapport 2022*, *Svenskt Vatten*. R2023-01. Available at: https://www.lbva.se/download/18.4ae4bf8818e5f8e814317097/1712054743341/revaq-arsrapport-2022.pdf.

Revaq (2025) *Regler för certifieringssystemet 2025*. Available at: https://www.svensktvatten.se/globalassets/dokument/avlopp-och-miljo/revaq/revaq-regler-2025-utgava-10.0-vit.pdf.

Sahlström, L., Sellström, U. and De Wit, C.A. (2012) 'Clean-up method for determination of established and emerging brominated flame retardants in dust', *Analytical and Bioanalytical Chemistry*, 404(2), pp. 459–466. Available at: https://doi.org/10.1007/s00216-012-6160-y.

Schultes, L., Vestergren, R., Volkova, K., Westberg, E., Jacobson, T. and Benskin, J.P. (2018) 'Per- and polyfluoroalkyl substances and fluorine mass balance in cosmetic products from the Swedish market: implications for environmental emissions and human exposure', *Environmental Science: Processes & Impacts*, 20(12), pp. 1680–1690. Available at: https://doi.org/10.1039/C8EM00368H.

Schultes, L., Peaslee, G.F., Brockman, J.D., Majumdar, A., McGuinness, S.R., Ngwenyama, R.A., Wilkinson, J.T., Sandblom, O., Ngwenyama, R.A. and Benskin, J.P. (2019) 'Total Fluorine Measurements in Food Packaging: How Do Current Methods Perform?', *Environmental Science & Technology Letters*, 6, pp. 73–78. Available at: https://doi.org/10.1021/acs.estlett.8b00700.

Schultes, L., Sandblom, O., Broeg, K., Bignert, A. and Benskin, J.P. (2020) 'Temporal Trends (1981–2013) of Per- and Polyfluoroalkyl Substances and Total Fluorine in Baltic cod (Gadus morhua)', *Environmental Toxicology and Chemistry*, 39(2), pp. 300–309. Available at: https://doi.org/10.1002/etc.4615.

Schymanski, E.L., Zhang, J., Thiessen, P.A., Chirsir, P., Kondic, T. and Bolton, E.E. (2023) 'Per- and Polyfluoroalkyl Substances (PFAS) in PubChem: 7 Million and Growing', *Environmental Science and Technology*, 57(44), pp. 16918–16928. Available at: https://doi.org/10.1021/acs.est.3c04855.

Schymanski, E.L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H.P. and Hollender, J. (2014) 'Identifying small molecules via high resolution mass spectrometry: Communicating confidence', *Environmental Science and Technology*, 48(4), pp. 2097–2098. Available at: https://doi.org/10.1021/es5002105.

Sellström, U., de Wit, C.A., Lundgren, N. and Tysklind, M. (2005) 'Effect of Sewage-Sludge Application on Concentrations of Higher-Brominated Diphenyl Ethers in Soils and Earthworms', *Environmental Science & Technology*, 39(23), pp. 9064–9070. Available at: https://doi.org/10.1021/es051190m.

Shelor, C.P., Warren, C., Odinaka, C. V. and Dumre, K. (2024) 'Comprehensive review of combustion ion chromatography for the analysis of total, adsorbable, and extractable organic fluorine', *Journal of Separation Science*, 47(15). Available at: https://doi.org/10.1002/jssc.202400235.

Shi, X., Langberg, H.A., Sobek, A. and Benskin, J.P. (2024) *Exploiting Molecular Ions for Screening Hydrophobic Contaminants in Sediments using Gas Chromatography-Atmospheric Pressure Chemical Ionization-Ion Mobility-Mass Spectrometry*. Available at: https://doi.org/10.26434/chemrxiv-2024-ft9cl.

Sichler, T.C., Adam, C., Montag, D. and Barjenbruch, M. (2022) 'Future nutrient recovery from sewage sludge regarding three different scenarios - German case study', *Journal of Cleaner Production*, 333(August 2021), p. 130130. Available at: https://doi.org/10.1016/j.jclepro.2021.130130.

Spaan, K.M., van Noordenburg, C., Plassmann, M.M., Schultes, L., Shaw, S., Berger, M., Heide-Jørgensen, M.P., Rosing-Asvid, A., Granquist, S.M., Dietz, R., Sonne, C., Rigét, F., Roos, A. and Benskin, J.P. (2020) 'Fluorine Mass Balance and Suspect Screening in Marine Mammals from the Northern Hemisphere', *Environmental Science & Technology*, 54. Available at: https://doi.org/10.1021/acs.est.9b06773.

SVOA (2019) *Miljörapport 2019 - Avloppsverksamenheten vid Stockholm Vatten och Avfall*. Stockholm.

Travis, S.C., Kordas, K. and Aga, D.S. (2021) 'Optimized workflow for unknown screening using gas chromatography high-resolution mass spectrometry expands identification of contaminants in silicone personal passive samplers', *Rapid Communications in Mass Spectrometry*, 35(8), pp. 1–13. Available at: https://doi.org/10.1002/rcm.9048.

UNEP (2018) *Listing of POPs in the Stockholm Convention*. Available at: http://chm.pops.int/TheConvention/ThePOPs/AllPOPs/tabid/2509/Default.aspx (Accessed: 24 February 2023).

UNEP (2023) *Stockholm Convention on Persistent Organic Pollutants*. Geneva.

Wan, Y., Jones, P.D., Wiseman, S., Cha, H., Chorney, D., Kannan, K., Zhang, K., Hu, J.Y., Khim, J.S., Tanabe, S., Lam, M.H.W. and Giesy, J.P. (2010) 'Contribution of synthetic and naturally occurring organobromine compounds to bromine mass in marine organisms', *Environmental Science and Technology*, 44(16), pp. 6068–6073. Available at: https://doi.org/10.1021/es100914r.

Wang, Z., Walker, G.W., Muir, D.C.G. and Nagatani-Yoshida, K. (2020) 'Toward a Global Understanding of Chemical Pollution: A First Comprehensive Analysis of National and Regional Chemical Inventories', *Environmental Science and Technology*, 54(5), pp. 2575–2584. Available at: https://doi.org/10.1021/acs.est.9b06379.

Xu, D., Zhong, W., Deng, L., Chai, Z. and Mao, X. (2003) 'Levels of extractable organohalogens in pine needles in China', *Environmental Science and Technology*, 37(1), pp. 1–6. Available at: https://doi.org/10.1021/es025799o.

Xu, D., Dan, M., Song, Y., Chai, Z. and Zhuang, G. (2005) 'Concentration characteristics of extractable organohalogens in PM2.5 and PM10 in Beijing, China', *Atmospheric Environment*, 39(22), pp. 4119–4128. Available at: https://doi.org/10.1016/j.atmosenv.2005.03.030.

Yeung, L., Eriksson, U. and Kärrman, A. (2017) *Time trend of unidentified poly- and perfluoroalkyl substances in sludge from wastewater treatment plants in Sweden*. National environmental monitoring report commisioned by the Swedish EPA, http://naturvardsverket.diva-portal.org/smash/record.jsf?pid=diva2%3A1138488&dswid=-9656 (accessed March 6, 2023). Available at: rn:nbn:se:naturvardsverket:diva-7164.

Yeung, L.W.Y., Miyake, Y., Wang, Y., Taniyasu, S., Yamashita, N. and Lam, P.K.S. (2009) 'Total fluorine, extractable organic fluorine, perfluorooctane sulfonate and other related fluorochemicals in liver of Indo-Pacific humpback dolphins (Sousa chinensis) and finless porpoises (Neophocaena phocaenoides) from South China', *Environmental Pollution*, 157(1), pp. 17–23. Available at: https://doi.org/10.1016/j.envpol.2008.08.005.

Yuan, B., Wang, Y.W., Fu, J.J., Zhang, Q.H. and Jiang, G. Bin (2010) 'An analytical method for chlorinated paraffins and their determination in soil samples', *Chinese Science Bulletin*, 55(22), pp. 2396–2402. Available at: https://doi.org/10.1007/s11434-010-3261-x.

Zhang, X., Di Lorenzo, R.A., Helm, P.A., Reiner, E.J., Howard, P.H., Muir, D.C.G., Sled, J.G. and Jobst, K.J. (2019) 'Compositional space: A guide for environmental chemists on the identification of persistent and bioaccumulative organics using mass spectrometry', *Environment International*, 132(May), p. 104808. Available at: https://doi.org/10.1016/j.envint.2019.05.002.