Faculteit wetenschappen

Departement Biologie

Toxicity of Perfluoralkyl Substances (PFASs) to terrestrial invertebrates and songbirds

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Toxiciteit van perfluoroalkylverbindingen (PFASs) voor terrestrische ongewervelden en zangvogels

Proefschrift voorgelegd tot het behalen van de graad van

Doctor in de Wetenschappen: Biologie

Te verdedigen door **Thimo GROFFEN**

Promotors:

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Prof. Dr. Marcel Eens

Antwerpen, 2019

“The only thing I know is that I know nothing”

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Socrates

This work was supported by the research project G038615N of the Research Foundation Flanders (FWO) to Lieven Bervoets and Marcel Eens.

Dit doctoraat werd gefinancierd door een doctoraatsbeurs G038615N van het Fonds Wetenschappelijk Onderzoek (FWO) aan Lieven Bervoets en Marcel Eens.

Dankwoord

Mijn eerste ervaring met ecotoxicologie, en dus ook met geperfluoreerde verbindingen (PFASs), was tijdens de Tropische stage in Tanzania in de derde bachelor Biologie. Als onderwerp voor de bachelor-thesis hadden we gekozen om verschillende polluenten (metalen en verschillende polluenten, waaronder PFASs te analyseren in de waterlopen rondom Morogoro in Tanzania. Ondanks de nodige problemen (nu weet ik dat onderzoek nooit gaat zoals gepland en dus soms heel erg frustrerend kan zijn), heeft de combinatie van veldwerk en labowerk en het gebrek aan informatie over PFASs in het milieu er toe geleid dat ik mijn master-thesis op PFASs in het aquatisch milieu van de Vaal Rivier in Zuid-Afrika ging doen. Tijdens mijn thesis kreeg ik al door dat ik de resultaten zou kunnen publiceren en dat dit de eerste stap zou kunnen zijn in de richting van een academische carrière. Ik werd door Lieven benaderd dat er een vacature open stond voor een project op PFASs in zangvogels in samenwerking met Marcel. Hierop heb ik gesolliciteerd en zo belandde ik in de SPHERE en BECO onderzoeksgroepen, waar ik mijn doctoraatsproject verder heb uitgewerkt. Ondanks de vele tegenslagen (en ook meevallers) zijn de jaren voorbij gevlogen. Dit alles heb ik te danken aan de steun en hulp van verschillende mensen, die ik daarom hartelijk wil bedanken.

Allereerst wil ik mijn promotoren Lieven en Marcel bedanken om mij de kans te geven om dit project tot een goed einde te brengen. Daarnaast wil ik hun bedanken voor hun raad en steun en voor de mogelijkheid om mijn eigen visie en ideeën los te laten op dit

project. Zonder hun steun en inbreng, had ik dit doctoraat niet tot een goed einde kunnen brengen.

Verder wil ik ook Els bedanken voor haar input, hulp en steun bij het ontwikkelen van de nieuwe extractiemethode voor PFASs in verschillende soorten stalen, en voor haar hulp en advies bij diverse andere publicaties. Ik heb ontzettend veel geleerd op gebied van methode ontwikkeling en alles wat daar bij komt kijken. Haar bijdrage aan het volledige project maakte haar als het ware mijn derde promotor en zonder haar hulp had ik nooit zo veel werk kunnen verrichten als ik nu heb gedaan. Graag wil ik ook Tim bedanken, omdat ik altijd bij hem terecht kon met vragen en problemen, maar ook voor zijn inbreng op het gebied van experimentele set-up, de ontwikkeling van de nieuwe extractiemethode, zijn kennis van UPLC en chemie, en uiteraard alle metingen die hij verricht heeft voor mij.

Ik wil ook Ana bedanken voor haar hulp met het veldwerk en het verzamelen van de stalen van de koolmezen. Ondanks de erg moeizame samenwerking, heb ik door haar de nodige ervaring opgedaan in het manipuleren van vogels en in de verwerking (vaak statistisch) van data. Bovendien wil ik Peter en Geert bedanken voor hun hulp met de staalname en het plaatsen van de nestkasten, waardoor een groot deel van dit project mogelijk werd. Verder wil ik Robin bedanken voor zijn hulp als thesis-student in het verzamelen en analyseren van de mezen stalen en zijn hulp bij de ontwikkeling van de extractiemethode, maar zeker ook daarna als collega voor zijn advies en voor de goede samenwerking en communicatie in zowel het labo als tijdens het schrijven van papers.

Helaas kan ik niet iedereen binnen de onderzoeksgroepen bedanken, anders zou het dankwoord veel te lang worden, maar ik wil zeker ook nog alle collega's bij SPHERE en BECO bedanken voor de gezellige samenwerking, gesprekken en activiteiten die we gehad hebben.

De goede en gezellige begeleiding tijdens mijn bachelor- en masterthesis hebben mij geënthousiasmeerd om verder te willen gaan in het onderzoek naar PFASs. Daarom wil ik Wendy ook enorm bedanken voor al haar hulp en voor haar kennis die ze op mij heeft overgedragen. Hierdoor had ik een erg goede basis om aan dit project te beginnen. Doordat ik nu zelf meerdere studenten heb begeleid, weet ik dat het voor haar niet altijd gemakkelijk geweest moet zijn om naast haar eigen werk, ook projecten van anderen te begeleiden. Ik wil dan ook alle studenten bedanken die ik heb mogen begeleiden, omdat ik daardoor gepassioneerd bleef in verschillende aspecten van het onderzoek naar PFAS. Ik hoop dat ik mijn kennis en ervaring ook op jullie heb kunnen overbrengen en dat ik jullie ook enthousiast heb kunnen maken voor een mogelijk vervolg in het onderzoek.

Dit hele project zou ook niet mogelijk zijn geweest zonder toestemming op de verschillende terreinen en gebieden waar we stalen hebben kunnen verzamelen. Daarom wil ik 3M, het Agentschap voor Natuur en Bos (ANB), Koes Maes, Wouter Melens en Godfried Bervoets bedanken.

Dan rest mij enkel nog een aantal mensen te bedanken, die erg veel voor mij betekenen

Allereerst mijn ouders Johan en Gina, om er altijd voor mij te zijn en om mij de kansen te geven om mijzelf te ontwikkelen tot de persoon die ik nu ben. Ik weet dat ik niet altijd even makkelijk ben geweest, maar ondanks dat hebben jullie altijd in mij geloofd en zonder die steun en jullie begrip was ik nooit geweest wie ik nu ben en had ik nooit gestaan waar ik nu sta. Ontzettend bedankt dat jullie er altijd voor mij zijn!

Tenslotte ben ik ontzettend dankbaar om mijn leven te mogen delen met Arina. Jij bent mijn steun en toeverlaat, niet alleen tijdens mijn doctoraat, maar vanaf het moment dat je in mijn leven kwam. Jouw positiviteit bracht vaak een glimlach op mijn gezicht in periodes dat ik erg gefrustreerd of chagrijnig was door vervelende situaties of tegenvallende resultaten. Ik weet dat afgelopen jaren voor jou ook niet altijd gemakkelijk zijn geweest en dat het voor jou ook vermoeiende en zware tijden waren. Ik hoop dan ook dat je weet dat ik je ontzettend dankbaar ben voor alles wat je voor me hebt gedaan en alles wat je nog altijd voor me doet!

Contents

1. General introduction

The earliest forms of environmental pollution were already reported from the moment humans were able to make fire, as the resulting smoke contributed to indoor air pollution. Environmental pollution is hence coeval with the appearance of humans. The long-lasting changes in the environment, which were the result of the harmful activities of ancient civilizations, can sometimes still be experienced today (Makra, 2015).

More intensive environmental pollution appeared simultaneously with the development of societies (Makra, 2015). During the Roman era, lead was the most popular metal, which was frequently used in numerous applications such as food preservation, birth control medicine and shipbuilding (Waldron, 1985). Furthermore, the copper production increased during the Roman times, as copper was used more intensively for military and civil purposes (Makra, 2015). During the Greco-Roman age, the lead and copper concentrations in the troposphere increased significantly as a result of their increased use (Hong et al., 1994, 1996), resulting in the first anthropogenic pollution on a hemispheric scale.

Environmental pollution through anthropogenic sources was also reported on the southern hemisphere in the 16th century, when the Spanish conquered South America. The mines were taken over and the Spanish began pumping clouds of lead dust over the Andes, making it the first industrial-scale toxic metal air pollution on the southern hemisphere (Uglietti et al., 2015). Although this mainly occurred on a relatively small scale, the global impact of environmental pollution and change during the industrial revolution in the 18th century, led to the concept of the Anthropocene, or the Age of Humans (Corlett, 2015; Rose, 2015). This period refers to the heavily increased emission of anthropogenic pollutants in the environment, but also to other major human impacts on the environment, such as e.g. climate change and deforestation (Zalasiewicz et al., 2015).

Since the last century, the development of organic chemical industries has also led to an increased production of a large number of anthropogenic chemicals. Many of these chemicals, that enter the environment, such as metals and persistent organic pollutants (POPs), hazardous organic chemicals that are resistant to degradation and thus remain in the environment for long time periods, may impact the health of biota in ecosystems. The investigation of the ecologically relevant effects of these pollutants at environmentally realistic concentrations in wild species is, however, still a great challenge in ecotoxicology. As a result of their global presence in nature, many of these pollutants have received worldwide scientific attention (Fernández and Grimalt, 2003). Therefore, some well-known POPs such as pesticides, polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) have been studied extensively (e.g. Ashraf 2017, Jaspers et al., 2014; Li et al., 2006; Ross and Birnbaum, 2010). However, much less is known about the accumulation, distribution and effects of more recently produced and detected per- and polyfluoroalkylated substances (PFASs) (Domingo and Nadal, 2017; Mudumbi et al., 2017), which have been produced for over six decades and have been detected globally in the environment, wildlife and even humans (e.g. Giesy and Kannan, 2001, 2002; Houde et al., 2006, Miller et al., 2015). The group of PFASs represents many different molecules, with similar structures, with a large number of applications and physicochemical properties.

Before giving a state of the art on accumulation and effects of PFASs in some environmental and biological matrices that are of interest in this thesis (i.e. soil, invertebrates and songbirds in chapters 1.5 – 1.7), I will first give a technical overview of the terminology (chapter 1.1), how PFASs could end up in the environment (production processes and applications in chapter 1.2 and 1.3) and how they behave in the environment (chapter 1.4).

1.1 Terminology and classification of fluorinated chemicals

The term ‘fluorinated chemicals’ describes a wide range of both organic and inorganic substances that contain at least 1 F atom and that contain a variety of physical, chemical and biological properties (Buck et al., 2011). Examples of fluorinated

chemicals are the highly fluorinated aliphatic substances that contain at least one C atom on which all H atoms, present in their non-fluorinated analogues from which these compounds are derived, are replaced by F atoms. This group is also referred to as perfluoroalkyl or polyfluoroalkyl substances, or PFASs, and contain the perfluoroalkyl moiety C_nF_{n2+1}- (Buck et al., 2011). The group of PFASs can be further classified into numerous subclasses, which are displayed in Figure 1.1.

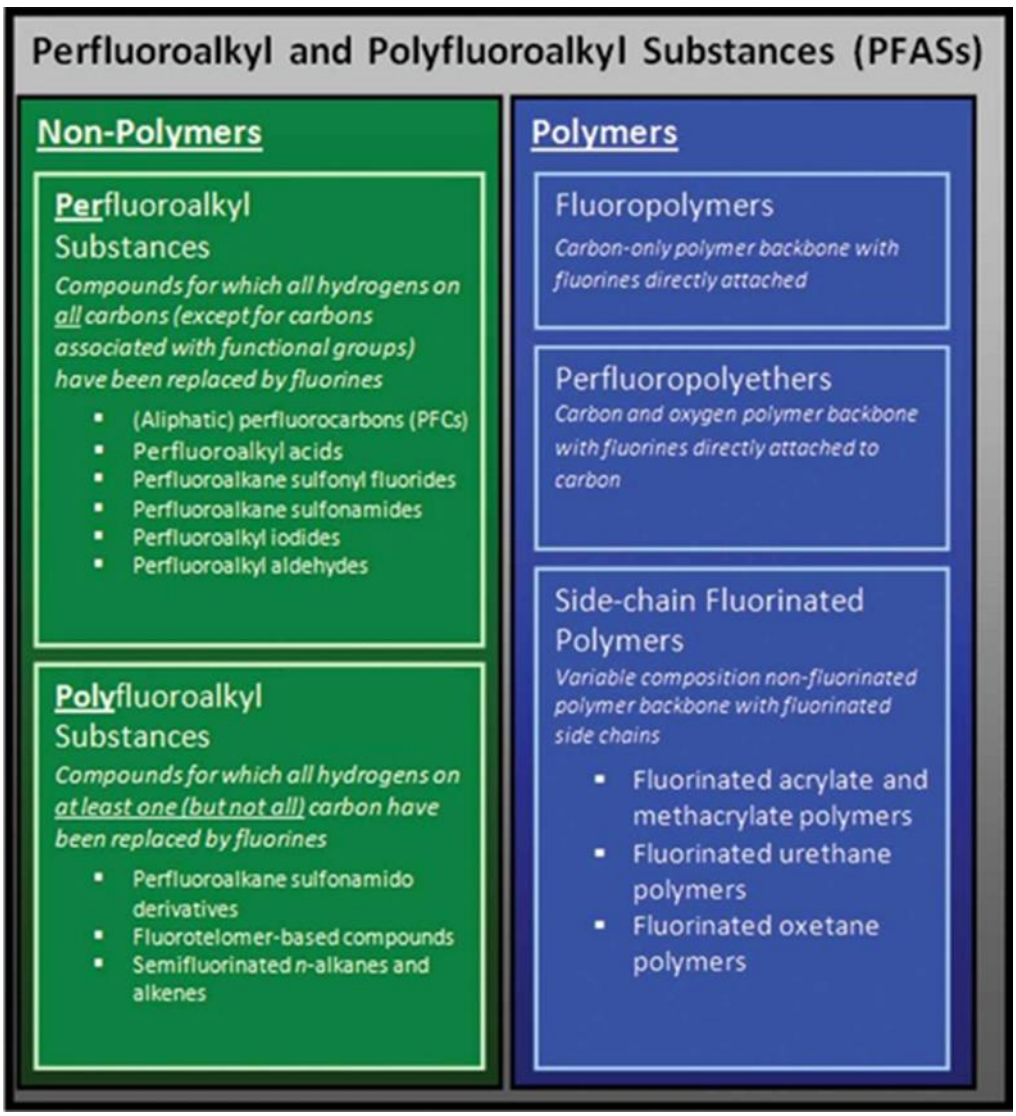


Figure 1.1. Classification hierarchy of environmentally relevant perfluoroalkyl and polyfluoroalkyl substances (PFASs). Adapted from Buck et al. (2011).

As the focus of this thesis is on perfluoroalkylated acids (PFAAs) and more specifically on perfluoroalkyl carboxylic acids (PFCAs) and perfluoroalkyl sulfonic acids (PFSAs) only these groups of PFAAs will be further discussed. Within the PFSAs and PFCAs a further distinction can be made based on the length of the carbon chain. The Organisation for Economic Co-operation and Development (OECD) refers to long-chain PFAAs as PFCAs with eight or more C atoms, and PFSAs with six or more C atoms (OECD, 2011).

1.1.1 Perfluoroalkyl carboxylic acids (PFCAs)

Perfluoroalkyl carboxylic acids (PFCAs) are a subfamily of PFAAs that can contain a perfluorinated carbon chain of between 2 and 16 C atoms in length with a terminal carboxylic acid group. Their general moiety is $C_nF_{2n+1}COOH$.

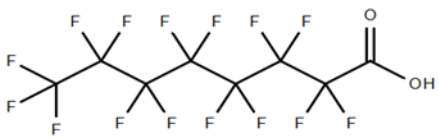


Figure 1.2 Chemical structure of perfluorooctanoic acid (PFOA)

The most commonly studied PFCA is perfluorooctanoic acid ($C_7F_{15}COOH$, PFOA, Figure 1.2), that is mostly manufactured as its ammonium salt, ammonium perfluorooctanoate (APFO). Similar to PFOA, perfluorononanoic acid ($C_8F_{17}COOH$, PFNA) is also mainly manufactured as its ammonium salt (ammonium perfluorononanoate, APFN) (Buck et al., 2011). Other PFCAs that are less frequently studied, but have been analyzed in this thesis, are displayed in Table 1.1.

Table 1.1 Full name, abbreviation and chemical formula of the perfluoroalkyl carboxylic acids (PFCAs) analyzed in this thesis.

Full name	*Abbreviation*	*Chemical formula*
Perfluorobutanoic acid	PFBA	C_3F_7COOH
Perfluoropentanoic acid	PFPeA	C_4F_9COOH
Perfluorohexanoic acid	PFHxA	$C_5F_{11}COOH$
Perfluoroheptanoic acid	PFHpA	$C_6F_{13}COOH$
Perfluorooctanoic acid	PFOA	$C_7F_{15}COOH$
Perfluorononanoic acid	PFNA	$C_8F_{17}COOH$
Perfluorodecanoic acid	PFDA	$C_9F_{19}COOH$
Perfluoroundecanoic acid	PFUnDA	$C_{10}F_{21}COOH$
Perfluorododecanoic acid	PFDoDA	$C_{11}F_{23}COOH$
Perfluorotridecanoic acid	PFTrDA	$C_{12}F_{25}COOH$
Perfluorotetradecanoic acid	PFTeDA	$C_{13}F_{27}COOH$

1.1.2 Perfluoroalkyl sulfonic acids (PFSAs)

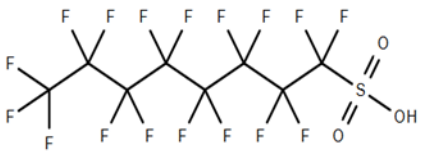


Figure 1.3 Chemical structure of perfluorooctane sulfonic acid (PFOS)

The second major group of PFAAs are the PFSAs, which generally contain 2 to 16 C atoms and a sulfonate group, resulting in the general moiety $C_nF_{2n+1}SO_3H$. The PFSA that gained the most attention from the moment it was first detected globally in biota (Giesy and Kannan, 2001) and humans (Hansen et al., 2001) is perfluorooctane sulfonic acid ($C_8F_{17}SO_3H$, PFOS, Figure 1.3), which is the most commonly encountered PFAA in the environment and wildlife (Giesy, 2010). In reality, PFOS is a mixture of both linear and branched isomers of PFOS, depending on the production process, which will be described in detail in chapter 1.2. Perfluoroalkyl sulfonic acids with shorter carbon chain lengths than PFOS, such as perfluorobutane sulfonate ($C_4F_9SO_3H$, PFBS) are nowadays used as replacement substances for PFOS. Besides the direct production of PFSAs, some PFSAs can also be present as impurities in the formation of perfluorooctane sulfonamide precursor substances (Buck et al., 2011).

Other frequently studied PFSAs, which have been studied in this research, are displayed in Table 1.2.

Table 1.2 Full name, abbreviation and chemical formula of the perfluoroalkyl sulfonic acids (PFSAs) analyzed in this thesis.

Full name	*Abbreviation*	*Chemical formula*
Perfluorobutane sulfonate	PFBS	$C_4F_9SO_3H$
Perfluorohexane sulfonate	PFHxS	$C_6F_{13}SO_3H$
Perfluorooctane sulfonate	PFOS	$C_8F_{17}SO_3H$
Perfluorodecane sulfonate	PFDS	$C_{10}F_{21}SO_3H$

1.1.3 Potential PFSA and PFCA precursor compounds

Both PFSAs and PFCAs may not only be produced directly, but can also be formed through abiotic or biotic transformation of less stable precursor compounds. These precursors are substances that have the potential to degrade to long-chain PFCAs or PFSAs. Among these precursors are side-chain fluorinated polymers, which are fluorinated polymers that consist of variable composition of non-fluorinated carbon backbones with polyfluoroalkyl or perfluoroalkyl side-chains (OECD, 2013).

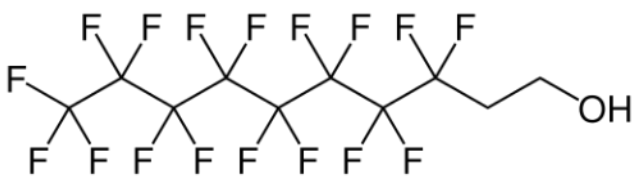


Figure 1.4 Chemical structure of fluorotelomer alcohol 8:2 FTOH

Perfluoroalkyl carboxylic acids may be the terminal degradation product of many precursor compounds including fluorotelomer alcohols (FTOHs; Figure 1.4), acrylates (FTACs), iodides (FTIs), olefins (FTOs), N-alkyl perfluoroalkane sulfonamides (FASAs), N-alkyl perfluoroalkane sulfonamidoethanols (FASEs) and polyfluoroalkyl phosphates (PAPs). Examples of precursor compounds for PFSAs are perfluoroalkane sulfonyl fluorides (PASFs) (Buck et al., 2011).

1.2 Production processes

It is important to describe the production processes of PFAAs for a better understanding of the environmental distribution and behaviour as well as the relationships between different families of PFASs. Two processes have been used for the production of PFASs, which result in different isomeric purities. These processes are electrochemical fluorination (ECF) and telomerization (TM).

1.2.1 Electrochemical fluorination (ECF)

When organic compounds undergo electrolysis in anhydrous hydrogen fluoride (aHF) and an organic feedstock with fluorine, all H atoms are replaced by F-atoms (Buck et al., 2011). This process is called electrochemical fluorination (ECF) or the Simons process (Ignat'ev and Sartori, 2000). During the electrochemical conversion of organo-fluorine compounds, active fluorine-containing species (e.g. radicals and anions), which may react to different substrates, are created (Ignat'ev and Sartori, 2000). These radicals cause carbon chains to rearrange into mixtures of linear and branched perfluorinated isomers and homologues (Buck et al., 2011). The ECF process generally leads to the production of even- and odd-numbered, branched and linear chains of PFAAs. For the manufacture of PFOS-related chemicals, perfluorooctane sulfonylfluoride (POSF, $C_8F_{17}SO_2F$), a precursor of PFOS (Buck et al., 2011), is used as a starting material, whereas PFOA can be produced through ECF using octanoyl fluoride as organic feedstock (Buck et al., 2011).

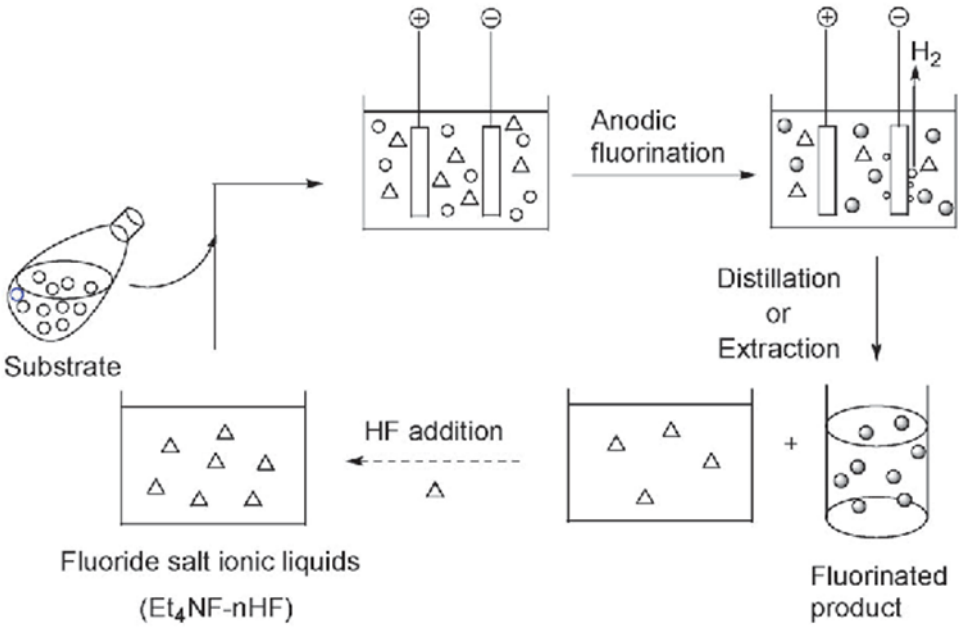


Figure 1.5 Solvent-free selective anodic fluorination in ionic liquids. Adapted from Fuchigami and Inagi, 2011

The procedure starts with the formation of organic radicals on the surface of an anode (Ignat'ev and Sartori, 2000). As described before, this radical may then react to different substrates and can therefore undergo fluorination, isomerization, cyclization and condensation (Ignat'ev and Sartori, 2000). Figure 1.5 illustrates a schematic overview a solvent-free selective anodic ECF procedure (Fuchigami and Inagi, 2011).

1.2.2 Telomerization (TM)

In the telomerization process a perfluoroalkyl iodide (PFAI) is reacting with tetrafluoroethylene (CF_2=CF_2, TFE) to successively add an ethyl group to the fluoroalkyl chain. The product mixture is often reacting further in a second step, in which ethylene is inserted to create a FTI. Both the PFAIs and FTIs are building blocks of FTOHs and a wide range of other products, including PFOA and PFNA (Fig. 1.6) (Buck et al., 2011).

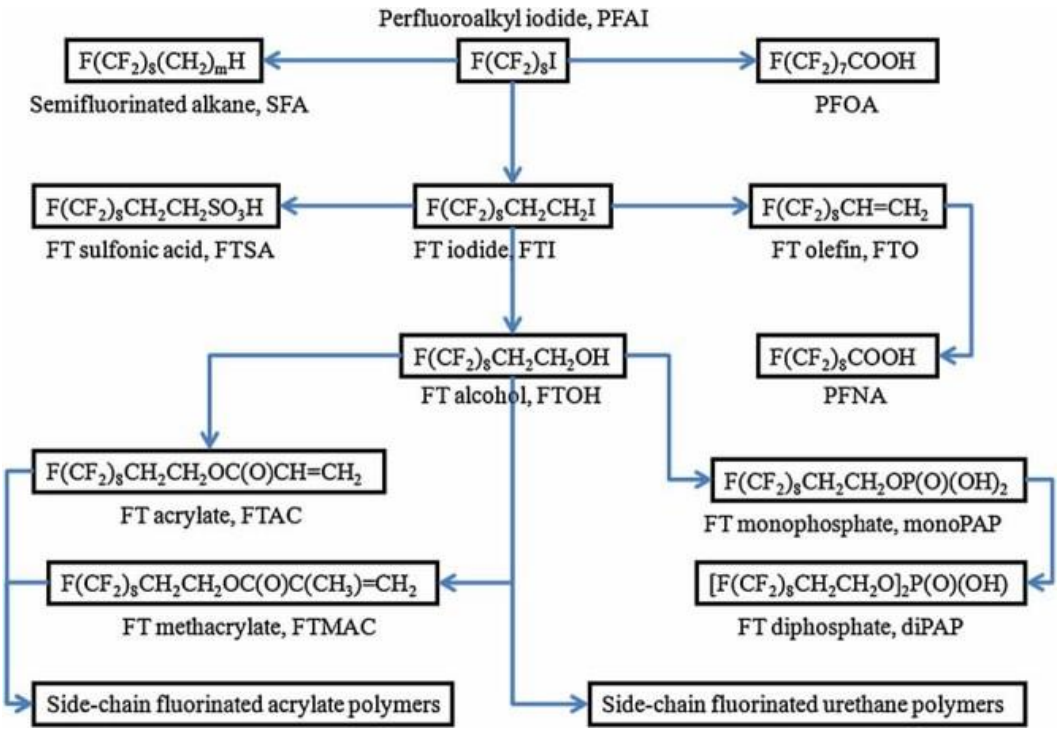


Figure 1.6 An example of PFCAs and fluorotelomer (FT) derivatives synthesized from perfluoroalkyl iodides (PFAIs) for a starting PFAI with 8C atoms. Names and acronyms for substance families are indicated. Adapted from Buck et al., 2011.

1.3 Use

PFASs have been used in a wide variety of products and production processes. The production and use of PFASs grew from the 1960s to the 1990s because of their distinct physicochemical properties, which will be described further in Chapter 1.4.

PFASs, their precursors and degradation products have been or are still used for numerous industrial and commercial applications, including food packaging (Schaider et al., 2017), textiles (Robel et al., 2017), chemicals such as paints and ink (Challener, 2008), metal plating (galvanic industry; Poulsen et al., 2001), printing plates and semi-conductors (photolithographic and electro industry; Brooke et al., 2004), insecticides (Manning et al., 1991; Grossman et al., 1992), cosmetics (Cassady et al., 2014), hydraulic fluids (aviation industry; Brooke et al., 2004), fire-fighting foams (e.g. Montagnolli et al., 2018), implants (Henry et al., 2018) and non-stick cookware (e.g. Sajid and Ilyas, 2017).

1.4 Properties, Fate and Behaviour

Both PFSAs and PFCAs are widely distributed in the global environment as a result of their outspoken physicochemical properties, including their high solubility in water (with exception of the compounds with the longest chain length), low/moderate sorption to soils and sediments and resistance to both biological and chemical degradation.

1.4.1 Physicochemical properties

The physicochemical properties of the target analytes of this thesis, reported in Table 1.3, are mainly caused by the properties of the individual atoms and the covalent carbon-fluorine bond, which is one of the strongest bonds in organic chemistry. The thermal, chemical, photolytic and biological stability of PFASs are likely the result of these bonds, as the dense packing of fluorine electrons protect PFASs from external attacks. In addition to the hydrophobic perfluorinated carbon chain, PFASs have a hydrophilic functional group, which makes them amphiphilic compounds.

Additionally, the fluorinated carbon-chain also has a lipophobic characteristic (Pancras et al., 2016). The physicochemical properties of PFASs may change non-linearly within a homologous PFASs series. This is most likely the result of a change in geometry with increasing chain length. PFASs molecules with more than eight fluorinated C-atoms can form a helix, which results in an increase in electron density. This increased electron density leads to changes in physicochemical properties (Wang Z et al., 2011).

Table 1.3 Physicochemical properties of the target analytes in this thesis. Data adapted from SGS (2019).

Name	Acronym	Mol. Weight (g/mol)	Water Solubility 20-25°C (g/L)	Vapor Pressure [Pa]
Perfluoroalkyl carboxylic acids (PFCAs)				
Perfluorobutanoic acid	PFBA	214.04	Miscible	1307
Perfluoropentanoic acid	PFPeA	264.05	112.6	1057
Perfluorohexanoic acid	PFHxA	314.05	21.7	457
Perfluoroheptanoic acid	PFHpA	364.06	4.2	158
Perfluorooctanoic acid	PFOA	414.07	3.4 – 9.5	4 – 1300
Perfluorononanoic acid	PFNA	464.08	9.5	1.3
Perfluorodecanoic acid	PFDA	514.09	9.5	0.2
Perfluoroundecanoic acid	PFUnDA	564.09	0.004	0.1
Perfluorododecanoic acid	PFDoDA	614.1	0.0007	0.01
Perfluorotridecanoic acid	PFTrDA	664.11	0.002	0.3
Perfluorotetradecanoic acid	PFTeDA	714.12	0.00003	0.1
Perfluoroalkyl sulfonic acids (PFSAs)				
Perfluorobutane sulfonate	PFBS	300.1	46.2 – 56.6	631
Perfluorohexane sulfonate	PFHxS	400.11	2.3	58.9
Perfluorooctane sulfonate	PFOS	500.13	1.52 – 1.57	6.7
Perfluorodecane sulfonate	PFDS	600.14	0.002	0.71

Table 1.3 (continued) Physicochemical properties of the target analytes in this thesis. Data adapted from SGS (2019).

Name	Acronym	Density (20°C) (g/mL)	Melting Point (°C)	Boiling Point (°C)	Dissociation Constant [pKa]
Perfluoroalkyl carboxylic acids (PFCAs)					
Perfluorobutanoic acid	PFBA	1.65	-17.5	121	-0.2 to 0.7
Perfluoropentanoic acid	PFPeA	1.7	---	124.4	-0.06
Perfluorohexanoic acid	PFHxA	1.72	14	143	-0.13
Perfluoroheptanoic acid	PFHpA	1.79	30	175	-0.15
Perfluorooctanoic acid	PFOA	1.8	37 – 60	188 – 192	-0.16 to 3.8
Perfluorononanoic acid	PFNA	1.75	56 – 59	218	-0.17
Perfluorodecanoic acid	PFDA	1.76	77 – 88	218	-0.17
Perfluoroundecanoic acid	PFUnDA	1.76	83 – 101	160 – 230	-0.17
Perfluorododecanoic acid	PFDoDA	1.77	107 – 109	245	-0.17 to 0.8
Perfluorotridecanoic acid	PFTrDA	1.77	---	---	---
Perfluorotetradecanoic acid	PFTeDA	1.78	---	276	---
Perfluoroalkyl sulfonic acids (PFSAs)					
Perfluorobutane sulfonate	PFBS	1.81	76 – 84	211	-6 to -5
Perfluorohexane sulfonate	PFHxS	---	---	---	-6 to -5
Perfluorooctane sulfonate	PFOS	---	54	>400	-6 to -2.6
Perfluorodecane sulfonate	PFDS	---	---	---	---

1.4.2 Fate and behaviour

The environmental fate of PFASs describes their transport, partitioning and transformation after their release in the environment (Figure 1.7). The release of PFASs into the environment occurs either through direct pollution, or through environmental degradation of precursor compounds (Buck et al., 2011; Prevedouros et al., 2006). In addition, gas- and particle-phase atmospheric long-range transport may also result in the release of PFASs in the environment (Barber et al., 2007; Ellis et al., 2003; Schenker et al., 2008). Precursor compounds are more volatile compared to PFASs and are therefore typically transported via the atmosphere and subsequently degraded (Ahrens and Bundschuh, 2014; Martin et al., 2006; Young and Mabury, 2010). They are subject to various transformation pathways in either the atmosphere or under aerobic and anaerobic conditions in environmental matrices (Butt et al., 2014). The environmental fate of PFASs depends on different environmental conditions and the physicochemical properties of the PFASs.

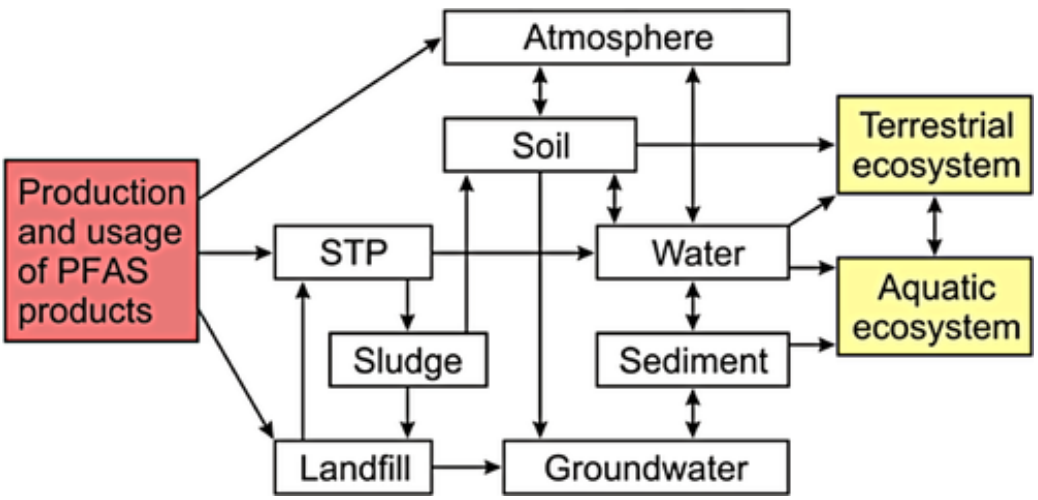


Figure 1.7 Pathways of PFASs into the environment and their fate. Adapted from Ahrens et al. (2011a). STP = sewage treatment plant.

Both PFCAs and PFSAs have relatively high solubility values (Table 1.3), caused by their hydrophilic functional groups. The hydrophobic carbon-chain, causes the water solubility of PFCAs and PFSAs to decrease with increasing chain length. (Ahrens et al., 2010; Martin et al., 2003). Dissociation is the process by which an electronegative

atom and a hydrogen atom, which are ionically bonded, separate into a proton (H^+) and a negative ion. The dissociation constant (pKa) describes the extent of dissociation in water and is a value at which half of the acid molecules dissociate into ions. A lower pKa value indicates a stronger acid (i.e. a lower value indicates that the acid dissociates more fully in water). Because of their very low (negative) dissociation constants, PFCAs and PFSAs will be present in their anionic forms in natural waters, which will eventually influence the sorption to solid matrices such as soils. As the focus of this manuscript is on the terrestrial environment, only soils will be further discussed.

1.4.2.1 Transport to soil

The environmental transport of PFASs to the soil will depend on the sorption of PFASs to soils during the transport. They can sorb to naturally-occurring organic carbon particles present in the soil. The sorption of PFCAs and PFSAs will increase with increasing chain length and with increasing solid phase fraction of organic carbon (Higgins and Luthy, 2006; Zareitalabad et al., 2013). Additionally, sorption increases with decreasing pH and increasing Ca^{2+} concentrations (Campos Pereira et al., 2018), which suggests that the degree of PFASs hydrophobic sorption to soils is a site-specific phenomenon. The maximum sorption capacity of soils is to a large extent influenced by organic carbon content ((T)OC; Miao et al., 2017; Milinovic et al., 2015; Wei et al., 2017), and more specifically the humin fraction (Chapter 1.5.2). As PFASs are relatively strong acids that exist as anions in natural waters, surface sorption may also occur to charged mineral surfaces that are naturally present in soils (e.g. Ferrey et al., 2012; Johnson et al., 2007; Tang et al., 2010).

1.4.2.2 Transformations

Less stable precursor compounds may undergo abiotic or biotic transformation to form PFSAs and PFCAs. The pathways of aerobic biodegradation and metabolic degradation for FTOHs have been well studied (Buck et al., 2011; Frömel and Knepper, 2010). A simplified scheme of the atmospheric degradation of N:2 FTOHs is illustrated in Figure 1.8. This figure also illustrated the atmospheric degradation pathways of FTIs, FTOs and FTACs, which all have parts of their degradation mechanisms in common. D'Eon

and Mabury (2007) have reported the pathways for the microbial degradation of PAPs by hydrolysis of the phosphor-ester bond to form the respective FTOH as a by-product, which may then be further degraded according to the pathways described in Figure 1.8.

Although these degradation pathways are the common pathways for many precursor compounds, it should be noted that the pathways and yields of the transformation products depends not only on the length of the perfluoroalkyl chain in the FTOH, but also on the matrix in which the environmental degradation or metabolism takes places (Dinglasan et al., 2004; Liu et al., 2010; Martin et al., 2005; Wang et al., 2009).

When PFCAs and PFSAs are formed, these compounds are generally considered to be highly resistant to biotransformation via microorganisms present in water or soil. Even though short-chained PFASs have been proposed as alternatives for long-chain PFASs, due to their lower bio-accumulative character and toxicity, Ochoa-Herrera et al. (2016) reported a high resistance to microbial degradation of both PFOS and PFBS. PFCAs and PFSAs are however susceptible to photolysis as long-chain PFAS can be dealkylated to short-chain PFAS under extreme reaction conditions (Giri et al., 2011; Hori et al., 2007). Photolytic degradation of PFASs has been reported in the field (Taniyasu et al., 2013), although it has been heavily criticized (e.g. Wang Z et al., 2015a).

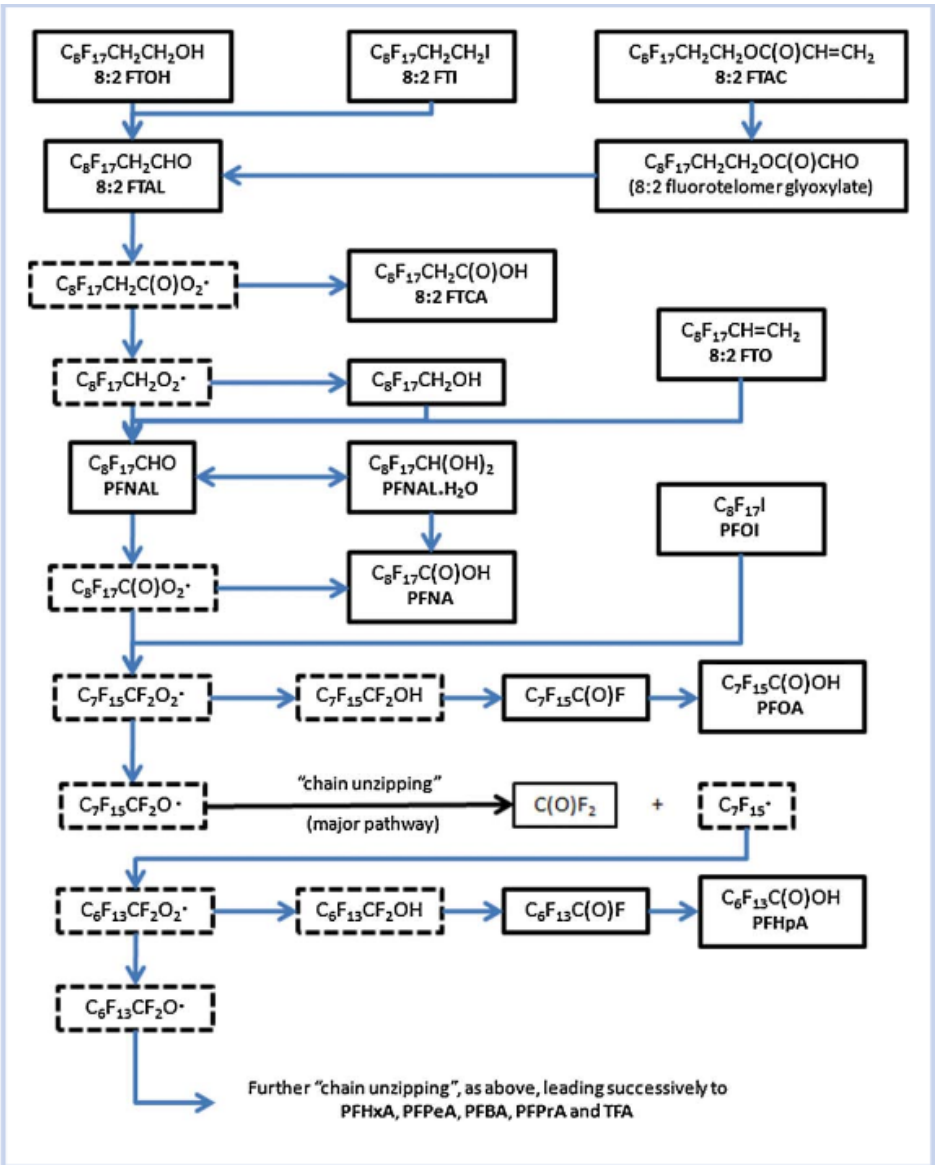


Figure 1.8 Simplified atmospheric degradation scheme for 8:2 fluorotelomer derivatives. Boxes with a dashed outline represent free-radical and transient molecular intermediates, while boxes with a solid outline contain more stable molecular intermediates and final products. The acronyms of some compounds are indicated in bold. An arrow may imply multiple elementary step, i.e. certain intermediates are omitted. Adapted from Buck et al., 2011.

1.5 PFASs in soils

Soil pollution can occur through many different processes. Air or water pollution may be settling into the topsoil, but soils can also be contaminated from the burial of toxic substances in an attempt to mitigate harmful effects (Fedotov et al., 2018; Lang et al., 2017). Due to the everyday use of PFAS and PFAS-containing products, pollution of soils may occur through leakage from industries and landfills, but also through waste water disposal (Gallen et al., 2018; Xiao et al., 2015). Soil pollution can have long-term effects. Contrary to e.g. water pollution, in which the natural flow will dilute and disperse toxic substances, soil pollutants may remain in the soil for long periods, posing risks for generations (Mapanda et al., 2005; Xiao et al., 2015). Due to rain and agricultural runoff, soil pollutants may wash into rivers or move to the groundwater, resulting in the potential spread of these contaminants over a large area.

1.5.1 Global distribution in soils

Soils are known sinks for many POPs, such as PBDEs, PCBs and polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) (Cetin et al., 2017; Maqsood and Murugan, 2017; Mueller et al., 2006; Rankin et al., 2016; Xiao et al., 2016). The sorption of PFAAs to solid matrices has been reported before (Ahrens et al., 2011b; Li YS et al., 2018; Miao et al., 2017; Qian et al., 2017; Rankin et al., 2016; Wei et al., 2017) and soils have been used frequently in studies on the effects of sludge- or biosolid-amendments, transformation processes, degradation of precursors, and more frequently remediation techniques. However, there are still a limited amount of studies that examine PFASs in natural soils worldwide. For example, a literature search on the term “perfluor* soil*” in large databases, such as Web of Science (WOS) and Google Scholar, resulted in less than 40 articles that report PFAAs in natural soils. Most of these studies were performed on the northern hemisphere and more particularly in Asia.

Within these studies a further distinction can be made between pathways of contamination. Although most studies in Asia focus on pollution through industrial activities, studies in Europe, North America and Australia mainly target sites that have been affected by aqueous film-forming foams (AFFFs. Fire-fighting foams; Figure 1.9).

Here, only natural soils will be discussed and previously described studies on e.g. sludge- or biosolid-amendments will not be mentioned further.

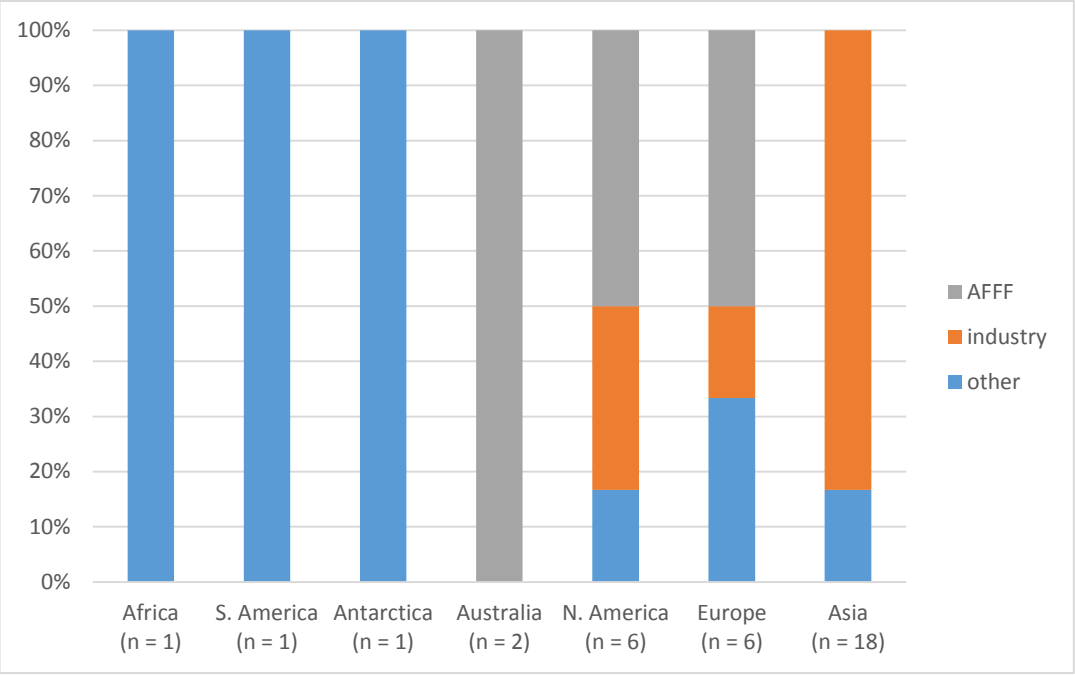


Figure 1.9 Contamination pathways reported in literature on PFASs in natural soils for each continent. Other is defined as every other possible pathway of contamination besides AFFFs and industrial pollution.

Rankin et al. (2016) determined the geometric mean ΣPFCA and ΣPFSA concentrations in soils from each continent (Table 1.4). Although they did not differentiate between natural soils and sludge- or biosolid-amended soils, it is clear that PFAS concentrations are much higher in soils from the northern hemisphere compared to the southern hemisphere, which is likely the result of a higher industrial activity on the northern hemisphere.

Table 1.4 Continental PFAS concentration ranges in pg/g dry weight with the continental geometric mean in parentheses. Table adopted from Rankin et al. (2016).

Continent	ΣPFCA	ΣPFSA
North America (NA)(n = 33)	145 – 6080 (1820)	35 – 1990 (410)
Europe (EU)(n = 10)	55 – 3640 (1000)	<LOD – 3270 (808)
Asia (AS)(n = 6)	129 – 14300 (4710)	79 – 421 (183)
Africa (AF)(n = 5)	124 – 1490 (548)	<LOD – 144 (67)
Australia (AU)(n = 4)	79 – 1260 (673)	44 – 297 (154)
South America (SA) (n = 3)	29 – 319 (138)	26 – 48 (36)
Antarctica (AN) (n = 1)	191	7

Studies in soils that have not been affected by PFASs-related industries or AFFFs are rather scarce. Despite the dominance of PFCAs compared to PFSAs, according to Rankin et al. (2016), multiple studies have reported the dominance of PFSAs such as PFOS in unaffected soils from Uganda (Dalahmeh et al., 2018), Argentina (Llorca et al., 2012), Canada (Cabrerizo et al., 2018), France (Gaspéri et al., 2018), Korea (Naile et al., 2010) and Antarctica (Llorca et al., 2012). However, the dominance of PFCAs has been confirmed by Naile et al. (2013) and Tan et al. (2014) who reported the dominance of PFOA in soils collected in Korea and Nepal, respectively. Plassmann and Berger (2013) examined only the PFCA concentrations in soil from a ski area in Sweden and reported much higher concentrations than the geometric means reported by Rankin et al. (2016).

Similarly, PFOS or PFSAs were also the dominant contributor to the total PFAS concentrations in soils that were affected by AFFFs in Australia (Bräunig et al., 2019; Das et al., 2015), USA (Anderson et al., 2016; Houtz et al., 2013), Sweden (Filipovic et al., 2015a) and Norway (Hale et al., 2017; Kärrman et al., 2011). Nevertheless, Mejia-Avendaño et al. (2017) observed a dominance of PFCAs in soils that were contaminated with AFFFs during a train accident in Canada.

In industrially polluted sites, the PFCA concentrations in the soil were often higher than those of PFSAs. This dominance of PFCAs has been reported in sites along a US highway

(Xiao et al., 2015). In China, this dominance has also been observed in rural areas (Chen et al., 2016; Li et al., 2010; Lu et al., 2018), near fluorochemical parks (Chen H et al., 2018; Shan et al., 2014; Wang P et al., 2013), around a heavily contaminated watershed (Meng et al., 2013, 2018), at Guanting Reservoir (Wang T et al., 2011a), in natural forest soils in the Hubei and Jiangxi provinces (Wang et al., 2018) and along estuarine and coastal areas (Wang T et al., 2011b). The dominance of PFOS or PFOA varied among sites in different coastal areas in North China (Meng et al., 2015). Contrary to the previously reported studies on industrially contaminated soils, where PFCAs were dominant, PFOS was dominant in soils from a highly contaminated industrialized area in China (Pan et al., 2011; Wang T et al., 2012) and in soils near fluorochemical plants in China (Wang et al., 2010). Although they did not analyze PFSAs, Zhu and Kannan (2019) reported much higher ΣPFCA concentrations in industrially polluted soils from North America than those reported by Rankin et al. (2016). D'Hollander et al. (2014), reported much higher PFOS concentrations than the geometric mean ΣPFSA concentrations in soils close to a fluorochemical manufacturing facility in Belgium.

1.5.2 Effects of physicochemical soil properties on the sorption of PFAAs

The sorption behaviour and bioaccumulation of PFASs is strongly influenced by their molecular structure. The hydrophobic perfluorinated carbon chain in combination with the hydrophilic, usually anionic, functional group results in a different environmental behaviour of PFASs compared to other POPs. The behaviour of PFASs is therefore not only governed by hydrophobic interactions, but electrostatic interactions also play a key role (Higgins and Luthy, 2007). As a result, the sorption of PFASs cannot be predicted from a single sorbent bulk property such as organic carbon (OC) content (Barzen-Hanson et al., 2017; Li YS et al., 2018). Consequently, there are still many uncertainties on how various sorbent properties, such as e.g. pH, interact to determine the binding of PFASs to soils (Li YS et al., 2018).

Organic carbon is one of the most important sorbents for PFASs in soils (e.g. Milinovic et al., 2015). The sorption of PFASs to soil organic matter (SOM) describes a nearly linear relation between sorption (described as the organic carbon-water partitioning

coefficient (K_{oc})) and the chain-length of the compounds (Labadie and Chevreuil, 2011). Besides chain-length, the functional group of the PFASs also affects the sorption (Ahrens et al., 2010; Campos Pereira et al., 2018; Higgins and Luthy, 2006). Nevertheless, chain-length is considered the dominating structural feature concerning the adsorption (Ahrens et al., 2010; Higgins and Luthy, 2006). In general, the sorption of PFASs increases with increasing chain length, which is attributed to an increase in the hydrophobicity with each CF_2 moiety. The hydrophobic effect of the carbon chain is stronger than the electrostatic negativity originating from the functional group, causing long-chained PFASs to sorb more strongly than short-chained ones (Du et al., 2014).

Despite the importance of SOM in the sorption of PFASs, it is still rather unclear what fractions of the organic matter may be important for binding of PFASs (Campos Pereira et al., 2018). Multiple studies reported that the humin fraction is the most important factor for the sorption of long-chained PFASs (e.g. Campos Pereira et al., 2018; Gunasekara and Xing, 2003; Zhang et al., 2015; Zhao et al., 2014), although the humic and fulvic acid fractions might also be important for the sorption of shorter-chained PFASs (Campos Pereira et al., 2018). The high sorption capacity of humin towards PFASs results from its highly condensed aliphatic and aromatic domains (Gunasekara and Xing, 2003; Chen et al., 2007).

Besides SOM, the sorption of PFASs is promoted by a decreased pH and increased cation concentration (e.g. Higgins and Luthy, 2006; Chen et al., 2009; Wang F et al., 2015; Zhang et al., 2013). The effects of pH on the adsorption of PFASs to soil are typically described as due to protonation or deprotonation of the organic acids (Higgins and Luthy, 2006). However, pH-dependent changes in the sorbent, such as surface charge of SOM, may also explain the pH effects (Higgins and Luthy, 2006). The decrease in adsorption with increasing pH is possibly caused by the decrease of electrostatic interactions, rather than protonation or deprotonation of the sorbate (Chen et al., 2009; Higgins and Luthy, 2006; Wang F et al., 2012). However, the influence of pH

changes in the presence of a sufficient amount of divalent cations (Chen et al., 2009; Du et al., 2014). Adsorbent surfaces develop more basic sites to bind these cations when the pH increases, resulting in increased sorption of PFASs (Du et al., 2014; Wang F et al., 2012).

Additionally, soils consisting of mainly large particles will have less functional groups (such as hydroxyl and carboxyl groups), which results in less binding sites to facilitate the sorption of PFASs to the soil (Qi et al, 2014).

1.6 PFASs in terrestrial invertebrates

The accumulation of substances, such as PFASs, in organisms is called bioaccumulation. It occurs when the uptake of a chemical goes at a faster rate than the loss of the substance through catabolism and excretion. This means that chemicals with a longer biological half-life will remain longer in the organism, potentially causing toxic effects even if environmental concentrations are rather low. PFASs are bioaccumulative and will mainly accumulate in protein-rich tissues (D'Eon and Mabury, 2011; Higgins et al., 2007; Zhao et al., 2013). Furthermore, it is known that short-chain PFCAs and PFSAs have lower bioaccumulation factors than long-chained ones (Buck et al., 2011; Lasier et al., 2011). In this chapter, the toxicokinetics and –dynamics of PFASs on terrestrial invertebrates will be discussed.

Invertebrates have been used in numerous field studies to test for bioaccumulation of PFASs. Nevertheless, most of these studies target aquatic invertebrates (e.g. Babut et al., 2017; Groffen et al., 2018; Lescord et al., 2015; Loi et al., 2011) and field data on terrestrial invertebrates remain scarce. Although there are numerous studies conducted on earthworms under laboratory conditions (e.g. Das et al., 2015; Zhao et al., 2013; Zhao Y et al., 2017), data on toxicokinetics or toxicodynamics of PFAS in terrestrial invertebrates is scarce (Zhao et al., 2013).

1.6.1 Toxicokinetics

Earthworms take up organic pollutants through pore water and ingestion of soil through the gut (Hallgren et al., 2006; Sijm et al., 2000). The accumulation of pollutants

consists mainly of three steps: 1) uptake from soil by ingestion, 2) desorption of the contaminants within the gut and 3) adsorption to the gut wall and absorption across the gut wall (Weston et al., 2000).

Exposure of earthworms to contaminated soils, showed that PFAS concentrations, with exception of PFDoDA, PFHxS and PFOS, reached an equilibrium after 30 days. The uptake coefficient (k_u) increased with increasing carbon chain length and PFSAs tended to have higher bioaccumulative potentials than PFCAs as PFSAs were taken up by earthworms at greater extent than PFCAs with the same chain length (Zhao et al., 2013).

Regarding the elimination, PFASs with longer chain length have been shown to display a smaller elimination coefficient (k_e) than short chained PFASs. As a result, biota-soil accumulation factors (BSAFs) of longer chain PFASs were higher than those of shorter ones (Zhao et al., 2013). In earthworms, the BSAFs were concentration-dependent and decreased with increasing concentrations (Zhao et al., 2013). This is likely the result of a saturation of binding sites in earthworms at higher concentrations (Liu et al., 2011). Das et al. (2015), who also reported that bioaccumulation factors in earthworms were highest from soils with the lowest PFOS concentrations, obtained similar results.

1.6.2 Toxicodynamics

Toxicodynamics describe the dynamic interactions of a pollutant within organisms and its biological effects. These effects may occur at different biological targets, such as binding proteins, ion channels or DNA. Pollutants may interact with these biological receptors and produce structural or functional alterations, causing potential harm to the organisms.

To assess the potential effects of pollutants, biomarkers are frequently used. These biomarkers can be enzymatic, such as superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and other antioxidant enzymes, or nonenzymatic. Antioxidant enzymes are often used to indicate the production of reactive oxygen species (ROS) and are involved in the detoxification of these ROS (Wen et al., 2011).

Oxidative stress (OS) is defined as the disturbance in the balance between the production of ROS and antioxidant defenses and may lead to tissue damage. Free radicals, which are highly reactive, can be formed as by-product of many biochemical processes, in response to electromagnetic radiation from the environment, or as oxidizing pollutants (Betteridge, 2000). Oxidative damage can occur in multiple tissues if antioxidant defenses are insufficient.

Superoxide dismutase has an important role in the protection of cells against oxygen free radicals by dismutation of a superoxide radical to hydrogen peroxide (H_2O_2) and oxygen. Zhao Y et al. (2017) reported a SOD increase after 14 days of exposure to PFOA in earthworms. From 21 to 28 days onwards, the SOD was inhibited again, indicating that PFOA causes the production of active radicals in earthworms. The increased activity of SOD prevented the organisms from experiencing oxidative damage. Concentrations of SOD decreased with increasing PFOA concentrations, which indicates that the antioxidant defense system could not tolerate high ROS concentrations, consequently leading to cell dysfunction. Xu et al. (2013) also reported that SOD activities were first activated and later inhibited in earthworms exposed to PFOS. They also report that the SOD activity was induced with increasing PFOS concentrations.

The main metabolite of the SOD process is H_2O_2, which is cytotoxic and further removed by CAT and peroxidase (POD). Catalase protects cells from damage by converting H_2O_2 to water and oxidizing it to molecular oxygen (Zamocky et al., 2008), whereas POD catalyzes the oxidation of H_2O_2 (Zhao Z et al., 2017). Both Xu et al. (2013) and Zhao Y et al. (2017) report a similar trend for POD as for SOD, with increased POD activity after 14 days, but a reduction after 28 days, due to increasing PFOA concentrations. Both studies also report an increased CAT activity after 14 days, indicating that earthworms could resist oxidative stress, as the increased H_2O_2 concentrations lead to a higher CAT activity. Catalase was inhibited after 28 days,

indicating that after long-term exposure to PFOA, the excessive ROS concentrations destroyed the antioxidant defense system of earthworms (Zhao Y et al. 2017).

Glutathione-S-transferase is a group of multifunctional enzymes involved in transformation and excretion of exogenous substances (Zhao Y et al., 2017). Functions of GST include e.g. the removal of ROS and the regeneration of S-thiolated protein (Sheehan et al., 2011). Zhao Y et al. (2017) reported an inhibition of GST in earthworms after 7 days exposure to PFOA, which might result from changes in enzyme synthesis and inactivation of glutathione and reduced glutathione (GSH). The GST activity was stimulated by PFOA from 14 to 28 days, but decreased hereafter, most likely due to the high concentrations of PFOA. Similarly, Xu et al. (2013) found that GSH was consumed in the PFOS-treated groups, suggesting that PFOS exposure might increase the vulnerability for oxidative stress.

The ultimate lipid peroxidation product of oxidative damage is malondialdehyde (MDA), which may cause a variety of cell damage. The MDA content in earthworms rose significantly after exposure to PFOS (Xu et al., 2013) and PFOA (Zhao Y et al., 2017), suggesting that treatment with these compounds led to an increase of ROS, which stimulated, although insufficient, the response of antioxidant defenses.

Finally, Xu et al. (2013) reported DNA damage due to oxidative stress after PFOS exposure. The ROS accumulation in tissues caused DNA damage by causing strand breaks, removing nucleotides and modifying the nucleotide bases.

Some studies have also investigated the lethality of PFAAs to earthworms (e.g. Joung et al., 2010; Xu et al., 2011). The lethality of xenobiotics is often reported in terms of lethal doses (LD/LC;lethal concentration). The LD_{50} is often used to describe the lethality of a toxic compound. The LD_{50} is the median lethal dose for 50% of the individuals in a certain population/group. Earthworms exposed to PFOS and PFOA died in a concentration dependent manner with 14-day LC_{50} values of 365 and 1000 mg/kg for PFOS and PFOA respectively (Joung et al., 2010). Xu et al. (2011) also reported the

death of earthworms exposed to PFOS and calculated a 14-day LC_{50} of 542.08 mg/kg in natural soils.

Finally, there are a few studies that reported other endpoints such as growth and behaviour. Although Joung et al. (2010) reported no behavioural changes or weight loss after PFOS and PFOA exposure in earthworms, earthworms exposed to PFOS showed a higher growth inhibition rate than those exposed to PFOA (Zheng et al., 2016). In addition, earthworms exposed to soils containing 160 mg/kg PFOS showed significant avoidance behaviour, proving that earthworms could perceive and avoid soils contaminated with higher PFOS concentrations (Xu et al., 2011).

1.6.3 Global distribution of PFASs in terrestrial invertebrates

As was reported previously, field studies on terrestrial invertebrates are very scarce. To the best of my knowledge, one study has been performed on isopods, millipedes, slugs and worms in Belgium (D'Hollander et al., 2014), one on adult Odonata in South Africa (Lesch et al., 2017) and one on earthworms in the US (Zhu and Kannan, 2019).

D'Hollander et al. (2014) reported PFOS concentrations in isopods, millipedes, slugs and worms, collected at Blokkersdijk (a site approximately 0.5 km from a fluorochemical plant in Belgium), of 497, 2570, 3090 and 2410 ng/g ww, respectively. At Galgenweel, approximately 3 km from the fluorochemical plant, the concentrations were 269, 280, 125 and 65 ng/g ww in these species. The highest median PFOS and PFOA concentrations in adult Odonata from South Africa were 16 ng/g ww and 0.89 ng/g ww, respectively (Lesch et al., 2017). Finally, Zhu and Kannan (2019) reported concentrations of multiple PFCAs in earthworms, collected at the Little Hocking well field in Ohio, USA, a site with a known historical contamination with PFASs due to a nearby fluorochemical manufacturing facility. The dominant PFCA was PFOA, with mean concentrations of 270 ng/g dw, followed by PFDoDA (200 ng/g dw; Zhu and Kannan, 2019).

1.7 Birds

Birds can play important roles as bioindicators for environmental pollution. They are relatively easy to observe, one of the best studied groups of organisms and in the focus of public interest. It is known that birds accumulate toxic chemicals (e.g. Giesy and Kannan, 2001; Holmström et al., 2005; Yoo et al., 2008), which affect parameters such as physiology, reproduction (e.g. Custer et al., 2012, 2014) and may even cause death. Environmental pollution has led to population declines and endangering of species. The most important examples of the value of birds as biomonitors include their use as accumulation indicators of pesticides and metals, based on non-destructive avian matrices, such as eggs, feathers or blood (e.g. Jaspers et al.,2004, 2006, 2007a,b, 2009, 2011; Løseth et al., 2019; Rattner et al., 2008; Svendsen et al., 2018; Van den Steen et al., 2006). Avian biomonitors have therefore been included into current research projects with the aims to indicate temporal and spatial trends in chemical pollution in terrestrial and aquatic ecosystems.

1.7.1 Toxicokinetics

There are only a few studies on kinetics of PFASs in birds and most studies focus on PFOS as a target analyte (Newsted et al., 2006; Tarazona et al., 2015; Yeung et al., 2009; Yoo et al., 2009) due to its higher retention times in the body than other perfluorinated anions such as PFOA (Yoo et al., 2009), which can be explained by a slower urinary excretion (Harada et al., 2005; Kowalczyk et al., 2012) and the recirculation of PFOS via the enterohepatic system (Lau et al., 2004).

Most studies on toxicokinetics in birds use the domestic chicken (*Gallus gallus domesticus*) as a model species (Tarazona et al., 2015; Yeung et al., 2009; Yoo et al., 2009). In all these studies, PFOS concentrations in serum and organs such as liver, kidney and brain, increased during the exposure period. Tarazona et al. (2015) described the daily uptake model in adult chickens, which were exposed to relatively low PFOS concentrations through their diet, as a pseudo first-order kinetics model, which means that the rate constant is only dependent on one reactant instead of multiple reactants, which is usually the case in dynamic biochemical processes.

The PFOS concentrations in serum and livers of juvenile mallard (*Anas platyrhynchos*) and northern bobwhite quail (*Colinus virginianus*) increased after a 22-day dietary exposure (Newsted et al., 2006). The accumulation of PFOS and PFDA occurred at a much higher rate than the accumulation of PFOA in dietary exposed one-day-old male chickens (Yeung et al., 2009).

The dissipation serum half-life and elimination rates in domestic chickens differ with increasing doses (Newsted et al., 2006; Tarazona et al., 2015; Yeung et al., 2009; Yoo et al., 2009). Tarazona et al. (2015) reported a first-order dissipation serum half-life of 230 days. A decreased PFOS concentration in serum and liver was also observed in juvenile mallard and quail, with exception for the quail serum concentrations, throughout the post-exposure recovery period (Newsted et al., 2006). The PFOS half-lives in mallard blood serum and liver were estimated at 6.86 and 17.5 days. The half-life in quail liver was estimated at 12.8 days (Newsted et al., 2006). Similarly, Yeung et al. (2009) observed dose-dependent half-lives of 15 and 17 days for PFOS, 11 and 16 days for PFDA and 3.9 days for PFOA in both the low and high dose groups. The lower half-lives of PFOA might be explained by the higher elimination rate constants of PFOA compared to PFOS. Yoo et al. (2009) reported a much higher elimination rate for PFOA than for PFOS in juvenile chickens that were exposed through subcutaneous implantation. The depuration half-lives for PFOA and PFOS were 4.6 and 125 days and the PFOS concentrations in organs (kidney, liver and brain) all decreased at a rate that fits a first-order kinetic model. The decreased concentrations during the depuration periods, are most likely the result of excretion, binding to non-exchangeable tissues and growth dilution (Newsted et al., 2006; Tarazona et al., 2015; Yoo et al., 2009).

1.7.2 (Sub-)lethal effects of PFASs on birds

Many potential health effects of PFASs have been frequently studied in mammals (e.g. Foresta et al., 2018; Hoff et al., 2004; Song et al., 2018). However, studies on birds are often scarce and most studies target different endpoints. Therefore, there is a relatively poor understanding of the toxicological effects of PFASs on birds. Below I give a brief summary of most frequently studied groups of endpoints.

1.7.2.1 Peroxisome proliferation and cytotoxicity

Some PFASs are known peroxisome proliferators. Peroxisome proliferator-activated receptors (PPARs) are nuclear receptor proteins that play essential roles in the regulation of multiple cellular processes such as development and metabolism of carbohydrates, lipids and proteins. Both PFOS and PFOA are known to activate peroxisome proliferator activated receptor alpha (PPARα), a ligand-dependent transcription factor of major importance for the regulation of lipid metabolism in the liver (Rakhshandehroo et al., 2007), in mammals (Ishibashi et al., 2008; Shipley et al., 2004). The activation of PPARα, promotes e.g. the uptake and catabolism of fatty acids. In birds, there are indications that PFOA and PFOS do not activate PPAR pathways in chicken eggs and embryos (Mattsson et al., 2015; Stromqvist et al., 2012).

Peroxisome proliferation can affect the activity of the cytochrome P450 (CYP) system (Hickey et al., 2009). These CYPs are mono-oxygenase enzymes that play a key role in the transformation of lipophilic compounds to more soluble derivatives (Hickey et al., 2009; Watanabe et al., 2009). Via a PPARα-independent action, PFOS increased CYP1A4 mRNA expressions in a concentration-dependent manner in chicken embryo hepatocytes (CEHs) (Watanabe et al., 2009). Hickey et al. (2009) observed an induction of CYP1A4/5 mRNA by PFHxS, PFPeA and PFHxA in CEHs. The effects of PFHxA were concentration-dependent. Long-chained PFASs with eight or more C atoms upregulated liver fatty acid-binding protein (L-FABP), a transport protein, in CEHs (Hickey et al., 2009). The induction of this CYP has been associated with various toxic syndromes, such as tumor promotion and immune dysfunction (Rifkind et al., 1994). Similarly, dietary exposure of cormorants to PFNA upregulated CYP1A4 mRNA levels (Nakayama et al., 2008).

1.7.2.2 Neurotoxicity

Neurotoxic effects of PFOS have been frequently studied in mammals (mainly mice). Neonatal PFOS exposure in mice resulted in a decreased habituation and hyperactivity in adulthood (Johansson et al., 2008) and increased norepinephrine, a chemical that functions as a hormone and neurotransmitter, concentrations have been reported in

rats, suggesting that PFOS can modulate the neuroendocrine system of rats (Austin et al., 2003). However, data on avian species is scarce. Peden-Adams et al. (2009) reported a higher frequency of brain asymmetry after *in ovo* exposure to PFOS in white leghorn chickens (*G. gallus domesticus*). Brain asymmetry has been associated with neurological disorders, such as dyslexia and autism, in children (Herbert et al., 2005; Leonard et al., 2008), showing the potential relation of PFASs exposure to altered learning and other behavioural and neurotoxic endpoints.

1.7.2.3 Immune response

Studies on effects of PFASs on immune systems of birds are also scarce. The immune system is often a target for xenobiotics and studies on other animals have shown that the immune function may be affected by PFASs (e.g. Peden-Adams et al., 2008). The PFOS concentrations significantly increased the plasma lysozyme activity, a marker of pro-inflammatory responses which also has antibacterial functions (Burton et al., 2002), in white leghorn chicks after *in ovo* exposure (Peden-Adams et al., 2009). The total sheep red blood cell (SRBC)-specific immunoglobulin (IgM + IgY) was significantly decreased at all treatment concentrations, indicating a decreased humoral immunity (Peden-Adams et al., 2009). Nevertheless, Sletten et al. (2016) reported no significant relationships between PFAS concentrations and plasma IgY levels in white-tailed eagle (*Haliaeetus albicilla*) nestlings. Smits and Nain (2013) reported a reduced T-cell mediated response in Japanese quails (*Coturnix coturnix japonica*) orally exposed to PFOA. However, the PFOA exposure did not affect antibody mediated or innate immunity and thus no increased morbidity or mortality after *Escherichia coli* infection.

1.7.2.4 Oxidative damage

Cells involved in the immune system are vulnerable targets to oxidative damage produced by xenobiotics (Monaghan et al., 2009). Studying the oxidative status of individuals is an important part of toxicological studies as organisms might need to use dietary antioxidants to deal with oxidative stress OS. In humans, PFOA is considered to be potentially carcinogenic as DNA damage secondary to OS could result in cancer (Tsuda, 2016). However, little is known on effects of PFASs on OS in birds. A study on

the transcriptional response of chicken hepatocytes exposed to PFOS pointed to OS as a possible cause of gene alteration (O'Brien et al., 2011). High blood levels of protein damage have been associated with high plasma concentrations of long-chained PFASs, such as PFDoDA, PFTrDA and PFTeDA, in Arctic black-legged kittiwakes (*Rissa tridactyla*) (Constantini et al., 2019). Additionally, the non-enzymatic antioxidant capacity (vitamins and carotenoids) of these birds was negatively associated with higher plasma concentrations of PFUnDA, PFTeDA and PFOS. Similarly, an altered transcriptional response of genes, involved in the antioxidant system, was observed in wild common cormorants (*Phalacrocorax carbo*) livers (Nakayama et al., 2008). However, not all studies have found relationships between PFAS concentrations and the activity of the antioxidant defense system. For example, PFAS concentrations in the liver of tree swallows (*Tachycineta bicolor*) were not associated with OS parameters (Custer et al., 2017) and Sletten et al. (2016) did not observe any relationship between PFAS concentrations and SOD in white-tailed eagle nestlings.

1.7.2.5 Reproduction and development

Only three field studies have studied the relationships between PFOS concentrations and hatching success. All of these studies were conducted in tree swallows. Custer et al. (2012) reported negative associations between PFOS concentrations starting at 150 ng/g ww in eggs and the hatching success of the remaining eggs. At PFOS concentrations of 283 ng/g ww in eggs, a 20% reduction in hatching success was observed (Custer et al., 2014). PFOS exposure was also significantly associated with embryo death in tree swallows (Custer et al., 2014). On the contrary, Custer et al. (2019) reported no demonstrable effects of PFASs exposure on reproduction in tree swallows nesting at Clarks Marsh, USA.

In chicken, hatching rates were not affected after *in ovo* exposure to PFOS under laboratory conditions (Peden-Adams et al., 2009). However, other studies have observed reproductive dysfunction after *in ovo* exposure to PFHxS, PFOS and PFOA (Cassone et al., 2012; Molina et al., 2006; Yanai et al., 2008). The hatching success was reduced by 20% and 63% after injection of 5000 ng/g ww PFOA and 38,000 ng/g ww

PFHxS (Cassone et al., 2012; Yanai et al, 2008). In addition, *in ovo* PFHxS, PFOS and PFOA exposure has led to a reduction of body weight, tarsus length and wing length (Cassone et al., 2012; Peden-Adams et al., 2009). Treatment-related mortalities or effects on body weight and reproductive parameters were not observed in northern bobwhite quail exposed to PFBS through diet (Newsted et al., 2008). Similarly, no effects of PFOS on body weight and reproductive performance were reported in mallard ducks (Newsted et al., 2007). Briels et al. (2018) recently reported that exposure to PFOS and its alternative F-53B decreased the heart rate of avian embryos before hatching. Additionally, F-53B significantly increased the liver mass of hatchlings.

1.7.2.6 Lethality

Lethality of PFASs in birds is still poorly understood and data is scarce. The LD_{50}s of PFOS were calculated over a 5-day period in juvenile mallards and quail based on average daily intake values and were 750 mg PFOS/kg body weight (bw) and 305 mg PFOS/kg bw (Newsted et al., 2006). In white leghorn chicken eggs, the PFOS LD_{50} was determined in eggs and was 4.9 μg/g ww (Molina et al., 2006).

1.7.3 PFASs in bird tissues

The accumulation and distribution of PFASs have been studied in many bird tissues. The majority of the studies has been performed on eggs (e.g. Ahrens et al., 2011c; Bouwman et al., 2015; Braune and Letcher, 2013; Lopez-Antia et al., 2017; Vicente et al., 2012), blood (e.g. Gebbink and Letcher, 2012; Rubarth et al., 2011) or liver (e.g. Chu et al., 2015; Gebbink and Letcher, 2012; Olivero-Verbel et al., 2006; Rubarth et al., 2011).

However, there are also studies that reported PFAS concentrations in the kidney (e.g. Olivero-Verbel et al., 2006; Rubarth et al., 2011), adipose tissue (e.g. Chu et al., 2015; Gebbink and Letcher, 2012;), brain (e.g. Gebbink and Letcher, 2012; Olivero-Verbel et al., 2006; Rubarth et al., 2011), feathers (e.g. Gómez-Ramírez et al., 2017; Herzke et al., 2011; Jaspers et al., 2013; Li Y. et al., 2017; Løseth et al., 2019; Meyer et al., 2009), gall bladder (e.g. Rubarth et al., 2011), heart (e.g. Olivero-Verbel et al., 2006; Rubarth et al., 2011), preen gland (Jaspers et al., 2013), lung (e.g. Olivero-Verbel et al., 2006;

Rubarth et al., 2011), muscle tissue (e.g. Chu et al., 2015; Gebbink and Letcher, 2012; Olivero-Verbel et al., 2006; Rubarth et al., 2011) and spleen (e.g. Olivero-Verbel et al., 2006; Rubarth et al., 2011).

Studies that compare PFAS concentrations in different organs and tissues of birds are rather scarce. In general, the highest PFAS concentrations have been observed in protein-rich tissues, such as the liver, whereas the lowest are observed in muscle tissue. This is most likely (partially) the result of the high affinity for protein binding of PFASs (as will be discussed below).

Rubarth et al. (2011) reported the highest PFAS concentrations in the liver and the lowest in the fatty and muscle tissue of red-throated divers (*Gavia stellata*). Similar results were observed in pelicans (*Pelecanus occidentalis*), where PFOS concentrations were the highest in the spleen and liver and the lowest in the muscle (Olivero-Verbel et al., 2006). Liver PFOS concentrations were also higher than those in feathers of grey heron (*Ardea cinerea*), Eurasian sparrowhawk (*Accipiter nisus*) and herring gulls (*Larus argentatus*), whereas the opposite was the case for the Eurasian magpie (*Pica pica*) and the Eurasian collared dove (*Streptopelia decaocto*) (Meyer et al., 2009). The liver PFSA concentrations in herring gulls from the Great Lakes were lower than those in adipose tissue, but still higher than all other tissues. The lowest PFSA concentrations were detected in the red blood cells (RBCs) and the brain. The results for PFCAs were different, as the highest PFCA concentrations were detected in the brain and plasma and the lowest in the RBCs, adipose and muscle tissue (Gebbink and Letcher, 2012). The higher concentrations in blood plasma compared to RBCs is most likely also the result of the affinity for proteins, as blood plasma is known to contain several proteins such as albumin and sex-hormone binding globulins (Chen and Guo, 2009; Jones et al., 2003). In Belgian barn owls (*Tyto alba*), the PFOS concentrations were the highest in the preen oil, followed by the liver, and the lowest in muscle tissue and tail feathers. The PFOA concentrations were, although likely due to external contamination, the highest in tail feathers, followed by liver and the lowest in the muscle tissue. The PFHxS

concentrations were also the highest in the liver and preen oil (Jaspers et al., 2013). The PFOS concentrations in glaucous gulls (*Larus hyperboreus*) were the highest in plasma, followed by the liver and eggs and the lowest in the brain. This pattern was also observed for the PFCAs (Verreault et al., 2005). Yoo et al. (2009) reported the highest PFOA concentrations in kidney, then liver and then brain, of domestic chickens after subcutaneous exposure. The PFOS concentrations were highest in liver, followed by kidney and brain.

Bird eggs have been used in numerous biomonitoring studies on PFASs in birds on a global scale (e.g. Gebbink and Letcher, 2012; Giesy and Kannan, 2001; Holmström et al., 2005; Miller et al., 2015; Yoo et al., 2008). Nevertheless, most of these studies target aquatic bird species, and only a very few of these studies have focused on terrestrial birds (Ahrens et al., 2011c; Custer et al., 2012; Holmström et al., 2010; Lopez-Antia et al., 2017; Rüdel et al., 2011; Yoo et al., 2008). Similarly, the extent of PFAS contamination in blood (plasma) of terrestrial bird species is poorly understood (Custer et al., 2012; Dauwe et al., 2007). To the best of my knowledge, in total only seven studies have been performed on PFASs in feathers (Gómez-Ramírez et al., 2017; Herzke et al., 2011; Jaspers et al., 2013; Li Y. et al., 2017; Løseth et al., 2019; Meyer et al., 2009; Sun et al., 2019). The majority of these studies have been performed on raptors such as the white tailed eagle (Gómez-Ramírez et al., 2017; Herzke et al., 2011; Løseth et al., 2019; Sun et al., 2019), barn owl (Jaspers et al., 2013) and *Accipiter sp*. (Li Y et al., 2017; Meyer et al., 2009), including the Eurasian sparrowhawk (Meyer et al., 2009). In addition, Meyer et al. (2009) also studied feathers of grey heron, herring gull, Eurasian magpie and Eurasian collared dove. This lack of knowledge on the terrestrial environment shows that there is a high need to monitor PFASs in terrestrial bird species.

1.8 Legislation and regulation

The global distribution and potential effects of PFASs have resulted in a global concern on these chemical since the late 1990s, especially after evidence had accumulated that PFOS and PFOA were not only ubiquitous in various biological and environmental matrices but also highly resistant and able to biomagnify (Giesy and Kannan, 2001). As a result of their global presence, the 3M company, the major PFASs manufacturer, phased-out the production of PFOS and related products (UNECE, 2006).

Within the European Union (EU) the main focus on legislation was on PFOS and its derivatives. PFOS is classified under REACH (registration, evaluation, authorization and restriction of chemicals) as a persistent, bioaccumulative and toxic (PBT) substance. In 2006, PFOS was banned in products. However, exemptions were made for certain industrial applications until PFOS was added to Annex B of the Stockholm Convention on POPs in 2009. This meant that measures had to be taken to restrict the production and use of PFOS. Hereafter there are still some exemptions for applications, e.g. photo imaging, aviation hydraulic fluids, fire fighting foam, etc., as sometimes no alternatives are present (Stockholm Convention, 2008). Very recently (in May 2019), PFOA, its salts and PFOA-related products were included in Annex A with specific excemptions.

Since 2013, PFUnDA, PFDoDA, PFTrDA and PFTeDA have been identified as chemicals of high concern (ECHA, 2017). Furthermore, PFHxS has been proposed for listing under the Stockholm Convention and is currently still under review.

After the phase-out and regulatory measures, PFAA substitutes have been developed and used as less bioacumulative, but still persistent, alternatives. For example, PFOA was used in the production of Teflon until 2012. Hereafter, PFOA has been replaced by the GenX technology (by Chemours/DuPont), using hexafluoropropylene oxide dimer acid

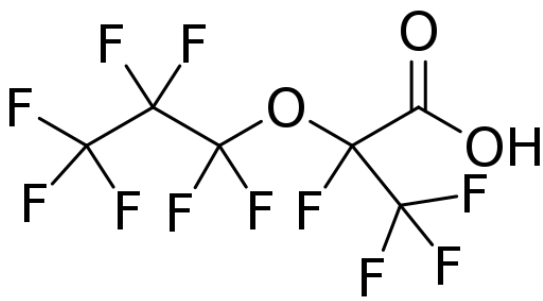


Figure 1.10 Chemical structure of HFPO-DA (GenX)

(HFPO-DA; Fig. 1.10). Due to the similarities between HFPO-DA and PFOA, risk limits have been determined by the Dutch National Institute for Public Health and the Environment (RIVM) for groundwater and soil (Rutgers et al., 2019). Other important alternatives, are dodecafluoro-3H-4,8-dioxanoate (ADONA; 3M) and a combination of 9-chlorohexadecafluoro-3-oxanonane-1-sulfonate and 11-chlororeicosafluoro-3-oxaundecane-1-sulfonic acid (F-53B).

To the best of my knowledge, no regulation or legislation of PFASs is present in Belgium specifically. The Belgian regulations are the same as the European ones and previous projects from e.g. the European Food Safety Authority (EFSA) showed that there was no exceedance of health risk guidelines for PFOS and PFOA in food items (EFSA 2012). Recently, the European Food Safety Authority (EFSA) evaluated the risks to human health related to the presence of PFOS and PFOA in food items. Based on this study, the tolerable weakly intake values were revised at 13 ng/kg body weight (bw) per week for PFOS and 6 ng/kg bw per week for PFOA (EFSA, 2018).

1.9 Aims and hypotheses

The lack of knowledge on the sorption of PFAAs to the soil (and how various sorbent properties interact to determine the binding of PFAAs), the PFAA concentrations in soils and the following bio-accumulation in terrestrial invertebrates and birds, including their potentially harmful effects on reproduction and oxidative stress, have led to the main objective of this research.

The main objective of this research was to study the exposure of PFAAs on terrestrial invertebrates (isopods) and songbirds (great tits and blue tits) along a distance gradient from a well-known fluorochemical hotspot and to determine their accumulation and possible effects on reproduction and oxidative status. To prevent the birds from being sacrificed, the focus was on eggs, blood plasma and feathers.

In order to understand the exposure pathways of PFAAs to invertebrates and songbirds and to predict potential health effects, we investigated the transfer of PFAAs between soil, invertebrates and songbirds. Furthermore, we looked at the influence of soil

physicochemical properties on the sorption of PFAAs to the soil and thus bioavailability. In addition, we examined the possibility of using an invertebrate species as bio-indicator for PFAA concentrations in songbird eggs. As females potentially deposit PFAA body burdens in their eggs, we examined the variation throughout the laying-sequence of PFAA concentrations in entire clutches along the distance gradient in great tits.

To realize the different aims of this research, four main hypotheses were formulated (Table 1.5). These hypotheses were investigated in different chapters. Hypotheses 1, 2 and 3 are mainly focused on accumulation and factors that might affect this, whereas hypothesis 4 is focused on the possible health effects for songbirds.

Table 1.5 The four main hypotheses of the current research, together with the chapter in which they are investigated.

Nr.	Hypothesis	Studied in
1	PFAAs present in the environment along a pollution gradient accumulate in the terrestrial foodchain and decrease with increasing distance from a fluorochemical hotspot	Chapters 3 - 9
2	Soil physicochemical properties play a key role in the sorption, distribution and bioavailability of PFAS	Chapters 3 and 4
3	Non-destructive sampling can be used to monitor environmental PFAA concentrations	Chapters 5 - 9
4	Accumulated concentrations of PFAAs under field condition in songbirds are related to toxic effects	Chapters 8 and 9

1.10 Study area and species

1.10.1 Study area

This research was conducted at multiple sites, along a distance gradient from an active 3M fluorochemical plant in Antwerp. The sampling sites are all illustrated in Figure 1.11.

The 3M site in Antwerp has been a focus area for the biomonitoring of PFASs in wildlife (Dauwe et al., 2007; D'Hollander et al., 2014; Hoff et al., 2005; Lopez-Antia et al., 2017). It is a known PFAS-hotspot and the concentrations detected in various biological matrices are among the highest concentrations ever found in wildlife. The 3M site contains the factory (mainly the western part of the site) and a small forest area with some open sandy spaces to the east. To the south there is a small forest between the 3M company and the E34 highway. The vegetation is dominated by deciduous forest but there are some conifers along the sides of the company terrain.

Starting at 3M, a distance gradient in the same direction was established, in order to limit the influence of wind direction on the PFAAs distribution. The selected sites included Vlietbos, Middenvijver-Rot, Burchtse Weel and Fort IV (Fort 4).

Vlietbos (approximately 1 km SE of 3M) is a sandy area that contains open spaces (two small lakes or dry sandy areas) and forest areas, which are primarily dominated by birch (*Betula sp.*) and willow (*Salix sp.*). The western part of Vlietbos is mainly dominated by the Canadian poplar (*Populus x canadensis*). Vlietbos has been included in previous studies on PFAA pollution in great tits (Dauwe et al., 2007; Lopez-Antia et al., 2017).

Middenvijver-Rot (shortly Rot; 2.3 km ESE from 3M) is an area that is connected to Vlietbos and is characterized by open pools, willows and a mixture of both deciduous and coniferous forest. The soil is primarily sandy, however, some parts in the center of Rot are more clayish. It is an important breeding site for birds, but also mammals and amphibians.

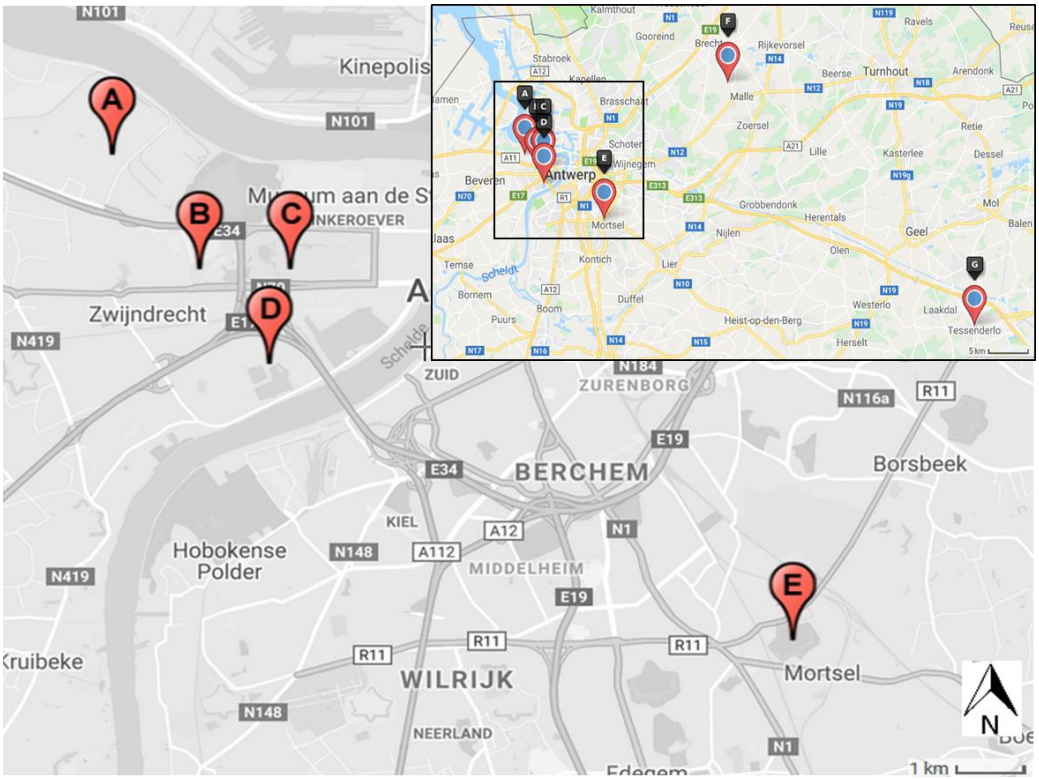


Figure 1.11 Overview of the study areas of this research. A = 3M; B = Vlietbos; C = Middenvijver-Rot (Rot); D = Burchtse Weel; E = Fort IV (Fort 4); F = Westmalle; G = Tessenderlo. Map created with Google Maps.

Burchtse Weel (3 km SE from 3M) was formed by a breach of a dyke. Hereafter the pool got enlarged and deepened. Nowadays the pond is a recreational tidal marsh area. The sides of this pond are characterized by deciduous forest dominated by willow, oak (*Quercus sp.*) and alder (*Alnus sp.*) and both the forests as well as the pond are of major importance for breeding birds. Burchtse Weel was included in a study on PFAA pollution in great tit eggs (Lopez-Antia et al., 2017).

Fort IV in Mortsel (11 km SE from 3M) is one of the eight forts near Antwerp. It is almost completely surrounded by water. The vegetation is dominated by deciduous forest or open grassland (used for recreational purposes) and a small part is a restricted nature conservation area. This site was selected as nestboxes, used in previous studies on metal pollution (e.g. Geens et al., 2010; Vermeulen et al., 2015) were already present

at the site. Fort IV was the site furthest away from both 3M as well as the main metal pollution source (UMICORE).

An earlier study, already performed in 2011 (chapter 5 of this thesis) used Tessenderlo as reference site as at that moment it was unclear how the PFAAs were spatially distributed in Flanders. As PFAAs were detected in Tessenderlo, and hence it was no reference site, we decided to restrict the gradient from 3M to Fort IV in most studies. In chapter 3 we used an organic farm as reference site. Some measurements in chicken eggs from this site (D'Hollander et al., 2011) showed very low PFAA contamination and it was therefore expected that soil concentrations would also be low.

Tessenderlo (18000 inhabitants) and Westmalle (15000 inhabitants) are cities/towns approximately 70 km (SE) and 25 km (NE), respectively, from Antwerp. The east part of Tessenderlo is a highly industrialized area, containing multiple chemical factories, such as Tessenderlo Chemie. Westmalle is surrounded by mainly agricultural lands, including some organic farmlands.

Table 1.6 illustrated the locations used in each chapter of this thesis.

Table 1.6. Locations used in each chapter of this thesis.

Chapter	3M	Vlietbos	Rot	Burchtse-Weel	Fort IV	Tessenderlo	Westmalle
3	X	X	X	X			X
4	X	X	X	X	X		
5	X	X	X			X	
6	X	X	X	X	X		
7	X	X	X	X	X		
8	X	X	X	X	X		
9	X	X	X	X	X		

1.10.2 Study species

We used multiple species in our research on the toxicity of PFASs in the terrestrial environment.

1.10.2.1 Invertebrates - isopods

As we wanted to investigate the accumulation of PFAAs in the terrestrial foodchain, invertebrates were collected. In order to investigate the potential relationships between PFAA concentrations in prey organisms and their predators, we were aiming to collect invertebrates that were part of the natural diets of great tits (our main species of interest for this thesis), such as caterpillars (see 1.10.2.2 for more details on the diet of great tits). Unfortunately we could not collect enough caterpillars during the breeding season in the close vicinity of the nestboxes of great tits and therefore we selected isopods, which were commonly found on the ground and trees. Although they are probably not part of the natural diet of great tits (1.10.2.2), we planned on using isopods to give an indication of concentrations we could expect in invertebrates in general and relate that to concentrations in great tits.

Terrestrial isopods mainly feed on plant litter from plants growing in the surrounding habitat, so they should give a good representation of local PFAA contamination. In addition, terrestrial isopods have been proven useful as biological indicators of environmental pollution (e.g. Dallinger et al., 1992; Drobne et al., 1997; Stroomberg et al., 2009; Van Brummelen et al., 1996).

1.10.2.2 Great tits

As a main species of interest, we selected great tits. The great tit is a passerine bird, which has been used frequently in ecotoxicological studies (e.g. Brahmia et al., 2013; Dauwe et al., 1999, 2002, 2004, 2005a; Eens et al., 1999, Eeva and Lehikoinen 1995, 1996; Eeva et al., 1998; Markowski et al., 2014; Rainio et al., 2013; Van den Steen et al., 2006, 2009b).

Great tits are known to feed on the ground during February and March, but during the breeding season they mainly feed in the canopy (e.g. Gibb, 1954; Betts, 1955; Royama,

1970). They are generalists, which feed primarily on caterpillars during the breeding season and warmer months (Grzędzicka, 2018; Naef-Daenzer and Keller, 1999; Rytkönen et al., 2018). Additionally, the low proportion of caterpillars in their diet is a characteristic for suboptimal habits (e.g. Blondel et al., 1991; Riddington and Gosler, 1995). However, individuals within a population may often specialize in the exploration of specific food sources, indicating that their diet may be variable between years and forests (Pagani-Núñez et al., 2016). Besides caterpillars, great tits are known to feed on spiders and to a small extent on Hymenoptera, Coleoptera and Gastropoda (Grzędzicka, 2018). During the winter, there is a deficiency in arthropods, caused by low air temperatures or snow covering (Robinson et al., 2007). In addition, they feed on moths, beetles and dipterans (Vel'ký et al., 2011). Great tits supplement the invertebrate part of their diet by consuming plant materials such as seeds, nuts and buds (Chamberlain et al., 2007).

Great tits are known to nest and sleep (during winter) in man-made nestboxes. As a result, they can be easily caught to collect samples. In addition, their nest-building process and reproduction can be studied relatively easily. Great tits are also abundant, which makes it relatively easy to obtain sufficient samples. Finally, great tits are known to lay remarkably large clutches, which can go up to 12 eggs. Therefore, the influence of the collection of one egg on the reproductive success is rather limited compared to species that lay fewer eggs. In addition, studying the PFAA concentrations in entire clutches, gives a better overview of the variation of these concentrations among eggs within a clutch, compared to clutches with fewer eggs.

1.11 Outline

In **chapter 2**, the development and validation of a novel extraction method using negative ion electrospray (ES (-)) operating on a liquid chromatography tandem mass spectrometer (LC-MS/MS) for the extraction of multiple PFCAs and PFSAs from both environmental and biological matrices was studied. We evaluated the recovery, sensitivity and reliability of the novel method with an existing and frequently used method. Additionally, we validated the use of internal-standards (ISTDs) to improve

the robustness and reliability by comparing different quantification methods. Finally, we investigated which ISTD was most suitable for (and should thus be used in) the quantification of analytes, for which no corresponding ISTD were available.

In **chapter 3**, we investigated the vertical distribution of multiple PFAAs in soils from five sites, representing a distance gradient from 3M. More specifically, we examined the associations between multiple physicochemical properties of the soil (TOC, clay content, pH and temperature) and the PFAA concentrations to reveal important factors in the sorption of PFAAs to the soil in these sites. Finally, we tested relationships between PFAA concentrations in the top soil with soil respiration and soil microbial parameters, such as microbial activity and microbial biomass.

The aim in **chapter 4** was to determine the concentrations of multiple PFAAs in the soil and isopods along a distance gradient from the 3M fluorochemical plant and to investigate whether the concentrations in these matrices could be used as an indicator for the PFAA concentrations in the eggs of great tits, which were collected at the same time and locations. In addition, the role of physicochemical soil properties (total organic carbon (TOC), and clay content) on the relationship between PFAA concentrations in soils and isopods were investigated.

The PFAA concentrations and composition profile of 12 PFAAs were determined in the eggs of great tits (*Parus major*), collected along a distance gradient (1 to 70 km) from the 3M fluorochemical plant, and described in **chapter 5**.

The variation of different PFAAs within and among clutches of great tits, possible laying sequence associations between egg parameters and PFAA concentrations and possible relationships of PFAAs among eggs from the same clutch were investigated in **chapter 6**. In addition, potential implications for future biomonitoring studies were assessed.

We investigated the PFAA concentrations and profile in great tit feathers in **chapter 7**. Furthermore, we examined whether tail feathers from adult great tits present a good matrix to monitor internal PFAA concentrations in blood plasma (reported in chapter 9).

The high PFAA concentrations reported in the study area gave rise to questions on potential effects of PFAAs on songbirds along the same site. In **chapter 8**, the influence of high PFAA concentrations on reproduction of great tits was examined.

In **chapter 9** the plasma concentrations and composition profile of multiple PFAAs were examined in nestling and adult great tits, settled along a distance gradient. Potentially pernicious effects of PFAAs on the birds were assessed by examining the associations between the measured PFAA concentrations and body condition and oxidative stress status. Moreover, as we sampled adult birds, their eggs (reported in chapter 8) and their nestlings, we could explore the maternal transfer of PFAAs to the offspring.

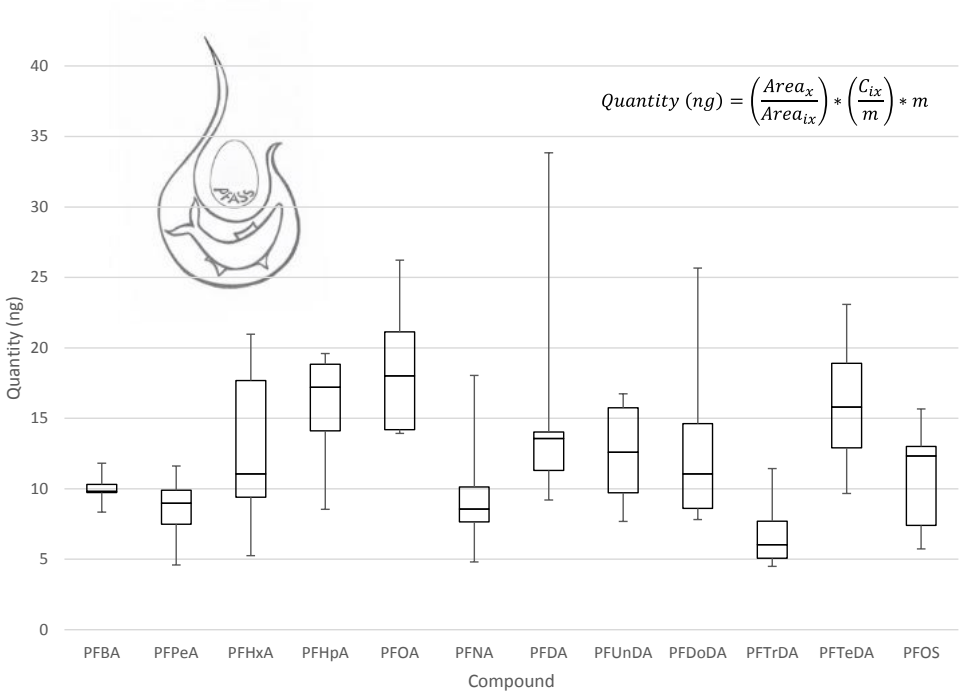
Finally, the most essential results of the present thesis are discussed in **chapter 10.** Additionally, some future perspectives and recommendations regarding future research are provided. At last, the general conclusions based on the main hypotheses of this PhD thesis are formulated in this chapter.

2. Development and validation of an extraction method for the analysis of perfluoroalkyl substances (PFASs) in environmental and biotic matrices

Based on:

Thimo Groffen, Robin Lasters, Filip Lemière, Tim Willems, Marcel Eens, Lieven Bervoets and Els Prinsen (2019a). Journal of Chromatography B 1116: 30 – 37. https://doi.org/10.1016/j.jchromb.2019.03.034

Tables were modified to fit the size of the pages. Fig. 2.4 now contains colour.



Abstract

Although long chained PFASs have been phased-out in several countries, their persistence in the environment and bioaccumulative potential cause the environmental and biotic concentrations to remain high, highlighting the need to further monitor these pollutants. Currently several methods are used for the quantification of perfluoroalkyl substances (PFASs) in biological matrices including different ways to correct for recovery losses, each with its specific pros and contras. With this paper we aim to re-evaluate current methodologies and to create an updated new analytical guideline that is applicable for both abiotic and biotic matrices. The developed LC/MS/MS method was validated and shown to be specific, selective, linear, robust and sensitive. Reliable results could still be obtained 6 days after extraction. The recoveries varied, depending on the matrix, between 1% and 100%, but nevertheless, a high accuracy was obtained even at the lowest recoveries. A reduction of sample mass could significantly increase method recoveries and therefore it is highly recommended to take less matrix. We confirmed that using the ISTD closest in terms of functional group and carbon chain length is a suitable method for the quantification of PFASs that lack a corresponding ISTD. The newly described method was, depending on the matrix, similar in terms of sensitivity and reliability compared to a frequently used method and could be used simultaneously in future monitoring studies. Therefore, we recommend to select the purification method based on the target analytes as well as the sample matrix.

2.1 Introduction

Perfluoroalkyl substances (PFASs) are chemical compounds, which have been produced and used in multiple consumer products and industrial processes, such as food packaging, firefighting foams, water and oil repellents, and waxes (Groffen et al., 2017; Kim and Oh, 2017). As a result of the widespread use of PFASs, they may end up in the environment through direct pollution or through environmental degradation of precursor compounds (Buck et al., 2011; Prevedouros et al., 2006).

Over the past decades, regulatory agencies and researchers mainly focused on long chain perfluoroalkyl carboxylic acids (PFCAs) and perfluoroalkyl sulfonic acids (PFSAs), because of their higher bio-accumulative potential (Buck et al., 2011). Although these groups comprise numerous compounds, the main attention of researchers has been on perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS). As a result of their potential toxicity to the environment and humans, the production and sale of long-chained PFASs have been phased out or banned in several countries (Groffen et al., 2017; Kim and Oh, 2017). In addition, some of these compounds have been registered as persistent organic pollutants (POPs) in 2009 (Groffen et al., 2017). Although environmental concentrations of these long-chained compounds appear to be decreasing since these measures were taken, concentrations of other PFASs are still rising (Ahrens et al., 2011c; Groffen et al., 2017; Miller et al., 2015), illustrating the importance of environmental monitoring of PFASs.

Although numerous methodologies have been developed for the determination of PFASs in different matrices, most of these studies target only one matrix (e.g. Holm et al., 2004; Kim and Oh, 2017; Mazzoni et al., 2015; Zhang et al., 2010) or focus only on either biotic or abiotic samples (e.g. Berger and Haukås, 2005; Lorenzo et al., 2015; Powley et al., 2005). Nevertheless, some of these methodologies, for example the method described by Powley et al. (2005), have been frequently used in monitoring studies either as a modification or as a full guideline (e.g. Groffen et al., 2017; Lauritzen et al., 2018; Loos et al., 2017). However, most environmental studies cover a wide range of matrices, highlighting the need for a method that works on both biotic and

abiotic samples. Nakayama et al. (in press) have also concluded this in a review on analytical techniques in aqueous, solid and biological matrices. Extraction procedures of aqueous matrices have been miniaturized by procedures such as dispersive liquid-liquid microextraction (DLLME), vortex-assisted liquid-liquid extraction (VALLE) and micro-solid phase extraction (micro-SPE), which have decreased sample volumes and amounts of extraction solvents needed. Nevertheless, the recoveries of these techniques, particularly for short-chain PFASs, is low due to ionization suppression (Nakayama et al., in press). In addition, Nakayama et al. (in press) reported that method development is needed to facilitate the analysis of PFASs exposure of wildlife and humans, including non- or less-invasive biological samples such as blood and eggs. Additionally, there are different methodologies to quantify the PFAA concentrations. Multiple studies use a linear fitted external calibration curve to quantify PFAA concentrations (e.g. Dauwe et al., 2007; Lopez-Antia et al., 2017; Meyer et al., 2009; Tao et al., 2006), whereas others use the ratio between the areas of the diagnostic ions of the labeled and unlabeled compounds (e.g. Groffen et al., 2017, 2018; Kim and Oh, 2017; Vicente et al., 2012, 2015). Therefore it should be investigated which method of quantification is most accurate and reliable and should thus be used in further monitoring studies.

Therefore, the aim of this study was to develop and validate an analytical method for measuring PFASs in both biotic and abiotic matrices, using negative ion electrospray (ES (−)) operating on a liquid chromatography tandem mass spectrometer (LC-MS/MS). The recovery, sensitivity and reliability of the newly described method were evaluated with an existing frequently used method. Additionally, the use of internal standards to improve robustness and reliability was investigated by comparing different ways of quantification.

2.2 Materials and Method

2.2.1 Chemicals and reagents

PFASs abbreviations were adapted from Buck et al. (2011). A PFASs solution (chemical purity > 98% for all PFASs), containing perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluordecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA), perfluorododecanoic acid (PFDoDA), perfluorotridecanoic acid (PFTrDA), perfluorotetradecanoic acid (PFTeDA), perfluorobutane sulfonate (PFBS), perfluorohexane sulfonate (PFHxS), perfluorooctane sulfonate (PFOS) and perfluorodecane sulfonate (PFDS) was purchased from Wellington Laboratories (Guelph, Canada). A mixture of isotopically mass-labeled PFASs (ISTD; Wellington Laboratories), with a chemical purity of >98% and isotopic purities of ≥99% or >94% per 13C or 18O respectively, was used as internal standards (Table 2.1). All solvents, including acetonitrile (ACN; LiChrosolv, Merck Chemicals, Belgium), ammonium acetate (VWR International, Belgium), ammonium hydroxide (Filter Service N.V., Belgium), and Milli-Q (18.2 mΩ; TOC: 2.0 ppb; Merck Millipore, Belgium) were HPLC grade.

2.2.2 Chemical extraction

Nine different matrices were used in this study. These samples were collected from different locations in Belgium and the Netherlands. Soil samples were collected at Vlietbos, approximately 1 km from a fluorochemical plant in Antwerp, Belgium. Additionally, at this location blood plasma and eggs of great tits (*Parus major*) and isopods were collected. Sediment was sampled in the Groot Schijn, a river in Antwerp, belonging to the Scheldt basin. Tapwater from the University of Antwerp was used in the analysis of the water samples. Chicken eggs were home-produced in the Netherlands. The chicken liver was sampled from a home raised chicken in Oud Turnhout (North of Flanders, Belgium). Finally, fish muscle tissue was taken from perch

(*Perca fluviatilis*), collected among multiple rivers in Flanders during a project of the Flemish Environment Agency (VMM) in 2017 (Teunen et al., 2018).

Homogenized samples (N = 5 for each matrix; 10 µL for blood plasma of great tits (collected at Vlietbos; approximately 1 km from a fluorochemical-plant in Antwerp, Belgium), 10 mL for tapwater and approximately 0.3 g for all other matrices (soil, sediment, chicken egg and liver, great tit egg and isopods)) were placed in 50 mL polypropylene (PP) tubes. Hereafter, the samples were spiked with 80 µL of an ISTD mixture, containing 125 pg/µL of each ISTD, and mixed thoroughly. After adding 10 mL of ACN, the samples were vortex-mixed, sonicated (3 × 10 min) and extracted overnight on a shaking plate (135 rpm) at room temperature. Subsequently, the samples were centrifuged (4 °C, 10 min, 2400 rpm, Eppendorf centrifuge 5804R) and the supernatant was transferred to a 14 mL PP tube.

Chromabond HR-XAW Solid Phase Extraction (SPE) cartridges (Application No 305200, SPE department, Macherey-Nagel, Germany, 2009) were conditioned and equilibrated with 5 mL of ACN and 5 mL of MQ water before the samples were loaded onto the columns. The cartridges were washed with 5 mL of a 25 mM ammonium acetate solution in MQ and 2 mL of ACN. PFASs were eluted with 2 × 1 mL of 2% ammonium hydroxide in ACN, stored in a 6 mL PP tube and dried completely using a rotational-vacuum-concentrator (Eppendorf concentrator 5301, Hamburg, Germany). The dried eluent was reconstituted with 200 µL of 2% ammonium hydroxide in ACN and vortex-mixed for at least 1 min. Hereafter, samples were filtrated through an Ion Chromatography Acrodisc 13 mm Syringe Filter with 0.2 µm Supor (polyethersulfone; PES) Membrane (VWR International, Leuven, Belgium) attached to a PP auto-injector vial.

2.2.3 UPLC-TQD analysis and quantification

Ultra-performance liquid chromatography-tandem ES (−) mass spectrometry (UPLC-MS/MS, ACQUITY, TQD, Waters, Milford, MA, USA) was used to analyze the PFASs. An ACQUITY BEH C18 column (2.1 × 50 mm; 1.7 µm, Waters, USA) was used to separate

the analytes. To retain any PFASs contamination originating from the system, an ACQUITY BEH C18 pre-column (2.1 × 30 mm; 1.7 µm, Waters, USA) was inserted between the solvent mixer and the injector. The mobile phase solvents were 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B), with a flow rate of 450 µL/min and an injection volume of 10 µL. The gradient started at 65% A, decreased to 0% A in 3.4 min and returned to 65% A at 4.7 min. PFASs were identified and quantified using multiple reaction monitoring (MRM) of two diagnostic transitions per target analyte. Diagnostic transitions, cone voltages and collision energy are shown in Table 2.1. The first product ions of PFCAs were the result of decarboxylation ($-CO_2$), whereas the second product ion was the result of a removal of the fluorinated carbon atoms ($-n*CF_2$), where n was selected based on the most optimal product ion spectrum. For the PFSAs, the first product ion (m/z = 80) was sulfur trioxide (SO_3-), whereas the second product ion was fluorosulfuric acid (SO_3F-).To validate the MRM transition for PFBA and PFPeA we used a

higher collision energy, as no second MRM transition was detected. The use of a higher collision energy resulted in no loss of stable fragment peaks at transitions 213 > 169 (PFBA) and 263 > 219 (PFPeA), a characteristic which was selected as additional evidence for the identification of the latter compounds.

2.2.4 Method validation

Calibration curves were prepared by adding a constant amount of the ISTD ($concentration_{ix}$, hereafter C_{ix}) to different concentrations of an unlabeled PFASs mixture ($concentration_x$, hereafter C_x). Dilutions of the unlabeled PFASs mixture were performed in ACN. After logarithmic transformation, the ratio of the concentrations (C_x/C_{ix}) was plotted against the ratio of the areas of the unlabeled ($Area_x$) and labeled ($Area_{ix}$) compounds. Linearity was assessed by observing the correlation coefficient values (R^2) of these linearity plots.

The robustness of the method was analyzed by repeatedly measuring an 1:1 non-extracted solution of native and heavy-labeled standards (125 pg/µL; 5000 pg of each

PFAS) for a period of six days. The variation in area units of the peak signals was determined, for each PFAS individually, with and without correction with internal standards. As no corresponding heavy labeled internal standard was available for PFBS, PFDS, PFPeA, PFHpA, PFTrDA and PFTeDA, the area of these compounds was corrected with each of the ISTDs available for the other PFASs. We determined which ISTD resulted in the smallest variation in area units and which should therefore be used for the quantification of these PFASs. As the synthetic standard solution only takes into account the ionization efficiency, we also determined which ISTD resulted in the smallest variation in area units when considering both the ionization and the extraction efficiency, by using procedural blanks (10 mL ACN) that were also 1:1 spiked with a solution of native and heavy-labeled standards (125 pg/µL; 5000 pg of each PFAS).

To validate the quality of the extraction method, we compared the recoveries of multiple matrices obtained after using the method reported here or the method by Powley et al. (2005), which is widely accepted. Five samples of each matrix were divided into three equal parts (0.3 g per part for solid matrices, 10 µL for blood plasma and 10 mL for water); one part to test for background contamination and one part for each of the extraction methods. Samples used in the comparison of the extraction methods were spiked with 40 µL of a 1:1 (125 pg/µL; 5000 pg of each PFAS) solution of native and heavy-labeled standards. To calculate the extraction recoveries for both methods, the $Area_{ix}$ of the extracted samples were compared with the $Area_{ix}$ of a non-extracted 1:1 native:labeled solution.

To validate the importance of internal standards in the quantitation of PFASs, two common quantitation methods were compared. On the one hand, we calculated concentrations based on the ratio between the areas of the diagnostic ions of the labeled and unlabeled compounds (Groffen et al., 2017, 2018; Kim and Oh, 2017; Vicente et al., 2012, 2015) (Formula (2.1)). On the other hand, these results were compared with a calculation using a linear fitted external calibration curve (Dauwe et

al., 2007; Lopez-Antia et al., 2017; Meyer et al. 2009; Tao et al., 2006) (Formula (2.2)). Ten chicken egg samples were spiked with a 1:1 10,000 pg non-extracted solution of native and heavy-labeled standards and extracted with the method described above.

$$C_x = \left(\frac{Area_x}{Area_{ix}}\right) * \left(\frac{C_{ix}}{m}\right) \quad (2.1)$$

$$C_x = \left(\frac{C_{cal} * V_{extract}}{m}\right) * \left(\frac{mean(Area_{ical})}{Area_{ix}}\right) \quad (2.2)$$

With C_x (pg/g) and $Area_x$ the concentration and peak area of native PFASs in the sample, C_{ix} (pg) and $Area_{ix}$ the concentration and peak area of the heavy-labeled standards, C_{cal} the concentration in the extract calculated by the external calibration curve (µg/µL), $V_{extract}$ the volume of the extract (µL), $Area_{ical}$ the peak area of the calibration points from the linear fitted external calibration curve, and m the mass of the sample (g).

For both quantification methods we multiplied the C_x with the mass of the sample to obtain PFASs quantities to prevent possible variations due to slightly varying sample masses. The limits of quantification (LOQ) for the different matrices were defined as a signal-to-noise (S/N) ratio of 10. Individual LOQs are displayed in Table S2.1 for each matrix, for both the method described in this study and the method described by Powley et al. (2005).

2.2.5 Statistical analysis

Statistical analyses were performed using R 3.1.3. The level of significance was set at $p \leq .05$. All data were tested for normality and homogeneity of variances prior to further analysis. Simple linear regression functions were used to test the linearity of the calibration curves. In the comparison of calculation methods, we used t-tests to test for deviations from the spiked concentrations.

Table 2.1. MRM transitions, internal standards (ISTDs), cone voltages (V) and collision energy (eV) for the target perfluoroalkyl substances and their internal standards.

Compound	Precursor ion (m/z)	Product ion (m/z)		Cone Voltage (V)	Collision energy (eV) for diagnostic transition1	Collision energy (eV) for diagnostic transition 2	Internal standard (ISTD) used for quantification
		Diagnostic product Ion 1	**Diagnostic product Ion 2**				
PFBA	213	169	169	19	19	50	$^{13}C_4$-PFBA
PFPeA	263	219	219	15	10	45	$^{13}C_4$-PFBA
PFHxA	313	269	119	19	21	65	[1,2-$^{13}C_2$]PFHxA
PFHpA	363	319	169	24	40	30	[1,2-$^{13}C_2$]PFHxA
PFOA	413	369	169	22	13	60	[1,2,3,4-$^{13}C_4$]PFOA
PFNA	463	419	169	28	17	20	[1,2,3,4,5-$^{13}C_5$]PFNA
PFDA	513	469	219	25	29	29	[1,2-$^{13}C_2$]PFDA
PFUnDA	563	519	169	18	30	35	[1,2-$^{13}C_2$]PFUnDA
PFDoDA	613	569	319	22	21	30	[1,2-$^{13}C_2$]PFDoDA
PFTrDA	663	619	319	26	21	30	[1,2-$^{13}C_2$]PFDoDA
PFTeDA	713	669	169	28	21	21	[1,2-$^{13}C_2$]PFDoDA
PFBS	299	80	99	40	65	45	$^{18}O_2$-PFHxS
PFHxS	399	80	99	22	30	60	$^{18}O_2$-PFHxS
PFOS	499	80	99	60	58	58	[1,2,3,4-$^{13}C_4$]PFOS
PFDS	599	80	99	29	63	63	[1,2,3,4-$^{13}C_4$]PFOS

Table 2.1 (continued). MRM transitions, internal standards (ISTDs), cone voltages (V) and collision energy (eV) for the target perfluoroalkyl substances and their internal standards.

Compound	Precursor ion (m/z)	Product ion (m/z)		Cone Voltage (V)	Collision energy (eV) for diagnostic transition1	Collision energy (eV) for diagnostic transition 2	Internal standard (ISTD) used for quantification
		Diagnostic product Ion 1	**Diagnostic product Ion 2**				
$^{13}C_4$-PFBA	217	172	172	19	19	50	
[1,2-$^{13}C_2$]PFHxA	315	269	119	19	21	65	
[1,2,3,4-$^{13}C_4$]PFOA	417	372	172	22	13	60	
[1,2,3,4,5-$^{13}C_5$]PFNA	468	423	172	28	17	20	
[1,2-$^{13}C_2$]PFDA	515	470	220	25	29	29	
[1,2-$^{13}C_2$]PFUnDA	565	520	170	18	32	35	
[1,2-$^{13}C_2$]PFDoDA	615	570	320	22	21	30	
$^{18}O_2$-PFHxS	403	84	103	22	30	60	
[1,2,3,4-$^{13}C_4$]PFOS	503	80	99	60	58	58	

2.3 Results and discussion

2.3.1 Linearity

A linear regression function (Fig. 2.1) described the relationship between C_x/C_{ix} and $Area_x/Area_{ix}$ of the compounds: PFBA ($R^2 = 0.999$), PFPeA ($R^2 = 0.992$), PFHxA ($R^2 = 0.989$), PFHpA ($R^2 = 0.988$), PFOA ($R^2 = 0.997$), PFNA ($R^2 = 0.998$), PFDA ($R^2 = 0.985$), PFUnDA ($R^2 = 0.984$), PFDoDA ($R^2 = 0.985$), PFTrDA ($R^2 = 0.985$), PFTeDA ($R^2 = 0.986$), PFBS ($R^2 = 0.996$), PFHxS ($R^2 = 0.999$), PFOS ($R^2 = 0.998$), PFDS ($R^2 = 0.997$). All compounds showed a highly significant linear fit (all $p < 0.001$).

Although calibration graphs for most PFASs remained linear in a range from a C_x/C_{ix} ratio of 1:1000 to 1000:1, this was not the case for PFHpA, PFDA, PFUnDA, PFBS, PFHxS and PFDS, which all showed a smaller linear range. In cases where PFBS concentrations exceeded the internal standard with a factor >10×, or <100×, the linear fit was not guaranteed. In the latter case, samples need to be diluted to fit within the linear range of the calibration graph. For PFHxS, the linear range is even narrower, as C_x/C_{ix} ratios should be in the range 1:10–10:1. Concentrations of PFHpA, PFDA, PFUnDA and PFDS should not be lower than 100× the concentrations of their corresponding ISTDs (Fig. 2.1).

2.3.2 Robustness

In previous studies (e.g. Groffen et al., 2017, 2018; Leat et al., 2012; Verreault et al., 2005) the PFASs that have no corresponding ISTD were quantified by using the ISTD of the compound closest in terms of functional group and carbon-chain length (i.e. PFBS and PFDS were quantified by using PFHxS and PFOS, PFPeA and PFHpA by using PFHxA and PFTrDA and PFTeDA by using the ISTD of PFDoDA).

To investigate whether these ISTDs are indeed the most optimal standards in the quantification of these compounds, we determined the variation in area units of these compounds corrected with each of the ISTDs in the previously described mixture. The results of the PFSAs and PFCAs of which no ISTD is commercially available, are displayed in Figs. S2.1 and S2.2 respectively. As these figures are based on synthetic standard solutions, they only take the ionization efficiency and not the extraction

efficiency into account. In case the extraction efficiency was taken into account (Figs. S2.3 and S2.4 for PFSAs and PFCAs respectively), we recommend to use the compounds closest in terms of functional group and carbon-chain length. This means that the most suitable standard to quantify the PFSAs PFBS and PFDS would be PFHxS and PFOS. PFPeA should be quantified with PFBA, PFHpA with PFHxA and PFTrDA, PFTeDA should be quantified using the standard of PFDoDA.

The robustness of the method is illustrated in Fig. 2.2 for all target analytes, using the most suitable ISTD based on both ionization and extraction efficiency. The inter-day variation in area units was reduced drastically after correcting with ISTDs, demonstrating that reliable results can still be obtained six days after extraction.

2.3.3 Extraction recovery

Concentrations (pg ± SE), LOQs, S/N ratios (mean ± SE) and recovery ranges for each PFAS were determined for different matrices with both quantification methods and the results are displayed in Table S2.1. The two methods did not differ much in terms of concentrations, with the exception of a few matrices. Differences in recoveries, S/N ratios and LOQs were, however, striking. Recoveries of the new method varied, depending on the matrix and target analyte between 1 and 133% and were generally higher for PFCAs and abiotic matrices. Nevertheless, despite the lower recoveries, the concentrations for most compounds and matrices did not differ from the spiked amount of 5000 pg showing the accuracy and quality of the new method even at low recoveries. Calculated concentrations only exceeded the spiked value in case the LOQ of that specific compound was high and did not exceed the LOQ in the blank. Background concentrations that were <LOQ would add up with the spiked concentrations, resulting in significantly higher concentrations in the samples. Concentrations that were significantly lower than the spiked concentration, could be explained by a loss of these compounds during the extraction procedure.

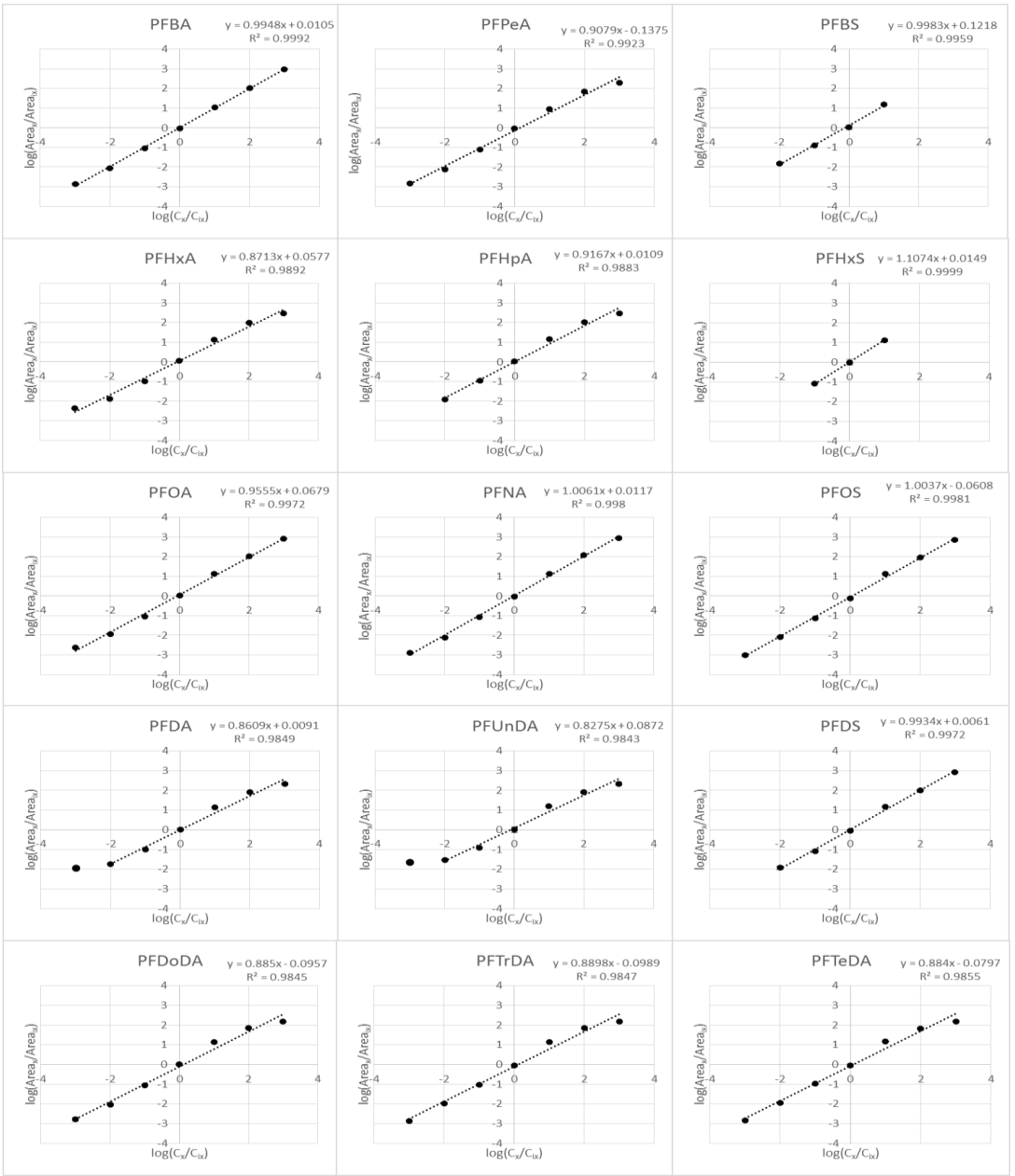


Fig. 2.1. Calibration graphs (±SE) of the 15 target analytes; 4 PFSAs (PFBS, PFHxS, PFOS and PFDS) and 11 PFCAs (PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoDA, PFTrDA and PFTeDA).

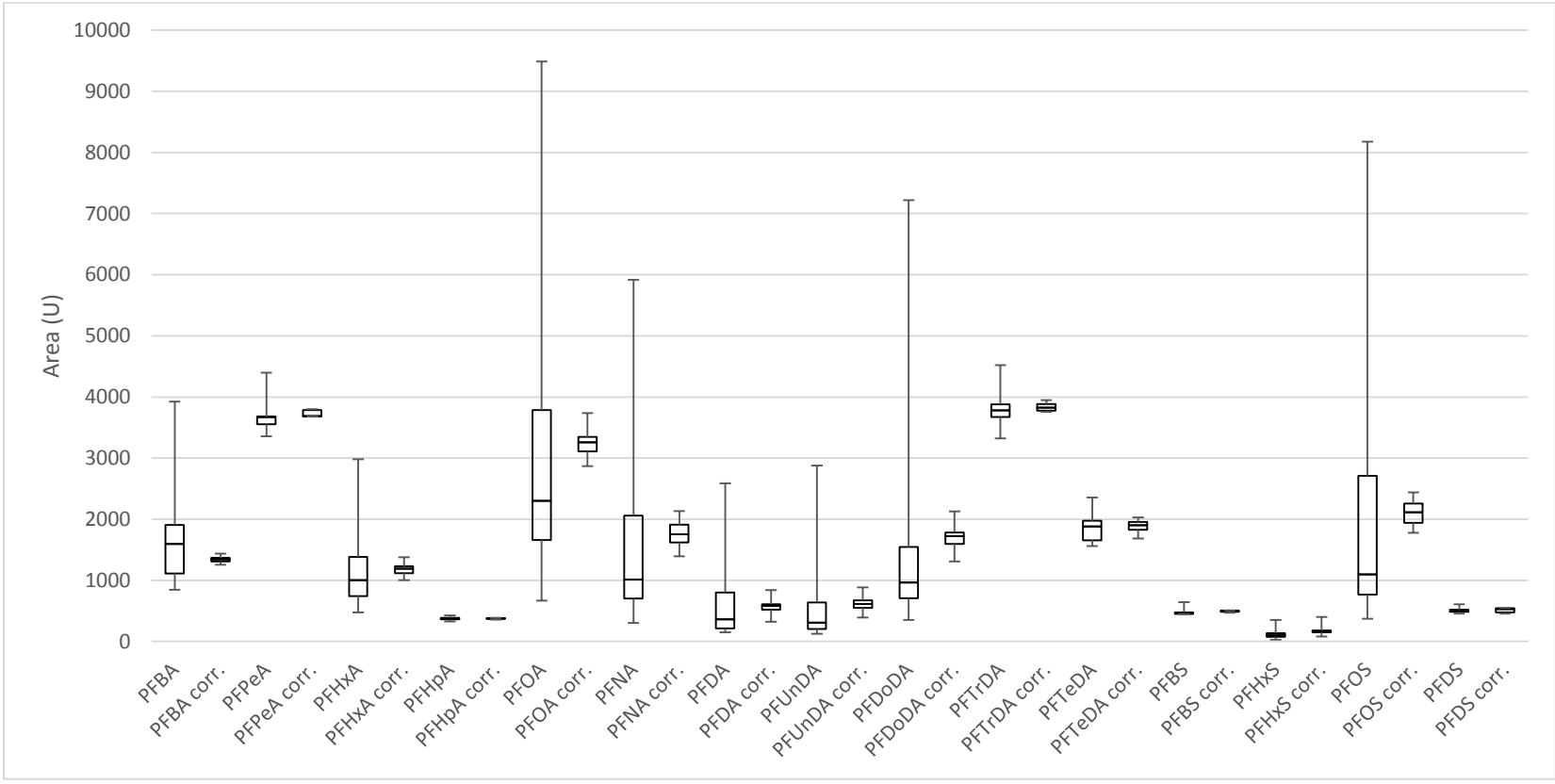


Fig. 2.2. Method robustness of all target analytes with and without correction (corr.) with the most suitable internal standards (N = 5). The variability displays the inter-day variation.

Despite the similarity in concentrations, there was a clear distinction between abiotic and biotic samples in terms of recoveries, S/N ratio, and LOQs. We observed that the method described in the present study had better recoveries in abiotic samples compared to the method described by Powley et al. (2005), whereas recoveries in biotic samples, with exception of blood plasma, were higher when using the Powley method (Table S2.1). Similar patterns were observed for S/N ratios and LOQs. In addition, recoveries of PFSAs were generally lower than those of PFCAs in the new method, whereas this was not necessarily true for the method by Powley et al. (2005). Spiked concentrations, especially for the PFSAs, were often around the LOQ. This indicates that a minor variation, due to matrix effects, could result in concentrations below the LOQ. Additionally, the recoveries of PFCAs might be better, as individual PFCA molecules might form hydrogen bonds. These bonds result in larger non-polar molecules, which dissolve better in a non-polar medium than the less non-polar PFSAs, which are present as their salts. Another factor that might cause the relatively low recoveries for biotic samples with the XAW method is the amount of sample used in the extraction. We tested the method using 0.3 g of each matrix, but higher sample mass could result in a higher matrix effect and thus lower recoveries.

Fig. 2.3 illustrates the recoveries in samples of chicken eggs in function of the mass of the sample. These results illustrate that using a lower sample mass would increase the recovery by 10–20%, depending on the compound. An increased sample mass would significantly reduce the recoveries of PFBA ($p < 0.001$), PFPeA ($p = 0.008$), PFHxA ($p = 0.005$), PFHpA ($p = 0.005$), PFHpA ($p = 0.005$), PFOA ($p = 0.015$), PFNA ($p = 0.002$), PFDA ($p < 0.001$), PFUndA ($p < 0.001$), PFDoDA ($p < 0.001$), PFTrDA ($p < 0.001$), PFTeDA ($p < 0.001$), PFOS ($p = 0.002$) and PFDS ($p = 0.002$) when using the method with the XAW-SPE cartridges. Therefore, it is recommended to take as less sample as possible in order to improve recoveries.

2.3.4 Comparison with other extraction methods

Compared to other PFASs extraction methods, the recoveries of the described method are generally lower. For example, Lorenzo et al. (2015) reported recoveries varying between 34 and 116% in soil and 44–125% in sediment using multiple methods with methanol (only methanol or in combination with acetic acid), whereas the recoveries in our method for matrices reached a maximum of 76% and 48% for soil and sediment, respectively. Increasing the sample size resulted in a clear reduction in recovery, reaching minima as low as 8% for soil and 1% for sediment. PFOS and PFOA extraction from blood plasma, using a C18 capillary column, resulted in recoveries of approximately 75% (Holm et al., 2004) and a method using solvent precipitation-isotope dilution-direct injection LC/MS/MS on serum and plasma samples obtained recoveries between 80 and 100% (Harrington, 2017). A highly selective MS-technique for the detection of PFASs in aqueous matrices resulted in recoveries between 92 and 134% (Wille et al., 2010).

The lower recoveries in the current method are likely the result of targeting both abiotic and biotic matrices, as well as both long and short chained PFASs. Problems with the use of a method developed for biological matrices on soil and sediment samples have been reported previously (Lorenzo et al., 2015). Furthermore, the range of compounds that can be extracted from e.g. water depends on the choice of the extraction method (Van Leeuwen and De Boer, 2007). Similarly, extraction techniques for soil and sediment give different outcomes, depending on how much they account for electrostatic and hydrophobic interactions (Van Leeuwen and De Boer, 2007). Finally, in the current method we have used the same solvent for both long and short-chained PFASs. Water soluble short chain PFASs might be better extracted with polar to medium solvents, whereas longer chained compounds require less polar solvents (Van Leeuwen and De Boer, 2007). Therefore, it is highly likely that recoveries of the currently described method will increase significantly when it targets individual matrices, as differences in matrix properties (e.g. fat content, electrostatic

interactions, etc.) can be accounted for. This has been confirmed in a field study which reports recoveries between 16% and 100% (Groffen et al., 2019b).

2.3.5 Comparison of calculation methods

The quantities of each compound in the spiked chicken eggs, calculated either using the ratio between the areas of the diagnostic ions of the labeled and unlabeled compounds (int.) or using an external calibration curve (ext.), are displayed in Fig. 2.4.

PFAS quantities were less variable when they were quantified using the internal standards. Secondly, PFAS quantities deviate more from the spiked 10,000 pg when quantifying the quantities using the external calibration curve (Formula (2.2)) compared to using the ratio of labeled and unlabeled compounds (Formula (2.1)). Quantities of PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFTeDA and PFOS, quantified using the external calibration curve, were all significantly higher (all $p < 0.05$) than 10,000 pg. When the internal standards were used, only PFHpA, PFOA, PFTrDA and PFTeDA deviated significantly from the spiked 10,000 pg (all $p < 0.05$). These results show the validity and effectivity of using internal standards for the quantification of PFASs concentrations in environmental matrices.

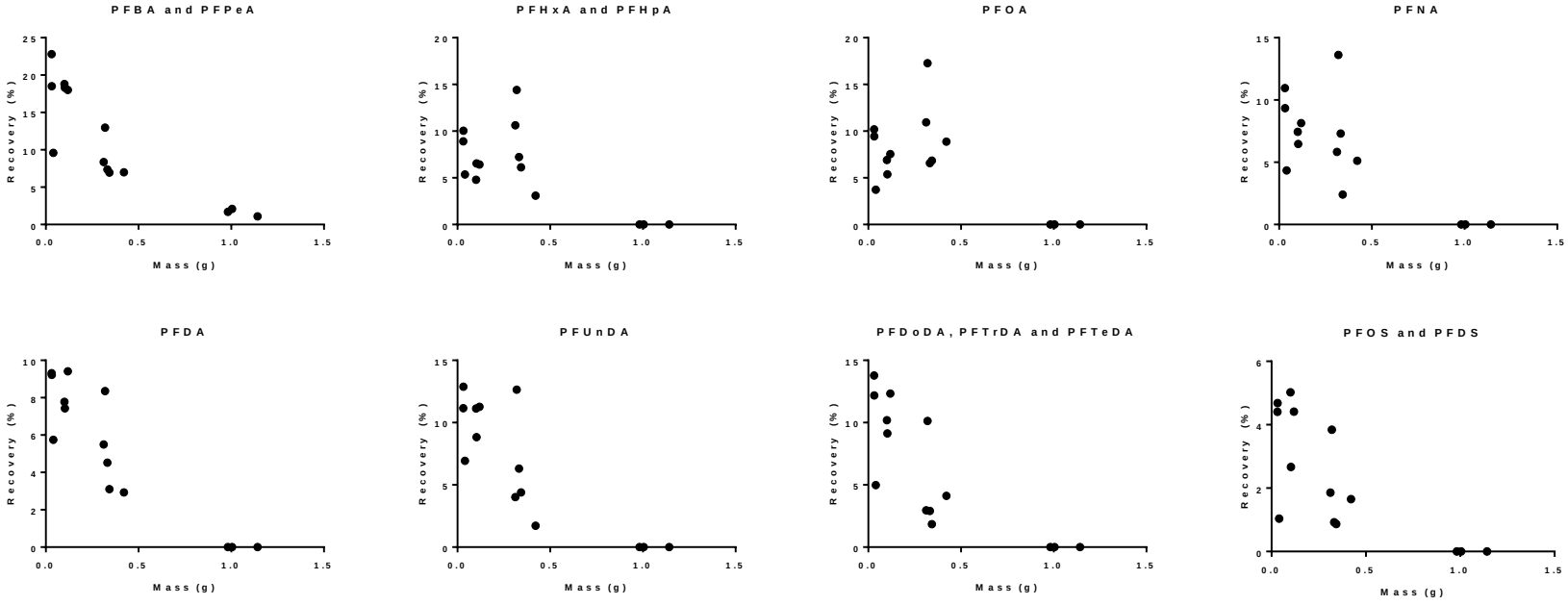


Fig. 2.3. Effect of sample mass (g) on recoveries (%) determined in chicken eggs. Compounds that were quantified using the same ISTD were grouped as the recoveries of the ISTD were the same.

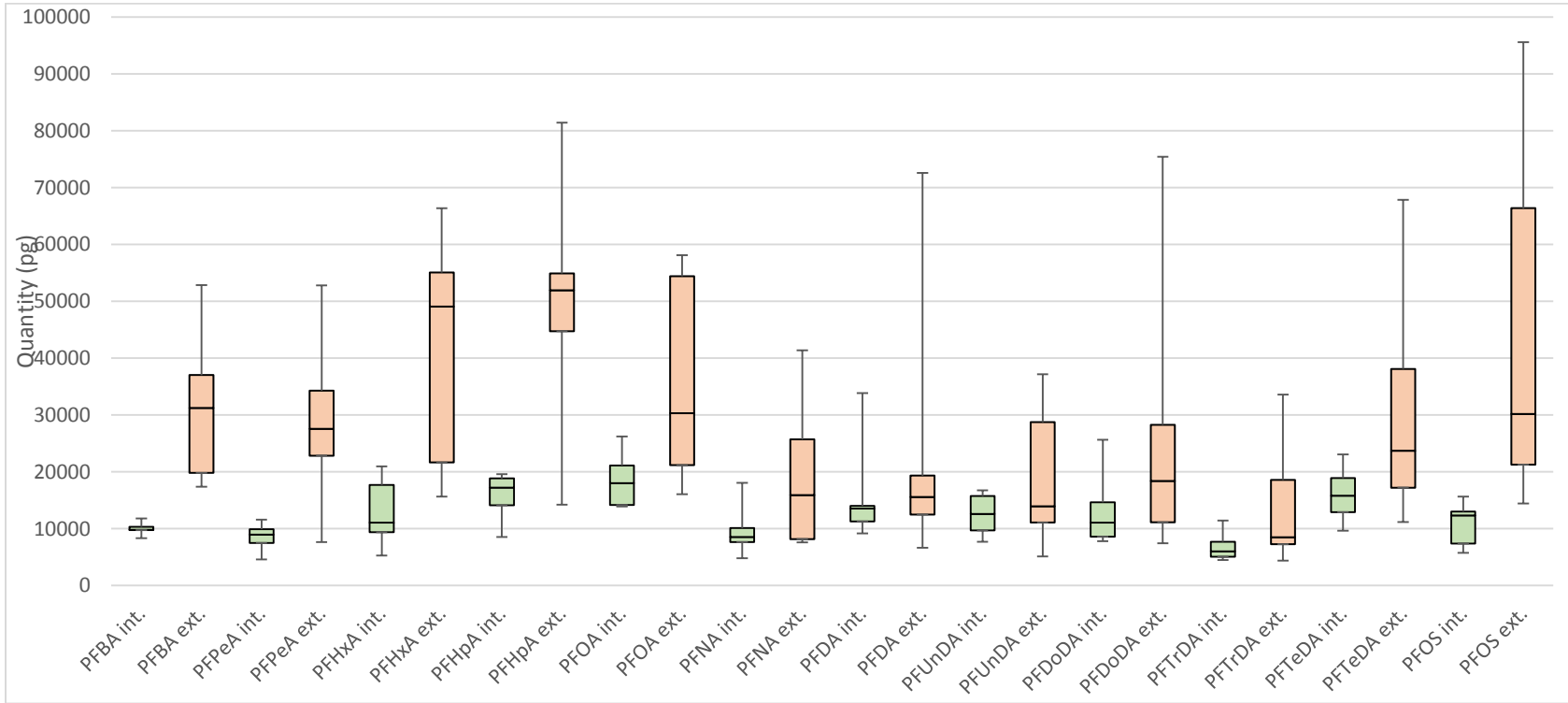


Fig. 2.4. Quantity (pg) of the spiked chicken egg samples calculated with two different quantitation methods: using internal standards (int.; N = 10; green), using an external calibration curve (ext.; N = 10; orange). To each sample 10000 pg was added. Quantities of PFBS, PFHxS and PFDS were <LOQ and were therefore excluded from the figure.

2.3.6 Application in real samples

We have successfully applied the method described here on eggs (n = 158) and blood plasma (n = 258) from a terrestrial songbird model species, the great tit, collected at a fluorochemical plant site and four other sites representing a distance gradient. Simultaneously we analyzed soil samples (n = 57) from the same study area using the described method. For more details regarding the PFASs concentrations in these samples, we refer to the corresponding publications (Groffen et al., 2019b,c; Lasters et al., 2019; Lopez-Antia et al., 2019). In several of these papers, associations between pollutant concentrations of PFAS and biological effects have been reported.

2.4 Conclusion

The developed LC/MS/MS method was validated and showed to be specific, selective, linear, robust and sensitive, even at low recoveries. Recoveries varied, depending on the matrix and target analyte between 1 and 100%, but could be significantly improved by using lower sample masses. We therefore recommend using low amounts of matrix, within the range of 30–100 mg as a compromise between the negative matrix effect in combination with the LOQ of the different analytes. We confirmed that using the ISTD closest in terms of functional group and carbon chain length is a suitable method for the quantification of PFASs that lack a corresponding ISTD. The newly developed method has been successfully used on numerous environmental and biotic matrices and can be applied for other matrices in the future. In addition, it should be examined in future studies whether this method could also be applied on other analytes, including precursor compounds and PFASs alternatives such as GenX or ADONA.

2.5 Acknowledgements

This work was supported by the research project G038615N of the Research Foundation Flanders (FWO) to LB and ME. The authors would like to thank L. Teunen and B. Slootmaekers for the perch muscle tissue and sediment samples. Finally, we would like to thank the Goris family for providing us the chicken liver.

2.6 Supplementary data

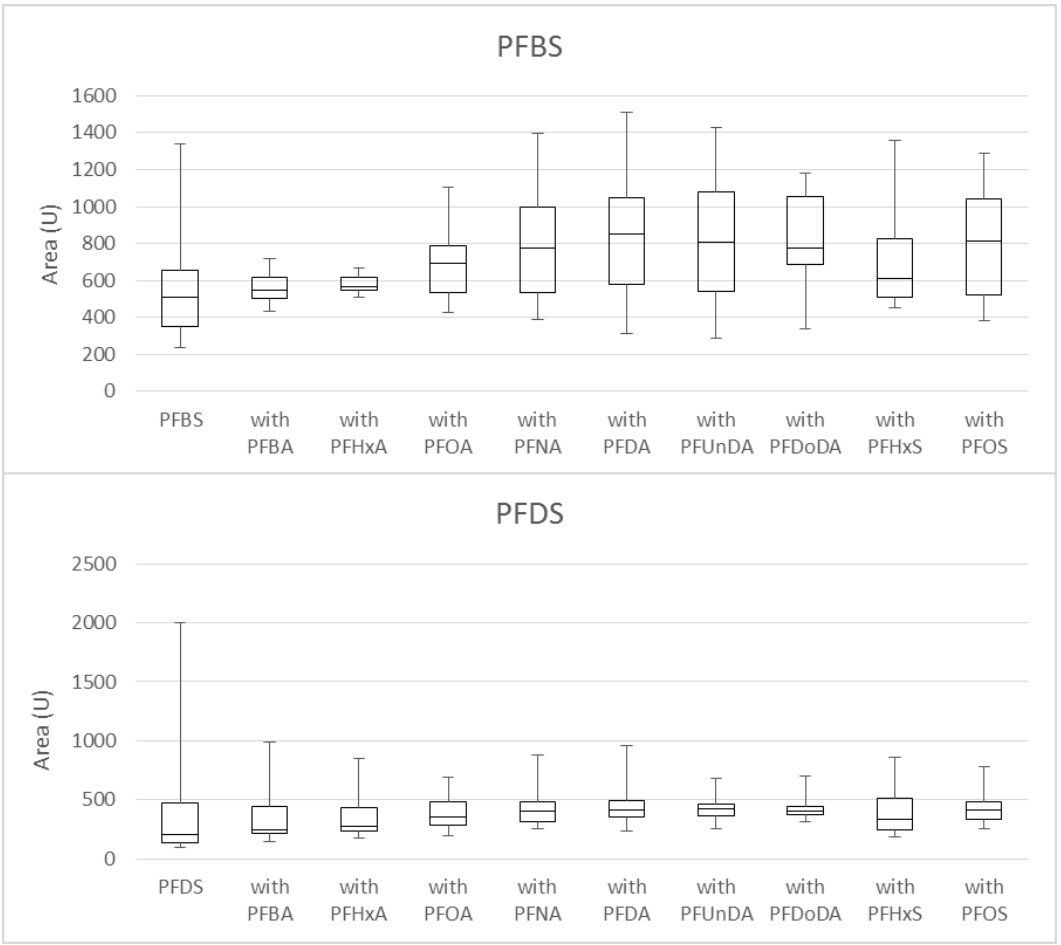


Fig. S2.1. Robustness of the method for the PFSAs that do not have a corresponding ISTD. PFSAs were quantified with all ISTDs from the mixture to determine the best suitable ISTD for the quantification and correction of the area units based on ionization efficiency.

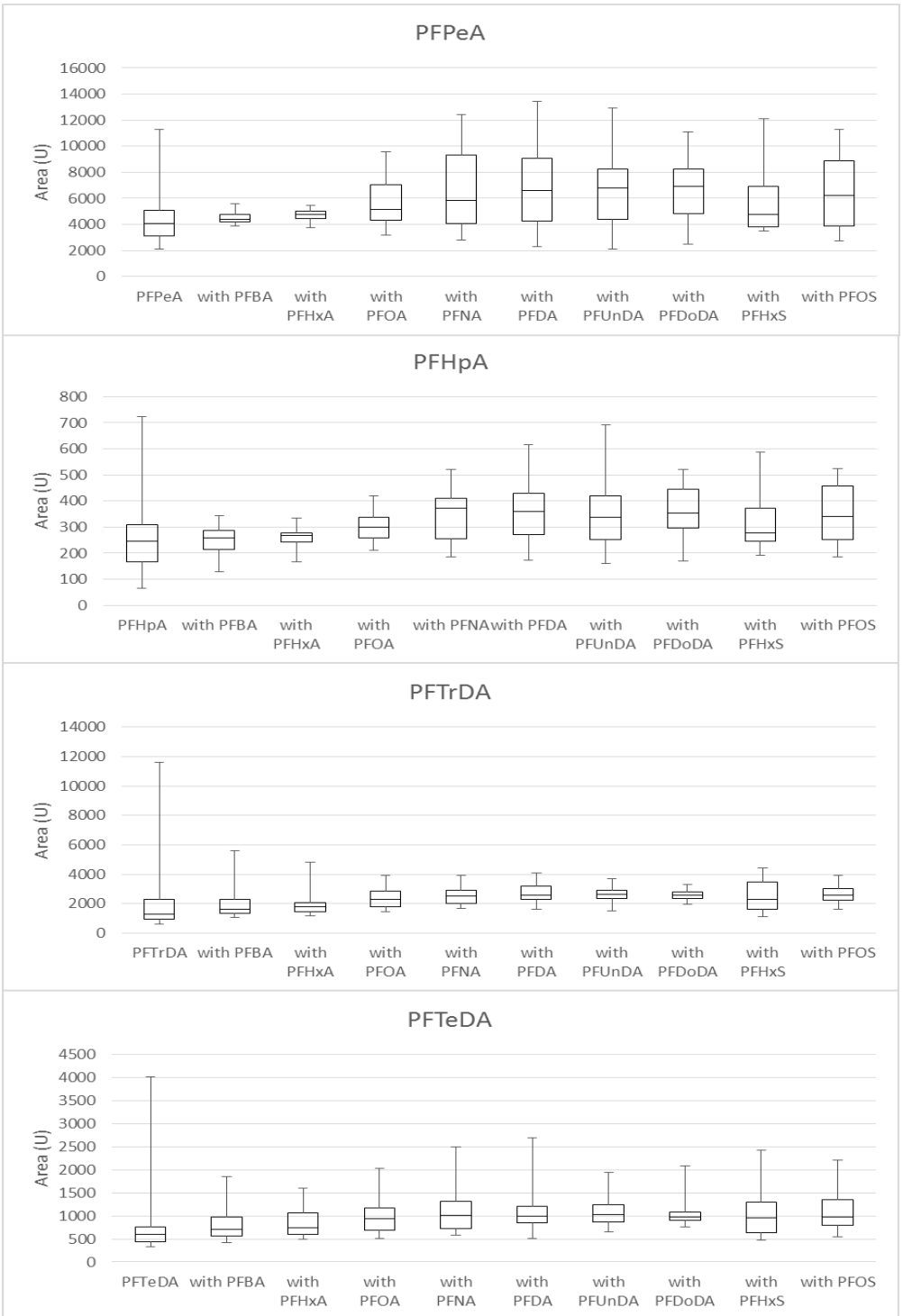


Fig. S2.2. Robustness of the method for the PFCAs that do not have a corresponding ISTD. PFCAs were quantified with all ISTDs from the mixture to determine the best suitable ISTD for the quantification and correction of the area units based on ionization efficiency.

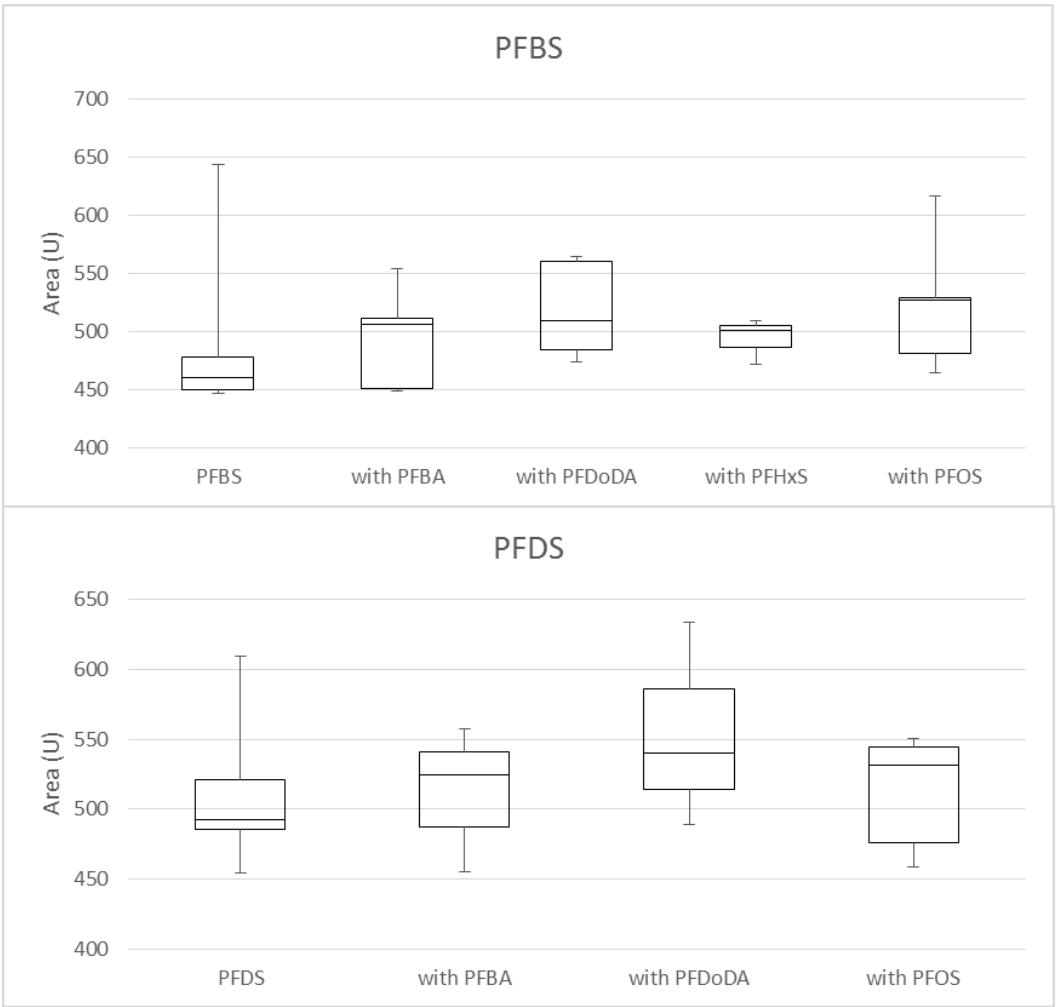


Fig. S2.3. Selection of the best suitable ISTD based on both ionization and extraction efficiency of the PFSAs that do not have a corresponding ISTD. Correction with PFOA resulted in an underestimation of the area units and is therefore removed from both figures. Correction with PFHxA, PFNA, PFDA and PFUdA resulted in an overestimation of the area units for both PFBS and PFDS. In addition, correction with PFHxS resulted in an overestimation for PFDS. These compounds are therefore removed from both figures.

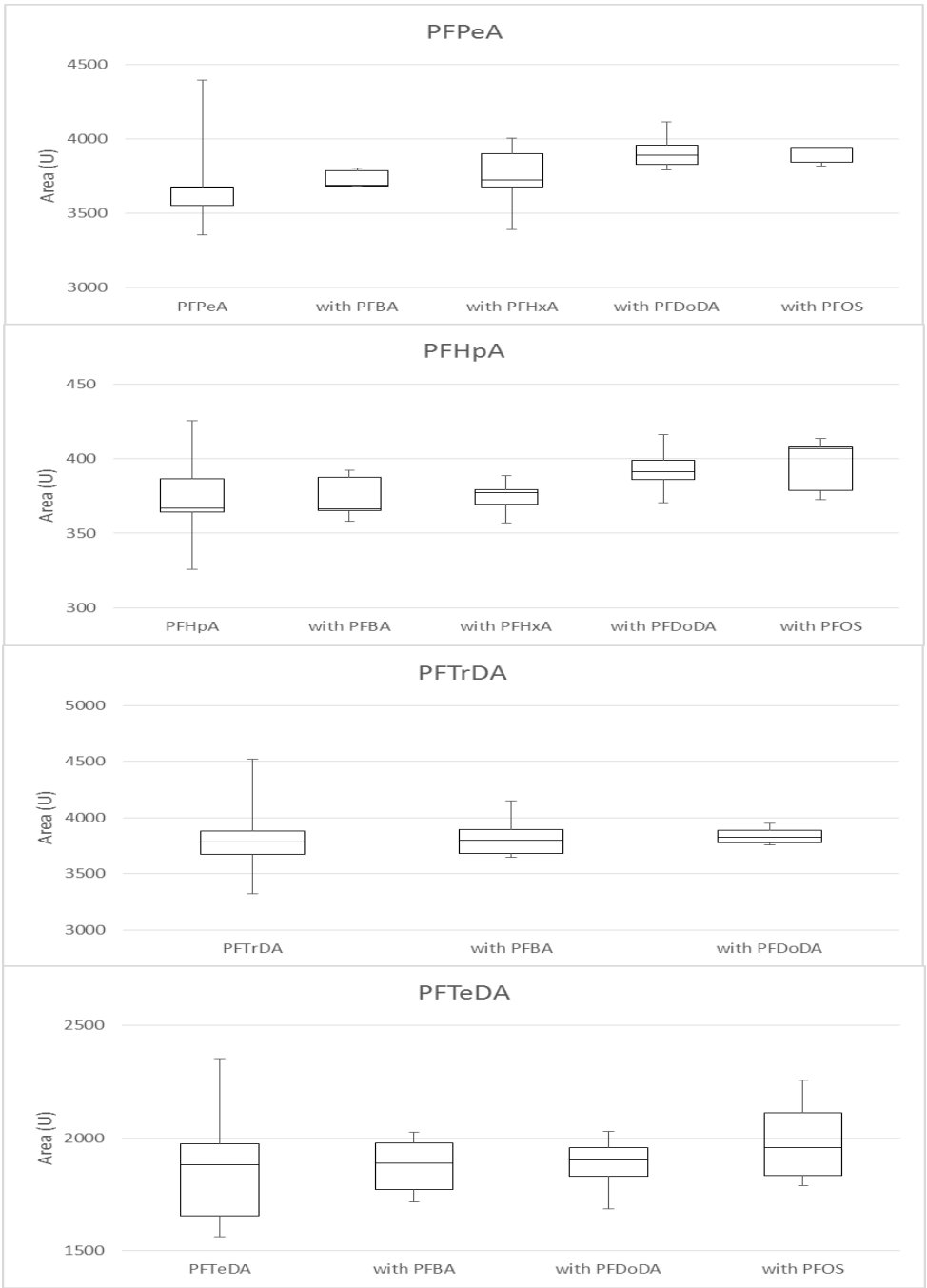


Fig. S2.4. Selection of the best suitable ISTD based on both ionization and extraction efficiency of the PFCAs that do not have a corresponding ISTD. Correction with PFOA resulted in an underestimation of the area units and is therefore removed from all figures. Correction with PFHxS, PFUnDA, PFDA and PFNA resulted in an overestimation for all compounds. In addition, correction with PFHxA resulted in an overestimation for PFTrDA and PFTeDA and a correction with PFOS resulted in an overestimation for PFTrDA. Therefore, all these compounds are removed from the corresponding figures.

Table S2.1. Mean quantities (± SE), mean signal-to-noise (S/N) ratios (± SE), recovery (range, %) and LOQs of all target analytes in procedural blanks and different environmental and biotic matrices. Two different extraction methodologies were used; the newly described procedure and a procedure described by Powley et al. (2005). Quantities are in pg for all samples. In cases where the internal standards where not quantifiable, the quantities, S/N-ratios and recoveries of these compounds are displayed as <LOQ. Letters behind the quantities indicate whether these quantities differ significantly from the spiked 5000 pg; A indicates significantly higher ($p < 0.05$), whereas a B indicates significantly lower ($p < 0.05$).

	Compound	This study				Powley et al. (2005)			
		LOQ (pg)	**Mean quantity (pg) ± SE**	**Mean S/N ratio ± SE**	**Recovery (%)**	**LOQ (pg)**	**Mean quantity (pg) ± SE**	**Mean S/N ratio ± SE**	**Recovery (%)**
Procedural blank (ACN)	PFBA	60	4800 ± 100	900 ± 200	82 – 100	230	4800 ± 60	200 ± 50	13 - 21
	PFPeA	90	5700 ± 200 A	600 ± 50	88 – 100	480	7000 ± 300 A	160 ± 20	12 – 28
	PFHxA	350	4900 ± 200	200 ± 50	88 – 100	870	5700 ± 400	80 ± 20	12 – 28
	PFHpA	210	5400 ± 80 A	300 ± 20	88 – 100	960	4200 ± 300	50 ± 10	12 – 28
	PFOA	90	5000 ± 50	700 ± 200	99 – 100	320	5700 ± 200 A	190 ± 30	9 – 27
	PFNA	90	4800 ± 300	600 ± 90	89 – 100	440	5300 ± 100	170 ± 50	9 – 22
	PFDA	230	4700 ± 300	300 ± 60	84 – 100	1300	5500 ± 700	40 ± 10	5 – 20
	PFUnDA	200	5800 ± 400	300 ± 20	53 – 100	1800	6600 ± 1100	30 ± 10	1 – 12
	PFDoDA	70	4800 ± 200	700 ± 90	88 – 100	700	4600 ± 300	80 ± 20	4 – 15
	PFTrDA	90	5700 ± 50 A	1200 ± 200	88 – 100	820	4700 ± 200	130 ± 30	4 – 15
	PFTeDA	80	5000 ± 200	600 ± 70	88 – 100	760	3700 ± 200 B	80 ± 20	4 – 15
	PFBS	190	5500 ± 80 A	300 ± 40	75 – 100	1100	5700 ± 1100	60 ± 10	12 – 29
	PFHxS	720	4800 ± 400	90 ± 10	75 – 100	2400	4100 ± 800	20 ± 10	12 – 29
	PFOS	50	4800 ± 90	1600 ± 40	97 – 100	300	4500 ± 300	310 ± 60	6 – 23
	PFDS	830	4800 ± 200	300 ± 20	97 – 100	4300	<LOQ	<LOQ	6 – 23

Table S2.1. (continued) Mean quantities (± SE), mean signal-to-noise (S/N) ratios (± SE), recovery (range, %) and LOQs of all target analytes in procedural blanks and different environmental and biotic matrices. Two different extraction methodologies were used; the newly described procedure and a procedure described by Powley et al. (2005). Quantities are in pg for all samples. In cases where the internal standards where not quantifiable, the quantities, S/N-ratios and recoveries of these compounds are displayed as <LOQ. Letters behind the quantities indicate whether these quantities differ significantly from the spiked 5000 pg; A indicates significantly higher ($p < 0.05$), whereas a B indicates significantly lower ($p < 0.05$).

	Compound	This study				Powley et al. (2005)			
		LOQ (pg)	**Mean quantity (pg) ± SE**	**Mean S/N ratio ± SE**	**Recovery (%)**	**LOQ (pg)**	**Mean quantity (pg) ± SE**	**Mean S/N ratio ± SE**	**Recovery (%)**
Water (tapwater)	PFBA	140	5300 ± 100	400 ± 30	34 – 48	1400	5300 ± 200	40 ± 10	4-6
	PFPeA	240	3800 ± 200 B	170 ± 30	37 – 68	1500	<LOQ	<LOQ	<LOQ
	PFHxA	850	4900 ± 200	80 ± 20	37 – 68	3600	<LOQ	<LOQ	<LOQ
	PFHpA	720	4500 ± 200 B	90 ± 20	37 – 68	3100	<LOQ	<LOQ	<LOQ
	PFOA	150	5700 ± 300	420 ± 60	42 – 71	2000	6900 ± 100 A	30 ± 10	1 – 2
	PFNA	350	5000 ± 200	300 ± 90	28 – 53	2100	<LOQ	<LOQ	<LOQ
	PFDA	680	4400 ± 400	70 ± 20	14 – 28	2900	<LOQ	<LOQ	<LOQ
	PFUnDA	2200	6700 ± 500 A	30 ± 10	3 – 11	6000	<LOQ	<LOQ	<LOQ
	PFDoDA	1900	5100 ± 1800	30 ± 10	2 – 4	5400	<LOQ	<LOQ	<LOQ
	PFTrDA	3000	4400 ± 1400	20 ± 10	1 – 2	5500	<LOQ	<LOQ	<LOQ
	PFTeDA	1900	<LOQ	<LOQ	1 – 4	2600	<LOQ	<LOQ	<LOQ
	PFBS	770	3600 ± 800	60 ± 20	16 – 56	3000	<LOQ	<LOQ	<LOQ
	PFHxS	2600	3100 ± 500 B	20 ± 10	16 – 56	6100	<LOQ	<LOQ	<LOQ
	PFOS	290	5000 ± 700	220 ± 60	6 – 28	1500	6300 ± 900	70 ± 20	1 – 6
	PFDS	5400	<LOQ	<LOQ	6 – 28	3800	<LOQ	<LOQ	1 – 6

Table S2.1. (continued) Mean quantities (± SE), mean signal-to-noise (S/N) ratios (± SE), recovery (range, %) and LOQs of all target analytes in procedural blanks and different environmental and biotic matrices. Two different extraction methodologies were used; the newly described procedure and a procedure described by Powley et al. (2005). Quantities are in pg for all samples. In cases where the internal standards where not quantifiable, the quantities, S/N-ratios and recoveries of these compounds are displayed as <LOQ. Letters behind the quantities indicate whether these quantities differ significantly from the spiked 5000 pg; A indicates significantly higher ($p < 0.05$), whereas a B indicates significantly lower ($p < 0.05$).

	Compound	This study				Powley et al. (2005)			
		LOQ (pg)	Mean quantity (pg) ± SE	Mean S/N ratio ± SE	Recovery (%)	LOQ (pg)	Mean quantity (pg) ± SE	Mean S/N ratio ± SE	Recovery (%)
Soil	PFBA	900	5400 ± 200	60 ± 10	8 – 14	1600	5400 ± 200	40 ± 3	2 – 3
	PFPeA	780	5300 ± 500	70 ± 10	10 – 19	2000	5400 ± 600	30 ± 3	4 – 7
	PFHxA	1500	6200 ± 900	40 ± 10	10 – 19	3200	5800 ± 1000	20 ± 4	4 – 7
	PFHpA	1600	5300 ± 500	40 ± 10	10 – 19	3800	5900 ± 1400	20 ± 1	4 – 7
	PFOA	330	4900 ± 300	180 ± 20	18 – 27	1600	5000 ± 700	60 ± 20	6 – 11
	PFNA	310	5100 ± 300	170 ± 10	17 – 29	1200	4800 ± 700	40 ± 5	4 – 10
	PFDA	1100	5100 ± 200	70 ± 20	18 – 29	3500	6200 ± 2000	20 ± 2	2 – 8
	PFUnDA	1000	5700 ± 400	60 ± 10	13 – 29	6100	<LOQ	<LOQ	<LOQ
	PFDoDA	220	5400 ± 700	250 ± 30	19 – 43	1800	5000 ± 700	40 ± 20	3 – 8
	PFTrDA	150	5300 ± 500	360 ± 40	19 – 43	1000	5400 ± 500	60 ± 10	3 – 8
	PFTeDA	640	7700 ± 700 A	130 ± 20	19 – 43	3800	10000 ± 1600 A	30 ± 4	3 – 8
	PFBS	310	5500 ± 700	190 ± 30	49 – 74	740	4700 ± 600	70 ± 10	17 – 40
	PFHxS	950	4700 ± 500	50 ± 10	49 – 74	2000	5100 ± 1000	30 ± 4	17 – 40
	PFOS	100	5600 ± 800	1720 ± 320	31 – 76	270	5500 ± 1000	690 ± 100	17 – 33
	PFDS	280	5500 ± 200 A	20 ± 10	31 – 76	970	4300 ± 600	50 ± 20	17 – 33

Table S2.1. (continued) Mean quantities (± SE), mean signal-to-noise (S/N) ratios (± SE), recovery (range, %) and LOQs of all target analytes in procedural blanks and different environmental and biotic matrices. Two different extraction methodologies were used; the newly described procedure and a procedure described by Powley et al. (2005). Quantities are in pg for all samples. In cases where the internal standards where not quantifiable, the quantities, S/N-ratios and recoveries of these compounds are displayed as <LOQ. Letters behind the quantities indicate whether these quantities differ significantly from the spiked 5000 pg; A indicates significantly higher ($p < 0.05$), whereas a B indicates significantly lower ($p < 0.05$).

	Compound	This study				Powley et al. (2005)			
		LOQ (pg)	**Mean quantity (pg) ± SE**	**Mean S/N ratio ± SE**	**Recovery (%)**	**LOQ (pg)**	**Mean quantity (pg) ± SE**	**Mean S/N ratio ± SE**	**Recovery (%)**
Sediment	PFBA	280	2700 ± 50 B	120 ± 30	26 – 48	730	2700 ± 100 B	50 ± 10	3 – 17
	PFPeA	830	4200 ± 700	70 ± 20	5 – 31	1800	4800 ± 700	40 ± 10	4 – 13
	PFHxA	1800	5000 ± 700	40 ± 10	5 – 31	2900	5600 ± 200	20 ± 4	4 – 13
	PFHpA	1600	4600 ± 600	30 ± 10	5 – 31	4100	7700 ± 800 A	30 ± 10	4 – 13
	PFOA	440	4300 ± 300	140 ± 50	4 – 27	900	6100 ± 200 A	90 ± 20	5 – 13
	PFNA	1200	4500 ± 500	50 ± 10	2 – 26	950	5900 ± 600	90 ± 20	5 – 18
	PFDA	2200	4700 ± 300	50 ± 20	2 – 25	1800	5800 ± 900	40 ± 10	4 – 15
	PFUnDA	1300	5000 ± 1300	50 ± 10	8 – 29	6200	9000 ± 2400	20 ± 10	1 – 15
	PFDoDA	1000	4400 ± 700	80 ± 30	2 – 24	890	4300 ± 600	60 ± 20	6 – 13
	PFTrDA	640	3800 ± 300 B	120 ± 50	2 – 24	600	2600 ± 300 B	50 ± 10	6 – 13
	PFTeDA	920	6500 ± 1500	100 ± 60	2 – 24	1500	5700 ± 1100	40 ± 10	6 – 13
	PFBS	5200	<LOQ	<LOQ	<LOQ	930	3400 ± 500 B	50 ± 10	12 – 36
	PFHxS	5400	<LOQ	<LOQ	<LOQ	1900	3300 ± 500 B	20 ± 3	12 – 36
	PFOS	1600	4500 ± 1000	150 ± 120	1 – 43	150	5000 ± 200	380 ± 50	10 – 32
	PFDS	720	3800 ± 500	60 ± 10	1 – 43	760	1200 ± 200 B	20 ± 1	10 – 32

Table S2.1. (continued) Mean quantities (± SE), mean signal-to-noise (S/N) ratios (± SE), recovery (range, %) and LOQs of all target analytes in procedural blanks and different environmental and biotic matrices. Two different extraction methodologies were used; the newly described procedure and a procedure described by Powley et al. (2005). Quantities are in pg for all samples. In cases where the internal standards where not quantifiable, the quantities, S/N-ratios and recoveries of these compounds are displayed as <LOQ. Letters behind the quantities indicate whether these quantities differ significantly from the spiked 5000 pg; A indicates significantly higher ($p < 0.05$), whereas a B indicates significantly lower ($p < 0.05$).

	Compound	This study				Powley et al. (2005)			
		LOQ (pg)	**Mean quantity (pg) ± SE**	**Mean S/N ratio ± SE**	**Recovery (%)**	**LOQ (pg)**	**Mean quantity (pg) ± SE**	**Mean S/N ratio ± SE**	**Recovery (%)**
Chicken egg	PFBA	550	5000 ± 200	90 ± 10	7 – 13	420	5000 ± 100	150 ± 30	10 – 19
	PFPeA	1300	6100 ± 300 A	50 ± 10	6 – 14	690	6500 ± 500 A	100 ± 20	9 – 20
	PFHxA	2800	5600 ± 1300	20 ± 3	3 – 14	1600	5000 ± 500	50 ± 10	9 – 20
	PFHpA	3700	4900 ± 1400	20 ± 2	3 – 14	1800	4400 ± 700	30 ± 5	9 – 20
	PFOA	480	6000 ± 400 A	140 ± 20	7 – 17	850	5800 ± 300 A	170 ± 40	9 – 28
	PFNA	1200	5500 ± 800	50 ± 10	2 – 14	800	5100 ± 200	90 ± 30	8 – 23
	PFDA	3300	5400 ± 900	20 ± 10	3 – 8	1200	4400 ± 1600	50 ± 10	3 – 25
	PFUnDA	4300	4700 ± 2100	20 ± 10	2 – 13	1400	4400 ± 1200	50 ± 20	8 – 28
	PFDoDA	2300	5500 ± 600	30 ± 10	2 – 10	730	6200 ± 800	120 ± 30	6 – 22
	PFTrDA	1300	4000 ± 500	40 ± 10	2 – 10	450	4900 ± 700	140 ± 30	6 – 22
	PFTeDA	5500	6300 ± 2100	20 ± 3	2 – 10	1600	7800 ± 1200	60 ± 10	6 – 22
	PFBS	4900	<LOQ	<LOQ	<LOQ	5800	<LOQ	<LOQ	<LOQ
	PFHxS	5200	<LOQ	<LOQ	<LOQ	4300	7500 ± 1600	20 ± 2	7 – 25
	PFOS	3500	6800 ± 1300	30 ± 10	2 – 4	220	5300 ± 200	280 ± 60	8 – 21
	PFDS	5600	<LOQ	<LOQ	1 – 4	1500	4300 ± 500	40 ± 7	8 – 21

Table S2.1. (continued) Mean quantities (± SE), mean signal-to-noise (S/N) ratios (± SE), recovery (range, %) and LOQs of all target analytes in procedural blanks and different environmental and biotic matrices. Two different extraction methodologies were used; the newly described procedure and a procedure described by Powley et al. (2005). Quantities are in pg for all samples. In cases where the internal standards where not quantifiable, the quantities, S/N-ratios and recoveries of these compounds are displayed as <LOQ. Letters behind the quantities indicate whether these quantities differ significantly from the spiked 5000 pg; A indicates significantly higher ($p < 0.05$), whereas a B indicates significantly lower ($p < 0.05$).

	Compound	This study				Powley et al. (2005)			
		LOQ (pg)	**Mean quantity (pg) ± SE**	**Mean S/N ratio ± SE**	**Recovery (%)**	**LOQ (pg)**	**Mean quantity (pg) ± SE**	**Mean S/N ratio ± SE**	**Recovery (%)**
Blood plasma (Great tit)	PFBA	250	2900 ± 40 B	200 ± 70	93 – 100	130	2900 ± 90 B	490 ± 100	14 – 100
	PFPeA	200	6400 ± 200 A	300 ± 20	40 – 76	550	8000 ± 300 A	290 ± 100	5 – 60
	PFHxA	410	6300 ± 200 A	200 ± 50	40 – 76	750	6200 ± 300 A	140 ± 40	5 – 60
	PFHpA	490	4600 ± 200	100 ± 20	40 – 76	1400	5300 ± 500	80 ± 20	5 – 60
	PFOA	130	6500 ± 200 A	600 ± 100	41 – 81	270	6100 ± 300 A	450 ± 100	6 – 68
	PFNA	140	5400 ± 200	420 ± 60	35 – 81	600	5300 ± 400	240 ± 100	6 – 68
	PFDA	480	5500 ± 300	170 ± 50	25 – 73	930	5800 ± 400	120 ± 30	4 – 51
	PFUnDA	1100	6700 ± 700	140 ± 40	19 – 72	2100	7200 ± 700 A	80 ± 30	2 – 49
	PFDoDA	100	5400 ± 200	580 ± 90	42 – 100	630	5600 ± 400	240 ± 100	6 – 78
	PFTrDA	60	4500 ± 200	800 ± 100	42 – 100	330	4400 ± 500	460 ± 200	6 – 78
	PFTeDA	240	7000 ± 900	320 ± 60	42 – 100	1300	6100 ± 600	190 ± 90	6 – 78
	PFBS	3400	4600 ± 500	20 ± 5	6 – 10	1300	6900 ± 800	100 ± 20	4 – 66
	PFHxS	6000	<LOQ	<LOQ	<LOQ	1800	6100 ± 1200	40 ± 10	4 – 66
	PFOS	470	6900 ± 800	360 ± 150	5 – 13	190	7200 ± 700 A	590 ± 140	5 – 73
	PFDS	3000	4800 ± 700	30 ± 10	5 – 13	960	3900 ± 300 B	90 ± 30	5 – 73

Table S2.1. (continued) Mean quantities (± SE), mean signal-to-noise (S/N) ratios (± SE), recovery (range, %) and LOQs of all target analytes in procedural blanks and different environmental and biotic matrices. Two different extraction methodologies were used; the newly described procedure and a procedure described by Powley et al. (2005). Quantities are in pg for all samples. In cases where the internal standards where not quantifiable, the quantities, S/N-ratios and recoveries of these compounds are displayed as <LOQ. Letters behind the quantities indicate whether these quantities differ significantly from the spiked 5000 pg; A indicates significantly higher ($p < 0.05$), whereas a B indicates significantly lower ($p < 0.05$).

	Compound	This study				Powley et al. (2005)			
		LOQ (pg)	**Mean quantity (pg) ± SE**	**Mean S/N ratio ± SE**	**Recovery (%)**	**LOQ (pg)**	**Mean quantity (pg) ± SE**	**Mean S/N ratio ± SE**	**Recovery (%)**
Chicken liver	PFBA	1200	2600 ± 200 B	20 ± 3	3 – 8	1000	2600 ± 200 B	30 ± 5	3 – 7
	PFPeA	3200	5400 ± 400	20 ± 5	2 – 5	6200	9000 ± 2600	20 ± 6	1 – 3
	PFHxA	4300	5300 ± 1400	20 ± 4	2 – 5	5400	<LOQ	<LOQ	<LOQ
	PFHpA	6200	<LOQ	<LOQ	<LOQ	6100	<LOQ	<LOQ	<LOQ
	PFOA	1700	3800 ± 1100	40 ± 10	2 – 5	4000	6800 ± 1800	40 ± 10	1 – 5
	PFNA	3500	5800 ± 600	20 ± 4	1 – 3	3400	3700 ± 500	10 ± 3	1 – 3
	PFDA	1100	<LOQ	<LOQ	<LOQ	1800	<LOQ	<LOQ	<LOQ
	PFUnDA	6200	<LOQ	<LOQ	<LOQ	5900	<LOQ	<LOQ	<LOQ
	PFDoDA	6500	<LOQ	<LOQ	1 – 2	2500	5500 ± 700	20 ± 5	1 – 3
	PFTrDA	2300	5400 ± 3100	20 ± 10	<LOQ – 1	1700	6300 ± 800	40 ± 10	1 – 3
	PFTeDA	6300	<LOQ	<LOQ	<LOQ	5800	9600 ± 1300 A	20 ± 3	1 – 3
	PFBS	5800	<LOQ	<LOQ	<LOQ	5500	<LOQ	<LOQ	<LOQ
	PFHxS	5400	<LOQ	<LOQ	<LOQ	6100	<LOQ	<LOQ	<LOQ
	PFOS	3900	<LOQ	<LOQ	<LOQ	1500	7300 ± 700 A	50 ± 7	1 – 4
	PFDS	4500	<LOQ	<LOQ	<LOQ	5000	7400 ± 1300 A	20 ± 1	1 – 4

Table S2.1. (continued) Mean quantities (± SE), mean signal-to-noise (S/N) ratios (± SE), recovery (range, %) and LOQs of all target analytes in procedural blanks and different environmental and biotic matrices. Two different extraction methodologies were used; the newly described procedure and a procedure described by Powley et al. (2005). Quantities are in pg for all samples. In cases where the internal standards where not quantifiable, the quantities, S/N-ratios and recoveries of these compounds are displayed as <LOQ. Letters behind the quantities indicate whether these quantities differ significantly from the spiked 5000 pg; A indicates significantly higher ($p < 0.05$), whereas a B indicates significantly lower ($p < 0.05$).

	Compound	This study				Powley et al. (2005)			
		LOQ (pg)	**Mean quantity (pg) ± SE**	**Mean S/N ratio ± SE**	**Recovery (%)**	**LOQ (pg)**	**Mean quantity (pg) ± SE**	**Mean S/N ratio ± SE**	**Recovery (%)**
Isopoda	PFBA	1100	3000 ± 200 B	30 ± 7	4 – 16	60	2900 ± 100 B	520 ± 70	59 – 93
	PFPeA	1500	6300 ± 1500	50 ± 10	4 – 11	360	14000 ± 4700	350 ± 30	<LOQ – 32
	PFHxA	1600	7400 ± 1000	50 ± 20	4 – 11	1800	6200 ± 400	60 ± 30	<LOQ – 32
	PFHpA	3300	5500 ± 800	20 ± 6	4 – 11	1600	8900 ± 3000	80 ± 50	<LOQ – 32
	PFOA	600	7500 ± 300 A	150 ± 30	8 – 22	370	5900 ± 200 A	400 ± 100	61 – 100
	PFNA	630	5600 ± 500	100 ± 20	9 – 16	100	5500 ± 200 A	600 ± 80	67 – 100
	PFDA	1200	6000 ± 700	50 ± 10	5 – 17	200	5500 ± 300	310 ± 60	46 – 91
	PFUnDA	2200	5800 ± 1000	30 ± 5	5 – 13	290	5600 ± 600	240 ± 60	53 – 76
	PFDoDA	640	5600 ± 600	110 ± 30	4 – 17	80	5500 ± 200 A	700 ± 50	55 – 100
	PFTrDA	390	5200 ± 1100	150 ± 20	4 – 17	80	5100 ± 400	850 ± 230	55 – 100
	PFTeDA	1500	4800 ± 800	40 ± 10	4 – 17	190	6200 ± 300 A	340 ± 40	55 – 100
	PFBS	6400	<LOQ	<LOQ	<LOQ	270	3300 ± 700	130 ± 10	27 – 58
	PFHxS	6200	<LOQ	<LOQ	<LOQ	2500	5700 ± 600	30 ± 10	27 – 58
	PFOS	2900	6400 ± 1500	60 ± 40	1 – 2	100	5700 ± 500	1100 ± 360	40 – 85
	PFDS	4800	<LOQ	<LOQ	1 – 2	300	4000 ± 200 B	160 ± 30	40 – 85

Table S2.1. (continued) Mean quantities (± SE), mean signal-to-noise (S/N) ratios (± SE), recovery (range, %) and LOQs of all target analytes in procedural blanks and different environmental and biotic matrices. Two different extraction methodologies were used; the newly described procedure and a procedure described by Powley et al. (2005). Quantities are in pg for all samples. In cases where the internal standards where not quantifiable, the quantities, S/N-ratios and recoveries of these compounds are displayed as <LOQ. Letters behind the quantities indicate whether these quantities differ significantly from the spiked 5000 pg; A indicates significantly higher ($p < 0.05$), whereas a B indicates significantly lower ($p < 0.05$).

	Compound	This study				Powley et al. (2005)			
		LOQ (pg)	**Mean quantity (pg) ± SE**	**Mean S/N ratio ± SE**	**Recovery (%)**	**LOQ (pg)**	**Mean quantity (pg) ± SE**	**Mean S/N ratio ± SE**	**Recovery (%)**
Fish muscle tissue	PFBA	240	3100 ± 10 B	160 ± 30	23 – 49	120	3100 ± 40 B	440 ± 100	38 – 94
	PFPeA	690	6500 ± 500 A	110 ± 10	10 – 25	260	6700 ± 600 A	290 ± 50	28 – 76
	PFHxA	1500	7800 ± 400 A	60 ± 10	10 – 25	670	7400 ± 300 A	160 ± 40	28 – 76
	PFHpA	1600	6700 ± 400 A	40 ± 3	10 – 25	630	6400 ± 100 A	130 ± 30	28 – 76
	PFOA	470	6600 ± 400 A	220 ± 70	6 – 31	100	6900 ± 300 A	760 ± 70	34 – 72
	PFNA	930	5800 ± 400	100 ± 20	5 – 30	470	6200 ± 200 A	330 ± 90	40 – 78
	PFDA	2700	6200 ± 1200	40 ± 20	5 – 31	390	6700 ± 500 A	190 ± 20	33 – 85
	PFUnDA	1100	7600 ± 1000	80 ± 20	2 – 23	520	7000 ± 700 A	200 ± 50	33 – 76
	PFDoDA	840	6900 ± 700	130 ± 50	4 – 25	140	6900 ± 300 A	540 ± 100	38 – 95
	PFTrDA	690	3100 ± 200 B	80 ± 30	4 – 25	90	4200 ± 300	500 ± 70	38 – 95
	PFTeDA	1400	4300 ± 800	30 ± 10	4 – 25	460	6300 ± 300	180 ± 50	38 – 95
	PFBS	5100	<LOQ	<LOQ	<LOQ	600	5400 ± 400	100 ± 20	20 – 53
	PFHxS	5000	<LOQ	<LOQ	<LOQ	3200	5300 ± 900	40 ± 10	20 – 53
	PFOS	2800	5600 ± 1200	30 ± 10	1 – 4	90	7600 ± 500 A	890 ± 130	27 – 51
	PFDS	5600	<LOQ	<LOQ	<LOQ	740	2200 ± 200 B	30 ± 6	27 – 51

Table S2.1. (continued) Mean quantities (± SE), mean signal-to-noise (S/N) ratios (± SE), recovery (range, %) and LOQs of all target analytes in procedural blanks and different environmental and biotic matrices. Two different extraction methodologies were used; the newly described procedure and a procedure described by Powley et al. (2005). Quantities are in pg for all samples. In cases where the internal standards where not quantifiable, the quantities, S/N-ratios and recoveries of these compounds are displayed as <LOQ. Letters behind the quantities indicate whether these quantities differ significantly from the spiked 5000 pg; A indicates significantly higher ($p < 0.05$), whereas a B indicates significantly lower ($p < 0.05$).

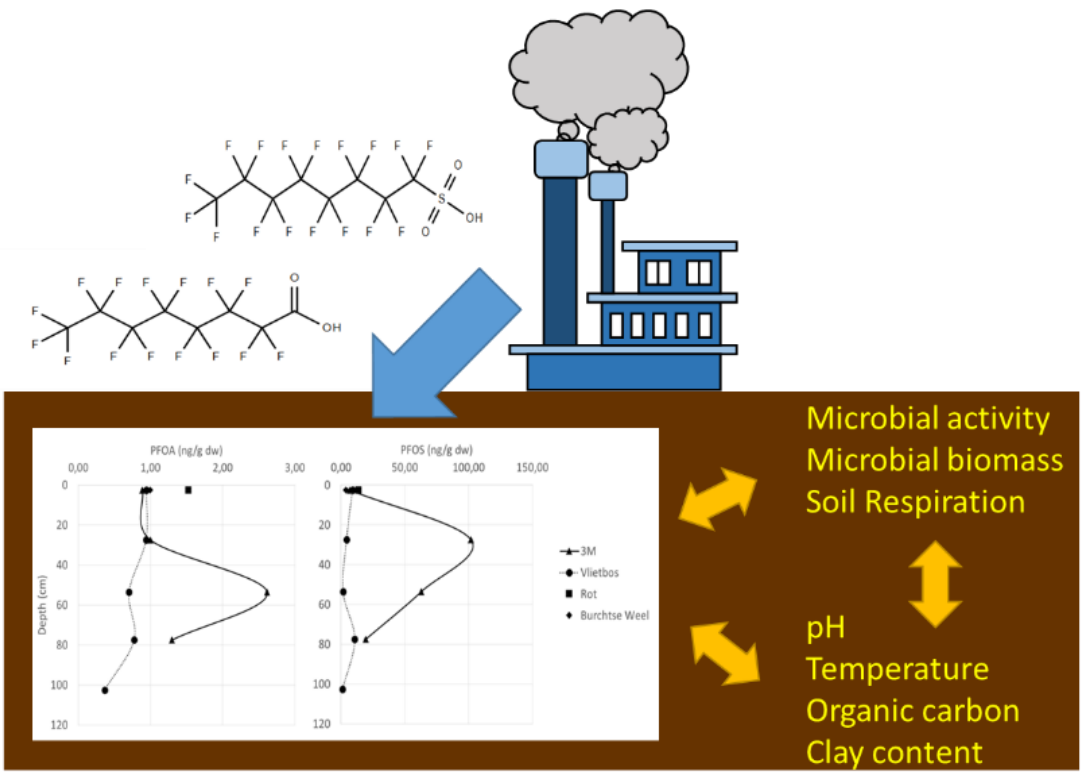
	Compound	This study				Powley et al. (2005)			
		LOQ (pg)	**Mean quantity (pg) ± SE**	**Mean S/N ratio ± SE**	**Recovery (%)**	**LOQ (pg)**	**Mean quantity (pg) ± SE**	**Mean S/N ratio ± SE**	**Recovery (%)**
Great tit egg	PFBA	230	3000 ± 50 B	200 ± 50	31 – 47	160	3000 ± 40 B	230 ± 50	20 – 73
	PFPeA	690	6700 ± 500 A	100 ± 10	13 – 23	650	7300 ± 500 A	150 ± 40	7 – 29
	PFHxA	970	6600 ± 300 A	70 ± 7	13 – 23	980	5400 ± 300	70 ± 10	13 – 23
	PFHpA	1500	4700 ± 800	30 ± 6	13 – 23	1700	4600 ± 400	40 ± 10	13 – 23
	PFOA	380	7200 ± 600 A	220 ± 40	12 – 23	1100	7400 ± 900	140 ± 40	4 – 27
	PFNA	840	6400 ± 400 A	120 ± 30	9 – 20	600	6300 ± 700	150 ± 30	5 – 31
	PFDA	2400	6400 ± 1200	40 ± 20	6 – 20	1700	7500 ± 1100	90 ± 40	3 – 36
	PFUnDA	2100	7700 ± 700 A	40 ± 3	3 – 14	3400	9900 ± 4800	30 ± 10	1 – 20
	PFDoDA	520	7100 ± 700 A	170 ± 40	6 – 27	900	6000 ± 200 A	160 ± 70	5 – 35
	PFTrDA	330	5300 ± 600	190 ± 40	6 – 27	310	4800 ± 200	250 ± 90	5 – 35
	PFTeDA	1400	5900 ± 600	60 ± 10	6 – 27	1700	5000 ± 500	50 ± 10	5 – 35
	PFBS	4500	<LOQ	<LOQ	<LOQ	770	4400 ± 800	50 ± 10	18 – 33
	PFHxS	5200	<LOQ	<LOQ	<LOQ	1900	5400 ± 1000	20 ± 6	18 – 33
	PFOS	2800	6600 ± 1800	350 ± 40	3 – 23	1400	6700 ± 1800	210 ± 60	15 – 43
	PFDS	5300	<LOQ	<LOQ	<LOQ	2500	9900 ± 4000	50 ± 10	3 – 30

3. Influence of soil physicochemical properties on the depth profiles of perfluoroalkylated acids (PFAAs) in soil along a distance gradient from a fluorochemical plant and associations with soil microbial parameters

Published in Chemosphere:

Thimo Groffen, Jet Rijnders, Niel Verbrigghe, Erik Verbruggen, Els Prinsen, Marcel Eens and Lieven Bervoets (2019). Influence of soil physicochemical properties on the depth profiles of perfluoroalkylated acids (PFAAs) in soil along a distance gradient from a fluorochemical plant and associations with soil microbial parameters. Chemosphere: doi: https://doi.org/10.1016/j.chemosphere.2019.124407.

Tables were modified to fit the size of the paper. No further alterations were made



Abstract

The widespread use of perfluoroalkylated acids (PFAAs) has led to a global presence in the environment, in which they accumulate and may cause detrimental effects. Although soils are known sinks for many persistent organic pollutants, still little is known on the behaviour of PFAAs in soils. Furthermore, studies that examine the relationships between PFAA concentrations and soil microbial parameters are scarce.

The 3 M fluorochemical plant near Antwerp has been characterized as a PFAAs hotspot. In the present study, we examined the vertical distribution of 15 PFAAs and their associations with multiple physicochemical soil properties along a distance gradient from this hotspot. Additionally, we tested the relationships between PFAA concentrations in the top soil with soil respiration, microbial activity and microbial biomass.

Our results show that both perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) concentrations were elevated in the subsurface layer (up to 50 cm), after which concentrations decreased again, suggesting a downward migration of both analytes in the soil. This downward movement might pose a potential threat for the contamination of the groundwater and, consequently, organisms that rely on this water for consumption. The soil concentrations were influenced by multiple physicochemical properties of the soil, which suggests differences in bioavailability and sorption/desorption capacities between different soil types. We did not observe any influence of PFAA contamination in the top soil on microbial activity and biomass nor soil respiration.

3.1 Introduction

Perfluoroalkylated acids (PFAAs) have been produced for almost seven decades, for use in a wide range of consumer products and industrial applications (Cousins et al., 2016). However, during the past decades, there has been a growing scientific attention and public concern towards these chemicals as a result of their toxicity in combination with their persistence and bioaccumulative potential (e.g. Conder et al., 2008; Giesy et al., 2010; Houtz et al., 2013). As a consequence, PFAAs have been detected globally in the environment, wildlife and even humans (e.g. Butt et al., 2010; Giesy and Kannan, 2001, 2002; Groffen et al., 2017, 2018, 2019b, 2019c, Houde et al., 2006; Lasters et al., 2019; Lopez-Antia et al., 2019; Miller et al., 2015).

Soils are known sinks for many persistent organic pollutants (POPs), including polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) (Cetin et al., 2017; Maqsood and Murugan, 2017; Mueller et al., 2006; Rankin et al., 2016; Xiao et al., 2016). However, the behaviour of PFAAs in the environment differs from other POPs due to their extreme surface-active properties (Goss and Bronner, 2006). PFAAs consist of a hydrophobic (oleophobic) perfluorinated carbon chain in combination with a hydrophilic functional group. This means that the behaviour of PFAAs, in contrast to other non-ionic POPs, is governed not only by hydrophobic interactions, but also by electrostatic interactions (Higgins and Luthy, 2007). Hence, the sorption of PFAAs cannot be predicted from a single soil property such as organic carbon (OC) content due to the complexity of the PFAAs chemistry and therefore there are still many uncertainties on how various physicochemical properties, such as pH, interact to determine the binding of PFAAs to soils (Li YS et al., 2018).

While it is not a sole linear predictor, organic carbon is one of the most important sorbents for PFAAs in soils (e.g. Milinovic et al., 2015). The sorption of PFAAs to soil organic matter (SOM) describes a nearly linear relation between sorption (described as the organic carbon-water partitioning coefficient (K_{oc})) and the chain-length of the compounds (Labadie and Chevreuil, 2011), which is attributed to an increase in the

hydrophobicity with each CF_2 moiety. Although the functional group of the PFAAs also affects the sorption (Ahrens et al., 2010; Campos Pereira et al., 2018; Higgins and Luthy, 2006), due to its electrostatic negativity (Du et al., 2014), chain-length is considered the dominating structural feature concerning the adsorption (Ahrens et al., 2010; Higgins and Luthy, 2006). Multiple studies have reported differences in PFAAs sorption between fractions of soil organic matter. For example, it has been reported that the humin fraction is the most important factor for the sorption of long-chained PFAAs (e.g. Campos Pereira et al., 2018; Zhang et al., 2015; Zhao et al., 2014), although the humic and fulvic acid fractions might also be important for the sorption of shorter-chained PFAAs (Campos Pereira et al., 2018).

Besides SOM, the sorption of PFAAs is promoted by a decreased pH and increased cation concentration (e.g. Higgins and Luthy, 2006; Chen et al., 2009; Wang F et al., 2015; Zhang et al., 2013). The effects of pH on the adsorption of PFAAs to soil are typically described as due to protonation or deprotonation of the organic acids (Higgins and Luthy, 2006). However, pH-dependent changes in the sorbent, such as surface charge of SOM, may also explain the pH effects (Higgins and Luthy, 2006). The decrease in adsorption with increasing pH is possibly caused by the decrease of electrostatic interactions, rather than protonation or deprotonation of the sorbate (Chen et al., 2009; Higgins and Luthy, 2006; Wang F et al., 2012). However, the influence of pH changes in the presence of a sufficient amount of divalent cations (Chen et al., 2009; Du et al., 2014). Adsorbent surfaces develop more basic sites to bind these cations when the pH increases, resulting in increased sorption of PFAAs (Du et al., 2014; Wang F et al., 2012).

The clay content, although poorly studied, might also play a role in the sorption of PFAAs as soils with smaller particles, such as clay, will have more functional groups (e.g. hydroxyl and carboxyl groups) and thus more binding sites to facilitate the sorption of contaminants (Qi et al., 2014).

The environmental threats posed by PFAAs have received an increasing attention in recent years. The composition and activity of the soil microbial community, important attributes of the soil ecosystems, are of great significance to the maintenance of soil fertility and can indicate changes in environmental quality of the soil (Qiao et al., 2018). Therefore, the assessment of the ecological toxicity of soil pollutants, such as PFAAs, is of great importance. However, studies that examine the effects of PFAAs on microbial communities in the environment are scarce. Pasquini et al. (2013) investigated the impact of PFOA and PFOS on a laboratory strain of *E. coli* and on activated sludge from an urban wastewater treatment plant. They observed a total absence of toxicity at high PFOA and PFOS concentrations (up to 10^2 μg/L). In addition, they observed that both PFOS and PFOA significantly induced an increase of bound extracellular polymeric substances (EPS), a polymeric network that enables microorganisms to live at high cell densities under adverse conditions and thus ensure their survival, adsorbing pollutants, nutrients and minerals (Finlayson et al., 1998; Flemming and Wingender, 2001), in the activated sludge at PFOS and PFOA concentrations ≥ 0.1 μg/L. This indicated that the biomass in the sludge had to cope with new conditions and that the microbial biomass was sensitive to these pollutants. Contradictory results were reported by Ochoa-Herrera et al. (2016), who observed no toxic effects of PFOS and short-chained PFAAs to the methanogenic activity of anaerobic wastewater sludge.

The main objective of this study was to assess the influence of multiple soil physicochemical properties (pH, temperature, total organic carbon (TOC) and clay content) on the vertical distribution of PFAAs in soils along a distance gradient from a fluorochemical plant. In addition, we examined the relationship between surface-layer PFAA pollution and soil respiration, microbial activity and biomass.

3.2 Materials and method

3.2.1 Study area and sample collection

Soil samples were collected during autumn 2018 at five sampling sites with increasing distance from a 3M fluorochemical plant in Antwerp. Based on prior monitoring

studies (e.g. Dauwe et al., 2007; Groffen et al., 2017, 2019b, 2019c, Lopez-Antia et al., 2019) we selected Vlietbos (1 km SE from 3M), Middenvijver-Rot (hereafter Rot; 2.3 km ESE from 3M) and Burchtse Weel (3 km SE from 3M) as sampling sites. As a reference site, Westmalle (25 km NE from 3M) was selected (Figure 3.1).

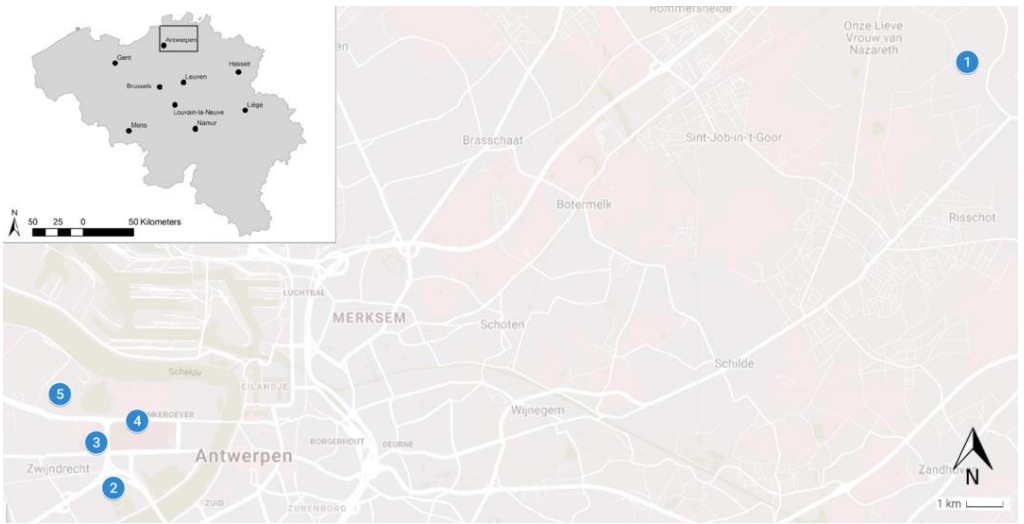


Figure 3.1. Overview of the study areas of this research. 1 = Westmalle (± 25 km from 3M), 2 = Burchtse Weel (± 3 km from 3M), 3 = Vlietbos (± 1 km from 3M), 4 = Middenvijver-Rot (± 2 km from 3M) and 1 = 3M. Produced with Google Maps.

At each site, five soil samples were collected at varying depths (0 – 5 cm, 25 – 30 cm, 50 – 55 cm, 75 – 80 cm and 100 – 105 cm) using an Edelman auger and a stainless steel shovel. Due to a rocky sublayer, at Rot and Burchtse Weel only the top layer could be collected and at 3M only four layers could be sampled. Of each sample the temperature and pH were recorded using a portable multimeter (HI9125, Hanna Instruments) prior to storing them into 50 mL polypropylene (PP) tubes. Additionally, we filled two 10 L plastic buckets, using a stainless steel shovel, with top soil (± 0 – 5 cm) from each site to use in further analyses on microbial biomass, activity and soil respiration. The 50 mL PP tubes were stored at -20 °C prior to PFAA analysis and determination of TOC and clay content. The buckets were stored, at a similar length of time, in a dark room at room temperature.

3.2.2 Chemical extraction and analysis

The used abbreviations are all according to Buck et al. (2011). Target analytes included four perfluorosulfonic acids (PFSAs) and eleven perfluorocarboxylic acids (PFCAs). The target analytes and the isotopically mass-labeled internal standards (ISTDs; Wellington Laboratories, Guelph, Canada) are displayed in Table 3.1. During the extraction HPLC grade acetonitrile (ACN; LiChrosolv, Merck Chemicals, Belgium), ammonium acetate (VWR International, Belgium), ammonium hydroxide (Filter Service N.V., Belgium) and Milli-Q water (MQ; 18.2 mΩ; TOC: 2.0 ppb; Merck Millipore, Belgium) were used.

Table 3.1. Target analytes, isotopically mass-labelled internal standards (ISTDs) used for quantification and MRM transitions. Table adapted from Groffen et al. (2019b).

Compound	Internal standard (ISTD) used for quantification	Precursor ion (m/z)	Product ion (m/z)	
			Diagnostic product ion 1	**Diagnostic product ion 2**
PFBS	$^{18}O_2$-PFHxS	299	80	99
PFHxS	$^{18}O_2$-PFHxS	399	80	99
PFOS	[1,2,3,4-$^{13}C_4$]PFOS	499	80	99
PFDS	[1,2,3,4-$^{13}C_4$]PFOS	599	80	99
PFBA	$^{13}C_4$-PFBA	213	169	169
PFPeA	$^{13}C_4$-PFBA	263	219	219
PFHxA	[1,2-$^{13}C_2$]PFHxA	313	269	119
PFHpA	[1,2-$^{13}C_2$]PFHxA	363	319	169
PFOA	[1,2,3,4-$^{13}C_4$]PFOA	413	369	169
PFNA	[1,2,3,4,5-$^{13}C_5$]PFNA	463	419	169
PFDA	[1,2-$^{13}C_2$]PFDA	513	469	219
PFUnDA	[1,2-$^{13}C_2$]PFUnDA	563	519	169
PFDoDA	[1,2-$^{13}C_2$]PFDoDA	613	569	319
PFTrDA	[1,2-$^{13}C_2$]PFDoDA	663	619	319
PFTeDA	[1,2-$^{13}C_2$]PFDoDA	713	669	169

The extraction procedure described and validated by Groffen et al. (2019a) was used. Soil samples were oven-dried at 60 °C prior to the analysis. To each sample (±0.3 g dw) 10 ng (80 μL, 125 pg/μL) of each ISTD and 10 mL of ACN was added. After vortex-mixing, the samples were sonicated for 3 x 10 min (Branson 2510) and left overnight on a shaking plate (135 rpm, room temperature, GFL 3020, VWR International, Leuven, Belgium). After centrifugation (4 °C, 2400 rpm, 10 min, Eppendorf centrifuge 5804R,

rotor A-4-44), the supernatant was transferred into a new PP tube. For the extraction we used Chromabond HR-XAW Solid Phase Extraction (SPE) cartridges, which are weakly basic secondary and tertiary ammonium polymeric anion exchangers. The cartridges (3mL, adsorbent weight 200 mg) were preconditioned and equilibrated with 5 mL of ACN and 5 mL of MQ, respectively, before loading the sample onto the cartridges. Hereafter, the SPE cartridges were washed with 5 mL of 25 mM ammonium acetate in MQ and 2 mL of ACN and eluted with 2 x 1 mL of 2% ammonium hydroxide in ACN. The eluent was completely dried using a rotational-vacuum-concentrator at 37 °C (Martin Christ, RVC-2-25, Osterode am Harz, Germany) and reconstituted with 200 μL of 2% ammonium hydroxide in ACN. Finally, the samples were vortex-mixed for at least 1 minute and filtrated through an Ion Chromatography Acrodisc 13 mm Syringe filter with 0.2 μm Super (PES) membrane (VWR International, Leuven, Belgium) into a PP auto-injector vial prior to the UPLC analysis.

3.2.3 UPLC-TQD analysis

We used ultra-performance liquid chromatography coupled tandem ES(-) mass spectrometry (ACQUITY, TQD, Waters, Milford, MA, USA) to analyze the target analytes. Individual PFAAs were separated using an ACQUITY BEH C18 column (2.1 x 50 mm; 1.7 μm, Waters, USA) and an ACQUITY BEH C18 pre-column (2.1 x 30 mm; 1.7 μm, Waters, USA) was inserted between the solvent mixer and injector to retain PFAA contamination from the system. As mobile phase solvents we used A) 0.1% formic acid in water and B) 0.1% formic acid in ACN. The injection volume was 10 μL at a flow rate of 450 μL/min. The gradient started at 65% A, decreased to 0% A in 3.4 min and returned to 65% A at 4.7 min. To identify and quantify the target PFAAs, multiple reaction monitoring (MRM) of two diagnostic transitions per target analyte (Table 3.1) was used.

3.2.4 Total organic carbon (TOC) and clay content

The loss on ignition method, as described by Heiri et al. (2001), was used to determine the organic carbon content of the soil. Approximately 10 g of the soil was oven-dried at 60 °C. Empty aluminum-foil bags were folded and dried at 105 °C for at least 2h after

which they were cooled down to room temperature in a desiccator and weighed. The bags where then filled with the dried soil, weighed and oven-dried in a muffle furnace at 105 °C for at least 24 h. Again, the samples were cooled down to room temperature in a desiccator, weighed and incinerated in the muffle furnace at 550 °C for at least 5 h. After cooling down in a desiccator, the weight loss was determined and the TOC was calculated using Formula 3.1.

$$TOC\ (\%) = \frac{\left(\frac{(DW_{105} - DW_{550})}{DW_{105}} * 100\right)}{1.742} \quad (3.1)$$

With DW the dry weight of the sample after heating at 105 °C or 550 °C.

A Malvern Mastersizer 2000 and Hydro 2000G were used to determine the clay content (particles with a size <2 μm) of the soil. The samples were pretreated with 40 mL 33% hydrogen peroxide (VWR Chemicals, Leuven, Belgium) and 9 mL 30% hydrochloric acid (VWR Chemicals, Leuven, Belgium) to destruct iron conglomerates and organic material in the samples. Additionally, the samples were boiled to speed up the destruction process and sieved over a 2.0 mm test sieve prior to the analysis.

3.2.5 Soil respiration, microbial biomass and microbial activity

Approximately 50 g of fresh soil was weighed, transferred into a glass jar (closed) and oven dried at 70 °C for 48 h to determine the dry mass. Hereafter the lid was replaced by a lid that was attached to an Environmental Gas Monitor (EGM-4). As CO_2 can be released from soil disturbance, the samples were rested for 5 min to return to their normal respiration rates. The CO_2 concentration (ppm) outside of the jars (reference concentration) was determined. The soil respiration was then determined by purging the reference air through the glass jar into the EGM at 350 mL/min (Pumpanen et al., 2004).

The soil microbial biomass was determined by fumigation of soil samples according to Brookes et al. (1985). Fumigation of the soil will kill and lyse microbial cells and thus a subtraction of the carbon content measured with fumigation from the total carbon content will give a measure for the microbial biomass in each sample. To determine

the carbon content approximately 2 g of each sample was transported to marked scintillation vials. After adding 250 mL of a 0.5 M potassium sulfate (K_2SO_4), the vials were placed on a shaking plate for 1 h. After the soil settled, the supernatant was filtered using a Whatmann no. 41 filter. Per 10 soil samples, one control sample (20 mL K_2SO_4) was added. Vials were closed and stored at -20 °C until further analysis. Another sample of approximately 2g was fumigated in a desiccator. Approximately 50 mL of chloroform was added to a measuring cup and placed in the desiccator, after which the desiccator was closed and vacuumed for 3 x 10 min. Hereafter, samples were rested in the dark for 24 – 48 h under vacuum. After removal of the chloroform, the samples were transferred into scintillation vials and the previously described K_2SO_4 extraction was performed. Each sample (10 mL) was diluted with 10 mL MQ prior to analysis with a Total Organic Carbon Analyzer (TOC-$V_{CPH/CPN}$, Shimadzu Corporation).

The soil microbial activity was assessed using the bait-lamina tests. This is an integrative method that consists of perforated PVC-strips (16 holes) filled with a standard substrate mixture and allows us to compare the feeding activity of soil organisms in soils from different sites, containing different PFAA concentrations. The disappearance of the bait material is directly associated with the activity of soil microorganisms living on the substrate (Bart et al., 2018; Hamel et al., 2007; Kratz, 1998). In each 10 mL bucket, five bait-lamina test strips (Terra Protecta GmbH, Berlin, Germany) were placed (in total 10 strips per site) and checked regularly to determine the feeding activity. In case the strip was fully consumed, a new strip was placed directly next to it. After four weeks, the number of consumed holes were counted and used as a measure of microbial activity (Kratz, 1998).

3.2.6 Quality assurance

Per batch of 20 samples, one procedural blank (10 mL of ACN) was analyzed as quality control for the PFAAs analysis. The concentrations in the blanks were all < limit of quantification (LOQ). The method recoveries for the target analytes ranged between 3% and 100% in the soil samples. Despite the low recoveries in some samples (Table S3.1), the previously described method was shown to be highly accurate even at low

recoveries of 1% (Groffen et al., 2019a). Therefore, we argue that the low detection frequencies for some analytes are not the result of the low recovery in these samples. Individual LOQs were determined based on a signal-to-noise (S/N) ratio of 10 and are displayed in Table 3.2, for the analytes with a detection frequency of at least 50% in at least one soil layer at a site (i.e. PFBA, PFOA, PFUnDA and PFOS).

3.2.7 Statistical analysis

Statistical analyses were performed using R Studio. We used the Shapiro-Wilk test to examine the validity of the models' assumptions and data were log-transformed when needed to fulfil the normality assumptions. The level of significance for all tests was set at $p \leq 0.05$. Concentrations that were below the LOQ were given a concentration of the LOQ/2 (Bervoets et al., 2004; Groffen et al., 2017; Lasters et al., 2019). Whenever the quantified concentrations of an analyte were below the LOQ in more than 50% of the samples at a certain layer or at a location, these data were excluded from the analyses.

Differences in concentrations between soil layers and between locations were examined using ANOVAs (in cases where comparisons could be made between multiple sites or layers) or t-tests (in cases where a comparison could only be made between two sites or layers). When significant differences were obtained, Tukey's post-hoc tests were used to compare mean PFAA concentrations among layers and sites. We used multiple linear regression to assess the relationships between soil physicochemical properties and PFAA concentrations in the soil and to assess relationships between PFAA concentrations and microbial parameters. We selected the best fitting model based on the Akaike Information Criterion (AIC). These models were only used as explanatory models and not predictive models.

3.3 Results

3.3.1 PFAA concentrations and vertical distribution

Only PFBA, PFOA, PFUnDA and PFOS were detected in more than 50% of the samples in at least one soil layer from at least one sampling site. Table 3.2 shows the concentrations and detection frequencies of these analytes.

PFBA was detected in the top soil (0 – 5 cm) at Vlietbos, Rot and Burchtse Weel with mean concentrations of 0.82, 2.78 and 1.11 ng/g dw respectively. In addition, PFBA was detected in the 25 – 30 cm layer at Vlietbos (0.38 ng/g dw). PFOA was detected in all layers at 3M, with concentrations up to 3.19 ng/g dw. At Vlietbos, PFOA was detected up to 80 cm deep at concentrations ranging from <LOQ to 1.52 ng/g dw. Mean PFOA concentrations in the top layer at Rot and Burchtse Weel were 1.53 and 0.99 ng/g dw, respectively. At Westmalle, PFOA was detected at low detection frequencies in all layers (<LOQ – 2.41). PFUnDA was only detected in the top layer at Vlietbos at a mean concentration of 0.52 ng/g dw. PFOS concentrations ranged up to 202 ng/g dw at 3M, 45 ng/g dw at Vlietbos, 21 ng/g dw at Rot and 4.46 ng/g dw at Burchtse Weel and were detected at all studied layers. At Westmalle, on the contrary, PFOS was only detected in 40% of the samples from the top layer and ranged from <LOQ up to 0.88 ng/g dw. As the detection frequencies at Westmalle, with exception of PFOA in the deepest layer, were <50% we will exclude this location from further analysis when looking at individual locations.

Table 3.2. Individual limits of quantification (LOQs; ng/g dw, determined as 10x the S/N ratio), median and mean concentrations (ng/g dw), range (ng/g dw) and detection frequencies (Freq. (%)) of PFAAs in soils collected at varying depths at five sampling sites: a perfluorochemical plant (3M) and at four other sites with increasing distance from 3M (i.e. Vlietbos 1km, Rot 2.3 km, Burchtse Weel 3 km and Westmalle 25 km).

Location	Layer (cm)		PFBA	PFOA	PFUnDA	PFOS
		LOQ	0.3	0.73	0.27	0.67
3M	0 – 5 (N = 5)	**Mean**	<LOQ	0.89	<LOQ	6.77
		Median		0.87		7.05
		Range		<LOQ – 1.34		3.93 – 8.46
		Freq.	0	80	0	100
	25 – 30 (N = 5)	**Mean**	<LOQ	1.00	<LOQ	102
		Median		0.93		70
		Range		<LOQ – 1.46		30 – 202
		Freq.	0	80	0	100
	50 – 55 (N = 5)	**Mean**	<LOQ	2.61	<LOQ	63
		Median		2.58		56
		Range		2.04 – 3.19		46 – 83
		Freq.	0	100	0	100
	75 – 80 (N = 5)	**Mean**	<LOQ	1.30	<LOQ	19
		Median		1.43		18
		Range		<LOQ – 1.88		1.85 – 39
		Freq.	0	80	0	100

Table 3.2. (continued) Individual limits of quantification (LOQs; ng/g dw, determined as 10x the S/N ratio), median and mean concentrations (ng/g dw), range (ng/g dw) and detection frequencies (Freq. (%)) of PFAAs in soils collected at varying depths at five sampling sites: a perfluorochemical plant (3M) and at four other sites with increasing distance from 3M (i.e. Vlietbos 1km, Rot 2.3 km, Burchtse Weel 3 km and Westmalle 25 km).

Location	Layer (cm)		PFBA	PFOA	PFUnDA	PFOS
		LOQ	0.3	0.73	0.27	0.67
Vlietbos	0 – 5 (N = 5)	**Mean**	0.82	0.94	0.52	8.91
		Median	1.00	1.04	0.59	9.47
		Range	<LOQ – 1.25	<LOQ – 1.20	<LOQ – 0.69	7.48 – 10
		Freq.	80	80	80	100
	25 – 30 (N = 5)	**Mean**	0.38	0.94	<LOQ	4.32
		Median	0.38	0.88		4.42
		Range	<LOQ – 0.50	0.85 – 1.13		2.88 – 5.80
		Freq.	80	100	0	100
	50 – 55 (N = 5)	**Mean**	<LOQ	<LOQ	<LOQ	1.58
		Median		<LOQ		1.84
		Range		<LOQ – 1.40		<LOQ – 2.27
		Freq.	0	40	0	80
	75 – 80 (N = 5)	**Mean**	<LOQ	0.77	<LOQ	11
		Median		0.77		2.27
		Range		<LOQ – 1.52		1.85 – 45
		Freq.	0	60	0	100
	100 – 105 (N = 3)	**Mean**	<LOQ	<LOQ	<LOQ	1.21
		Median				1.14
		Range				<LOQ – 2.15
		Freq.	0	0	0	67
Rot	0 – 5 (N = 5)	**Mean**	2.78	1.53	<LOQ	14
		Median	2.97	1.50		13
		Range	2.06 – 3.67	1.11 – 2.27		9.50 – 21
		Freq.	100	100	0	100

Table 3.2. (continued) Individual limits of quantification (LOQs; ng/g dw, determined as 10x the S/N ratio), median and mean concentrations (ng/g dw), range (ng/g dw) and detection frequencies (Freq. (%)) of PFAAs in soils collected at varying depths at five sampling sites: a perfluorochemical plant (3M) and at four other sites with increasing distance from 3M (i.e. Vlietbos 1km, Rot 2.3 km, Burchtse Weel 3 km and Westmalle 25 km).

Location	Layer (cm)		PFBA	PFOA	PFUnDA	PFOS
		LOQ	0.3	0.73	0.27	0.67
Burchtse Weel	0 – 5 (N = 5)	**Mean**	1.11	0.99	<LOQ	3.95
		Median	1.11	0.94		3.89
		Range	<LOQ – 2.01	<LOQ – 1.55		3.65 – 4.46
		Freq.	80	80	0	100
Westmalle	0 – 5 (N = 5)	**Mean**	<LOQ	<LOQ	<LOQ	<LOQ
		Median		<LOQ		<LOQ
		Range		<LOQ – 0.95		<LOQ – 0.88
		Freq.	0	40	0	40
	25 – 30 (N = 5)	**Mean**	<LOQ	<LOQ	<LOQ	<LOQ
		Median		<LOQ		
		Range		<LOQ – 1.15		
		Freq.	0	20	0	0
	50 – 55 (N = 4)	**Mean**	<LOQ	<LOQ	<LOQ	<LOQ
		Median		<LOQ		
		Range		<LOQ – 1.72		
		Freq.	0	25	0	0
	75 – 80 (N = 5)	**Mean**	<LOQ	<LOQ	<LOQ	<LOQ
		Median		<LOQ		
		Range		<LOQ – 0.90		
		Freq.	0	40	0	0
	100 – 105 (N = 3)	**Mean**	<LOQ	1.04	<LOQ	<LOQ
		Median		1.02		
		Range		<LOQ – 2.41		
		Freq.	0	60	0	0

The vertical distribution of PFOA and PFOS could only be determined at 3M and Vlietbos as only top-soil was collected at Rot and Burchtse Weel (see M&M). Figure 3.2 illustrates the vertical distribution in the soils from these locations. Significant differences in PFOA concentrations were observed between the soil layers at 3M ($p < 0.001$, $F_{3,16} = 15.2$). The concentrations in the third layer (50 – 55 cm) were significantly higher than those in the other layers (all $p < 0.020$). At Vlietbos, the PFOA concentrations did not differ between layers ($p = 0.229$, $F_{4,18} = 1.6$). Concentrations of PFOS at 3M also differed significantly between layers ($p < 0.001$, $F_{3,16} = 14.3$), with PFOS concentrations being significantly lower in the top soil compared to the second (25 – 30 cm) and third (50 – 55 cm) layer (both $p < 0.001$). Similarly, the concentrations in the deepest layer (75 – 80 cm) were lower than those in the second ($p = 0.005$) and third ($p = 0.020$) layers. At Vlietbos, PFOS concentrations differed between layers ($p = 0.005$, $F_{4,18} = 5.3$), as concentrations in the top layer were significantly higher ($p = 0.011$) than in the deepest layer (100 – 105 cm), but no other significant differences were observed. PFBA concentrations at Vlietbos did not differ between the first two layers ($p = 0.319$, $F_{4,18} = 1.4$).

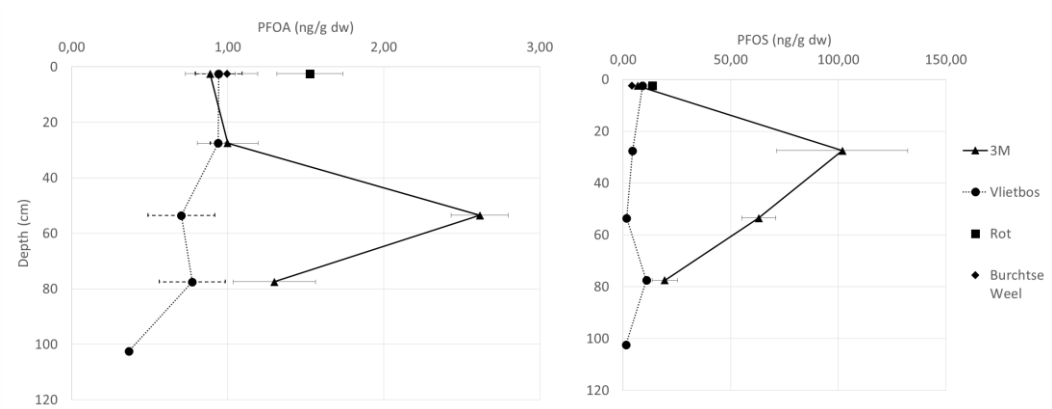


Figure 3.2. Vertical distribution of PFOA and PFOS (ng/g dw ± st. error) in soils from 3M (triangles), Vlietbos (circles), Rot (squares) and Burchtse Weel (diamonds). The soil layers used in the analysis were; 0 – 5, 25 – 30, 50 – 55, 75 – 80 and 100 – 105 cm.

PFBA concentrations in the surface soil differed between locations ($p < 0.001$, $F_{2,12} = 15.0$) were significantly higher at Rot compared to Vlietbos ($p < 0.001$) and Burchtse Weel ($p = 0.003$). PFOA concentrations in the top layer did not differ significantly

between sites ($p = 0.084$, $F_{3,16} = 2.7$). The second ($p = 0.558$) and fourth ($p = 0.157$) layers at 3M and Vlietbos did not differ either. However, soil at 3M showed significantly higher PFOA concentrations in the third layer than Vlietbos ($p < 0.001$). PFOS concentrations in the surface layer did differ between sites ($p < 0.001$, $F_{3,16} = 13.2$) and were significantly higher at Rot compared to 3M ($p = 0.001$) and Burchtse Weel ($p < 0.001$). Additionally, the PFOS concentrations in the surface soil at Burchtse Weel were significantly lower than those at 3M ($p = 0.014$) and Vlietbos ($p < 0.001$). PFOS concentrations in the second ($p = 0.008$) and third ($p = 0.001$) layer were higher at 3M than at Vlietbos ($p = 0.008$). Concentrations in the fourth layer were not different between sites ($p = 0.548$).

Significant differences in PFOA concentrations have been observed between the soil layers at 3M ($p < 0.001$). The concentrations in the third layer (50 – 55) were significantly higher than those in the other layers (all $p < 0.020$). At Vlietbos, the PFOA concentrations did not differ between layers ($p = 0.229$). PFOS concentrations at 3M were significantly lower in the top soil compared to the second (25 – 30 cm) and third (50 – 55 cm) layer (both $p < 0.001$). Similarly, the concentrations in the deepest layer (75 – 80 cm) were lower than those in the second ($p = 0.005$) and third ($p = 0.020$) layers. At Vlietbos, PFOS concentrations in the top layer were significantly higher ($p = 0.011$) than in the deepest layer (100 – 105 cm), but no other significant differences were observed. PFBA concentrations at Vlietbos did not differ between the first two layers ($p = 0.319$).

PFBA concentrations in the surface soil were significantly higher at Rot compared to Vlietbos ($p < 0.001$) and Burchtse Weel ($p = 0.003$). PFOA concentrations in the top layer did not differ significantly between sites ($p = 0.084$). The second ($p = 0.558$) and fourth ($p = 0.157$) layers at 3M and Vlietbos did not differ as well. However, soil at 3M showed significantly higher PFOA concentrations in the third layer than Vlietbos ($p < 0.001$). PFOS concentrations in the surface layer were significantly higher at Rot compared to 3M ($p = 0.001$) and Burchtse Weel ($p < 0.001$). Additionally, the PFOS

concentrations in the surface soil at Burchtse Weel were significantly lower than those at 3M ($p = 0.014$) and Vlietbos ($p < 0.001$). PFOS concentrations in the second ($p = 0.008$) and third ($p = 0.001$) layer were higher at 3M than at Vlietbos ($p = 0.008$). Concentrations in the fourth layer were not different between sites ($p = 0.548$).

3.3.2 Relationships with physicochemical soil properties

The average physicochemical properties of the soil are displayed in Table 3.3 for each location and each soil layer. When all locations were combined, PFBA concentrations in the soil were significantly positively related to TOC ($p < 0.001$, $R^2 = 0.27$) and there was a negative trend with clay content ($p = 0.052$, $R^2 = 0.05$). PFOA concentrations were, on the other hand, positively related to the soil temperature ($p = 0.042$, $R^2 = 0.07$) and there was a positive trend with pH ($p = 0.059$, $R^2 = 0.06$). Similarly, PFOS was only positively related to the soil pH ($p = 0.001$, $R^2 = 0.15$) and there was a positive trend with temperature ($p = 0.075$, $R^2 = 0.04$). When distinguishing the individual locations, at 3M the PFOA concentrations were not related to any soil property (all $p > 0.100$), whereas PFOS concentrations were negatively related to the temperature ($p = 0.011$, $R^2 = 0.23$) and positively to the TOC ($p < 0.001$, $R^2 = 0.48$). At Vlietbos, the PFBA concentrations were positively related to the TOC ($p < 0.001$, $R^2 = 0.72$), the PFOA concentrations were positively related to the clay content ($p = 0.040$, $R^2 = 0.24$) and PFOS concentrations were not related to any soil property (all $p > 0.440$).

Table 3.3. Average physicochemical soil properties in each layer at the sampling sites.

Location	Depth (cm)	pH	Temperature (°C)	TOC (%)	Clay content (%)
3M	0 – 5	6.7	21.3	1.47	0.07
	25 – 30	7.0	18.7	2.89	0.06
	50 – 55	6.9	18.2	0.75	0.05
	75 – 80	6.9	18.7	0.95	0.11
Vlietbos	0 – 5	6.8	17.3	6.05	0.42
	25 – 30	6.8	16.3	0.73	0.05
	50 – 55	6.8	16.0	0.93	0.12
	75 – 80	6.8	15.9	0.93	0.02
	100 – 105	6.7	15.9	0.40	0.0
Rot	0 – 5	7.0	17.5	5.44	0.64
Burchtse Weel	0 – 5	6.9	18.8	3.86	0.41
Westmalle	0 – 5	6.7	13.8	3.99	1.77
	25 – 30	6.6	14.9	3.24	1.25
	50 – 55	6.6	15.0	2.10	2.38
	75 – 80	6.7	14.9	1.83	3.21
	100 - 105	6.7	14.7	1.07	0.87

3.3.3. Associations with microbial parameters

All data on soil respiration, microbial biomass and microbial activity (determined with the bait-lamina) are displayed in Table 3.4. No significant associations between concentrations of PFBA, PFOA and PFOS were observed with soil respiration, microbial activity and microbial biomass (all $p > 0.100$). Neither were any of these microbial parameters associated with soil physicochemical properties (all $p > 0.230$).

Table 3.4. Microbial activity (average number of holes of the bait-lamina sticks consumed after 4 weeks), soil respiration and microbial biomass in the top soil at each location.

Location	Microbial activity (# holes consumed after 4 w)	Soil respiration (µg/h*g dw)	Microbial biomass (µg C/ g soil)
3M	2.0	-0.83	-3.23
Vlietbos	3.2	46.34	3.86
Rot	7.5	13.76	1.19
Burchtse Weel	0.6	0.143	4.01
Westmalle	7.4	11.93	4.54

3.4 Discussion

3.4.1 Soil concentrations

Our results showed no clear gradient of decreasing PFAA concentrations with increasing distance from the fluorochemical plant. This was contradictory to a previous study, performed by Groffen et al. (2019b) on soil and isopods in the same study area. This gradient has also been reported by other studies on PFAA concentrations in wildlife at this site (e.g. Groffen et al., 2017, 2019c; Lopez-Antia et al., 2019).

In order to compare the PFAA concentrations in the soil with literature, a few examples of PFAA concentrations in the surface soils around fluorochemical plants are shown in Table 3.5. In 2016, many PFAAs were detected in the surface soils at 3M, Vlietbos, Rot and Burchtse Weel, often at higher concentrations than those in the present study (Groffen et al., 2019b). Surprisingly, the PFBA and PFOS concentrations at Rot were higher in the present study, which was likely the result of differences in physicochemical soil properties and thus sorption. The TOC was much lower in the study by Groffen et al. (2019b) and TOC is known to play an important role in the sorption of PFAAs (e.g. Milinovic et al., 2015). Groffen et al. (2019b) also observed a higher contribution of PFBA to the total PFAA concentrations in songbird eggs with increasing distance from the fluorochemical plant and suggested that this was likely the result of different pollution pathways or different pollution sources. It is possible that concentrations close to the plant are mainly influenced by the direct industrial pollution, whereas further away the atmospheric degradation of volatile precursor compounds could play a role. The differences between both studies could also be explained by differences in sampling strategy. In the present study, the soil samples were collected in a small area, all relatively close to each other, whereas Groffen et al. (2019b) collected soil samples in the vicinity of nestboxes that were placed across the entire study sites. In addition, we collected soil samples approximately 50 – 100 m south-west from the 3M plant, whereas samples in 2016 were collected in the east of the plant (but still at the plant site). The dominant wind direction in Belgium is from the south-west (Royal Meteorological Institute Belgium (KMI), 2018), indicating that

aerial deposition of PFAAs should mainly affect areas north to east from the fluorochemical plant. This hypothesis could also explain the higher PFOS concentrations observed by D'Hollander et al. (2014) at Blokkersdijk, approximately 0.5 – 1.5 km east from 3M (69 ng/g dw). PFBA concentrations at a fluorochemical industrial park in China (0.6 ng/g dw, Lu et al., 2018) were similar to the concentrations we observed at Vlietbos, but lower to those at Rot and Burchtse Weel. Both PFOA (50.1 ng/g dw) and PFOS (2583 ng/g dw) concentrations at a fluorochemical manufacturing facility in Wuhan, China were much higher than those reported in the present study (Wang et al., 2010). However, concentrations of PFOA (0.79 ng/g dw) and PFOS (7.06 ng/g dw) near a fluorochemical manufacturing facility in Hubei Province, China (Wang et al., 2010), were similar to the ones detected at 3M. Nevertheless, it must be noted that, in most of the studies reported in Table 4.5, it is often unclear which soil layer (i.e. how deep) was collected and defined as top/surface layer. Therefore, it is possible that these comparisons were based on different soil layers.

Elevated concentrations of PFOS and PFOA were mainly observed in subsurface layer, up to approximately 50 cm depth. Hereafter, the concentrations decreased again, suggesting a downward movement of both compounds. This downward movement might result in a contamination of the groundwater and thus pose a potential source of exposure for organisms and communities that rely on this water. The downward movement of PFOS and PFOA has also been reported by Xiao et al. (2015) in soils at a U.S. metropolitan area with both PFOS and PFOA concentrations generally increasing with depth. Similarly, concentrations of ammonium perfluorooctanoate (APFO), the salt of PFOA, were highest in the surface soil and decreased with depth in soils on an alluvial floodplain of the Ohio River (Davis et al., 2007). Sepulvado et al. (2011) also reported the downward migration of PFOA and PFOS in biosolid-amended soils to depths of 120 cm.

Table 3.5. Mean PFAA concentrations (ng/g) in soils published in literature.

Location	Year	PFBA	PFOA	PFUnDA	PFOS	Reference
3M fluorochemical plant, Belgium	2016	1.92	24.0	8.89	1700	Groffen et al., 2019b
	2018	<LOQ	0.89	<LOQ	6.77	The present study
Vlietbos, Belgium	2016	1.33	2.05	<LOQ	22	Groffen et al., 2019b
	2018	0.82	0.94	0.52	8.91	The present study
Rot, Belgium	2016	<LOQ	2.71	<LOQ	3.26	Groffen et al., 2019b
	2018	2.78	1.53	<LOQ	14	The present study
Burchtse Weel, Belgium	2016	0.77	203	<LOQ	7.82	Groffen et al., 2019b
	2018	1.11	0.99	<LOQ	3.95	The present study
Blokkersdijk, Belgium	2006				69	D'Hollander et al., 2014
Daikon Co, Lit, Fluorochemical Industrial Park, China	2015	0.6	62.5	0.2	64.6	Lu et al., 2018
Fluorochemical manufacturing facility in Wuhan, China	2009		50.1		2583	Wang et al., 2010
Fluorochemical manufacturing facility in Hubei Province China	2009		0.79		7.06	

3.4.2 Associations with soil physicochemical properties

As was already described before, TOC plays a key role in the sorption of PFAAs to soils (Campos Pereira et al., 2018; Miao et al., 2017; Milinovic et al., 2015). Therefore, the positive relationship between TOC and PFOS concentrations at 3M was expected. Although chain-length is considered a dominant factor concerning the adsorption to

soils (Ahrens et al., 2010; Higgins and Luthy, 2006), we also observed a positive relationship between TOC and PFBA concentrations when all locations were combined and at Vlietbos separately, which might be caused by larger humic and fulvic acid fractions, which are considered important for the sorption of short-chained PFAAs, such as PFBA (Campos Pereira et al., 2018).

Soils with a higher clay content will have more functional groups to facilitate the sorption of PFAAs (Qi et al., 2014). Although clay content itself is not a measure of the surface charge of the soil, it is considered an indication of potential binding sites for electrostatic interactions (Li YS et al., 2018). Therefore, it was expected that clay content would be positively associated with PFAA concentrations, as was the case for PFOA concentrations at Vlietbos. By contrast, we observed a negative trend between PFBA and clay content when all locations were combined, which could potentially be explained by the difference in water solubility between long-chained and short-chained compounds. For example, Ahrens et al. (2010) reported a variation in PFBA concentrations in water at varying depths, indicating that concentrations can vary depending upon sampling depth of the water column. As short-chained compounds are less hydrophobic, they are more water soluble (e.g. Deng et al., 2012) and as a result it is less likely that they will bind to solid matrices such as soil.

The positive relationship between pH and PFOS concentrations, as well as the positive trend between pH and PFOA concentrations were unexpected as PFAAs are weak acidic chemicals and the proportion of anionic molecule increases with increasing pH, resulting in a decreased sorption to soils (Li YS et al., 2018). In addition, the pH can affect surface properties of the sorbent as an increased pH results in a less positive surface charge on mineral particles (Johnson et al., 2007). The increased PFOA and PFOS concentrations at a higher pH could be the result of the development of more basic surface sites, consisting of carboxyl, alcoholic, phenolic and quinone groups (Schwarzenbach et al., 2003). These surface sites may increase the sorption of calcium

(II) ions onto the soil, which further enhances the sorption of PFOS through electrostatic effects and Ca-bridging mechanisms (You et al., 2010).

Jia et al. (2010) investigated the effect of temperature on the sorption of PFOS on humic acid, an important part of TOC, and found that the sorption capacity was doubled when the temperature increased from 5 to 35°C. This could potentially also explain the positive trend between PFOS concentrations and soil temperature. However, it was expected that the concentrations would decrease with increasing temperature, as we observed for PFOS at 3M, as adsorption is often an exothermic reaction (Zhou et al., 2010).

Despite the significant correlations between PFAA concentrations and soil physicochemical properties, these correlations were often weak. This is likely the result of a high similarity in soil properties at the study sites. Therefore, future studies should include sites that contain distinct differences in physicochemical properties to further improve our understanding of these associations.

3.4.3 Associations with soil microbial parameters

PFAAs have a dual effect on stimulating the growth of some soil bacteria, while inhibiting the growth of others and the extents of this effect varies among analytes (Qiao et al., 2018). Although we did not observe any associations between PFAA concentrations and soil microbial parameters, it is likely that other factors, which we did not measure, affected these results. For example, seasonal variations in microbial respiration and activity have been reported in turfgrass systems, where a lower microbial biomass and activity in September were associated with lower soil available nitrogen (Yao et al., 2011). To minimize the influence of environmental conditions, we recommend investigating the associations between microbial parameters and PFAA concentrations under controlled conditions. Studies that investigate the effects of PFAAs on soil microbial communities are scarce and often only investigate species abundance, richness and diversity (e.g. Bao et al., 2018; Li BX et al., 2017). The highest bacterial abundance was found in the top soil and was potentially influenced by PFHxS,

at a site with a long exposure to severe PFAA pollution (Li BX et al., 2017). The links between PFHxS concentrations and bacterial abundance were not observed by Bao et al. (2018), who reported that soil TOC might be a key determinant of bacterial abundance. Similarly, Li BX et al. (2017) reported that archaeal abundance could be affected by PFHxS, whereas Bao et al. (2018) did not observe such associations. Ochoa-Herrera et al. (2016) also observed no toxic effects of PFOS and short-chained PFAAs on the methanogenic activity (performed exclusively by archaea) of anaerobic wastewater sludge.

3.5 Conclusion

Our results show that both PFOA and PFOS concentrations were elevated in the subsurface layer (up to 50 cm), after which they decreased again, suggesting a downward migration of both analytes in the soil. This downward movement might pose a potential threat for the contamination of the groundwater and, consequently, people and organisms who rely on this water. The soil concentrations were influenced by multiple physicochemical properties of the soil, including TOC, pH, clay content and soil temperature, which suggests differences in bioavailability and sorption/desorption capacities between different soil types. Although expected, we did not observe any influence of PFAA contamination in the top soil on microbial activity and biomass nor soil respiration.

3.6 Acknowledgements

This work was supported by the Research Foundation Flanders (FWO; research project G038615N to LB and ME). The authors would like to thank T. Willems, who performed the UPLC analysis, and T. van der Spiet for his help with the particle size analysis.

3.7 Supplementary data

Table S3.1. Recoveries (%) for each target analyte in all soil samples (N = 75) and the number of samples with a recovery less than 10% for a specific analyte.

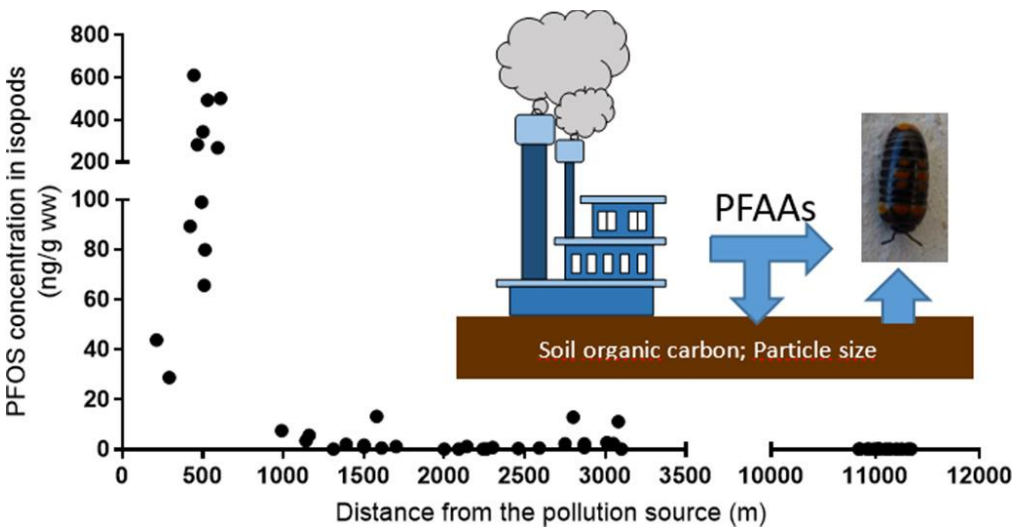
Analyte	Recovery (%)	# samples with recovery <10%
PFBA	4 – 100	3
PFPeA	4 – 100	7
PFHxA	4 – 100	5
PFHpA	4 – 100	5
PFOA	6 – 100	5
PFNA	5 – 100	4
PFDA	3 – 100	5
PFUnDA	4 – 100	7
PFDoDA	3 – 100	6
PFTrDA	3 – 100	7
PFTeDA	3 – 100	8
PFBS	7 – 100	3
PFHxS	7 – 100	3
PFOS	3 – 100	2
PFDS	3 – 100	3

4. Do concentrations of perfluoroalkylated acids (PFAAs) in isopods reflect concentrations in soil and songbirds? A study using a distance gradient from a fluorochemical plant

Based on:

Thimo Groffen, Marcel Eens and Lieven Bervoets (2019b). Science of the Total Environment 657: 111 - 123. https://doi.org/10.1016/j.scitotenv.2018.12.072

Tables were modified to fit the size of the paper. One sentence has been removed. No further alterations were made.



Abstract

Perfluoroalkylated acids (PFAAs) are persistent chemicals that have been detected globally in the environment and in wildlife. Although it is known that PFAAs sorb to solid matrices, little is known on PFAA concentrations in soils. PFAA pollution has often been studied in aquatic invertebrates. However, this has rarely been done on terrestrial species. In the present study, we examined whether the concentrations of 15 PFAAs in isopods (Oniscidae), collected at a fluorochemical plant and in four other areas, representing a gradient in distance from the pollution source (1 km to 11 km), were related to those in the soil and in eggs of a songbird, the great tit (*Parus major*), collected in the same areas. Additionally, we examined the effect of physicochemical properties such as total organic carbon (TOC) and clay content on the relationship between the concentrations in soil and isopods. Finally, we examined the composition profile in the soil and isopods.

Mean PFOS and PFOA concentrations of 1700 ng/g dw and 24 ng/g dw were detected in the soil at the plant. PFOS and PFPeA were the dominant PFAAs in isopods and were detected at mean concentrations of 253 and 108 ng/g ww, respectively. The great tit eggs showed elevated mean PFOS concentrations of 55,970 ng/g ww. In most cases, PFAA concentrations decreased with increasing distance from the plant.

As PFAA concentrations in isopods were correlated with concentrations in the soils, isopods could serve as a bioindicator for PFAA concentrations in soils. Additionally, there were indications that isopods could also serve as a bioindicator for PFAA concentrations in eggs of great tits. However, these indications were only the case at two locations, showing the need to further monitor the possibilities of using isopods as a bioindicator for PFAA concentrations in songbird eggs.

4.1 Introduction

The global distribution of perfluoroalkylated acids (PFAAs) over the past decades has led to a growing scientific attention and public concern towards these chemicals. The strong carbon-fluorine bonds and their hydrophobic and lipophobic character result in outspoken physicochemical properties, which make them suitable for numerous applications such as soil repellents, food-contact paper and fire-fighting foams (Buck et al., 2011; Kissa, 2001). These applications may cause PFAAs to end up in the environment either through direct pollution or via environmental degradation of precursor compounds (Buck et al., 2011; Prevedouros et al., 2006). Additionally, gas- and particle-phase atmospheric long-range transport may also result in the distribution of PFAAs in the environment (Barber et al., 2007; Ellis et al., 2003; Schenker et al., 2008). PFAAs have been reported globally in the environment, wildlife and humans (Butt et al., 2010; D'Hollander et al., 2010; Giesy and Kannan, 2001, Giesy and Kannan, 2002; Groffen et al., 2017, Groffen et al., 2018; Houde et al., 2006; Miller et al., 2015), which shows their bioaccumulative potential.

Due to their high bioaccumulative potential and toxicity (Conder et al., 2008), there has been a growing scientific concern towards long-chain perfluoroalkyl sulfonic acids (PFSAs) and perfluoroalkyl carboxylic acids (PFCAs) over the past decades (Conder et al., 2008). In 2002, the major manufacturer of PFAAs, 3M, phased-out the production of perfluorooctane sulfonate (PFOS, $C_8F_{17}SO_3H$), perfluorooctanoic acid (PFOA, $C_7F_{15}COOH$) and related compounds, based on their persistence in the environment, widespread distribution and potential health effects. Additionally, PFOS was included in the Stockholm Convention on Persistent Organic Pollutants in 2009, which allows limited on-going use of PFOS. Although these measures appear to have reduced environmental PFOS concentrations, concentrations of other PFAAs are sometimes still rising (Ahrens et al., 2011c; FIlipovic et al., 2015b; Groffen et al., 2017, Groffen et al., 2019c; Miller et al., 2015). Furthermore, short-chain PFAAs, which are widely used as alternatives to long-chain PFAAs, are known to have extremely persistent final degradation products resulting in a permanent exposure of organisms to these

compounds (Brendel et al., 2018). Therefore, it is still necessary to further monitor PFAAs in the environment.

Soils are important sinks for many persistent organic pollutants (POPs), such as polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) (Cetin et al., 2017; Maqsood and Murugan, 2017; Mueller et al., 2006; Rankin et al., 2016; Xiao et al., 2016). Although it is known that PFAAs sorb to solid matrices (Ahrens et al., 2011b; Li YS et al., 2018; Miao et al., 2017; Qian et al., 2017; Rankin et al., 2016; Wei et al., 2017), there is limited knowledge on the possible role of soils as sinks for PFAAs.

Invertebrates have been used in numerous field studies that monitor PFAA concentrations. However, most of these studies target aquatic invertebrates (e.g. Babut et al., 2017; Groffen et al., 2018; Lescord et al., 2015; Loi et al., 2011), whereas field data on terrestrial invertebrates remain scarce. Only one field study has been performed on isopods in Belgium (D'Hollander et al., 2014), one on adult Odonata in South Africa (Lesch et al., 2017) and one on earthworms in the USA (Zhu and Kannan, 2019). Other studies on terrestrial invertebrates, were often performed on earth worms under laboratory conditions (e.g. Das et al., 2015; Zhao et al., 2013; Zhao Y et al., 2017). Furthermore, the relationships between PFAA concentrations in the soil and invertebrates, and the effects of physicochemical properties on these relationships, have rarely been studied (Das et al., 2015). Finally, information on trophic transfer in the terrestrial food chain, from soil to invertebrates and eventually vertebrates, is scarce (D'Hollander et al., 2014).

In the present study we measured the concentrations of multiple PFAAs in the soil and isopods along a distance gradient from a fluorochemical plant, and investigated whether the concentrations in the isopods were correlated to the PFAA concentrations in the soil and in the eggs of great tits (*Parus major*), which were collected at the same time and locations by Groffen et al. (2019c). Additionally, we examined the role of physicochemical properties of the soil such as total organic carbon (TOC) and clay

content on the relationship between PFAA concentrations in the soil and isopods. Finally, the composition profiles in the soil and isopods were determined.

4.2 Materials and method

4.2.1 Sample collection

Soil and invertebrate samples were collected in June 2016. Five sampling sites (Fig. 4.1), representing a gradient from the 3M fluorochemical plant in Antwerp, Belgium, were selected based on prior biomonitoring studies in the vicinity of this plant (Dauwe et al., 2007; D'Hollander et al., 2014; Groffen et al., 2017; Hoff et al., 2005; Lopez-Antia et al., 2017): 3 M, Vlietbos (1 km SE from 3M), Rot-Middenvijver (hereafter Rot; 2.3 km ESE from 3M), Burchtse Weel (3 km SE from 3M) and Fort 4 (11 km SE from 3M). At each location approximately 10 soil samples were collected, within a 3 m radius of nest boxes that were used in multiple biomonitoring studies (e.g. Groffen et al., 2019c), by using a stainless steel shovel. Samples were sieved through an ASTM E 11-81 Test Sieve (1.7 mm) and stored in 50 mL polypropylene (PP) tubes until further analysis.

At the same sites where the soil samples were collected, isopods (Oniscidae) were collected by picking them off the ground, trunks of trees and rotting wood, and pooled ($N \geq 10$) into 50 mL PP tubes. As variation in PFOS concentrations within a clutch has been demonstrated for Audouin's gulls (*Larus audouinii*, Vicente et al., 2015), we expected that sampling a fixed egg of each nest would reduce the variation among nests at a site compared to random sampling (Groffen et al., 2019c). Therefore, we collected the third egg of great tit nests, before incubation had started during the breeding season of 2016.

The PFAA concentrations in bird eggs in the present study are a part of a larger dataset, reported by Groffen et al. (2019c). All samples were stored at −20 °C prior to further analyses.

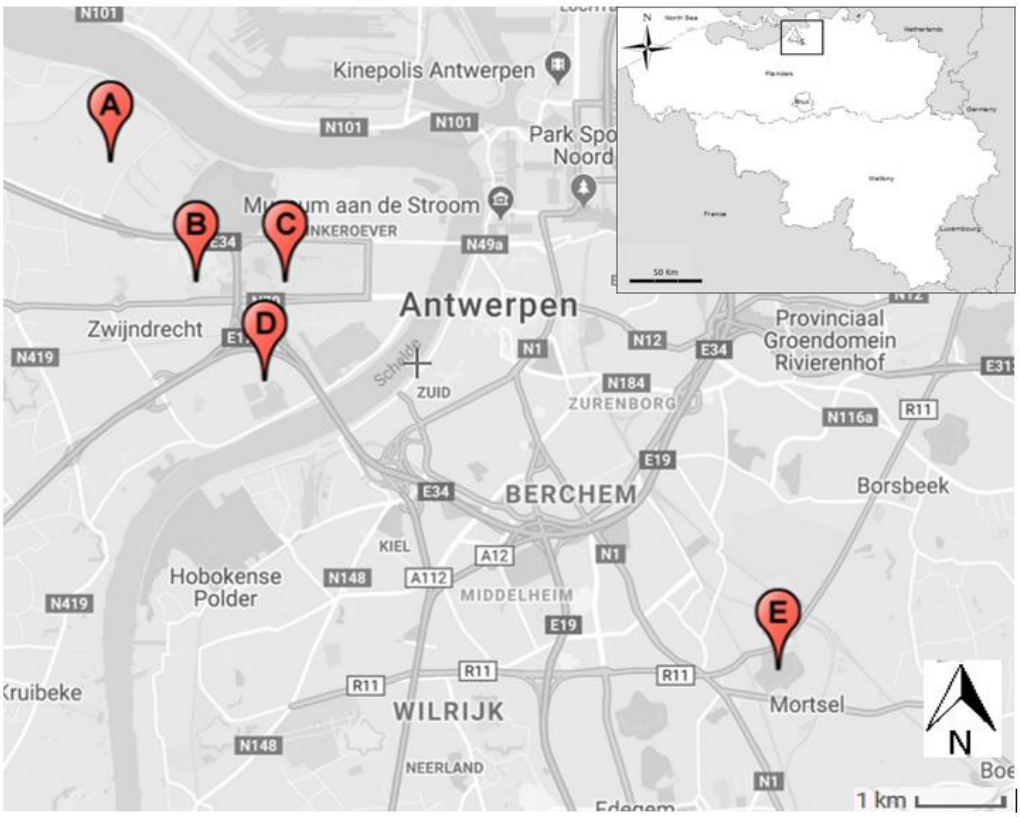


Figure 4.1. Overview of the study area in Antwerp, Belgium. Sampling locations are indicated as letters: A. Fluorochemical plant 3M, B. Vlietbos, C. Middenvijver-Rot, D. Burchtse Weel, E. Fort 4. Figure adopted from Groffen et al. (2019c).

4.2.2 Sample extraction

Prior to the analysis, soil samples were air-dried and eggs were homogenized by repeatedly sonicating and vortexing. To each sample, 10,000 pg (80 µL, 125 pg/µL) of the ISTD mixture was added. After mixing, 10 mL of ACN was added and samples were sonicated (3 × 10 min, Branson 2510) and left overnight on a shaking plate (135 rpm, room temperature, GFL 3020, VWR International, Leuven, Belgium). After centrifugation (4 °C, 2400 rpm, 10 min, Eppendorf centrifuge 5804R, rotor A-4-44), the supernatant was transferred to a 14 mL PP tube. Chromabond HR-XAW Solid Phase Extraction (SPE) cartridges (Application No. 305200, SPE department, Macherey-Nagel, Germany, 2009) were conditioned with 5 mL of ACN. After equilibration with 5 mL of

MQ, the samples were loaded onto the cartridges. The cartridges were washed with 5 mL of 25 mM ammonium acetate in MQ and 2 mL of ACN and eluted with 2 × 1 mL of 2% ammonium hydroxide in ACN. The eluent was dried completely using a rotational-vacuum-concentrator (Eppendorf concentrator 5301, Hamburg, Germany) and reconstituted with 200 µL of 2% ammonium hydroxide in ACN. Samples were vortex-mixed for at least 1 min and filtrated through an Ion Chromatography Acrodisc 13 mm Syringe Filter with 0.2 µm Supor (PES) Membrane (VWR International, Leuven, Belgium) attached to a PP auto-injector vial.

The extraction procedure for the isopods was based on a method described by Powley et al. (2005) with minor modifications. The isopods were homogenized using a TissueLyser LT (Qiagen GmbH, Germany) with stainless steel beads (5 mm; Qiagen GmbH, Germany). The protocol follows the same steps as described previously for the method in soil and eggs until the samples were centrifuged (4 °C, 2400 rpm, 10 min, Eppendorf centrifuge 5804R, rotor A-4-44). Hereafter, the supernatant was transferred to a 15 mL PP tube and dried to approximately 0.5 mL in the rotational-vacuum-concentrator. To eliminate pigments, the concentrated extract was transferred to a PP Eppendorf tube containing 50 mg of graphitized carbon powder (Supelclean ENVI-Carb, Sigma-Aldrich, Belgium) and 50 µL of glacial acetic acid. In addition, 2 × 250 µL of ACN, used to rinse the 15 mL tubes, was added to the Eppendorf tubes. These tubes were vortexed and centrifuged (4 °C, 10000 rpm, 10 min, Eppendorf centrifuge 5415R; Rotor F 45-24-11) and the supernatant was treated equal as the eluent from the method described for soil and egg samples.

4.2.3 Chemical analysis

All used abbreviations are according to Buck et al. (2011). Fifteen target analytes were selected, including 4 PFSAs and 11 PFCAs. All target analytes and the isotopically mass-labelled internal standards (ISTDs; Wellington Laboratories, Guelph, Canada) used in the quantification of these analytes are illustrated in Table 4.1. In addition, HPLC grade Acetonitrile (ACN; LiChrosolv, Merck Chemicals, Belgium), ammonium acetate (VWR

International, Belgium), ammonium hydroxide (Filter Service N.V., Belgium) and Milli-Q (MQ; 18.2 mΩ; TOC: 2.0 ppb; Merck Millipore, Belgium) were used.

4.2.4 UPLC-TQD analysis and quantification

To analyze the PFAAs, we used ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS, ACQUITY, TQD, Waters, Milford, MA, USA). Target analytes were separated using an ACQUITY BEH C18 column (2.1 × 50 mm; 1.7 µm, Waters, USA) and an ACQUITY BEH C18 pre-column (2.1 × 30 mm; 1.7 µm, Waters, USA) was inserted between the solvent mixer and the injector to retain any PFAAs contamination originating from the system. The mobile phase solvents were A) 0.1% formic acid in water and B) 0.1% formic acid in ACN. The flow rate was set at 450 µL/min with an injection volume of 10 µL. The gradient started at 65% A, decreased in 3.4 min to 0% A and returned to 65% A at 4.7 min. Multiple reaction monitoring (MRM) of two diagnostic transitions per target analyte was used to identify and quantify the PFAAs. The diagnostic transitions are displayed in Table 4.1.

Table4.1. Target analytes, isotopically mass-labelled internal standards (ISTDs) used for quantification and MRM transitions.

Compound	Internal standard (ISTD) used for quantification	Precursor ion (m/z)	Product ion (m/z)	
			Diagnostic product ion 1	**Diagnostic product ion 2**
PFBS	$^{18}O_2$-PFHxS	299	80	99
PFHxS	$^{18}O_2$-PFHxS	399	80	99
PFOS	[1,2,3,4-$^{13}C_4$]PFOS	499	80	99
PFDS	[1,2,3,4-$^{13}C_4$]PFOS	599	80	99
PFBA	$^{13}C_4$-PFBA	213	169	169
PFPeA	$^{13}C_4$-PFBA	263	219	219
PFHxA	[1,2-$^{13}C_2$]PFHxA	313	269	119
PFHpA	[1,2-$^{13}C_2$]PFHxA	363	319	169
PFOA	[1,2,3,4-$^{13}C_4$]PFOA	413	369	169
PFNA	[1,2,3,4,5-$^{13}C_5$]PFNA	463	419	169
PFDA	[1,2-$^{13}C_2$]PFDA	513	469	219
PFUnDA	[1,2-$^{13}C_2$]PFUnDA	563	519	169
PFDoDA	[1,2-$^{13}C_2$]PFDoDA	613	569	319
PFTrDA	[1,2-$^{13}C_2$]PFDoDA	663	619	319
PFTeDA	[1,2-$^{13}C_2$]PFDoDA	713	669	169

4.2.5 Physicochemical properties of the soil

To determine the organic carbon content (TOC) of the soil, the loss on ignition method, as described by Heiri et al. (2001), was used. Approximately 1 g of the soil was oven-dried at 60 °C. Empty aluminum-foil bags were dried at 105 °C for at least 2 h, cooled to room temperature in a desiccator and weighed. Hereafter, the bags were filled with the dried soil, weighed and oven-dried at 105 °C for at least one day. After cooling down, the samples were weighed again and incinerated in a muffle furnace at 550 °C for at least 5 h. Finally, after cooling down in a desiccator, weight loss was determined and TOC was calculated using (Formula 4.1), (Formula 4.2).

$$LOI_{550}(\%) = \frac{(DW_{105} - DW_{550})}{DW_{105}} * 100 \quad (4.1)$$

$$TOC\ (\%) = LOI_{550}/1.742 \quad (4.2)$$

With LOI, the loss on ignition after 550 °C and DW the dry weight of the sample after drying at 105 °C or 550 °C.

The clay content (particles with a size <4 µm) of the soil was assessed by using a Malvern Mastersizer 2000 and Hydro 2000G. Prior to the analysis the samples were pretreated with 40 mL 33% hydrogen peroxide (VWR Chemicals, Leuven, Belgium) and 9 mL 30% hydrochloric acid (VWR Chemicals, Leuven, Belgium) to destruct organic material and iron conglomerates in the soil. In addition, the samples were boiled to speed up the destruction process, and sieved over a 2.0 mm test sieve prior to the analysis.

4.2.6 Quality assurance

Per 10 samples, one procedural blank was analyzed as quality control. Concentrations in the blanks were all <LOQ. Method recoveries for the target analytes varied between 4% and 50% in the isopod samples and between 16% and 100% in soil samples. Individual limits of quantification (LOQs) were determined based on a signal-to-noise (S/N) ratio of 10 and are displayed in Table 4.2 for soil, Table 4.3 for isopods and Table 4.4 for great tit eggs.

4.2.7 Statistical analysis

Statistical analyses were performed using R Studio and Graphpad Prism 7.04. To obtain a normal distribution, PFAA concentrations and TOC values were log-transformed. Compounds with a detection frequency below 50% at a location were excluded from further analysis.

A reverse Kaplan-Meier (KM) analysis and a Mantel-Cox pairwise comparison test were used to evaluate differences between locations in PFAA concentrations in soil and in isopods. As this test is nonparametric, untransformed data was used to perform the analysis. The reverse KM test is commonly used in the survival analysis of left censored data (Gillespie et al., 2010) and is a useful tool to cope with concentrations below the LOQ (Groffen et al., 2017; Jaspers et al., 2013). In all other analyses, concentrations <LOQ were substituted with a value of LOQ/2 (Bervoets et al., 2004; Custer et al., 2000). Relationships between PFAA concentrations in the soil and in isopods and the role of the physicochemical properties on these relationships were tested using multiple linear regressions. Spearman correlation tests were used to test for associations between PFAA concentrations in the soil and physicochemical characteristics of the soil and for associations between TOC and clay content. Similarly, concentrations in the soil and isopods were correlated (spearman correlation test) with concentrations in third egg of great tits (*Parus major*) collected from the nest boxes. Differences between locations in TOC and clay content were assessed using a One-way ANOVA.

4.3 Results

4.3.1 PFAA concentrations in soil, isopods and songbird eggs

Table 4.2 shows an overview of the median and mean concentrations, ranges and detection frequencies of PFAAs in the soil. Fig. 4.2 shows the concentrations of PFBA, PFOA, PFDoDA, PFBS and PFOS in the soil in function of the distance from the pollution source. The center of the fluorochemical plant was considered as the pollution source (0 m).

Only PFBA, PFOA, PFDoDA, PFBS and PFOS were detected at >50% of the sites. Therefore, only these PFAAs have been used in further statistical analysis. PFBA concentrations did not differ among study sites (all $p > 0.05$). PFOA concentrations at the plant site were significantly higher than those at all other locations (all $p < 0.001$). PFOA concentrations at Rot were also significantly higher than those at Burchtse Weel ($p = 0.025$) and Fort 4 ($p = 0.05$). Similarly, PFDoDA concentrations were significantly higher at the plant site compared to Vlietbos and Fort 4 (both $p < 0.001$). Concentrations of PFBS were significantly higher at the plant site compared to Vlietbos ($p = 0.031$) and finally, PFOS concentrations at the plant site were significantly higher compared to all other locations (all $p < 0.001$). At Vlietbos, the PFOS concentrations were significantly higher than all locations further away (all $p < 0.001$), whereas the PFOS concentrations at Rot were significantly lower than those further away at Burchtse Weel and Fort 4 (both $p < 0.005$).

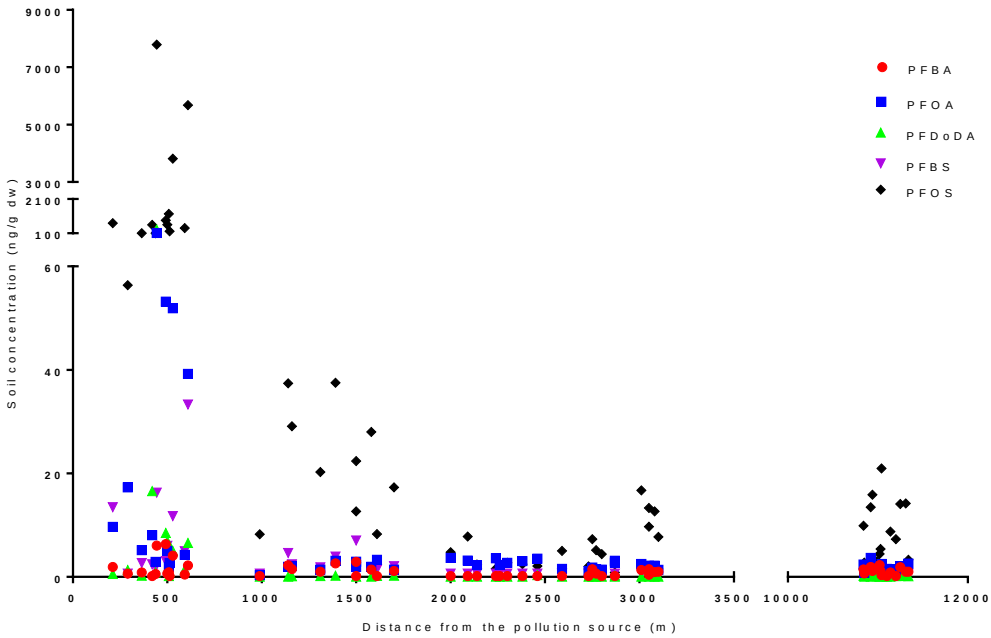


Figure 4.2. PFAA concentrations (ng/g dw) in soil collected along the distance gradient from the pollution source. The center of the fluorochemical plant is selected as the start of the gradient (0 m).

An overview of median and mean concentrations, ranges and detection frequencies of PFAAs in isopods is given in Table 4.3. PFOS was the only PFAA with detection frequencies >50% at all of the sites. Therefore, only PFOS was included in further statistical analysis. Fig. 4.3 shows the PFOS concentrations in isopods in function of the distance from the pollution source. PFOS concentrations were significantly higher at 3M compared to all other locations (all $p < 0.001$). In addition, the PFOS concentrations at Vlietbos and Burchtse Weel were both significantly higher than those at Rot ($p = 0.003$ and 0.001, respectively).

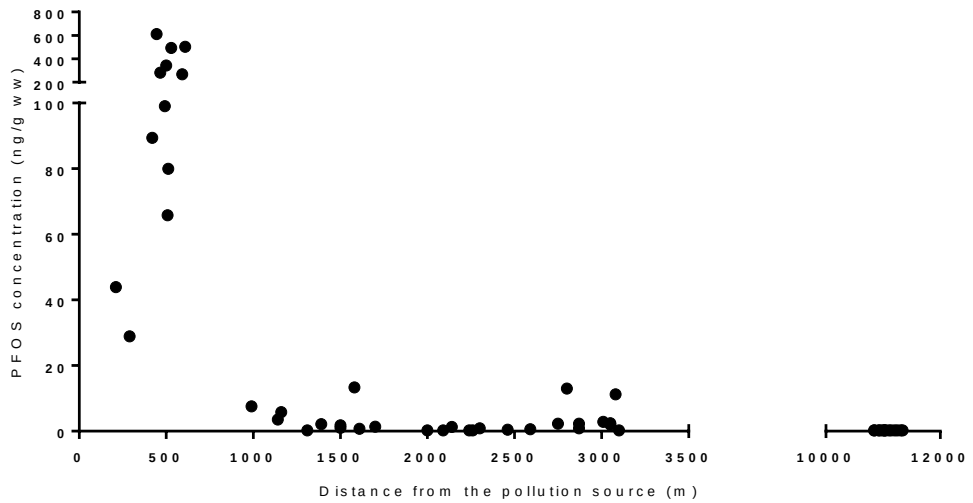


Figure 4.3. PFOS concentrations (ng/g ww) in isopods, collected along the distance gradient from the pollution source. The center of the fluorochemical plant is selected as the start of the gradient (0 m).

Finally, an overview of median and mean concentrations, ranges and detection frequencies in great tit eggs is given in Table 4.4. The results in Table 4.4 are a part of a larger dataset reported by Groffen et al. (2019c). The concentrations in bird eggs in function of the distance from the pollution source are displayed in Fig. 4.4. PFOS concentrations were significantly higher at 3M compared to all other locations (all $p < 0.001$). Furthermore, PFOS concentrations at Rot were significantly higher than those at Vlietbos ($p = 0.019$) and Fort 4 ($p < 0.001$). Similarly, concentrations of PFNA

were also significantly higher at 3M compared to the other locations (all $p < 0.001$). Songbird eggs at 3M also contained significantly higher concentrations of PFOA ($p = 0.030$), PFDA ($p < 0.001$), PFDoDA ($p < 0.001$) and PFTrDA ($p = 0.002$), PFTeDA ($p = 0.028$) than at Fort 4. Concentrations of PFDA and PFDoDA were significantly higher at 3M compared to Rot (both $p < 0.001$). Finally, PFDoDA and PFTrDA concentrations were significantly higher at 3M compared to Burchtse Weel (both $p < 0.001$) and PFTrDA concentrations at 3M were also higher compared to Vlietbos ($p = 0.003$).

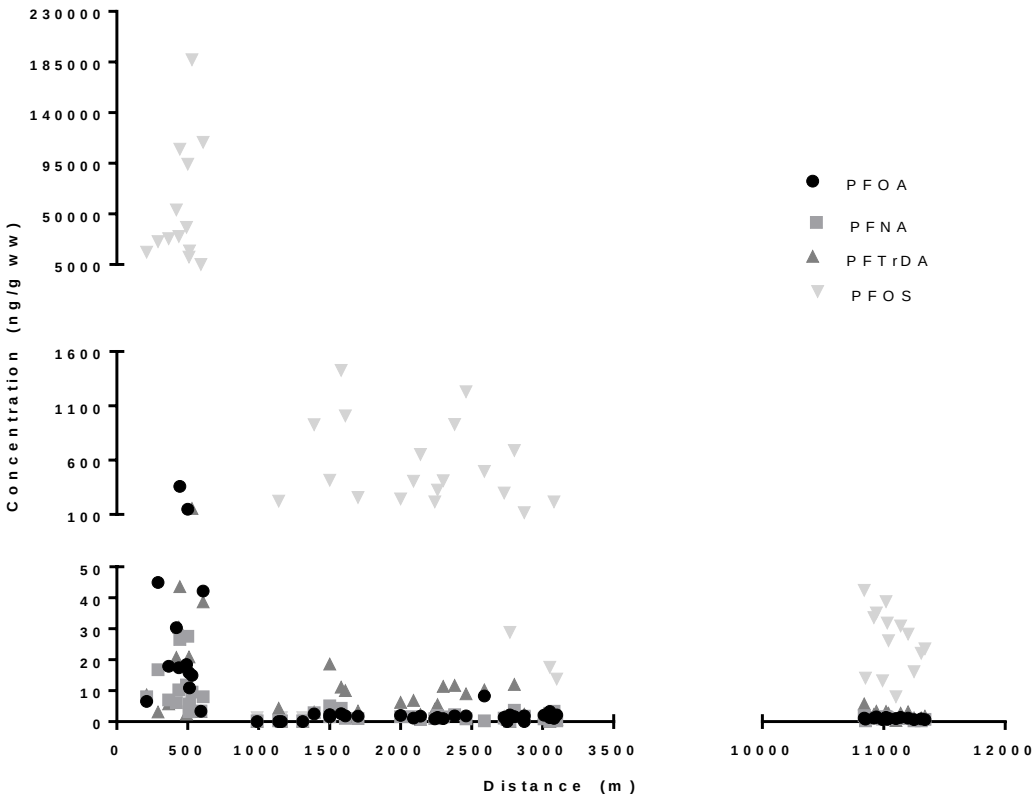


Figure 4.4. PFAA concentrations (ng/g ww) in great tit eggs collected along the distance gradient from the pollution source. The center of the fluorochemical plant is selected as the start of the gradient (0 m). The presented data is part of a larger dataset, reported by Groffen et al. (2019c).

Table 4.2. Individual limits of quantification (LOQs; ng/g dw, determined as 10x the S/N ratio), median and mean concentrations (ng/g dw), range (ng/g dw) and detection frequencies (Freq) of PFAAs in soil collected at the five sampling sites: a perfluorochemical plant and at four sites with increasing distance from the plant site (i.e. 1 km Vlietbos, 2.3 km Rot, 3 km Burchtse Weel and 11 km Fort 4). Different letters indicate significant differences ($p \leq 0.05$) between sampling sites in PFAA concentrations with a detection frequency > 50%.

		PFCAs										
		PFBA	**PFPeA**	**PFHxA**	**PFHpA**	**PFOA**	**PFNA**	**PFDA**	**PFUnDA**	**PFDoDA**	**PFTrDA**	**PFTeDA**
LOQ		0.30	0.97	0.31	1.67	0.73	0.26	0.33	0.27	0.08	0.04	0.04
Plant (n = 13)	Median	0.85 A	<LOQ	0.42	<LOQ	8.07 A	0.34	<LOQ	0.43	1.39 A	0.59	0.06
	Mean	1.92	5.54	2.11	1.43	24	0.83	1.05	8.89	28	12	1.18
	Range	<LOQ – 6.33	<LOQ – 26	<LOQ – 11	<LOQ – 4.75	1.97 – 114	<LOQ – 2.53	<LOQ – 7.28	<LOQ – 105	0.16 – 316	0.05 – 126	<LOQ – 12
	Freq	85	46	62	23	100	69	46	77	100	100	54
Vlietbos (n = 10)	Median	1.28 A	<LOQ	<LOQ	<LOQ	1.94 BC	<LOQ	<LOQ	<LOQ	0.09 B	<LOQ	<LOQ
	Mean	1.33	<LOQ	<LOQ	<LOQ	2.05	<LOQ	<LOQ	<LOQ	0.12	<LOQ	<LOQ
	Range	<LOQ – 2.92	<LOQ – 1.03	<LOQ – 0.37	<LOQ	<LOQ – 3.30	<LOQ – 0.44	<LOQ – 0.48	<LOQ – 0.47	<LOQ – 0.24	<LOQ – 0.09	<LOQ
	Freq	70	10	10	0	90	30	10	30	50	0	0

Table 4.2. (continued) Individual limits of quantification (LOQs; ng/g dw, determined as 10x the S/N ratio), median and mean concentrations (ng/g dw), range (ng/g dw) and detection frequencies (Freq) of PFAAs in soil collected at the five sampling sites: a perfluorochemical plant and at four sites with increasing distance from the plant site (i.e. 1 km Vlietbos, 2.3 km Rot, 3 km Burchtse Weel and 11 km Fort 4). Different letters indicate significant differences ($p \leq 0.05$) between sampling sites in PFAA concentrations with a detection frequency > 50%.

		PFCAs										
		PFBA	**PFPeA**	**PFHxA**	**PFHpA**	**PFOA**	**PFNA**	**PFDA**	**PFUnDA**	**PFDoDA**	**PFTrDA**	**PFTeDA**
LOQ		0.30	0.97	0.31	1.67	0.73	0.26	0.33	0.27	0.08	0.04	0.04
Rot (n = 10)	Median	<LOQ	<LOQ	<LOQ	<LOQ	2.89 C	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Mean	<LOQ	<LOQ	<LOQ	<LOQ	2.71	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Range	<LOQ	<LOQ	<LOQ	<LOQ	1.12 – 3.69	<LOQ	<LOQ	<LOQ	<LOQ – 0.36	<LOQ	<LOQ
	Freq	0	0	0	0	100	0	0	0	10	0	0
Burchtse Weel (n = 10)	Median	0.78 A	<LOQ	<LOQ	<LOQ	1.98 B	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Mean	0.77	<LOQ	<LOQ	<LOQ	2.03	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Range	<LOQ – 1.48	<LOQ	<LOQ	<LOQ	1.37 – 3.12	<LOQ – 0.38	<LOQ	<LOQ – 0.54	<LOQ – 0.28	<LOQ	<LOQ
	Freq	70	0	0	0	100	10	0	20	10	0	0
Fort 4 (n = 14)	Median	1.08 A	<LOQ	<LOQ	<LOQ	1.83 B	<LOQ	<LOQ	<LOQ	0.13 B	<LOQ	<LOQ
	Mean	1.12	<LOQ	<LOQ	<LOQ	1.95	<LOQ	<LOQ	<LOQ	0.12	0.06	<LOQ
	Range	<LOQ – 2.27	<LOQ – 1.65	<LOQ	<LOQ	0.82 – 3.66	<LOQ – 0.53	<LOQ – 0.47	<LOQ – 0.41	<LOQ – 0.29	<LOQ – 0.19	<LOQ
	Freq	86	21	0	0	100	29	14	14	57	36	0

Table 4.2. (continued) Individual limits of quantification (LOQs; ng/g dw, determined as 10x the S/N ratio), median and mean concentrations (ng/g dw), range (ng/g dw) and detection frequencies (Freq) of PFAAs in soil collected at the five sampling sites: a perfluorochemical plant and at four sites with increasing distance from the plant site (i.e. 1 km Vlietbos, 2.3 km Rot, 3 km Burchtse Weel and 11 km Fort 4). Different letters indicate significant differences ($p \leq 0.05$) between sampling sites in PFAA concentrations with a detection frequency > 50%.

		PFSAs			
		PFBS	**PFHxS**	**PFOS**	**PFDS**
LOQ		1.31	4.91	0.67	3.26
Plant (n = 13)	Median	4.01 A	<LOQ	606 A	3.34
	Mean	7.84	6.88	1700	33
	Range	<LOQ – 33	<LOQ – 32	56 – 7800	<LOQ - 282
	Freq	92	31	100	54
Vlietbos (n = 10)	Median	2.13 B	<LOQ	21 B	<LOQ
	Mean	2.79	<LOQ	22	<LOQ
	Range	<LOQ – 7.04	<LOQ	8.24 – 37	<LOQ
	Freq	90	0	100	0

Table 4.2. (continued) Individual limits of quantification (LOQs; ng/g dw, determined as 10x the S/N ratio), median and mean concentrations (ng/g dw), range (ng/g dw) and detection frequencies (Freq) of PFAAs in soil collected at the five sampling sites: a perfluorochemical plant and at four sites with increasing distance from the plant site (i.e. 1 km Vlietbos, 2.3 km Rot, 3 km Burchtse Weel and 11 km Fort 4). Different letters indicate significant differences ($p \leq 0.05$) between sampling sites in PFAA concentrations with a detection frequency > 50%.

		PFSAs			
		PFBS	**PFHxS**	**PFOS**	**PFDS**
LOQ		1.31	4.91	0.67	3.26
Rot (n = 10)	Median	<LOQ	<LOQ	2.41 C	<LOQ
	Mean	<LOQ	<LOQ	3.26	<LOQ
	Range	<LOQ	<LOQ	1.57 – 7.81	<LOQ
	Freq	0	0	100	0
Burchtse Weel (n = 10)	Median	<LOQ	<LOQ	7.51 D	<LOQ
	Mean	<LOQ	<LOQ	7.82	<LOQ
	Range	<LOQ	<LOQ	<LOQ – 17	<LOQ
	Freq	0	0	90	0
Fort 4 (n = 14)	Median	<LOQ	<LOQ	8.03 D	<LOQ
	Mean	<LOQ	<LOQ	8.84	<LOQ
	Range	<LOQ	<LOQ	<LOQ – 21	<LOQ
	Freq	0	0	93	0

Table 4.3. Individual limits of quantification (LOQs; ng/g dw, determined as 10x the S/N ratio), median and mean concentrations (ng/g dw), range (ng/g dw) and detection frequencies (Freq) of PFAAs in isopods collected at the five sampling sites: a perfluorochemical plant and at four sites with increasing distance from the plant site (i.e. 1 km Vlietbos, 2.3 km Rot, 3 km Burchtse Weel and 11 km Fort 4). Different letters indicate significant differences ($p \leq 0.05$) between sampling sites in PFAA concentrations with a detection frequency > 50%.

		PFCAs										
		PFBA	**PFPeA**	**PFHxA**	**PFHpA**	**PFOA**	**PFNA**	**PFDA**	**PFUnDA**	**PFDoDA**	**PFTrDA**	**PFTeDA**
LOQ		1.34	1.84	4.80	6.92	0.74	0.31	0.98	1.20	0.96	0.30	1.40
Plant (n = 12)	Median	12	87	<LOQ	<LOQ	7.56	<LOQ	<LOQ	<LOQ	2.17	1.41	3.23
	Mean	12	108	<LOQ	32	18	<LOQ	<LOQ	6.25	68	20	8.8
	Range	2.51 – 30	<LOQ – 292	<LOQ	<LOQ – 313	1.58 – 121	<LOQ – 1.18	<LOQ – 1.74	<LOQ – 66	<LOQ – 729	<LOQ – 193	<LOQ – 62
	Freq	100	92	0	17	100	17	33	25	83	92	67
Vlietbos (n = 10)	Median	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Mean	1.79	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	1.46	<LOQ	<LOQ	<LOQ
	Range	<LOQ – 6.95	<LOQ	<LOQ	<LOQ	<LOQ – 1.72	<LOQ	<LOQ – 1.25	<LOQ – 5.26	<LOQ	<LOQ – 0.32	<LOQ
	Freq	40	0	0	0	10	0	10	40	0	10	0

Table 4.3 (continued). Individual limits of quantification (LOQs; ng/g dw, determined as 10x the S/N ratio), median and mean concentrations (ng/g dw), range (ng/g dw) and detection frequencies (Freq) of PFAAs in isopods collected at the five sampling sites: a perfluorochemical plant and at four sites with increasing distance from the plant site (i.e. 1 km Vlietbos, 2.3 km Rot, 3 km Burchtse Weel and 11 km Fort 4). Different letters indicate significant differences ($p \leq 0.05$) between sampling sites in PFAA concentrations with a detection frequency > 50%.

		PFCAs										
		PFBA	**PFPeA**	**PFHxA**	**PFHpA**	**PFOA**	**PFNA**	**PFDA**	**PFUnDA**	**PFDoDA**	**PFTrDA**	**PFTeDA**
LOQ		1.34	1.84	4.80	6.92	0.74	0.31	0.98	1.20	0.96	0.30	1.40
Rot (n = 8)	Median	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Mean	1.79	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	1.22	<LOQ	<LOQ	<LOQ
	Range	<LOQ – 8.21	<LOQ	<LOQ	<LOQ	<LOQ – 1.02	<LOQ	<LOQ – 0.99	<LOQ – 3.28	<LOQ	<LOQ	<LOQ
	Freq	25	0	0	0	13	0	13	25	0	0	0
Burchtse Weel (n = 10)	Median	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Mean	<LOQ	<LOQ	<LOQ	<LOQ	0.86	<LOQ	1.11	<LOQ	<LOQ	<LOQ	<LOQ
	Range	<LOQ – 5.04	<LOQ	<LOQ	<LOQ	<LOQ – 3.28	<LOQ	<LOQ – 3.13	<LOQ – 3.08	<LOQ – 1.26	<LOQ – 0.77	<LOQ
	Freq	30	0	0	0	40	0	30	20	10	10	0
Fort 4 (n = 14)	Median	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Mean	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Range	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ – 0.75	<LOQ	<LOQ – 1.56	<LOQ	<LOQ	<LOQ	<LOQ
	Freq	0	0	0	0	7	0	7	0	0	0	0

Table 4.3 (continued). Individual limits of quantification (LOQs; ng/g dw, determined as 10x the S/N ratio), median and mean concentrations (ng/g dw), range (ng/g dw) and detection frequencies (Freq) of PFAAs in isopods collected at the five sampling sites: a perfluorochemical plant and at four sites with increasing distance from the plant site (i.e. 1 km Vlietbos, 2.3 km Rot, 3 km Burchtse Weel and 11 km Fort 4). Different letters indicate significant differences ($p \leq 0.05$) between sampling sites in PFAA concentrations with a detection frequency > 50%.

		PFSAs			
		PFBS	**PFHxS**	**PFOS**	**PFDS**
LOQ		2.66	6.74	0.45	0.99
Plant (n = 12)	Median	6.52	<LOQ	185 A	<LOQ
	Mean	8.31	9.33	253	39
	Range	<LOQ – 26	<LOQ – 26	29 – 611	<LOQ – 388
	Freq	67	42	100	50
Vlietbos (n = 10)	Median	<LOQ	<LOQ	1.98 B	<LOQ
	Mean	<LOQ	<LOQ	3.75	<LOQ
	Range	<LOQ – 5.96	<LOQ	<LOQ – 13	<LOQ
	Freq	30	0	90	0

Table 4.3 (continued). Individual limits of quantification (LOQs; ng/g dw, determined as 10x the S/N ratio), median and mean concentrations (ng/g dw), range (ng/g dw) and detection frequencies (Freq) of PFAAs in isopods collected at the five sampling sites: a perfluorochemical plant and at four sites with increasing distance from the plant site (i.e. 1 km Vlietbos, 2.3 km Rot, 3 km Burchtse Weel and 11 km Fort 4). Different letters indicate significant differences ($p \leq 0.05$) between sampling sites in PFAA concentrations with a detection frequency > 50%.

		PFSAs			
		PFBS	**PFHxS**	**PFOS**	**PFDS**
LOQ		2.66	6.74	0.45	0.99
Rot (n = 8)	Median	<LOQ	<LOQ	<LOQ C	<LOQ
	Mean	<LOQ	<LOQ	0.53	<LOQ
	Range	<LOQ	<LOQ	<LOQ – 1.31	<LOQ
	Freq	0	0	50	0
Burchtse Weel (n = 10)	Median	<LOQ	<LOQ	2.31 B	<LOQ
	Mean	<LOQ	<LOQ	4.13	<LOQ
	Range	<LOQ – 2.97	<LOQ	<LOQ – 13	<LOQ
	Freq	10	0	90	0
Fort 4 (n = 14)	Median	<LOQ	<LOQ	<LOQ	<LOQ
	Mean	<LOQ	<LOQ	<LOQ	<LOQ
	Range	<LOQ	<LOQ	<LOQ	<LOQ
	Freq	0	0	0	0

Table 4.4. Individual limits of quantification (LOQs; ng/g dw, determined as 10x the S/N ratio), median and mean concentrations (ng/g dw), range (ng/g dw) and detection frequencies (Freq) of PFAAs in eggs of great tit collected at the five sampling sites: a perfluorochemical plant and at four sites with increasing distance from the plant site (i.e. 1 km Vlietbos, 2.3 km Rot, 3 km Burchtse Weel and 11 km Fort 4). Different letters indicate significant differences (p ≤ 0.05) between sampling sites in PFAA concentrations with a detection frequency > 50%. The presented data are a part of a larger dataset reported by Groffen et al.(2019c).

		PFCAs						
		PFBA	**PFOA**	**PFNA**	**PFDA**	**PFDoDA**	**PFTrDA**	**PFTeDA**
LOQ		0.261	0.045	0.586	0.425	0.444	0.256	0.355
Plant (n = 13)	Median	<LOQ	18A	8.09A	13A	18A	21A	3.13A
	Mean	1.61	56	11	23	35	32	4.97
	Range	<LOQ – 11	3.36 – 359	3.25 – 28	3.46 – 102	2.21 – 133	2.53 – 155	<LOQ – 22
	Freq	31	100	100	100	100	100	69
Vlietbos (n = 10)	Median	<LOQ	1.65AB	1.07B	<LOQ	<LOQ	3.33B	<LOQ
	Mean	0.31	1.26	1.72	0.54	1.95	5.44	1.14
	Range	<LOQ – 1.42	<LOQ – 2.61	<LOQ – 5.12	<LOQ – 2.08	<LOQ – 6.37	<LOQ – 19	<LOQ – 3.93
	Freq	20	60	60	20	40	70	40
Rot (n = 10)	Median	<LOQ	1.56AB	1.33B	1.17B	2.40B	6.58AB	1.40AB
	Mean	0.32	2.16	1.31	1.22	2.56	6.90	1.26
	Range	<LOQ – 1.03	0.90 – 8.33	<LOQ – 2.26	<LOQ – 2.72	<LOQ – 5.05	1.71 – 12	<LOQ – 2.26
	Freq	30	100	90	70	90	100	80
Burchtse Weel (n = 10)	Median	<LOQ	1.75AB	0.81B	<LOQ	0.69B	2.12B	<LOQ
	Mean	<LOQ	1.57	1.30	1.36	1.71	2.73	0.6
	Range	<LOQ	<LOQ – 3.26	<LOQ – 3.66	<LOQ – 5.49	<LOQ – 6.94	<LOQ – 12	<LOQ – 3.58
	Freq	0	80	60	30	50	80	20
Fort 4 (n = 14)	Median	<LOQ	1.01B	1.0B	1.54B	1.51B	2.39B	0.66B
	Mean	0.31	1.01	1.0	1.59	1.54	2.39	0.58
	Range	<LOQ – 0.86	0.65 – 1.53	<LOQ – 1.91	<LOQ – 4.90	<LOQ – 3.14	0.38 – 5.74	<LOQ – 0.96
	Freq	50	100	79	79	93	100	71

Table 4.4 (continued). Individual limits of quantification (LOQs; ng/g dw, determined as 10x the S/N ratio), median and mean concentrations (ng/g dw), range (ng/g dw) and detection frequencies (Freq) of PFAAs in eggs of great tit collected at the five sampling sites: a perfluorochemical plant and at four sites with increasing distance from the plant site (i.e. 1 km Vlietbos, 2.3 km Rot, 3 km Burchtse Weel and 11 km Fort 4). Different letters indicate significant differences ($p \leq 0.05$) between sampling sites in PFAA concentrations with a detection frequency > 50%. The presented data are a part of a larger dataset reported by Groffen et al.(2019c).

		PFSAs	
		PFOS	**PFDS**
LOQ		2.55	5.92
Plant (n = 13)	Median	29958A	73
	Mean	55970	415
	Range	5111 – 187032	9.44 – 1489
	Freq	100	100
Vlietbos (n = 10)	Median	241B	<LOQ
	Mean	426	<LOQ
	Range	<LOQ – 1427	<LOQ
	Freq	60	0
Rot (n = 10)	Median	409C	<LOQ
	Mean	521	<LOQ
	Range	217 – 1230	<LOQ
	Freq	100	0
Burchtse Weel (n = 10)	Median	79BC	<LOQ
	Mean	140	<LOQ
	Range	14 - 690	<LOQ
	Freq	100	0
Fort 4 (n = 14)	Median	27B	<LOQ
	Mean	26	<LOQ
	Range	8.08 – 42	<LOQ
	Freq	100	0

4.3.2 Correlations between PFAA concentrations in isopods, soil and songbird eggs

The PFOS concentrations in the isopods were related to those in the soil when all locations were combined (Fig. 4.5. Solid line; $p < 0.001$, $R^2 = 0.75$). Although, not significant (both $p = 0.06$), there was an indication that both TOC and clay content of the soil had a positive effect on this association. Further analysis on the individual sites revealed that only at 3M the PFOS concentrations in isopods were associated with only the PFOS concentrations in the soil (Fig. 4.5. Dashed line; $p = 0.005$, $R^2 = 0.64$) and clay content and TOC played no role in this.

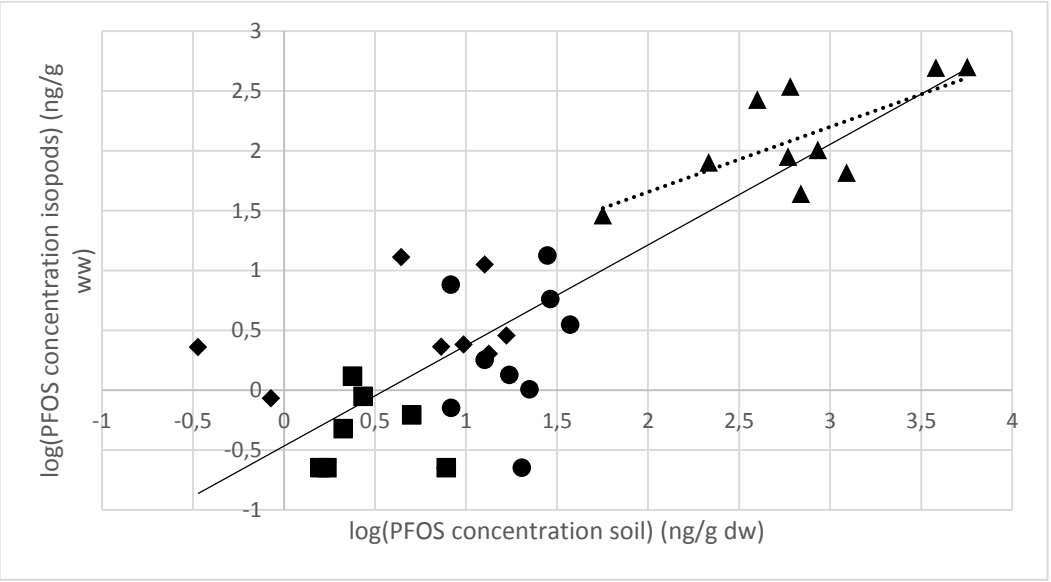


Figure 4.5. Associations between PFOS concentrations in isopods and PFOS concentrations in the soil. Different symbols resemble different sampling locations: triangles = 3M, dots = Vlietbos, squares = Rot and diamonds = Burchtse Weel. The solid line is the regression curve of the entire dataset ($p < 0.001$, $R^2 = 0.75$), the dashed line is the regression curve for 3M ($p = 0.005$, $R^2 = 0.64$).

Furthermore, at 3M, the PFOA concentrations in isopods were not related to those in the soil. The PFDoDA concentrations in the isopods, on the other hand, were related to those in the soil ($p = 0.017$; $R^2 = 0.75$) and to the clay content ($p = 0.035$). Similarly, PFTrDA concentrations in isopods were positively related to those in the soil ($p = 0.007$; $R^2 = 0.83$) and clay content ($p = 0.015$). PFTeDA concentrations were also positively related between isopods and soil ($p < 0.001$; $R^2 = 0.94$). PFBS concentrations

were not related in the isopods and soil. Finally, PFDS concentrations in isopods were positively related to those in the soil ($p = 0.01$; $R^2 = 0.63$).

As mentioned before, soil and invertebrate samples were collected in the immediate vicinity of nest boxes that were used in previous biomonitoring studies. We found no significant correlations between PFAA concentrations in the soil and those in the third egg of great tits (all $p > 0.05$). However, there were significant positive correlations between PFDoDA ($p = 0.010$, $\rho = 0.711$), PFTrDA ($p = 0.040$, $\rho = 0.608$), PFOS ($p = 0.009$, $\rho = 0.734$) and PFDS ($p = 0.008$, $\rho = 0.720$) concentrations in isopods and the third egg at 3M. In addition, PFOA ($p = 0.071$, $\rho = 0.546$) and PFTeDA ($p = 0.067$, $\rho = 0.545$) concentrations in the eggs and isopods showed a trend at 3M. Finally, PFOS concentrations in the eggs and isopods were also positively correlated at Rot ($p = 0.028$, $\rho = 0.761$).

4.3.3 Associations with physicochemical properties of the soil

The organic carbon content (TOC) and clay content of the soil at each location are reported in Table 4.5. TOC differed significantly among locations ($p < 0.001$), caused by a significantly lower TOC at Rot compared to all other locations (all $p < 0.003$). The clay content was significantly different among locations ($p < 0.001$), which was the result of a significantly higher clay content at Burchtse Weel and Fort 4 compared to 3M (both $p < 0.004$) and Rot (both $p < 0.001$). Furthermore, the clay content was significantly higher at Fort 4 compared to Vlietbos ($p = 0.007$). When all locations were combined, there was a significant positive correlation between TOC and clay content of the soil ($p < 0.001$, $\rho = 0.773$). When looking at the individual locations, similar correlations were observed at 3M ($p < 0.001$, $\rho = 0.853$), Burchtse Weel ($p = 0.021$, $\rho = 0.733$) and Fort 4 ($p = 0.006$, $\rho = 0.731$).

Table 4.5. Physicochemical properties of the soil at each location; mean total organic carbon (TOC) content in % ± st. dev. and clay content (% ± st. dev.)

	3M	Vlietbos	Rot	Burchtse Weel	Fort 4
TOC (%)	3.4 ± 3.0	5.8 ± 2.2	0.7 ± 0.2	4.2 ± 2.8	6.2 ± 3.6
Clay content (%)	1.1 ± 0.9	1.5 ± 0.6	0.4 ± 0.3	2.8 ± 1.1	3.1 ± 1.7

A significant positive correlation between PFBA concentrations in the soil and TOC content was only observed at 3M ($p = 0.034$, $\rho = 0.613$) and Burchtse Weel ($p = 0.01$, $\rho = 0.767$) and a marginally significant correlation was observed at Vlietbos ($p = 0.071$, $\rho = 0.627$). PFNA concentrations at 3M were also positively correlated with TOC content ($p = 0.001$, $\rho = 0.822$). PFBS concentrations at 3M were significantly correlated with TOC ($p = 0.005$, $\rho = 0.776$) and at Vlietbos these concentrations showed a trend with TOC content ($p = 0.070$, $\rho = 0.627$). PFOS concentrations were strongly correlated with TOC content at 3M ($p < 0.001$, $\rho = 0.909$) and Burchtse Weel ($p < 0.001$, $\rho = 0.818$), but not at the other locations. PFHxA, PFOA, PFUnDA, PFDoDA, PFTrDA, PFTeDA and PFDS concentrations in the soil were not correlated with TOC content at sites where these compounds had a detection frequency >50%. At 3M, positive correlations were observed between the clay content and the soil concentrations of PFNA ($p = 0.029$, $\rho = 0.626$), PFBS ($p = 0.004$, $\rho = 0.790$) and PFOS ($p = 0.019$, $\rho = 0.678$). PFOS concentrations in the soil at Burchtse Weel were also correlated to the clay content of the soil ($p = 0.027$, $\rho = 0.709$).

4.4 Discussion

4.4.1 PFAA concentrations

For most PFAAs, detected in >50% of the samples, concentrations decreased with increasing distance from the fluorochemical plant in both isopods and soil samples. However, PFOS concentrations at Rot were lower than those detected further away at Burchtse Weel and Fort 4. This latter result is likely the outcome of differences in physicochemical properties of the soil between locations, which will be discussed later. Previous studies conducted near the same fluorochemical plant also revealed that

PFAA concentrations decreased with increasing distance from the plant in wildlife such as invertebrates (D'Hollander et al., 2014) birds (Dauwe et al., 2007; Groffen et al., 2017, Groffen et al., 2019b; Hoff et al., 2005; Lopez-Antia et al., 2017) and mammals (D'Hollander et al., 2014).

To be able to compare the PFAA concentrations in the soil with literature, some examples of PFAA concentrations reported in soils are shown in Table 4.6.

The mean PFOS concentration in the soil at 3M in the present study (1700 ng/g dw) was much higher than those detected at Blokkersdijk (69 ng/g ww; D'Hollander et al., 2014), which is approximately 0.5–1.5 km east from the 3M fluorochemical plant in Antwerp. Compared to the PFOS concentrations in the soil at Vlietbos (1 km SE from the plant; 22 ng/g dw), the concentrations at Blokkersdijk in 2006 were higher. This is likely the result of the voluntary phase-out by 3M of PFOS, PFOA and related products in 2002, which appear to have reduced environmental PFOS concentrations, whereas concentrations of other PFAAs are still rising (Ahrens et al., 2011c; Filipovic et al., 2015b; Groffen et al., 2017, Groffen et al., 2019c; Miller et al., 2015). In addition, the wind in Belgium is mainly coming from the south-west (Royal Meteorological Institute Belgium (KMI), 2018), which would indicate that aerial deposition of PFAAs should mainly affect areas north to east from the fluorochemical plant, which include Blokkersdijk.

Table 4.6. PFAA concentrations (ng/g) in soils published in literature. *Mean concentrations; $Active fluorochemical plant; ranges are illustrated by '-'; ND = not detected; Blanks = analyte was not included in the study.

Location	Year	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnDA	PFTrDA	Publication
*Blokkersdijk, Belgium**	2006										D'Hollander et al., 2014
*Galgenweel, Belgium**	2006										D'Hollander et al., 2014
Main firefighting training facility, Air force base F18, Tullinge Riksten, Sweden[a]	2011					6.98 – 287					Filipovic et al. 2015a
*Daikin Co, Lit, Fluorochemical Industrial Park, China**	2015	0.6	0.3	0.9	<0.5	62.5	0.2	<0.5	0.2	<0.2	Lu et al., 2018
*Estuarine and coastal areas along the west coast, South Korea.**	2009		ND	ND	ND	2.2	ND	ND	ND		Naile et al., 2013
Fluorochemical manufacturing facility in Wuhan, China[$,*]	2009					50.1					Wang et al., 2010
*Fluorochemical manufacturing facility, Hubei Province, China**	2009					0.79					Wang et al., 2010
*Hubei Province, China**	2009					<LOD					Wang et al., 2010

Table 4.6 (continued). PFAA concentrations (ng/g) in soils published in literature. *Mean concentrations; $Active fluorochemical plant; ranges are illustrated by '-'; ND = not detected; Blanks = analyte was not included in the study.

Location	Year	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnDA	PFTrDA	Publication
Liaodong Bay, China	2008					<LOD – 0.32		<LOD – 0.06	<LOD – 0.30	<LOD – 0.46	Wang P et al., 2013
Highway 10, Cottage Grove to Big Lake, USA	2012					5.5 – 125.7					Xiao et al., 2015
*Little Hocking well field, Washington County, Ohio, USA**	2009				2.0	130	2.7	4.3	7.6		Zhu and Kannan 2019
*3M fluorochemical plant, Belgium**	2016	1.92 (<LOQ – 6.33)	5.54 (<LOQ – 26)	2.11 (<LOQ – 11)	1.43 (<LOQ – 4.75)	24.0 (1.97 – 114)	0.83 (<LOQ – 2.53)	1.05 (<LOQ – 7.28)	8.89 (<LOQ – 105)	12.0 (0.05 – 126)	The present study

[a]Concentrations were the result of aqueous firefighting foams rather than those of an industrial source.

Table 4.6 (continued). PFAA concentrations (ng/g) in soils published in literature. *Mean concentrations; $Active fluorochemical plant; ranges are illustrated by '-'; ND = not detected; Blanks = analyte was not included in the study.

Location	Year	PFBS	PFHxS	PFOS	Publication
*Blokkersdijk, Belgium**	2006			69	D'Hollander et al., 2014
*Galgenweel, Belgium**	2006			<LOD (2.4)	D'Hollander et al., 2014
Main firefighting training facility, Air force base F18, Tullinge Riksten, Sweden[a]	2011			118 – 8520	Filipovic et al. 2015a
*Daikin Co, Lit, Fluorochemical Industrial Park, China**	2015	<0.5	<0.5	64.6	Lu et al., 2018
*Estuarine and coastal areas along the west coast, South Korea.**	2009	ND	ND	0.82	Naile et al., 2013
Fluorochemical manufacturing facility in Wuhan, China[$,*]	2009		35.3	2583	Wang et al., 2010
*Fluorochemical manufacturing facility, Hubei Province, China**	2009		0.11	7.06	Wang et al., 2010
*Hubei Province, China**	2009		0.01	0.65	Wang et al., 2010
Liaodong Bay, China	2008			<LOD – 0.42	Wang P et al., 2013
Highway 10, Cottage Grove to Big Lake, USA	2012			0.2 – 28.2	Xiao et al., 2015
*Little Hocking well field, Washington County, Ohio, USA**	2009				Zhu and Kannan 2019
*3M fluorochemical plant, Belgium**	2016	7.84 (<LOQ – 33)	6.88 (<LOQ – 32)	1700 (56 – 7800)	The present study

[a]Concentrations were the result of aqueous firefighting foams rather than those of an industrial source.

Soil PFOA and PFOS concentrations at the plant site were similar to those at a firefighting training facility in Sweden (6.98–287 ng/g for PFOA and 118–8520 ng/g for PFOS), where PFAA-contaminated aqueous film fighting foams were used (Filipovic et al., 2015a). Rankin et al. (2016) reviewed the continental PFAA concentration ranges in soils. Geometric means of the ΣPFCAs and ΣPFSAs in Europe were 1000 pg/g dw and 808 pg/g dw, respectively. PFCA and PFSA concentrations in the present study were much higher than the European mean. The ΣPFSA concentrations were higher than the ΣPFCA concentrations in Europe (Rankin et al., 2016), which is in agreement with our results, as PFOS concentrations were much higher than those of any other analyte. The PFOA concentrations in the soil (62.5 ng/g) collected from a fluorochemical industrial park in China (Lu et al., 2018) were higher than those at 3M in Antwerp. However, concentrations of other PFCAs were much lower in China than in the present study. PFOA, PFHxS and PFOS concentrations at 3M were much lower than those measured in the soil near an active fluorochemical manufacturing facility in Wuhan, China (Wang et al., 2010), where concentrations of 50.1, 35.3 and 2583 ng/g PFOA, PFHxS and PFOS, respectively, were detected. However, compared to an inactive plant in the same area in China (0.79, 0.11 and 7.06 ng/g for PFOA, PFHxS and PFOS; Wang et al., 2010), the concentrations at 3M in the present study were much higher. PFOA and PFOS concentrations at Fort 4, approximately 11 km from the fluorochemical plant, were similar to those near the inactive plant in China. PFOA and PFOS concentrations (2.2 and 0.82 ng/g respectively) in the soil along the estuaries and coastal areas along the South Korean west coast, an area which is highly industrialized and urbanized (Naile et al., 2013), were also much lower than those reported at 3M. PFOA concentrations were, however, similar to those at the other sampling sites. Wang P et al. (2013) reported PFOA, PFDA, PFUnDA, PFTrDA and PFOS concentrations in soils from Liaodong Bay, China, which is an area with concentrated fluorine industry parks, that were all much lower than those detected in most of the sites in the present study. Although PFOS concentrations in the soils of a U.S. metropolitan area, near Cottage Grove, where a former PFAA manufacturer is located, were much lower than those at

3M, the range of PFOA concentrations observed in the US was similar to the one observed at 3M in Belgium. PFOS concentrations in the US were very similar to those detected at Vlietbos, approximately 1 km away from the fluorochemical plant (Xiao et al., 2015). Finally, Zhu and Kannan (2019) determined PFCA concentrations in the soil of the Little Hocking well field in Washington County, Ohio, USA. With exception of PFUnDA, the concentrations in Washington County were all higher than those reported at 3M in the present study.

When we compare the PFOS concentrations in isopods during the present study with a previous study near the Antwerp hot-spot by D'Hollander et al. (2014), median PFOS concentrations in isopods collected at Blokkersdijk (497 ng/g ww) and Galgenweel (3 km SE; 269 ng/g ww) were higher than the median PFOS concentration is isopods collected at the plant site in the present study (185 ng/g ww). Again, this could possibly be explained by the voluntary phase-out of PFOS in 2002. The study performed by D'Hollander et al. (2014) was the only study that examined PFAA concentrations in isopods. Only two other field studies were performed on terrestrial invertebrates. Lesch et al. (2017) detected PFAAs in adult Odonata from South Africa. Median PFOS (highest median of 16 ng/g ww) and PFOA (highest median of 0.89 ng/g ww) concentrations in the Odonata were much lower than those detected at the plant site in the present study (185 ng/g ww and 7.56 ng/g ww for PFOS and PFOA, respectively), but were higher than the concentrations in the adjacent sites. Zhu and Kannan (2019) reported concentrations of multiple PFCAs in earthworms, collected at the Little Hocking well field, Ohio, USA. Similarly to the study area in the present study, this site is historically contaminated by a nearby fluorochemical manufacturing facility. Although the mean PFPeA concentrations detected in earthworms (1.2 ng/g dw) were much lower than those reported at the plant site in the present study (108 ng/g ww), concentrations of PFOA (270 ng/g dw), PFNA (13 ng/g dw), PFDA (26 ng/g dw), PFUnDA (110 ng/g dw) and PFDoDA (200 ng/g dw) in the earthworms were much higher (Zhu and Kannan, 2019).

As was mentioned before, the concentrations determined in great tit eggs were part of a larger dataset. A comparison of these concentrations with literature has already been done by Groffen et al. (2019c).

4.4.2 Correlations between PFAA concentrations in isopods, soil and songbird eggs

We observed a positive relationship between PFOS concentrations in isopods and soil when all locations were combined, and at 3M individually. Furthermore, we found evidence that PFDoDA, PFTrDA, PFTeDA and PFDS concentrations in isopods reflect the concentrations of these compounds in the soil. For PFDoDA and PFTrDA the concentrations in the isopods were not only positively related to the concentrations in the soil, but also to the clay content of the soil. These results were expected as isopods are exposed to soils and therefore, soils are most likely an important pathway of PFAA exposure to these invertebrates.

We also correlated PFAA concentrations in isopods and soil with those in the eggs of great tit, to determine the possibility of trophic transfer as a pathway of the PFAA concentrations in the songbirds and eventually in their eggs. In general, PFAA concentrations in the soil were not correlated with those in the eggs of great tit, indicating that soil concentrations were not representative of the concentration in the eggs. This was expected, as great tits are insectivorous songbirds that mainly feed on invertebrates (mainly caterpillars), berries and seeds, depending on the season (del Hoyo et al., 2007). This might also explain the positive correlations between concentrations in the eggs and those in isopods at 3M and Rot.[1]

4.4.3 Associations with physiochemical properties of the soil

Our results, that PFOS soil concentrations at Rot were significantly lower than those at Burchtse Weel and Fort 4, both further away from the fluorochemical plant, could be explained by a lower TOC content at Rot. The maximum sorption capacity of the soil is to a large extent influenced by soil organic carbon content (Miao et al., 2017). Soil

[1] A sentence from the original paper has been removed here

organic carbon content has shown to be positively correlated with the sorption capacity of soils (Milinovic et al., 2015; Wei et al., 2017). This indicates that more PFAAs will adsorb to the soil when the TOC is higher. Soils with smaller particles, such as clay, will have more functional groups like hydroxyl and carboxyl groups than bigger particles, which results in more binding sites to facilitate the sorption of the contaminants (Qi et al., 2014). Therefore, it was expected that PFAA concentrations in the soil would be higher in areas where the clay content is higher. The positive correlations between clay content and soil concentrations of PFNA, PFBS and PFOS at 3M and Burchtse Weel were therefore expected.

4.4.4 PFAA profile

Fig. 4.6 shows the PFAA profiles in soil, invertebrates and songbird eggs at all the sampling sites. Similarly to previous analyses, only analytes with a detection frequency of at least 50% were taken into account. Consequently, locations with no analyte detected in >50% of the samples, or only one PFSA and/or PFCA detected in frequencies higher than 50% (e.g. PFOA and PFOS in soil collected from Rot) were not included in the figures, as their profile would result in 100% contribution of these compounds.

PFOS was the major contributor to the PFSA concentrations in the soil (Fig. 4.6b) at the plant site (97 ± 1%) and at Vlietbos (89 ± 2%), in isopods (88 ± 4% at 3 M; Fig. 4.6c) and in bird eggs at all locations (100% with exception of 3M: 99.5%). Furthermore, due to the high PFOS concentrations in both matrices, PFOS can be considered to be the dominant contributor to the total PFAA concentrations in the soil, eggs and isopods. With regard to the PFCA profile, PFOA was the dominant contributor in soil (Fig. 4.6a), whereas PFPeA became more dominant in isopods. In bird eggs, the PFCA profile at 3M was dominated by PFOA, whereas PFTrDA had a higher contribution at the other sites (Fig. 4.6d).

These patterns are generally in agreement with other studies on PFSAs in soil and invertebrates and PFCAs in soil. Rankin et al. (2016) performed a global survey on the

distribution patterns and mode of occurrence of PFSAs and PFCAs and they reported that, in general, PFOA and PFOS were the most abundant analytes in the soil. The dominance of PFOS in the soil was also reported by Naile et al. (2013) as the PFOS concentrations in soil samples, collected along the west coast of Korea, were also higher than those of the other PFSAs. The dominance of PFOA in soils was similar to a study by Filipovic et al. (2015a) in which the relative contribution of PFOA in the soil, polluted due to historical usage of aqueous film forming foam, was higher than the one of PFHxA. PFOA was also the dominant PFCA in soils, collected near a fluorochemical industrial park in eastern China (Lu et al., 2018).

No studies have been performed on the pollution of multiple PFAAs in isopods. Although D'Hollander et al. (2014) reported PFOS concentrations in isopods, they did not study other PFAAs and were therefore unable to determine PFAA profiles. Similar to our results, Zhao et al. (2013) observed that PFOS concentrations were higher in earthworms, exposed for 30 days to a soil contaminated with a 200 ng/g PFAAs mixture, than those of other PFSAs. PFOS was also the dominant PFSA in earthworms exposed in biosolid amended soils (Navarro et al., 2016). PFCA profiles in isopods were different from those in earthworms, exposed to mixtures of PFAAs. Zhao et al. (2013) observed that PFDoDA was the dominant contributor to the ΣPFCAs, whereas PFPeA had the second lowest concentrations. Similar results were obtained by Zhao et al. (2016), where biota accumulation factors (BAFs) increased with the increase in carbon chain length in earthworms exposed to spiked soils. These results suggest different exposure pathways for earthworms and isopods, which is possibly the result from differences in diet and feeding behaviour.

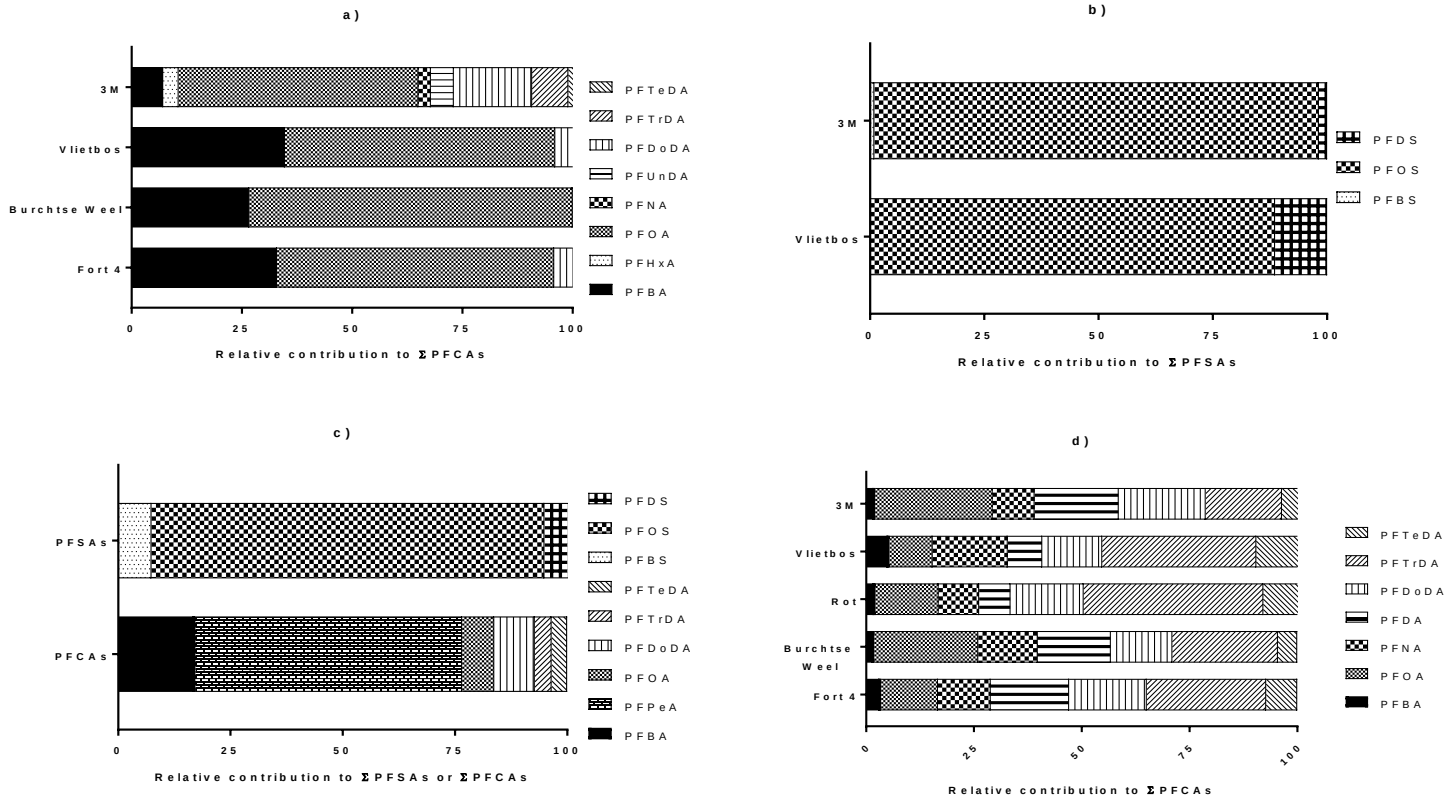


Figure 4.6. Composition profile of PFAAs in soil and isopods. A) PFCA composition profile in soil samples. B) PFSA composition profile in soil samples. C) Composition profile of PFSAs and PFCAs in isopods, collected at the 3M fluorochemical plant. D) Composition profile of PFCAs in song bird eggs.

The dominance of PFOS in the PFAA profile of the bird eggs was in agreement with literature (e.g. Ahrens et al., 2011c; Custer et al., 2012; Groffen et al., 2017). Similarly, Groffen et al. (2017) already mentioned that the dominance of PFOA to the ΣPFCAs at the plant site and the dominance of PFTrDA at sites further away could possibly be explained by the direct deposition of PFOA close to the plant, whereas further away atmospheric and biological degradation of volatile polyfluorinated precursor compounds might explain the dominance of PFTrDA.

Surprisingly, the contribution of PFBA increased with increasing distance from the fluorochemical plant, which is likely the result of different ways of pollution or a different pollution source. The PFCA profile close to the plant site could be explained by the influence of a direct pollution source, where PFOA is the main product (Prevedouros et al., 2006), whereas further away from the plant atmospheric degradation of volatile precursor compounds such as fluorotelomer alcohols (FTOHs) could play a role.

4.5 Conclusions

The PFOS concentrations in soil in the present study were often much higher than those reported in literature, with exception of those measured in soils at an active fluorochemical manufacturing facility in China. Compared to the European geometric means, the concentrations of PFOS and PFOA in soils were much higher in the present study. In isopods, the concentrations have decreased compared to a previous study conducted in the same area, which might be the result of the voluntary phase-out by 3M in 2002. PFOS and PFOA concentrations in all matrices were elevated at the plant site and decreased with increasing distance from the fluorochemical plant. However, there were some deviations in this pattern, which were likely the result of differences in physicochemical properties of the soil.

4.6 Acknowledgements

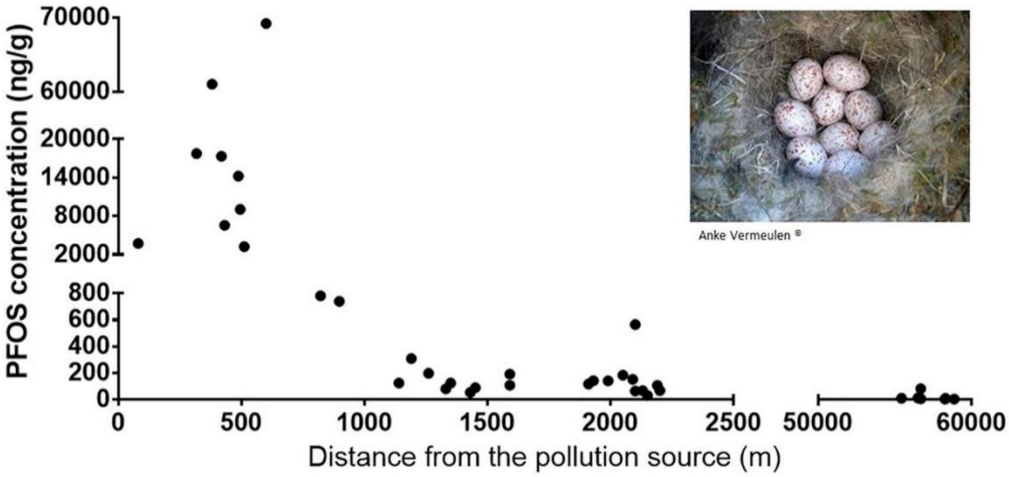
The authors would like to thank the FWO for the funding (FWO: G038615N). Furthermore, we would like to express our sincere thanks to 3M for the opportunity to conduct this research at their chemical plant. We would also like to acknowledge A. Lopez-Antia for her help during the fieldwork, T. van der Spiet for his help with the particle size analysis and T. Willems, who performed the UPLC analysis. Finally, we are extremely thankful to the Agency for Nature and Forest (ANB), K. Maes and W. Melens for providing us access to the sampling sites and the possibility to store field materials at these sites.

5. Perfluoroalkylated acids in the eggs of great tits (*Parus major*) near a fluorochemical plant in Flanders, Belgium

Published in Environmental Pollution:

Thimo Groffen, Ana Lopez-Antia, Wendy D'Hollander, Els Prinsen, Marcel Eens and Lieven Bervoets (2017). Environmental Pollution 228: 140 – 148. https://doi.org/10.1016/j.envpol.2017.05.007

Tables have been modified to fit the size of the page. The term 'levels' has been replaced by 'concentrations' in terms of PFAA concentrations. No further modifications have been made.



Abstract

Perfluoroalkyl acids (PFAAs) are highly persistent substances which have been detected in wildlife around the world, including birds. Although bird eggs have often been used to determine and monitor PFAAs concentrations in the marine environment, this has rarely been done in the terrestrial environment. In the present study we examined the concentrations and composition profile of 12 PFAAs (4 perfluoroalkyl sulfonic acids (PFSAs) and 8 perfluoroalkyl carboxylic acids (PFCAs) in the eggs of great tits (*Parus major*) collected at a fluorochemical plant and in three other areas, representing a gradient in distance from the pollution source (from 1 to 70 km), in Antwerp, Belgium.

The PFSA concentrations measured at the site of the fluorochemical plant were among the highest ever reported in eggs with median concentrations of 10380 ng/g (extrapolated), 99.3 ng/g and 47.7 ng/g for PFOS, PFHxS and PFDS respectively. Furthermore, the median concentration of 19.8 ng/g for PFOA was also among the highest ever reported in bird eggs. Although these concentrations decreased sharply with distance from the fluorochemical plant, concentrations found in the adjacent sites were still high compared to what has been reported in literature. Moreover, based on what is known in literature, it is likely that these concentrations may cause toxicological effects. PFOS was the dominant contributor to the PFSA and PFAAs (63.4–97.6%) profile at each site, whereas for PFCAs this was PFOA at the plant site and the nearest locations (41.0–52.8%) but PFDoDA (37.7%) at the farthest location. Although there is some evidence that PFAAs concentrations close to the plant site are decreasing in comparison with earlier measurements, which may be due to the phase out of PFOS, more research is necessary to understand the extent of the toxicological effects in the vicinity of this PFAAs hotspot.

5.1 Introduction

Perfluoroalkyl acids (PFAAs) have been produced for more than 50 years. The strength and stability of the C-F binding in combination with the hydrophobic and lipophobic character of PFAAs lead to unique physicochemical properties. PFAAs applications include fire-fighting foams, fast food packaging and surface coatings for carpets (Buck et al., 2011, Kissa, 2001). PFAAs are highly persistent and may enter the environment either directly or indirectly from environmental degradation of precursors (Buck et al., 2011, Prevedouros et al., 2006). The widespread use of PFAAs has resulted in a global presence in the environment, wildlife and even humans as described in many studies (e.g., Butt et al., 2010, D'Hollander et al., 2010, Giesy and Kannan, 2001, Giesy and Kannan, 2002, Houde et al., 2006, Miller et al., 2015).

The attention of regulatory agencies and researchers has focused on long chain perfluoroalkyl carboxylic acids (PFCAs) and perfluoroalkyl sulfonic acids (PFSAs), because of their higher bioaccumulative potential compared to their short chain analogues (Buck et al., 2011). They are particularly interested in the two most widely known ones: PFOA ($C_7F_{15}COOH$) and PFOS ($C_8F_{17}SO_3H$).

PFOS, PFOA and related compounds have been phased out by 3M, the major global manufacturer, in 2002, due to their persistence, potential health effects and global distribution. Furthermore, PFOS was included in the Stockholm Convention on Persistent Organic Pollutants (POPs) in 2009. These measures, in most cases, appear to be reducing PFOS environmental concentrations while concentrations of other PFAAs are still rising (Ahrens et al., 2011c, Filipovic et al., 2015b, Miller et al., 2015).

Bird eggs have been used in multiple studies to monitor PFAAs concentrations in many regions of the world (e.g., Gebbink and Letcher, 2012, Giesy and Kannan, 2001, Holmström et al., 2005, Miller et al., 2015, Yoo et al., 2008). However, the majority of these studies have been performed on aquatic birds, whereas data on terrestrial birds, especially passerine birds, remain scarce (Ahrens et al., 2011c, Custer et al., 2012, Holmström et al., 2010, Rüdel et al., 2011, Yoo et al., 2008).

Previous studies conducted near a fluorochemical plant in Antwerp, Belgium, revealed the highest PFOS concentrations ever found in wildlife (Dauwe et al., 2007, D'Hollander et al., 2014, Hoff et al., 2005, Lopez Antia et al., 2017). Liver PFOS concentrations measured in great tits (*Parus major*) and blue tits (*Cyanistes caeruleus*) from this area were higher than those measured in top predators in other regions worldwide, and were also above the benchmark concentrations for the possible risk levels of avian species (Dauwe et al., 2007). Furthermore, PFOS concentrations in eggs were among the highest ever reported in bird eggs worldwide (Lopez Antia et al., 2017). These studies conducted nearby the fluorochemical plant in Antwerp have demonstrated that PFOS concentrations measured in wildlife decreased significantly at relatively short distances from the plant site (from 3 to 10 km) on the one hand, and that concentrations found at these distances are still very high on the other hand. Monitoring PFAAs concentrations and composition profile in this hot spot and its surroundings is therefore extremely important.

In the present study, concentrations of multiple PFAAs were measured in eggs of a terrestrial songbird, the great tit, at a fluorochemical plant in Antwerp. Additionally eggs from three other areas were analyzed, representing a gradient in distance from the pollution source. It is important to compare the concentrations and composition profile of PFAAs along this distance gradient to better understand the environmental dynamics of PFAAs. Moreover, the outcome of the present study can be used for further monitoring studies, to investigate temporal changes in PFAAs concentrations using 1) minimally invasive sampling, namely eggs (Furness and Greenwood, 1993), and 2) a species that has demonstrated to be useful as sentinel species for local contamination of Persistent Organic Pollutants (Dauwe et al., 2003, Dauwe et al., 2007, Van den Steen et al., 2006, Van den Steen et al., 2009c). Finally, detected concentrations were used to assess the potential risk to birds based on the current toxicological benchmark concentrations.

5.2 Materials and method

5.2.1 Study species and sample collection

Great tits, insectivorous songbirds, are increasingly being used in biomonitoring studies because they readily nest in man-made nestboxes, are abundant and can even be attracted to polluted areas (Eens et al., 1999, Eeva and Lehikoinen, 1995, Eeva and Lehikoinen, 1996, Eeva et al., 1998, Dauwe et al., 1999, Dauwe et al., 2004, Dauwe et al., 2005a, Van den Steen et al., 2006).

During the winter of 2011, nestboxes were placed at four sampling sites. Three locations were situated in the vicinity of a perfluorochemical plant (3M) in Antwerp, Belgium. These locations were the perfluorochemical plant itself (32 nestboxes), Vlietbos (1 km SE from the plant site; 23 nestboxes) and Rot-Middenvijver (shortly Rot; 2.3 km ESE from the plant site; 16 nestboxes). As a reference site, Tessenderlo-Ham (20 nestboxes), approximately 70 km ESE from the plant site was selected, as it is an area without a known perfluorochemical point source in the direct environment.

Nestboxes were checked weekly or daily just before laying to be able to determine the laying date and clutch size. At each site one egg per clutch was collected randomly by hand from 10 to 12 different nestboxes before the incubation had started (early April).

5.2.2 Chemical analysis

The used abbreviations of PFAAs are according to Buck et al. (2011). The target analytes included 4 PFSAs (PFBS, PFHxS, PFOS and PFDS) and 8 PFCAs (PFBA, PFHxA, PFOA, PFNA, PFDA, PFDoDA, PFTrDA and PFTeDA). The isotopically mass-labelled internal standards (ISTDs) comprised [1,2-$^{13}C_2$]PFHxA, $^{13}C_8$-PFOA, $^{13}C_9$-PFNA, [1,2,3,4,5,6-$^{13}C_6$]PFDA, [1,2,3,4,5,6,7-$^{13}C_7$]PFUnDA, [1,2,3,4,5,6,7-$^{13}C_7$]PFDoDA, $^{18}O_2$-PFHxS and $^{13}C_8$-PFOS and were purchased by Wellington Laboratories (Guelph, Canada). HPLC-grade Acetonitrile (ACN) and water (Acros Organics, New Jersey, USA) were used.

5.2.3 Sample extraction

After removal of the shell, the content of the egg was homogenized with an Ultra Turrax mixer (T25, Staufen, Germany) in a polypropylene (PP) tube and divided into two parts of approximately 0.5 g.

The extraction procedure was based on a method described by Powley et al. (2005) with minor modifications. Samples were spiked with an internal standard mixture (ISTD, 80 µL, 125 pg/µL), containing 125 pg/µL of each ISTD and mixed thoroughly. Hereafter 10 mL acetonitrile was added, samples were sonicated (3 × 10 min) and left overnight at room temperature on a shaking plate. After centrifugation (4 °C, 10 min, 2400 rpm, Eppendorf centrifuge 5804R), the supernatant was transferred to a 15 mL PP tube and reduced to approximately 0.5 mL by using a rotational-vacuum-concentrator at 20 °C (Martin Christ, RVC 2-25, Osterode am Harz, Germany). The concentrated extract and 2 times 250 µL acetonitrile, which was used to rinse the tubes, were transferred to a PP micro centrifuge tube containing 50 mg graphitized carbon powder (Supelclean ENVI-Carb, Sigma-Aldrich, Belgium) and 70 µL glacial acetic acid merely to eliminate pigments. These tubes were vortex-mixed during at least one minute and centrifuged (4 °C, 10 min, 10 000 rpm, Eppendorf centrifuge 5415R). The cleaned-up supernatants were stored at −20 °C until analysis. Before analyses, 70 µL of extract was diluted with 130 µL 2 mM aqueous ammonium acetate and filtrated through an Ion Chromatography Acrodisc 13 mm Syringe Filter with 0.2 µm Supor (PES) Membrane (Leuven, Belgium) attached into a PP auto-injector vial.

5.2.4 UPLC-TQD analysis

We analyzed PFAAs by UPLC coupled tandem ES(-) mass spectrometry (ACQUITY, TQD, Waters, Milford, MA, USA) using an ACQUITY BEH C18 column (2.1 × 50 mm; 1.7 µm, Waters, USA), mobile phase: 0.1% formic acid in water(A), 0.1% formic acid in acetonitrile(B), solvent gradient: from 65% A to 0% A in 3.4 min and back to 65%A at 4.7 min, flow rate: 450 µL/min, injection volume: 10 µl. To retain any PFAA contamination originating from the system, we inserted an ACQUITY BEH C18 pre-column (2.1 × 30 mm; 1.7 µm, Waters, USA) between the solvent mixer and the

injector. Identification and quantification was based on multiple reaction monitoring (MRM) of the following diagnostic transitions: 213 → 169 (PFBA), 313 → 296 (PFHxA), 315 → 270 ($^{13}C_2$-PFHxA), 413 → 369 (PFOA), 421 → 376 ($^{13}C_8$-PFOA), 463 → 419 (PFNA), 472 → 427 ($^{13}C_9$-PFNA), 513 → 469 (PFDA), 519 → 474 ($^{13}C_6$-PFDA), 613 → 569 (PFDoDA), 613 → 319 (PFDoDA), 615 → 169 ($^{13}C_7$PFDoDA), 615 → 570 ($^{13}C_7$PFDoDA), 570 → 525 ($^{13}C_7$PFUnDA), 663 → 619 (PFTrDA), 713 → 669 (PFTeDA), 713 → 369 (PFTeDA), 299 → 99 (PFBS), 399 → 99 (PFHxS), 403 → 103 ($^{18}O_2$-PFHxS), 599 → 80 (PFDS), 499 → 80 (PFOS), 499 → 99 (PFOS) and 507 → 80 ($^{13}C_8$-PFOS).

5.2.5 Calibration

Non-labelled standards of all the target analytes were used to construct ten-level calibration curves ($R^2 > 0.99$) covering the entire linear range (0.0125 till 16 ng/mL) in HPLC-grade ACN and water. Labeled internal standards were added to each calibration point in the same amount as in samples. Each PFAA was quantified using the corresponding internal standard with the exception of PFBS, PFDS, PFTrDA and PFTeDA of which no labelled standards were available. PFBS and PFDS were quantified using $^{18}O_2$-PFHxS and $^{13}C_4$-PFOS respectively, whereas for both PFTrDA and PFTeDA, $^{13}C_2$-PFDoDA was used. The internal standards allowed us to correct for matrix effects and recovery losses for the corresponding compounds.

5.2.6 Quality assurance

One procedural blank per 10 samples was analyzed as quality control. Minor concentrations of contamination (<0.4 pg/µL) of PFOA and PFOS were subtracted from the correspondent concentrations found in the samples. For PFOA and PFOS, the quality of the applied method was evaluated by 3 laboratories on spiked egg samples; a triplicate analysis of a sample, spiked with linear (61.7 ng/g and 63.2 ng/g for PFOA and PFOS respectively) or branched (32.2 ng/g and 32.0 ng/g for PFOA and PFOS respectively) isomers of PFOS and PFOA, was performed in each laboratory (Table S5.1). No significant differences were detected between the laboratories. For the spiked samples, an accuracy of 93–107% was achieved. The precision of the applied method varied between 2 and 4% (Table S5.1). The limit of quantifications (LOQs),

corresponding to a signal-to-noise ratio 10, ranged from 0.02 ng/g to 1 ng/g for PFBS, PFHxS, PFOS, PFDS, PFOA, PFDoDA and PFTrDA. Due to some high noise levels the LOQs for PFBA, PFHxA, PFNA, PFDA and PFTeDA are considerably higher and ranged from 1.4 ng/g to 4.3 ng/g. Individual LOQs are displayed in Table 5.1. For all samples, of which concentrations were within the linear range of the calibration curve, recoveries of the ISTDs were calculated. The samples were corrected for recoveries, which were between 92% and 110%. At two locations, some PFOS concentrations were outside the linear range of the calibration curve and therefore the samples were 10–800 times diluted. As a consequence, the internal standards were no longer visible and therefore a correction based on the recoveries was extrapolated.

5.2.7 Statistical analysis

Statistical analyses were performed using SPSS 23. Samples with a bad recovery were excluded from the analyses. PFAAs concentrations were log transformed to obtain a normal distribution.

Differences in concentrations between the different sampling locations were evaluated in two ways. First of all, we performed a one way ANOVA using Least Significant Difference (LSD) test for Post-hoc analysis for PFAAs found in all samples, i.e. PFOS and PFOA. Secondly, for PFAAs with at least one value above the LOQ (i.e. PFDoDA, PFTrDA, PFHxS and PFDS), we used a reverse Kaplan Meier (KM) analysis and a Mantel-Cox test for pairwise comparisons among sampling sites. This analysis is commonly used for survival analysis of left censored data (Gillespie et al., 2010) and has been proven useful to cope with concentrations below the LOQ (Jaspers et al., 2013). Details about how to perform this analysis with SPSS are provided in Gillespie et al. (2010). As reverse KM is a nonparametric analysis we used untransformed data to perform the analysis. To study correlations between the individual compound concentrations, and between the ∑PFSAs and ∑PFCAs in each study site Spearman rank correlation analyses were performed. For each site the composition profiles were determined by calculating the proportions of individual compounds to the total concentrations of PFAAs, PFSAs and PFCAs in each egg and then averaging the

percentages for all the eggs at a site. For this calculation, values below the LOQ were replaced with a value of LOQ/2 (Bervoets et al., 2004, Custer et al., 2000).

5.3 Results

5.3.1 PFAA concentrations

An overview of median concentrations, ranges and detection frequencies of PFAAs in the eggs is given in Table 5.1. Some PFOS concentrations at 3M and Vlietbos exceeded the linear range of the calibration curve and were thus higher than 16 ng/mL. Although these concentrations were already very high, the extrapolated concentrations have been used in this study.

PFOS, PFOA, PFDoDA and PFTrDA were detected at all the sampling sites. PFHxS and PFDS were only detected at 2 sampling sites (at the plant site and 1 km away from the plant site, at Vlietbos). PFDA and PFNA were only detected at the plant site. PFBS, PFBA, PFHxA and PFTeDA were not detected in any of the samples at any of the sites. The overall detection frequencies of the analyzed PFAAs decreased in following other: both PFOS and PFOA were detected in all the samples (100%), followed by PFDoDA (60%), PFTrDA (56%), PFHxS (38%), PFDS (33%), PFDA (16%) and PFNA (11%). The detection frequencies should be interpreted with caution as there were relatively large differences between the LOQs.

Table 5.1. Individual limits of quantification (LOQ: ng/g, determined as 10 times the signal to noise ratio), median and mean concentrations (ng/g ww), range (ng/g ww) and detection frequencies (Freq) of PFAAs in eggs of great tit at the four sampling sites: a perfluorochemical plant and at three sites with an increasing distance from the plant site (i.e. 1 km Vlietbos, 2.3 km Rot and 70 km Tessenderlo). ND = not detected. Different letters indicate significant differences ($p \leq 0.05$) between sampling sites in each compound concentration and in each compound prevalence respectively.

		PFCAs								PFSAs			
		PFOA	PFDA	PFNA	PFDoDA	PFTrDA	PFHxA	PFBA	PFTeDA	PFOS	PFHxS	PFDS	PFBS
LOQ		0.02	1.4	1.8	0.32	0.38	2.9	4.3	2.1	0.02	0.45	0.2	1.1
Plant (n = 11)	Median	19.8A	12.0	<LOQ	13.7A	5.6A	ND	ND	ND	10380A	99.3A	47.7A	ND
	Mean	26.9	12.3	4.2	22.0	7.9	ND	ND	ND	20122	162.3	100.8	ND
	Range	2.7 - 56.3	<LOQ - 37.2	<LOQ - 20.5	2.0 – 103.9	<LOQ – 32.3				3237 – 69218	36.9 – 354.6	<LOQ – 426.3	
	Freq	100	58.3	41.6	100	91.7				100	100	91.6	
Vlietbos (n = 11)	Median	0.9B	<LOQ	<LOQ	1.0B	<LOQB	ND	ND	ND	125B	<LOQB	<LOQB	ND
	Mean	1.0	<LOQ	<LOQ	0.7	0.4	ND	ND	ND	254	1.6	0.7	ND
	Range	0.3 – 1.9	/	/	<LOQ – 1.50	<LOQ – 0.9				55.1 – 782	<LOQ – 5.6	<LOQ – 2.9	
	Freq	100			50	25				100	50	25	
Rot (n=11)	Median	0.8B	<LOQ	<LOQ	<LOQB	<LOQB	ND	ND	ND	107.1B	<LOQ	<LOQ	ND
	Mean	0.7	<LOQ	<LOQ	1.0	0.8	ND	ND	ND	133.2	<LOQ	<LOQ	ND
	Range	0.3 – 1.3	/	/	<LOQ – 6.0	<LOQ – 4.8				4.3 – 565.3	/	/	
	Freq	100			54.5	54.5				100			
Tessenderlo (n = 8)	Median	0.3B	<LOQ	<LOQ	0.6B	0.5B	ND	ND	ND	9.4C	<LOQ	<LOQ	ND
	Mean	0.3	<LOQ	<LOQ	0.8	0.6	ND	ND	ND	17.6	<LOQ	<LOQ	ND
	Range	0.1 – 0.8	/	/	<LOQ – 1.9	<LOQ – 1.6				4.3 – 82.2	/	/	
	Freq	100			40	50				100			

Significant differences between sampling sites in PFOS ($F_{3,44}$ = 114.15, P < 0.001) and PFOA ($F_{3,44}$ = 77.14, P < 0.001) concentrations were observed. Post hoc test revealed that concentrations were significantly higher at the plant site compared to Vlietbos, 't Rot and Tessenderlo (all P < 0.001). For PFOS, significant differences were found also between Vlietbos and Tessenderlo (all P < 0.001) and between Rot and Tessenderlo (all P ≤ 0.001) but not between Vlietbos and Rot. Concentrations of PFOA were significantly higher at the plant than all of the other sites (all P < 0.001). For PFDoDA and PFTrDA concentrations were significantly higher at the plant site compared with all the other sampling sites (all $\chi^2 \geq 24.79$, all P < 0.05) but no significant differences existed among the other sampling sites. Finally, significantly higher concentrations of PFHxS (χ^2 = 24.7, P < 0.001) and PFDS (χ^2 = 19.9, all P < 0.001) were found at the plant site compared to Vlietbos.

Figure 5.1 shows the PFAAs concentrations in function of the distance from the pollution source the center of the plant site is considered to be the pollution source (0 m).

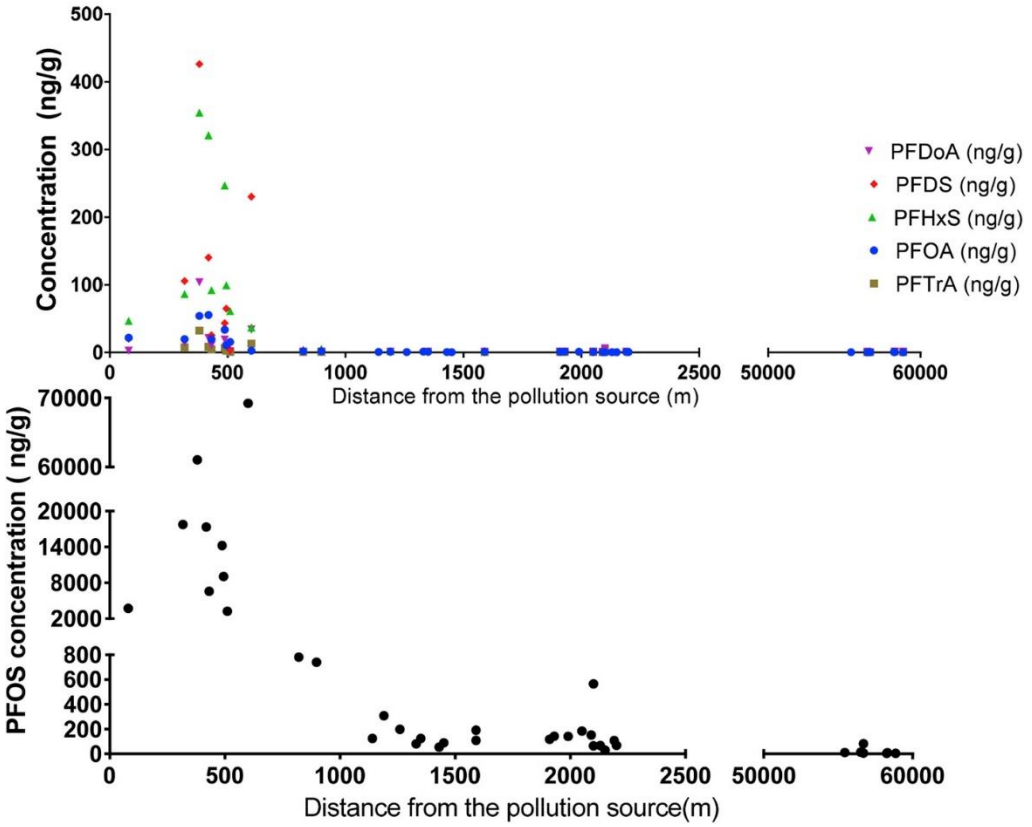


Figure 5.1. a) PFAAs concentrations along the distance gradient from the pollution source. The center of the fluorochemical plant is considered to be the pollution source (0m). b) PFOS concentrations along the distance gradient from the pollution source.

5.3.2 PFAA profile

For all the sampling sites PFOS was the dominant contributor to the ∑PFSAs (Fig. S5.1) and to the ∑PFAAs as its contribution to the ∑PFAAs ranged between 97.6 ± 0.3% (mean ± SE) at the plant site and 63.4 ± 6.4% at Tessenderlo. For ∑PFCAs, the major compound was PFOA at the plant site (43.2 ± 6.5%), Vlietbos (52.8 ± 4.3%) and Rot (41 ± 5.6%), but not at Tessenderlo where it accounted for the 27.0 ± 7.5% and where PFDoDA and PFTrDA represented 37.7 ± 6.5% and 35.2 ± 5.2% respectively (Fig. 5.2).

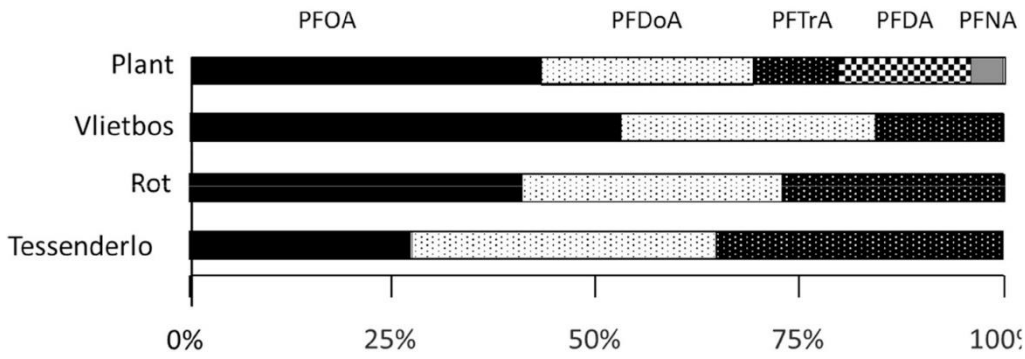


Figure 5.2. Composition profile of PFCAs in eggs of great tit at the four sampling sites; a fluorochemical plant and at three sites with increasing distance from the plant site: 1 km (Vlietbos), 2.3 km (Rot) and 70 km (Tessenderlo, reference site).

5.3.3 Correlations

All correlations found among PFAAs at the different sampling sites are summarized in Table 5.2.

Significant correlations were observed mostly at the plant site (14 significant correlations), followed by Vlietbos (13), Rot (3) and Tessenderlo (where no significant correlations were observed). All significant correlations were positive. At the plant site PFOS, PFDS, PFDoDA and PFTrDA were all correlated with each other, whereas PFOA, PFHxS, PFNA and PFDA were also correlated with each other. However, PFNA was also correlated with PFDoDA. At Vlietbos PFOS concentrations were correlated with concentrations of all other compounds. Although many of these compounds were also related with each other, no correlations were observed between PFTrDA and PFOA, PFHxS and PFDS. At Rot PFOS was only correlated with PFDoDA, which was also correlated with PFTrDA. Overall PFCA concentrations (∑PFCA) were correlated with overall PFSA concentrations (∑PFSA) at the plant site, Vlietbos and Rot (Fig. 5.3).

Table 5.2. Correlations found between different PFAAs in the different sampling sites. Values in bold are significant correlations.

		Plant site (n = 11)		Vlietbos (n = 11)		Rot (n = 11)		Tessenderlo (n = 8)	
		p-value	Rho	p-value	Rho	p-value	Rho	p-value	Rho
PFOS	PFOA	0.654	0.155	**0.016**	0.702	0.818	0.082	0.233	-0.7
	PFHxS	0.451	0.255	**0.008**	0.745	/	/	/	/
	PFDS	**<0.001**	0.891	**0.005**	0.776	/	/	/	/
	PFNA	0.296	0.347	/	/	/	/	/	/
	PFDA	0.614	0.172	/	/	/	/	/	/
	PFDoDA	**<0.001**	0.927	**<0.001**	0.898	**0.009**	0.739	0.858	-0.112
	PFTrDA	**<0.001**	0.900	**0.015**	0.707	0.097	0.525	0.614	0.308
PFOA	PFHxS	**0.031**	0.664	**0.038**	0.630	/	/	/	/
	PFDS	0.435	0.264	**0.024**	0.671	/	/	/	/
	PFNA	**0.013**	0.719	/	/	/	/	/	/
	PFDA	**0.031**	0.648	/	/	/	/	/	/
	PFDoDA	0.341	0.318	**0.015**	0.706	0.620	0.169	0.718	-0.224
	PFTrDA	0.755	0.109	0.062	0.579	0.481	0.238	0.219	-0.667
PFHxS	PFDS	0.503	0.227	**0.009**	0.744	/	/	/	/
	PFNA	**0.007**	0.758	/	/	/	/	/	/
	PFDA	**0.022**	0.677	/	/	/	/	/	/
	PFDoDA	0.214	0.409	**0.038**	0.629	/	/	/	/
	PFTrDA	0.341	0.318	0.052	0.597	/	/	/	/

Table 5.2. (continued) Correlations found between different PFAAs in the different sampling sites. Values in bold are significant correlations.

		Plant site (n = 11)		**Vlietbos (n = 11)**		**Rot (n = 11)**		**Tessenderlo (n = 8)**	
		p-value	Rho	p-value	Rho	p-value	Rho	p-value	Rho
PFDS	PFNA	0.197	0.421	/	/	/	/	/	/
	PFDA	0.427	0.267	/	/	/	/	/	/
	PFDoDA	**0.003**	0.827	**0.002**	0.825	/	/	/	/
	PFTrDA	**0.010**	0.755	0.089	0.536	/	/	/	/
PFNA	PFDA	**<0.001**	0.858	/	/	/	/	/	/
	PFDoDA	**0.038**	0.630	/	/	/	/	/	/
	PFTrDA	0.113	0.506	/	/	/	/	/	/
PFDA	PFDoDA	0.210	0.410	/	/	/	/	/	/
	PFTrDA	0.462	0.248	/	/	/	/	/	/
PFDoDA	PFTrDA	**<0.001**	0.945	**0.009**	0.740	**0.038**	0.641	0.199	0.688
∑PFSA	∑PFCA	**0.034**	0.6	**<0.001**	0.882	**0.006**	0.791	0.683	0.3

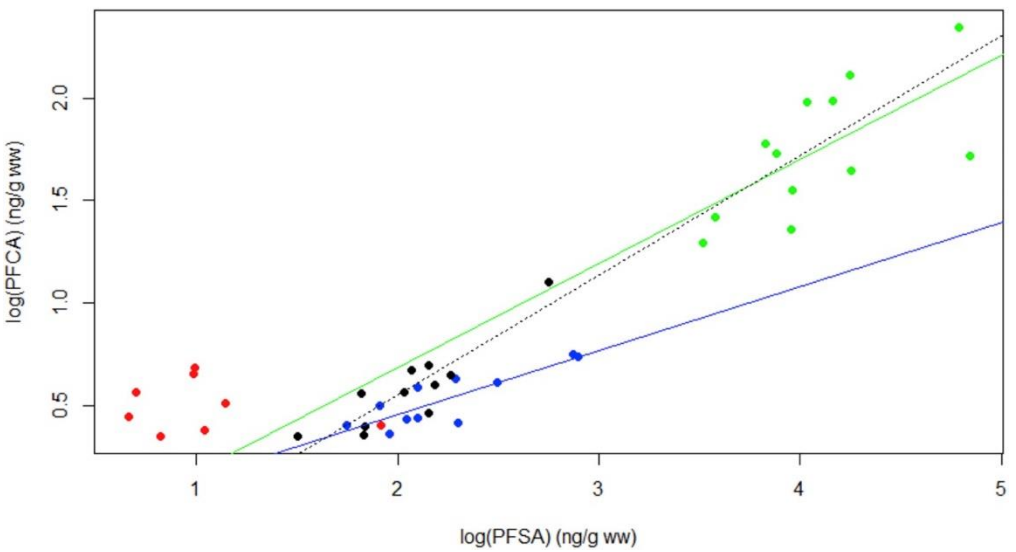


Figure 5.3. Correlations between ∑PFSA and ∑PFCA concentrations amongst sites. Green = 3M, Blue = Vlietbos, Black = Middenvijver-Rot and Red = Tessenderlo.

5.4 Discussion

5.4.1 PFAA concentrations

At the plant site, the observed concentrations of the detected PFSAs (PFOS, PFHxS and PFDS) were among the highest ever reported in bird eggs with median concentrations of 10380 ng/g, 99.3 ng/g and 47.7 ng/g respectively. The median PFOA concentration (19.8 ng/g) was also among the highest ever reported in bird eggs.

Compared to a study in 2006 on PFOS in eggs and blood of great tit, northern lapwing and Mediterranean gull, near the same fluorochemical plant (Lopez Antia et al., 2017), ranges of PFOS concentrations at Vlietbos were approximately 6.5 times lower in the present study. However, the highest concentration reported in northern lapwing (90 m from the fluorochemical plant) was 1.5 times lower than the highest concentration in great tit at the fluorochemical plant in the present study, but 4 times higher than the median PFOS concentration at the plant.

To be able to make comparisons among species, some examples of PFAAs concentrations found previously in terrestrial and marine bird eggs are shown in Table 5.3.

To the best of our knowledge only four papers on PFAAs in passerine birds have been published. Until now, the highest concentrations in passerine birds were found by Yoo et al. (2008) in parrot bill (*Paradoxornis webbiana*) eggs collected around the shores of a lake in Korea that receives wastewaters from an industrial complex. Mean PFOS concentrations detected in that study (314 ng/g) were slightly higher than the one found in Vlietbos but more than 60 times lower than the one found at the plant site in the current study. Interestingly, ∑PFCAs concentrations measured in the study in Korea are much higher than the one found in the present study, mainly due to PFNA (40 ng/g) and PFDA (114.2 ng/g) concentrations, suggesting a different type of contamination between both places.

The highest mean PFOS concentration detected at the plant site in the present study is more than 18 times higher than the highest mean concentration reported in Great Blue Heron (*Ardea herodias*) eggs (1014 ng/g) in 1993 in the Mississippi river, in a colony located approximately 20 km from a 3M fluorochemical plant site (Custer TW et al., 2010). In the same study, concentrations of the other reported PFAAs (PFDS, PFHxS, PFDA, PFNA, and PFOA) were also, at least two times, lower than the ones we measured in the present study. It must be considered that PFOS was still produced in the plant when the Great Blue Heron eggs were collected. Mean concentrations found in the same Great Blue Heron colony in 2010 (465 ng/g; Custer et al., 2013) remain among the highest ever found, however, they are 41 times lower than the one reported at the fluorochemical plant in the present study. Mean PFOS (109 ng/g), PFHxS (0.52 ng/g) and PFOA (0.9 ng/g) concentrations found in free range chicken eggs (*Gallus gallus*) collected at a distance of less than 500 m from a fluorochemical manufacturing plant in China (Wang et al., 2010), were lower than those found in the present study in Vlietbos and very similar to the ones found in Rot, located at 1 and 2.3 km from the plant site respectively. In fact, PFOS and PFOA concentrations found 1 km away from the fluorochemical plant in China (9.8 and 0.15 ng/g respectively) were lower than those found in our reference site, Tessenderlo, 70 km away from the plant site. We must consider that when the study of Wang et al. (2010) was performed the

plant in China still produced PFOS and related compounds. A study on PFOS concentrations in chicken eggs in Belgium also showed high concentrations of PFOS (highest mean concentration of 3500 ng/g) in the vicinity of the same fluorochemical plant as in the present study (D'Hollander et al., 2011). However, this mean concentration is similar to the lower PFOS concentrations at the plant site in the present study.

The present study site contains one of the highest PFOS concentrations ever reported in wildlife worldwide. This was confirmed by previous studies performed in the surroundings of this hot-spot in Antwerp in which PFOS concentrations measured in wood mice (*Apodemus sylvaticus*) livers (D'Hollander et al., 2014, Hoff et al., 2004), great and blue tit nestlings livers (Hoff et al., 2005) and great tit blood and plasma (Dauwe et al., 2007) were all the highest ever reported in these matrices in wildlife.

We found a steep decrease in concentrations of all the detected compounds with distance from the plant site. However, significant differences in concentrations were not detected between the sites 1 km or 2.3 km away from the plant and only for PFOS and PFOA significantly lower concentrations were found at the reference site (70 km away from the plant site) comparing with the two other points outside the plant. This decrease with distance from the pollution source was also observed for PFOS concentrations in the aforementioned studies conducted in this area (Dauwe et al., 2007, D'Hollander et al., 2014, Hoff et al., 2004, Hoff et al., 2005). In these studies, as in ours, despite the decrease in concentrations with distance (between 3 and 10 km away from the plant site), concentrations found in the furthest sites remained high comparing with the literature. Also in China, in the surroundings of a perfluorochemical manufacturing facility, a decreasing trend of PFOA, PFOS and PFHxS concentrations in soils, water, and chicken eggs with increasing distance from the plant was observed (Wang et al., 2010).

Table 5.3. Median PFAAs concentrations in bird eggs (ng/g ww) published in the literature. All studies on concentrations in terrestrial birds and four studies with higher concentrations in sea birds have been included. *Geometric means; ** median concentrations; * range; NP=concentrations were measured but were not provided; NA = not assessed; § Concentrations found in active fluorochemical plant † Concentrations found in a fluorochemical plant not used since 2002.**

Species	Country	Year	PFOA	PFNA	PFDA	PFDoA	PFTrA	PFTeA	Publication
Corvus frugilegus **	Germany	2009	0.5	2.1	0.8	NA	NA	NA	Rüdel et al., 2011
Paradoxornis webbiana	Korea	2006	0.8	40	114.2	25.6	NA	NA	Yoo et al. 2008
Strix aluco*	Norway	1986-2009	<LOQ	<LOQ	0.20	0.12	0.36	NA	Ahrens et al. 2011c
Falco peregrinus	Sweden	2006	<LOD	1.6	3.1	3.2	7.3	2.7	Holmström et al. 2010
Tachycineta bicolor	Minnesota (USA)	2008-2009	<LOD	NP	5.51	NP	NA	NA	Custer et al. 2012
Tachycineta bicolor*	Minnesota (USA)	2007-2011	18.7	3.10	5.47	1.96	NA	NA	Custer et al. 2014
Gallus gallus §	China	2009	0.9	<LOD	<LOD	<LOD	NA	NA	Wang et al. 2010
Gallus gallus†	China	2009	0.76	<LOD	<LOD	<LOD	NA	NA	Wang et al. 2010
Ardea herodias*	Minnesota (USA)	1993	<LOD	0.9	3.6	3.7	NA	NA	Custer TW et al. 2010
Ardea herodias*	Minnesota (USA)	2010-2011	0.6	2.55	22	12.9	NA	NA	Custer et al. 2013
Phalacrocorax auritus	San Francisco (USA)	2009	ND-24.3	13.4	13.8	7.08	NA	NA	Sedlak and Greig 2012
Phalacrocorax carbo **	Sweden	2007-2009	4.05	20.7	44.8	23.9	23.7	4.08	Nordén et al.2013
Phalacrocorax carbo **	Germany	2009	1.1	2.7	10.4	1.0	NA	NA	Rüdel et al., 2011
Parus major* **	Belgium	2006	NA	NA	NA	NA	NA	NA	Lopez Antia et al., 2017
Vanellus Vanellus* **	Belgium	2006	NA	NA	NA	NA	NA	NA	Lopez Antia et al., 2017
Ichthyaetus melanocephalus* **	Belgium	2006	NA	NA	NA	NA	NA	NA	Lopez Antia et al., 2017

Table 5.3. (continued) Median PFAAs concentrations in bird eggs (ng/g ww) published in the literature. All studies on concentrations in terrestrial birds and four studies with higher concentrations in sea birds have been included. *Geometric means; ** median concentrations; * range; NP=concentrations were measured but were not provided; NA = not assessed; § Concentrations found in active fluorochemical plant † Concentrations found in a fluorochemical plant not used since 2002.**

Species	Country	Year	PFHxS	PFOS	PFDS	Publication
Corvus frugilegus **	Germany	2009	<LOQ	5.3	NA	Rüdel et al., 2011
Paradoxornis webbiana	Korea	2006	1.3	314.1	1.1	Yoo et al. 2008
Strix aluco*	Norway	1986-2009	0.05	10.9	0.06	Ahrens et al. 2011c
Falco peregrinus	Sweden	2006	0.80	83	0.66	Holmström et al. 2010
Tachycineta bicolor	Minnesota (USA)	2008-2009	NP	141	NA	Custer et al. 2012
Tachycineta bicolor*	Minnesota (USA)	2007-2011	0.95	270	NA	Custer et al. 2014
Gallus gallus §	China	2009	0.52	109	NA	Wang et al. 2010
Gallus gallus†	China	2009	0.24	85.2	NA	Wang et al. 2010
Ardea herodias*	Minnesota (USA)	1993	1.5	940	33	Custer TW et al. 2010
Ardea herodias*	Minnesota (USA)	2010-2011	0.65	342	8	Custer et al. 2013
Phalacrocorax auritus	San Francisco (USA)	2009	LOD-25.2	483.7	NA	Sedlak and Greig 2012
Phalacrocorax carbo **	Sweden	2007-2009	2.5	552	2.06	Nordén et al.2013
Phalacrocorax carbo **	Germany	2009	2.8	400	NA	Rüdel et al., 2011
Parus major* **	Belgium	2006	NA	19 – 5635	NA	Lopez Antia et al., 2017
Vanellus Vanellus* **	Belgium	2006	NA	143 - 46182	NA	Lopez Antia et al., 2017
Ichthyaetus melanocephalus* **	Belgium	2006	NA	150 – 916	NA	Lopez Antia et al., 2017

Within each site, the variability between the concentrations of the individual PFAAs of the different nestboxes was considerably high. The highest variability was observed at the plant site where the minimum and maximum concentrations varied up to 20 times. The variability of the PFSA concentrations was higher compared to those observed for the PFCAs. These differences could be indicating variations in concentrations in the laying females. For example, higher PFOS concentrations were found in young great tits (<one-year old) than in old ones (>1 year-old) in a study performed in the same study area than ours (Dauwe et al., 2007). Unfortunately we know neither the age of the laying females in the present study, nor the origin of these birds (locally born versus immigrant females). Therefore, we do not know the degree of prior exposure. On the other hand, this variability found at the plant site may also be due to variations in egg concentrations within the clutches. Variations within the clutch have been demonstrated in a study about PFOS concentrations in eggs of tree swallow, in a PFAAs contaminated area in Minnesota, where a 4-fold difference between the highest and lowest concentration within a clutch was found (Custer et al., 2012). Moreover, a study of PFAAs concentrations in entire clutches of Audouins' gulls demonstrated that PFOS concentrations decreased with the laying order of the eggs (Vicente et al., 2015). Unfortunately, in the present study, the eggs were randomly collected before incubation so we could not evaluate the effect of the laying order.

5.4.2 PFAA profile

Our results showed that PFOS is the major contributor to the total PFAAs. This is in agreement with the literature on PFAAs in bird eggs (e.g., Ahrens et al., 2011c, Custer et al., 2012, Nordén et al., 2013, Rüdel et al., 2011). Regarding the PFCAs composition profile, PFOA, followed by PFDoDA, is the major contributor at the plant site, Vlietbos and Rot whereas at PFDoDA and PFTrDA are the major contributors at Tessenderlo. Moreover, a trend can be observed for PFOA to reduce and PFTrDA to increase their relative concentrations with the increase in distance (Fig. 6.2). In the plant site and surrounding areas the PFCAs composition profile could be explained by the influence of a direct contamination source, where PFOA is the main product (Prevedouros et al.,

2006), whereas 70 km away from the plant site, in Tessenderlo, the composition profile could be explained by the atmospheric and biological degradation of the volatile polyfluorinated precursor compounds (fluorotelomer alcohols; FTOH), and the fact that long chain fluorinated compounds (PFTrDA and PFDoDA) are more bioaccumulative than shorter chain ones (PFOA) (Armitage et al., 2009, Conder et al., 2008, Ellis et al., 2004, Houde et al., 2006). The composition profile found in Tessenderlo is similar to the ones found in eggs of tawny owl in Norway (Ahrens et al., 2011c) and peregrine falcon in Sweden (Holmström et al., 2010), where PFTrDA and PFUnDA were the major contributors to ∑PFCAs.

5.4.3 Toxicological implications

The toxicological and biological effects of PFAAs on avian species are not well characterized but several laboratory studies have verified developmental toxicity (Cassone et al., 2012, Jiang et al., 2012, Molina et al., 2006, O'Brien et al., 2009a, O'Brien et al., 2009b, Pinkas et al., 2010). Furthermore, negative effects on the neuroendocrine system (Cassone et al., 2012, Smits and Nain, 2013, Vongphachan et al., 2011) and histology (Molina et al., 2006) have been suggested. Most of these studies focus on the effects of PFOS and PFOA while information on other PFAAs is limited. Additionally, there is a considerable variation in the effect concentrations. For example, *in ovo* exposure to PFOS in chicken eggs determined an LD50 based on hatchability of 4.9 µg/g (Molina et al., 2006) whereas O'Brien et al. (2009a) established it as 93 µg/g. These concentrations are in the same order of magnitude as the concentrations found in the present study. However, most of the effects on PFAAs in the laboratories have been established after egg injection which strongly differs from the exposure route of the eggs in the present study.

Regarding PFOS, both laboratory and field studies are present. Molina et al. (2006) indicated that PFOS caused a significant reduced hatchability of the chicken embryo after *in ovo* exposure at a dose as low as 0.1 µg/g egg. Pathological changes in the liver, including bile duct hyperplasia, periportal inflammation and necrosis were observed at a dose of 1.0 µg/g after *in ovo* exposure. Peden-Adams et al. (2009) observed

increased spleen mass, increased lysozyme activity, suppressed total sheep red blood cell -specific immunoglobulin production, shorter right wings and increased frequency and severity of brain symmetry in chickens at *in ovo* exposure concentration of 1 µg/g. Newsted et al. (2005) derived a predicted-no-effect concentration of 1 µg/ml PFOS in egg yolk based on chronic and acute dietary exposures of northern bobwhite quail (*Colinus virginianus*) and mallard (*Anas platyrhynchos*). According to these values, the PFOS concentrations observed at the plant site may cause physiological and neurological effects on great tits if we assume equal sensitivity between species. Moreover, in a PFAAs contaminated area in east central Minnesota, USA, reduced hatching success was associated with PFOS concentrations as low as 150 ng/g in eggs of tree swallow (*Tachycineta bicolor*) (Custer et al., 2012, Custer et al., 2014). If great tit have the same sensitivity as tree swallow, the current PFOS contamination at all the sampling locations, except Tessenderlo, would result in reduced hatchability.

The available studies on the toxicity of other PFAAs to birds are limited. The toxic effects of PFOA, PFUnDA and PFDS on hatching success and liver mRNA expression in chicken embryos after *in ovo* exposure were evaluated by O'Brien et al. (2009b). Even at the highest exposed group of 10 µg/g these PFAAs did not influence the hatching success. Furthermore, Smits and Nain (2013) evaluated the immunotoxicity of subchronic exposure to PFOA via drinking water in Japanese quail and they found that although birds exposed to the highest dose (10 µg/g) presented a reduced T cell immune response. This reduced response did not translate into an increased disease susceptibility. However, they also found that the highest dose of PFOA reduced thyroid hormone levels and increased the growth rate of exposed Japanese quail (*Coturnix coturnix japonica*).

Reduced hatching success and a decrease in tarsus length and embryo mass have been observed in chickens that were exposed *in ovo* to PFHxS concentrations up to 38 µg/g (Cassone et al., 2012). Furthermore, a reduction in plasma thyroid hormone levels was

observed at concentrations up to 0.89 µg/g (Cassone et al., 2012), a concentration about 5 times higher than the one found at the plant site in the present study.

We have to consider that while most of these toxicological studies were focussed on the effects of a single compound, free-living animals such as the great tits in the present study are exposed to a mixture of PFAAs and other contaminants in combination with natural stressors and therefore more research on toxicological effects under real conditions is urgently needed.

5.5 Conclusion

Even though PFOS concentrations have been decreased since the phase out in 2002, the PFAAs concentrations, especially these of PFHxS, PFOS, PFDS and PFOA, in the eggs of great tit at the plant site in 2011 were still among the highest ever reported in wild birds. Furthermore, concentrations in adjacent sites decreased with distance from the fluorochemical plant, but remained high compared to what has been reported in literature. It is therefore expected that concentrations have decreased further since the present study, although this remains to be tested.

More research on PFAAs toxicological effects along with studies on other bird species and biota (to cover the entire food chain) is needed to understand the extent of the problem in this PFAAs contamination hot spot and its surroundings.

5.6 Acknowledgements

The authors wish to thank 3M and the FWO (FWO nr: G034117N) for funding, G. Eens, P. Scheys, R. Pinxten and L. Demongin for their assistance with fieldwork and T. Willems for UPLC-MS/MS analysis.

5.7 Supplementary data

Table S5.1. Results obtained for spiked egg samples in the inter-laboratory study between SGS, University of Antwerp and the University of Amsterdam.

Spiked isomers	Trial	SGS		University of Antwerp		University of Amsterdam		theoretical values	
		PFOA (ng/g)	PFOS (ng/g)	PFOA (ng/g)	PFOS (ng/g)	PFOA (ng/g)	PFOS (ng/g)	PFOA (ng/g)	PFOS (ng/g)
Linear	1	55.6	59	57.0	57.4	56	66	61.7	63.2
	2	50.1	62.8	60.3	57.1	57	67		
	3	53.7	59.2	61.4	60.9	55	68		
Branched	1	28.5	30.1	34.5	34.9	28	30	32.2	32
	2	28.4	29.5	32.1	33.5	26	28		
	3	29.1	30.5	33.4	34.8	28	29		

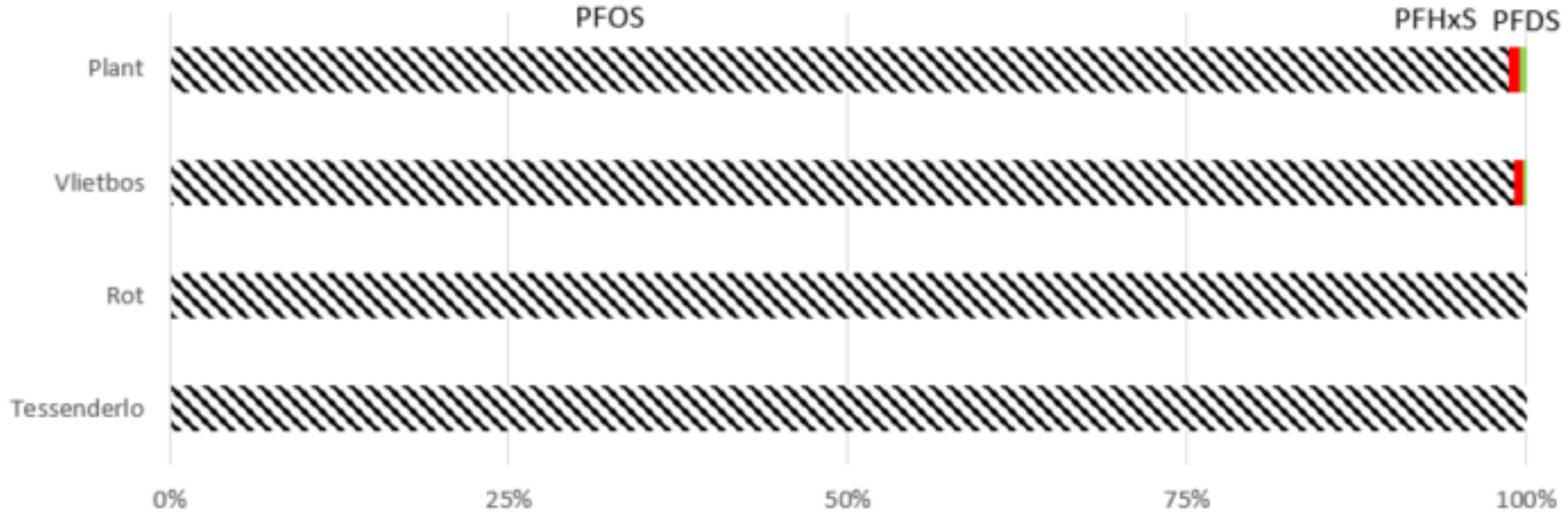


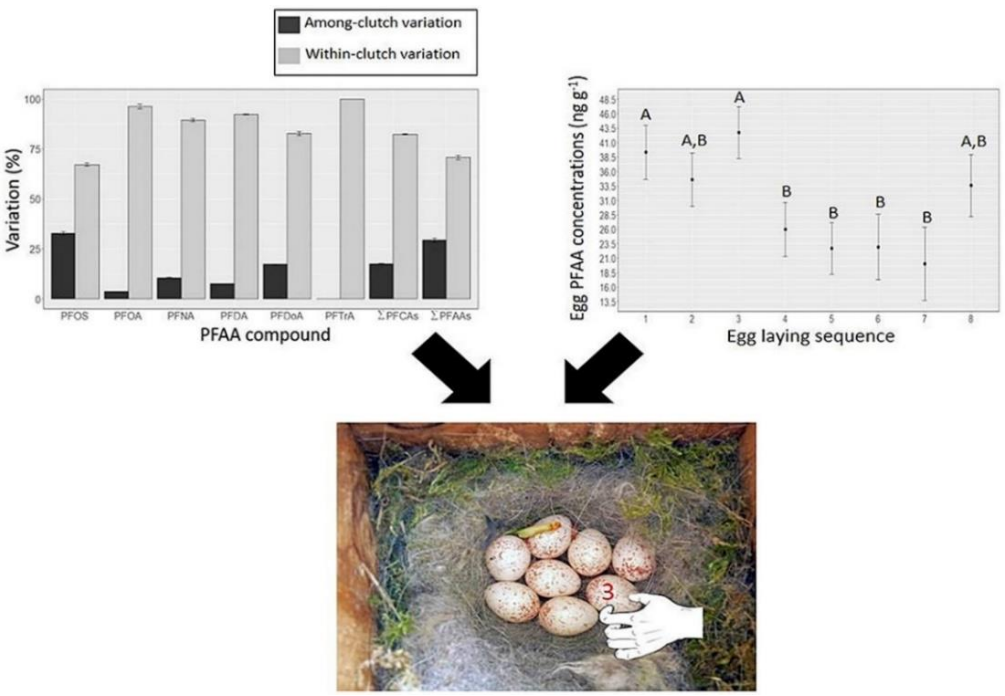
Figure S5.1. Composition profile of ∑PFSA in eggs of great tit at the four sampling sites; a fluorochemical plant and at three sites with increasing distance from the plant site: 1 km (Vlietbos), 2.3 km (Rot) and 70 km (Tessenderlo, reference site).

6. Variation in PFAA concentrations and egg parameters throughout the egg-laying sequence in a free-living songbird (the great tit, *Parus major*): Implications for biomonitoring studies.

Published in Environmental Pollution:

Robin Lasters, Thimo Groffen, Ana Lopez-Antia, Lieven Bervoets and Marcel Eens (2019). Environmental Pollution 246: 237 – 248. https://doi.org/10.1016/j.envpol.2018.12.014

Tables were modified to fit the size of the page. No further modifications were made.



Abstract

Over the past decades, there has been growing scientific attention and public concern towards perfluoroalkyl acids (PFAAs), due to their widespread presence in the environment and associations with adverse effects on various organisms. Bird eggs have often been used as less-invasive biomonitoring tools for toxicological risk assessments of persistent organic pollutants, including some PFAAs. Hereby, it is typically assumed that one random egg is representative for the PFAA concentrations of the whole clutch. However, variation of PFAA concentrations within clutches due to laying sequence influences can have important implications for the egg collection strategy and may impede interpretations of the quantified concentrations. Therefore, the main objective of this paper was to study variation patterns and possible laying sequence associations with PFAA concentrations in eggs of the great tit (Parus major). Eight whole clutches (4–8 eggs) were collected at a location in the Antwerp region, situated about 11 km from a known PFAA point source. The ∑ PFAA concentrations ranged from 8.9 to 75.1 ng g−1 ww. PFOS concentrations ranged from 6.7 to 55.1 ng g−1 ww and this compound was the dominant contributor to the total PFAA profile (74%), followed by PFDoA (7%), PFOA (7%), PFDA (5%), PFTrA (4%) and PFNA (3%). The within-clutch variation (70.7%) of the ∑PFAA concentrations was much larger than the among-clutch variation (29.3%) and concentrations decreased significantly for some PFAA compounds throughout the laying sequence. Nevertheless, PFAA concentrations were positively and significantly correlated between some egg pairs within the same clutch, especially between egg 1 and egg 3. For future PFAA biomonitoring studies, we recommend to consistently collect the same egg along the laying sequence, preferably the first or third egg if maximizing egg exposure metrics is the main objective.

6.1 Introduction

Since industrialization took place in the 18th century, human-induced environmental change has led to the concept of "the Anthropocene" (Corlett, 2015; Rose, 2015). Particularly, it also refers to the dramatically increased emission of persistent organic pollutants (POPs) into the environment (Zalasiewicz et al., 2015). After detection of their global presence in nature, these pollutants have received worldwide research attention (Fernández and Grimalt, 2003; Jaspers et al., 2014). Therefore, well-known POPs such as pesticides, polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) have been studied extensively for toxic effects among various wildlife and humans (Li et al., 2006; Ross and Birnbaum, 2010; Jaspers et al., 2014; Ashraf, 2017). However, much less is known about the environmental impact of the more recently detectable perfluoroalkyl acids (PFAAs) (Domingo and Nadal, 2017; Mudumbi et al., 2017).

PFAAs are a diverse family of synthetic, organic compounds that consist of a perfluoroalkyl chain with strong C-F bonds and a functional acid group (Buck et al., 2011). These physicochemical properties make them extremely resistant to different types of degradation (Beach et al., 2006; Surma et al., 2017). As a result of these physicochemical properties, combined with both their lipophobic and hydrophobic character, they have been produced at large-scale for more than 60 years and used for diverse applications. These include surface coating for textiles, soil repellents, food contact paper, cleaning products, fire-fighting foams and pesticides (Buck et al., 2011; Zhou et al., 2013; Ulrich et al., 2016). Consequently, PFAAs can enter the environment directly via industrial production and usage but also via degradation of precursor compounds as an indirect pathway (Martin et al., 2010; Butt et al., 2014).

Biomonitoring of PFAAs has shown that they bioaccumulate and can biomagnify through the trophic chain (Conder et al., 2008; Fang et al., 2014; Groffen et al., 2018). Hence, PFAAs have been globally reported from 2000 onwards in the environment, in various organisms (Giesy and Kannan, 2001, 2002; Butt et al., 2010; Rodriguez-Jorquera et al., 2016) and in humans (Hansen et al., 2001; Roosens et al., 2010; Olesen

et al., 2016). Over the past decades, there has been growing scientific attention and public concern towards long-chain perfluoroalkyl sulfonic acids (PFSAs) and perfluoroalkyl carboxylic acids (PFCAs), due to their higher bioaccumulation potential and toxicity to various organisms (Conder et al., 2008). As a consequence, the production of these long-chain PFAAs has been regulated in Europe and North-America (Gebbink et al., 2015; Kim and Oh, 2017). Despite these regulatory measures, global PFAA concentrations are still high and even increasing in several countries (Ahrens et al., 2011c; Miller et al., 2015; Groffen et al., 2017), highlighting that it remains crucial to continuously monitor these PFAAs.

Although PFAAs exposure in terrestrial animals occurs generally via inhalation of dust and air and ingestion of contaminated food and water (Gebbink et al., 2015), the latter two are thought to be the most important intake routes for biota (D'Hollander et al., 2015). Unlike the majority of POPs, which generally bind to fatty tissues, PFAAs show high affinity toward protein-rich organs such as blood serum, liver and kidney (Jones et al., 2003; Lau et al., 2007). Therefore, these matrices have often been used as biomonitoring method for PFAAs (Dauwe et al., 2007; D'Hollander et al., 2014; Jaspers et al., 2014). Nevertheless, because of ethical and practical reasons, the application of non-destructive biomonitoring methods is highly recommended.

Bird eggs have already been successfully used in many studies as a less-invasive biomonitoring method for PFAAs in various regions (Gebbink and Letcher, 2012; Nordén et al., 2013; Custer et al., 2014; Lopez-Antia et al., 2017; Sedlak et al., 2017), although very few of these studies have focused on terrestrial birds (Ahrens et al., 2011c; Groffen et al., 2017; Gewurtz et al., 2018). Most of the previously mentioned studies determined PFAAs in one random egg per nest. It is well known that pollutant concentrations are deposited in eggs, but still very little is known on the overall variation of PFAA release across entire clutches and which factors (eg. diet, age, PFAA chemical properties) are contributing to this process.

Surprisingly, there exist only very few studies to date that investigated within-clutch variation of PFAA concentrations in birds (Custer et al., 2012; Vicente et al., 2015). Nevertheless, this information can be very useful in further biomonitoring studies, e.g. to know whether a single egg represents the PFAA contamination of an entire clutch and if so, to minimize the impact of biomonitoring on the population. Furthermore, Vicente et al. (2015) demonstrated that PFOS concentrations decreased with laying sequence in Audouin's gull (*Larus audouini*) eggs. It should be noted that females in this species have relatively small clutch sizes of maximum three eggs per nest which may make it more difficult to detect influences of laying sequence on PFAA concentrations.

In comparison, passerines may be more appropriate candidate birds to study PFAA clutch variation and laying sequence influences. They generally have large clutch sizes which makes them more suitable to study pollutant variation and associations with laying sequence (Van den Steen et al., 2006). The great tit, *Parus major*, can be considered as a promising model species to study the accumulation and possible effects of PFAAs. Great tits are terrestrial, insectivorous passerine birds which are common and abundant in nearly every urban or wooded area throughout Europe (Van den Steen et al., 2009a). They are very useful as biomonitors of local contamination, because of their small home ranges (Eens et al., 1999). They are cavity-nesting birds and make readily use of nest boxes (Dauwe et al., 2007), which makes it easy to collect samples such as eggs.

Great tits have relatively large clutch sizes of 6–12 eggs (Van den Steen et al., 2009a), making them suitable to study PFAA accumulation patterns and egg laying sequence influences. Great tits lay eggs daily and need replenishment of endogenous resources with exogenous resources for the eggs. Maternal resources are used for the first eggs and energy from the daily diet for the later eggs, which might contain lower PFAA concentrations than maternal resources, due to less accumulated time within the mother (Van den Steen et al., 2009a). Therefore, a relatively large within-clutch

variation and decrease in PFAA concentrations along the laying sequence can be expected.

To the best of our knowledge, there are no studies which have examined possible egg laying sequence influences on PFAAs in a terrestrial bird species. Nevertheless, this information is crucial with respect to future biomonitoring studies, especially for a bird species which has been frequently used in this context. Recent measurements close to a fluorochemical plant near Antwerp reported PFOS concentrations up to 69 218 ng g^{-1} in great tit eggs (Groffen et al., 2017), despite the phase-out of this compound among others in 2002 by 3M (3M Company, 2000). Therefore, biomonitoring of PFAAs in proximity of the fluorochemical plant in Antwerp is extremely important.

The main objective of this study was to assess the variation of different PFAAs, i.e. PFCAs and PFSAs, both within and among clutches of the great tit. Furthermore, possible laying sequence associations between egg parameters (egg weight, egg volume and shell thickness) and PFAA concentrations were studied. In addition, possible relationships of PFAAs among eggs from the same clutch were investigated to infer potential implications for future biomonitoring studies.

6.2 Materials and method

6.2.1 Study site

Figure 6.1. Map of the study area (Fort IV) showing the distribution of the great tit nestboxes (black dots) from which whole clutches of great tit eggs were collected (n = 46) near Antwerp, Belgium in 2016. The right bottom map depicts the distance of the study area (rectangle) from the fluorochemical plant (star) in Antwerp.

The data collection was conducted at Fort IV (51°10'24"N, 4°27'37"E), which is a park characterized by loam soil and groves dominated with deciduous trees (Hoff et al., 2005). This study site is situated about 11 km from a known PFAAs pollution source in Antwerp (Fig. 6.1). One of the largest perfluorochemical plants (3M) is located in Antwerp near the river Scheldt and has been a primary production site for PFAAs since 1971. Its importance as a major point source of perfluoroalkyl substances has recently become clear, as a range of monitoring studies have reported among the highest PFAA concentrations in wildlife from 2003 onwards (Hoff et al., 2005; Dauwe et al., 2007; D'Hollander et al., 2014; Groffen et al., 2017; Lopez-Antia et al., 2017).

6.2.2 Data sampling

In total 56 nestboxes were installed at least six months prior to the breeding season and at similar distances from each other to minimalize local differences in great tit densities. Whole clutches were collected from eight nests during March–May 2016 at Fort IV (clutch size: 4–8 eggs ± 1.3 (min - max ± SD); n = 47). The nest-building phases of each nest were followed-up and advanced nests were checked daily in sequence to determine the egg laying date and identify the eggs individually. Every egg was then cautiously numbered with a non-toxic marker according to the laying sequence. After an entire clutch was completed, all the present eggs with known egg laying sequence were collected before incubation started and stored in 50 mL polypropylene (PP) tubes in a freezer (−20 °C) for later chemical analysis. The collection of the eggs was approved by the Ethical Committee for Animal Testing of the University of Antwerp (2014 - 90).

6.2.3 Egg parameters

Prior to chemical analysis, egg length (EL) and width (EW) were measured to the nearest 0.01 mm with digital callipers (Mitutoyo Belgium NV, Kruibeke, Belgium). The egg volume (EV) was then estimated using the equation (7.1) described in Ojanen et al. (1978), which can be applied as a specific measure for determining volume of great tit eggs:

$$EV = 0.042 + 0.4673 \times EL \times EW^2 \quad (6.1)$$

Afterwards, the content of every egg sample was weighed on a precision balance to the nearest 0.01 mg (Mettler Toledo, Zaventem, Belgium). Shell thickness was measured following the methodology described in Lopez-Antia et al. (2013). Briefly, three small shell pieces (approximately 0.5 cm^2 each) were obtained from the equatorial region and were dried. Then, the thickness of these pieces was measured with a micrometer (Mitutoyo Belgium NV, Kruibeke, Belgium) to the nearest 0.01 mm and egg shell thickness was then calculated as the average value of all pieces.

6.2.4 Chemical analysis

Samples were analyzed for a range of PFAAs and the mentioned abbreviations are conforming to Buck et al. (2011). In total four target PFSAs (PFBS, PFHxS, PFOS and PFDS) and 11 PFCAs (PFBA, PFPeA, PFHxA, PFHpA, PFNA, PFOA, PFDA, PFUdA, PFDoA, PFTrA and PFTeA) were examined (Table S6.1). Analyses were conducted by using isotopically mass-labelled internal standards (ISTDs) including $^{18}O_2$-PFHxS, [1,2,3,4–$^{13}C_4$]PFOS, $^{13}C_4$-PFBA, [1,2–$^{13}C_2$]PFHxA, [1,2,3,4–$^{13}C_4$]PFOA, [1,2,3,4,5–$^{13}C_5$]PFNA, [1,2–$^{13}C_2$]PFDA, [1,2–$^{13}C_2$]PFUdA and 1,2[$^{13}C_2$]PFDoA which were purchased from Wellington Laboratories (Guelph, Canada). The stock ISTD mixture (1.2 mL solution containing 2000 pg μL^{-1} of each previously mentioned mass-labelled internal standard with chemical purities of >98%) was diluted in a 50:50 mixture of HPLC grade acetonitrile (ACN) and water (VWR International, Leuven, Belgium) at a concentration of 125 pg μL^{-1} to spike the samples.

6.2.5 Sample extraction

The extraction was performed using solid-phase extraction based on the principle of weak-anion exchange. Whole egg content was transferred into a new PP tube and homogenized by alternatively vortex-mixing and sonicating. About 0.3 g of homogenized sample was weighed with a precision balance (±0.01 mg, Mettler Toledo, Zaventem, Belgium) and used for analysis. Briefly, the homogenates were spiked with 80 µL of 125 pg μL^{-1} ISTD mixture. Subsequently, 10 mL of ACN was added and the samples were sonicated (three times 10 min) with vortex-mixing in between periods. Then, the samples were left overnight on a shaking plate (±135 rpm, 20 °C, GFL 3020, VWR International, Leuven, Belgium) for approximately 16 h. Afterwards, the samples were centrifuged for 10 min in a type 5804R centrifuge (2400 rpm, 4 °C, Eppendorf centrifuge, rotor A-4-44) to precipitate and remove insoluble particles. The supernatant was transferred into a 14 mL PP tube.

PFAA extraction was performed by solid phase extraction (SPE) using Chromabond HR-XAW columns (Application-No 305200, SPE department, Macherey-Nagel, Germany, 2009). The column cartridges were conditioned with 5 mL ACN and 5 mL Milli-Q (MQ)

water. After the samples were loaded, the column cartridges were washed with 5 mL 25 mM ammonium acetate and 2 mL ACN. Then, the columns eluted with 2 × 1 mL 2% ammonium hydroxide and the purified extract was completely evaporated with an Eppendorf concentrator (30 °C, type 5301, Hamburg, Germany). The dried extract was dissolved in 200 µL 2% ammonium hydroxide in ACN and filtered through a 13 mm Acrodisc Ion Chromatography Syringe Filter with 0.2 µm Supor (PES) membrane (VWR International, Leuven, Belgium). Finally, the extract was transferred into a PP injector vial before instrumental analysis.

6.2.6 Instrumental analysis

PFAA measurements were conducted by UPLC coupled tandem mass spectrometry (ACQUITY, TQD, Waters, Milford, MA, USA) using negative electrospray ionization. Separation of the different PFAA target compounds was performed on an ACQUITY UPLC BEH C18 VanGuard Pre-column (2.1 × 5 mm; 1.7 µm, Waters, USA). The mobile phase consisted of HPLC grade water and ACN, both solvents dissolved in 0.1% HPLC grade formic acid. Then, the mobile phase was set up in a concentration gradient, initially consisting of 65% MQ and 35% ACN in 3.5 min and then changed to 10% MQ and 90% in nearly 1.5 min. This was followed by a return to the initial conditions for 2 min up to the end and the flow rate was set at 450 µL min^{-1} throughout the whole sample run time. An ACQUITY BEH C18 pre-column (2.1 × 30 mm, 1.7 µm, Waters, USA) was inserted between the injector and the solvent mixer. In this way, any PFAA contamination from the system could be retained.

The mass spectrometer operated in multiple reaction monitoring (MRM) mode, which enables detection and quantification of the selected target PFAA analytes, based on their corresponding diagnostic transitions (Table S6.1).

6.2.7 Quality control

Two types of blanks were used to assure proper analysis and extraction method. One spiked blank of 10 mL ACN was used as procedural blank after each batch of ten samples to detect any contamination. The same extraction and filtration procedure as described earlier was applied to these blanks. A second type of blank, consisting of

300 µL ACN, was immediately transferred into the injector vial every ten samples to prevent cross-over contamination between samples during detection in the UPLC-MS/MS.

A linear calibration curve was made by adding the same concentration of ISTD (125 pg μL^{-1}) to different concentrations of an unlabeled PFAAs mixture of each PFAA compound in ACN. This calibration curve consisted of 15 calibration points. The relationship between the ratio of concentrations of unlabeled and labeled PFAAs was described by a linear regression function with a highly significant linear fit ($R^2 > 0.99$; $P < 0.001$) for all the target PFAA analytes.

6.2.8 Statistical analysis

All statistical analyses were conducted in R (version 3.2.3) and graphs were created with the package "ggplot2". Validity of the models' assumptions was examined with Shapiro-Wilks test and data were log-transformed when needed to fulfil normality assumptions. The level of significance for all statistical tests was set at $P \leq 0.05$. Unless stated otherwise, reported means are expressed as least square means ± standard errors (SEs). Statistical differences among variable levels were denoted with different letters. Limits of quantification (LOQs) were determined on a signal to noise ratio of 10. PFAA concentrations that were below the LOQ were given a concentration of LOQ/2 (Bervoets et al., 2004; Loppi et al., 2015; Groffen et al., 2017). Whenever quantified concentrations of a given PFAA compound were below the LOQ in more than 50% of the samples, the compound was excluded from analyses.

Possible relationships of the laying sequence with the egg parameters and PFAA concentrations were tested with linear mixed-effect models using the package "lmerTest". The egg number (egg 1–8) according to the laying sequence in the clutch was considered as a fixed factor and the clutch identity as a random factor. These statistical models nested the individual eggs within their respective clutch and thus consider the dependency of the data. When significant differences were obtained, corrected Tukey's post-hoc tests were used to compare mean PFAA concentrations

and mean egg content weight among different egg numbers. The laying date was converted into a continuous variable by considering the first registered laying date (April 1) as day 1. Subsequently, linear regressions were used to identify the relationship between the egg parameters and PFAA concentrations considering the laying date of the 1ste egg as continuous covariate. PFAA variation within and among clutches was studied by estimating variance components using the restricted maximum likelihood estimation method. Correlations in PFAA concentrations among eggs from the same clutch were determined by Pearson's correlation coefficient.

6.3 Results

6.3.1 General accumulation profile PFAAs in eggs

An overview of the mean egg concentrations, range, detection frequency and limit of quantification for each detected PFAA compound is given in Table 6.1. PFOS was the only detected PFSA and showed the highest PFAA concentrations ranging from 6.7 ng g^{-1} to 55.1 ng g^{-1}. Hence, this compound accounted for a dominant contribution of 74% to the ∑ PFAAs (Fig. S7.1). Regarding the PFCAs, PFOA and PFDoA were detected in all samples with concentrations ranging from 0.72 ng g^{-1} to 3.7 ng g^{-1} and 0.90 ng g^{-1} to 4.78 ng g^{-1}, respectively. Both PFCAs contributed 7% of the ∑ PFAAs, followed by PFDA, PFTrA and PFNA (Table 6.1; Fig. S6.1). None of the following target PFAAs were detected in any sample and were therefore omitted from further analyses: all the short-chain PFAAs (PFBS, PFBA, PFPeA, PFHxA and PFHpA), some long-chain PFSAs (PFHxS and PFDS) and long-chain PFCAs (PFUnA and PFTeA).

Table 6.1. Mean ± standard error (SE) concentrations with minimum-maximum ranges and limit of quantification (LOQ) in pooled great tit eggs from eight clutches near Antwerp, Belgium in 2016, expressed in ng g^{-1} wet weight, along with the detection frequency, expressed in %, for each PFAA compound. Note that the ∑ PFSAs only includes PFOS as other PFSAs were not detected.

COMPOUND	MEAN ± SE	MIN.	MAX.	DET. FREQ.	LOQ
PFOA	2.0 ± 0.2	0.72	3.7	100	0.05
PFNA	1.0 ± 0.1	0.29	2.4	80.9	0.59
PFDA	1.5 ± 0.2	0.21	3.5	95.7	0.43
PFDOA	2.1 ± 0.3	0.9	4.8	100	0.44
PFTRA	0.97 ± 0.22	0.13	5.7	93.6	0.26
∑ PFCAS	7.8 ± 0.2	3.2	12.8		
PFOS	22.7 ± 3.8	6.7	55.1	100	2.6
∑ PFAAS	30.2 ± 0.8	8.9	75.1		

6.3.2 Within -and among-clutch variation in PFAA concentrations

Based on the estimated variance components, most of the variation in individual PFAA concentrations in the eggs could be explained by variation within clutches (Fig. 6.2). The within-clutch variation (WCV) of the ∑ PFAA concentrations was higher than the among-clutch variation (ACV), contributing for respectively 70.7% and 29.3% of the total variation. Likewise, the WCV component of the ∑ PFCAs was higher than the ACV component, accounting for 82.4% and 17.6% of the total variation, respectively. For PFOS, the WCV and ACV accounted for 67.2% and 32.8% of the total variation in PFOS concentrations, respectively (Fig. 6.2). Most of the variation in PFOA concentrations could be explained by the WCV component, which contributed for 96.3% of the total variation, whereas the ACV component only accounted for 3.7%. For PFNA, PFDA, PFDOA and PFTrA, the WCV accounted respectively for 89.4%, 92.3%, 82.7% and 99.9% of the total variation in PFAA concentrations (Fig. 6.2).

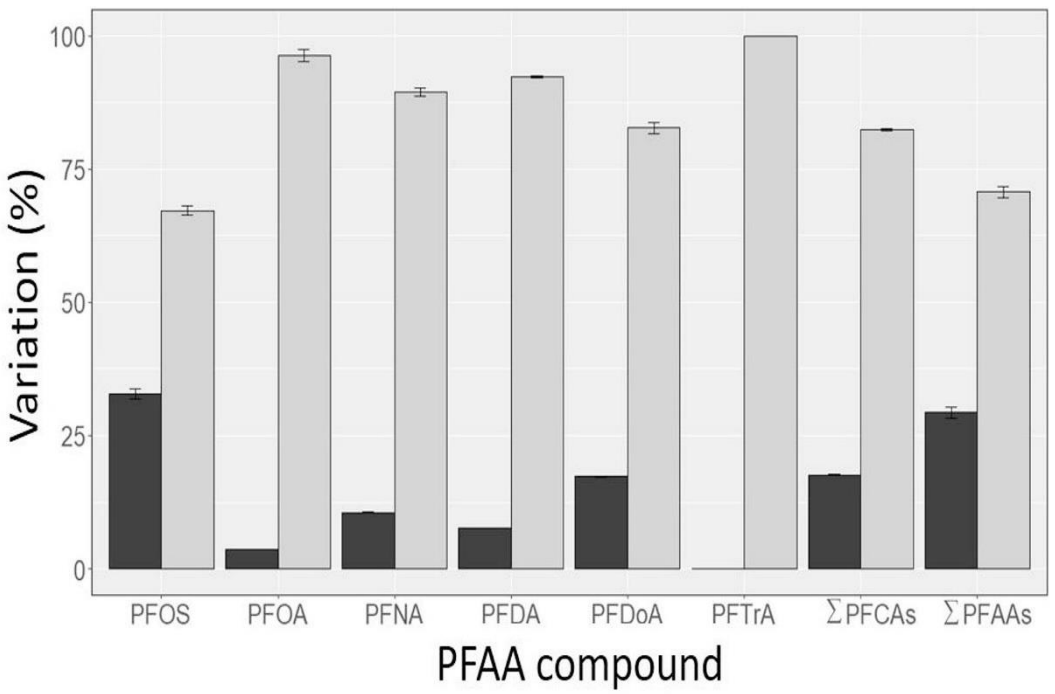


Figure 6.2. The estimated variance components, expressed in %, of the within-clutch variation (WCV: grey bar) and between-clutch variation (BCV: black bar) in eggs of whole clutches from great tit, nesting near Antwerp, Belgium in 2016. Error bars represent standard errors.

6.3.3 Relationships of egg laying sequence with PFAA concentrations

The large variation within clutches was reflected in significant changes of PFAA concentrations throughout the egg-laying sequence, both for PFOS and ∑ PFCAs (Fig. 6.3). Marked egg-laying sequence differences were observed for the detected PFAA compounds. For PFOS, egg 1 and egg 3 showed significantly higher concentrations compared to, respectively, egg 4, egg 5, egg 6 and egg 7 (all $P < 0.01$, $F_{7,38} = 5.7$; Fig. 6.4A). However, egg 8 was not different from egg 1, egg 2 or egg 3. Based on the total amount of PFOS transferred in each clutch, the mean percentage in each egg is presented in Table S6.2.

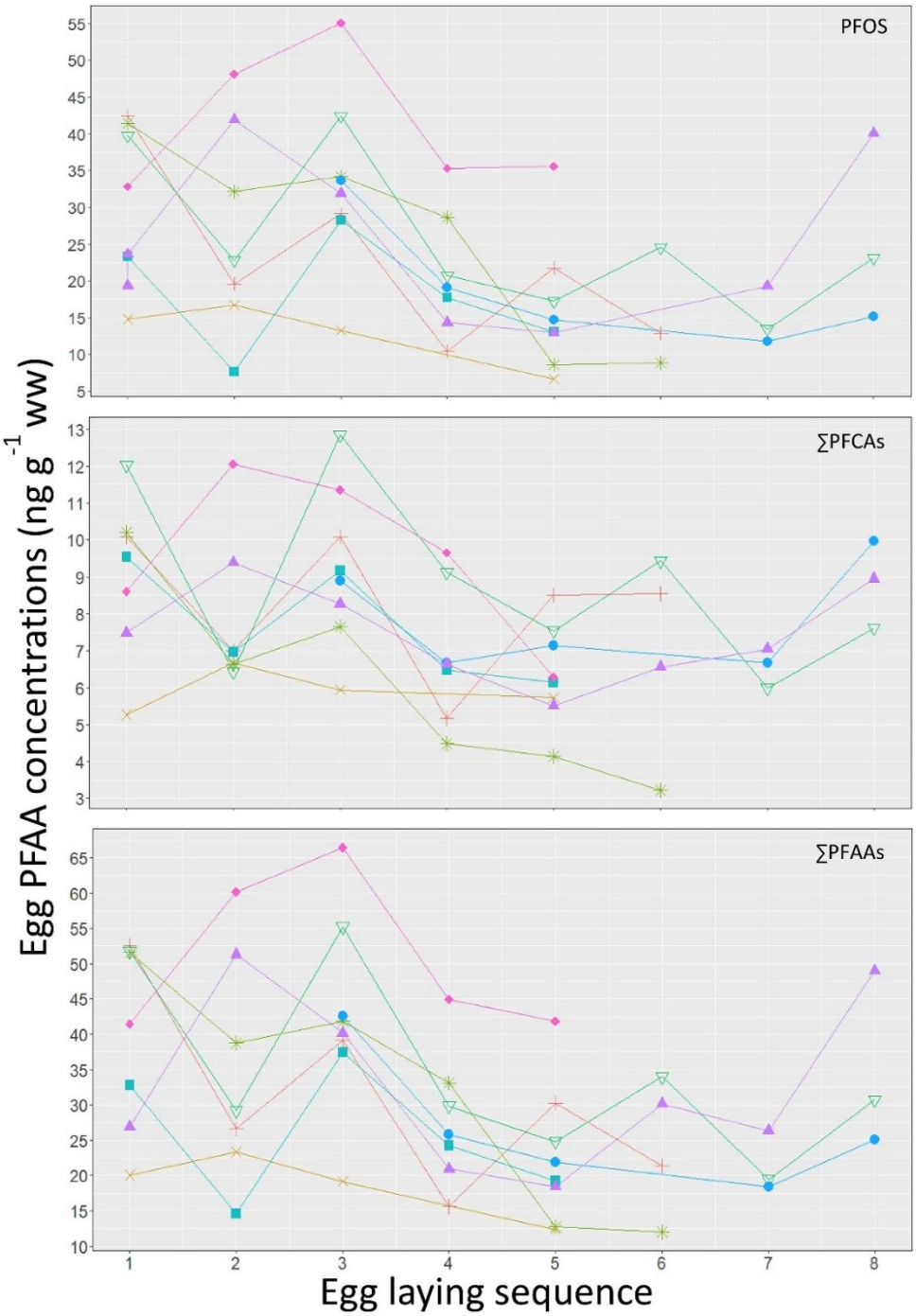


Figure 6.3. The change in absolute PFAA concentrations, expressed in ng g^{-1} wet weight (ww), in sequentially laid great tit eggs within the same nestbox for PFOS (upper graph), ∑ PFCAs (middle graph) and the ∑ PFAAs (lower graph) near Antwerp, Belgium in 2016. Note that the PFSAs only includes PFOS as other target PFSAs were not detected. Colors represent different nestbox identities. Egg 1: n = 7, egg 2: n = 7, egg 3: n = 8, egg 4: n = 7, egg 5: n = 8, egg 6: n = 4, egg 7: n = 3, egg 8: n = 3.

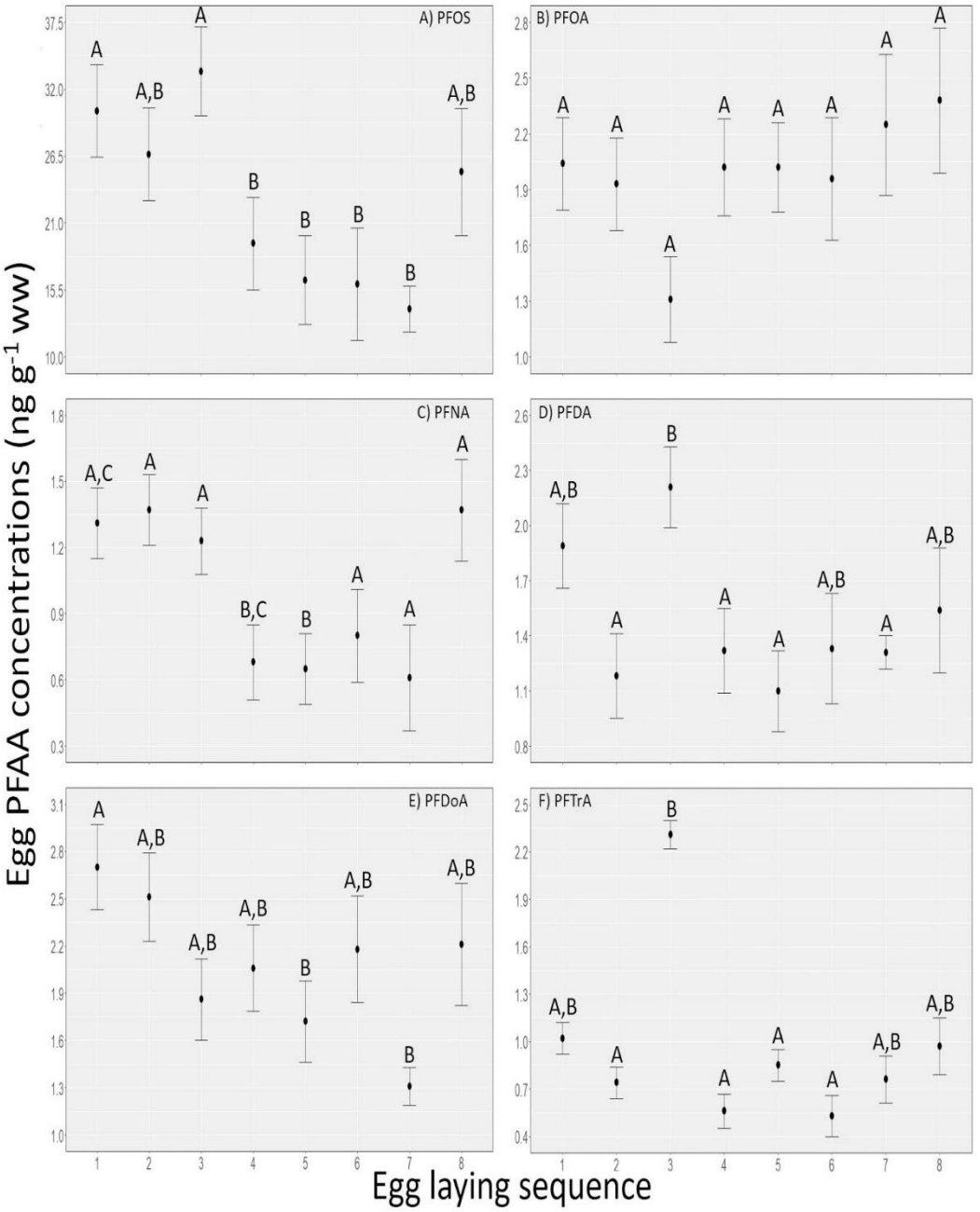


Figure 6.4. Mean PFAA concentrations, expressed in ng g^{-1} wet weight (ww), in sequentially laid great tit eggs from whole clutches near Antwerp, Belgium in 2016 for PFOS (A), PFOA (B), PFNA (C), PFDA (D), PFDoA (E) and PFTrA (F). Different letters denote significant (P < 0.05) differences among egg numbers in the laying order and the error bars represent standard errors. Egg 1: n = 7, egg 2: n = 7, egg 3: n = 8, egg 4: n = 7, egg 5: n = 8, egg 6: n = 4, egg 7: n = 3, egg 8: n = 3.

Approximately half of the clutches had similar PFOS concentrations in egg 8 as in egg 1 and three of the seven clutches showed a steady PFOS concentration decline throughout the egg laying period. The ∑ PFCAs showed an equally variable pattern with four of the seven clutches containing lower concentrations in later laid eggs, but three of the seven clutches containing higher or equal concentrations of ∑ PFCAs (Fig. 6.3). PFOA concentrations were not significantly associated with laying sequence ($P = 0.47$, $F_{7,38} = 1.3$; Fig. 6.4B). PFNA concentrations were significantly lower in egg 5 than in, respectively, egg 1 and egg 2 while egg 4 contained lower PFNA concentrations compared to egg 2 ($P = 0.001$, $F_{7,38} = 4.5$; Fig. 6.4C), but overall no clear egg order differences were observed. For PFDA, concentrations were significantly higher in egg 3 compared to egg 2, egg 4, egg 5 and egg 7 ($P < 0.01$, $F_{7,38} = 3.5$; Fig. 6.4D), but no clear trend throughout the laying order could be observed. Moreover, significantly higher PFDoA concentrations were found in egg 1 compared to egg 5 and egg 7 ($P < 0.05$, $F_{7,38} = 2.8$; Fig. 6.4E), but no egg order trend was found. Lastly, PFTrA concentrations were significantly higher in egg 3 than in egg 2, egg 4 and egg 5 ($P < 0.001$, $F_{7,38} = 5.6$; Fig. 6.4F).

6.3.4 Correlations PFAA concentrations among egg numbers

Correlations between PFAA concentrations in different egg numbers are shown in the correlation matrix (Table 6.2) and significantly positive correlations were observed between different egg pairs within the same clutch. PFOS, PFDA, PFTrA and ∑ PFCA concentrations were positively correlated between egg 1 and egg 3 (PFOS: $R = 0.66$, $P = 0.05$; PFDA: $R = 0.67$, $P < 0.05$; PFTrA: $R = 0.74$, $P < 0.05$; ∑ PFCAs: $R = 0.83$, $P < 0.05$, Table 6.2) whereas positive correlations could be found for ∑ PFAAs and PFNA, although marginally significant ($R \geq 0.60$, all $0.05 > P < 0.1$). PFDA concentrations were positively correlated between egg 2 and egg 4 ($R = 0.73$, $P < 0.05$, Table 6.2).

Table 6.2. Overview of the correlations in PFAA concentrations for all detected PFAA compounds between egg pairs of the same clutch near Antwerp, Belgium in 2016. Values represent Pearson correlation coefficients and bold values denote significant associations (significance levels: * = 0.05 > P < 0.10; ** = P < 0.05). Note that the ∑ PFSAs only includes PFOS as other PFSAs were not detected. Egg numbers 6, 7 and 8 were excluded from the statistical analysis as these data were missing from some clutches.

	PEARSON CORRELATION COEFFICIENT							
EGG-LAYING SEQUENCE	**PFOA**	**PFNA**	**PFDA**	**PFDoA**	**PFTrA**	**∑PFCAs**	**PFOS**	**∑PFAAs**
1 VS 2	0.43	0.12	0.46	-0.13	-0.5	-0.58	-0.49	0.05
1 VS 3	-0.36	**0.6***	**0.67****	0.04	**0.74****	**0.83****	**0.66****	**0.71***
1 VS 4	-0.07	**-0.92***	0.48	0.09	-0.21	0.31	-0.27	-0.2
1 VS 5	0.17	0.33	-0.18	0.12	-0.14	0.25	0.51	0.46
2 VS 3	**-0.84***	0.06	0.49	0.61	-0.48	-0.11	-0.02	0.26
2 VS 4	0.24	0.11	**0.73****	-0.53	0.3	0.32	-0.04	0.47
2 VS 5	0.5	0.01	**-0.83***	-0.02	0.26	0.1	-0.06	-0.07
3 VS 4	-0.58	-0.49	0.38	-0.15	-0.11	-0.05	-0.34	-0.27
3 VS 5	-0.3	0.2	-0.01	0.15	0.19	**0.66***	0.51	0.53
4 VS 5	-0.55	-0.52	**-0.79***	-0.05	-0.63	-0.61	-0.64	-0.65

6.3.5 Relationships between PFAA concentrations and egg parameters throughout the laying order

The mean egg weight ranged from 1.38 g (mean max.) to 0.57 g (mean min.), with egg 1 weighing significantly heavier than egg 2, egg 4, egg 5 and egg 8 ($P < 0.05$, $F_{7,38} = 6.9$; Fig. 6.5). Egg 8 weighed significantly lighter compared to all the other recorded eggs in the laying sequence ($P < 0.05$; Fig. 6.5). There was a significant interaction between PFOS and the laying sequence ($P < 0.05$, $F_{7,25} = 3.1$), while the interaction term was not significant between ∑ PFCAs and the laying sequence ($P = 0.89$, $F_{7,25} = 0.37$). Specifically, there was a positive association between PFOS concentrations and the weight of egg 1 ($P = 0.01$, $t_{25} = 2.3$), egg 3 ($P = 0.03$, $t_{27} = 2.6$) and egg 8 ($P = 0.002$,

t_{25} = 3.4). The egg volume and egg shell thickness (0.208 ± 0.004 mm) did not change significantly throughout the egg laying sequence (egg volume: P = 0.88, $F_{7,32}$ = 0.41; egg shell thickness: P = 0.56, $F_{7,39}$ = 0.84).

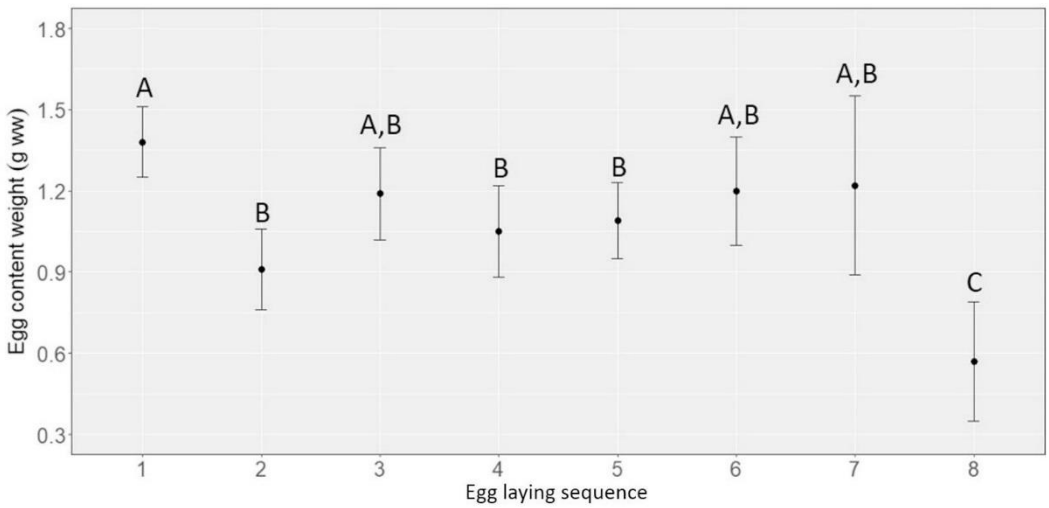


Figure 6.5. Mean egg content weight, expressed in g wet weight (ww), in sequentially laid great tit eggs from whole clutches near Antwerp, Belgium in 2016. Different letters denote significant (P < 0.05) differences among egg numbers in the laying order and the error bars represent standard errors. Egg 1: n = 7, egg 2: n = 7, egg 3: n = 8, egg 4: n = 7, egg 5: n = 8, egg 6: n = 4, egg 7: n = 3, egg 8: n = 3.

6.3.6 Correlations between egg-laying date of the 1st egg and PFAA concentrations

The laying date of the 1st egg was negatively and positively correlated with, respectively, the mean PFOS concentrations and mean egg weight of the clutches (Fig. 6.6). Clutches that were initiated later in the breeding season contained on average significantly higher PFOS concentrations (R^2 = 0.58, P < 0.05, Fig. 6.6), whereas a positive relationship was found between egg laying date and egg content weight (R^2 = 0.49, P < 0.05, Fig. 6.6). There was no significant association of 1st egg laying date with other PFAA concentrations and egg parameters (all P > 0.05).

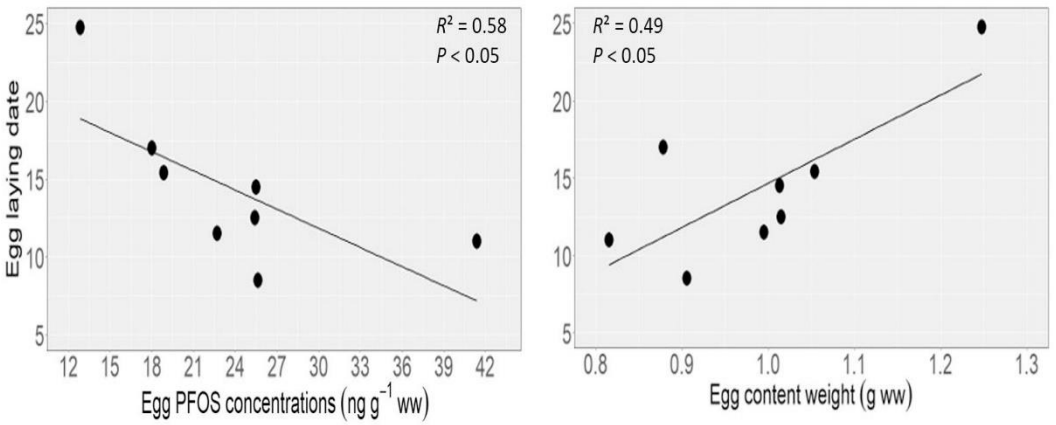


Figure 6.6. Linear regression plots showing the significantly (P < 0.05, R^2 = 0.58) negative relationship between egg laying date and egg PFOS concentrations, in ng g wet weight (left graph) and the positive (P < 0.05, R^2 = 0.49) relationship between egg laying date and egg content weight, in g wet weight (right graph) of great tit clutches near Antwerp, Belgium in 2016. The data points represent the mean egg PFOS concentrations and egg laying date for each clutch (n = 8).

6.4 Discussion

6.4.1 General accumulation profile PFAAs in eggs

The measured PFAA concentrations in the present study are relatively low in comparison with those found in previous monitoring studies conducted on bird eggs near Antwerp (Groffen et al., 2017; Lopez-Antia et al., 2017). The closest known PFAA point source (plant site, Fig. 6.1) is located about 11 km from Fort IV and previous monitoring studies in bird eggs at Antwerp indicated that PFAA concentrations follow a clear pollution gradient, with concentrations decreasing steeply from the plant site (D'Hollander et al., 2014; Groffen et al., 2017). However, mean PFOS and PFOA concentrations (resp. 12.0 ng g^{-1} and 0.45 ng g^{-1}) detected in free-range chicken eggs (*Gallus gallus*) only 1 km away from a fluorochemical plant in China (Wang et al., 2010) were lower than those found in the present study (resp. 22.7 ng g^{-1} and 2.0 ng g^{-1}). Multiple factors might explain the differences between the present study including indirect pathways, such as environmental and biological degradation (Liu and Avendaño, 2013; Gebbink et al., 2015; Brendel et al., 2018), which probably become more important drivers of PFAA concentrations compared to direct pathways at distant sites from the point source. Furthermore, given the ubiquitous presence of PFAAs in numerous consumer products (Buck et al., 2011; Zhou et al., 2013; Ulrich

et al., 2016), the possible influence of local, unknown PFAA sources on the exposure to birds and their eggs cannot be completely excluded. Great tits are free-living birds that live in much more variable conditions than domestic chickens and could therefore be more likely exposed to these unknown PFAA sources (e.g. dietary intake). This could ultimately lead to higher PFOS and PFOA concentrations in great tits compared to domestic chickens.

The contribution profile was dominated by PFOS (Fig. S6.1), which is in accordance with other studies conducted on PFAAs in eggs of terrestrial birds (Ahrens et al., 2011c; Custer et al., 2014; Groffen et al., 2017), aquatic birds (Nordén et al., 2013) and other wildlife (Butt et al., 2010; Fang et al., 2014; Groffen et al., 2018). PFOS tends to bioaccumulate in the liver due to the high amount of protein-rich tissue in this organ (Lau et al., 2007; Gebbink and Letcher, 2012). These proteins are synthesized in the liver of the mother and then transferred via the blood to the ovary and the eggs (Bertolero et al., 2015), which explains the dominant pattern of PFOS in eggs. Besides, the prevalent spatial presence of PFOS can generally be explained due to its high bioaccumulation potential as PFOS is a terminal degradation product of many perfluorinated compounds (Conder et al., 2008; Buck et al., 2011; Mudumbi et al., 2017).

None of the target short-chain PFAAs were detected, while the majority of target long-chain PFCAs (PFOA, PFNA, PFDA, PFDoA and PFTrA) could be detected in ≥80% of the egg samples. The dominance of PFOA and PFDoA to the ∑ PFCAs is in accordance with some PFAA biomonitoring studies in bird eggs (Haukas et al., 2007; Groffen et al., 2017), whereas other studies have not confirmed this dominant ∑ PFCA contribution of PFOA and PFDoA (Ahrens et al., 2011c; Custer et al., 2012; Nordén et al., 2013).

Generally, long-chain PFAAs have greater bioaccumulation potential than their short-chain homologues (Conder et al., 2008; Olesen et al., 2016) and thus are more likely to be transferred from the mother to her respective eggs. Moreover, the frequent detection of long-chain PFAAs in this study is in contrast with other reports in eggs

from whole clutches of gulls (*Larus* sp.), in which long-chain PFAAs were not or only sporadically detected (Vicente et al., 2012, 2015). On the other hand, Custer et al. (2012) also detected long-chain PFAAs in the eggs of tree swallows (*Tachycineta bicolor*). Compared to gulls, great tits and tree swallows invest much larger amounts of resources in eggs relative to their body weight and due to their large clutch size (Van den Steen et al., 2009b). Consequently, it could be that the persistent and bioaccumulative long-chain PFAAs are more prone to transfer into the eggs of small passerines compared to gulls.

6.4.2 Within -and among-clutch variation in PFAAs

A remarkable variation in PFOS and in the sum of PFAAs concentrations within clutches was observed (Fig. 6.2). Despite that the absolute PFOS concentrations were relatively low, WCV for all PFAAs was consistently higher than the ACV. For the ∑ PFAAs, WCV of was still greater than the ACV. These results support those of the few other studies in which PFAA clutch variation was assessed (Custer et al., 2012; Vicente et al., 2015).

However, Van den Steen et al. (2006; 2009a; 2009b) found higher ACV than WCV for other classes of organic pollutants (PCBs and PBDEs) in great and blue tits at Fort IV, although egg laying sequence influences were present (Table S6.3). Different chemical properties (PCBs and PBDEs: lipophilic versus PFAAs: both lipo- and hydrophobic) and hence different environmental transport mechanisms could explain these diverging results. It could be that the PCBs and PBDEs are spatially distributed in a more heterogeneous way than PFAAs. In contrast with PCBs and PBDEs, PFAAs have high water solubility and are more volatile (Siddiqi et al., 2003; Mudumbi et al., 2017). As wind and water are mainly responsible for a relatively homogeneous distribution pattern of most contaminants in general (Fernández and Grimalt, 2003), PCBs and PBDEs might be restricted to only some places within a location due to their more limited transport via these media. Lastly, natal dispersal distances of females in tit species sometimes exceed more than 3 km (Greenwood et al., 1979). Females that dispersed from varying PCB and PBDE polluted sites to Fort IV could also contribute in explaining the relatively large ACV contaminated sites in the former studies.

The very large WCV found in the present study is most likely related to the large clutch size of great tits and the fact that they are 'income' breeders (Ward and Bryant, 2006; Van den Steen et al., 2009a). Because of their large clutch size, great tits invest relatively large amounts of resources (e.g. proteins and lipids) in their eggs and most likely use resources from current, rather than stored nutrients. Consequently, large variations in PFAA concentrations, which are associated with these nutrients, could be expected. In addition, tits lay eggs on a daily basis and therefore rely on daily replenishment of maternal resources with resources of their diet (Van den Steen et al., 2009b). Therefore, the large WCV is probably also a consequence of the large PFAAs variation in prey items or variation in the types of prey being consumed.

During the breeding season, the diet of great tits mainly consists of caterpillars (Lepidoptera) (Dauwe et al., 2007). Variation in prey preferences for females throughout the days and local shifts in availability of insects throughout the breeding season can also be contributing mechanisms to increase WCV in great tits (Longcore et al., 2007; Custer CM et al., 2010). In order to understand better how variation in prey items is translated in variation of PFAA egg concentrations, stable isotope analysis of nitrogen and carbon could be a promising tool in future field studies investigating PFAA clutch variation.

6.4.3 Relationships of egg laying sequence with PFAA concentrations

The results of the present study show marked egg laying sequence variation of PFAAs throughout the clutch and an overall significant decrease on an average basis could be observed in the sum of PFAA concentrations (Figs. 6.3 and 6.4). While this was true especially for PFOS, it was not the case for all PFAA compounds. For example, PFOA, PFNA, PFDA, PFDoA and PFTrA showed basically no difference across the egg laying sequence (Fig. 6.4). On an individual clutch basis (Fig. 6.3), only three of the seven clutches declined in these PFAA concentrations while two clutches increased and then started to decline in pollutant concentrations. Three clutches alternately declined and increased in PFAA concentrations throughout the laying order and had similar concentrations in egg 8 as in egg 1.

Vicente et al. (2015) demonstrated that PFOS decreased with the laying sequence of the eggs in gulls (*Larus sp.*) and these results are also in line with studies assessing other organic pollutants in tits (Van den Steen et al., 2006, 2009a; 2009b). This observed pattern is likely due to decreasing concentrations of PFAAs in the mother during the laying period. Tits rely on daily replenishment of endogenous maternal resources with exogenous resources of their diet (Van den Steen et al., 2009b). Throughout the egg-laying period, these maternal resources are thus used for production of the first eggs, while dietary lipids and proteins for the later eggs probably contain lower PFAA concentrations (Van den Steen et al., 2009a). Likewise, maternal tissues from the liver probably store higher amounts of proteins to which PFAAs strongly bind and may therefore be more present in the endogenous maternal resources than in the exogenous dietary resources. Hence, a decreasing pattern in PFAA concentrations can be observed throughout the clutch.

Remarkably, the results of this study also show a significant increase in PFAA concentrations in egg 3 for PFDA and PFTrA. Although speculative, this result is best explained by a shortage in food availability and could possibly reflect mother birds which laid their third under lower food conditions. Instead, endogenous maternal reserves are exploited which may contain higher PFAA concentrations (Braune and Norstrom, 1989). The precise reason or proximate mechanism why this result was only expressed for these three mentioned compounds, remains to be elucidated. Notice that the sample sizes for the last eggs were relatively low. Ideally, more data should be collected to reveal whether this result is a spurious statistical relationship or there is a real biological mechanism behind this result.

6.4.4 Correlations PFAA concentrations among egg numbers

Despite the substantial within clutch variation, strong positive correlations were found in PFAA concentrations between eggs from the same clutch, particularly between egg 1 and egg 3. In other words, those clutches which contained high PFOS, PFDA and PFTrA concentrations in egg 1 also had high concentrations of these compounds in egg 3 (Table 6.2, Fig. 6.3). Apart from PFTrA, concentrations in egg 3 did not significantly

differ from those found in egg 1. Therefore, taking into account that WCV was large and marked egg-laying sequence associations with the PFAA concentrations were present, we recommend two alternative sampling strategies depending on the main research goal.

For great tits (and other passerine species provided that our results can be generalized for other species), the first or third egg for future biomonitoring studies should be collected. Indeed, when maximizing egg exposure metrics is the goal of biomonitoring, collection of the first or third egg is recommended. Alternatively, two or three random eggs could be collected from a clutch to even out the large variation in PFAA concentrations and hence obtaining a more representative sample. Importantly, this should not interfere with collecting other data in another context, for instance studying associations between pollutant concentrations and reproductive parameters (see Groffen et al., 2019c). From a practical point of view, random egg collection also prevents the need to visit the nest daily to identify and mark specific eggs in the laying sequence which may be practically beneficial.

Despite the present study being one of the very first in which PFAA variation along the egg-laying sequence is investigated, the previous statements concerning the sampling strategy are further supported by findings in other studies. In tree swallows (*Tachycineta bicolor*), up to 4-fold differences in PFOS concentrations within two clutches were found (Custer et al., 2012). Moreover, Vicente et al. (2015) demonstrated considerable PFAA variation in eggs of Audouin gulls (*Larus audouini*). In contrast to passerines, gulls lay small clutches of mostly three eggs and also differ in terms of trophic position and feeding habits. However, both species are income breeders and therefore depend on exogenous resources for egg formation (Hobson, 1995; Meijer and Drent, 1999). Interestingly, Vicente et al. (2015) reported decreasing concentrations of PFAAs along the laying sequence and PFOS concentrations between egg 1 and egg 3 were also strongly and positively correlated. This finding not only enhances the general importance of considering the sampling strategy when

monitoring PFAAs in bird eggs, but also suggests that the way of resource assimilation is a major proximate mechanism in explaining egg laying sequence associations with PFAAs, regardless of other life-history traits.

6.4.5 Relationships between PFAA concentrations and egg parameters throughout the laying order

The mean egg weight declined throughout the laying sequence (Fig. 6.5) and the significant interaction term between egg laying sequence and PFOS showed that concentrations of this compound were positively associated with the weight of egg 1, egg 3 and egg 8. Interestingly, these eggs also contained among the highest absolute PFOS concentrations. This result suggests that PFAA exposure may alter the egg composition which may change on its turn the egg weight, although it could equally well be that the egg composition changes the PFAA concentrations. For PCBs, higher concentrations in eggs of American kestrels (*Falco sparverius*) and blue tits (*Cyanistes caeruleus*) were also associated with heavier eggs (Fernie et al., 2000; Van den Steen et al., 2009b).

Recent studies demonstrated that PFOS shows high affinity towards very low-density lipoproteins, for instance phosvitin and lipovitellin, which are mainly present in egg yolk (Nordén et al., 2013; Bertolero et al., 2015). These egg nutrients are all synthesized in the liver of the mother bird before they get transferred to the egg yolk via the ovaries (Bertolero et al., 2015). Following this reasoning, heavier eggs presumably have higher lipoprotein concentrations and therefore might result in higher PFOS concentrations. Future studies on laying sequence associations with PFOS concentrations should assess nutrient concentrations in eggs to further examine the plausibility of this hypothesis.

In addition, the significant decrease in PFOS concentrations throughout the laying sequence has direct relevance to the potential toxicity of PFOS to the embryos. In many altricial bird species including great tits, hatching asynchrony is a ubiquitous life-history treat which results in higher survival rates of chicks from earlier laid eggs compared to those hatched from later laid eggs (Pijanowski, 1992; De Heij et al., 2006).

However, if the first laid eggs also contain higher PFOS concentrations, this general life-history pattern may be disrupted in heavily polluted habitats as PFAAs have been associated with reduced hatching success and growth rate of chicks (Molina et al., 2006; Yanai et al., 2008; Cassone et al., 2012; Custer et al., 2012, 2014).

6.4.6 Correlations between egg-laying date first egg and PFAA concentrations

Interestingly, the egg-laying date of the first egg was significantly and negatively correlated with egg PFOS concentrations, while heavier eggs were associated with late-breeding females (Fig. 6.6). This result is in contrast with studies conducted on other POPs. For pesticides, higher concentrations were associated with later breeding (Bustnes et al., 2007; Lopez-Antia et al., 2015a, 2015b) while no effect was observed for PCBs (Van den Steen et al., 2009b). To the best of our knowledge, this is the first study reporting associations between laying date and PFAA concentrations.

It could be possible that these associations reflect an age effect, given that early-breeding females are generally older than late-breeding females (Sydeman et al., 1991; De Forest and Gaston, 1996; Tartu et al., 2014a). Given the high bioaccumulation potential of PFOS, older birds would experience a higher lifetime exposure to PFOS compared to younger birds and this may be reflected in the transfer of higher concentrations to their eggs. On the other hand, Blévin et al. (2017) found in black-legged kittiwakes (*Rissa tridactyla*) that higher PFOS concentrations were correlated with longer telomere lengths, which is considered to be a measure of quality. Therefore, the authors proposed that PFAAs may stimulate self-maintenance mechanisms in birds bearing the highest PFAA concentrations. If so, older birds which presumably accumulate higher PFOS concentrations may invest more energy in these self-sustaining mechanisms in favour of their own individual fitness. This could be at the expense of energy investment in the eggs and the fitness of their offspring, which could explain the found association between lighter eggs and early breeding (Fig. 6.6).

Although the above explanation is plausible, it should also be emphasized that the sample size for testing this hypothesis was rather low and that the age of the birds was

unfortunately not known in the current study. Furthermore, to the best of our knowledge, the possible causal link of age with breeding date and egg weight, taking into account the PFAA pollution context, has never been examined. Future field studies on PFAAs in birds should include these variables.

6.5 Conclusions

In our study on great tits, an important model species in environmental research, the within-clutch variation was much higher for all PFAA compounds compared to the among-clutch variation, which is probably related with traits on (i) the species level (clutch size, variability in PFAA concentrations between exogenous and endogenous resources) (ii) individual level (e.g. age and variation in prey items), and (iii) the pollutant level (heterogeneous distribution of PFAAs). Significant and negative laying sequence relationships with both egg weight and PFAA concentrations were detected. These laying sequence associations may have important toxicological implications for developing embryos with potential disruption of general life-history patterns in bird species.

Regarding PFAA biomonitoring implications, one of the key findings is that in biomonitoring studies using bird eggs the sampling strategy chosen should depend on the main research objective. When maximizing egg exposure metrics is the main goal, one should sample consistently the same egg of each nest. In the case of great tits, the sampling of the first or third egg is recommended. Finally, it should be noted that research focusing on possible egg-laying sequence influences on PFAAs remains very scarce and sample sizes are often rather small. Therefore, further biomonitoring studies on the same species and other species with similar large clutch sizes should be conducted to validate the reported results in this study.

6.6 Acknowledgements

The authors are thankful to the Fund for Scientific Research Flanders (FWO-Flanders; grant nr. G038615N) and the University of Antwerp for supporting this research. We also acknowledge Wouter Melens, the park guard of Fort IV, for providing access to the study area and storage of field materials. We also express many thanks to Peter Scheys for assistance with the placement and maintenance of the nestboxes in the study area and Tim Willems for the support during the chemical analyses.

6.7 Supplementary data

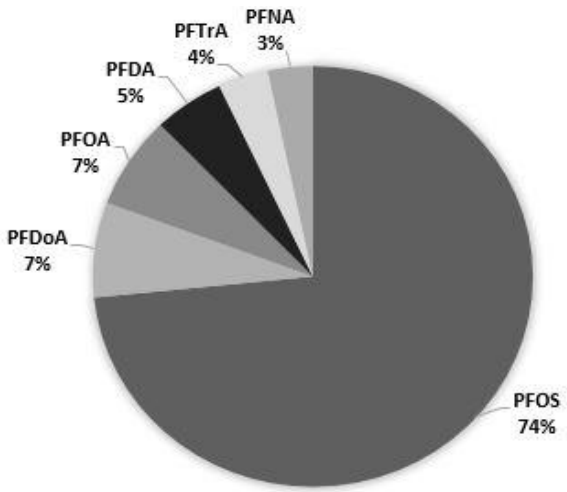


Figure S6.1. Relative mean contribution of each PFAA compound, expressed in %, to the total amount of PFAAs in great tit eggs.

Table S6.1. The multiple reaction monitoring (MRM) transitions of the target compounds and the used isotopically mass-labelled internal standards (ISTD) to quantify the compounds. The TQD tandem quadrupole mass spectrometer (MS) conditions, including collision energy (eV) and cone voltage (V) were adjusted to optimize detection of each compound. Adopted from Groffen et al. (2019a).

Compound	Precursor ion (m/z)	Product ion (m/z)		Cone Voltage (V)	Collision energy (eV) for diagnostic transition1	Collision energy (eV) for diagnostic transition 2	Internal standard (ISTD) used for quantification
		Diagnostic product Ion 1	**Diagnostic product Ion 2**				
PFBA	213	169	169	19	19	50	$^{13}C_4$-PFBA
PFPeA	263	219	219	15	10	45	$^{13}C_4$-PFBA
PFHxA	313	269	119	19	21	65	[1,2-$^{13}C_2$]PFHxA
PFHpA	363	319	169	24	40	30	[1,2-$^{13}C_2$]PFHxA
PFOA	413	369	169	22	13	60	[1,2,3,4-$^{13}C_4$]PFOA
PFNA	463	419	169	28	17	20	[1,2,3,4,5-$^{13}C_5$]PFNA
PFDA	513	469	219	25	29	29	[1,2-$^{13}C_2$]PFDA
PFUnDA	563	519	169	18	30	35	[1,2-$^{13}C_2$]PFUnDA
PFDoDA	613	569	319	22	21	30	[1,2-$^{13}C_2$]PFDoDA
PFTrDA	663	619	319	26	21	30	[1,2-$^{13}C_2$]PFDoDA
PFTeDA	713	669	169	28	21	21	[1,2-$^{13}C_2$]PFDoDA
PFBS	299	80	99	40	65	45	$^{18}O_2$-PFHxS
PFHxS	399	80	99	22	30	60	$^{18}O_2$-PFHxS
PFOS	499	80	99	60	58	58	[1,2,3,4-$^{13}C_4$]PFOS
PFDS	599	80	99	29	63	63	[1,2,3,4-$^{13}C_4$]PFOS

Table S6.1 (continued). The multiple reaction monitoring (MRM) transitions of the target compounds and the used isotopically mass-labelled internal standards (ISTD) to quantify the compounds. The TQD tandem quadrupole mass spectrometer (MS) conditions, including collision energy (eV) and cone voltage (V) were adjusted to optimize detection of each compound. Adopted from Groffen et al. (2019a).

Compound	Precursor ion (m/z)	Product ion (m/z)		Cone Voltage (V)	Collision energy (eV) for diagnostic transition1	Collision energy (eV) for diagnostic transition 2	Internal standard (ISTD) used for quantification
		Diagnostic product Ion 1	**Diagnostic product Ion 2**				
$^{13}C_4$-PFBA	217	172	172	19	19	50	
[1,2-$^{13}C_2$]PFHxA	315	269	119	19	21	65	
[1,2,3,4-$^{13}C_4$]PFOA	417	372	172	22	13	60	
[1,2,3,4,5-$^{13}C_5$]PFNA	468	423	172	28	17	20	
[1,2-$^{13}C_2$]PFDA	515	470	220	25	29	29	
[1,2-$^{13}C_2$]PFUnDA	565	520	170	18	32	35	
[1,2-$^{13}C_2$]PFDoDA	615	570	320	22	21	30	
$^{18}O_2$-PFHxS	403	84	103	22	30	60	
[1,2,3,4-$^{13}C_4$]PFOS	503	80	99	60	58	58	

Table S6.2 Transfer percentage of PFOS concentrations according to the egg laying sequence of each clutch. NA = data not available.

	Transfer percentage PFOS (%)							
Clutch ID	1	2	3	4	5	6	7	8
1	31.2	14.4	21.4	7.7	16.0	9.4	NA	NA
2	28.8	32.5	25.8	NA	13.0	NA	NA	NA
3	26.9	20.9	22.2	18.6	5.6	5.7	NA	NA
4	0.2	15.2	11.2	12.4	10.4	13.2	7.9	13.4
5	25.9	8.5	31.4	19.7	14.5	NA	NA	NA
6	NA	NA	35.7	20.3	15.6	NA	12.4	16.0
7	9.5	20.6	15.7	7.0	6.4	11.6	9.5	19.7
8	15.9	23.3	26.6	17.1	17.2	NA	NA	NA
Mean ± SE	19.7 ± 2.7	16.4 ± 2.9	24.9 ± 2.1	12.6 ± 2.0	12.1 ± 1.5	4.9 ± 1.3	3.6 ± 1.4	5.9 ± 2.0

Table S6.3: Overview of studies in which the laying order patterns and/or clutch variation (within-clutch variation (WCV) and among-clutch variation (ACV)) in concentrations of perfluorooctane sulfonic acid (PFOS) or other persistent organic pollutants (polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs)) were reported. NA = data not available.

Publication	Species	Sampling year	Pollutant(s)	Laying order effects	WCV (%)	ACV (%)	N clutches
Present study	Great tit (*Parus major*)	2016	PFOS	Yes	67.2	32.8	8
Vicente et al. (2015)	Audouin's gull (*Larus audouinii*)	2009	PFOS	Yes	NA, but ± 3.5-fold difference within clutch	NA	10
Custer et al. (2012)	Tree swallow (*Tachycineta bicolor*)	2008-2009	PFOS	NA	NA, but 4-fold difference within clutch	NA	2
Van den Steen et al. (2009b)	Blue tit (*Cyanistes caeruleus*)	2006	PCBs	Yes	40	60	10
			PBDEs	Yes	39	61	10
Van den Steen et al. (2009a)	Great tit (*Parus major*)	2006	PCBs	Yes	40	60	8
			PBDEs	Yes	30	70	8
Van den Steen et al. (2006)	Great tit (*Parus major*)	2000	PCBs	No	7	93	10
			PBDEs	No	3	97	10

7. Are feathers of a songbird model species (the great tit: *Parus major*) suitable for monitoring perfluoroalkylated acids (PFAAs) in blood plasma?

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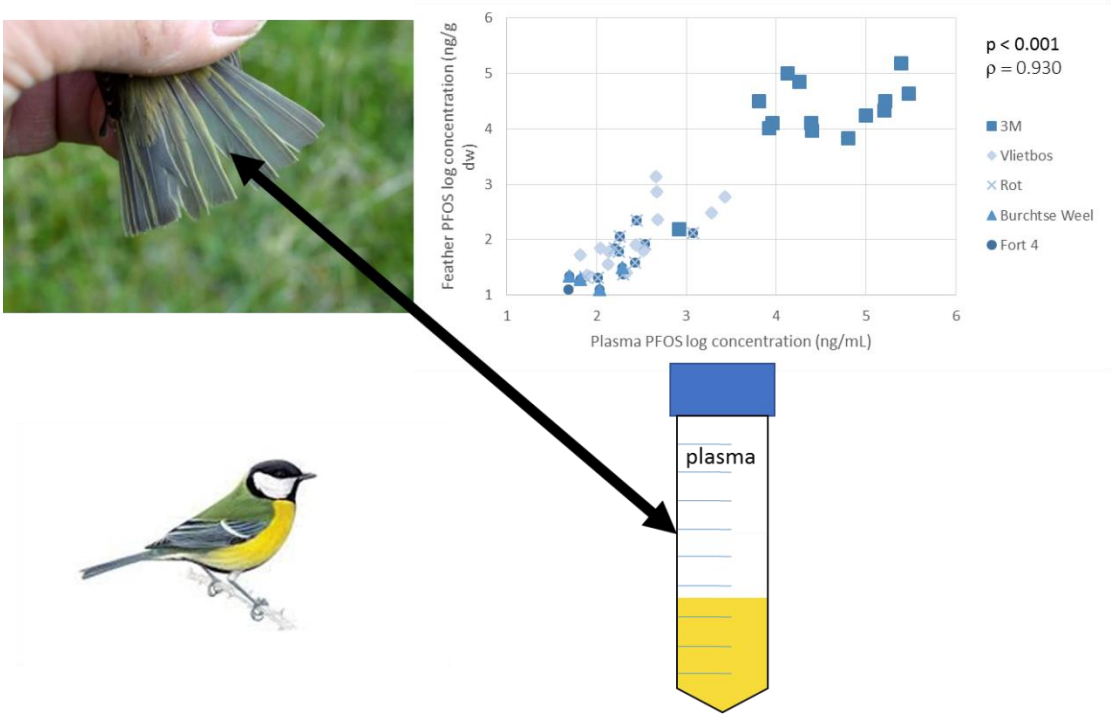


Abstract

Wild birds have been used in numerous biomonitoring studies on environmental contaminants, such as perfluoroalkylated acids (PFAAs). Due to both ethical and practical aspects, the use of non- or less-destructive sampling methods has increased. Feathers have been proven useful in the biomonitoring of environmental contaminants, such as metals and persistent organic pollutants. However, only little is known on PFAAs concentrations in feathers and their applicability as biomonitoring matrix for these compounds. In the present study, we evaluated to which extent feathers can serve as alternative to blood plasma for the biomonitoring of PFAAs in an insectivorous songbird model species, the great tit (*Parus major*), along a well-known distance gradient from a fluorochemical hotspot in Antwerp, Belgium.

We determined the concentrations of 11 perfluoroalkyl carboxylic acids (PFCAs; C4 – C14) and four perfluoroalkyl sulfonic acids (PFSAs; C4, C6, C8 and C10). The concentrations of all target analytes at the fluorochemical plant were the highest ever reported in feathers of wild birds and generally decreased with increasing distance from the plant. The dominant PFAA was perfluorooctane sulfonate (PFOS), for which concentrations ranged between 158 – 155000 ng/g ww (median 21700 ng/g ww) at the plant. The PFOS and perfluorooctanoic acid (PFOA) concentrations in feathers and blood plasma were significantly positively correlated. Concentrations of PFOA differed between both matrices at certain locations and these differences varied between individual birds at a certain location. Therefore we do not recommend using feathers to estimate internal PFOA concentrations in the plasma. However, our results indicate that feathers can be used to estimate PFOS concentrations in blood plasma. Feathers are likely useful in the biomonitoring of PFAAs as more compounds were detected in the feathers (13 compounds) than in the blood plasma (7 compounds), showing that feather PFAA concentrations and profiles are a better indication of the total PFAA exposure from the environment.

7.1 Introduction

Perfluoroalkylated acids (PFAAs) are compounds that have been produced and used for over 60 years in numerous industrial applications and consumer products, such as fire-fighting foams, surface coatings for carpets and fast-food packaging (Buck et al., 2011; Kissa, 2001). Some are classified as persistent organic pollutants (POPs), with hydrophobic and lipophobic properties, which are resistant to both chemical and biological degradation (Liu and Avendano, 2013; Ochoa-Herrera et al., 2016; Parsons et al. 2008). Consequently, PFAAs end up in the environment, either through direct emission or via the degradation of precursor compounds (Buck et al., 2011; Prevedouros et al. 2006) and are now widely distributed in the environment, biota and humans (e.g. Butt et al., 2010; D'Hollander et al., 2010; Giesy and Kannan, 2001, 2002; Houde et al., 2006; Miller et al., 2015).

Due to their persistence, potential (human) health effects and global distribution in the environment, the major global manufacturer of PFAAs, 3M, phased-out the production of perfluorooctane sulfonate (PFOS, $C_8F_{17}SO_3H$) and perfluorooctanoic acid (PFOA, $C_7F_{15}COOH$) and related products. These two PFAAs are the most frequently studied perfluoroalkyl sulfonic acid (PFSA) and perfluoroalkyl carboxylic acid (PFCA) respectively. In addition, PFOS was included in the Stockholm Convention on POPs in 2009. As a result, PFOS concentrations appear to be decreasing in most cases in the environment. Nevertheless, concentrations of PFOS and other PFAAs are sometimes still high in the environment and biota (Ahrens et al., 2011c; Filipovic et al., 2015b; Groffen et al., 2017; Miller et al., 2015).

Wild birds have been proven to be important biomonitors of environmental contaminants (e.g. Custer et al., 2012; Furness and Greenwood, 1993; Groffen et al., 2017; Holmström et al., 2010; Lasters et al., 2019). In multiple studies, non- or less-destructive sampling methods have been used due to both ethical and practical aspects. The use of feathers in biomonitoring studies is increasing. During formation and growth, feathers are connected to the blood circulation. Consequently, the internal contaminant concentrations may be transferred and deposited into the

feather (García-Fernández et al., 2013; Jaspers et al., 2006; Løseth et al., 2019). Although feathers have been used for numerous decades to monitor environmental metal concentrations (e.g. Dauwe et al., 2002, 2003; Eens et al., 1999; Golden et al., 2003; Jaspers et al., 2004; Ramos et al., 2009; Rattner et al., 2008), they have only been used since the early 21st century to study POPs (e.g. Dauwe et al., 2005a; Eulaers et al., 2011; Jaspers et al., 2006, 2007a, 2007b, 2009, 2011; Løseth et al., 2019; Svendsen et al., 2018). Only during the last decade, feathers have been used for the monitoring of PFAA concentrations (Gómez-Ramírez et al., 2017; Herzke et al., 2011; Jaspers et al., 2013; Li Y et al., 2017; Løseth et al., 2019; Meyer et al., 2009; Sun et al., 2019). Consequently, there is only limited data on the exposure and deposition of PFAAs into feathers and at present there is still discussion about whether feathers are suitable for the biomonitoring of PFAAs (Jaspers et al., 2019; Løseth et al., 2019).

The usefulness of feathers as a biomonitoring matrix is often tested by correlating the feather PFAA concentrations with PFAA concentrations in internal tissues and organs, such as blood and liver (e.g. Gómez-Ramírez et al., 2017; Løseth et al., 2019). To the best of our knowledge, only seven studies have measured PFAAs in feathers, of which only one having focused on a passerine bird species. In most of these studies, PFAA concentrations were lower in feathers compared to other tissues and organs (Gómez-Ramírez et al., 2017; Jaspers et al., 2013). However, Meyer et al. (2009) found differences in this pattern for PFOS depending on the target species. Similarly, most of these studies reported positive correlations between PFAA concentrations in feathers and liver (Gómez-Ramírez et al., 2017; Jaspers et al., 2013; Løseth et al., 2019; Meyer et al., 2009) and between feathers and preen oil (Herzke et al., 2011). Of these seven studies, only two studies have investigated the correlations between blood (plasma) and feather PFAA concentrations (Gómez-Ramírez et al., 2017; ; Løseth et al., 2019). Although significant positive correlations between both matrices were reported for concentrations of multiple compounds (PFHxS, PFDA, PFDoDA and PFTrDA) in white-tailed eagle nestlings (Gómez-Ramírez et al., 2017), this was not the case for PFOS,

PFOA, PFNA and PFUnDA (Gómez-Ramírez et al., 2017; ; Løseth et al., 2019), showing the difficulty to confirm or rule out the utility of feathers in PFAA biomonitoring.

In this study, we aim to evaluate if tail feathers from adult great tits (*Parus major*) present a valid matrix to monitor internal PFAA concentrations in plasma, for which the results have been reported by Lopez-Antia et al. (2019), by 1) correlating the PFAA concentrations in both matrices and 2) investigating differences in PFAA concentrations between both matrices. Additionally, we examined whether the PFAA concentrations in feathers decrease with increasing distance from a fluorochemical plant ,taking into account the age and sex of the birds.

7.2 Materials and method

7.2.1 Study species and sample collection

Great tits (*Parus major*) can be considered a model species for ecotoxicological studies. They breed in man-made nestboxes, are abundant and are known to live in polluted areas (Dauwe et al., 1999, 2004, 2005b; Eens et al., 1999; Eeva and Lehikoinen, 1995, 1996; Eeva et al., 1998; Van den Steen et al., 2006). The diet of great tits consists mainly of caterpillars during the breeding season (Grzędzicka, 2018; Naef-Daenzer and Keller, 1999; Rytkönen et al., 2018) and seeds, nuts and buds in the winter (Chamberlain et al., 2007).

During the autumn of 2015 we placed nestboxes at five sampling sites (Fig. 7.1), which represent a distance gradient from a fluorochemical plant (3M) in Antwerp, Belgium. Besides the 3M fluorochemical plant (28 nestboxes), which is a known PFAA hotspot containing the highest PFOS concentrations ever reported in different matrices (Dauwe et al., 2007; D'Hollander et al., 2014; Groffen et al., 2017; Groffen et al., 2019b, 2019c; Hoff et al., 2005; Lopez-Antia et al., 2017, 2019), Vlietbos (24 nestboxes; 1 km SE from 3M), Rot-Middenvijver (further called Rot; 20 nestboxes; 2.3 km ESE from 3M), Burchtse Weel (21 nestboxes; 3 km SE from 3M) and Fort 4 in Mortsel (58 nestboxes; 11 km SE from the plant) were chosen as sampling sites.

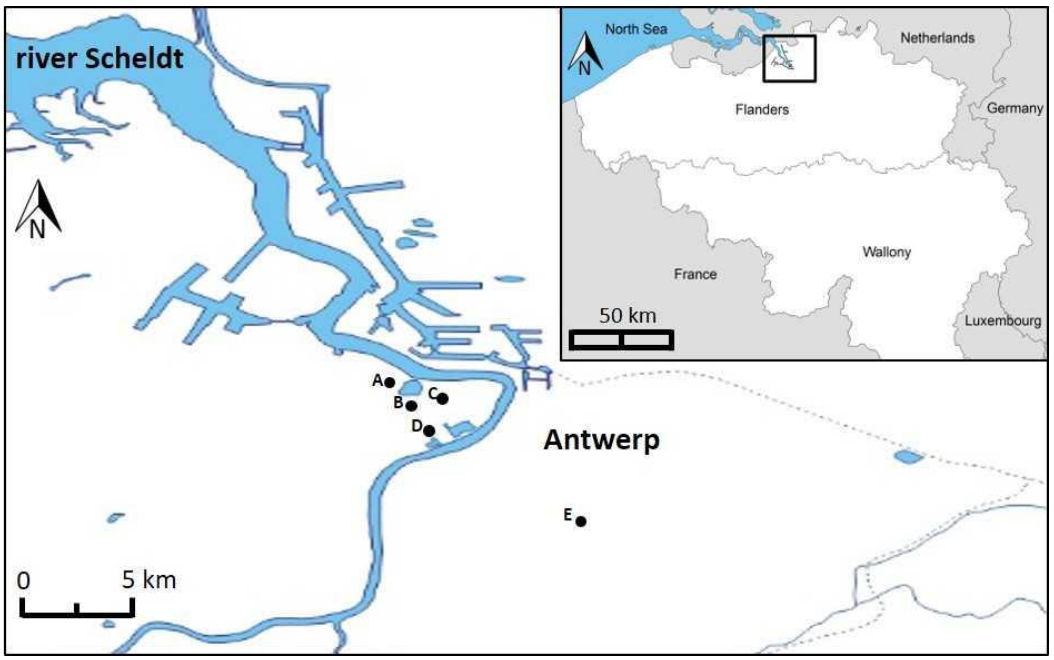


Figure 7.1. Overview of the study area in Antwerp, Belgium. Sampling locations are indicated as letters: A. Fluorochemical plant 3M, B. Vlietbos, C. Middenvijver-Rot, D. Burchtse Weel, E. Fort 4.

The outermost tail feathers (two feathers per individual) were collected during the winter of 2016. In total 75 birds were sampled (N = 15 for 3M and Vlietbos, N = 14 for Rot and Burchtse Weel and N = 17 for Fort 4). The sampling of these feathers occurred simultaneously with the sampling of the blood plasma from these birds (N = 14 for 3M, Vlietbos, Rot and Burchtse Weel, N = 17 for Fort 4; N = 73 in total), which has been reported by Lopez-Antia et al. (2019). Additionally, the birds were ringed and the sex and age class (yearling: <1 year old; older: > 1 year old) were determined. The tail feathers were stored in dark conditions at room temperature, to protect them from UV radiation, and the blood plasma at -80°C.

7.2.2 Chemicals and reagents

PFAAs are abbreviated according to Buck et al. (2011). The target analytes for feathers and blood plasma consisted of 11 PFCAs (PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoDA, PFTrDA and PFTeDA) and four PFSAs (PFBS, PFHxS, PFOS and PFDS). Isotopically mass-labelled internal standards (ISTDs) comprised $^{13}C_4$-PFBA, [1,2-$^{13}C_2$]PFHxA, [1,2,3,4-$^{13}C_4$]PFOA, [1,2,3,4,5-$^{13}C_5$]PFNA, [1,2-$^{13}C_2$]PFDA, [1,2-$^{13}C_2$]PFUnDA,

1,2[$^{13}C_2$]PFDoDA, $^{18}O_2$-PFHxS and [1,2,3,4-$^{13}C_4$]PFOS and were purchased from Wellington Laboratories (Guelph, Canada). Furthermore, HPLC grade acetonitrile (ACN; LiChrosolv, Merck Chemicals, Belgium), methanol (VWR International, Belgium), ammonium hydroxide (Filter Service N.V., Belgium) and Milli-Q (18.2 mΩ; TOC: 2.0 ppb; Merck Millipore, Belgium) were used.

7.2.3 Chemical extraction

The feathers were not washed prior to the analysis, as no external contamination was observed in a washing-test (with methanol) on outdoor free-range chicken feathers, with a known PFAA-contamination history, collected approximately 1 km from 3M (Table S7.1). Therefore, we are of opinion that PFAA concentrations in the feather samples represent mainly internal concentrations rather than external contamination from dust or other sources.

One of the two unwashed tailfeathers (8.3 ± 2.9 mg) were cut into small pieces (1 mm), using PFAA-free scissors, and placed into 50 mL PP tubes. After adding 10 mL of methanol, the samples were vortex-mixed during 1 min and left in the dark for 48 h at room temperature. Hereafter, the samples were centrifuged (4°C, 5 min, 2400 rpm, Eppendorf centrifuge 5804R, rotor A-4-44). The supernatant was transferred into a 15 mL PP tube, spiked with 80 μL of a 125 pg/μL ISTD solution and dried completely using a rotational-vacuum-concentrator (Martin Christ, RVC 2-25, Osterode am Harz, Germany). Finally, the samples were reconstituted with 2 mL of a 2% ammoniumhydroxide solution in ACN, vortex-mixed, and filtrated through an Ion Chromatography Acrodisc 13 mm Syringe Filter with 0.2 μm Supor (PES) Membrane (VWR International, Leuven, Belgium) attached to a PP auto-injector vial.

7.2.4 UPLC-TQD analysis and quantification

We used ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS, ACQUITY, TQD, Waters, Milford, MA, USA), with electrospray in negative ion mode (ES(-)), to analyze the target analytes. To separate the analytes, an ACQUITY BEH C18 column (2.1 x 50 mm; 1.7 μm, Waters, USA) was used. Additionally, an ACQUITY

BEH C18 pre-column (2.1 x 30 mm; 1.7 μm, Waters, USA) was inserted between the injector and the solvent mixer, to retain any PFAAs contamination from the system. 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B) were used as mobile phase solvents, with a flow rate of 450 μL/min and an injection volume of 10 μL. The gradient started at 65% A, decreased in 3.4 min to 0% A and returned to 65% A at 4.7 min. The analytes were identified and quantified using multiple reaction monitoring (MRM) of two diagnostic transitions per analyte. MRM transitions, cone voltages and collision energy of each target analyte, including the ISTDs, are displayed in Table S7.2, and were validated by Groffen et al. (2019a).

7.2.5 Quality assurance

Per batch of 10 samples, one procedural blank (10 mL methanol) was added as quality control. The blanks contained low concentrations of PFOA (3.4 pg/mL), PFDA (9.8 pg/mL), PFUnDA (20 pg/mL), PFDoDA (1.0 pg/mL) and PFTeDA (0.5 pg/mL), which were subtracted from concentrations in the samples in the same batch. Method recoveries for the target analytes varied between 60% and 95%. Concentrations of PFHpA did not exceed the LOQ in any of the samples. The individual limits of quantification (LOQs) for feathers were determined based on a signal-to-noise (S/N) ratio of 10 and are displayed in Table 7.1. Blood plasma PFAA concentrations in great tits and LOQs were part of a larger dataset (Lopez-Antia et al., 2019) and are displayed in Table 7.2.

7.2.6 Statistical analyses

Statistical analyses were performed in R Studio (version 3.2.2) and the level of significance was set at $p \leq 0.05$ (adjusted p-values). Normality assumptions of the used statistical models were examined using the Shapiro-Wilk test and running diagnostic plots. The data were log-transformed when needed to meet the normality assumptions of the residuals. The concentrations below the LOQ were given a value of LOQ/2 (Bervoets et al., 2004; Custer et al., 2000). Locations were excluded from statistical analyses when the detection frequencies of a certain compound at that location were below 50% (for example, Burchtse Weel and Fort 4 were not included when comparing PFBA concentrations among sites, as their detection frequencies

were <50%). To investigate the potential of feathers as alternative to blood plasma, we tested for correlations between the PFAA concentrations in both matrices using Spearman's correlations test for all sites together and also at each site separately. Furthermore, we compared the plasma and feather PFAA concentrations within sites using paired t-tests or non-parametric Wilcoxon rank sum tests (the latter only for PFOS at 3M). Differences in PFAA concentrations among locations were assessed using general linear models, followed by a backward elimination, with the location, sex and age of the bird and the two-way interactions between them as factors.

7.3 Results

7.3.1 PFAA concentrations and detection frequencies in feathers and blood plasma

Concentrations of the individual PFAA compounds in feathers and blood plasma are displayed in Tables 7.1 and 7.2, respectively. Concentrations of compounds that were detected in more than 50% of the samples at a site are also illustrated in Figures 7.2 and 7.3. For this reason, differences in feather PFAA concentrations among locations were only tested for PFBA, PFHxA, PFOA, PFDA, PFUnDA, PFDoDA and PFOS. In blood plasma, we could only compare PFAA concentrations among locations for PFOA, PFUnDA, PFDoDA and PFOS.

Table 7.1. Individual limits of quantifications (LOQs; ng/g ww), mean and median concentrations (ng/g ww), concentrations range (ng/g ww) and detection frequencies (DF; %) in feathers from great tits at the five study sites. Different letter indicate significant differences between locations in PFAA concentrations. PFHpA was excluded from the Table as concentrations did not exceed the LOQ in any of the samples.

		PFCAs									
		PFBA	PFPeA	PFHxA	PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTrDA	PFTeDA
LOQ (ng/g)		6.1	21	25	15	12	12	7.3	2.2	1.2	4.5
3M (N = 15)	Mean	334A	65	206A	897A	30	87A	32A	14A	3.6	<LOQ
	Median	392	50	192	698	19	79	26	9.5	1.7	<LOQ
	Range	22 – 627	<LOQ – 182	<LOQ – 561	27 – 2345	<LOQ – 99	16 – 297	7.8 – 63	<LOQ – 53	<LOQ – 17	<LOQ – 13
	DF	100	80	93	100	80	100	100	80	67	27
Vlietbos (N = 15)	Mean	29B	<LOQ	26B	44B	<LOQ	17B	28A	2.3	<LOQ	<LOQ
	Median	27	<LOQ	28	23	<LOQ	16	24	<LOQ	<LOQ	<LOQ
	Range	<LOQ – 93	<LOQ	<LOQ – 40	<LOQ – 175	<LOQ	<LOQ – 32	12 – 69	<LOQ – 6.3	<LOQ	<LOQ
	DF	93	0	60	73	0	80	100	33	0	0
Rot (N = 14)	Mean	17B	<LOQ	<LOQ	<LOQ	<LOQ	14B	28A	<LOQ	<LOQ	<LOQ
	Median	13	<LOQ	<LOQ	<LOQ	<LOQ	15	29	<LOQ	<LOQ	<LOQ
	Range	<LOQ – 48	<LOQ	<LOQ – 29	<LOQ – 26	<LOQ	<LOQ – 31	12 – 46	<LOQ – 5.0	<LOQ – 1.4	<LOQ
	DF	93	0	7	36	0	57	100	36	7	0

Table 7.1. (continued). Individual limits of quantifications (LOQs; ng/g ww), mean and median concentrations (ng/g ww), concentrations range (ng/g ww) and detection frequencies (DF; %) in feathers from great tits at the five study sites. Different letter indicate significant differences between locations in PFAA concentrations. PFHpA was excluded from the Table as concentrations did not exceed the LOQ in any of the samples.

		PFCAs									
		PFBA	PFPeA	PFHxA	PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTrDA	PFTeDA
LOQ (ng/g)		6.1	20	25	15	12	12	7.3	2.2	1.2	4.5
Burchtse Weel (N =14)	Mean	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	17B	25A	2.7B	<LOQ	<LOQ
	Median	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	18	21	2.3	<LOQ	<LOQ
	Range	<LOQ – 11	<LOQ	<LOQ – 40	<LOQ – 32	<LOQ	<LOQ – 38	10 – 68	<LOQ – 7.1	<LOQ	<LOQ
	DF	43	0	29	36	0	71	100	64	0	0
Fort 4 (N = 17)	Mean	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	19B	26A	3.4B	<LOQ	<LOQ
	Median	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	16	25	2.5	<LOQ	<LOQ
	Range	<LOQ	<LOQ	<LOQ – 45	<LOQ – 20	<LOQ	<LOQ – 61	13 – 78	<LOQ – 19	<LOQ – 2.1	<LOQ
	DF	0	0	12	24	0	65	100	53	6	0

Table 7.1. (continued). Individual limits of quantifications (LOQs; ng/g ww), mean and median concentrations (ng/g ww), concentrations range (ng/g ww) and detection frequencies (DF; %) in feathers from adult great tits at the five study sites. Different letter indicate significant differences between locations in PFAA concentrations. PFHpA was excluded from the Table as concentrations did not exceed the LOQ in any of the samples.

		PFSAs			
		PFBS	PFHxS	PFOS	PFDS
LOQ (ng/g)		8.7	73	3.0	39
3M (N = 15)	Mean	209	972	37121A	254
	Median	134	821	21737	89
	Range	<LOQ – 1251	<LOQ – 2769	158 – 154526	<LOQ - 1297
	DF	93	93	100	67
Vlietbos (N = 15)	Mean	9.7	<LOQ	275B	<LOQ
	Median	<LOQ	<LOQ	80	<LOQ
	Range	<LOQ – 30	<LOQ – 124	23 – 1359	<LOQ
	DF	27	7	100	0
Rot (N = 14)	Mean	<LOQ	<LOQ	219B	<LOQ
	Median	<LOQ	<LOQ	76	<LOQ
	Range	<LOQ	<LOQ	20 – 223	<LOQ
	DF	0	0	100	0
Burchtse Weel (N =14)	Mean	<LOQ	<LOQ	58C	<LOQ
	Median	<LOQ	<LOQ	11	<LOQ
	Range	<LOQ	<LOQ	<LOQ – 33	<LOQ
	DF	0	0	93	0
Fort 4 (N = 17)	Mean	<LOQ	<LOQ	27C	<LOQ
	Median	<LOQ	<LOQ	5.2	<LOQ
	Range	<LOQ	<LOQ	<LOQ – 23	<LOQ
	DF	0	0	88	0

Significant differences in feather PFAA concentrations between locations were observed for investigated analytes, with exception of PFUnDA ($F_{4,70}$ = 0.45, p = 0.736). The concentrations of PFBA ($F_{2,41}$ = 52.4, p < 0.001), PFHxA ($F_{1,28}$ = 44.1, p < 0.001), PFOA ($F_{1,28}$ = 60.9, p < 0.001), PFDA ($F_{4,68}$ = 16.1, p < 0.001), PFDoDA ($F_{2,43}$ = 7.17, p < 0.001) and PFOS ($F_{4,70}$ = 134, p < 0.001) were all significantly higher at 3M compared to the other locations. Furthermore, the PFOS concentrations at both Vlietbos and Rot were significantly higher than those at Burchtse Weel and Fort 4 ($F_{4,70}$ = 134, p < 0.001).

Concentrations of PFOS ($F_{3,50}$ = 71.9, p < 0.001), PFOA ($F_{4,68}$ = 5.74, p < 0.006) and PFDoDA ($F_{2,39}$ = 4.70, p < 0.033) in the blood plasma were significantly higher at 3M compared to all other locations. In addition, the PFOS concentrations at Vlietbos were higher than those at Burchtse Weel ($F_{3,50}$ = 71.9, p = 0.005). No significant differences were observed between PFUnDA concentrations at Vlietbos and 3M ($F_{1,24}$ = 1.62, p = 0.215).

Differences in feather PFAA concentrations between sexes were only observed for PFDA, with significantly higher concentrations in males compared to females ($F_{1,73}$ = 4.40, p = 0.036). For the other compounds, no differences between sexes were observed ($F_{1,73}$ = 0.12 – 2.26, p = 0.137 – 0.731). In blood plasma, no significant differences were observed between sexes ($F_{1,71}$ = 0.33 - 0.65, p = 0.422 - 0.565). No differences between older and yearling birds and no interactions between location, sex and age were observed in both feathers ($F_{1,73}$ = 0.03 – 1.27, p = 0.263 – 0.862) and blood plasma ($F_{1,71}$ = 0.02 - 0.79, p = 0.377 - 0.891).

The detection frequencies of most compounds were generally higher at 3M in both matrices compared to the other locations (Fig. 7.2 and 7.3, Tables 7.1 and 7.2). In feathers more compounds could be detected than in blood plasma, as only PFHpA was not detected in any sample and PFTeDA was only detected in a few samples, whereas in blood plasma no concentrations of PFHpA, PFTeDA, PFBS, PFHxS and PFDS could be detected.

Table 7.2. Individual limits of quantifications (LOQs; ng/mL), mean and median concentrations (ng/mL), concentrations range (ng/mL) and detection frequencies (DF; %) in blood plasma from great tits at the five study sites. Different letter indicate significant differences between locations in PFAA concentrations. The data is adapted from a larger dataset reported by Lopez-Antia et al. (2019). PFHpA, PFTeDA, PFBS, PFHxS and PFDS have been excluded, as they were not detected in any of the samples.

		PFCAs									PFSAs
		PFBA	PFPeA	PFHxA	PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTrDA	PFOS
LOQ (ng/mL)		6.5	52	8.2	2.6	4.1	5.5	6.4	1.8	1.4	47
3M (N = 14)	Mean	13	<LOQ	<LOQ	109A	27	124	18A	20A	5.81	80439A
	Median	<LOQ	<LOQ	<LOQ	92	16	7.6	6.5	16	3.1	24592
	Range	<LOQ – 133	<LOQ	<LOQ	22 – 233	<LOQ – 81	<LOQ – 477	<LOQ – 57	<LOQ – 60	<LOQ – 25	818 – 294052
	DF	7	0	0	100	79	50	50	71	50	100
Vlietbos (N = 14)	Mean	<LOQ	<LOQ	<LOQ	53B	<LOQ	<LOQ	8.1A	4.2AB	<LOQ	528B
	Median	<LOQ	<LOQ	<LOQ	50	<LOQ	<LOQ	<LOQ	3.3	<LOQ	244
	Range	<LOQ	<LOQ	<LOQ – 8.6	28 – 95	<LOQ – 5.5	<LOQ – 19	<LOQ – 24	<LOQ – 17	<LOQ – 2.3	<LOQ – 2712
	DF	0	0	7	100	7	21	64	64	29	71
Rot (N = 14)	Mean	<LOQ	55	<LOQ	68B	<LOQ	<LOQ	9.7	3.6B	<LOQ	219BC
	Median	<LOQ	<LOQ	<LOQ	43	<LOQ	<LOQ	10	3.5	<LOQ	168
	Range	<LOQ	<LOQ – 203	<LOQ – 9.8	29 – 405	<LOQ – 12	<LOQ – 11	<LOQ – 19	<LOQ – 8.2	<LOQ – 2.3	<LOQ – 1182
	DF	0	36	7	100	21	29	43	64	36	93

Table 7.2. (continued) Individual limits of quantifications (LOQs; ng/mL), mean and median concentrations (ng/mL), concentrations range (ng/mL) and detection frequencies (DF; %) in blood plasma from great tits at the five study sites. Different letter indicate significant differences between locations in PFAA concentrations. The data is adapted from a larger dataset reported by Lopez-Antia et al. (2019). PFHpA, PFTeDA, PFBS, PFHxS and PFDS have been excluded, as they were not detected in any of the samples.

		PFCAs									PFSAs
		PFBA	PFPeA	PFHxA	PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTrDA	PFOS
LOQ (ng/mL)		6.5	52	8.2	2.6	4.1	5.5	6.4	1.8	1.4	47
Burchtse Weel (N =14)	Mean	<LOQ	<LOQ	<LOQ	49B	<LOQ	<LOQ	<LOQ	2.7	<LOQ	58C
	Median	<LOQ	<LOQ	<LOQ	48	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Range	<LOQ	<LOQ	<LOQ	18 – 85	<LOQ – 8.4	<LOQ	<LOQ – 21	<LOQ – 6.6	<LOQ	<LOQ – 195
	DF	0	0	0	100	14	0	14	29	0	57
Fort 4 (N = 17)	Mean	<LOQ	<LOQ	<LOQ	51B	<LOQ	<LOQ	<LOQ	1.9	<LOQ	<LOQ
	Median	<LOQ	<LOQ	<LOQ	49	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Range	<LOQ – 17	<LOQ	<LOQ	32 – 75	<LOQ – 12	<LOQ – 11	<LOQ – 27	<LOQ – 8.4	<LOQ – 4.9	<LOQ – 61
	DF	6	0	0	100	29	6	12	41	6	12

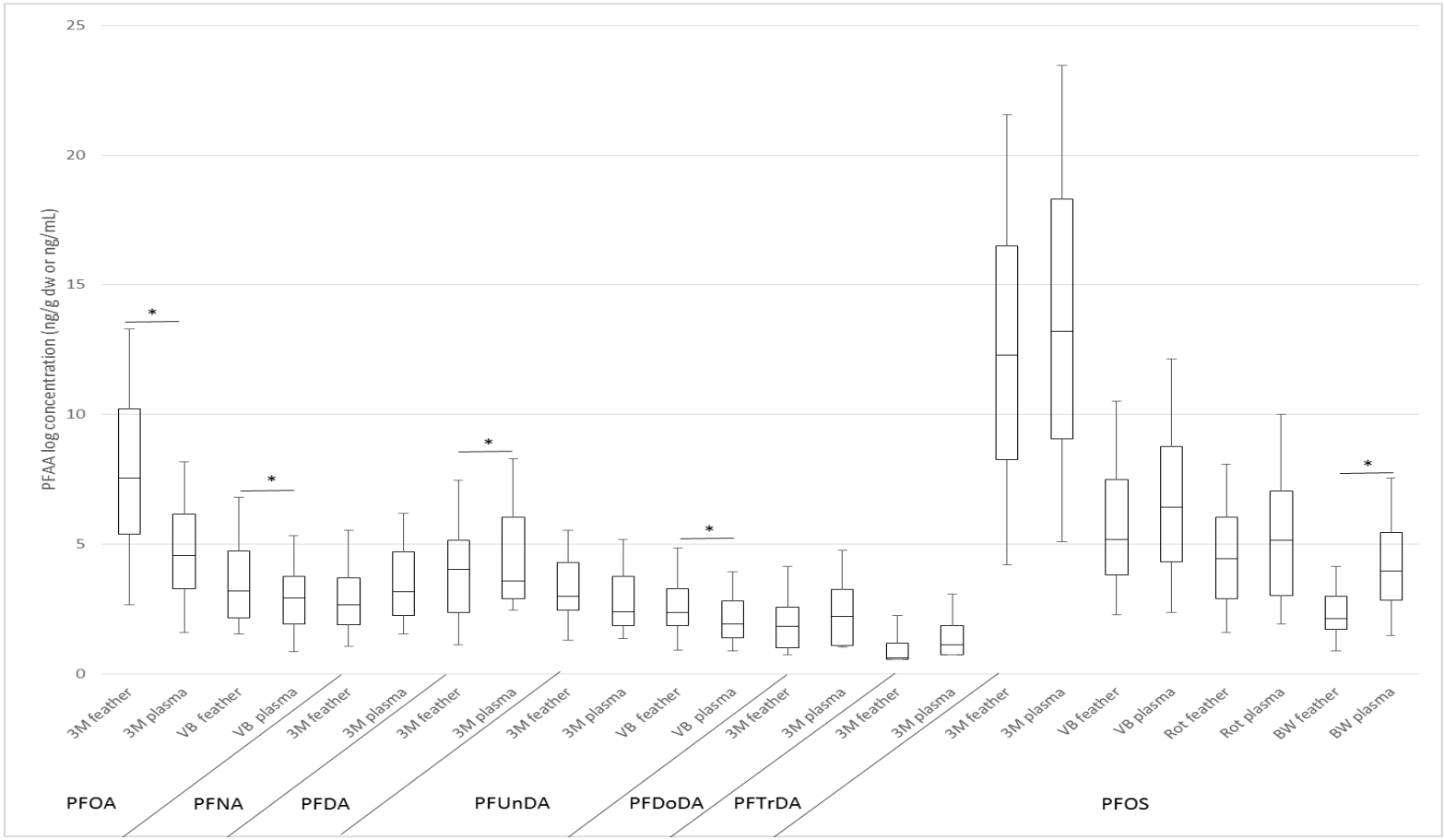


Figure 7.2. Concentrations (log-transformed) of PFCAs and PFOS in feathers (ng/g ww) and blood plasma (ng/mL) at different locations (3M, VB = Vlietbos, Rot and BW = Burchtse Weel). Significant differences between matrices at a certain location are indicated with an asterisk.

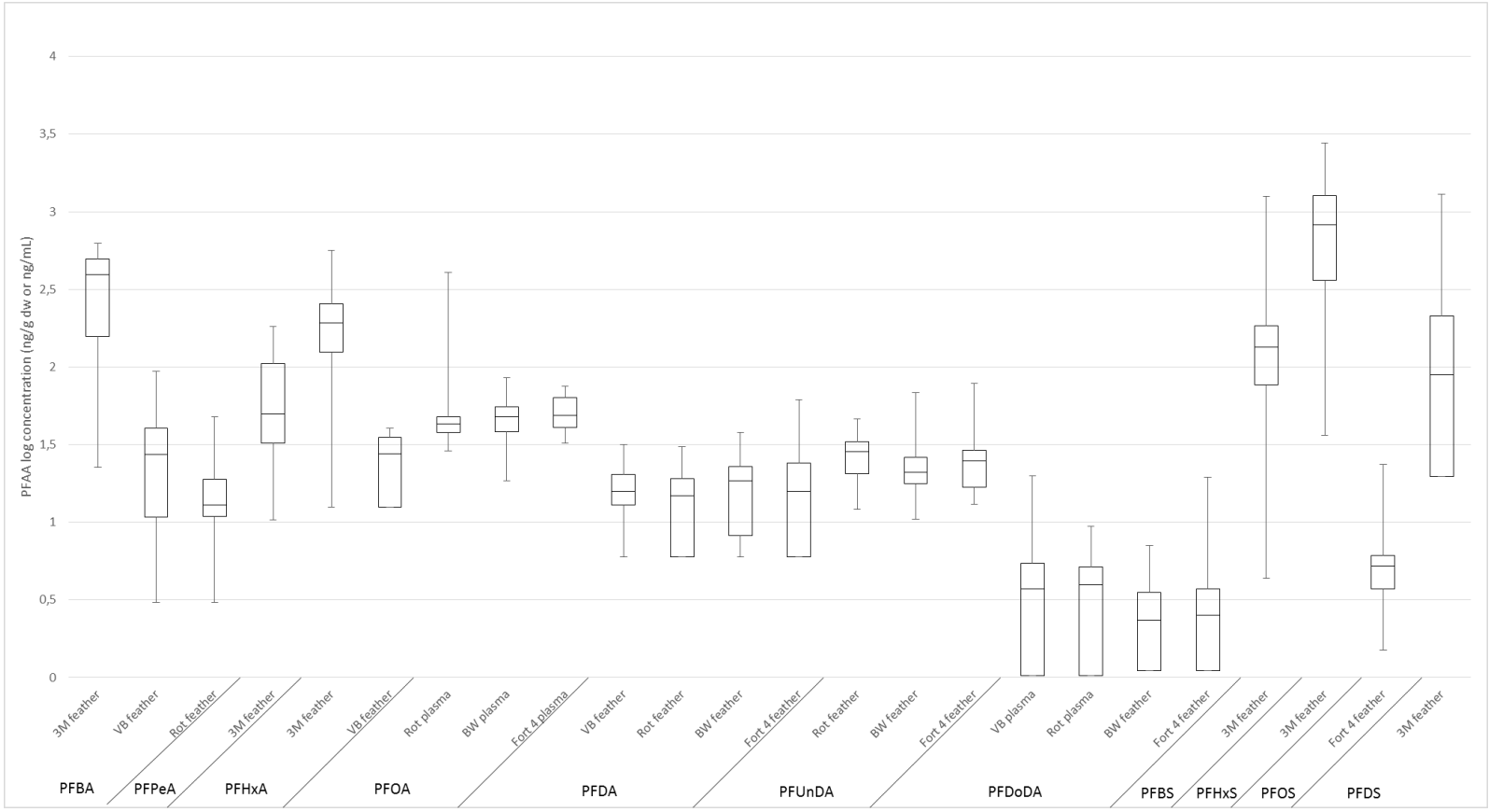


Figure 7.3. Concentrations (log-transformed) of PFCAs and PFSAs that were detected either in feathers (ng/g ww) or blood plasma (ng/mL) at different locations (3M, VB = Vlietbos, Rot, BW = Burchtse Weel and Fort 4). Only compounds with a detection frequency >50% at a site were taken into account.

7.3.2 Associations and differences between plasma and feather concentrations

When grouping all sites together, highly significant positive correlations were observed between feather and blood plasma concentrations of PFOA (N = 40, $p < 0.001$, $\rho = 0.639$, Fig. 7.4a) and PFOS (N = 46, $p < 0.001$, $\rho = 0.930$, Fig 7.4b.). At the individual sites, no significant correlations were observed for PFOA (N = 14, $p = 0.502$, $\rho = 0.196$) , PFNA (N = 8, $p = 0.389$, $\rho = 0.357$), PFDA (N = 7, $p = 0.236$, $\rho = 0.536$), PFUnDA (N = 7, $p = 0.783$, $\rho = 0.143$), PFDoDA (N = 8, $p = 0.752$, $\rho = -0.143$) and PFOS (N = 14, $p = 0.186$, $\rho = 0.376$) at 3M. Significant positive correlations were observed between feather and blood plasma concentrations of PFOA (N = 10, $p < 0.001$, $\rho = 0.791$, Fig. 7.4a) and PFOS (N = 13, $p = 0.021$, $\rho = 0.618$, Fig. 7.4b) at Vlietbos. Finally, at Rot, no significant correlations were observed for PFUnDA (N = 9, $p = 0.194$, $\rho = -0.483$), but a significant positive correlation was observed for PFOS (N = 10, $p = 0.028$, $\rho = 0.709$, Fig. 7.4b).

Figure 7.2 illustrates the PFCA and PFOS concentrations in both matrices at different sites. Compounds that were detected in only one of the two different matrices at a detection frequency of more than 50% are illustrated in Fig. 7.3. Significant differences between feather and blood plasma PFAA concentrations at 3M were observed for PFOA ($t_{13} = 4.25$, $p < 0.001$) and PFDA ($t_{13} = 2.28$, $p = 0.040$), with in both cases concentrations being higher in the feathers than in the blood plasma. Concentrations of PFUnDA were marginally significantly higher in the feathers compared to the plasma ($t_{13} = 2.10$, $p = 0.056$). No differences between both matrices were observed for PFNA ($t_{13} = 0.75$, $p = 0.466$), PFDoDA ($t_{13} = -0.46$, $p = 0.652$), PFTrDA ($t_{13} = -0.329$, $p = 0.748$) and PFOS ($p = 0.533$). At Vlietbos, the PFOA concentrations were significantly higher in plasma ($t_{13} = -3.06$, $p = 0.009$), whereas PFUnDA concentrations were higher in feathers ($t_{13} = 6.45$, $p < 0.001$). Concentrations of PFOS were marginally significantly higher in blood plasma ($t_{13} = -2.00$, $p = 0.066$). Finally, the PFOS concentrations did not differ between matrices at Rot ($t_{13} = -1.43$, $p = 0.178$) and were significantly higher in blood plasma at Burchtse Weel ($t_{13} = -4.71$, $p < 0.001$).

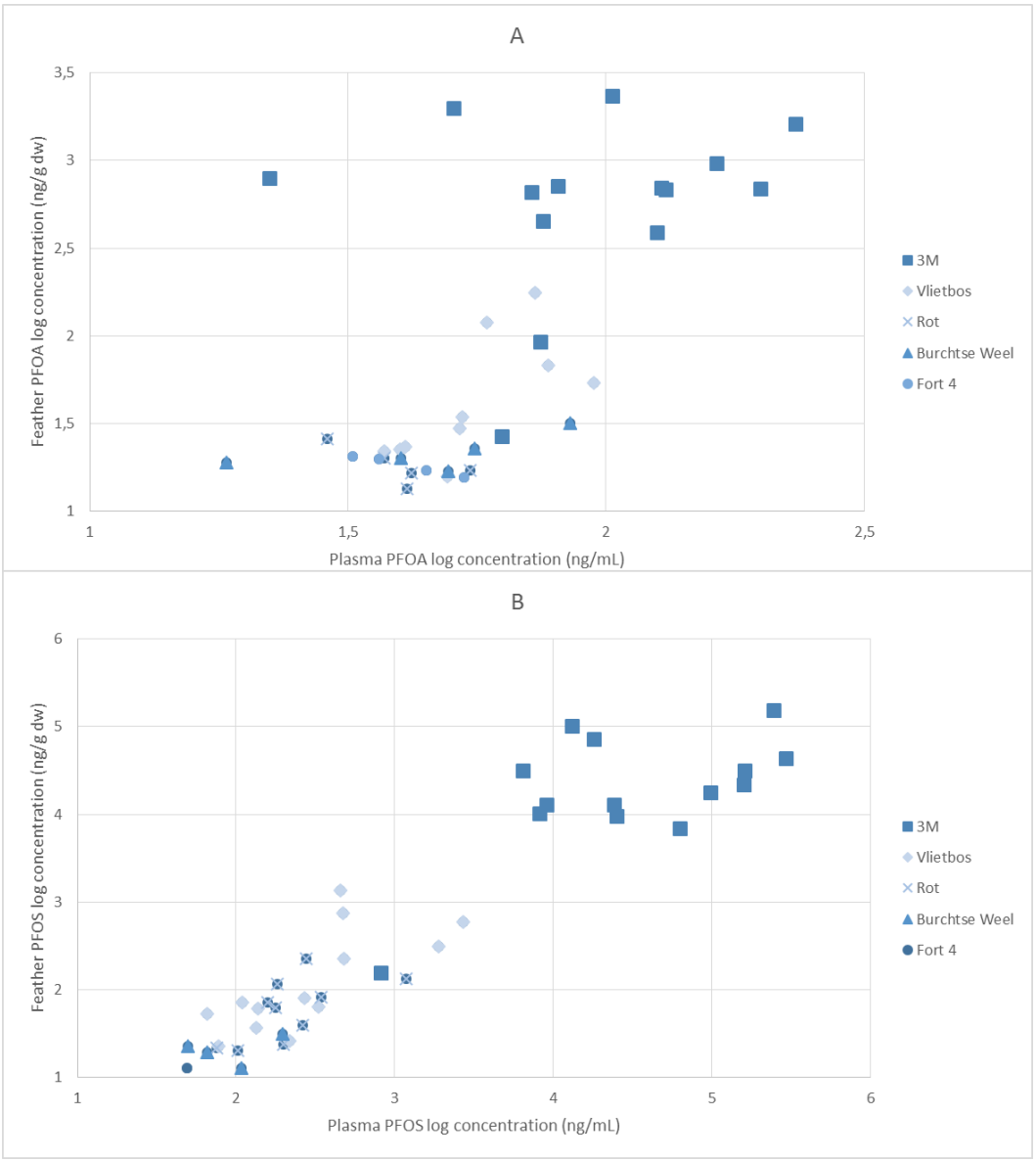


Figure 7.4. Correlations between feather (ng/g ww) and blood plasma (ng/mL) concentrations (log-transformed) of a) PFOA and b) PFOS at different sites.

7.4 Discussion

7.4.1 PFAA concentrations and detection frequencies in feathers and blood plasma

With exception of PFUnDA, concentrations of PFAAs were generally higher at 3M and tended to decrease with increasing distance from the fluorochemical plant. This particular trend was also reported in previous studies conducted in the same study area (Groffen et al., 2017, 2019b, 2019c; Lopez-Antia et al., 2019) and confirms that the 3M fluorochemical plant in Antwerp can be considered a PFAAs hotspot.

The concentrations of all PFAAs in feathers at 3M were the highest ever reported in wild birds. In order to compare the feather PFAA concentrations with already existing literature, an overview of PFAA concentrations in bird feathers is given in Table 7.3. In previous studies on PFAAs in birds, the highest mean PFOS concentration reported was 247 ng/g dw in grey heron (*Ardea cinerea*) tail feathers (Meyer et al., 2009), which is slightly lower than those reported at Vlietbos in the present study. However, concentrations at 3M were almost 150 times higher than those reported by Meyer et al. (2009). Similarly, mean and median concentrations of all other PFAAs were much higher close to the point source in the present study compared to the previously known highest concentrations.

Table 7.3. PFAA concentrations (ng/g ww) reported in bird feathers in literature and at 3M in the present study. Different feather types were abbreviated as following: body (B), tail (T) and wing (W). NM = not mentioned. [1]median concentrations, [2]mean concentrations, [3]Concentration in one sample, [4]Concentrations were not reported in the text, but were estimated from barplots.

	White-tailed eagle, Haliaeetus albicilla							
Country	Norway[1]	Norway	Norway[1]	Norway[1]	Greenland[1]			Sweden[1]
Year	2014	2015 – 2016	1997 – 2009	2006 – 2015	1997 – 2009		2006 – 2015	2006 – 2015
Type	B	B	T	B	B	W	B	B
PFBS								
PFHxS	0.05							
PFOS	6.18		12.5	4.2	8.3	9.4	2.8	20.1
PFBA								
PFHxA								
PFOA	0.3							
PFNA	0.76		<LOQ		<LOQ	0.6		
PFDA	0.43							
PFUnDA	0.92	0.05 – 1.07						
PFDoDA	0.25							
PFTrDA	1.00							
PFTeDA	0.01							
Publication	Gómez-Ramírez et al., 2017	Løseth et al., 2019	Herzke et al., 2011	Sun et al., 2019	Herzke et al., 2011	Herzke et al., 2011	Sun et al., 2019	Sun et al., 2019

Table 7.3 (continued). PFAA concentrations (ng/g ww) reported in bird feathers in literature and at 3M in the present study. Different feather types were abbreviated as following: body (B), tail (T) and wing (W). NM = not mentioned. [1]median concentrations, [2]mean concentrations, [3]Concentration in one sample, [4]Concentrations were not reported in the text, but were estimated from barplots.

	Barn owl, *Tyto alba*	Grey Heron, *Ardea cinerea*	Herring gull, *Larus argentatus*	Eurasian Sparrowhawk, *Accipiter nisus*	Eurasian magpie, *Pica pica*	Eurasian Collared Dove, *Streptopelia decaocto*	*Accipiter sp.*	Great tit, *Parus major*
Country	Belgium[1]	Belgium[2,4]	Belgium[2,4]	Belgium[2,4]	Belgium[2,4]	Belgium[2,4]	China[2]	Belgium[1]
Year	2008 – 2009	NM	NM	NM	NM	NM	NM	2016
Type	T	T	T	T	T	T	NM	T
PFBS							1.91	134
PFHxS	<1.9	20	20	30	ND	ND	0.43	821
PFOS	16.9	250	100	80	40	60	4.67	21737
PFBA							1.65	392
PFHxA	128.8[3]						0.27	192
PFOA	85.9							698
PFNA		ND	ND	ND	ND	ND		19
PFDA							0.30	79
PFUnDA							0.37	26
PFDoDA								9.5
PFTrDA								1.7
PFTeDA								<4.5
Publication	Jaspers et al., 2013	Meyer et al., 2009	Meyer et al., 2009	Meyer et al., 2009	Meyer et al., 2009	Meyer et al., 2009	Li Y et al., 2017	The present study

Differences between sexes were observed only for PFDA, asconcentrations in feathers were higher in males compared to females. Sturm and Ahrens (2010) reviewed that most studies on PFAA concentrations in birds do not report differences between sexes. The studies that did observe differences, always reported higher concentrations in males (Bertolero et al., 2015; Blévin et al., 2017; Bustnes et al., 2008; Hitchcock et al., 2019; Sinclair et al., 2006). It is possible that females have lower PFAA concentrations due to maternal deposition in the eggs (Lopez-Antia et al., 2019; Newsted et al., 2007), however, the extent to which maternal transfer influences concentrations in the mother, due to excretion via the eggs, is still discussed (Hitchcock et al., 2019). Studies on mammals reported that differences in PFAA concentrations between sexes may be the result of differences in elimination half-lives in males and females, which is likely influenced by a hormonal regulation of the elimination (Lau et al., 2007). Furthermore, differences in foraging strategies (Milligan et al., 2017) could explain the potential differences between sexes. Surprisingly, we did not observe any age-related differences in PFAA concentrations. Age differences in niche use and exploratory behaviour, including foraging habits, of male great tits have been reported before (Pagani-Núñez et al., 2018; Verbeek et al., 1994).

7.4.2 Are feathers suitable alternatives for blood plasma?

Feathers have been proven suitable in the monitoring of environmental pollutants (e.g. Dauwe et al., 2002, 2003, 2005b; Eulaers et al., 2011; Jaspers et al., 2004, 2006, 2007a, 2007b, 2009, 2011). However, there is ongoing discussion whether this is also the case for emerging contaminants, such as PFAAs (Jaspers et al., 2019; Løseth et al., 2019). The concentrations measured in feathers may not always be related to internal tissue concentrations as feathers can also be exposed to external sources, such as preen oil or contact with the environment (air, dust and water) (Jaspers et al., 2019), whereas concentrations in blood are mainly the result of uptake via diet. Furthermore, detection frequencies are often low (e.g. Løseth et al., 2019) and correlations between feathers and internal organs have only sporadically been observed. For example, PFHxS and PFOS concentrations were positively related between feathers and liver of

Belgian Barn owl (*T. alba*; Jaspers et al., 2013). Meyer et al. (2009) examined the concentrations of PFHxS, PFOS, PFOA and PFNA in multiple tissues of five bird species and reported a significant positive correlation between feather and liver PFOS concentrations when they grouped all species. However, the correlations between these matrices were not significant when considering the individual species separately. Positive associations between feathers and plasma of white-tailed eagle nestlings (*Haliaeetus albicilla*) have been reported for PFHxS, PFDA, PFDoDA and PFTrDA, but not for PFOS, PFOA and PFNA (Gómez-Ramírez et al., 2017). However, as no correlations were observed for PFUnDA in white-tailed eagles, Jaspers et al. (2019) and Løseth et al. (2019) suggested prioritizing plasma for PFAAs analyses.

Although we also did not observe an association between blood and feather PFUnDA concentrations, we observed highly significant positive correlations for PFOA and PFOS between these matrices when data from all sites were pooled, but in most cases not for the individual locations, which is likely the result of a smaller sample size at each area. The feathers and blood were both collected during the winter, and it is possible that PFAA concentrations circulating in the blood at that time were different from those that were available at the time of feather formation, which could potentially explain the lack of correlations between concentrations in blood plasma and feathers for the other PFAAs. Nevertheless, in most cases there were significant differences between both matrices in PFOA concentrations. These differences did vary between individuals at the same locations as in some individuals concentrations in blood plasma were higher, whereas in others those in feathers were higher. Therefore, we do not recommend estimating internal PFOA concentrations in blood plasma from feather concentrations. The significant difference between feather and plasma PFOS concentrations at Burchtse Weel can be explained by a much higher LOQ in plasma. Substituting the non-detects with a value of LOQ/2 will result in higher concentrations than those in feathers, causing significant differences. Therefore, we argue that feathers from great tit in this area can be used to estimate the PFOS concentrations in the blood plasma if there is enough variation in PFOS concentrations.

However, feathers could also be useful in the biomonitoring of PFAAs, as 1) the correlations showed that high blood plasma concentrations can be expected in cases where feather concentrations are high and 2) more compounds were detected in the feathers compared to the blood plasma.

7.5 Conclusion

Feathers are an interesting matrix in the non-destructive biomonitoring of environmental pollutants in birds. Although they have been proven useful for the determination of legacy contaminants, there is an ongoing discussion as to whether they are also suitable for emerging compounds such as PFAAs. Our results show that feathers can be used to estimate the internal PFOS concentrations in the blood plasma, as a strong significantly positive correlation was observed together with no significant differences between both matrices. However, despite the correlations between PFOA concentrations in feathers and blood plasma, feathers are not suitable for estimating the internal PFOA concentrations in blood plasma, as concentrations differed between both matrices and these differences varied between individuals. Feathers may be useful in the biomonitoring of PFAAs as more target analytes were detected in feathers than in blood, thus resulting in a better representation of the total PFAA exposure from the environment. The reported PFAA concentrations in feathers at 3M were the highest ever reported in wild birds. Although these concentrations decreased away from the fluorochemical plant, they remained high in the adjacent sites.

7.6 Acknowledgements

The authors would like to thank the FWO for funding this study (FWO: G038615N). Furthermore, we would like to express our sincere gratitude to 3M, the Agency for Nature and Forest (ANB), K. Maes and W. Melens for providing us access to all the sampling sites and for the possibility to store field materials at these sites. Finally, we would like to acknowledge T. Willems for the UPLC measurements and P. Scheys and A. Lopez-Antia for their help with the collection of the samples.

7.7 Supplementary data

Table S7.1. MRM transitions, internal standards (ISTDs), cone voltages (V) and collision energy (eV) for the target perfluoroalkyl substances and their internal standards.

Compound	Precursor ion (m/z)	Product ion (m/z)		Cone Voltage (V)	Collision energy (eV) for diagnostic transition1	Collision energy (eV) for diagnostic transition 2	Internal standard (ISTD) used for quantification
		Diagnostic product Ion 1	**Diagnostic product Ion 2**				
PFBA	213	169	169	19	19	50	$^{13}C_4$-PFBA
PFPeA	263	219	219	15	10	45	$^{13}C_4$-PFBA
PFHxA	313	269	119	19	21	65	[1,2-$^{13}C_2$]PFHxA
PFHpA	363	319	169	24	40	30	[1,2-$^{13}C_2$]PFHxA
PFOA	413	369	169	22	13	60	[1,2,3,4-$^{13}C_4$]PFOA
PFNA	463	419	169	28	17	20	[1,2,3,4,5-$^{13}C_5$]PFNA
PFDA	513	469	219	25	29	29	[1,2-$^{13}C_2$]PFDA
PFUnDA	563	519	169	18	30	35	[1,2-$^{13}C_2$]PFUnDA
PFDoDA	613	569	319	22	21	30	[1,2-$^{13}C_2$]PFDoDA
PFTrDA	663	619	319	26	21	30	[1,2-$^{13}C_2$]PFDoDA
PFTeDA	713	669	169	28	21	21	[1,2-$^{13}C_2$]PFDoDA
PFBS	299	80	99	40	65	45	$^{18}O_2$-PFHxS
PFHxS	399	80	99	22	30	60	$^{18}O_2$-PFHxS
PFOS	499	80	99	60	58	58	[1,2,3,4-$^{13}C_4$]PFOS
PFDS	599	80	99	29	63	63	[1,2,3,4-$^{13}C_4$]PFOS

Table S7.1. (continued). MRM transitions, internal standards (ISTDs), cone voltages (V) and collision energy (eV) for the target perfluoroalkyl substances and their internal standards.

Compound	Precursor ion (m/z)	Product ion (m/z)		Cone Voltage (V)	Collision energy (eV) for diagnostic transition1	Collision energy (eV) for diagnostic transition 2	Internal standard (ISTD) used for quantification
		Diagnostic product Ion 1	**Diagnostic product Ion 2**				
$^{13}C_4$-PFBA	217	172	172	19	19	50	
[1,2-$^{13}C_2$]PFHxA	315	269	119	19	21	65	
[1,2,3,4-$^{13}C_4$]PFOA	417	372	172	22	13	60	
[1,2,3,4,5-$^{13}C_5$]PFNA	468	423	172	28	17	20	
[1,2-$^{13}C_2$]PFDA	515	470	220	25	29	29	
[1,2-$^{13}C_2$]PFUnDA	565	520	170	18	32	35	
[1,2-$^{13}C_2$]PFDoDA	615	570	320	22	21	30	
$^{18}O_2$-PFHxS	403	84	103	22	30	60	
[1,2,3,4-$^{13}C_4$]PFOS	503	80	99	60	58	58	

Table S7.2. PFAA concentrations in the washing step (ng/g ww) and chicken feathers (N = 2) (ng/g dw) , collected approximately 1 km from 3M, used to examine the extent of external contamination of PFAAs.

	PFBA	PFPEA	PFHXA	PFHPA	PFOA	PFNA	PFDA	PFUNDA	PFDODA	PFTRDA	PFTEDA	PFBS	PFHXS	PFOS	PFDS
WASHING STEP (10 ML MEOH)	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
FEATHER	10	<LOQ	6.0	<LOQ	5.4	<LOQ	4.7	6.0	0.5	<LOQ	<LOQ	4.1	<LOQ	65	<LOQ

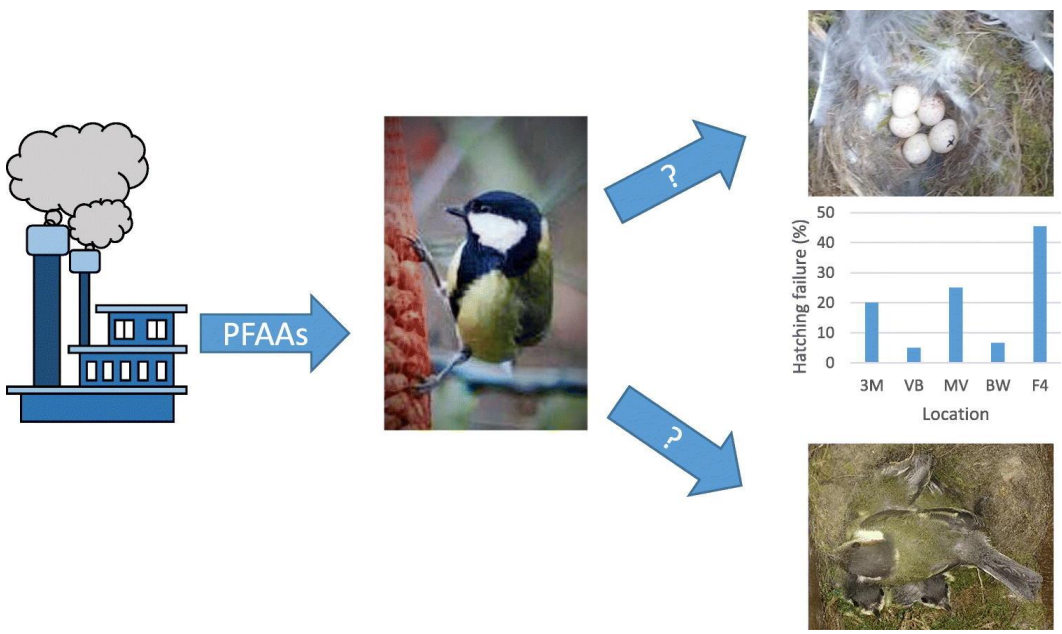
8. Limited reproductive impairment in a passerine bird species exposed along a perfluoroalkyl acid (PFAA) pollution gradient

Published in Science of the Total Environment:

Thimo Groffen*, Robin Lasters*, Ana Lopez-Antia, Els Prinsen, Lieven Bervoets and Marcel Eens (2019c). Science of the Total Environment 652: 718 – 728. https://doi.org/10.1016/j.scitotenv.2018.10.273

Tables were modified to fit the size of the pages. No further modifications were made.

*Both authors contributed equally to this work



Abstract

Although bird eggs have been used in biomonitoring studies on perfluoroalkyl acids (PFAAs), effects of environmental concentrations on reproduction remain largely unknown in wild birds. In the present study we examined the associations between the concentrations of 4 perfluoroalkyl sulfonic acids (PFSAs) and 11 perfluoroalkyl carboxylic acids (PFCAs) in the eggs of great tits (*Parus major*), collected along a distance gradient from a pollution source, and multiple reproductive parameters (including the start of egg laying, clutch size, hatching success, fledging success and total breeding success) along with egg shell thickness and body condition of the nestlings.

The PFAA concentrations measured at the plant site were among the highest ever reported in wild bird eggs. PFAA concentrations decreased sharply with increasing distance (0–11 km) from the plant, but remained relatively elevated in the adjacent sites. PFAAs were grouped into principal components (PCs) to prevent collinearity. High concentrations of PFOS, PFDS, PFDoDA, PFTrDA and PFTeDA (grouped as PC1) were associated with a reduced hatching success of nests where at least one egg hatched, thinner egg shells and increased survival of the hatched chicks. High concentrations of PFDA (PC2) were associated with a reduced hatching success, especially in nests where no eggs hatched, an earlier start of egg laying and a reduction of total breeding success, mainly caused by the failure in hatching.

Although the major manufacturer of PFAAs phased out the production of perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and related products in 2002, concentrations appear to have increased since previous measurements. Surprisingly, despite the very high concentrations close to the fluorochemical plant, there was no clear evidence for reproductive impairment as the observed associations between PFAA concentrations and reproductive parameters were rather limited compared to previous studies in songbirds. These findings also suggest potential differences in sensitivity between species.

8.1 Introduction

Perfluoroalkyl acids (PFAAs) are chemicals with distinctive physicochemical properties, which result from the strong C-F binding and the hydrophobic and lipophobic character that make them highly persistent and bioaccumulative in the environment. They have been produced and used since 1950 for numerous applications, such as textile stain and soil repellents, food-contact paper and fire-fighting foams (Buck et al., 2011; Kissa, 2001). Consequently, PFAAs have been detected globally in the environment, wildlife and humans (Butt et al., 2010; D'Hollander et al., 2010; Giesy and Kannan, 2001, 2002; Groffen et al., 2017, 2018; Houde et al., 2006; Miller et al., 2015), which can all be polluted either directly or via environmental degradation of precursor compounds (Buck et al., 2011; Martin et al., 2010; Prevedouros et al., 2006). During the last decades, regulatory agencies and researchers have mainly focused on long chain perfluoroalkyl carboxylic acids (PFCAs) and perfluoroalkyl sulfonic acids (PFSAs), rather than their short-chained analogues, which have a lower bioaccumulative potential. Particularly perfluorooctanoic acid (PFOA, $C_7F_{15}COOH$) and perfluorooctane sulfonate (PFOS, $C_8F_{17}SO_3H$) have been studied often (Buck et al., 2011).

Based on their persistence, widespread distribution and potential health effects, the major manufacturer of PFAAs, 3M, phased-out the production of PFOS, PFOA and related compounds in 2002. Furthermore, PFOS was included in the Stockholm Convention on Persistent Organic Pollutants in 2009. These measures appear to have reduced environmental PFOS concentrations in many cases, whereas concentrations of other PFAAs are rising (Ahrens et al., 2011c; Filipovic et al., 2015b; Miller et al., 2015).

Although bird eggs have been used in numerous studies to monitor PFAA concentrations on a global scale (e.g., Gebbink and Letcher, 2012; Giesy and Kannan, 2001; Holmström et al., 2005; Miller et al., 2015; Yoo et al., 2008), only very few of these studies have focused on terrestrial birds (Ahrens et al., 2011c; Custer et al., 2012; Groffen et al., 2017; Holmström et al., 2010; Lopez-Antia et al., 2017; Rüdel et al., 2011; Yoo et al., 2008).

Previous studies on PFAA concentrations in wildlife near a fluorochemical plant in Antwerp, Belgium, revealed the highest concentrations ever found in wildlife (Dauwe et al., 2007; D'Hollander et al., 2014; Groffen et al., 2017; Hoff et al., 2005; Lopez-Antia et al., 2017). PFOS concentrations in liver from great tits (*Parus major*) and blue tits (*Cyanistes caeruleus*) were higher in this area than those measured in top predators in other regions (Dauwe et al., 2007). In addition, PFOS concentrations in great tit eggs were among the highest ever reported in bird eggs worldwide (Groffen et al., 2017; Lopez-Antia et al., 2017). Furthermore, studies on the effects of PFAAs in the vicinity of this plant in Antwerp were restricted to PFOS and mainly reported biochemical effects in wood mice (Hoff et al., 2004) and great tits (Hoff et al., 2005; Lopez-Antia et al., 2017). Hoff et al. (2004, 2005) observed significantly positive associations with liver weight in both species and lipid peroxidation level in liver of mice. Plasmatic biochemical biomarkers in great tits were not affected by PFAA concentrations (Lopez-Antia et al., 2017). Biomonitoring of PFAA concentrations and their composition profile in the surroundings of the fluorochemical plant in Antwerp is therefore extremely important.

Reproductive effects of PFAAs have been studied in a wide variety of taxa, including nematodes (e.g. Chen FJ et al., 2018), arthropods (e.g. Princz et al., 2018), fish (e.g. Lee et al., 2017; Xia and Niu, 2017) and humans (e.g. Foresta et al., 2018; Song et al., 2018). Despite the ubiquity of PFAAs, not much is known about their effects on the individual and population level in terrestrial bird species. To the best of our knowledge, only a few studies investigated the associations between PFAA concentrations and reproductive parameters in birds. Most of these studies were performed under laboratory conditions, where bird eggs were injected with PFAAs or where birds were exposed to PFAAs through their diet, whereas field studies remain scarce. In addition, the majority of these studies only focus on PFOS as their target analyte.

Two field studies have studied the relationship between PFOS concentrations and hatching success in tree swallows (*Tachycineta bicolor*; Custer et al., 2012, 2014).

Custer et al. (2012) have reported negative associations between PFOS concentrations starting from 150 ng/g ww in eggs of tree swallows and the hatching success of the remaining eggs in the nest. Furthermore, a 20% decrease in hatching success at PFOS concentrations of 283 ng/g in eggs has been observed (Custer et al., 2014).

In ovo exposure to PFOS, under laboratory conditions, did not affect hatching rate in white leghorn chickens (*Gallus gallus domesticus*), but did cause a reduced body and wing length (Peden-Adams et al., 2009). However, other laboratory studies have observed reproductive dysfunction after *in ovo* exposure to perfluorohexane sulfonic acid (PFHxS), PFOS and PFOA (Cassone et al., 2012; Molina et al., 2006; Yanai et al., 2008). A significant reduction in hatching success by 20% and 63% was observed after injection of 5000 ng/g PFOA and 38,000 ng/g PFHxS, respectively (Cassone et al., 2012; Yanai et al., 2008). In addition, tarsus length and body weight were reduced at the same concentrations (Cassone et al., 2012). Treatment-related mortalities or effects on body weight and reproductive parameters were not observed in a study in which northern bobwhite quail (*Colinus virginianus*) were exposed to perfluorobutane sulfonic acid (PFBS) through diet (Newsted et al., 2008). Furthermore, no effects of PFOS on body weight and reproductive performance have been found in mallard ducks (*Anas platyrhynchos*; Newsted et al., 2007).

In the present study, we investigated possible relationships between multiple PFAA concentrations in great tit eggs and multiple reproductive parameters (including the start of egg laying, clutch size, hatching success, fledging success and total breeding success), egg shell thickness and body condition of the nestlings along a distance gradient, starting from a fluorochemical plant in Antwerp. This study can help to understand possible effects of these pollutants on wild birds.

8.2 Materials and method

8.2.1 Study species and sample collection

Great tits (*Parus major*) are insectivorous songbirds that feed mainly on caterpillars during the breeding season and berries and seeds during the winter (Del Hoyo et al., 2007; Lopez-Antia et al., 2017). They are considered to be a model species for ecotoxicological studies as they nest in man-made nestboxes, are abundant and can be attracted to polluted areas (Dauwe et al., 1999, 2004, 2005a; Eens et al., 1999; Eeva and Lehikoinen, 1995, 1996; Eeva et al., 1998; Van den Steen et al., 2006).

Nestboxes were placed during autumn of 2015 at five sampling sites (Fig. 8.1), representing a gradient from a fluorochemical plant (3M) in Antwerp, Belgium. These sites were the 3M fluorochemical plant (28 nestboxes), Vlietbos (24 nestboxes; 1 km SE from 3M), Rot-Middenvijver (further called Rot; 20 nestboxes; 2.3 km ESE from 3M), Burchtse Weel (21 nestboxes; 3 km SE from the plant) and Fort 4 in Mortsel (58 nestboxes; 11 km SE from the plant).

From just before egg laying until incubation, nestboxes were checked every other day or daily to be able to determine the start of the egg laying period and clutch size. From each nest, the third egg was collected by hand before the incubation had started. From 10 days after incubation onwards, nests were daily checked for hatching to determine hatching success. Body condition, determined according to the scales mass index of Peig and Green (2009) of the nestlings was determined 14 days after hatching. Finally, nestboxes were checked after approximately 25 days to determine the number of fledglings.

Figure 8.1. Overview of the study area in Antwerp, Belgium. Sampling locations are indicated as letters: A. Fluorochemical plant 3M, B. Vlietbos, C. Middenvijver-Rot, D. Burchtse Weel, E. Fort 4.

8.2.2 Egg parameters

Prior to the chemical analysis eggs were weighed (±0.1 g, Mettler Toledo, Zaventem, Belgium) and their length and width were measured using a digital caliper (±0.01 mm, Mitutoyo Belgium NV, Kruibeke, Belgium). Shell thickness was measured using the methodology described by Lopez-Antia et al. (2013). Three small pieces of shell from the equatorial region were collected and dried. Hereafter, the thickness of these pieces was measured with a micrometer (±0.01 mm, Mitutoyo Belgium NV, Kruibeke, Belgium).

8.2.3 Chemical analysis

All used abbreviations of PFAAs are according to Buck et al. (2011). Target PFAAs included 11 PFCAs (PFBA, PFPeA, PFHxA, PFHpA, PFNA, PFOA, PFDA, PFUnDA, PFDoDA, PFTrDA and PFTeDA) and 4 PFSAs (PFBS, PFHxS, PFOS and PFDS). Isotopically mass-labeled internal standards (ISTDs) were purchased by Wellington Laboratories (Guelph, Canada) and comprised $^{13}C_4$-PFBA, [1,2-$^{13}C_2$]PFHxA, [1,2,3,4-$^{13}C_4$] PFOA, [1,2,3,4,5-$^{13}C_5$]PFNA, [1,2-$^{13}C_2$]PFDA, [1,2-$^{13}C_2$]PFUnDA, [1,2-$^{13}C_2$]PFDoDA, $^{18}O_2$-PFHxS and [1,2,3,4-$^{13}C_4$]PFOS. HPLC grade Acetonitrile (ACN) and water (VWR International, Leuven, Belgium) were used.

8.2.4 Sample extraction

Egg content was transferred into a polypropylene (PP) tube and homogenized by repeatedly sonicating and vortex-mixing. The extraction procedure was based on solid-phase-extraction. Approximately 0.4 g of homogenized egg was used for the analysis. Samples were spiked with 10 ng of each ISTD (in 50:50 ACN/HPLC grade water). Hereafter, 10 mL ACN was added and the samples were sonicated (3 × 10 min, Branson 2510) and left overnight on a shaking plate (135 rpm, room temperature, GFL 3020, VWR International, Leuven, Belgium). After centrifugation (4 °C, 10 min, 2400 rpm, Eppendorf centrifuge 5804R, rotor A-4- 44), the supernatant was transferred into a 14 mL PP tube. Chromabond HR-XAW SPE cartridges (Application No 305200, SPE department, Macherey-Nagel, Germany, 2009) were conditioned with 5 mL ACN and equilibrated with 5 mL Milli-Q (MQ) water. After loading the samples, the columns were washed with 5 mL 25 mM ammonium acetate and 2 mL ACN. The elution was performed with 2 × 2 mL 2% ammonium hydroxide in ACN and the eluent was completely dried using a rotational-vacuum-concentrator at 30 °C (Eppendorf concentrator 5301, Hamburg, Germany). The dried eluent was reconstituted with 200 µL 2% ammonium hydroxide in ACN and vortex-mixed during 1 min. Samples were filtered through an Ion Chromatography Acrodisc 13 mm Syringe Filter with 0.2 µm Supor (PES) Membrane (VWR International, Leuven, Belgium) and collected into a PP auto-injector vial before analysis.

8.2.5 UPLC-TQD analysis

PFAAs were analyzed by UPLC coupled tandem ES(−) mass spectrometry (ACQUITY, TQD, Waters, Milford, MA, USA). An ACQUITY BEH C18 column (2.1 × 50 mm; 1.7 µm, Waters, USA) was used to separate the analytes. The mobile phase solvents were 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B), with a solvent gradient starting at 65% A to 0% A in 3.4 min to 65% A at 4.7 min and a flow rate of 450 µL/min and an injection volume of 10 µL. An ACQUITY BEH C18 pre-column (2.1 × 30 mm; 1.7 µm, Waters, USA) was inserted, between the solvent mixer and injector, to retain any PFAAs contamination originating from the system.

PFAAs were identified and quantified based on multiple reaction monitoring (MRM) of the following diagnostic transitions: 213 → 169 (PFBA), 217 → 172 (^{13}C4PFBA), 263 → 219 (PFPeA), 313 → 269 (PFHxA), 313 → 119 (PFHxA), 315 → 269 ($^{13}C_2$PFHxA), 315 → 119 ($^{13}C_2$PFHxA), 363 → 319 (PFHpA), 363 → 169 (PFHpA), 413 → 369 (PFOA), 413 → 169 (PFOA), 417 → 372 ($^{13}C_4$PFOA), 417 → 172 ($^{13}C_4$PFOA), 463 → 419 (PFNA), 463 → 169 (PFNA), 468 → 423 ($^{13}C_5$PFNA), 468 → 172 ($^{13}C_5$PFNA), 513 → 469 (PFDA), 513 → 219 (PFDA), 515 → 470 ($^{13}C_2$PFDA), 515 → 220 ($^{13}C_2$PFDA), 563 → 519 (PFUnDA), 563 → 169 (PFUnDA), 565 → 520 ($^{13}C_2$PFUnDA), 565 → 170 ($^{13}C_2$PFUnDA), 613 → 569 (PFDoDA), 613 → 319 (PFDoDA), 615 → 570 ($^{13}C_2$PFDoDA), 615 → 320 ($^{13}C_2$PFDoDA), 663 → 619 (PFTrDA), 663 → 319 (PFTrDA), 713 → 669 (PFTeDA), 713 → 169 (PFTeDA), 299 → 99 (PFBS), 299 → 80 (PFBS), 399 → 99 (PFHxS), 399 → 80 (PFHxS), 403 → 103 ($^{18}O_2$PFHxS), 403 → 84 ($^{18}O_2$PFHxS), 499 → 80 (PFOS), 499 → 99 (PFOS), 503 → 80 ($^{13}C_4$PFOS), 503 → 99 ($^{13}C_4$PFOS), 599 → 99 (PFDS) and 599 → 80 (PFDS).

Table 8.1. Individual limits of quantification (LOQ: ng/g determined as 10 times the signal to noise ratio), median and mean concentrations (ng/g ww), range (ng/g ww) and detection frequencies (Freq; %) of PFAAs in eggs of great tit at the five sampling sites with increasing distance from the fluorochemical plant of 3M: Vlietbos (1 km), Rot (2.3 km), Burchtse Weel (3 km) and Fort 4 (11 km). Compounds that are not detected (i.e. PFPeA, PFHxA, PFHpA, PFUnDA, PFBS and PFHxS) are excluded from the table.

		PFCAs						
		PFBA	**PFOA**	**PFNA**	**PFDA**	**PFDoDA**	**PFTrDA**	**PFTeDA**
LOQ		0.261	0.045	0.586	0.425	0.444	0.256	0.355
3M (n = 23)	**Median**	<LOQ	18	7.7	13	18	14	1.3
	Mean	1.7	39	9.1	25	29	25	3.4
	Range	<LOQ – 11	3.4 – 359	2.1 – 28	1.6 – 102	1.1 – 133	<LOQ – 156	<LOQ – 22
	Freq	39	100	100	100	100	91	61
Vlietbos (n = 21)	**Median**	<LOQ	1.3	1.4	<LOQ	<LOQ	4.1	<LOQ
	Mean	<LOQ	1.8	1.8	0.7	1.8	6.0	1.2
	Range	<LOQ – 1.7	<LOQ – 3.5	<LOQ – 5.7	<LOQ – 4.1	<LOQ – 7.8	<LOQ – 22	<LOQ – 4.1
	Freq	29	71	71	29	38	76	38
Rot (n = 18)	**Median**	<LOQ	1.4	1.4	1.4	2.9	6.6	1.2
	Mean	0.4	1.4	1.4	1.6	3.4	7.9	1.4
	Range	<LOQ – 1.0	0.6 – 8.3	<LOQ – 2.3	<LOQ – 4.0	<LOQ – 12	1.7 – 26	<LOQ – 4.2
	Freq	39	100	94	78	94	100	83
Burchtse Weel (n = 16)	**Median**	<LOQ	1.5	1.2	<LOQ	1.6	2.5	<LOQ
	Mean	<LOQ	1.4	1.4	1.1	1.9	3.2	0.6
	Range	<LOQ	<LOQ – 3.3	<LOQ – 3.7	<LOQ – 5.5	<LOQ – 6.9	<LOQ – 12	<LOQ – 3.6
	Freq	0	81	69	25	63	81	31
Fort 4 (n = 33)	**Median**	<LOQ	1.2	1.3	1.9	1.6	2.5	0.7
	Mean	<LOQ	1.2	1.2	2.0	1.8	2.5	0.7
	Range	<LOQ – 0.9	<LOQ – 6.9	<LOQ – 4.5	<LOQ – 5.7	<LOQ – 6.7	<LOQ – 8.3	<LOQ – 2.0
	Freq	27 76	97		73	88	91	67

Table 8.1. (continued) Individual limits of quantification (LOQ: ng/g determined as 10 times the signal to noise ratio), median and mean concentrations (ng/g ww), range (ng/g ww) and detection frequencies (Freq; %) of PFAAs in eggs of great tit at the five sampling sites with increasing distance from the fluorochemical plant of 3M: Vlietbos (1 km), Rot (2.3 km), Burchtse Weel (3 km) and Fort 4 (11 km). Compounds that are not detected (i.e. PFPeA, PFHxA, PFHpA, PFUnDA, PFBS and PFHxS) are excluded from the table.

		PFSAs	
		PFOS	**PFDS**
LOQ		2.55	5.92
3M (n = 23)	**Median**	34251	82
	Mean	48056	315
	Range	5111– 187032	9.4 – 1489
	Freq	100	100
Vlietbos (n = 21)	**Median**	416	<LOQ
	Mean	830	<LOQ
	Range	<LOQ – 4035	<LOQ
	Freq	81	0
Rot (n = 18)	**Median**	454	<LOQ
	Mean	764	<LOQ
	Range	207 – 3806	<LOQ
	Freq	100	0
Burchtse Weel (n = 16)	**Median**	87	<LOQ
	Mean	130	<LOQ
	Range	18 – 690	<LOQ
	Freq	100	0
Fort 4 (n = 33)	**Median**	30	<LOQ
	Mean	32	<LOQ
	Range	<LOQ – 73	<LOQ
	Freq	97	0

8.2.6 Calibration

Calibration curves were prepared by adding a constant amount of internal standard to varying amounts of unlabeled standards. The dilutions of these standards were performed in ACN and water. The relationship between the ratio of concentrations of unlabeled and labeled PFASs and the area of unlabeled and labeled PFASs was described by a linear regression function with a highly significant linear fit for all target analytes (all $p \leq 0.001$; $R_2 > 0.98$).

Individual PFAAs were quantified using the corresponding ISTD with exception of PFPeA, PFHpA, PFTrDA, PFTeDA, PFBS and PFDS which were quantified using the ISTD of the compound closest in terms of functional group and size, i.e. the ISTD of PFBA to quantify PFPeA, PFHxA to quantify PFHpA, PFDoDA to quantify both PFTrDA and PFTeDA, PFHxS to quantify PFBS and PFOS to quantify PFDS.

8.2.7 Quality assurance

Procedural blanks were regularly (one per batch of 10 samples) analyzed and contained contamination of PFBA (<0.25 ng/µL). Concentrations observed in blanks were subtracted from the concentrations found in samples in the same batch. The limit of quantification (LOQ) was calculated based on a signal-to-noise ratio of 10 and ranged from 0.045 ng/g to 0.59 ng/g for PFBA, PFOA, PFNA, PFDA, PFDoDA, PFTrDA and PFTeDA. LOQs were considerably higher for PFOS and PFDS and were 2.55 ng/g and 5.92 ng/g respectively. LOQs for PFPeA, PFHxA, PFHpA, PFUnDA, PFBS and PFHxS could not be determined as these PFAAs were not detected in any sample. Individual LOQs of the detected compounds are displayed in Table 8.1. Recoveries for each sample were determined based on the ISTD of the corresponding sample and an ISTD solution. Detection frequencies of the detected compounds varied between 25% and 100% and should be interpreted with caution due to high variation in LOQs.

8.2.8 Statistical analysis

Statistical analyses were performed using SPSS 23. To obtain a normal distribution, data were log transformed. The level of significance was set at $p \leq 0.05$. PFAA

concentrations below the LOQ were given a value of LOQ/2 (Bervoets et al., 2004; Custer et al., 2000).

Differences in PFAA concentrations among sampling sites were evaluated by using a one way ANOVA, followed by Tukey's honest significant differences Post-hoc analysis. Correlations between individual compounds and between ∑PFSA and ∑PFCA were assessed in each study site using Spearman rank correlation analyses. PFAAs composition profiles were calculated as the proportions of individual compounds to the total PFAAs, PFSA and PFCA concentration in each egg. These percentages were averaged for all the eggs at a site.

Generalized Linear Models (GLMz) were used to test for correlations between PFAA concentrations and reproductive parameters. In order to reduce the number of covariates and to account for collinearity among them, we conducted a Principal Component Analysis (PCA) on the 9 detected PFAAs, i.e. PFBA, PFOA, PFNA, PFDA, PFDoDA, PFTrDA, PFTeDA, PFOS and PFDS. To study the correlations between PFAAs and the different reproductive parameters, we used the following distributions: a Poisson distribution to study correlations with the clutch size, a normal distribution to study correlations with the egg laying date, egg parameters (length, width and shell thickness) and the mean condition of the chicks. For ratios, we used a binary logistic distribution; hatching success (number of hatched eggs divided by the number of incubated eggs), fledging success (number of fledglings divided by the number of hatched eggs), overall breeding success (number of fledglings divided by the number of incubated eggs). Finally we studied the total failure of hatching (those nests where any egg hatched) and the total failure of reproduction (nests where incubation did not occur or hatching or fledging success completely failed), both based on a negative binomial distribution type.

8.3 Results

8.3.1 PFAA concentrations

Table 8.1 gives an overview of median concentrations, ranges and detected frequencies of PFAAs in great tit eggs. PFOA, PFNA, PFDA, PFDoDA, PFTrDA, PFTeDA and PFOS were detected at all locations, whereas PFDS was only detected at 3M and PFBA was not detected at Burchtse Weel. With exception of PFBA, short-chained PFSAs (PFBS) and PFCAs (PFPeA, PFHxA, PFHpA) were not detected in any sample from any location. In addition, PFHxS and PFUnDA were not detected as well.

Significant differences among sampling sites were observed for PFBA, PFOA, PFNA, PFDA, PFDoDA, PFTrDA, PFTeDA, PFOS and PFDS (all $p < 0.007$; Fig. 8.2). Eggs collected at the 3M site showed significantly higher concentrations of PFOA, PFNA, PFDA, PFDoDA, PFOS and PFDS compared to all other locations (all $p < 0.001$). In addition, PFBA concentrations were higher at 3M than at Burchtse Weel ($p = 0.002$) and Fort 4 ($p = 0.011$), PFTrDA concentrations were higher at 3M than at Vlietbos ($p = 0.009$), Burchtse Weel ($p = 0.003$) and Fort 4 ($p < 0.001$) and PFTeDA concentrations were higher at 3M than at Burchtse Weel ($p = 0.015$). PFOA and PFDoDA concentrations were higher at Rot compared to Vlietbos ($p = 0.046$ and $p = 0.003$, respectively). Furthermore, PFOS concentrations at Rot were significantly higher than those measured at the sites furthest away from the plant, i.e. Burchtse Weel ($p = 0.004$) and Fort 4 ($p < 0.001$). PFOS concentrations at Fort 4 were also lower than those at Vlietbos ($p < 0.001$). Eggs taken from Rot also contained higher PFTrDA concentrations compared to Fort 4 ($p = 0.019$) and PFTeDA concentrations compared to Burchtse Weel ($p = 0.048$). Finally, PFDA concentrations were higher at Fort 4 than at Vlietbos ($p = 0.005$) and Burchtse Weel ($p = 0.032$).

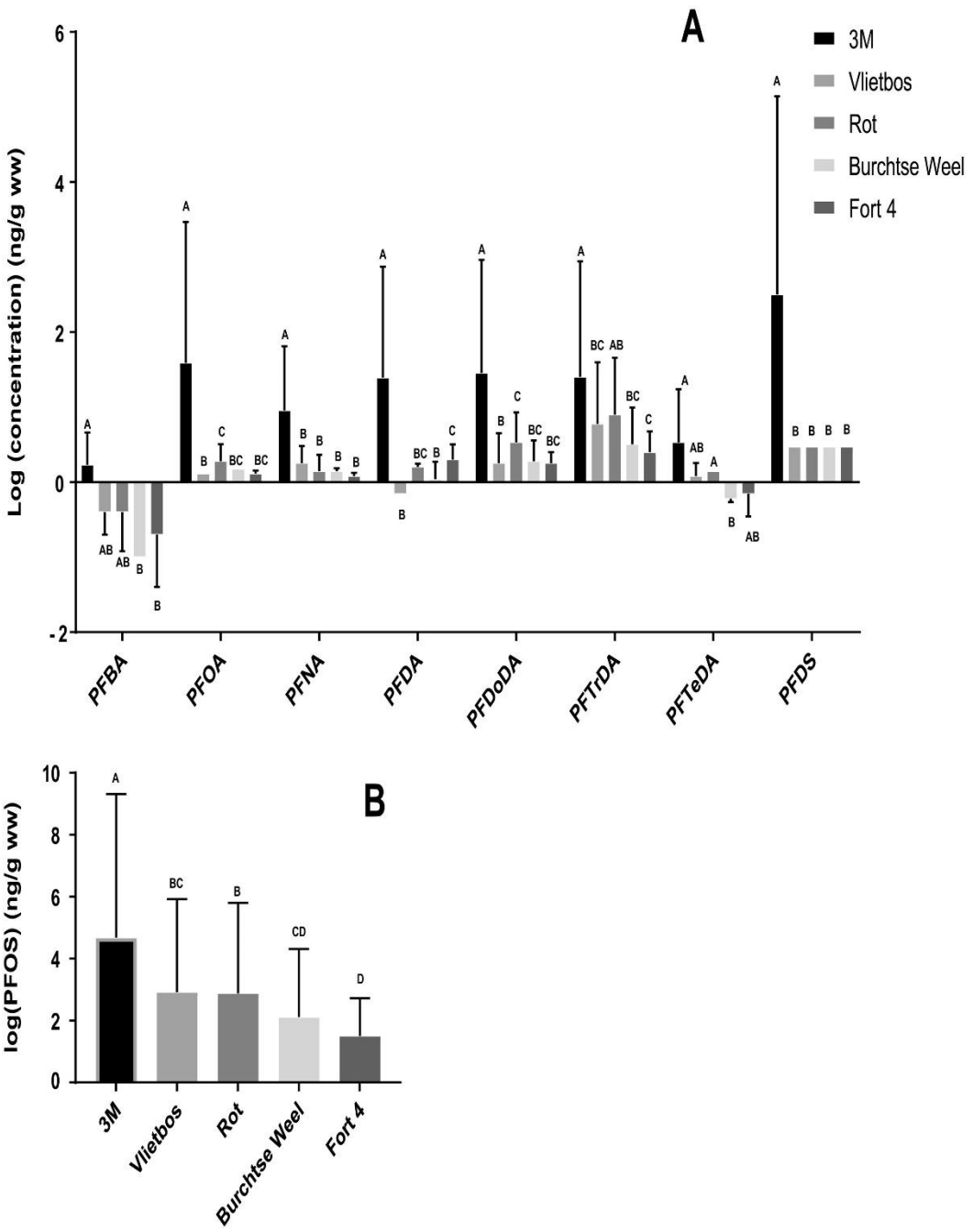


Figure 8.2. Concentrations of multiple PFAAs at each study site. A) Mean concentrations (logarithmic) of PFBA, PFOA, PFNA, PFDA, PFDoDA, PFTrDA, PFTeDA and PFDS (+standard deviation). B) Mean concentrations (logarithmic + standard deviation) of PFOS at each site. Different letters indicate significant differences in PFAA concentrations between the different locations.

8.3.2 PFAA profile

PFOS was the dominant contributor to both the ∑PFSAs (Fig. S8.1) and the ∑PFAAs at each sampling site. Its contribution to the ∑PFSAs was lowest at Vlietbos (82.9%) and highest at 3M (99.5%). The major contributor to the ∑PFCAs was PFOA at 3M (25.5%) and PFTrDA at all other locations (37.0% at Vlietbos, 41.4% at Rot, 28.5% at Burchtse Weel and 24.5% at Fort 4; Fig. 8.3).

8.3.3 PFAAs correlations

Table S8.1 summarizes the correlations found among PFAAs at the different sampling locations. All significant correlations were positive. 3M had the highest number of significant correlations (20), followed by Vlietbos (18), Fort 4 (17), Rot (14) and Burchtse Weel (11). These results show a high degree of collinearity between PFAA compounds and thus confirm the use of PCs in further analysis.

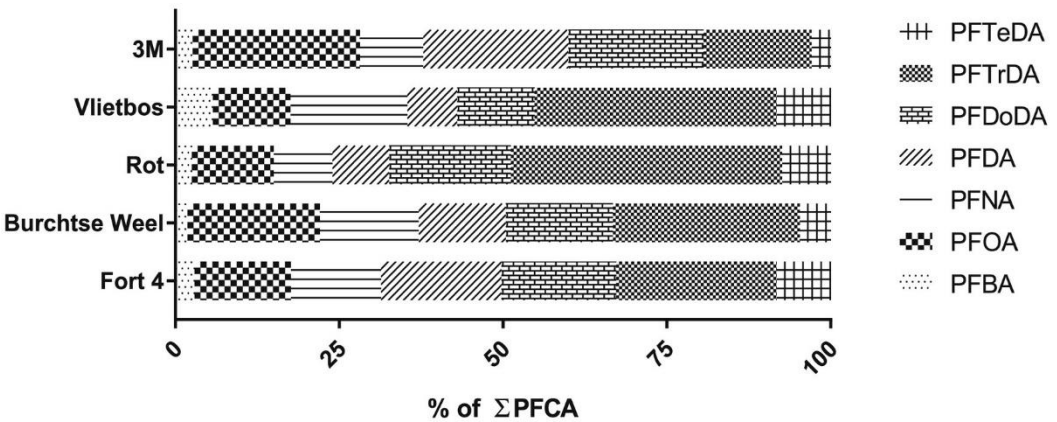


Figure 8.3. Composition profile of PFCAs in eggs of great tit at the five sampling sites, with increasing distance from the fluorochemical plant of 3M: Vlietbos (1 km), Rot (2.3 km), Burchtse Weel (3 km) and Fort 4 (11 km).

8.3.4 Reproductive parameters

Reproductive parameters among the different sampling sites are reported in Table 8.2. The day of the first egg and the hatching success did not differ significantly among locations. The shell thickness of the eggs was significantly lower at 3M compared to all other locations (all $p < 0.02$). In addition, the thickness was also lower at Rot and Fort

4 compared to Burchtse Weel (both $p < 0.001$). Furthermore, the breeding success differed significantly among locations, which was caused by a significantly lower success at Fort 4 compared to Vlietbos and Burchtse Weel (both $p < 0.001$). Similarly, the condition of the chicks was significantly higher at Vlietbos and Burchtse Weel compared to 3M and Rot (all $p < 0.001$) and the survival of the chicks was higher at Vlietbos and Burchtse Weel than at Fort 4 ($p = 0.050$ and $p = 0.016$, respectively). Finally, the clutch size was significantly lower at Fort 4 than at 3M ($p = 0.010$) and Burchtse Weel ($p = 0.036$).

Only a few reproductive parameters were correlated (Table S8.2). At all locations, breeding success was positively correlated with hatching success and chicks' survival. In addition, at Vlietbos a significant negative correlation was observed between clutch size and the day of the first egg and at Fort 4, we observed a significant negative correlation between clutch size and chicks' survival.

Two principal components were selected according to Kaiser criterion (eigenvalue higher than 1; Kaiser, 1960). The first Principal Component (PC1) explained 61.61% of the variance and the second Principal Component (PC2) explained a further 14.38% (Table S8.3). PC1 was mainly influenced by PFOS, PFDS, PFDoDA, PFTrDA and PFTeDA and to minor extent by PFOA and PFNA; high concentrations of these compounds corresponded with high values of PC1. PC2 was mainly influenced by PFDA, therefore high values of PC2 mainly indicated high PFDA concentrations (Table S8.3).

Table 8.2. Mean values and standard deviations for the different reproductive parameters at each location. Different letters indicate significant differences in this parameter between the locations.

REPRODUCTIVE PARAMETER	LOCATION				
	3M	Vlietbos	Rot	Burchtse Weel	Fort 4
AVERAGE DAY 1ST EGG	11 ± 8A	8 ± 4A	12 ± 6A	8 ± 4A	8 ± 5A
SHELL THICKNESS (MM)	0.16 ± 0.03A	0.22 ± 0.03BC	0.19 ± 0.05C	0.23 ± 0.03B	0.19 ± 0.02C
HATCHING SUCCESS[A]	0.80 ± 0.21A	0.92 ± 0.13A	0.78 ± 0.15A	0.89 ± 0.13A	0.87 ± 0.17A
FAILURE OF TOTAL HATCHING (%)[B]	20	5	25	7	45
BREEDING SUCCESS[C]	0.54 ± 0.42AB	0.81 ± 0.31B	0.51 ± 0.42AB	0.83 ± 0.26B	0.30 ± 0.40A
TOTAL FAILURE OF REPRODUCTION (%)[D]	39	18	41	13	55
MEAN CHICK CONDITION	15 ± 2A	17 ± 2B	15 ± 2A	16 ± 2B	16 ± 2AB
CHICKS' SURVIVAL[E]	0.84 ± 0.34AB	0.93 ± 0.23A	0.83 ± 0.39AB	1.0 A	0.63 ± 0.43AB
CLUTCH SIZE	9 ± 3 A	9 ± 2 AB	8 ± 2 AB	9 ± 3 A	7 ± 2 B

[a] Number of hatched eggs divided by the number of incubated eggs (we considered only those nests with at least one hatched egg). 3M (N = 16), Vlietbos (N = 19), Rot (N = 12), Burchtse Weel (N = 14), Fort 4 (N = 18).

[b] Percentage of nests where no egg hatched.

[c] Number of fledglings divided by the number of incubated eggs.

[d] Percentage of nests where incubation, hatching or chicks survival failed.

[e] Number of fledglings divided by the number of hatched eggs. Only nests where at least one egg hatched were included

Table 8.3 summarizes the results (Wald $\chi 2$, p, beta and standard error of the beta) of the GLMz for the reproductive parameters. Both PC1 ($p = 0.003$) and PC2 ($p < 0.001$) were negatively associated with the hatching success. Further analysis demonstrated that high values of PC2 were significantly associated with the total failure of hatching ($p = 0.025$; nests where no egg hatched, Fig. 8.4D) while PC1 was negatively associated with the hatching success in those nests where at least one egg hatched ($p < 0.001$; Fig. 8.4A). Moreover, high values of PC1 (PFOS, PFDS, PFDoDA, PFTrDA and PFTeDA) were associated with thinner egg shells ($p < 0.001$; Fig. 6.4B), but also with an increased survival of hatched chicks ($p = 0.027$). PC2 (influenced mainly by PFDA) was negatively associated with the day of the 1st egg ($p = 0.005$; Fig. 8.4C). Finally, high PC2 values were significantly associated with a reduction of the total breeding success ($p = 0.05$; Fig. 8.4D), which was mainly due to a failure in hatching success.

Table 8.3. Overview of the results of the GLMz (Wald χ^2 , p, beta and standard error of the beta) for the significant PCs at each reproductive parameter assessed.

PARAMETER	SIG. PC	WALD χ**2**	P	BETA	SE
DAYS FOR THE 1ST EGG	PC2	4.660	0.031	-0.063	0.0290
SHELL THICKNESS	PC1	22.19	0.00	-0.016	0.0035
HATCHING SUCCESS[A]	PC1	14.58	0.00	-0.270	0.0707
TOTAL FAILURE OF HATCHING[B]	PC2	5.046	0.025	0.595	0.2649
CHICKS SURVIVAL	PC1	4.868	0.027	0.603	0.273
BREEDING SUCCESS[C]	PC2	7.955	0.005	-0.198	0.07
TOTAL FAILURE OF REPRODUCTION[D]	PC2	3.181	0.074	-0.438	0.246
EGG WEIGHT					
EGG LENGTH					
EGG WIDTH					
CHICKS' SURVIVAL[E]		Not significant			
MEAN CHICK CONDITION					
CLUTCH SIZE					

[a] Number of hatched eggs divided by the number of incubated eggs (we considered only those nests with at least one hatched egg).

[b] Binomial variable, nests where any egg hatched vs. nests where at least one egg hatched.

[c] Number of fledglings divided by the number of incubated eggs.

[d] Binomial variable, nests where incubation, hatching or chicks survival failed vs nests with at least one fledgling.

[e] Number of fledglings divided by the number of hatched eggs.

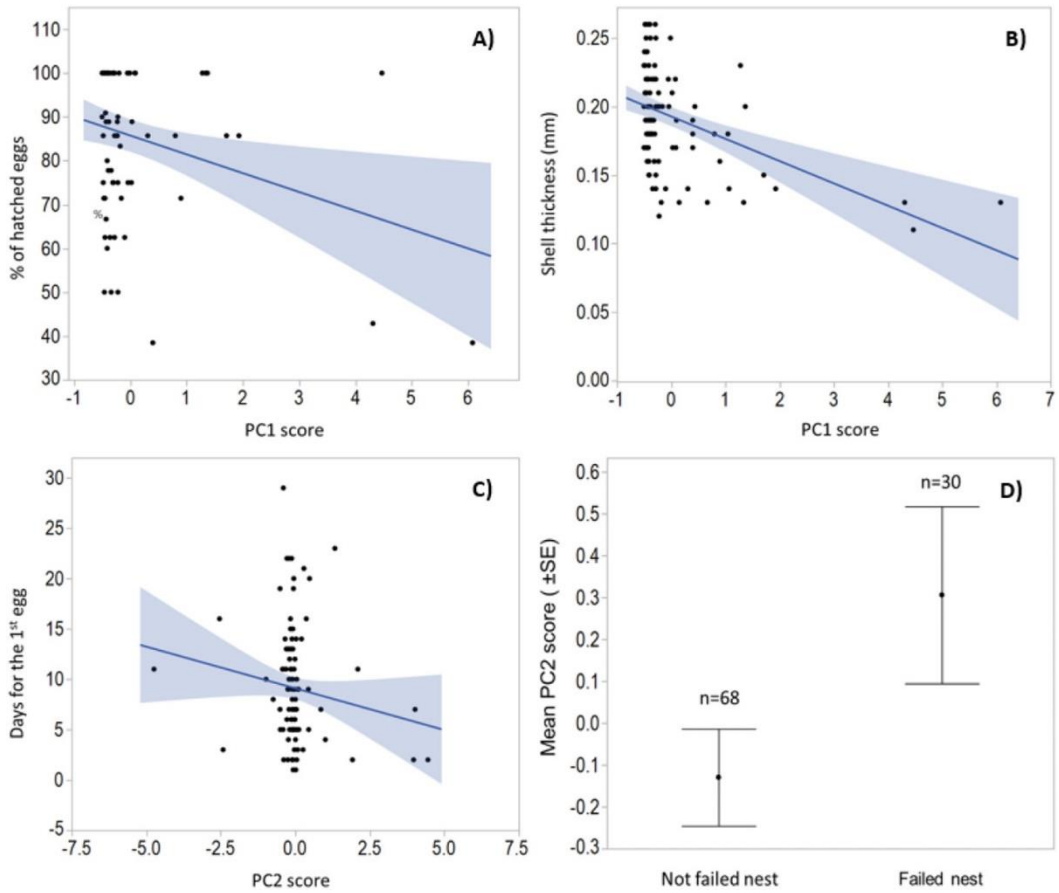


Figure 8.4. Correlations between the Principal Components (PCs) and reproductive parameters. A) Negative correlation between PC1 factor scores and the percentage of hatched eggs in a nest. B) Negative correlation between PC1 factor scores and shell thickness (mm). C) Negative association between PC2 factor scores and the egg laying date. D) Effects of factor scores of PC2 on reproduction total failure. The blue band represents the 95% confidence intervals of the correlation coefficients.

8.4 Discussion

8.4.1 PFAA concentrations

PFOS, PFOA and PFDS concentrations at the 3M fluorochemical plant were among the highest ever reported in eggs of free-living birds with median concentrations of 48,056 ng/g ww, 18 ng/g ww and 315 ng/g ww, respectively.

To the best of our knowledge, only six papers on PFAAs in passerine bird eggs have been published. Table 8.4 shows some the PFAA concentrations detected in these studies. The highest PFCA concentrations have been observed by Yoo et al. (2008) in parrot bill (*Paradoxornis webbiana*) eggs collected around the shores of a lake that receives wastewater from an industrial complex in Korea. Yoo et al. (2008) observed median PFNA and PFDA concentrations of 40 ng/g and 114.2 ng/g, respectively. In the present study, PFNA (7.7 ng/g ww) and PFDA (13 ng/g ww) concentrations were much lower, suggesting different types of contamination between both places. Until now, the highest PFSA concentrations, have been detected by Groffen et al. (2017) in great tit eggs collected near the same fluorochemical plant as in the present study, with median concentrations of 10,380 ng/g ww PFOS, 99.3 ng/g ww PFHxS and 47.7 ng/g ww PFDS. As a result of the phase-out of PFOS, PFOA and related products in 2002, it was expected that environmental concentrations of long-chained PFAAs would decrease, whereas those of short-chained alternatives would increase (Ahrens et al., 2011c; Filipovic et al., 2015b; Miller et al., 2015). Lopez-Antia et al. (2017) detected PFOS concentrations ranging from 19 ng/g ww to 5635 ng/g ww in great tit eggs collected at Vlietbos and Burchtse Weel in 2006. In the present study, PFOS concentrations at these locations were lower and ranged from <LOQ to 4035 ng/g ww at Vlietbos and 17.6 to 690 ng/g ww at Burchtse Weel. On the contrary, median PFOS concentrations in great tit eggs from the fluorochemical plant were approximately 3.5 times higher in the present study compared to 2011 (Groffen et al., 2017). With exception of PFDS, concentrations of other PFAAs were comparable to concentrations detected in 2011 (Groffen et al., 2017). However, the PFOS concentrations in the study by Groffen et al. (2017) exceeded the linear range of the calibration curve and where therefore extrapolated. Therefore, these concentrations should be treated with caution, as real concentrations may have been higher.

Table 8.4. Median PFAA concentrations in eggs (ng/g ww) of passerine birds. * range; NP = concentrations were analyzed but not reported; NA = not assessed. # = concentrations detected at a fluorochemical plant.

Species	Country	Year	PFHxS	PFOS	PFDS	PFOA	PFNA	PFDA	PFDoDA	PFTrDA	Publication
Corvus frugilegus	Germany	2009	<LOQ	5.3	NA	0.5	2.1	0.8	NA	NA	Rüdel et al., 2011
Paradoxornis webbiana	Korea	2006	1.3	314.1	1.1	0.8	40	114.2	25.6	NA	Yoo et al., 2008
Tachycineta bicolor	USA	2008 – 2009	NP	141	NA	<LOD	NP	5.51	NP	NA	Custer et al., 2012
Tachycineta bicolor	USA	2007 – 2011	0.95	270	NA	18.7	3.10	5.47	1.96	NA	Custer et al., 2014
Parus major*	Belgium	2006	NA	19 – 5635	NA	NA	NA	NA	NA	NA	Lopez Antia et al., 2017
***Parus major**#*	Belgium	2011	99.3	10380[a]	47.7	19.88	<LOQ	12.0	13.7	5.6	Groffen et al., 2017

[a] Extrapolated concentration.

Our earlier studies support the present one, reporting high PFOS concentrations in the liver of wood mice (*Apodemus sylvaticus*; D'Hollander et al., 2014; Hoff et al., 2004), livers of great tit and blue tit nestlings (Hoff et al., 2005) and great tit blood and plasma (Dauwe et al., 2007) near this fluorochemical hotspot.

Although there was a steep decrease for most PFAAs with increasing distance from the fluorochemical plant, differences between Vlietbos and Rot were less evident, as PFOA and PFDoDA concentrations were higher at Rot. In addition, PFDA concentrations were higher at Fort 4, furthest away from the fluorochemical plant, than at Vlietbos and Burchtse Weel. As was mentioned before, this could be explained by direct exposure closer to the fluorochemical plant and indirect exposure due to degradation of fluorotelomer alcohols (FTOHs) further away. Nevertheless, the decrease of PFAA concentrations with increasing distance from the fluorochemical plant was also observed in previous studies conducted in the area (Dauwe et al., 2007; D'Hollander et al., 2014; Groffen et al., 2017; Hoff et al., 2004, 2005; Lopez-Antia et al., 2017) and in chicken eggs, soil and water near a fluorochemical plant in China (Wang et al., 2010).

Variation in PFAA concentrations within a nest has been demonstrated before in the Audouin's gull (*Larus audouinii*), where PFOS concentrations decreased with laying order (Vicente et al., 2015). Therefore, it was expected that sampling a fixed egg from each nest would reduce the variation among nests at a site compared to randomized sampling. However, the variation in PFAA concentrations among nests remained as large as in our previous study (Groffen et al., 2017), in which eggs were collected randomly. This suggests that other factors such as the age or dispersal status of the bird, might cause this variation. Unfortunately, we do not have information about the origin of most female birds.

8.4.2 PFAA profile

A similar PFAA profile was observed in the present study compared to a study conducted in the same area in 2011 (Groffen et al., 2017), with PFOS being the major contributor to the total PFAAs and ∑PFSA concentrations. The dominance of PFOS was

in agreement with literature on PFAAs in bird eggs (Ahrens et al., 2011c; Custer et al., 2012; Nordén et al., 2013; Rüdel et al., 2011). At the plant site, PFOA was the dominant contributor to the ∑PFCAs in both the present study and the study performed by Groffen et al. (2017), whereas further away from the plant, PFTrDA and PFDoDA were more dominant in both studies. This profile can be explained by a direct deposition of PFOA close to the plant, whereas further away atmospheric and biological degradation of volatile polyfluorinated precursor compounds could explain the dominance of PFTrDA. In addition, the bioaccumulative potential of PFAAs increases with increasing chain length, indicating that longchained PFAAs such as PFDoDA and PFTrDA are more bioaccumulative than the shorter-chained PFOA (Armitage et al., 2009; Conder et al., 2008; Ellis et al., 2004; Houde et al., 2006).

8.4.3 Are high PFAA concentrations associated with reproductive impairment?

In the present study, hatching success was negatively correlated with PC1 (influenced by PFOS, PFDS, PFDoDA, PFTrDA and PFTeDA and to minor extent by PFOA and PFNA) in nests where at least one egg hatched. Fledging success, on the other hand, was positively correlated with PC1, which was probably caused by a higher chance of survival of the chicks in nests with a lower number of hatched eggs. In addition, PC2 (PFDA) was negatively correlated with both hatching success, in nests where no egg hatched, and total breeding success. A possible explanation for the negative correlation between PFAAs and hatching success and hatching and fledging probability might be that parents have a reduced fertility or that toxic effects on the development of the embryo occur (Molina et al., 2006; Yanai et al., 2008). Custer et al. (2014) observed an association between PFOS exposure and embryo death in tree swallows.

Studies on the associations of PFAAs on reproductive parameters of birds remain scarce. To the best of our knowledge, only two studies (Custer et al., 2012; Custer et al., 2014) suggest effects of environmental PFAA concentrations on reproduction. Similar outcomes were observed by Custer et al. (2012, 2014), who also observed negative associations between PFOS concentrations and hatching success in tree swallows. However, the effects they observed started from 150 ng/g ww, which is

approximately 1000 times lower than the PFOS concentrations detected in the present study. In addition, the associations with reproductive parameters of great tits (present study) were less evident compared to the associations in tree swallows. This suggests that differences in sensitivity between species may exist. Karchner et al. (2006) have reported species-specific differences in sensitivity to other POPs between chickens and free-living wild birds. These species-specific differences in sensitivity to pollutants have also been observed for PFAAs. Nordén et al. (2016) reported that the toxic effects of PFOS and PFOA, after *in ovo* injection, were higher in White Leghorn chicken (*Gallus gallus domesticus*) compared to herring gull (*Larus argentatus*) and great cormorant (*Phalacrocorax carbo sinensis*). Another explanation for the differences between the great tits and the tree-swallows, but highly speculative, is that great tits near the fluorochemical plant in Antwerp may have adapted to PFAAs pollution, as fast trait changes in response to changing environmental factors, including toxics, have been reported before (Marzluff, 2016).

A reduced hatching success was also observed in multiple laboratory studies (e.g., Cassone et al., 2012; Molina et al., 2006; O'Brien et al., 2009b). *In ovo* PFHxS exposure resulted in a reduction of hatching success, tarsus length and embryo mass at concentrations of 38,000 ng/g in white leghorn chicken (Cassone et al., 2012). Hatchability of eggs of white leghorn chicken was reduced after *in ovo* exposure to PFOS concentrations of 0.1 µg/g and higher (Molina et al., 2006). However, no effects of PFOA, PFUnDA and PFDS on hatching success were observed at concentrations up to 10 µg/g after *in ovo* exposure in white leghorn chicken (O'Brien et al., 2009b). Median PFOS concentrations in the present study were higher at 3M than reported by Molina et al. (2006), indicating that hatching success at 3M might be influenced by high PFOS concentrations.

Surprisingly, higher values of PC2 (mainly influenced by PFDA) were correlated with the earlier start of the egg laying period, which is contradictory to studies on pesticides (Bustnes et al., 2007; Helberg et al., 2005; Lopez-Antia et al., 2015a,b), where a delayed

start of the egg laying period was observed at higher concentrations. However, for metals, often no effects are observed (Dauwe et al., 2005a; Eeva and Lehikoinen, 1995; Janssens et al., 2003). Early breeding is typically associated with a higher reproductive output and quality. The positive association with early egg laying may therefore indicate that PFAAs stimulate selfmaintenance mechanisms in birds. This hypothesis has also been suggested by Blévin et al. (2017) who reported a positive relationship between PFAAs contamination and telomere dynamics, which is also a measure of quality. A decline in reproductive output, could be the result of direct or indirect effects of differences in the quality of breeders (Kwon et al., 2018; Stenseth and Mysterud, 2002), but also other factors such as seasonal timing (Kwon et al., 2018). This could possibly explain the negative correlation between the start of the egg laying period and the clutch size at Vlietbos as higher quality breeding will start laying earlier and will thus have larger clutch sizes. At Fort 4, the quality of the breeders is possible lower, which might explain the negative correlation between clutch size and chicks' survival there. To the best of our knowledge, this is the first study that reports the correlation between PFAA concentrations and the timing of egg laying.

The reduced shell thickness of the eggs may have also resulted in a reduced hatching, especially for nests where at least one egg hatched, since both parameters were negatively correlated with PC1, and overall breeding success. Contaminant-induced eggshell thinning is a major threat to populations of avian species, as it reduces the survival of the embryos and the hatchability (Miljeteig et al., 2012). Studies that relate eggshell thickness with PFAA concentrations are scarce. Miljeteig et al. (2012) observed no significant association between PFAAs and eggshell thickness in ivory gulls. Similarly, Vicente et al. (2015) observed no associations between PFAA concentrations and egg dimensions in yellow legged gulls. However, concentrations in both these studies were much lower compared to the present study. Eggshell formation is a complex process and disruption of this process, or any of its steps, may lead to alterations in eggshell thickness. Some of the steps in eggshell formation are under hormonal control, indicating that compounds influencing estrogens, androgens

and thyroid hormones may alter eggshell thickness (Miljeteig et al., 2012). Nost et al. (2012) reported positive associations between total thyroxin and PFAA concentrations in black-legged kittiwake and northern fulmar chicks, which indicates that PFAAs may affect the hormones that control eggshell formation.

Finally, our results show that reproductive output of great tits were not necessarily related to the distance from the pollution source, as reproduction, in terms of the studied reproductive parameters, tended to be better at Vlietbos and Burchtse Weel compared to the other locations. To the best of our knowledge, only three studies, which all focused on metals, have been performed that associate environmental pollutant concentrations with reproduction in great tits. Similar results were observed by Eeva and Lehikoinen, who did not observe a variation in clutch size, hatching success and egg shell thickness at different distances from a copper smelter in Finland. However, at the same study site, Eeva and Lehikoinen (1996) reported an increased breeding success with increasing distance from the factory complex. Finally, the hatching and breeding success were lower in great tits closer to a large nonferrous smelter in Belgium compared to lesser-polluted sites (Janssens et al., 2003).

8.5 Conclusion

Although PFAA concentrations in the area surrounding the fluorochemical plant in Antwerp were among the highest ever reported in wildlife, there were no severe declines in reproductive output of great tits. Only a few, weak, associations between PFAA concentrations and reproductive parameters, such as hatching success and total breeding success, were observed. Stronger associations have been reported for other species at lower PFAA concentrations, suggesting either a lower sensitivity to PFAAs of great tits compared to other species, or an adaptation to PFAAs contamination. The association with a reduced hatching success, shows the possibility of PFAAs to affect populations of great tits and implies the need for future monitoring of PFAA concentrations in the environment, as well as monitoring of the population dynamics of this species. Nevertheless, other environmental factors and/or other pollutants, which have not been studied in the present study, could also play an important role in

explaining the differences in reproduction among sites. Therefore, future research should try to include other important factors that could affect the reproduction of free-living bird species.

The outcome of this study can be used in other monitoring studies that use both minimally invasive sampling (eggs; Furness and Greenwood, 1993) and a species that has demonstrated to be a useful sentinel species for local contamination of Persistent Organic Pollutants (Dauwe et al., 2003, 2007; Van den Steen et al., 2006, 2009c). But as the great tit does not seem to be very sensitive to PFAAs (which ideally should be confirmed in additional studies, given that the current study only covers one breeding season), other bird species may be preferable. More research on PFAAs pollution in the vicinity of the fluorochemical plant is necessary to understand 1) the environmental distribution of PFAAs in multiple matrices along the terrestrial food chain, 2) the possible effects at different levels of biological organization both in a wild population, as well as under controlled laboratory conditions.

8.6 Acknowledgements

The authors would like to thank FWO-Flanders for funding this research (FWO nr: G038615N). Furthermore, we would like to express our sincere thanks to 3M for the opportunity to conduct this research at their chemical plant. We would also like to acknowledge Peter Scheys and Geert Eens for their help during the fieldwork and Tim Willems, who performed the UPLC analysis. Finally, we are extremely thankful to the Agency for Nature and Forest (ANB), Koen Maes and Wouter Melens for providing us access to the sampling sites and the possibility to store field materials at these sites.

8.7 Supplementary data

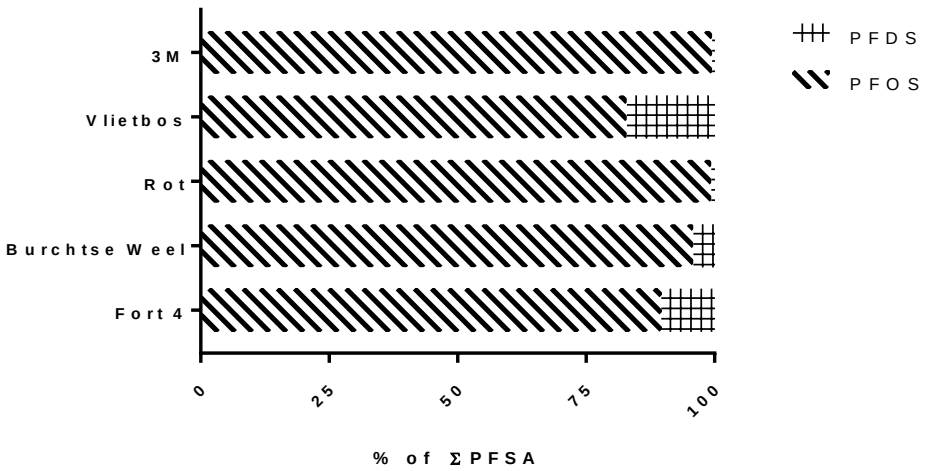


Figure S8.1. Composition profile of PFSAs in eggs of great tit at the five sampling sites, with increasing distance from the fluorochemical plant of 3M: Vlietbos (1 km), Rot (2.3 km), Burchtse Weel (3 km) and Fort 4 (11 km).

Table S8.1. Correlations found between different PFAAs in the different sampling sites. Values in bold are significant correlations.

		3M (n = 23)		Vlietbos (n = 21)		Rot (n = 18)		Burchtse Weel (n = 16)		Fort 4 (n = 33)	
		P-value	**Rho**	**P-value**	**Rho**	**P-value**	**Rho**	**P-value**	**Rho**	**P-value**	**Rho**
PFOS	**PFBA**	0.921	0.022	0.484	0.162	**0.037**	-0.494	-	-	0.096	0.294
	PFNA	**0.009**	0.537	**<0.001**	0.733	0.850	0.049	**<0.001**	0.761	**<0.001**	0.832
	PFOA	**0.003**	0.602	**<0.001**	0.666	**0.040**	0.492	0.118	-0.407	**0.024**	0.394
	PFDA	**<0.001**	0.718	**0.007**	0.568	0.094	0.407	0.887	0.039	0.058	0.333
	PFDoDA	**<0.001**	0.839	**0.003**	0.608	**0.004**	0.659	**0.031**	0.539	**<0.001**	0.762
	PFTrDA	**<0.001**	0.723	**<0.001**	0.847	**0.005**	0.643	**0.015**	0.593	**<0.001**	0.716
	PFTeDA	0.206	0.274	**<0.001**	0.728	0.092	0.408	**0.027**	0.551	**0.001**	0.616
	PFDS	**<0.001**	0.882	-	-	-	-	-	-	-	-
PFBA	**PFNA**	0.766	-0.066	0.598	0.122	0.083	0.419	-	-	0.225	0.217
	PFOA	0.489	-0.152	0.578	-0.129	0.106	-0.393	-	-	0.718	0.065
	PFDA	0.321	-0.217	0.891	-0.032	0.292	0.263	-	-	0.524	0.115
	PFDoDA	0.637	0.104	0.485	0.161	0.886	-0.036	-	-	0.051	0.342
	PFTrDA	0.282	0.234	0.548	0.139	0.708	-0.095	-	-	**<0.001**	0.599
	PFTeDA	0.071	0.384	0.454	0.173	0.599	0.133	-	-	0.135	0.266
	PFDS	0.343	0.207	-	-	-	-	-	-	-	-
PFNA	**PFOA**	**0.010**	0.530	**<0.001**	0.781	0.163	-0.344	0.179	-0.353	**0.037**	0.365
	PFDA	**0.021**	0.482	0.103	0.366	**0.009**	0.599	0.532	-0.169	0.179	0.240
	PFDoDA	0.138	0.319	0.089	0.380	**0.021**	0.546	**0.039**	0.520	**<0.001**	0.571
	PFTrDA	0.153	0.308	**0.002**	0.633	0.110	0.391	**0.002**	0.707	**0.001**	0.542
	PFTeDA	0.418	0.177	**0.018**	0.511	**0.027**	0.520	**0.017**	0.588	0.053	0.340
	PFDS	0.116	0.337	-	-	-	-	-	-	-	-

Table S8.1 (continued). Correlations found between different PFAAs in the different sampling sites. Values in bold are significant correlations.

		3M (n = 23)		Vlietbos (n = 21)		Rot (n = 18)		Burchtse Weel (n = 16)		Fort 4 (n = 33)	
		P-value	**Rho**	**P-value**	**Rho**	**P-value**	**Rho**	**P-value**	**Rho**	**P-value**	**Rho**
PFOA	**PFDA**	0.127	0.328	0.083	0.387	0.731	-0.087	0.265	-0.296	**0.023**	0.394
	PFDoDA	**0.019**	0.488	0.139	0.334	0.412	0.205	0.755	0.085	**0.008**	0.452
	PFTrDA	**0.029**	0.454	**0.024**	0.489	0.451	0.189	0.785	-0.074	0.125	0.273
	PFTeDA	0.908	0.025	**0.012**	0.535	0.990	-0.003	0.569	0.154	0.504	0.121
	PFDS	**0.018**	0.492	-	-	-	-	-	-	-	-
PFDA	**PFDoDA**	**0.034**	0.446	**0.018**	0.510	**0.002**	0.667	0.417	-0.218	0.108	0.285
	PFTrDA	0.271	0.240	**0.004**	0.605	**0.007**	0.610	0.547	-0.162	**0.001**	0.541
	PFTeDA	0.839	0.045	**0.005**	0.592	**0.005**	0.629	0.151	-0.376	**0.009**	0.445
	PFDS	**0.025**	0.469	-	-	-	-	-	-	-	-
PFDoDA	**PFTrDA**	**<0.001**	0.933	**<0.001**	0.769	**<0.001**	0.742	**<0.001**	0.877	**<0.001**	0.688
	PFTeDA	**0.013**	0.508	**<0.001**	0.855	**<0.001**	0.841	**0.001**	0.738	**0.002**	0.521
	PFDS	**<0.001**	0.965	-	-	-	-	-	-	-	-

Table S8.1 (continued). Correlations found between different PFAAs in the different sampling sites. Values in bold are significant correlations.

		3M (n = 23)		Vlietbos (n = 21)		Rot (n = 18)		Burchtse Weel (n = 16)		Fort 4 (n = 33)	
		P-value	**Rho**	**P-value**	**Rho**	**P-value**	**Rho**	**P-value**	**Rho**	**P-value**	**Rho**
PFTrDA	**PFTeDA**	**0.002**	0.619	**<0.001**	0.822	**<0.001**	0.756	**0.007**	0.649	**<0.001**	0.702
	PFDS	**<0.001**	0.902	-	-	-	-	-	-	-	-
PFTeDA	**PFDS**	**0.012**	0.512	-	-	-	-	-	-	-	-
∑PFSA	**∑PFCA**	**<0.001**	0.924	**0.001**	0.658	**0.003**	0.664	**0.017**	0.594	**<0.001**	0.733

Tabel S8.2. Correlations found between different reproductive parameters at each sampling site. Values in bold represent the significant correlations.

		3M		Vlietbos		Rot		Burchtse Weel		Fort 4	
		P-value	**Rho**	**P-value**	**Rho**	**P-value**	**Rho**	**P-value**	**Rho**	**P-value**	**Rho**
Day 1st egg	**Clutch size**	0.656	-0.098	**0.037**	**-0.447**	0.786	-0.071	0.755	-0.085	0.058	-0.356
	Breeding success	0.170	0.319	0.895	0.031	0.414	0.219	0.568	-0.160	0.658	0.095
	Hatching success	0.105	0.373	0.81	0.057	0.241	0.311	0.568	-0.160	0.922	0.021
	Chicks survival	0.373	-0.239	0.507	0.162	0.414	-0.260	NA[a]	NA	0.248	0.287
	Shell Thickness	0.433	0.172	0.562	-0.138	0.421	-0.209	0.872	-0.045	0.098	-0.346
	Chick body condition	0.724	-0.104	0.361	-0.229	0.165	-0.476	0.652	-0.139	0.773	0.085
Clutch size	**Breeding success**	0.509	-0.157	0.163	-0.324	0.369	0.241	0.623	0.138	0.129	-0.319
	Hatching success	0.365	-0.214	0.168	-0.321	0.321	0.265	0.622	0.138	0.871	-0.035
	Chicks survival	0.955	-0.015	0.585	-0.134	0.466	0.233	NA[a]	NA	**0.003**	**-0.667**
	Shell Thickness	0.268	-0.241	0.673	0.101	0.062	0.462	0.206	0.346	0.986	0.004
	Chick body condition	0.761	0.090	0.964	0.012	0.351	0.330	0.673	-0.130	0.539	0.180
Breeding success[b]	**Hatching success**	**<0.001**	**0.893**	**<0.001**	**0.786**	**<0.001**	**0.931**	**<0.001**	**1**	**0.004**	**0.561**
	Chicks survival	**0.002**	**0.718**	**0.004**	**0.631**	**0.022**	**0.652**	NA[a]	NA	**<0.001**	**0.904**
	Shell Thickness	0.311	0.238	0.177	0.323	0.464	0.197	0.679	-0.121	0.723	0.078
	Chick body condition	0.092	-0.467	0.789	-0.068	0.736	0.122	0.062	-0.532	0.651	-0.133

Tabel S8.2 (continued). Correlations found between different reproductive parameters at each sampling site. Values in bold represent the significant correlations.

		3M		Vlietbos		Rot		Burchtse Weel		Fort 4	
		P-value	**Rho**	**P-value**	**Rho**	**P-value**	**Rho**	**P-value**	**Rho**	**P-value**	**Rho**
Hatching success[c]	**Chicks survival**	0.130	0.394	0.567	0.140	0.170	0.424	NA[a]	NA	0.998	<0.001
	Shell Thickness	0.324	0.233	0.919	0.025	0.388	0.232	0.679	-0.121	0.94	-0.017
	Chick body condition	0.064	-0.508	0.561	-0.147	0.736	0.122	0.062	-0.532	0.766	0.088
Chicks survival[d]	**Shell Thickness**	0.751	0.086	0.072	0.434	0.413	0.261	NA[a]	NA	0.458	0.193
	Chick body condition	0.588	0.159	0.754	-0.080	NA[e]	NA	NA[a]	NA	0.467	-0.212
Shell Thickness	**Chick body condition**	0.196	-0.368	0.330	-0.252	0.243	0.407	0.227	-0.360	0.933	-0.026

[a]Survival was 100% for all the nests were at least one egg hatched.

[b]Number of fledged chicks divided by the number of incubated eggs. Only nests where incubation started were taken into account.

[c]Number of hatched eggs weighed by the number of incubated eggs. Only nests where incubation started were taken into account.

[d]Number of fledged chicks weighed by the number of hatched eggs. Only nests where at least one egg hatched were taken into account.

[e]Chicks body condition was only determined in nests where all chicks survived.

Table S8.3. Explained variance in PFAA concentrations (%) of PC1 and PC2 and factor loadings of the nine detected PFAAs for PC1 and PC2. Values in bold represent significant factor loadings (>0.60).

	PFBA	*PFNA*	*PFOA*	*PFOS*	*PFDA*	*PFDS*	*PFDoDA*	*PFTrDA*	*PFTeDA*	*Explained variance in PFAAs (%)*
PC1	0.391	**0.764**	**0.644**	**0.950**	0.413	**0.953**	**0.959**	**0.908**	**0.818**	61.61
PC2	-0.464	0.497	0.442	0.052	**0.624**	-0.081	-0.156	-0.336	-0.317	14.38

9. Perfluoroalkyl acids (PFAAs) concentrations and oxidative status in two generations of great tits inhabiting a contamination hotspot

Published in Environmental Science and Technology:

Ana Lopez-Antia*, Thimo Groffen*, Robin Lasters, Hamada AbdElgawad, Jiachen Sun, Han Asard, Lieven Bervoets and Marcel Eens (2019). Environmental Science and Technology 53: 1617 – 1626. https://doi.org/10.1021/acs.est.8b05235

Tables were modified to fit the size of the pages. References were added as text instead of numbers. No further modifications were made.

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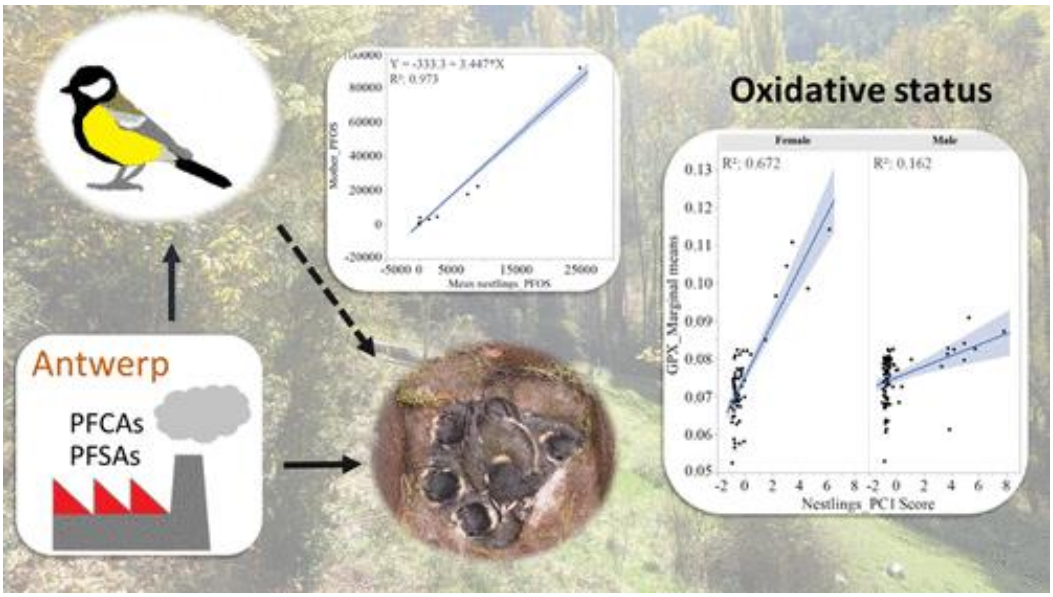

Abstract

The ubiquity of perfluoroalkyl acids (PFAAs) contrasts with the limited information about their effects. We report here PFAA plasma concentrations in wild populations of great tits (*Parus major*) settled at and in the vicinity of a fluorochemical plant in Antwerp (Belgium). Using two generations we obtained novel results on some poorly known issues such as differences between sexes, maternal transfer of the compounds and potential associations with the oxidative status. For five out of the 11 detected PFAAs, the concentrations were the highest ever reported in birds' plasma, which confirms that Antwerp is one of the main hotspots for PFAAs pollution. Contrary to other studies conducted in birds, we found that females presented higher mean concentrations and detection frequencies for two compounds (perfluorooctanesulfonic acid (PFOS) and perfluoroundecanoic acid (PFUnDA)) than males. Maternal transfer and the dietary intake appear to be the main route of exposure for nestlings to PFOS but not to other compounds. Finally, PFAA concentrations tended to correlate positively with protein damage in adult birds while in nestlings they positively correlated with higher activity of antioxidant enzymes (glutathione peroxidase and catalase). Experimental work is needed to confirm oxidative stress as a pathway for the pernicious effects of PFAAs.

9.1 Introduction

Perfluoroalkyl acids (PFAAs) are highly persistent substances produced and extensively used for more than six decades. Historically, long chain (LC) perfluoroalkyl carboxylic acids (PFCAs; with ≥7 perfluorinated carbons) and perfluoroalkyl sulfonic acids (PFSAs; with ≥6 perfluorinated carbons) have been the most used ones, concretely perfluorooctanoic acid (PFOA, $C_7F_{15}COOH$) and perfluorooctanesulfonic acid (PFOS, $C_8F_{17}SO_3H$). The widespread use of these PFAAs, together with their persistence and bioaccumulation potential, have resulted in a global contamination of the environment, wildlife and humans (D'Hollander et al., 2010; Giesy and Kannan, 2001; Houde et al., 2006; Kissa, 2001).

Since 2000, the widespread distribution and potential health effects of LC-PFAAs (reviewed by OECD, 2013), led the industry and regulators to take action by reducing the use and the release of these compounds. In 2002, the 3M company voluntarily phased out the production of PFOS and in 2009 PFOS and related substances were listed under Annex B (restriction of production and use) of the Stockholm Convention on Persistent Organic Pollutants. Other LC-PFCAs and PFSAs have been recently included in the Candidate List of Substances of Very High Concern for Authorization under the European Chemicals Regulation (REACH (ECHA, 2017)). Due to these actions, a transition is taking place in the industry to replace LC-PFAAs with short chain (SC) PFAAs and polyfluorinated substitute compounds (Scheringer et al., 2014; Wang Z et al., 2013). However, for many of these alternatives, information on actual releases and exposures is missing. Moreover, their risks and potential toxicity to various biota remain largely unexplored (OECD/UNEP, 2013; Scheringer et al., 2014; Wang Z et al., 2013, 2015b).

Previous studies on the bioaccumulation and effects in birds (Dauwe et al., 2007; Groffen et al., 2017, 2019c; Hoff et al., 2005; Lasters et al., 2019; Lopez-Antia et al., 2017) have been conducted near the 3M fluorochemical plant in Antwerp using great tits and blue tits (*Cyanistes caeruleus*), lapwing (*Vanellus vanellus*), and the Mediterranean gull (*Larus melanocephalus)*. These studies have revealed the highest

PFOS concentrations ever found in wildlife (e.g., mean concentration of 48 056 ng/g found in the eggs of great tits breeding at the fluorochemical plant (Groffen et al., 2019c). Furthermore, concentrations of other PFSA such as perfluorodecanesulfonic acid (PFDS) and perfluorohexanesulfonic acid (PFHxS) and concentrations of PFOA were also the highest reported in bird eggs (Groffen et al., 2017, 2019c)

Previous studies performed in birds described negative effects of PFAAs on reproduction (Custer et al., 2014; Groffen et al., 2019c) chick survival (Custer et al., 2014) and the immune system (Peden-Adams et al., 2009; Smits and Nain, 2013). The oxidative status of individuals could be used as an indicator of the pernicious effects of PFAAs (Custer et al., 2017). Immune system cells or sperm cells are vulnerable targets to the oxidative damage produced by many pollutants (Monaghan et al., 2009). Furthermore, organisms might need to use dietary antioxidants to deal with oxidative stress (OS), which causes an imbalance of the trade-off in the allocation of these substances among physiological functions (e.g., reproduction, sexual signaling (Lopez-Antia et al., 2015c; Monaghan et al., 2009). Therefore, studying the oxidative status is a key element in toxicological studies. Nevertheless, not much is known about PFAAs effects on birds' antioxidant system. The study of the transcriptional response of chicken hepatocytes exposed to PFOS pointed to OS as a cause of gene alteration (O'Brien et al., 2011). Similarly, wild common cormorants (*Phalacrocorax carbo*) livers, naturally exposed to PFAAs, presented an altered transcriptional response of genes involved in the antioxidant system (Nakayama et al., 2008). Despite this, a study performed in white-tailed eagle (*Haliaeetus albicilla*) nestlings, did not find any relationship between PFAA concentrations and the activity of the antioxidant enzyme superoxide dismutase (SOD) in plasma (Sletten et al., 2016).

In the present study we examined plasma concentrations and the composition profile of 15 PFAAs (11 PFCAs and 4 PFSAs) in wild populations of great tits (*Parus major*) settled along a distance gradient of 11 km from an active fluorochemical plant in Antwerp (Belgium). We studied differences in PFAA concentrations and composition

profile along the gradient. We also examined the association between the measured PFAAs concentrations, the body condition and the OS status of the birds. Moreover, we sampled adult birds, their eggs and their nestlings, which enabled us to explore the maternal transfer of PFAAs to the offspring (Custer et al., 2012, 2014). The outcome of this study will reveal the current exposure status of wildlife to PFAAs in one of the main hotspots in the world. It will also improve our understanding of OS as a potential underlying mechanism for pernicious effects of PFAAs and predicting the exposure consequences for wild bird populations.

9.2 Materials and method

9.2.1 Sample collection

Nestboxes were placed during autumn of 2015 at five sampling sites, representing a distance gradient from a fluorochemical plant in Antwerp (Supporting Information (SI) Figure S9.1). These sites were the fluorochemical plant (25 nestboxes), Vlietbos (22 nestboxes; 1 km SE from the plant), Rot-Middenvijver (shortly Rot; 18 nestboxes; 2.3 km ESE from the plant), Burchtse Weel (19 nestboxes; 3 km SE from the plant) and Fort 4 in Mortsel (31 nestboxes; 11 km SE from the plant).

The first blood sampling period was performed before the start of the breeding season between the eighth of February and the ninth of March of 2016. During this period, all nestboxes were visited after sunset and roosting birds were captured. Captured birds were ringed (if not already ringed), tarsus length and body mass were measured and age (yearlings versus older) and sex were determined following Svensson (1992). Body condition was calculated according to the scaled mass index (Peig and Green, 2009). We also took a blood sample (maximum 150 µL) from the brachial vein using microhematocrit heparinized capillary tubes (Microvette). These samples were kept refrigerated and centrifuged at 10 000*g* for 10 min at 4 °C to separate plasma from the red blood cells (RBC), which were stored separately at −80 °C for later analysis. The number of sampled birds was 79 (between 13 and 18 per location).

From just before egg laying until incubation, nestboxes were checked every other day or daily to determine the start of the egg-laying period. From each nest, the third egg was collected before the incubation started. Later, in the nestling period, the second blood sampling was performed, in May and June 2016. When nestlings were 10 days old, parents (mostly the female) were captured inside the nestbox, using a trap door in the entrance hole, and we proceeded as was explained above. In this way we sampled 60 birds (45 females and 15 males). Finally, when nestlings were 14 days old, all nestlings in each nest were ringed, measured (tarsus length and body mass) and a blood sample was taken. A total of 441 nestlings from 79 nests were sampled, from which 179 samples were selected for PFAAs and OS parameters analyses: (1) we selected two nestlings per nest (the lightest and the heaviest); (2) we selected one complete brood per site. A small portion of nestlings' RBC (≈1 µL) was used to determine the sex genetically following the method described by Griffiths et al. (1998) with minor modifications (Vermeulen et al., 2016).

Due to great tits being highly resident with birds staying close to or in their breeding area during the winter, we have repeated measurements from 18 individuals (birds sampled both in winter and in the nestling period).

9.2.2 PFAAs analysis in plasma

The used abbreviations for PFAA compounds are according to Buck et al. (2011) (SI Table S9.1). Eleven PFCAs (PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoDA, PFTrDA and PFTeDA) and four PFSAs (PFBS, PFHxS, PFOS, and PFDS) were selected as target analytes. A mixture of isotopically mass-labeled internal standards (ISTDs) were used (SI).

9.2.3 Sample extraction

Samples were extracted using a solid-phase extraction technique, which is based on the chemical principle of weak-anion exchange described by Groffen et al. (2019c) (SI). Briefly, 80 µL of isotopically mass-labeled internal standard mixture and 10 µL of acetonitrile was added to each sample (10 µL of plasma/∼0.4 g of homogenized egg).

After sonication, samples were left overnight on a shaking plate. After centrifugation the supernatant was transferred into a 14 mL tube and loaded on HR-XAW columns.

9.2.4 UPLC-TQD analysis

UPLC coupled tandem ES(−) mass spectrometry (ACQUITY, TQD, Waters, Milford, MA) was used to analyze PFAAs.

Individual LOQs of the detected compounds are shown in SI Table S9.2 (SI Table S9.3 shows those compounds with detection frequency <20%). Based on the ratio of the mean ISTD area of the sample over the area of a blank ISTD solution, recoveries were determined. PFBS and PFHxS recoveries were too low and therefore they were excluded from further analysis. Further details about the analysis conditions, the calibration method and the quality assurance can be found in the SI and in Groffen et al. (2019c).

9.2.6 Statistical analysis

To perform statistical analyses we used JMP Pro 14. In each location, we only considered those compounds with detection frequency ≥50%, values below LOQ were replaced by LOQ/2 (Bervoets et al., 2004; Custer et al., 2014). All PFAA concentrations were log transformed to obtain a normal distribution. Temporal data from adult birds were pooled (adults from both the late winter and the spring) and analyzed separately from nestlings' data. To study the maternal transfer we used a database that included data from mothers captured in the nestling period (spring) and data from their eggs and their offspring.

We performed linear mixed models to compare the concentrations of the different PFAAs and levels of different oxidative stress parameters among locations. For adult birds, we included the location, sex and age of the bird, the sampling period and the interactions between them as factors and we followed a backward elimination. We included bird identity (determined by ring number) as a random effect. To calculate the mean, median, range and detection frequency values of PFAA compounds in adults in each sampling site (SI Table S9.2), each bird was only considered once (the winter

measurement in case the bird was captured both in winter and in spring). For nestlings, we included nest identity (determined by nestbox number) as a random effect and we included clutch size as a factor in the models. To compare the detection frequency of the different PFAAs among locations we performed a Generalized Linear Model (GLMz) with binomial distribution and we proceeded as for the concentrations but only including each bird once. When significant results were found ($p \leq 0.05$) posthoc analyses (Tukey test) were conducted for pairwise comparison. To compare the distribution of single PFAA compounds in the mothers, their eggs and the offspring, data were treated using methods of survival analyses for left-censored data, that is, reverse Kaplan–Meier method (Gillespie et al., 2010; Jaspers et al., 2013). To compare ∑PFAA and ∑PFCA concentrations and to test for effects of the body condition on PFAA concentrations we used an ANOVA analysis including the type of sample as a factor and the body condition as acovariate. Relationship between compound concentrations in each location and relationships between mothers', eggs' and nestlings' concentrations were investigated using Spearman's correlation test.

To study the relationship between PFAAs and OS parameters or body condition, first, in order to reduce the number of covariates and to account for collinearity among them, we conducted principal component analysis (PCA). In this analysis we included all those compounds with a detection frequency ≥20% (seven compounds for adults and four compounds for nestlings; SI Table S8.4), for these compounds values below LOQ were replaced by LOQ/2. The number of significant principal components was selected according to the Kaiser criterion (i.e., eigenvalue higher than 1 (Kaiser, 1960)). Two principal components (PCs) were selected for adults (hereafter adults-PC1 and adults-PC2) and one for nestlings (hereafter nestlings-PC1). Each compound loading and variance explained by each PC are shown in SI Table S9.4. Adults-PC1 explained 62% of the variance and adults-PC2 explained a further 19%. Nestlings-PC1 explained 73% of the variance.

We performed linear mixed models for each OS parameter and for the body condition of the birds. OS parameters were log transformed to obtain a normal distribution when necessary. For adults, the age and sex of the bird, the season, the PCs and the interactions between them were included as explanatory variables and followed a backward elimination, while including the ring number as a random effect. For nestlings we proceeded as for adults but including the nestbox number as random effect.

9.3 Results

9.3.1 Spatial PFAAs contamination in adult birds

PFOS concentration in the plasma of adult birds decreased significantly with the distance from the plant (all $p < 0.001$; Figure 9.1, SI Table S9.2). PFDoDA concentration at the plant site was significantly higher than at the other sites ($p < 0.001$). For PFOA there is a season-dependent site effect (site × season interaction $p = 0.05$), with significantly higher concentrations at the plant ($p < 0.0001$) but only in winter. There was not significant difference between sites for PFUnDA ($p > 0.13$; Figure 9.1).

For PFOS ($p = 0.01$) and PFUnDA ($p = 0.02$), females had significantly higher concentrations than males (SI Figure S9.2). This sex effect was independent of the sampling site (site*sex interactions $p > 0.09$) but it was more apparent at 3M where mean concentrations (±SE) were (females/males): PFOS 94153 ± 33531/46337 ± 17596 pg/uL, PFUnDA 21.3 ± 6.2/12.3 ± 4.1 pg/uL. The age of the birds did not affect the concentrations of PFAAs.

Differences between periods were found for PFOA and PFDoDA (all $p < 0.01$). Concentrations (mean ± SE) were higher in winter for PFOA (winter = 60.2 ± 4.2 pg/uL, spring = 42.2 ± 5.2 pg/uL; these differences were significant in Vlietbos and Fort 4 (both $p \leq 0.003$)) whereas for PFDoDA higher concentrations were found in spring (winter = 6.1 ± 1.2 pg/uL, spring = 9.6 ± 2.9 pg/uL) regardless of the sampling site (site*season interactions $p = 0.54$).

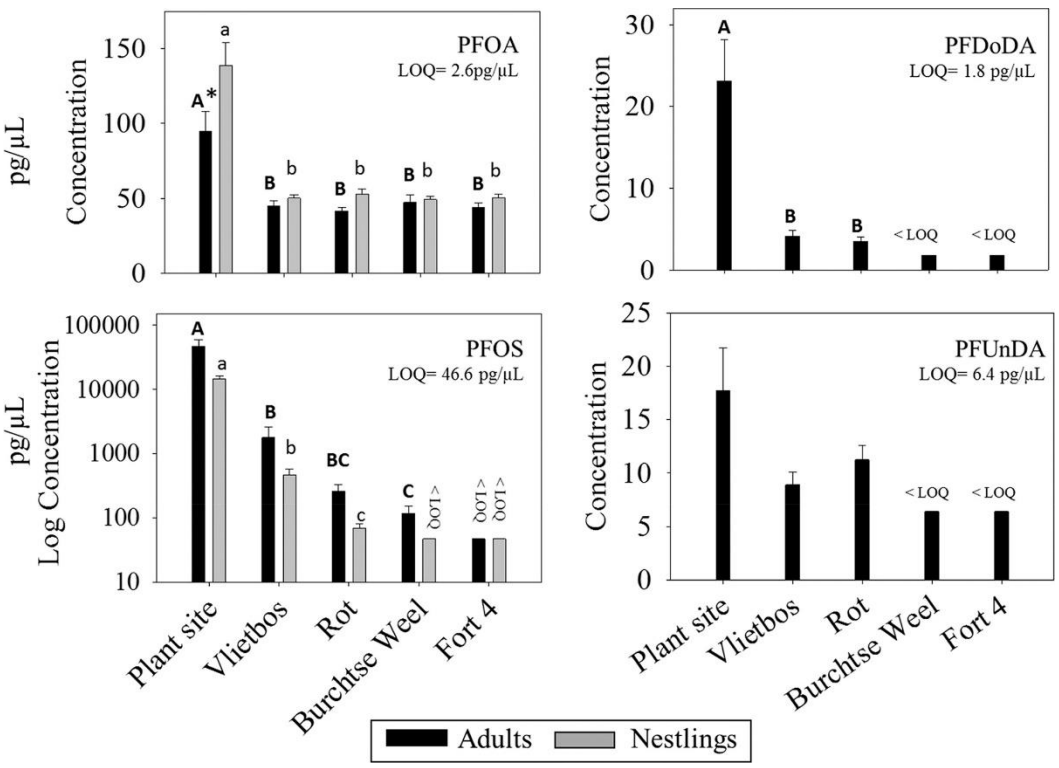

Figure 9.1. Mean concentrations (±SE) of different PFAAs found in adult birds'(black) and nestlings'(gray) plasma at the five sampling sites in 2016: a fluorochemical plant in Antwerp and four sites with an increasing distance from the plant site (i.e., 1 km Vlietbos, 2.3 km Rot, 3 Km Burchtse Weel and 11 km Fort 4). Different upper case letters and lower case letters indicate different concentrations among sampling sites in adults birds and nestlings, respectively. Additionally, mean concentrations (±SE) could be calculated (detection frequency ≥50%) at the plant site in adults for PFNA (21.8 ± 5.3) and PFTrDA (8.5 ± 2.3) and in nestlings for PFBA (24.1 ± 4.6) and PFDoDA (12.2 ± 1.9). Temporal data from adult birds were pooled together (adults from both the late winter and the spring). *For PFOA, concentration at the plant site was only significantly higher in winter (no differences in spring). <LOQ; detection frequency was below 50% and mean values were not calculated (the value given is the LOQ).

Regarding the detection frequencies of the different PFAA compounds (SI Table S9.2), PFOA was found above its LOQ at 99% of the samples. PFOS was detected above its LOQ at 72% of the samples. Significant differences existed in the detection frequency of PFOS among locations ($p < 0.0001$) appearing less frequently at Burchtse Weel (60%) than at the plant and Vlietbos (100%), and at Fort 4 (25%) than at all the other locations. PFOS detection frequency was higher in females (80.0%) than in males (62.3%; $p = 0.03$) regardless of the sampling site (site × sex interaction $p = 0.85$). PFUnDA overall detection frequency was 48% and significant differences existed

among locations ($p \leq 0.01$) with lower detection frequency at Burchtse Weel (32%) and Fort 4 (26%) than at Vlietbos (62%) and Rot (70%) and similar than at the plant (50%). PFUnDA appeared more often in female (61%) than in male (32%) birds ($p = 0.001$) regardless of the sampling site (site × sex interaction $p = 0.38$). PFDoDA overall detection frequency was 63% with no significant differences among locations. PFDoDA was detectable more often ($p = 0.001$) in spring (80%) than in winter (52%). Detection frequencies of PFNA (overall 27%), PFDA (overall 24%) and PFTrDA (overall 33%) were only at the plant site ≥50% (70, 50, and 55%, respectively).

An overview of the correlations found among compounds (with a detection frequency of ≥50%) at the different locations is given in SI Table S9.5. When a value was < LOQ it was substituted by LOQ/2. Almost all the compounds were correlated with each other at the plant site except for the following pairs: PFOA/PFUnDA, PFOA/PFTrDA, PFNA/PFTrDA, and PFDA/PFTrDA. By contrast, no correlations were found for Vlietbos. At Rot PFDoDA was significantly correlated with PFUnDA and PFOS and at Burchtse Weel PFOA and PFOS were significantly correlated.

The PFAAs profile was clearly dominated by PFOS at the plant (93% of the PFAAs) but this percentage decreased with the distance from the plant to only 30% at Fort 4. On the other hand, the contribution of PFOA to the total of PFAAs increased from 1% at the plant to 41% at Fort 4. The PFCAs profile was dominated by PFOA at all the locations (from 46 to 58%) followed by PFDA at the plant site and by PFUnDA at the other locations (SI Table S9.2).

For PFHpA and PFDS, concentrations in all samples were below the LOQ (7.4 and 5.1 pg/µL, respectively). Moreover, values above the LOQ were only detected in four samples for PFHxA (range 8.5–9.7 pg/µL), five samples for PFBA (9.4–133.2 pg/µL), seven samples for PFTeDA (1.4–2.4 pg/µL) and 15 samples for PFPeA (52–202 pg/µL). LOQ and detection frequencies of these compounds in each sampling site are shown in SI Table S9.3.

9.3.2 Spatial PFAAs contamination in nestlings

Mean concentrations of PFAAs found in nestlings are shown in Figure 9.1 (median concentrations, LOQs, ranges and detection frequencies shown in SI Table S9.6).

Significantly different concentrations were found among locations for all compounds (all $p < 0.000$; Figure 9.1) and post hoc analysis revealed that differences occurred between the plant and all the other locations. No significant differences in concentrations of PFAAs between sexes were found (all $p > 0.185$). Clutch size did not have a significant effect on nestling concentrations (all $p > 0.08$).

For PFOS, among nests, concentrations varied up to 58-fold at the plant, 143-fold at Vlietbos, 9-fold at Rot, 11-fold at Burchtse Weel, and 6 fold at Fort 4. For ∑PFCAs, concentrations varied up to 15-fold at the plant and around 3-fold at the other locations. The maximum variation of PFOS concentrations within nests was similar at the plant, Vlietbos and Burchtse Weel (around 4-fold) and slightly higher at Rot (7-fold) and at Fort 4 (6 fold). For ∑PFCAs, the maximum variation within nest was around 3-fold at the plant site and Vlietbos and around 2-fold at the other locations.

PFOA (LOQ: 2.9 pg/mL) was detected in all samples whereas PFOS (LOQ: 46.6 pg/mL), PFDoDA (LOQ: 1.8 pg/mL) and PFBA (LOQ: 6.5 pg/mL) were detected in 61%, 34%, and 20% of the samples, respectively. Differences exist in the detection frequencies of these compounds among locations (all $p < 0.001$; SI Table S9.6). The detection frequency of PFOS decreased with the distance from the plant because many samples fell below the LOQ. PFOS appeared more often in males (64%)than in females (52%; $p = 0.014$), mainly due to the differences found at Rot ($p = 0.005$).

All the studied compounds in nestlings from the plant were correlated with each other (SI Table S9.7). PFOA was correlated with PFOS at Vlietbos but not at Rot.

PFOS dominated the PFAAs profile at the plant (98%) and at Vlietbos (70%) but at Rot it represented only 47% of the ∑PFAAs, exactly the same as PFOA. PFOS ratio decreased in farther locations where PFOA was the dominant compound. Regarding

the PFCAs profile, PFOA was the dominant compound at all the locations (ratio range from 81 to 91%).

PFPeA, PFHxA, PFHpA, and PFDS concentrations were below their respective LOQ in all the samples. For PFNA we found 16 samples with concentrations above the LOQ (range 4.70–18.4 pg/µL), with 15 of these samples belonging to nestlings sampled at the plant. Moreover, these nestlings belonged only to eight nestboxes (out of 14 with nestlings at the plant). Similarly, for PFTrDA we found 11 samples above the LOQ (2.92–12.1 pg/µL), most of them at the plant site. We found 10 samples above the LOQ for PFDA (6.9–14.7 pg/µL) and PFUnDA (8.6–24.7 pg/µL). Finally, we found three samples with concentrations above the LOQ of PFTeDA (1.7–6.7 pg/µL): LOQ and detection frequencies of these compounds in each sampling site are shown in SI Table S9.8.

9.3.3 Relationships between mothers, eggs and offspring concentrations

Concentrations in females plasma in spring (mothers), and in the plasma of offspring (the heaviest and the lightest nestlings in the nest) were compared (SI Table S9.9, Figure S9.3). We only compared those compounds with a detection frequency ≥50% in at least one of the sampling sites (i.e., PFOA, PFDoDA, and PFOS). PFOA concentrations were significantly higher in the nestlings than in the mothers ($p < 0.001$). PFOS concentrations were higher in the mothers than in the nestlings ($p \leq 0.04$). No differences between sample types were found for PFDoDA. ∑PFAA and ∑PFCA concentrations were higher in the mothers than in the nestlings ($p < 0.01$ and $p < 0.0001$, respectively). No significant differences were found in the concentration of any compound between siblings. Body condition did not explain the differences between mothers and offspring for any of the compounds (all $p > 0.14$).

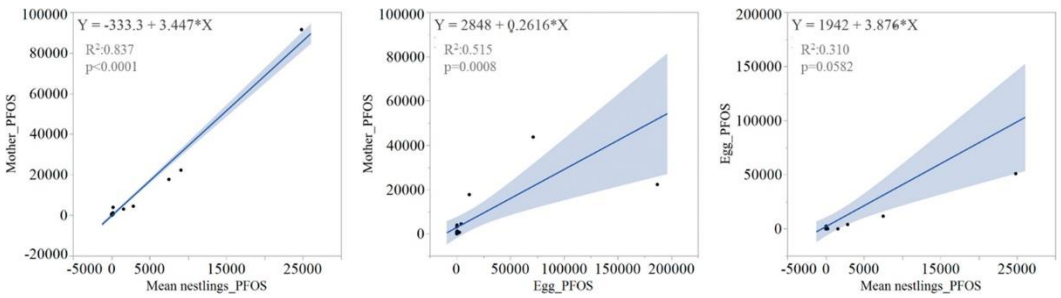

Figure 9.2. Correlations between mother, offspring (pg/µL;mean values were calculated for the lightest and the heaviest nestlings in the nest) and egg (ng/g) PFOS concentrations. Spearman correlation values, ps and the regression equations are given. Regression lines are shown with 95% confidence bands shaded.

Correlations were studied between mother, the third egg and offspring concentrations. We found significant correlations in ∑PFAA concentrations between mothers and eggs ($p < 0.001$; $r = 0.52$), mothers and offspring (all $p < 0.0001$; $r \geq 0.70$) and between siblings (the heaviest and the lightest; $p < 0.0001$; $r = 0.71$), but not between eggs and nestlings (all $p > 0.11$; $r < 0.26$). Very similar correlations were found in PFOS concentrations with slightly higher correlations between eggs and nestlings (both $p = 0.06$, $r = 0.31$; Figure 9.2). PFOA concentrations in mothers were correlated with the concentrations in the eggs ($p < 0.01$; $r = 0.41$) but no correlations were found between mothers/eggs and offspring, nor between siblings. For PFDoDA and ∑PFCAs no correlations were found.

9.3.4 Correlation of PFAA concentrations with body condition and the oxidative status

Body condition and OS parameters' results at the five sampling sites for adults and nestlings are shown in SI Table S9.10 and S9.11 respectively (differences between sites on these parameters are also indicated).

Adults-PC1 was mainly influenced by PFOS, PFDA, PFNA, PFOA, PFUnDA and PFDoDA; high concentrations of these compounds corresponded with high values of adults-PC1. Adults-PC2 was mainly influenced by PFTrDA, with high values of aduts-PC2 mainly indicating high concentrations of PFTrDA (SI Table S9.4).

A trend existed in adult birds to present higher levels of protein carbonyls with higher values of Adults-PC1 (p = 0.08) and Adults-PC2 (p = 0.07; SI Figure S9.4). This means that birds with higher concentrations of PFAAs tended to have also higher oxidative protein damage. There was also a significant effect of the sampling season on protein carbonyls' concentrations ($p < 0.0001$) with higher concentrations in winter, although the interaction between Adults-PCs and the season was not significant ($p \geq 0.26$). We did not find significant correlations (all $p > 0.14$) between Adults-PCs and the body condition or between Adults-PCs and the other measured stress parameters (GSH and GSSG concentrations or the ratio between them, SOD, CAT, and GPX activity or the measurement of the TAC) in adult birds.

Nestlings-PC1 was influenced by PFOS, PFOA, PFDoDA, and PFBA: therefore high concentrations of these compounds corresponded with high values of nestlings-PC1.

Nestlings' GPX activity was positively correlated with Nestlings-PC1 (p = 0.006; Figure 9.3) and the body condition of the chick (p = 0.007). There was also a marginally significant result in the interaction between Nestlings-PC1 and the sex (p = 0.06). When we performed a separate analysis for males and females, the relationship between Nestlings-PC1 and GPX activity was only significant in females (p = 0.002; Figure 9.3) with increased enzyme activity detected in higher exposed females.

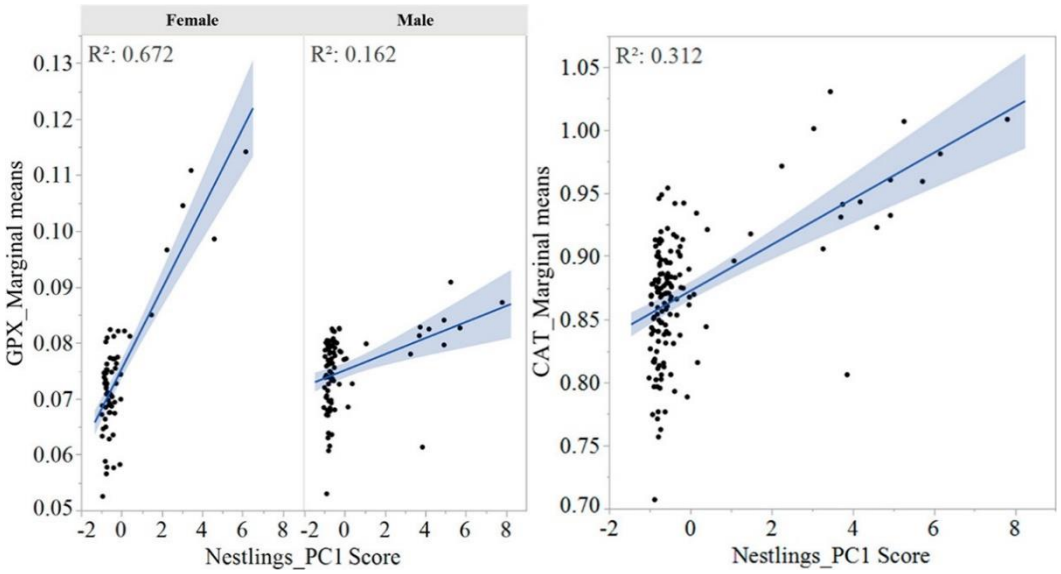

Figure 9.3. Relationship between nestlings-PC1 and the glutathione peroxidase (separated by sex) and catalase activity (marginal means as obtained in the mix models when considering body condition as a covariate and nestbox as random effect) in 2016 at five sites in the vicinity of Antwerp, Belgium. Nestlings-PC1 was influenced by PFOS, PFOA, PFDoDA, and PFBA: therefore high concentrations of these compounds corresponded with high values of nestlings-PC1. Regression lines are shown with 95% confidence bands shaded.

Nestlings' CAT activity was positively correlated with Nestlings-PC1 (p = 0.05; Figure 9.3), body condition (p = 0.012) and marginally affected by the sex of the chick (p = 0.06), with higher activity in females. We did not find significant correlations between PFAA concentrations and the other stress parameters or the body condition in nestlings (all p > 0.23).

9.4 Discussion

9.4.1 PFAA concentrations in adults and nestlings

Concentrations found in this study are, like in previous studies performed in the area (Dauwe et al., 2007; D'Hollander et al., 2014; Groffen et al., 2017, 2019c; Hoff et al., 2005; Lopez-Antia et al., 2017), among the highest ever reported in wildlife. According to previous studies (Dauwe et al., 2007; Groffen et al., 2017, 2019c; Hoff et al., 2005; Lopez-Antia et al., 2017) a pollution gradient was detected for PFOS but this decrease was not so evident for other PFAA compounds (Groffen et al., 2017, 2019c). Considering the literature on plasmatic PFAAs concentrations in birds (SI Table S9.12)

it is evident that the entire study area is influenced by the presence of the fluorochemical plant (Groffen et al., 2017, 2019c; Hoff et al., 2005).

For five of the detected compounds (PFBA, PFOA, PFDA, PFDoDA, and PFOS) concentrations found in the present study (in adults and in most cases also in nestlings) were the highest ever reported in birds' plasma. Concentrations of other four compounds (PFNA, PFUnDA, PFTrDA, and PFTeDA) were only surpassed by concentrations found in bald eagle (*Haliaeetus leucocephalus*) nestlings sampled in the upper midwest of the U.S. (Route et al., 2014), a region with several sources of PFAAs, including a 3M fluorochemical plant. PFDA concentrations in nestlings of the present study were also surpassed by those found in bald eagle nestlings (Route et al., 2014). PFAA compounds are highly bioaccumulative (especially LC ones) and, at similar exposure condition, higher concentrations would be expected in a top predator (bald eagle) compared to a small passerine (great tit).

We only found two studies that measured PFAA concentrations in blood of passerine species. In a study by Custer et al. (2012) tree swallow (*Tachycineta bicolor*) nestlings that hatched very close to a U.S. 3M fluorochemical plant were analyzed. Compared to that study, concentrations in the nestlings hatched at the Antwerp 3M plant were higher for all the compounds measured in both studies (PFOA, PFNA, PFDA, PFUnDA, PFDoDA, and PFOS). Moreover, even when comparing concentrations in tree swallows with the ones we found in the nestlings from Fort 4 (10 km away from the plant), they were higher for all the compounds but PFOS in the present study. Remarkable is the difference in PFOA concentrations, which were around 50 times higher in our study.

The second study that measured PFAAs in blood of passerine birds was by Dauwe et al. (2007) who measured PFOS concentrations in adult great tits in 2005. The study area was the same as in the current study but birds were only sampled in Vlietbos, Rot, and Burchtse Weel (concentrations ranges were 173–1625, 154–234, and 24.3–123 pg/µL respectively). Whole blood was used as the matrix. When comparing both studies we found that the mean concentrations of PFOS were higher in the present

study: 3-, 1.2-, and 1.7-fold higher at Vlietbos, Rot, and Burchtse Weel, respectively. These differences are very probably due to the different matrix used, whole blood vs plasma. It has been calculated that concentrations measured in whole blood are 2 to 5-fold lower than in plasma (Kannan et al., 2001). Taking this into account, the concentrations we found are very similar to the ones found by Dauwe et al. (2007) This, or even a decrease in the concentrations, was expected as 3M plant has phased out PFOS production since 2002. Other studies performed in the same area have detected a decrease in PFOS concentrations measured in great tit eggs (Groffen et al., 2017) from 2006 to 2011 and also in wood mice liver from 2002 to 2006 (D'Hollander et al., 2014). In other places in Europe and the U.S. the same decrease has been detected since 2000 in several bird species (Ahrens et al., 2011c; Holmström et al., 2010; Route et al., 2014; Sedlak et al., 2017).

PFAA concentrations and profiles found in the present study appear to correspond with both a high historical contamination, with high concentration of PFOS and LC-PFCA, and a recent contamination, with the presence of SC-PFCAs such as PFBA and PFHxA, all related to current fluorinated compounds production (as final compounds, degradation products, or impurities (Wang Z et al., 2013). In the future, the analytical method should be improved to increase the recoveries, and thus the detection possibilities, of SC-PFSAs in blood, including PFBS and PFHxS. Also other currently used per- and polyfluoroalkyl substances such as 3H-perfluoro-3-[(3-methoxy-propoxy)propanoic acid], ammonium salt (ADONA) or dodecafluoro-2-methylpentan-3-one (3M Novec 1230) should be included in future analyses.

A remarkable result is the higher concentrations and detection frequencies of PFUnDA and PFOS found in adult females compared with males (SI Figure S9.2), although most pronounced at the plant site, these differences were consistent at all sites and in both sampling periods. Most of the studies on PFAA concentrations in birds did not observe differences between sexes (reviewed in Sturm and Ahrens (2010)), and the ones that did always have reported higher concentrations in males (Bertolero et al., 2015; Blévin

et al., 2017; Bustnes et al., 2008; Sinclair et al., 2006). Moreover, in two studies performed previously in great tits' blood (Dauwe et al., 2007) and liver (Hoff et al., 2005) in the same area, no differences in PFOS concentrations between sexes were found. In general for PFAA compounds, as for other contaminants, females could present lower concentrations due to the excretion of these compounds through the eggs (Newsted et al., 2007). We know that female great tits actually excreted PFOS through the eggs, as very high concentrations of PFOS were detected in the eggs analyzed in this study. On the other hand, no PFUnDA concentrations were found in those eggs which can sometimes be due to low exposure and modest detection limits.

In mammals, sex differences in the elimination half-life of some PFAA compounds have been observed, and the elimination is not always faster in females (Lau et al., 2007). The reason for the differences in elimination is not well understood but some studies pointed to a hormonal regulation of the elimination (Lau et al., 2007). These sex differences could also be explained by behavioral reasons such as differences in foraging strategies (Milligan et al., 2017), or ecological reasons such a greater longevity and thus higher accumulation in females. Further research is therefore necessary to better understand the sex differences and their consequences.

9.4.2 Relationship between mothers, eggs and offspring concentrations

The distribution of PFOS in mothers, their eggs, and nestlings, and the fact that concentrations in mothers and nestlings (and to a lesser extent in eggs and nestlings) correlated with each other, are suggesting that the main exposure of nestlings to this compound is through maternal transfer and/or the diet (provided by the parents). The transfer of PFOS from females to the eggs was previously described in birds (Bertolero et al., 2015; Custer et al., 2014; Gebbink and Letcher, 2012; Lasters et al., 2019) but as far as we know this is the first study that correlates plasmatic concentrations in the mother with plasmatic concentrations in the offspring. On the other hand, for ∑PFCAs, the lack of correlation between mothers, eggs and nestlings, and even between siblings, could be indicating that maternal transfer or the diet is not the main route of exposure for these compounds. Moreover, higher concentrations of PFOA (~1.6 times)

and PFBA were found in the offspring while ∑PFCAs were higher in the mothers (SI Table S9.9, Figure S9.3). These differences in PFCAs profile could be explained in two nonexclusive ways: (1) Mothers and offspring were exposed differently during the nestling period. (2) Birds were exposed to precursor substances (e.g., fluorotelomer alcohols) and these compounds follow different biotransformation pathways in adults and nestlings (Butt et al., 2014). This second hypothesis is supported by correlations found between PFNA concentrations in the mothers and PFOA concentrations in the offspring and between LC-PFCAs (PFOA, PFDA, and PFTrDA) in the mothers and PFBA in the offspring. Future studies to be performed in this hot-spot should include the study of "precursors" together with the study of PFAAs.

9.4.3 Associations between PFAA concentrations and the oxidative status

In adult great tits, a trend existed for more exposed birds to have higher levels of protein damage (measured as protein carbonyls). This could mean that the antioxidant defenses failed in neutralizing the extra reactive oxygen species (ROS) generated because of the pollutants, and thus oxidative damage occurred. Similarly, a recent study performed in Artic black-legged kittiwakes (*Rissa tridactyla*)(Constantini et al., 2019) found that high blood levels of protein damage were associated with high plasma concentrations of certain LC-PFAA compounds (i.e., PFDoDA, PFTrDA, and PFTeDA). Additionally, they found negative associations between the nonenzymatic antioxidant capacity (i.e., vitamins, carotenoids, glutathione) of these birds and high plasma concentrations of other LC-PFAAs such as PFUnDA, PFTeDA, or PFOS. .

In nestlings, we detected a positive correlation between PFAAs load and antioxidant defenses. More exposed nestlings presented higher activity of GPX and CAT enzymes, both part of the first line of defense against ROS. Their increased activity seemed to efficiently neutralized ROS, as no changes in other endogenous antioxidants (glutathione) or oxidative damage were detected.

Due to the presumably long duration of the physiological stress (Constantini et al., 2011) and the susceptibility of early stages of life to oxidative damage (Metcalfe and

Alonso-Alvarez, 2010), detrimental effects could occur in these birds. We also must consider that, due to limitations in sample volume, we were not able to measure exogenous antioxidant concentrations (i.e., vitamins and carotenoids) or other oxidative damage parameters. Therefore, consequences for the birds are difficult to predict.

Finally, it is also important to note that the tissue we used (RBC) is not the main target of PFAAs (Lau et al., 2007), therefore, we hypothesize that the effects of oxidative damage produced by PFAAs would be more evident in other tissues (e.g., liver or adipose tissue) but this remains to be tested. A previous study performed in tree shallows nestlings from the Great Lakes did not find any association between oxidative stress parameters measured in the liver and PFAAs concentrations in the plasma (Custer et al., 2017). On the other hand, a previous study performed in wood mice living in the vicinity of the fluorochemical plant in Antwerp, found positive associations between PFOS concentrations and the level of lipid peroxidation in the liver of these mice (Hoff et al., 2004).

Our study provides evidence that OS is a possible pathway for the pernicious effects of PFAAs, however, the causal relationship has to be proven. In humans, PFOA has been recently classified by the Agency for Research on Cancer as “possibly carcinogenic”; DNA damage secondary to oxidative stress has been pointed out as the cause of this carcinogenic effect (Tsuda, 2016).

The obtained data represent an important step towards the understanding of the consequences of exposure to these compounds for wild birds at the individual and the population levels. Continuous monitoring of exposure and effects in these populations will give us longitudinal and multigenerational data, which are essential for PFAAs risk assessment.

9.5 Acknowledgements

We thank Peter Scheys and Geert Eens for their help during the fieldwork and Tim Willems for the UPLC analysis. We also thank 3M for allowing us to perform part of the sampling inside their plant. Ana Lopez Antia is a postdoctoral researcher of the Research Foundation − Flanders (FWO; 12T4118N). Hamada AbdElgawad is supported by a postdoctoral fellowship from the FWO (12U8918N). This study was supported by FWO project number 42/FA070400/20/6811. We also thank University of Antwerp for financial support. The map in Figure S9.1 was created using ArcGIS software by Esri. ArcGIS and ArcMap are the intellectual property of Esri and are used herein under license. Copyright © Esri. All rights reserved. For more information about Esri software, please visit www.esri.com.

9.6 Supporting Information

DETAILED DESCRIPTION OF THE METHODS

We provide below a more detailed description of some of the methods used in this study.PFAAs analysis in plasma. A mixture of isotopically mass-labelled internal standards (ISTDs), comprising $^{13}C_4$-PFBA, [1,2-$^{13}C_2$]PFHxA, [1,2,3,4-$^{13}C_4$]PFOA, [1,2,3,4,5-$^{13}C_5$]PFNA, [1,2-$^{13}C_2$]PFDA, [1,2-$^{13}C_2$]PFUnDA, [1,2-$^{13}C_2$]PFDoDA, $^{18}O_2$-PFHxS and [1,2,3,4-$^{13}C_4$]PFOS, were purchased by Wellington Laboratories (Guelph, Canada). HPLC grade acetonitrile (ACN; Merck Chemicals, N.V./S.A. (Millipore), Overijse, Belgium) and water (VWR International, Leuven, Belgium) were used.

Sample extraction

Egg content was transferred into a polypropylene (PP) tube and homogenized by repeatedly sonicating and vortex-mixing. Blood plasma (10 µL), or homogenized egg (~0.4g) samples were transferred into PP tubes. Hereafter, 80 µL of the previously described ISTD mixture, containing 125 pg/µL of each ISTD (in 50:50 ACN:HPLC grade water), and 10 mL ACN was added to each sample. After sonication (3x10 min), samples were left overnight on a shaking plate at room temperature. After centrifugation (4°C, 10 min, 2400 rpm, Eppendorf centrifuge 5804R) the supernatant

was transferred into a 14 mL tube and loaded on HR-XAW columns that were preconditioned and equilibrated with 5 mL ACN and 5 mL Milli-Q (MQ) water, respectively. Samples were washed with 5 mL 25 mM ammonium acetate and 2 mL ACN. Finally, samples were eluted from the SPE columns using 2 x 1 mL 2% ammonium hydroxide in ACN. The eluent was completely dried using a rotational-vacuum-concentrator at 30°C (Eppendorf concentrator 5301, Hamburg, Germany), reconstituted with 200 µL 2% ammonium hydroxide in ACN and vortex-mixed for at least 1 minute. Prior to the analysis, samples were filtrated through an Ion Chromatography Acrodisc 13 mm Syringe Filter with 0.2 µm Supor (PES) Membrane (VWR International, Leuven, Belgium) attached to a PP auto-injector vial.

UPLC-TQD analysis

To separate PFAAs, an ACQUITY BEH C18 column (2.1 x 50 mm; 1.7 µm, Waters, USA) was used. Mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B). Solvent gradients were 65% A to 0% A in 3.4 min and 65% A at 4.7 min. The injection volume was 10 µL at a flow rate of 450 µL/min, with a total run time of 6.7 min.An ACQUITY BEH C18-pre-column (2.1 x 30 mm; 1.7 µm, Waters, USA) was inserted between the solvent mixer and injector, to retain any PFAAs contamination originating from the system. Identification and quantification of individual PFAAs was based on multiple reaction monitoring (MRM) of two diagnostic transitions per analyte or ISTD.

Calibration

A constant amount of ISTD was added to varying amounts of non-labelled standards, ACN and water, to construct calibration curves. A linear regression function with a highly significant linear fit for all target analytes (all $p < 0.001$; $R^2 > 0.98$) described the relationship between the ratio of unlabelled and labelled PFAA concentrations and the ratio of the area of the unlabelled and labelled PFAAs. With exception of PFPeA, PFHpA, PFTrDA, PFTeDA, PFBS and PFDS, which were all quantified using the ISTD of

the compound closest in terms of functional group and size (individual PFAAs were quantified using their corresponding ISTD.

Quality assurance

The quality of the method was assured by regular analysis of procedural blanks (one per batch of 20 samples) and contained no contamination. The limit of quantification (LOQ) was determined, based on a signal-to-noise ratio of 10 and ranged from 1.1 to 8.2 pg/µL for all compounds with exception of PFHxS (129.2 pg/µL), PFOS (LOQ = 46.6 pg/µL) and PFPeA (52.4 pg/µL) and which had considerably higher LOQs due to high noise.

Antioxidant and oxidative stress parameters measurement in red blood cells

For the detection of molecular antioxidants in red blood cells: reduced glutathione (GSH) and oxidised glutathione (GSSG), high-performance liquid chromatography with electro-chemical detection by a reversed-phase HPLC of Shimadzu (Shimadzu, 's Hertogenbosch, The Netherlands) was used, following the protocol as described by Sinha et al. (2014). Approximately, 10 mg of RBC per sample were used. The ratio between GSH/ GSSG was used as an index of redox state with lower values indicating higher oxidative stress (Jones 2006). Activity of antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) were determined from haemolysates of red blood cells. Approximately, 10 mg of RBC were homogenized by MagNALyser (Roche, Vilvoorde, Belgium) in 250 µl of extracting buffer (pH 7.4; 1.15% KCl and 0.02 M EDTA in 0.01 M PBS). All measurements were scaled down for semi-high throughput using a micro-plate reader (Multiskan RC plate reader type 351; Synergy Mx, Biotek Instruments Inc., Vermont, USA). SOD activity was determined by measuring the inhibition of nitroblue tetrazolium (NBT) reduction at 560 nm (ε530 = 12.8 mM^{-1} cm^{-1}), following Dhindsa et al. (1981). CAT activity was measured following Aebi (1984), by monitoring the rate of decomposition of H_2O_2 (ε240 = 39.4 $M^{-1}cm^{-1}$). Activity of GPX was determined following Drotar et al. (1985) by measuring the decrease in NADPH absorbance measured at 340 nm and calculated from the 6.22

mM^{-1} cm^{-1} extinction coefficient. A modified ferric ion reducing antioxidant power (FRAP) assay was used to estimate the total antioxidant capacity (TAC) (Benzie and Strain 1996). Homogenised red blood cells were mixed with the FRAP reagent, and the absorption was measured at 600 nm after 30 min. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used as the standard.Finally, we measured protein carbonyls (marker of protein damage) in red blood cells as oxidative damage markers. We followed the procedure explained in the "Protein Carbonyl Colorimetric Assay Kit" by Cayman Chemical's (Ann Arbor, MI, USA; see also Levine et al. 1990) to measure protein carbonyl content after samples had been diluted with buffer extract to 2 mg protein ml^{-1}.

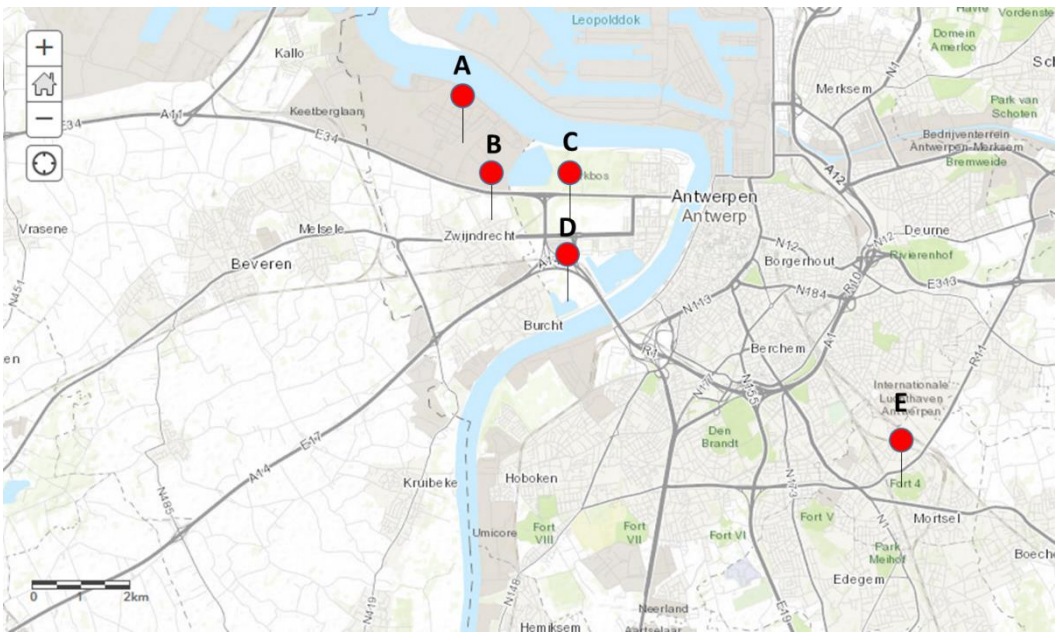

Figure S9.1. Overview of the different study sites located along a distance gradient of 11 km from an active fluorochemical plant in Antwerp (Belgium). A: Fluorochemical plant; B: Vlietbos; C: Rot; D: Burchtse Weel; E: Fort 4. (Map created using ArcGIS® software by Esri).

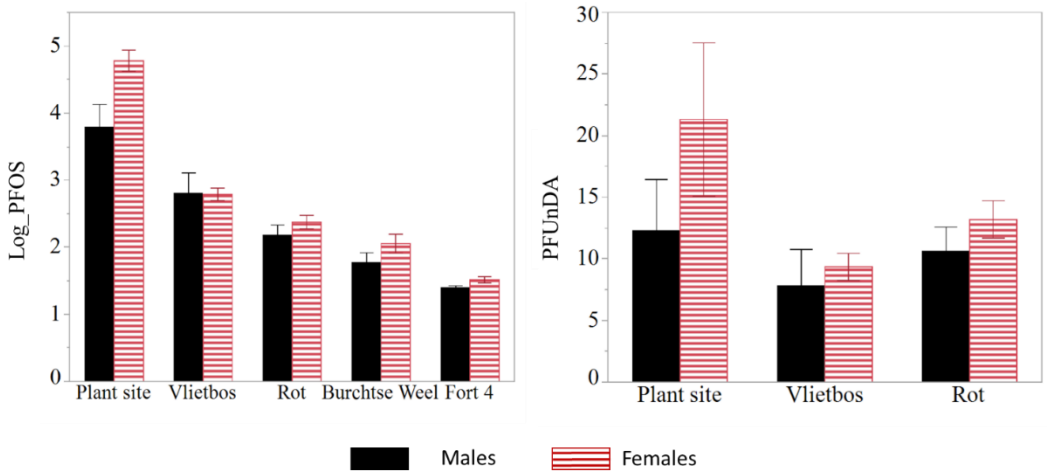

Figure S9.2. Mean concentrations (pg/µL) of PFUnDA and logPFOS (±SE) found in adult birds' plasma (temporal data were pooled together (adults from both the late winter and the spring)), at the five sampling sites, separated by sex. Sample sizes are (F/M): Plant site=16/8; Vlietbos=7/24; Rot=14/8; Burchtse Weel=12/11; Fort 4=12/19.

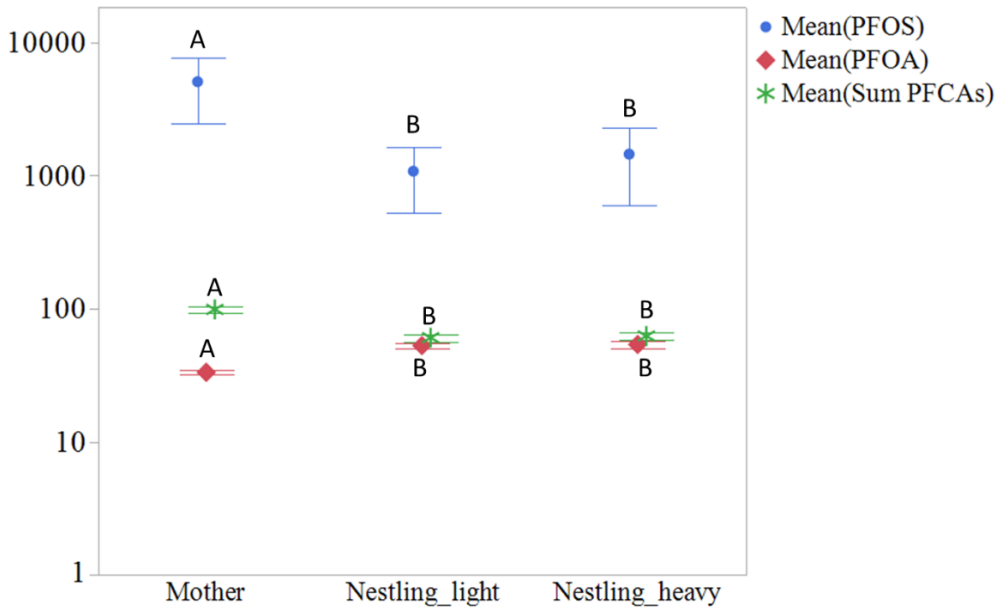

Figure S9.3. Mean concentrations (pg/µL ±SE) of PFOA, PFOS and ∑PFCAs found in the blood of the mother and the offspring (the lightest and the heaviest nestlings in the nest, n=40 nests). Different letters indicate significantly different concentrations between different sample types.

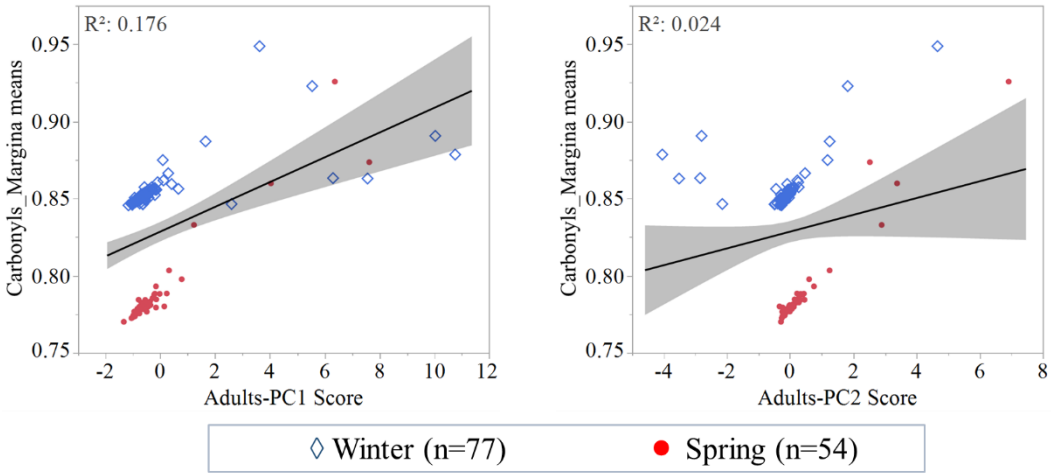

Figure S9.4. Relationship between Adults-PC1 and Adults-PC2 and protein carbonyl (marginal means as obtained in the mix model when considering season as a factor and ring number as random effect) content in blood of adult birds sampled in winter and spring. Regression lines are shown with 95% confidence bands shaded.

Table S9.1 Target PFAA compounds (11 perfluoroalkyl carboxylic acids and 4 perfluoralkyl sulfonic acids), chemical formula and their acronyms (the used abbreviations for PFAA compounds are according to Buck et al. 2011)

Family	Compound	Formula	Acronym
Perfluoroalkyl carboxylic acids $C_nF_{2n+1}COOH$ (PFCAs)	Perfluorobutanoic acid	C_3F_7COOH	PFBA
	Perfluoropentanoic acid	C_4F_9COOH	PFPeA
	Perfluorohexanoic acid	$C_5F_{11}COOH$	PFHxA
	Perfluoroheptanoic acid	$C_6F_{13}COOH$	PFHpA
	Perfluorooctanoic acid	$C_7F_{15}COOH$	PFOA
	Perfluorononanoic acid	$C_8F_{17}COOH$	PFNA
	Perfluorodecanoic acid	$C_9F_{19}COOH$	PFDA
	Perfluoroundecanoic acid	$C_{10}F_{21}COOH$	PFUnDA
	Perfluorododecanoic acid	$C_{11}F_{23}COOH$	PFDoDA
	Perfluorotridecanoic acid	$C_{12}F_{25}COOH$	PFTrDA
	Perfluorotetradecanoic acid	$C_{13}F_{27}COOH$	PTeDA
Perfluoroalkyl sulfonic acids $C_nF_{2n}+SO_3H$ (PFSAs)	Perfluorobutane sulfonic acid	$C_4F_9SO_3H$	PFBS
	Perfluorohexane sulfonic acid	$C_6F_{13}SO_3H$	PFHxS
	Perfluorooctane sulfonic acid	$C_8F_{17}SO_3H$	PFOS
	Perfluorodecane sulfonic acid	$C_{10}F_{21}SO_3H$	PFDS

Table S9.2 Limits of quantification (LOQ: pg/µL), mean and median concentrations (pg/µL), range and prevalence (%) of PFAAs in plasma of adult great tits sampled at the five sampling sites. Different letters indicates significantly different prevalences among locations. =

		PFCAs						PFSAs
		PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTrDA	PFOS
LOQ		2.6	4.1	5.5	6.4	1.8	1.4	46.6
Detection frequency		99	26.7	24.1	47.8	62.9	32.7	71.7
Plant site (n =24)	Median	75.1	12.8	5.57	9.99	15.7	5.60	20168
	Mean	94.9^{A*}	21.8	89.2	17.7	23.1^{A}	8.54	43428^{A}
	Range	<LOQ -244	<LOQ – 81.0	<LOQ - 477	<LOQ – 57.2	<LOQ – 122	<LOQ – 40.0	<LOQ - 161333
	Freq	95	70 ^{a}	50	50 ^{ab}	75	55	100 ^{a}
Vlietbos (n =31)	Median	40.0	<LOQ	<LOQ	8.15	3.38	<LOQ	488
	Mean	44.8^{B}	<LOQ	<LOQ	8.92	4.16^{B}	<LOQ	1780^{B}
	Range	25.5 – 94.7	<LOQ - 23.9	<LOQ - 19.1	<LOQ - 24.5	<LOQ – 17.4	<LOQ - 3.17	65.4 - 21139
	Freq	100	17 ^{b}	28	62 ^{a}	76	38	100 ^{a}
Rot (n=22)	Median	41.1	<LOQ	<LOQ	10.3	3.44	<LOQ	178
	Mean	41.5	<LOQ	<LOQ	11.2	3.46^{B}	<LOQ	260^{BC}
	Range	28.8 - 69.1	<LOQ - 11.7	<LOQ - 11.0	<LOQ – 22.8	<LOQ – 8.25	<LOQ - 4.18	<LOQ - 1182
	Freq	100	20 ^{b}	20	70 ^{a}	70	35	84 ^{ab}
Burchtse Weel (n =23)	Median	40.2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	59.6
	Mean	47.1^{B}	<LOQ	<LOQ	<LOQ	<LOQ	1.56	118^{D}
	Range	18.4 - 104	<LOQ - 8.36	<LOQ - 20.4	<LOQ - 22.1	<LOQ - 9.15	<LOQ - 9.95	<LOQ - 657
	Freq	100	15 ^{b}	15	32 ^{b}	45	25	60 ^{b}
Fort 4 (n=31)	Median	41.3	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Mean	44.0^{B}	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Range	7.63 - 75.4	<LOQ - 12.2	<LOQ - 10.5	<LOQ - 27.4	<LOQ - 9.87	<LOQ - 6.20	<LOQ - 91.22
	Freq	100	18 ^{b}	11	26 ^{b}	48	15	25 c

Table S9.3 Limits of quantification (LOQ: pg/µL) and detection frequencies (%) of PFAA compounds rarely found (less than 20% of the samples above the LOQ) in plasma of adult great tits sampled at the five sampling sites.

		PFCAs			
		PFBA	PFPeA	PFHxA	PFTeDA
LOQ		6.5	52.4	8.2	1.3
Plant site	Freq	12.5	0	0	8.3
Vlietbos	Freq	0	22.6	9.7	3.2
Rot	Freq	0	22.7	4.5	4.5
Burchtse Weel	Freq	0	4.3	0	4.3
Fort 4	Freq	6.4	6.4	0	6.4

Table S9.4 Limits of quantification (LOQ: pg/µL) and detection frequencies (%) of PFAA compounds rarely found (less than 20% of the samples above the LOQ) in plasma of adult great tits sampled at the five sampling sites.

	Adults		Nestlings
	PC1	PC2	PC1
PFBA			**0.738**
PFOA	**0.758**	-0.136	**0.856**
PFNA	**0.841**	-0.419	
PFDA	**0.858**	-0.355	
PFUnDA	**0.756**	0.185	
PFDoDA	**0.751**	0.586	**0.855**
PFTrDA	0.555	**0.763**	
PFOS	**0.933**	-0.260	**0.950**
Variance explained			
Proportion	61.91	19.26	72.76
Cumulative		81.17	

Table S9.5 Coefficient (r) and probability (p) of the correlations found between different PFAA compounds (those with detection frequency ≥ 50% in each site) at the five sampling sites in the plasma samples of the adults.

		Plant site (n = 24)		Vlietbos (n = 31)		Rot (n = 22)		Burchtse Weel (n = 23)		Fort 4 (n = 31)	
		r	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**
PFOA	**PFNA**	0.83	**<.0001**								
	PFDA	0.55	**0.005**								
	PFUnDA	0.27	0.197	0.03	0.851	0.32	0.144				
	PFDoDA	0.46	**0.027**	-0.19	0.322	0.33	0.130				
	PFTrDA	0.16	0.451								
	PFOS	0.58	**0.003**	-0.13	0.488	0.20	0.373	-0.50	**0.015**		
PFNA	**PFDA**	0.65	**0.001**								
	PFUnDA	0.49	**0.018**								
	PFDoDA	0.44	**0.041**								
	PFTrDA	0.16	0.475								
	PFOS	0.84	**<.0001**								
PFDA	**PFUnDA**	0.49	**0.014**								
	PFDoDA	0.53	**0.009**								
	PFTrDA	0.25	0.252								
	PFOS	0.74	**<.0001**								
PFUnDA	**PFDoDA**	0.60	**0.002**	-0.21	0.266	0.66	**<.001**				
	PFTrDA	0.47	**0.022**								
	PFOS	0.55	**0.005**	0.05	0.806	0.37	0.0880				
PFDoDA	**PFTrDA**	0.81	**<.0001**								
	PFOS	0.70	**<.001**	-0.05	0.786	0.56	**0.006**				
PFTrDA	**PFOS**	0.50	**0.014**								

Table S9.6 Limits of quantification (LOQ: pg/µL), mean and median concentrations (pg/µL), range (pg/µL) and detection frequencies (%) of most frequently found PFAA compounds (PFCAs; perfluoroalkyl carboxylic acids. PFSAs; perfluoroalkyl sulfonic acids) in plasma of great tits nestlings (14 days old) at the five sampling sites. Different upper case letters indicate significantly different mean concentrations among locations. Different lower case letters indicate significantly different detection frequencies between locations.

		PFCAs			PFSAs
		PFBA	PFOA	PFDoDA	PFOS
LOQ		6.5	2.6	1.8	46.6
Plant site (n =38nestlings /14 nests)	Median	16.4	93.3	10.2	17137
	Mean	24.1	139^{A}	12.2	14514^{A}
	Range	<LOQ - 112	32.1 - 438.7	<LOQ - 49.8	612 -35624
	Freq	60.5^{a}	100	80.5^{a}	100^{a}
Vlietbos (n =47nestlings /18 nests)	Median	<LOQ	48.4	<LOQ	123
	Mean	<LOQ	50.1^{B}	1.9	464^{B}
	Range	<LOQ -10.8	20.2 - 81.0	<LOQ -9.39	<LOQ -3292
	Freq	10.4^{b}	100	19.1^{b}	91.3^{a}
Rot (n=22nestlings / 10 nests)	Median		54.1	<LOQ	47.3
	Mean		52.9^{B}	<LOQ	68.3^{C}
	Range	All <LOQ	27.8 - 83.2	<LOQ - 7.74	<LOQ - 198
	Freq	0^{c}	100	30.4^{b}	52.2^{b}
Burchtse Weel (n =35nestlings / 14 nests)	Median	<LOQ	46.5	<LOQ	<LOQ
	Mean	<LOQ	49.1^{B}	<LOQ	<LOQ
	Range	<LOQ - 7.16	23.9 - 83.4	<LOQ - 7.58	<LOD - 247
	Freq	5.7^{bc}	100	14.3^{b}	28.6^{b}
Fort 4 (n=28nestlings /14 nests)	Median	<LOQ	51.6	<LOQ	<LOQ
	Mean	<LOQ	50.3^{B}	<LOQ	<LOQ
	Range	<LOQ - 8.36	26.2 - 72.9	<LOQ - 10.9	<LOQ - 138
	Freq	17.9^{b}	100	25^{b}	7.14^{c}

Table S9.7 Coefficient (r) and probability (p) of the correlations found between different PFAA compounds (those with detection frequency ≥ 50% in each site) at the five sampling sites in the plasma samples of the nestlings.

		Plant site (n = 38)		Vlietbos (n = 47)		Rot (n = 22)		Burchtse Weel (n = 35)		Fort 4 (n = 28)	
		r	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**
PFBA	**PFOA**	0.38	**0.020**								
	PFDoDA	0.37	**0.030**								
	PFOS	0.62	**<.0001**								
PFOA	**PFDoDA**	0.71	**<.0001**								
	PFOS	0.67	**<.0001**	0.47	**<.001**	0.0984	0.6630				
PFDoDA	**PFOS**	0.73	**<.0001**								

Table S9.8 Limits of quantification (LOQ: pg/µL) and detection frequencies (%) of PFAA compounds rarely found (detection frequency < 20%) in plasma of great tit nestlings at the five sampling sites.

		PFCAs				
		PFNA	PFDA	PFUnDA	PFTrDA	PFTeDA
LOQ		4.1	5.5	6.4	1.4	1.1
Plant site (n =38nestlings /14 nests)	Freq	39	10	0	21	5
Vlietbos (n =47nestlings /18 nests)	Freq	0	4	4	2	2
Rot (n=22nestlings / 10 nests)	Freq	0	0	4	0	0
Burchtse Weel (n =35nestlings / 14 nests)	Freq	0	6	3	3	0
Fort 4 (n=28nestlings /14 nests)	Freq	4	7	11	4	0

Table S9.9 ∑PFAAs, ∑PFCAs, PFOA, PFDoDA and PFOS mean (± SE) concentrations in mothers, eggs and both nestlings (the lightest and the heaviest in the nest; mean ± SE) at the five sampling sites (n=40 nests)

		Location				
		Plant site	**Vlietbos**	**Rot**	**Burchtse Weel**	**Fort 4**
Mother (pg/µL)	∑PFAAs	43902 ± 16891	1391 ± 323	530 ± 153	354 ± 60	130 ± 12
	∑PFCAs	145 ± 22	102 ± 7.3	90.0 ± 3.5	77.4 ± 4.1	87.2 ±8.0
	PFOA	44.8 ± 3.4	33.1 ± 1.6	36.3 ± 3.0	27.5 ± 2.3	30.2 ± 3.0
	PFDoDA	23.5 ± 10	3.6 ±0.6	3.5 ±0.4	5.0 ±1.3	4.6 ±1.0
	PFOS	43757 ± 16870	1289 ± 324	410 ± 156	276 ±62	43 ± 8.0
Egg (ng/g)	∑PFAAs	81032 ± 38056	908 ± 253	363 ± 104	89.8 ± 9.0	41.7 ± 8.1
	∑PFCAs	171 ± 67	14.2 ± 3.1	9.7 ± 1.8	6.8 ± 1.1	9.5 ± 2.2
	PFOA	18.2 ± 2.1	1.3 ± 0.2	1.2 ± 0.2	1.1 ± 0.3	1.0 ± 0.1
	PFDoDA	57.5 ± 26	<LOD	1.7 ± 0.3	<LOD	2.0 ± 0.7
	PFOS	80231 ± 37684	890 ± 251	351 ± 104	80.0 ± 9.1	29.2 ± 6.0
Chick light (pg/µL)	∑PFAAs	8517 ± 3980	464 ± 208	128 ± 37	94 ± 8.7	90 ± 15
	∑PFCAs	115 ± 12	55.6 ± 4.0	55.2 ± 8.2	50.7 ± 3.2	52.5 ± 4.8
	PFOA	88.0 ± 4.1	50.2 ± 3.8	49.2 ± 7.2	44.5 ± 3.0	48.0 ± 5.1
	PFDoDA	7.3 ± 3.7	<LOD	2.8 ± 1.1	<LOD	<LOD
	PFOS	11203 ± 3997	409 ± 206	73 ± 42	<LOD	<LOD
Chick heavy (pg/µL)	∑PFAAs	12419 ± 6509	372 ± 146	84 ± 15	123 ± 22	82 ± 7.2
	∑PFCAs	115 ± 27	54.7 ± 3.5	52.3 ± 10.4	62.3 ± 4.9	55.3 ± 6.4
	PFOA	85.2 ± 19	48.3 ± 3.2	45.9 ± 9.1	57.4 ± 4.8	49.2 ± 5.8
	PFDoDA	6.8 ± 3.0	<LOD	<LOD	<LOD	<LOD
	PFOS	16406 ± 7113	336 ± 151	<LOD	60 ± 18	<LOD

Table S9.10 Mean (± SE) values of body condition and oxidative stress biomarkers (in red blood cells) in adult great tits at the five sampling sites. Different letters indicate significant differences between locations at the p < 0.05 level according to Tukey test results.

	Location				
	Plant site	**Vlietbos**	**Rot**	**Burchtse Weel**	**Fort 4**
Body condition	17.6 ± 0.3	17.0 ±0.2	17.2 ± 0.2	17.7 ± 0.2	17.4 ± 0.2
TAC	10.5 ± 0.5A	10.9 ± 0.5A	10.7 ± 0.33A	10.9 ± 0.5A	8.8 ± 0.5B
GPX	0.29 ± 0.03BC	0.38 ± 0.02AB	0.30 ± 0.02BC	0.46 ± 0.04A	0.27 ± 0.03C
SOD	0.82 ± 0.09	0.81 ± 0.12	0.98 ± 0.29	0.83 ± 0.11	1.14 ± 0.12
Protein carbonyls	7.71 ± 0.21A	5.90 ± 0.22C	7.87 ± 0.16A	6.11 ± 0.28BC	7.05 ± 0.29AB
CAT	13.2 ± 1.1	15.6 ± 1.2	14.6 ± 1.40	19.9 ± 2.2	12.7 ± 1.30
GSH	1.25 ± 0.18AB	0.80 ± 0.16B	1.36 ± 0.17A	1.37 ± 0.23AB	0.76 ± 0.11AB
GSSG	0.57 ±0.07A	0.99 ± 0.12B	0.74 ± 0.09AB	1.19 ± 0.33AB	0.55 ± 0.08A
GSH/GSSG ratio	3.36 ±1.08A	0.73 ± 0.13B	3.28 ± 0.87A	1.70 ± 0.52AB	2.43 ± 0.55A

Body condition (Scaled mass index);TAC (Total antioxidant capacity (µmol trolox/g)); GPX (glutathione peroxidase (µmol NADPH/mg protein)); SOD(Superoxide dismutase (U/mg protein)); Protein carbonyls (nmol/mg protein); CAT (catalase (µmol H_2O_2/mg protein)); GSH (Reduced and total glutathione (µmol/g)); GSSG (oxidized glutathione (µmol/g))..

Table S9.11 Mean (± SE) values of body condition and oxidative stress biomarkers (in red blood cells) in nestlings at the five sampling sites. Different letters indicate significant differences between locations at the p < 0.05 level according to Tukey test results.

	Location				
	Plant site	**Vlietbos**	**Rot**	**Burchtse Weel**	**Fort 4**
Body condition	15.6 ± 0.3	16.6 ± 0.2	15.63 ± 0.4	15.9 ± 0.3	15.0 ± 0.3
TAC	5.33 ± 0.60AB	8.18 ± 0.65A	3.87 ± 0.54B	6.06 ± 0.47AB	7.74 ± 0.60A
GPX	0.18 ± 0.01AB	0.21 ± 0.01A	0.14 ± 0.01B	0.17 ± 0.01AB	0.19 ± 0.01AB
SOD	0.66 ± 0.05A	0.45 ± 0.03B	0.54 ± 0.03AB	0.53 ± 0.07AB	0.68 ± 0.07A
Protein carbonyls	6.60 ± 0.29AB	7.91 ± 0.28A	5.84 ± 0.37B	7.39 ± 0.36A	6.81 ± 0.38AB
CAT	7.65 ± 0.64	7.64 ± 0.43	6.33 ± 0.54	6.59 ± 0.53	6.05 ± 0.65
GSH	1.15 ± 0.11	1.23 ± 0.09	1.02 ± 0.15	1.37 ± 01.7	1.39 ± 0.11
GSSG	1.05 ± 0.09	1.15 ± 0.09	0.84 ± 0.08	1.02 ± 0.13	0.98 ± 0.07
GSH/GSSG ratio	1.37 ± 0.22	1.45 ± 0.18	1.75 ± 0.54	2.64 ± 0.80	1.57 ± 0.15

Body condition (Scaled mass index);TAC (Total antioxidant capacity (µmol trolox/g)); GPX (glutathione peroxidase (µmol NADPH/mg protein)); SOD(Superoxide dismutase (U/mg protein)); Protein carbonyls (nmol/mg protein); CAT (catalase (µmol H_2O_2/mg protein)); GSH (Reduced and total glutathione (µmol/g)); GSSG (oxidized glutathione (µmol/g)).

Table S9.12 PFAA concentration (range; pg/µL) measured in plasma of different bird species around the world. < Concentrations below the LOQ.

Species	Place	Year	PFCAs								REF
			PFBA	PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTrDA	PFTeDA	
Great tit Adults	Belgium	2015	<-133	<-232	<–81	<-477	<–57	<–61	<-25	<-2.4	Current
Great tit nestlings			<-112	20-438	<–19	<–15	<–25	<–50	<–12	<–6.7	
Bald eagle	USA	90s									Giesy and Kannan, 2001
Albatrosses	North Pacific Ocean	1992-1996		0.1-0.30							Tao et al., 2006
†Carrion crow	Japan	2002									Taniyasu et al., 2003
Glaucous gull	Norwegian Arctic	2004		0.70-0.74	2.3-6.3	3.1-15		2.9-24	3.6-30		Verreault et al., 2005
*†Black-backed gull	Norway	2005						1.2±0.08	2.4±0.17		Bustnes et al., 2008
†Griffon vulture	Israel	2007		1.4–3.5							Shlosberg et al., 2011
Tree swallow	Minnesota	2008-2009		2.1–3.5	1.8–7.6	3.4–13		0.7-4.3			Custer et al., 2012
Bald eagle nestlings	Midwestern USA	2006-2011		<–15	0.3-160	0.1–85	1.3-110	0.04–33	0.13–63	<-310	Route et al., 2014
Kittiwakes	Svalbard	2012	<-78	0.03–0.12	0.8–3.0	1.3–2.8		1.5-4.0	4.5-29.7		Tartu et al., 2014b
*Kittiwakes	Svalbard	2012-2014			1.2±0.1	2.2 ± 0.1	12± 0.6	2.5± 0.1	11 ± 1.4		Blévin et al., 2017
†Calonectris shearwaters	Mediterranean and Atlantic	2014					0.9–9.3	0.2-3.8	0.1-2.5		Escoruela et al., 2018
*Kittiwakes	Svalbard	2016			2.0±0.9	2.9±1.2	10.3±3.7	1.7±0.8	8.6±3.1	1.0±0.8	Constantini et al., 2019

Table S9.12 (continued) PFAA concentration (range; pg/µL) measured in plasma of different bird species around the world. < Concentrations below the LOQ.

Species	Place	Year	PFSA			REF
			PFBS	PFHxS	PFOS	
Great tit Adults Great tit Nestlings	Belgium	2015			<-161333 <-35624	Current
Bald Eagle	USA	90s			1-2570	Giesy and Kannan, 2001
Albatrosses	North Pacific Ocean	1992-1996			0.310-13.4	Tao et al., 2006
†Carrion Crow	Japan	2002	<45		68-1200	Taniyasu et al., 2003
Glaucous Gull	Norwegian Arctic	2004		0.3-2.7	48-349	Verreault et al., 2005
*†Black-backed Gull	Norway	2005		1.0±0.06	37±2.7	Bustnes et al., 2008
†Griffon vulture	Israel	2007			2.2–7.4	Shlosberg et al., 2011
Tree swallow	Minnesota	2008-2009		4.5–19.2	75-190	Custer et al., 2012
Bald eagle nestlings	Midwestern USA	2006-2011	<-4100	<–47	6.6-4200	Route et al., 2014
Kittiwakes	Svalbard	2012		0.01-0.22	6.8–14	Tartu et al., 2014b
*Kittiwakes	Svalbard	2012-2014			11±0.6	Blévin et al., 2017
†Calonectris shearwaters	Mediterranean and Atlantic	2014			3.2 - 53	Escoruela et al., 2018
*Kittiwakes	Svalbard	2016			13±6.2	Constantini et al., 2019

*Mean concentrations ± standard error. † Measured in whole blood (concentrations expressed in plasma would be 2 to 5-fold higher (Kannan et al. 2001))

10. General discussion and future perspectives

10.1 Summary of PFAA studies near Antwerp

Over the past 15 years, numerous studies have been conducted in the vicinity of the 3M plant site or in Flanders on PFAAs. The present studies were a part of a long-term study on the distribution and potential effects of PFAAs near a hotspot. In Table 10.1 an overview is given on the field studies conducted in the SPHERE lab that focused on the terrestrial environment.

Table 10.1. Overview of studies conducted on the terrestrial environment near Antwerp.

Location(s)	No. of investigated analytes	Investigated species	Matrix / tissue / sample type	Reference
Surface water				
Blokkersdijk, Galgenweel	1			D'Hollander et al. (2014)
Soil				
Blokkersdijk, Galgenweel	1			D'Hollander et al. (2014)
3M, Vlietbos, Rot, Burchtse Weel, Fort IV	15			Groffen et al. (2019b)
				Groffen et al. (2019d)
Plants/fruits				
Blokkersdijk, Galgenweel	1	Common Blackberry	Fruit	D'Hollander et al. (2014)
		European Elder		
Invertebrates				
Blokkersdijk, Galgenweel	1	Earthworms		D'Hollander et al. (2014)
		Slugs		
		Millipedes		
		Isopods		
3M, Vlietbos, Rot, Burchtse Weel, Fort IV	15			Groffen et al. (2019b)

Table 10.1 (continued). Overview of studies conducted on the terrestrial environment near Antwerp.

Location(s)	No. of analytes	Investigated species	Matrix / tissue / sample type	Reference
Birds				
Not specified	4	Grey Heron	Liver	Meyer et al. (2009)
			Tail feather	
			Spleen	
		Herring Gull	Liver	
			Tail feather	
		Eurasian Sparrowhawk	Liver	
			Tail feather	
		Eurasian Magpie	Liver	
			Tail feather	
		Eurasian Collared Dove	Liver	
			Tail feather	
Not specified	1	Northern Lapwing	Eggs	Lopez-Antia et al. (2017)
Zandvliet	1	Mediterranean Gull	Liver	
			Blood plasma	
Blokkersdijk, Fort IV	1	Blue Tit	Liver	Hoff et al. (2005)
		Great Tit	Liver	
Vlietbos, Rot, Burchtse Weel	1			Dauwe et al. (2007)
Vlietbos, Burchtse Weel			Eggs	Lopez-Antia et al. (2017)
3M, Vlietbos, Rot, Tessenderlo	12			Groffen et al. (2017)
3M, Vlietbos, Rot, Burchtse Weel, Fort IV	15			Groffen et al. (2019b)
				Groffen et al. (2019c)
Fort IV	15			Lasters et al. (2019)
Vlietbos, Rot, Burchtse Weel	1		Blood plasma	Dauwe et al. (2007)
Vlietbos, Burchtse Weel	1			Lopez-Antia et al. (2017)
3M, Vlietbos, Rot, Burchtse Weel, Fort IV	15			Lopez-Antia et al. (2019)

Table 10.1 (continued). Overview of studies conducted on the terrestrial environment near Antwerp.

Location(s)	No. of investigated analytes	Investigated species	Matrix / tissue / sample type	Reference
Mammals				
Blokkersdijk, Galgenweel	6	Wood mice	Liver	Hoff et al. (2004)
	1			D'Hollander et al. (2014)
			Spleen	
			Pancreas	
			Lungs	
			Kidneys	
Not specified	8	Human	Cord blood plasma	Roosens et al. (2010)
			Serum	
			Milk	
Blokkersdijk	1	Bank vole	Liver	D'Hollander et al. (2014)

Over the past years the number of investigated analytes has increased, most likely caused by further development of extraction protocols and the fact that nowadays more internal standards are commercially present. However, there are most likely much more PFAS, which might be of risk, still left undetermined in the environment around 3M. The evaluation of these PFAS, for example by using the Total Oxidisable Precursor (TOP) Assay, may offer a clearer view of the total amount of PFAS present in the samples and is therefore highly recommended for future studies.

10.2 Detection and quantification of PFAAs in environmental and biological matrices

Regulatory actions have been taken to restrict the use of some PFAAs. For example, the production of specific long-chained PFAAs have been phased-out or banned in several countries (Groffen et al., 2017; Kim and Oh, 2017) and some compounds have been included to the Stockholm Convention on POPs in 2009 (Groffen et al., 2017). As a result of these regulatory measures, environmental concentrations of these PFAAs are often decreasing, whereas those of other PFAAs are still rising (Ahrens et al., 2011c,

Groffen et al., 2017, Miller et al., 2015). Therefore, it remains important to continue the environmental monitoring of PFAAs.

For the determination of PFAAs in different environmental and biological matrices, numerous methods exist. However, most of these analytical studies target only one specific matrix (e.g. Holm et al., 2004; Kim and Oh, 2017; Mazzoni et al., 2015) or focus on either biotic or abiotic samples (e.g. Lorenzo et al., 2015; Powley et al., 2005). Most environmental studies, on the other hand, cover a wide range of matrices, highlighting the need for an extraction procedure that works on both abiotic and biotic samples (Nakayama et al., in press). Furthermore, there is a need to facilitate the analysis of PFASs exposure of wildlife and humans, including non- or less-invasive biological samples (Nakayama et al., in press). Finally, internal standards might not be available for all target analytes. Concentrations of analytes that do not have their own ISTD, were previously quantified using the ISTD closest in terms of both functional group and carbon chain length (e.g. the ISTD of PFOS was used to quantify PFDS and the ISTD of PFBA was used to quantify PFPeA; Groffen et al., 2017, 2018, Leat et al., 2012, Verreault et al., 2005). However, it was still unclear whether these ISTDs were indeed the most suitable for the quantification of the target analytes.

The present study clearly described an extraction protocol that appeared to be suitable for both biological and environmental samples, although some differences among matrices exist. The method was specific and highly accurate, even at low recoveries. We confirmed that the ISTD closest in terms of functional group and carbon chain length is most suitable for the quantification of analytes that have no corresponding ISTD. The low recoveries can be explained by the use of too much matrix, as we confirmed that, in terms of the effect of sample mass on recoveries, less is more, as lower sample masses significantly increased the recoveries of the method.

It should, however, be noted that ISTDs of the compounds used in this study are commercially available, but, in order to reduce the costs and to validate both previous as well as recent studies using the ISTD of an other PFAA compound in the

quantification of specific PFAAs, we decided to use an ISTD mixture. Although the results of these compounds are still reliable (as was shown by the high accuracy for these compounds), using their own ISTD should be preferred as this will limit the differences in behaviour caused by differences in physicochemical properties. This might also explain why concentrations were sometimes lower than the spiked concentrations as this was mainly the case at compounds that were quantified using an other ISTD.

However, some significant differences in recoveries existed between the newly developed method and a frequently used method by Powley et al. (2005). However, the accuracy of both methods was similar. Therefore, both methods can be used simultaneously in studies that target both abiotic and biological samples. The method developed in the current study appeared to be more suitable for abiotic samples, whereas Powley's method was more suitable for biological matrices. The Powley et al. (2005) method was developed for abiotic matrices. It was therefore expected that the recoveries would be better for abiotic matrices, which was not the case according to our results. Powley's method uses activated carbon powder in the clean up step, which is known to sorb organic compounds. Although speculative, we argue that in samples that contain lower concentrations of organic compounds (including lipids, etc.), the PFAAs will likely sorb to the carbon powder, resulting in a lower recovery. Samples that contain a lot of lipids, proteins and other organic compounds may saturate the carbon powder, causing PFAAs not to sorb (or to a lesser extent), hence resulting in higher recoveries. This might explain why Powley's method appeared to work better on biotic matrices compared to abiotic matrices.

It should also be noticed that our results might not be so optimal in terms of recoveries, due to the conditions we performed the extractions in. All samples were extracted at neutral pH and a slight difference in pH might change the solubility of certain compounds (Van Leeuwen et al., 2006). Different PFAAs might behave differently in certain matrices and therefore method development studies should focus either on

groups of PFAAs that behave similar, or on finding the best suitable compromise between different groups.

New matrices, that have not been tested before, or have been tested only sporadically (e.g. feathers), should be tested using multiple methods, to determine which is the most suitable. To continue on the example of feathers, we tested both the method described in Chapter 2, the method by Powley et al. (2005), as well as the method used by Jaspers et al. (2013) and none of these appeared to work as ISTDs were often absent after the extractions. As a result, we had to develop a new extraction protocol for feathers.

10.3 PFAAs in soils and terrestrial invertebrates

10.3.1 Distribution and behaviour in soils

The behaviour and accumulation of PFAAs in soils has been reported before (e.g. D'Hollander et al., 2014; Lu et al., 2018; Wang et al., 2010). Nevertheless, there are still many uncertainties on how soil physicochemical properties might influence the behaviour and sorption of PFAAs to soils. Soil has not been studied often around the hotspot in Antwerp and therefore it was interesting to not only monitor the soil concentrations, but also investigate the behaviour and distribution of PFAAs within the soil.

Our studies from chapters 3 and 4 show that multiple PFAAs, including both PFCAs and PFSAs, accumulate in the soil. The concentrations at the plant site were low or intermediate compared to literature and decreased with increasing distance from the 3M site in chapter 4, whereas there was no gradient observed in chapter 3. These differences might be the result of differences in sampling strategy as well as soil physicochemical properties. In the study of chapter 4, soil samples were collected in the vicinity of nestboxes that were distributed across the study sites, whereas in chapter 3 the soil samples were collected much closer to each other. This resulted in a lower TOC at 3M and a higher TOC at Rot in chapter 3 compared to chapter 4. Consequently, this affected the sorption of PFAAs and hence the PFAA concentrations

at 3M were lower in chapter 3 compared to chapter 4, whereas at Rot the concentrations were higher.

We also observed a clear difference between long- and short-chained PFAAs in terms of detection frequencies. In general, the PFAA profiles in soils were dominated by long-chained compounds whereas short-chained ones were often not detected. Due to the high solubility of short-chained compounds, they are likely flushed out to the groundwater by rainwater percolation. Long-chained compounds are less soluble and will hence sorb more strongly to the soil particles. Therefore, it would be interesting for future studies to also investigate the groundwater concentrations to determine which PFAAs are present and hence might accumulate in organisms that rely on this groundwater, such as plants.

We did observe some correlations between PFAA concentrations and soil physicochemical properties, including TOC, clay content, pH and temperature. Although generally the associations between PFAA concentrations and TOC were stronger than those with other properties, our results confirm that the sorption behaviour of PFAAs cannot be predicted from a single soil property. It is also still unclear how different soil properties interact to determine the binding of PFAAs to soils and future studies should pay more attention to these interactions. Furthermore, our results show a downward migration of PFAAs in the soil, which might result in a potential contamination of the groundwater and consequentially pose a potential threat for wildlife and humans who depend on this groundwater for e.g. consumption. This downward migration was different for PFOA and PFOS as the concentrations of PFOA 'peaked' deeper down in the soil than for PFOS. This can be explained by differences in solubility between both compounds as PFOA has a slightly higher solubility (Eschauzier et al., 2013). Therefore, in future studies differences in physicochemical properties of the PFAAs should also be taken into account.

Although unexpected, we did not observe any significant relationship between PFAA concentrations and soil microbial parameters, which could be the result of a low

sensitivity of the microbial communities to PFAAs, but also of the sampling period. Microbial activities are often lower in autumn (Yao et al., 2011) due to low temperatures and sampling in the summer might change the outcomes of our results. Furthermore, microbial activity is influenced by many environmental factors (e.g. Li L et al., 2018), which were not all assessed in our study. It is therefore recommended to test the effects of PFAAs on microbial communities in controlled environments, or to include more sampling sites.

10.3.2 Accumulation in invertebrates

Together with the new insights on PFAAs contamination in soils near the 3M fluorochemical plant, this thesis also investigated the accumulation in terrestrial invertebrates and their potential role as bioindicator for PFAAs contamination in soils and songbird eggs. More specifically, isopods were selected as target organisms, due to their presence in the vicinity of the nestboxes, used to monitor the great tits, at all the sampling sites.

The PFAA concentrations were mainly high in the direct surroundings of the 3M fluorochemical plant and were often higher than those reported in literature (with exception of Zhu and Kannan et al. (2019), who reported higher PFOA, PFNA, PFDA, PFUnDA and PFDoDA concentrations in earthworms near a fluorochemical manufacturing facility in China). Multiple studies, including some in this thesis, have reported a higher bioaccumulation potential for long-chained compounds in animals (e.g. Groffen et al., 2017; Müller et al., 2011; Zhao et al., 2013), which is contradictory to our findings that PFPeA, a short-chained PFCA, was dominant in isopods. This contradiction could be explained by the diet of isopods in combination with the physicochemical properties of the investigated PFAAs. As a result of their high water solubility and low adsorption potential, short-chain PFAAs are flushed out into the groundwater, where they can be taken up by plants in which they will mainly accumulate in leaves and fruits (Blaine et al., 2013; Felizeter et al., 2012, 2014). Terrestrial isopods feed primarily on plant litter, which might explain the uptake and dominance of short-chained PFAAs such as PFPeA. These results show that PFAAs

accumulate in the terrestrial foodchain. PFAAs are known to biomagnify, resulting in even higher concentrations in organisms at higher trophic levels. Although isopods are not a part of the natural diet of great tits, the concentrations in great tits discussed in this thesis were much higher than those in isopods, validating the biomagnification potential of PFAAs. The accumulation and biomagnification in the terrestrial foodchain might pose a risk regarding human exposure, for example when consuming vegetables.

Additionally, our results show that isopods may serve as bioindicator for soil and to some extent also for great tit eggs. However, as we only found a few correlations between PFAA concentrations in isopods and songbird eggs, we believe that other invertebrate species, which are a natural part of their diet, such as caterpillars, will be better indicators for the PFAA concentrations in the eggs. When these types of experiments are repeated in the future, a better option would be to further intensify the sampling campaign and really look for species that are a part of the natural diet of great tits. Furthermore, there is a need for future studies, on both the distribution as well as the toxicity of PFAAs in terrestrial invertebrates, to also target other invertebrate groups or species, as studies on potential effects of PFAAs on terrestrial invertebrates are scarce and often only target worm species (e.g. Das et al., 2015; Zhao et al., 2013; Zhao Y et al., 2017).

10.4 PFAAs in songbirds

10.4.1 Accumulation, maternal transfer and variation within clutches

When the exposure and bioaccumulation of PFAAs to birds is studied, it is crucial to understand the different exposure pathways and bioavailability. Our results show that PFAAs accumulate in multiple bird tissues, including blood plasma, eggs and tail feathers. The concentrations in the direct surroundings of the 3M fluorochemical plant were (among) the highest ever reported in wild birds in all these matrices. Although we did not specifically test for different exposure pathways, it is often suggested that the exposure occurs mainly through diet (D'Hollander et al., 2015). However, as diet is often dependent on environmental (such as vegetation) and behavioural factors (e.g.

foraging behaviour and diet selection), it is important to also have detailed information on as many factors as possible.

Besides the diet, the maternal transfer of PFAAs can be considered an important exposure pathway for nestlings (e.g. Bertolero et al., 2015; Custer et al., 2014; Gebbink and Letcher, 2012; Lasters et al. 2019) and might result in detrimental effects on survival and development of the chicks. The distribution of PFOS in mothers, their eggs, and nestlings, and the fact that concentrations in mothers and nestlings (and to a lesser extent in eggs and nestlings) correlated with each other, are suggesting that the main exposure of nestlings to this compound is through maternal transfer and/or the diet (provided by the parents). On the other hand, for ∑PFCAs, the lack of correlation between mothers, eggs and nestlings, and even between siblings, could be indicating that maternal transfer or the diet is not the main route of exposure for these compounds. Possible explanations are that mothers and offspring were exposed differently during the nesting period, or that precursor substances follow different biotransformation pathways in adults and nestlings. Therefore, it would be interesting to investigate the presence and distribution of precursor compounds together with the target PFAAs in future studies performed in this hotspot.

Finally, PFAAs may enter the body by ingestion of dust and dirt particles (Haug et al., 2011a; Goosey and Harrad, 2011) or by inhalation of PFAAs in air (Haug et al., 2011b; Huber et al., 2011; Langer et al., 2011). The uptake of PFOS and PFOA from air was considered negligible in comparison to the dietary uptake in humans (EFSA, 2008). To the best of our knowledge, no studies are present on the PFAA exposure via air or dust in birds. However, a review by Sanderfoot and Holloway (2017) on the impact of inhalation exposure on avian species, reported that adverse health impacts on birds have been attributed to the exposure to gas-phase and air pollutants, including carbon monoxide (CO), oxone (O_3), sulfur dioxide (SO_2) and metals. Therefore, air pollution should be considered as a potential exposure route for PFAAs in birds.

Within clutches, the PFAA concentrations generally decreased, most likely caused by differences in resources used in the production of the eggs. It is likely that the mother uses maternal resources for the first eggs and dietary resources, with lower PFAA concentrations, for the later eggs. These variations in PFAA concentrations within a clutch should be taken into account when conducting a similar experiment in the future. Depending on the research question, random eggs or fixed eggs can be sampled, resulting in different concentrations. For example, sampling the first egg will often give the maximum egg concentration in that clutch and consequentially at that location, whereas sampling a random egg will result in a more average concentration per site.The differences in PFAA accumulation between individuals are hence not only depending on the locations in terms of distance to the PFAAs source, but also on the major routes of exposure and the degree of exposure, as well as on environmental and behavioural factors at these locations.

10.4.2 Sequestration

Organisms often use sequestration mechanisms to detoxify pollutants and prevent interactions with biomolecules. Birds are known to deposit environmental pollutants, such as metals in their feathers (García-Fernández et al., 2013; Jaspers et al., 2006; Løseth et al., 2019) and females can also sequester pollutants in their eggs.

Our results show that multiple PFAAs were detected in the eggs and feathers of great tits. More specifically, our results demonstrate that great tit mothers deposit PFOS concentrations in their eggs. As PFOS concentrations in the eggs decreased with laying order, it is likely that the internal PFOS concentrations in the mother also decreased during laying. Unfortunately, we only collected data from the mothers once during the breeding season and future studies should therefore focus on temporal variations in PFAA concentrations in the mothers throughout the egg-laying period.

Elevated PFAA concentrations were detected in feathers and these concentrations were only related to those in blood plasma for a few PFAAs. The feathers and blood where both collected during the winter, and it is possible that PFAA concentrations

circulating in the blood at that time were different from those that were available at the time of feather formation, resulting in a lack of correlations for most PFAAs. Therefore, it would be ideal to sample feathers that were recently grown or still connected to the blood circulation. In addition, the removal of feathers induces the formation of new feathers which can be collected around 6 – 7 weeks after the removal, when they are fully grown (Jaspers et al., 2004). In this case the collection of feathers can be standardized and individual differences due to different exposure times might be limited. Furthermore, we did not distinguish between internally accumulated and externally deposited PFAAs in feathers, as we did not observe any PFAA contamination of chicken feathers (of which we were certain that they contained PFAAs) collected in the close proximity of the 3M site. It is possible that our washing solvent (methanol) was not ideal to dissolve the external contamination and only washed away the dust rather than the preen oil on the feathers. Therefore, other washing solvents, for example hexane, should be tested. However, even with a specific washing protocol it will always remain unclear whether all external contamination has been removed and secondly, whether no internally accumulated PFAAs have been extracted during the washing.

10.4.3 Effects

When animals are exposed to high environmental PFAA concentrations, health effects can be expected. Nevertheless, we observed only limited reproductive impairment and limited effects on oxidative status at PFAA concentrations that were (among) the highest ever reported in wild bird eggs and plasma. Although it appears, based on these results, that PFAAs are not harmful for great tits, these results of the thesis should be treated carefully. Since we only collected data during one breeding season, it is possible that environmental conditions (e.g. food availability) were very favourable for breeding during that year. Furthermore, our results show that the breeding success at the reference site, Fort IV was rather low. This could (partially) be explained by the degree of urbanization or other differences between study sites, for example in food availability. The main factor in the urban environment is chemical pollution and many

of these pollutants may affect reproduction. Urban areas usually have a lower diversity of trees and it has been shown that urban trees of birch and oak produce less carotenoids (Isaksson, 2009). Carotenoids are important nutrients for birds and a lack of carotenoids has been shown to affect breeding success as clutch sizes were smaller and fledging success was reduced (Blount et al., 2002; Ewen et al., 2009). Urbanization might also affect the reproductive timing and mating behaviours as birds originating from the city developed their reproductive system earlier than those form dark forest areas (Dominoni et al., 2013). Additionally, many urban air pollutants may cause oxidative damage, so taking the degree of urbanization into account may also result in a better reflection of the actual risks of PFAAs in these areas. Therefore, future studies on the potential toxicity of PFAAs on reproduction should 1) be conducted during multiple years, 2) take as many environmental factors, such as food availability, degree of urbanization and disturbance through light or noise into account and 3) try to limit the differences in these factors between sampling sites.

Furthermore, it is possible that the great tits are not sensitive to PFAAs pollution or, although very speculative, that great tits adapted to the PFAAs pollution. Species-specific differences in sensitivity to other POPs have been reported between chickens and free-living wild birds (Karchner et al., 2006). Additionally, Nordén et al. (2016) reported that toxic effects of PFOS and PFOA were higher in white leghorn chicken compared to herring gull and great cormorant after *in ovo* injection. Regarding the adaptation, no studies have been reported before on PFAAs specifically, but fast trait changes in response to changing environmental factors, including pollutants, have been reported (Marzluff et al., 2016). Hence, future studies should also investigate differences in sensitivity between species to determine whether there is an impact of the high degree of pollution at the fluorochemical plant on the terrestrial ecosystem.

10.5 General conclusions

The outcomes of this thesis revealed new insights on the distribution of PFAAs in the terrestrial environment near a fluorochemical plant. During this current study, we investigated four main hypotheses.

Hypothesis 1: PFAAs present in the environment along a pollution gradient accumulate in the terrestrial foodchain and decrease with increasing distance from a fluorochemical hotspot.

Perfluoroalkylated acids were detected in multiple environmental and biological matrices, including soil, isopods, songbird eggs and feathers, indicating that PFAAs present in the environment accumulate in biota. In almost all of the reported studies in this thesis, the PFAA concentrations decreased sharply with increasing distance from the 3M fluorochemical plant, indicating that this plant is a point source of PFAAs to the environment around Antwerp. In most studies, the concentrations detected in the environmental or biological matrices were (among) the highest ever reported in those matrices. Therefore, the 3M fluorochemical plant in Belgium can still be mentioned as a PFAAs-hotspot, despite the phase-out of long-chained PFAS such as PFOS and PFOA, in the early 2000s.

Hypothesis 2: Soil physicochemical properties play a key role in the sorption, distribution and bioavailability of PFAS

The complexity of the PFAA chemistry resulted in many uncertainties on how various soil physicochemical properties interact to determine the binding of PFAAs to soils. Our results suggested that PFAA concentrations in the soils are associated with multiple soil physicochemical properties, including TOC, clay content, pH and temperature. The major contributor to the sorption of PFAAs in the soil was the TOC, as it was most strongly correlated with PFAA concentrations. However, as this was mainly the case for long-chained PFAAs, the physicochemical properties of the PFAAs

also play a large role in explaining their sorption and hence distribution and bioavailability.

The results of the vertical distribution of PFAAs suggested that the PFAAs distribution in soils is also influenced by soil physicochemical properties, as concentrations appeared to migrate through the soil depending on its properties, and that this downward migration of PFAAS should be considered when studying soil concentrations as surface layer concentrations are not necessarily representative of the soil concentrations.

Hypothesis 3: Non-destructive sampling can be used to monitor environmental PFAA concentrations

In addition to internal PFAA concentrations in the songbirds, we investigated the potential use of non-destructive samples, such as eggs and feathers. Our results show that eggs can be used to determine PFAA concentrations in the blood of adult great tits, as PFAA concentrations in the eggs were related to those in the blood of the mother and nestlings. Similarly, we found that tail feather PFOS concentrations were correlated with those in blood plasma, showing that tail feathers can be useful to estimate PFOSconcentrations in blood of great tits. Furthermore, as more compounds were observed in feathers than in blood plasma, I argue that feathers give a more accurate overview of the total exposure to PFAAs in great tits. Overall, feathers are likely useful in the biomonitoring of PFAAs in birds. Nevertheless, the eggs and feathers were collected invasively during my thesis and more research is necessary to investigate the possibility of using unhatched eggs and shed feathers in biomonitoring.

Hypothesis 4: Accumulated concentrations of PFAAs under field condition in songbirds are related to toxic effects

Perfluoroalkylated acids are known to cause detrimental effects on wildlife. As the environmental concentrations at the study sites were often among the highest ever reported in wild birds, it was expected that these concentrations would result in severe effects on reproduction and oxidative stress in great tits. Although our studies suggest some effects of PFAAs on reproduction (e.g. reduced hatching success, eggshell thinning, reduced breeding success and earlier breeding) and oxidative stress (e.g. protein damage and higher activity of antioxidant enzymes), the effects were rather limited despite the high, accumulated, concentrations. The outcomes of these studies can potentially be explained by a lower sensitivity of great tits to PFAAs compared to other songbird species. However, it should be noted that we only investigated the effects on reproduction once and reproduction is influenced by numerous environmental factors. Furthermore, environmental factors could also play a role in the oxidative stress and therefore, laboratory experiments under controlled conditions should be performed to really examine the impact of PFAA pollution on these parameters. Nevertheless, the potential effects were minor despite the very high accumulated concentrations.

11. Nederlandstalige samenvatting

11.1 Probleemstelling

Humane activiteiten hebben de omgeving op verschillende manieren beïnvloed. Klimaatverandering, ontbossing, de verhoogde emissie van antropogene chemicaliën in het milieu en andere activiteiten hebben ernstige gevolgen voor het milieu (Zalasiewicz et al., 2015). Tijdens de laatste eeuw hebben ontwikkelingen in de chemische industrie geleid tot een verhoogde productie van antropogene chemische producten, zoals polychloorbifenyl ethers (PCBs), polygebromeerde difenylethers (PBDEs) en perfluoralkaanzuren (PFAAs). Deze PFAAs zijn gedurende de laatste 60 jaar geproduceerd en gebruikt voor diverse toepassingen en eindproducten, resulterend in een globale pollutie van het milieu en organismen, inclusief mensen (e.g. Giesy en Kannan, 2001, 2002; Houde et al., 2006; Miller et al., 2015). Ondanks dat zowel de wetenschappelijke als publieke bezorgdheid omtrent PFAAs toeneemt en deze stoffen ook frequenter onderzocht worden, is er nog altijd weinig gekend over hoe PFAAs zich gedragen in het milieu en welke schade ze daar aanrichten. Bovendien focussen de meeste wetenschappelijke studies zich op het aquatisch milieu, terwijl data over het terrestrisch milieu vaak ontbreekt. Zo is er bijvoorbeeld nog weinig gekend over PFAAs in bodems (concentraties, sorptie mechanismen en gedrag in de bodem) en biota. Daarom is het van essentieel belang om PFAAs te bestuderen in het terrestrische milieu, inclusief de mogelijke effecten die ze daar veroorzaken op biota.

11.2 Doelstelling en hypothesen

Het gebrek aan kennis over de concentraties en het gedrag van PFAAs in bodems en de daaruit volgende accumulatie in terrestrische invertebraten en vogels, inclusief de mogelijke effecten op reproductie en oxidative status, hebben geleid tot de doelstelling van dit onderzoek.

Het voornaamste doel was om de blootstelling van terrestrische invertebraten (pissebedden) en zangvogels (koolmezen) aan PFAAs te bestuderen langsheen een afstandsgradiënt van een fluorochemische fabriek. Bovendien onderzochten we de accumulatie en mogelijke effecten op reproductie en oxidative status in de vogels. Om te voorkomen dat de vogels opgeofferd moesten worden, lag de focus van dit onderzoek op eieren, bloed plasma en veren.

Om de verschillende manieren van blootstelling van invertebraten en zangvogels beter te begrijpen, hebben we de transfer onderzocht tussen bodem en invertebraten. Daarnaast hebben we gekeken naar de invloed van fysicochemische bodemeigenschappen op de sorptie van PFAAs in de bodem en de mogelijkheid om een invertebraten soort te gebruiken als bio-indicator voor PFAA concentraties in eieren van koolmezen. Tenslotte hebben we de variatie van PFAA concentraties met legvolgorde bestudeerd in volledige nesten van koolmezen, langsheen de afstandsgradient.

Om de verschillende doelstellingen van dit onderzoek te realiseren, werden vier hypothesen geformuleerd (Tabel 11.1).

Tabel 11.1 De vier hypothesen van het onderzoek en de hoofdstukken van de Nederlandstalige samenvatting waarin ze besproken worden.

Nr.	Hypothese	Hoofdstuk
1	PFAAs in het milieu accumuleren in de terrestrische voedselketen en de concentraties nemen af met toenemende afstand van een fluorochemische hotspot	11.5.1, 11.6 & 11.7.1
2	Fysicochemische bodem eigenschappen spelen een rol in de sorptie, distributie en beschikbaarheid van PFAAs	11.5.2
3	Niet-destructieve weefsels kunnen gebruikt worden om PFAA concentraties in het milieu en in organismen te monitoren	11.7.1
4	Geaccumuleerde PFAA concentraties zijn gerelateerd aan toxische effecten in mezen in veldomstandigheden	11.7.3

11.3 Studiegebied

Dit onderzoek vond plaats in verschillende gebieden, langsheen een afstandsgradiënt started bij een actieve fluorochemische fabriek (3M) in Antwerpen. De locaties zijn weergegeven in Figuur 11.1.

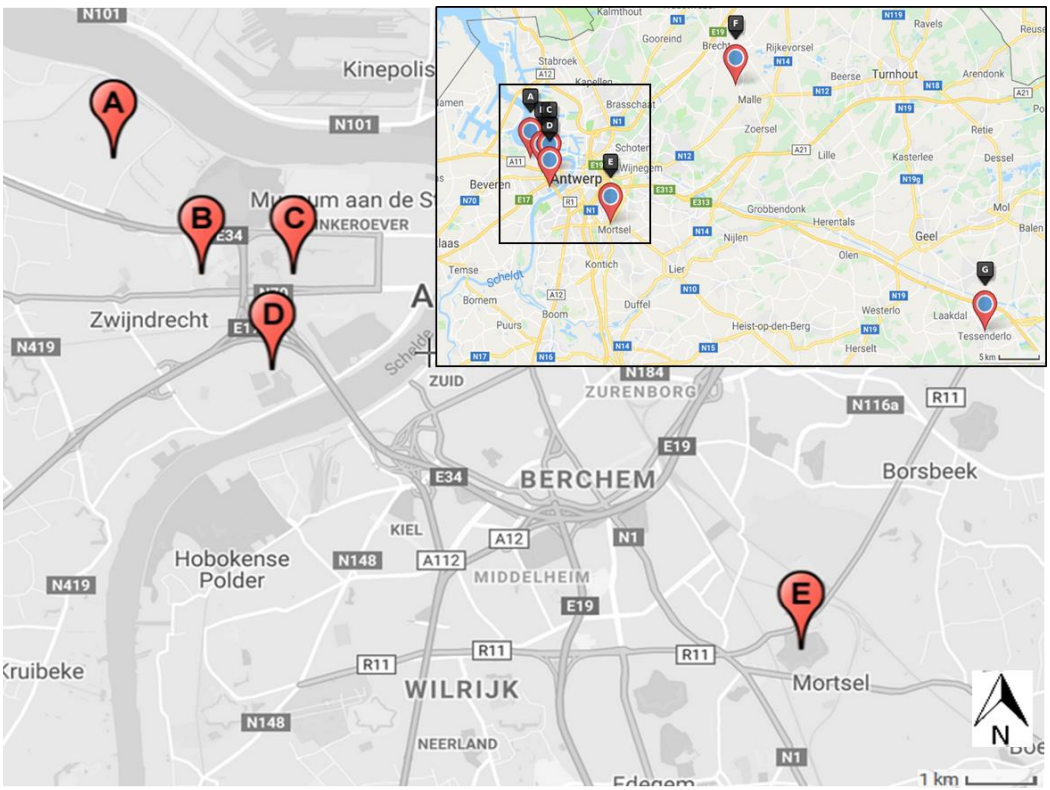

Figure 11.1 Overzicht van het studiegebied van dit onderzoek. A = 3M; B = Vlietbos; C = Middenvijver-Rot; D = Burchtse Weel; E = Fort IV; F = Westmalle; G = Tessenderlo. Map is gemaakt met Google Maps.

3M in Antwerpen is het studiegebied geweest voor diverse onderzoeken naar PFAAs in biota (Dauwe et al., 2007; D'Hollander et al., 2014; Hoff et al., 2005; Lopez-Antia et al., 2017) en is een bekende PFAAs-hotspot. De concentraties gedetecteerd in de diverse biologische matrixen zijn een van de hoogste ooit gemeten in biota. De 3M site bevat zowel de fabriek (voornamelijk het westelijke deel) en een klein bosachtig gebied met wat open zandvlaktes in het oosten. In het zuiden is er een klein bos tussen

3M en de E34 snelweg. De vegetatie is gedomineerd door loofbos maar er zijn ook een aantal coniferen langs de randen van het terrein.

Vlietbos ligt ongeveer 1 km ten zuid-oosten van 3M en is een zandig gebied dat diverse open vlakten (twee kleine meren en droge zandvlakten) en beboste gebieden bevat. De bossen zijn voornamelijk gedomineerd door berk (*Betula sp.*) en wilg (*Salix sp.*). In het westelijke deel van Vlietbos groeit voornamelijk Canadese populier (*Populus x canadensis*).

Middenvijver-Rot (in het kort Rot) is een gebied dat verbonden is met Vlietbos en dat gekarateriseerd wordt door open meertjes, wilgen en een mix van zowel loof- als naaldbos. De bodem is voornamelijk zandig, hoewel sommige delen meer kleiig zijn. Het gebied is gelegen op ongeveer 2.3 km oost-zuid-oost van 3M.

Op ongeveer 3 km ten zuidoosten van 3M ligt Burchtse Weel, een gebied dat gevormd is door een dijkdoorbraak. De plas is daarna vergroot en verdiept en tegenwoordig is het een overstromingsgebied en recreatievijver. De randen van de plas worden gekarakteriseerd door loofbos, gedomineerd door wilg, eik (*Quercus sp.*) en els (*Alnus sp.*).

Fort 4 in Mortsel ligt op ongeveer 11 km van 3M en is één van de acht forten die een gordel vormen rond Antwerpen. Het wordt volledig omgeven door water en de vegetatie wordt gedomineerd door loofbos of open grasland, wat gebruikt wordt voor recreatie. Een klein stuk van het fort heeft als bestemming natuurgebied.

Als referentiegebieden zijn Tessenderlo en Westmalle gekozen. Dit zijn steden/dorpen op ongeveer 70 km (zuidoost) en 25 km (noordoost) afstand van 3M. Het oostelijke deel van Tessenderlo is een erg geïndustrialiseerd gebied, waar diverse chemische fabrieken staan. Tessenderlo is gekozen, omdat het in dezelfde windrichting ligt als de gradient A-E. Westmalle is daarentegen omgeven door voornamelijk landbouwgronden. In Westmalle is gekozen voor een biologisch landbouwbedrijf waar

tijdens testen geen PFAA contaminatie was gedetecteerd in kippeneieren. Op basis daarvan verwachtte ik geen of weinig pollutie in bijvoorbeeld de bodem.

11.4 Detectie van PFAAs in biotische en abiotische matrixen (Groffen et al., 2019a)

Gedurende de laatste decennia lag de focus van onderzoekers en beleidsmakers vooral op perfluoroalkyl carboxyl zuren (PFCAs) en perfluoroalkyl sulfonzuren (PFSAs) met lange koolstofketens (>6), vanwege hun hogere bio-accumulatieve potentie. Binnen deze groepen lag de voornaamste focus op perfluorooctaanzuur (PFOA) en perfluorooctaan sulfonzuur (PFOS). Als gevolg van hun toxiciteit en globale verspreiding (Giesy en Kannan, 2001), is de productie en het gebruik van PFAAs met lange keten verboden of gelimiteerd in verschillende landen. Ondanks deze beleidsmaatregelen zijn de concentraties van deze en van andere PFAAs de laatste jaren aan het toenemen (Ahrens et al., 2011c; Groffen et al., 2017). Dit geeft het belang aan om door te gaan met het monitoren van PFAAs in het milieu.

Momenteel bestaan er diverse methoden om PFAS te kwantificeren in verschillende biologische en abiotische matrices, met verschillende manieren om te corrigeren voor verliezen in rendement en elke met hun eigen plus- en minpunten. Wij hebben getracht om een nieuwe methode te ontwikkelen die toegepast kan worden op zowel biotische als abiotische stalen. De methode is specifiek, selectief, lineair, robuust en gevoelig. Zelfs na 6 dagen, na de extractie, konden nog betrouwbare resultaten verkregen worden. De rendementen varieerden, afhankelijk van de matrix, tussen de 1% en 100%, maar desondanks was er een zeer hoge nauwkeurigheid, zelfs bij de laagste rendementen. Een afname van de massa van het staal zou de rendementen significant kunnen verbeteren en het is daarom ook aangeraden om minder matrix te gebruiken. In veel studies werd de interne standaard (ISTD) gebruikt van die component die het meest overeenkwam in termen van functionele groep en koolstofketen lengte om PFAAs te kwantificeren die geen eigen interne standaard hebben. Wij hebben bevestigd dat dit de meest geschikte methode is voor deze PFAAs. Uiteraard zou het beter zijn om de eigen interne standaard te gebruiken indien deze

beschikbaar zijn. Dit beperkt de fysicochemische verschillen tussen de verschillende componenten en ik verwacht daarom dat dit resulteert in betrouwbaardere resultaten en hogere rendementen. De nieuwe methode is, afhankelijk van de matrix, vergelijkbaar, in termen van gevoeligheid en betrouwbaarheid, met een veel gebruikte methode en zou tegelijkertijd gebruikt kunnen worden in monitoring studies. Daarom raden we aan om de extractiemethode te selecteren op basis van zowel de matrix als de doel componenten.

11.5 PFAAs in de bodem

Bodemvervuiling kan op verschillende manieren gebeuren. Zo kan vervuiling, aanwezig in de lucht of in het water, zich afzetten in de toplaag van de bodem en kunnen bodems gecontamineerd worden door het begraven van toxische substanties om zo schadelijke effecten te voorkomen (Fedotov et al., 2018; Lang et al., 2017). Als gevolg van het dagelijks gebruik van PFAAs en PFAAs-bevattende producten, kan de bodem ook vervuild worden door lekkage vanuit industrie of stortplaatsen, maar ook door afvalwater (Gallen et al., 2018; Xiao et al., 2015). Bodemverontreiniging kan langdurige effecten veroorzaken. In tegenstelling tot bijvoorbeeld waterverontreiniging, waarbij de natuurlijke stroom de toxische substanties verdund en verspreid, kunnen bodempolluenten gedurende een lange tijd in de bodem blijven, waarmee ze een risico vormen voor verschillende generaties (Mapanda et al., 2005; Xiao et al., 2015). Door regen en oppervlakte-afstroming kunnen bodempolluenten in rivieren of aquifers terechtkomen, wat mogelijk resulteert in een verspreiding over een groter gebied, maar ook in een mogelijk gezondheidsrisico voor consumenten van dit water.

De volgende paragrafen zijn een samenvatting van Groffen et al. (2019b) en Groffen et al. (2019d) en hoofdstukken 3 en 4 van deze thesis.

11.5.1 Verspreiding in de bodem

Tijdens deze studies werden bodemstalen verzameld langsheen een afstandsgradiënt van de 3M fluorochemische fabriek in Antwerpen. In de eerste studie (staalname in 2016) werd enkel de bovenlaag verzameld in de directe nabijheid (max. 3 m) van nestkasten die gebruikt werden om kool- en pimpelmezen te monitoren. In de tweede studie (staalname in 2018) werden bodemstalen verzameld op verschillende dieptes om zo de verticale profielen te kunnen onderzoeken en de invloed van fysicochemische bodemeigenschappen na te gaan op deze profielen. Het studiegebied bestond in beide studies uit 3M, Vlietbos, Middenvijver-Rot en Burchtse Weel. Als referentiegebied werd in de eerste studie gekozen voor Fort 4 in Mortsel en in de tweede studie voor een biologisch landbouwbedrijf in Westmalle.

Voor de meeste PFAAs namen de concentraties in de bovenlaag af met toenemende afstand van 3M. De gemiddelde PFOS concentraties in de toplaag bij 3M (1700 ng/g dw) waren in 2016 veel hoger dan deze gemeten in een eerdere studie in een gebied ongeveer 0.5 km van 3M (D'Hollander et al., 2014). De concentraties waren in 2016 overwegend hoger dan deze gerapporteerd in literatuur wereldwijd, wat aangeeft dat 3M een echte hotspot is voor PFAAs vervuiling. In 2018 lagen de concentraties in de toplaag een stuk lager, wat het gevolg kan zijn van een andere plek van staalname (net buiten het terrein van 3M). De PFOA en PFOS concentraties namen toe in een onderlaag (tot 50 cm), waarna ze weer afnamen. Dit suggereert een neerwaartse migratie in de bodem en dus een mogelijk risico voor de vervuiling van het grondwater en dus ook voor organismen die gebruik maken van dit grondwater.

11.5.2 Invloed van fysicochemische bodemeigenschappen

In beide jaren waren de PFAA concentraties in de bodem beïnvloed door fysicochemische bodemeigenschappen zoals organisch koolstofgehalte (TOC; totaal organisch koolstof), kleigehalte, pH en temperatuur. Zo waren de PFAA concentraties positief gecorreleerd met TOC, kleigehalte en temperatuur. Desondanks waren de PFOS concentraties in 2018 negatief gerelateerd aan de temperatuur. De positive verbanden met TOC en korrelgrootte waren logisch, aangezien organisch koolstof

gehalte een van de meest belangrijke sorbenten is voor PFAAs in bodems (Milinovic et al., 2015) als gevolg van elektrostatische en hydrofobe interacties tussen PFAAs en diverse functionele groepen in de bodem (Higgins en Luthy, 2007). Bodems met kleinere partikels, zoals klei, hebben meer functionele groepen (zoals hydroxyl en carboxyl groepen) en daardoor dus ook meer bindingsplaatsen voor polluenten (Qi et al., 2014). Bodemtemperatuur heeft een effect op de sorptie van o.a. PFOS op humuszuur, een belangrijk onderdeel van TOC (Jia et al., 2010).

11.5.3 Associaties met microbiële bodemparameters

In 2018 hebben we in de toplaag diverse microbiële bodemparameters bepaald, zoals bodemrespiratie, microbiële biomassa en microbiële activiteit. Geen enkele van deze parameters was gerelateerd aan de PFAA concentraties. Dit was tegenstrijdig met onze verwachting, aangezien PFAAs een stimulans of remming kunnen zijn voor de groei van bepaalde bacteriën (Qiao et al., 2018). Microbiële activiteit en respiratie worden echter beïnvloed door diverse omgevingsfactoren en seizoenale variatie in respiratie en activiteit zijn eerder gerapporteerd voor turfgras systemen, waarbij een lagere microbiële biomassa en activiteit in september waren geassocieerd met een lagere beschikbaarheid van stikstof in de bodem (Yao et al., 2011).

11.6 PFAAs in terrestrische invertebraten

Invertebraten zijn in diverse veldstudies gebruikt om PFAA concentraties te monitoren. Desondanks zijn het merendeel van deze studies uitgevoerd op aquatische invertebraten (bijv. Babut et al., 2017; Groffen et al., 2018; Lescord et al., 2015; Loi et al., 2011) en data over terrestrische invertebraten is nog erg schaars. Diverse laboratorium onderzoeken zijn uitgevoerd op wormen (bijv. Das et al., 2015; Zhao et al., 2013; Zhao Y et al., 2017), terwijl er slechts enkele veldstudies op terrestrische invertebraten zijn uitgevoerd (bijv. D'Hollander et al., 2014; Lesch et al., 2017; Zhu en Kannan, 2019). Bovendien zijn de relaties tussen PFAA concentraties in bodems en invertebraten en de invloed van fysicochemische eigenschappen op deze relaties nauwelijks onderzocht (Das et al., 2015). Tenslotte is er nog weinig gekend over de trofische transfer van bodem via invertebraten naar uiteindelijk vertebraten.

In juni 2016 werden bodemstalen (dezelfde als vermeld in 11.5.1) en pissebedden verzameld in de directe omgeving (max. 3 m) van nestkasten die gebruikt werden om de reproductie van kool- en pimpelmezen te monitoren (hoofdstuk 11.7). De stalen werden verzameld in 3M, Vlietbos, Middenvijver-Rot, Burchtse Weel en Fort 4. Enkel PFOS was detecteerd in meer dan 50% van de pissebed stalen in alle gebieden. De PFOS concentraties waren significant hoger bij 3M vergeleken met de overige gebieden en leken af te nemen met afstand van 3M (m.u.v. Rot, waar de concentraties lager waren dan op Burchtse Weel). De PFOS concentraties in pissebedden bij 3M (185 ng/g) waren lager in de huidige studie vergeleken met een eerdere studie bij Blokkersdijk (ongeveer 0.5 km van 3M), waar concentraties van 497 ng/g waren gedetecteerd (D'Hollander et al., 2014). Dit kan het gevolg zijn van de uitfasering van PFOS en gerelateerde producten door 3M in 2002. De concentraties waren, met uitzondering van perfluoropentaanzuur (PFPeA), lager dan deze in wormen in een historisch vervuild gebied in Ohio, VS (Zhu en Kannan, 2019). De PFOS concentraties in pissebedden waren positief gerelateerd aan deze in bodems als alle locaties samen werden genomen en enkel op 3M als we keken naar individuele locaties. Bovendien vonden we bewijs dat andere PFAA concentraties in pissebedden ook gerelateerd waren aan deze in bodem. Dit was te verwachten, aangezien pissebedden blootgesteld zijn aan bodems en bodems daardoor gezien kunnen worden als een belangrijke bron van PFAAs opname in deze invertebraten. De PFAA concentraties in pissebedden waren enkel gerelateerd aan deze in koolmeeseieren op 3M en Rot.

11.7 PFAAs in zangvogels

Vogels kunnen een belangrijke rol spelen als bio-indicator voor milieuvervuiling. Ze zijn relatief gemakkelijk om te observeren en één van de best bestudeerde groepen van organismen. Het is gekend dat vogels toxische chemicaliën accumuleren (bijv. Giesy en Kannan, 2001; Holmström et al., 2005; Yoo et al., 2008), wat resulteert in diverse effecten op fysiologie en reproductie (bijv. Custer et al., 2012, 2014) en zelfs kan leiden tot de dood. Milieuvervuiling is één van de bedreigingen van vogelpopulaties. Vogels zijn frequent gebruikt in studies die de accumulatie van pesticiden en metalen

onderzoeken in (vaak niet-invasieve of niet-destructieve) stalen (bijv. Jaspers et al., 2004, 2006, 2007a,b,2009,2011; Løseth et al., 2019; Rattner et al., 2008; Svendsen et al., 2018; Van den Steen et al., 2006). Vogels worden daarom ook vaak gebruikt in hedendaagse monitoring programma's waarbij het doel is om temporele en spatiële trends in chemische pollutie in zowel het terrestrisch als het aquatisch milieu aan te tonen.

De volgende paragrafen zijn een samenvatting van Groffen et al. (2017, 2019c, *subm.*), Lasters et al. (2019) en Lopez-Antia et al. (2019), of hoofdstukken 5 – 9 van deze thesis.

11.7.1 Accumulatie in zangvogels

In dit onderzoek zijn matrices geselecteerd waarvoor de vogels niet opgeofferd dienden te worden. Het gaat hierbij om eieren, bloed plasma en veren.

11.7.1.1 Eieren

Vogeleieren zijn al diverse keren gebruikt om PFAAs the monitoren in diverse regio's van de wereld (bijv. Gebbink en Letcher, 2012; Giesy en Kannan, 2001; Holmström et al., 2005; Miller et al., 2015; Yoo et al., 2008). Echter zijn het merendeel van deze studies uitgevoerd op aquatische vogels en is informatie over terrestrische vogels, en specifieker zangvogels, schaars (Ahrens et al., 2011c; Custer et al., 2012; Holmström et al., 2010; Rüdel et al., 2011; Yoo et al., 2008).

Tijdens de winter van 2011 zijn eieren van koolmezen (één willekeurig ei per nest) verzameld in 3M, Vlietbos, Rot en Tessenderlo. Op 3M werden toen één van de hoogste PFOA, PFOS, perfluorhexaan sulfonzuur (PFHxS) en perfluordecaan sulfonzuur (PFDS) concentraties gemeten in vogeleieren met een mediaan van respectievelijk 19.8 ng/g, 10380 ng/g, 99.3 ng/g en 47.7 ng/g. De concentraties namen allemaal sterk af met toenemende afstand van 3M, maar Vlietbos en Rot verschilden nauwelijks van elkaar in PFAA concentraties. Tussen nestkasten zat een grote variatie in PFAA concentraties, wat mogelijk het gevolg is van verschillen in PFAA concentraties in de moeder vogels als gevolg van migratie of leeftijdsverschillen. Een andere reden is dat

er een hoge variatie binnen legsels kan zijn, met als gevolg een hogere variatie tussen legsels.

Tijdens het broedseizoen in 2016 werden opnieuw eieren verzameld van koolmezen. In tegenstelling tot 2011, waar een willekeurig ei per nest was verzameld, is er gekozen om steeds het derde ei van elk koolmees-nest te gebruiken. Van een aantal nesten zijn alle eieren mee genomen om de variatie in PFAA concentraties binnen legsels te vergelijken (11.7.2). Dit is zowel voor pimpelmezen als koolmezen gedaan. Ondanks de uitfasering van PFOS en gerelateerde producten door 3M, waren de concentraties van o.a. PFOS (mediaan van 48056 ng/g), maar ook PFOA (mediaan 18 ng/g) en PFDS (315 ng/g) opnieuw bij de hoogste concentraties ooit gemeten in vogel eieren. De mediaan van de PFOS concentraties lag nu zelfs 4.5 keer hoger dan in 2011. Mogelijke verklaringen hiervoor zijn dat in 2011 de PFOS concentraties het lineair bereik van de ijklijn overschreden en deze concentraties dus geëxtrapoleerd zijn, maar ook een verschil in sampling strategie kan een rol spelen, aangezien legsel variatie eerder is aangetoond in meeuwen (Vicente et al., 2015).

11.7.1.2 Bloed plasma

Tijdens de winter van 2015 en het broedseizoen van 2016 zijn bloedstalen verzameld van zowel volwassen als jonge (enkel tijdens het broedseizoen) koolmezen. Voor perfluorbutaanzuur (PFBA), PFOA, perfluordecaanzuur (PFDA), perfluordodecaanzuur (PFDoDA) en PFOS vonden we de hoogste concentraties terug die wereldwijd in bloedplasma van vogels gerapporteerd werden. PFOS concentraties namen af met toenemende afstand van 3M, maar voor de andere componenten was dit minder duidelijk. Concentraties van vier andere PFCAs waren enkel hoger in jonge Amerikaanse zeearenden (Route et al., 2014).

11.7.1.3 Veren

Veren zijn eerder gebruikt in studies naar polluenten als metalen en persistente organische polluenten. Desondanks is er slechts weinig gekend over PFAAs in veren en of veren geschikt zijn in de biomonitoring van PFAAs. In de herfst en winter van 2015 – 2016 zijn nestkasten geplaatst in vijf gebieden: 3M, Vlietbos, Rot, Burchtse Weel en

Fort 4. In elk gebied zijn veren en bloedstalen verzameld van vogels die sliepen in deze nestkasten. Het doel van dit onderzoek was om te evalueren in welke mate veren kunnen dienen als alternatief voor bloedplasma in de biomonitoring van PFAAs in koolmezen.

De concentraties van vrijwel alle doelcomponenten bij 3M waren de hoogste ooit gemeten in veren van wilde vogels en namen over het algemeen af met toenemende afstand van de fabriek. De meest dominante PFAA was PFOS. Zowel PFOS als PFOA concentraties in veren waren significant positief gecorreleerd met de concentraties in bloedplasma. Verder waren de PFOS concentraties in bloedplasma en veren niet significant verschillend, waaruit blijkt dat koolmeesveren wellicht geschikt zijn als matrix om interne PFOS concentraties in het bloed te schatten. Voor PFOA waren er echter wel significant verschillen tussen beide matrices. De wijze waarop deze verschilden, was afhankelijk van het individu, aangezien de ene keer bloedplasma concentraties hoger waren en de andere keer veer concentraties. Daarom raden we af om veren te gebruiken om de interne PFOA concentraties te schatten. Hier moet echter wel vermeld worden dat zowel de veren als het bloed verzameld zijn tijdens de winter en het is daarom mogelijk dat de PFAA concentraties die circuleren in het bloed anders waren op dat moment dan dat ze zouden zijn tijdens de vorming van de veren.

Veren zijn echter wel zeer nuttig in de biomonitoring van PFAAs in het milieu, aangezien meer componenten zijn gedetecteerd dan in bloedplasma. Dit geeft aan dat veren zelfs een betere matrix zijn dan bloedplasma om een indicatie te krijgen van de PFAA blootstelling vanuit het milieu.

11.7.2. Maternale transfer en variatie binnen legsels

Aangezien er tijdens het broedseizoen in 2016 zowel bloed plasma van moeders en jongen, als eieren in hetzelfde nest zijn verzameld, konden we nagaan of er maternale transfer is van PFAAs. Met andere woorden, we onderzochten of PFAAs vanuit de moeder doorgegeven konden worden via het ei om zo in het jong terecht te komen. We vonden een sterk significante correlatie tussen de PFOS concentraties in moeders

en jongen, maar ook, in mindere mate, tussen eieren en jongen, wat aangeeft dat de voornaamste route van PFOS blootstelling maternale transfer is en/of het dieet wat gegeven wordt door de ouders. Voor overige componenten vonden we geen correlaties, wat mogelijk aangeeft dat maternale transfer of dieet voor deze componenten niet de belangrijkste blootstellingsroutes zijn. Jongen zouden bijvoorbeeld blootgesteld kunnen zijn aan precursor componenten die een verschillende biotransformatie ondergaan in ouders en jongen.

Variatie in PFAA concentraties binnen en tussen legsels is onderzocht in legsels van koolmezen. Bij koolmezen was de variatie binnen een legsel groter dan tussen legsels voor alle PFAAs, wat mogelijk het gevolg is van hun fysicochemische eigenschappen. Wind en water zijn de voornaamste media voor de homogene verspreiding van wateroplosbare polluenten zoals PFAAs. Een andere reden voor de hoge variatie binnen legsels is de legselgrootte. Mezen investeren relatief grote hoeveelheden aan voedingsstoffen in hun eieren en gebruiken zeer waarschijnlijk eerder recent opgenomen voedingsstoffen dan opgeslagen nutrienten. Hierdoor worden grote variaties in PFAA concentraties, die geassocieerd zijn met deze voedingsstoffen, verwacht. Bovendien leggen koolmezen elke dag een ei en daarvoor zijn ze afhankelijk van het dagelijks aanvullen van hun nutrienten via het dieet (Van den Steen et al., 2009b). Een grote variatie aan PFAA concentraties in de prooien of in het type van prooien kan dan een verklaring geven voor de grote variatie aan PFAA concentraties binnen legsels.

De totale PFAA concentratie neemt af met legvolgorde binnen een legsel bij koolmezen. Dit was vooral duidelijk bij PFOS maar niet zo zeer bij de overige PFAAs. Dit is zeer waarschijnlijk het gevolg van afnemende concentraties in de moeder gedurende de legperiode.

Binnen een legsel van koolmezen waren de concentraties in diverse eieren gecorreleerd. Zo waren PFAA concentraties gecorreleerd in het eerste en derde ei. Dit geeft implicaties voor monitoringstudies, aangezien deze eieren ook de hoogste

concentraties bevatten. In biomonitoring van koolmezen is het beter om het eerste of derde ei te verzamelen indien het doel is om een maximale concentratie te krijgen binnen een nest. Wanneer het doel van het onderzoek is om een gemiddelde concentratie te krijgen, raden we aan om twee of drie willekeurige eieren te verzamelen per nest.

11.7.3 Mogelijke effecten op zangvogels

De toxicologische en biologische effecten van PFAAs op vogels zijn nog niet duidelijk. Diverse laboratoria hebben toxische effecten gerapporteerd op de ontwikkeling (Cassone et al., 2012; Jiang et al., 2012; Molina et al., 2006). Bovendien zijn negatieve effecten op het neuroendocriene systeem (Cassone et al., 2012; Smits en Nain, 2013; Vongphachan et al., 2011) en histologie (Molina et al., 2006) gekend. De meeste studies focussen echter op PFOS en PFOA en informatie over andere PFAAs ontbreekt vaak nog. Daarnaast is er een grote variatie in de effectconcentraties. Zo is er bijvoorbeeld een LD50 van 4.9 μg/g, gebaseerd op uitkippen, gerapporteerd in kippeneieren na *in ovo* injectie (Molina et al., 2006), terwijl een andere studie een zelfde LD50 rapporteerd op 93 μg/g (O'Brien et al., 2009a). Deze studies zijn veelal gebaseerd op injectie in eieren en resultaten na natuurlijke blootstelling kunnen anders zijn. In de huidige studie hebben we onderzoek gedaan naar de mogelijke effecten van PFAAs op reproductie en oxidatieve stress bij koolmezen.

11.7.3.1 Reproductie

Zoals eerder vermeld zijn er tijdens het broedseizoen van 2016 koolmeeseieren verzameld. Tijdens de staalname hebben we ook diverse reproductieve parameters onderzocht, waaronder de start van de broedperiode, legselgrootte, uitkipsucces, overleving en uitvliegsucces. Bovendien hebben we de schaaldikte van de eieren en de lichaamsconditie van de jongen bepaald. Omdat de PFAA concentraties onderling aan elkaar gerelateerd waren, hebben we ze moeten groeperen in principal components (PCs). Deze PCs werden gerelateerd aan de eerder genoemde parameters en daaruit vonden we een negative correlatie tussen uitkipsucces en PC1 (die voornamelijk beïnvloed werd door hoge concentraties van PFOS, PFDS, PFDoDA,

perfluorotridecaanzuur (PFTrDA) en perfluorotetradecaanzuur (PFTeDA) en in mindere mate door PFOA en perfluorononaanzuur (PFNA)) in nesten waar minstens één ei uitgekomen was. Daarnaast was PC1 positief gecorreleerd met uitvliegsucces, wat waarschijnlijk het gevolg was van een hogere overleving van jongen in nesten waar minder jongen uitkipten. PC2 (enkel beïnvloed door PFDA), was negatief gecorreleerd met zowel uitkipsucces (in nesten waar geen ei uitkwam) als totaal broedsucces. Dit kan verklaard worden doordat ouders mogelijk een gereduceerde vruchtbaarheid hebben of doordat toxische effecten op de ontwikkeling van het embryo plaats hebben gevonden (Molina et al., 2006; Yanai et al., 2008). Hogere waarden van PC2 waren ook gecorreleerd met een eerder begin van de eilegperiode. Vogels die vroeg broeden hebben over het algemeen een hogere reproductieve output en kwaliteit. De schaaldikte van de eieren was gereduceerd met toenemende PC1 waarden. Het verdunnen van de eischalen is een grote bedreiging voor vogelpopulaties, aangezien het de overleving van embryo's en het uitkipsucces reduceert (Miljeteig et al., 2012). Tenslotte geven onze resultaten aan dat het reproductief succes van koolmezen niet zo zeer gerelateerd zijn aan de afstand tot 3M, vermits het reproductief succes op Vlietbos en Burchtse Weel hoger was dan op de andere locaties. Dit geeft aan dat mogelijk andere omgevingsfactoren en/of polluenten, die niet bestudeerd zijn in de huidige studie, een rol spelen in het reproductief succes van koolmezen in deze gebieden.

11.7.3.2 Oxidatieve stress (OS)

De oxidatieve status van individuen kan dienen als indicator voor de schadelijke effecten van PFAAs. Cellen van het immuunsysteem of zaadcellen zijn kwetsbare doelwitten voor oxidatieve schade die veroorzaakt wordt door verschillende polluenten. Bovendien hebben organismen antioxidanten vanuit het dieet nodig om oxidatieve stress te bestrijden, wat resulteert in een onbalans van de trade-off tussen de allocatie van deze substanties tussen verschillende fysiologische functies, zoals bijv. reproductie. Daarom is het bestuderen van oxidatieve stress erg belangrijk in

toxicologische studies. Desondanks is er slechts weinig gekend over de mogelijke effecten van PFAAs op het antioxidant systeem van vogels.

Gedurende de winter van 2015 en het broedseizoen van 2016 zijn er bloedstalen verzameld van twee generaties koolmezen; tijdens de winter enkel van volwassen vogels en tijdens het broedseizoen van volwassen vogels en hun jongen. De PFAA concentraties gemeten in het plasma werden gerelateerd aan diverse OS parameters die gemeten zijn in de rode bloedcellen. In volwassen koolmezen was er een trend zichtbaar waarbij meer blootgestelde vogels meer eiwitschade hadden, wat betekent dat het antioxidant systeem faalde in het neutraliseren van reactieve zuurstofcomponenten (reactive oxygen species; ROS) die gegenereerd werden door de polluenten. In jongen was een positief verband geobserveerd tussen PFAA concentraties en antioxidante afweer. Meer blootgestelde jongen hadden een verhoogde activiteit van glutathionperoxidase en catalase enzymen, die beiden een rol spelen in de eerste verdediging tegen ROS. Onze studie leverde dus het bewijs dat PFAAs een mogelijk (vermits causaliteit niet is aangetoond) schadelijk effect hebben op de oxidatieve status van koolmezen.

11.8 Algemene conclusies

De uitkomst van deze thesis gaf nieuwe inzichten in de verspreiding van PFAAs in het terrestrische milieu in de nabijheid van een fluorochemische fabriek. Tijdens deze studies werden vier hypothesen onderzocht

Hypothese 1: PFAAs in het milieu accumuleren in de terrestrische voedselketen en de concentraties nemen af met toenemende afstand van een fluorochemische hotspot.

Perfluoroalkaan zuren werden gedetecteed in verschillende matrices, waaronder bodem, pissebedden, eieren van zangvogels, bloed plasma van koolmezen en veren van koolmezen, wat aangeeft dat PFAAs, aanwezig in het milieu, accumuleren in biota. In vrijwel alle studies van deze thesis namen de PFAA concentraties sterk af met toenemende afstand van de 3M fluorochemische fabriek, wat aantoont dat deze fabriek een puntbron is van PFAAs vervuiling in het milieu rond Antwerpen. In de

meeste studies waren de concentraties gemeten in de diverse matrices (één van) de hoogste ooit gemeten wereldwijd. Daarom kan de 3M fabriek nog altijd gezien worden als PFAAs-hotspot, ondanks de uitfasering van diverse componenten in 2002.

Hypothese 2: Fysicochemische bodem eigenschappen spelen een rol in de sorptie, distributie en beschikbaarheid van PFAAs

De complexiteit van de PFAAs chemie resulteert in diverse onzekerheden over hoe verschillende fysicochemische bodemeigenschappen interageren om zo de sorptie van PFAAs aan bodems te bepalen. Onze resultaten tonen aan dat PFAA concentraties in de bodem geassocieerd zijn met diverse bodemeigenschappen, zoals organisch koolstof en kleigehalte, pH en temperatuur. De grootste bijdrager aan de sorptie van PFAAs in bodems was het organisch koolstofgehalte, aangezien dat het sterkst gecorreleerd was met de PFAA concentraties. De verticale distributie van PFAAs in bodems is zeer waarschijnlijk ook het gevolg van verschillen in fysicochemische bodemeigenschappen tussen verschillende bodemlagen. De neerwaartse migratie van PFAAs in de bodem moet mee in rekening gebracht worden bij volgende studies op de bodem, aangezien de toplaag niet altijd een representatief beeld geeft van de bodem PFAA concentraties.

Hypothese 3: Niet-destructive staalname kan gebruikt worden om PFAA concentraties in het milieu en in organismen te monitoren

Naast de interne concentraties in bloed plasma van de koolmezen, hebben we gebruik gemaakt van weefsels, zoals eieren en veren. Ondanks we in deze studie de eieren en veren op invasieve en destructieve manier verkregen hebben, bieden beide matrices wel mogelijkheden voor niet-invasieve staalname. Zo kunnen veren verzameld worden die op de grond zijn gevallen en zouden eieren gebruikt kunnen worden die niet uitgekomen zijn. Onze resultaten tonen aan dat de concentraties in de eieren gebruikt kunnen worden om de concentraties in het bloed van de moeders en in de jongen te voorspellen. Dit geeft aan dat het (zeer waarschijnlijk, vermits we enkel niet

geïncubeerde eieren hebben getest) mogelijk is om niet-uitgekomen eieren te gebruiken, als niet-destructieve methode, om zo de PFAA concentraties in het bloed van de moeders te schatten. Vergelijkbaar met deze resultaten, vonden we dat voor PFOS de concentraties in de veren gecorreleerd waren aan deze in het bloed plasma. Dit toont aan dat veren gebruikt kunnen worden om de PFOS concentraties in het bloed van koolmezen te schatten. Bovendien zijn veren een meer geschikte biomonitoring matrix aangezien ze een beter overzicht geven van de totale blootstelling doordat er meer componenten in gedetecteerd werden dan in bloed plasma.

Hypothese 4: Geaccumuleerde PFAA concentraties zijn gerelateerd aan toxische effecten in veldomstandigheden

Er zijn diverse effecten gekend van PFAAs op biota. Aangezien de concentraties in deze studies (één van) de hoogste concentraties ooit gemeten wereldwijd zijn in zangvogels, was de verwachting dat deze concentraties zouden resulteren in effecten op reproductie en oxidatieve stress in koolmezen. Ondanks dat onze studies een aantal effecten op reproductie (bijv. lager uitkipsucces, verdunnen van eischalen, lager broedsucces en een eerdere start van het broeden) en oxidatieve stress (eiwitschade, hogere activiteit van antioxidante enzymen) aantoonden, waren deze effecten erg beperkt. Dit kan mogelijk deels verklaard worden door een lagere gevoeligheid van koolmezen voor PFAAs vergeleken met andere soorten, waar ernstigere effecten werden geobserveerd bij lagere concentraties. Het moet echter opgemerkt worden dat we de mogelijke effecten op reproductie slechts éénmalig hebben bestudeerd, waarbij we niet alle omgevingsfactoren mee in rekening konden brengen die reproductie ook kunnen beïnvloeden. Deze factoren kunnen ook een rol spelen bij oxidatieve stress en daarom moeten experimenten onder gecontroleerde omstandigheden uitgevoerd worden om causaliteit aan te kunnen tonen.

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13. List of Abbreviations

ACN	Acetonitrile
ACV	Among clutch variation
ADONA	3H-perfluoro-3-[(3-methoxy-propoxy)propanoic acid]
AFFF	Aqueous film-forming foam
aHF	Anhydrous hydrogen fluoride
AIC	Akaike Information Criterion
ANB	Agency for Nature and Forest
APFN	Ammonium perfluorononanoate
APFO	Ammonium perfluorooctanoate
BAF	Biota accumulation factor
BSAF	Biota-sediment accumulation factor
bw	Body weight
CAT	Catalase
CEH	Chicken embryo hepatocyte
CYP	Cytochrome P450 enzyme system
DLLME	Dispersive liquid-liquid microextraction
dw	Dry weight
ECF	Electrochemical fluorination
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EGM	Environmental Gas Monitor
EPS	Extracellular polymeric substances

ES(-)	Electrospray (negative)
F-53B	A combination of 9-chlorohexadecafluoro-3-oxanonane-1-sulfonate and 11-chloroeicosafluoro-3-oxaundecane-1-sulfonic acid
FASA	N-alkyl perfluoroalkane sulfonamide
FASE	N-alkyl perfluoroalkane sulfonamidoethanol
FRAP	Ferric ion reducing antioxidant power
FTAC	Fluorotelomer acrylate
FTI	Fluorotelomer iodide
FTO	Fluorotelomer olefin
FTOH	Fluorotelomer alcohol
FWO	Research Foundation Flanders
GLM	Generalized linear model
GPX	Gluthathione peroxidase
GSH	Reduced gluthatione
GSSG	Oxidized gluthathione
GST	Gluthatione-S-transferase
HFPO-DA	Hexafluoropropylene oxide dimer acid
Ig(M/Y)	Immunoglobulin (M / Y)
ISTD	Internal standard
K_e	Elimination coefficient
KM	Kaplan Meier
KMI	Royal Meteorological Institute Belgium
K_{oc}	Octanol-water partitioning coefficient

K_u	Uptake coefficient
LC	Long chain
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
L(D/C)50	Median lethal dose/concentration for 50% of the individuals in a group
L-FABP	Liver fatty acid-binding protein
LOI	Loss on ignition
LOQ	Limit of quantification
LSD	Least Significant Difference
MDA	Malondialdehyde
MQ	Milli-Q
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NBT	Nitroblue tetrazolium
OC	Organic carbon
OECD	Organisation for Economic Co-operation and Development
OS	Oxidative stress
PAP	Polyfluoroalkyl phosphate
PASF	Perfluoroalkane sulfonyl fluoride
PBDE	Polybrominated diphenyl ether
PBT	Persistent, Bioaccumulative and Toxic
PC	Principal component
PCA	Principal component analysis

PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo-*p*-dioxin
PCDF	Polychlorinated dibenzofuran
PES	Polyethersulfone
PFAA	Perfluoroalkylated acid
PFAI	Perfluoroalkyl iodide
PFAS	Perfluoroalkyl substance
PFBA	Perfluorobutanoic acid
PFBS	Perfluorobutane sulfonate
PFCA	Perfluorocarboxylic acids
PFDA	Perfluorodecanoic acid
PFDoDA	Perfluorododecanoic acid
PFDS	Perfluorodecane sulfonate
PFHpA	Perfluoroheptanoic acid
PFHxA	Perfluorohexanoic acid
PFHxS	Perfluorohexane sulfonate
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonate
PFPeA	Perfluoropentanoic acid
PFSA	Perfluorosulfonic acid
PFTeDA	Perfluorotetradecanoic acid
PFTrDA	Perfluorotridecanoic acid
PFUnDA	Perfluoroundecanoic acid

pKa	Acid dissociation constant
POD	Peroxidase
POP	Persistent organic pollutant
POSF	Perfluorooctane sulfonylfluoride
PP	Polypropylene
PPAR(α)	Peroxisome proliferator-activated receptor (alpha)
RBC	Red blood cell
ROS	Reactive oxygen species
S/N	Signal-to-noise
SE	Standard error
SI	Supplementary Index
SOD	Superoxide dismutase
SOM	Soil organic matter
SPE	Solid phase extraction
SRBC	Sheep red blood cell
TAC	Total antioxidant capacity
TFE	Tetrafluoroethylene
TM	Telomerization
TOC	Total organic carbon
TOP	Total Oxidisable Precursor
TQD	Triple quadrupole
UPLC	Ultra performance liquid chromatography
VALLE	Vortex-assisted liquid-liquid extraction
VMM	Flemish Environment Agency

WCV	Within clutch variation
WOS	Web of science
ww	wet weight

14. Curriculum Vitae (CV)

Personal information

Name: Thimo Groffen

Date of Birth (d/m/y): 07-04-1992

Place of Birth: Zevenbergen, The Netherlands

Mobile phone: 0031 (0)6 41 20 90 80

E-mail: Thimo.Groffen@hotmail.com

Education and work experience

2015 – 2019	PhD researcher at the Systemic Physiological & Ecotoxicological Research (SPHERE) and Behavioural Ecology and Ecophysiology (BECO) groups, University of Antwerp: '*Toxicity of Perfluoroalkyl Substances (PFAAs) to terrestrial invertebrates and songbirds'.*
April 2015	Student job at SPHERE, University of Antwerp. Guiding of an internship student with the extraction and analysis of PFAAs in sediment.
2013 – 2015	Degree: Master of Science in Biology, University of Antwerp. Masterproject: '*Distribution and effects of Perfluorinated compounds on an aquatic food chain in the Vaal River, South Africa'.*
2010 – 2013	Degree: Bachelor of Science in Biology, University of Antwerp. Bachelorproject: '*Analysis of pollutants in water, sediment, fish and invertebrates in the water reservoirs of Morogoro, Tanzania'.*

Publications

- Groffen T, Lopez-Antia A, D'Hollander W, Prinsen E, Eens M and Bervoets L (2017). Perfluoroalkylated acids in the eggs of great tits (*Parus major*) near a fluorochemical plant in Flanders, Belgium. Environmental Pollution 228: 140 – 148.

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- Lopez-Antia A, Groffen T, Lasters R, AbdElgawad H, Sun J, Asard H, Bervoets L and Eens M (2019). Perfluoroalkyl acids (PFAAs) concentrations and oxidative status in two generations of great tits inhabiting a contamination hotspot. Environmental Science and Technology 53: 1617 – 1626.
- Groffen T, Lasters R, Lemière F, Willems T, Eens M, Bervoets L and Prinsen E (2019). Development and validation of an extraction method for the analysis of perfluoroalkyl substances (PFASs) in environmental and biotic matrices. Journal of Chromatography B 1116: 30 – 37.
- Groffen T, Rijnders J, Verbrigghe N, Verbruggen E, Prinsen E, Eens M and Bervoets L (2019). Influence of soil physicochemical properties on the depth profiles of perfluoroalkylated acids (PFAAs) in soil along a distance gradient from a fluorochemical plant and associations with soil microbial parameters. Chemosphere 236: 124407.

- Fauconier G, Groffen T, Wepener V and Bervoets L (*accepted in Science of the Total Environment*). Perfluorinated compounds in the aquatic food chains of two sub-tropical estuaries.
- Groffen T, Lasters R, Bervoets L, Prinsen E and Eens M (*subm.*). Are feathers of great tits (Parus major) suitable alternatives for monitoring perfluoroalkylated acids (PFAAs) in blood plasma? Environmental Science and Technology (*subm.*).
- Groffen T, Rijnders J, Van Doorn L, Jorissen C, Mortier de Borger S, Oude Luttikhuis D, De Deyn L and Bervoets L (*in prep*). Preliminary study on the distribution of metals and persistent organic pollutants (POPs), including perfluoroalkylated acids (PFAAs), in the aquatic environment near Morogoro, Tanzania.
- Lasters R, Groffen T, Eens M and Bervoets L (*in prep.*). Distinct differences in PFAA concentrations and profile between two closely related tit species in function of important life-history traits.
- Lasters R, Groffen T, Eens M and Bervoets L (*in prep.*). Home-produced eggs: an important human exposure pathway of perfluoroalkylated acids (PFAAs) nearby a fluorochemical plant in Antwerp.
- Groffen T, Bervoets L, Willems T, Eens M and Prinsen E (*in prep.*). A novel method for the detection and quantification of perfluoroalkylated acids in feathers using UPLC-MS/MS.
- Rijnders J, Bervoets L, Prinsen E, Eens M, Beemster G, AbdElgawad H and Groffen T (*in prep.*). Influence of perfluoroalkylated acids (PFAAs) on the oxidative status of snails (*Cepaea sp.*) exposed to nettles (*Urtica sp.*) along a distance gradient from a fluorochemical plant.
- Carteny CC, Groffen T, Willems T, Prinsen E, Bervoets L and Blust R (*in prep.*). Simple, fast, and economical method for long-chain PFAS extraction from small volumes of seawater.

- Padilha JA, Groffen T, Willems T, Eens M, Prinsen E, Bervoets L, Dorneles P and Das K (*in prep.*). Perfluoroalkylated compounds in the eggs and feathers of resident and migratory seabirds from Antarctic Peninsula.

Presentations at congresses

- Groffen T, Wepener V, Malherbe W and Bervoets L (2016). Distribution of perfluorinated alkyl substances (PFAAs) in an aquatic food chain in the Vaal River, South Africa. 23rd Zoology Congress, Antwerp, Belgium. Poster presentation.
- Groffen T, Bervoets L and Eens M (2016). Toxicity of perfluoroalkyl substances (PFAAs) to a terrestrial songbird, the great tit (*Parus major*). 2nd Biology Research Day, Antwerp, Belgium. Oral presentation, speaker.
- Groffen T, Lopez-Antia A, Bervoets L and Eens M (2017). Distribution of perfluorinated compounds (PFAAs) in great tits (*Parus major*) along a pollution gradient in Antwerp, Belgium, and their effects on reproduction. SETAC Europe 27th annual meeting, Brussels, Belgium. Poster presentation.
- Groffen T, Lopez-Antia A, Lasters R, Prinsen E, Bervoets L and Eens M (2017). Reproductive effects of perfluoroalkyl acids (PFASs) on great tits (*Parus major*) near a PFASs hotspot in Flanders, Belgium. 3rd Biology Research Day, Antwerp, Belgium. Poster presentation.
- Groffen T, Eens M and Bervoets L (2018). Perfluoroalkylated acids (PFAAs) in soil and invertebrates collected along a distance gradient starting at a fluorochemical plant in Antwerp, Belgium. SETAC Europe 28th annual meeting, Rome, Italy. Poster presentation.
- Groffen T, Lasters R, Lemière F, Willems T, Eens M, Bervoets L and Prinsen E (2018). Development and validation of an extraction method for the analysis of perfluoroalkyl substances (PFASs) in environmental matrices. 7th EuCheMS Chemistry Congress, Liverpool, United Kingdom. Poster presentation.

- Groffen T, Lasters R, Lemière F, Willems T, Eens M, Bervoets L and Prinsen E (2018). Development and validation of an extraction method for the analysis of perfluoroalkyl substances (PFASs) in environmental matrices. 4th Biology Research Day, Antwerp, Belgium. Poster presentation.
- Groffen T, Eens M and Bervoets L (2018). Do concentrations of perfluoroalkylated acids (PFAAs) in isopods reflect concentrations in soil and songbirds? A study along a distance gradient from a fluorochemical plant in Antwerp. 4th Biology Research Day, Antwerp, Belgium. Oral presentation, speaker.
- Groffen T, Eens M and Bervoets L (2019). PFAS in the terrestrial environment near a hotspot in Belgium. Remediation Technology Summit (REMTEC), Denver, United States. Oral presentation, invited speaker.
- Groffen T, Rijnders J, Verbrigghe N, Verbruggen E, Prinsen E, Eens M and Bervoets L (2019). Influence of soil physicochemical properties on the depth profiles of perfluoroalkylated acids (PFAAs) in soil along a distance gradient from a fluorochemical plant. SETAC Europe 29th annual meeting, Helsinki, Finland. Poster presentation.

General skills

- **Communicational**

Give presentations and talks in different languages to (international) audiences, writing scientific papers in English.

- **Networking and teamwork**

Work in international research groups, report scientific results in- and outside the research group by attending conferences, guiding thesis students. Contact with partners (e.g. 3M company) to organize the fieldwork and sampling. Helping with the organization of international and national congresses.

- **Time management and flexibility**

Planning and follow-up of the PhD project, adjust research objectives and planning to changing results, conduct labour intensive fieldwork in sometimes difficult weather conditions at day or night.

- **Leadership**

Coaching of both master- and bachelorstudents with their theses. Aiding in practical courses (supervision and guiding of students), taking initiative to conduct projects, invent new strategies and techniques.

- **Problem solving and creativity**

Invent new research strategies and techniques (e.g. analytical methods), make a critical analysis of research outcomes and questions, finding and solving problems when things did not go as planned (e.g. measurements were delayed by defect devices, etc.). Invent new research opportunities and small projects.

- **Practical skills and technical knowledge**

Organizing and conducting sampling in the field, experience in handling and manipulating songbirds, create and elaborate sampling and research protocols, prepare and conduct measurements in the laboratory, develop novel analytical techniques

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Papers reviewed: 4

Marine Pollution Bulletin	1
Pakistan Journal of Zoology	1
Science of the Total Environment	1
Environmental Pollution	1