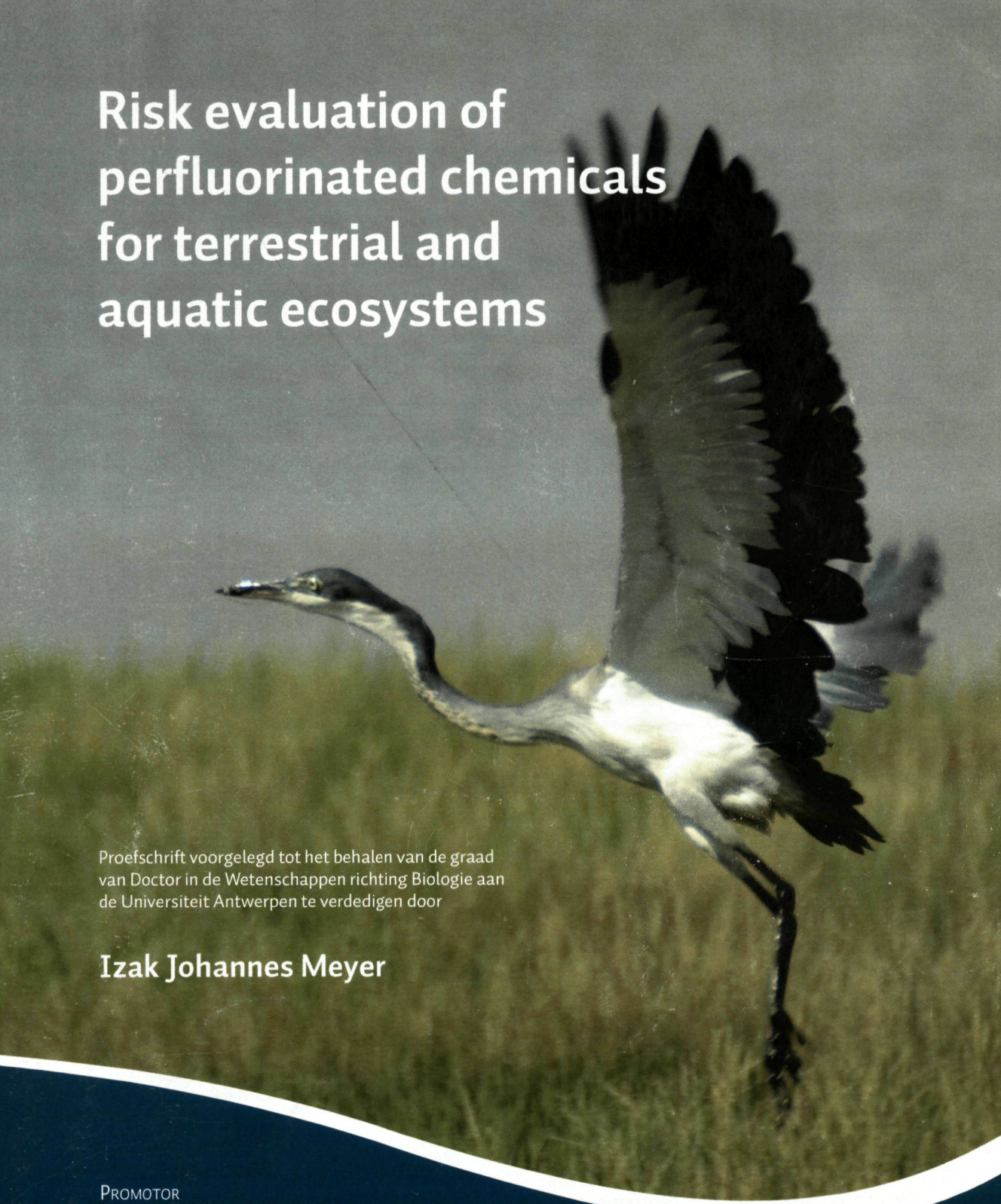
Risk evaluation of perfluorinated chemicals for terrestrial and aquatic ecosystems



Proefschrift voorgelegd tot het behalen van de graad van Doctor in de Wetenschappen richting Biologie aan de Universiteit Antwerpen te verdedigen door

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Faculteit Wetenschappen
Departement Biologie
Antwerpen, oktober 2008

Universiteit Antwerpen

Each moment spent searching
is also a moment spent finding

Paulo Coelho
"The Alchemist"

Pollution: The result of civilization, development, expansion, growth and prosperity. Too many gases in the air, too much smoke, too few trees, and people, people, people…

Environment: If you've planted a tree that will only be at its best years after you've died, you'll understand Environment. If you chop down trees because they are in the way, you won't know what we're talking about.

Evita Bezuidenhout
"The Essential Evita Bezuidenhout"

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For Patrick,
ets el meu mon i la meva anima

and my parents,
dankie vir al julle liefde en ondersteuning deur my hele lewe.

Contents

Introduction

1.1 Perfluorinated Chemicals

Perfluorinated chemicals (PFCs) form part of a diverse group of chemicals where all of the C-H bonds have been replaced by C-F bonds. The two PFC groups that are found in the highest concentrations in the environment are the perfluorosulfonates and the perfluorocarboxylic acids. In this thesis, the focus will be on perfluorooctane sulfonate (PFOS), a perfluorosulfonate, since this is the PFC found in the highest levels in wildlife. Three other PFCs will also be studied, namely perfluorohexane sulfonate (PFHxS), perfluorooctanoic acid (PFOA) and perfluorononanoic acid (PFNA). PFHxS is a perfluorosulfonate, whereas PFOA and PFNA are perfluorocarboxylic acids. PFOS is the PFC on which the most data is available; therefore, the introduction will mainly contain information on PFOS. The molecular formulas of the four PFCs studied in this thesis are given in Table 1.

Table 1. The molecular formulas of the PFCs studied

PFC	Molecular Formula
PFOS	$C_8F_{17}SO_3$
PFHxS	$C_6F_{13}SO_3$
PFOA	$C_8HF_{15}O_2$
PFNA	$C_9HF_{17}O_2$

1.2 Characteristics of PFCs

Very little is known about the physical properties of many PFCs. PFOS is an amphipatic molecule consisting of a hydrophobic perfluorinated carbon tail consisting of eight carbon atoms and a hydrophilic sulfonyl group (Figure 1).

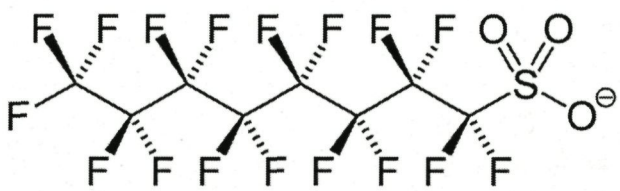

Figure 1. The structure of PFOS

It is the strength of the F-H bonds that give PFCs extreme stability and its unique properties. PFOS forms three layers in octanol/water, therefore an n-octanol/water (Kow) partitioning coefficient cannot be determined. Therefore, many other physiochemical properties that are estimated using Kow equations cannot be estimated for PFOS. Unlike other organic compounds, PFCs do not bioconcentrate in the lipid fraction, but tend to bind to certain proteins, for example albumin the blood stream (Jones et al., 2003). In pure water (24 –

25°C) PFOS has a solubility of between 549 mg/L and 570 mg/L. The solubility of PFOS in natural seawater is lower (12.4 mg/L at 22 – 23 °C). The melting point of the potassium salt of PFOS is larger or equal to 400 °C and its vapour pressure is 3.31×10^{-4} Pa at 20°C (OECD, 2002).

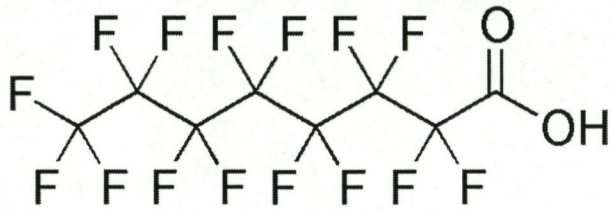

Figure 2. The structure of PFOA

PFOA is a totally fluorinated organic acid (Figure 2). PFOA is more soluble in pure water (3.4 – 9.5 g/L at 22 – 25°C) than PFOS. Like PFOS, the Kow of PFOA cannot be measured. The melting point of PFOA is 45 – 50 °C and its vapour pressure is 0.1 kPa at 20°C (EFSA, 2008).

1.3 Production and use of PFCs

PFOS is produced by either of two processes, namely electrochemical fluorination and telomerisation. 3M used the electrochemical fluorination process to produce PFOS, which gave a mixture of branched and straight-chained molecules. Most other companies use the telomerisation process, which primarily produces straight-chained products (Renner, 2001). Historical data on the production of PFOS exists only for the United States of America (USA). About 1848 metric tonnes of perfluorooctane sulphonyl fluoride (PFOSF), which is the basic building block of PFOS, were either manufactured or imported into the USA. This figure was slightly lower in 2000 (1820 metric tonnes), the year 3M announced it will phase out the production of PFOS (OSPAR, 2005). In 2000, the volume of PFOS and its salts that were sold as finished products was less than 91 metric tons (3M, 2000). It is now generally accepted that the production of PFOS is zero.

PFCs have a wide variety of applications in industry due to both their lipophobic and hydrophobic properties and have been produced for over 50 years (Renner, 2001). PFCs are used as surface-active agents in different applications. Due to their extreme persistence, PFCs are suitable for high temperature applications and for applications in contact with strong acids or bases. PFCs have numerous uses including fire-fighting foams, paper grease proofing treatments, carpet and leather treatment and textile (Giesy and Kannan, 2002). A more detailed overview is given in Table 2.

Table2. An overview of the uses of PFCs (taken from OSPAR, 2005)

Group	Applications	End Product
Surface Treatment Applications	Treatment of Fabrics (water/oil/soil repellence)	Apparel/Textile Fabric/upholstery Carpets Automotive interiors
	Treatment of metal and glass	Metal/glass
	Leather treatment (water/oil/solvent repellence)	Leather
	Mist suppressant Corrosion inhibitors	Metal plating baths
Paper Protection Applications	Water/oil/grease/solvent repellence	Plates and food containers Bags and wraps Folding cartons Containers Carbonless forms Masking papers
Performance Chemical Applications	Surfactants	Surfactant in fire fighting foams Surfactants in alkaline cleaners Mine and oil well surfactants
	Cleaning agents	Denture cleaners Shampoos Carpet spot cleaners Mould release agents
	Waxes and polishes	Emulsifier in wax and floor polishes
	Coatings	Coating additives
	Photography	Antistatic agents Surfactants for paper, films, photographic plates
	Photolithography	Coatings for semiconductors anti-reflective coatings
	Pesticides/insecticides	Pesticides active ingredient Active ingredient for ant bait traps
	Chemical synthesis	Chemical intermediates
	Medical applications	Waterproofing casts/wound dressings
	Hydraulic fluids	Hydraulic fluid agents

1.4 Regulation of PFCs in the EU

Most regulations focus on PFOS and PFOA. In the European Union PFOS is regulated by the European Union Directive 2006/122/EC3, which has been applied since June 2008. The directive lays down restrictions on the marketing and use of PFOS for new non-food products. Ongoing risk assessment activities for PFOA are also compulsorily under this directive. There is currently no legislation at the EU level on PFCs in food or feed (EFSA, 2008). PFOS is also being reviewed for inclusion in the UNECE-CLRTAP protocol on persistent organic pollutants (POPs).

1.5 Toxicologic effects in animals

Toxicity data of PFCs on animals have recently increased. However, most of the data is on rats and monkeys in laboratory studies (Seacat et al., 2002; Seacat et al., 2003). Some studies have also been performed on fish, both in the laboratory and in the field. Studies dealing with the effects of PFCs on birds have mainly been performed on only two species, the Northern Bobwhite (*Colinus virginianus*) and Mallard (*Anas platyrhynchus*) (Newsted et al., 2006; Newsted et al., 2007)

1.5.1 Toxicological Effects in Mammals

Most of the laboratory studies on the effects of PFOS on animals have been performed on rodents. Toxicity induced effects in laboratory rats and mice include breathing disturbance, body weight loss, a decrease in food intake and general poor condition (Austin et al., 2003; Rusch et al., 1979; Hu et al., 2002).

Field data on the effects of PFCs on mammals are limited. As in the laboratory studies, wild Wood Mice (*Apodemus sylvaticus*) from a PFOS polluted area experienced an increase in liver weight, relative liver weight and the liver microsomal lipid peroxidation level and a decrease in the serum alanine aminotransferase activity (Hoff et al., 2004).

1.5.2 Toxicological Effects in Birds

The number of studies on the effects of PFC exposure in birds is very limited. Only two bird species, namely the Mallard and Northern Bobwhite, have been used in laboratory studies to assess the effects of PFCs on birds. Effects included increased liver weight, decrease in body weight and a decrease in testes length in males (Newsted et al., 2006; Newsted et al., 2007). In field studies, Hoff et al. (2005b) indicated that there is a significant increase in serum alanine aminotransferase activity (ALT), which is a biomarker of liver damage, with an increase in liver PFOS levels in wild tits. They also found a decrease in serum cholesterol and triglyceride levels with an increase in PFOS levels, which suggests that PFOS influences the lipid metabolism of exposed organisms.

1.6 Environmental exposure

Not all PFCs occur with the same frequency and levels in wildlife samples. PFOS is not biodegradable in nature (Key et al., 1998). Since a large number of PFCs degrade to PFOS as a final metabolite, PFOS is normally found at the highest levels and frequency in wildlife samples. Although PFOA is the PFC usually measured in the highest levels in water samples, PFOA is normally found in very low levels in biota or the PFOA levels are below the detection limit. Both PFHxS and PFNA levels are usually below the detection limits in wildlife samples, although in some studies very low levels were measured. (Kannan et al., 2002a; Kannan et al., 2002b; Bossi et al., 2005; Holström et al., 2005; Haukås et al., 2007; Senthilkumar et al., 2007).

Various studies have also been performed on the distribution of PFOS in the different components of an ecosystem. All of these studies have been performed in the aquatic environment, either freshwater or marine. De Vos et al. (2008) investigated the accumulation of PFOS in a food chain of the Western Scheldt estuary in the Netherlands. They found that the uptake of PFOS is comparable to moderately hydrophobic compounds and elimination is comparable to the elimination kinetics of metals, thus the accumulation behaviour of PFOS is comparable to that of short and medium chained fatty acids. Stable isotopes have also been used to determine the transfer of PFCs through the trophic levels in the ecosystem. In a food web from the Barents Sea, consisting of Sea Ice Amphipods (*Gammarus wilkitzkii*), Polar Cod (*Boreogadus saida*), Black Guillemot (*Cepphys grille*) and Glaucous Gull (*Larus hyperboreus*), the bioaccumulation factors (BAFs) were all above 1 (Haukås et al., 2007). Van den Vijver et al. (2003) showed that marine mammals with the highest trophic positions contained the highest levels of PFOS in their livers. A bioconcentration factor (BFC) of about 1000 was found in benthic invertebrates from the Great Lakes region in the USA. A BAF of between 10 and 20 were also determined for American Mink (*Mustela vison*) and Bald Eagles (*Haliaeetus leucocephalus*) from the same area (Kannan et al., 2005). In a food chain in Lake Ontario, Canada, the diet weighted BAFs for Lake Trout (*Salvelinus namaycush*) was 2.9 for PFOS, 0.41 for PFOA and 2.3 for PFNA (Martin et al., 2004a). The biomagnification factors (BMF) for PFOS in a marine food web from the eastern Arctic were also higher than the BMFs for PFOA (Tomy et al., 2004). BAFs factors up to 156 for PFOS in the food web of the Bottlenose Dolphin (*Tursiops truncatus*) have been reported (Houde et al., 2006). This all indicates that PFCs accumulate and biomagnify in the ecosystem.

1.6.1 PFCs in different matrices

Due to their lipophobic characteristics, PFCs do not accumulate in the adipose tissue of animals. Instead, once in the blood, PFCs bind unspecific to albumin (Jones et al., 2003). Liver has mainly been used to determine animals' exposure to PFCs. Several studies have also successfully used eggs of fish and birds as alternative, less invasive matrices to measure PFC exposure. Laboratory studies have shown that female birds transfer perfluorooctane sulfonate (PFOS), the PFC most commonly found in wildlife, to their eggs. The greatest portion of the PFOS burden in the eggs was associated with very low-density lipoproteins in the yolk (Newsted et al., 2007). Various studies have found a correlation between the PFC levels in blood and liver and in egg and liver, indicating that these matrices give an accurate picture of the PFC exposure of the animal. No data is available on the PFC levels in feathers of birds. Dauwe et al., 2007 showed that there is a significant positive correlation (Pearson, $r = 0.80$, $p < 0.001$) between the PFOS levels in the blood and livers of Great Tits (*Parus major*). The PFOS levels in the eggs, plasma and liver of Glaucous Gulls were all within the same range (89 ng/g – 150 ng/g ww), but the mean plasma PFOS levels were slightly higher (Verreault et al., 2005). The PFOS levels in the eggs of Common Terns (*Sterna hirundo*) were on average slightly higher than the liver PFOS levels of the mother bird, although this difference was not significant (Van den Brink et al., 2007). In mammals, the highest PFCs levels are usually found in the liver (Houde et al., 2006; Van den Vijver et al., 2007). However, in the Harbour Seal (*Phoca vitulina*) the mean PFOS levels in the kidney were higher than those in the liver (Van den Vijver et al., 2003).

1.5.1 Mammals

Earlier studies of PFOS in wildlife focused to a large degree on marine mammals. For various seal species, the liver PFOS concentrations were mainly below 100 ng/g ww (Bossi et al., 2005; Giesy and Kannan, 2001; Tao et al., 2006). The levels of PFOS found in dolphins were slightly higher (Bossi et al., 2005; Giesy and Kannan, 2001; Kannan et al., 2002b). However, Martin et el. (2004a) measured 3100 ng/g ww in the liver of Polar Bears (*Ursus maritimus*), which are very high in the food chain, feeding on seals that already show relatively high PFOS exposure. Polar Bears from east Greenland also contained high levels of PFCs, with a mean of 24700 ng/g ww in their liver and PFHxS and PFNA levels over the 100 ng/g ww. The PFOA liver levels were around 10 ng/g ww (Smithwick et al., 2005b). In a circumpolar study of liver PFC levels of Polar Bears, PFOS levels of up to 6340 ng/g ww, 4430 ng/g ww for PFHxS, 57 ng/g ww for PFOA and 540 ng/g ww for PFNA were measured (Smithwick et al., 2005a). Although American Mink are opportunistic predators, Giesy and Kannan (2002) measured PFOS levels in their liver as high as 3680 ng/g ww. This shows that smaller animals, which are lower in the food chain,

Belangrijk om te onderzoeken hoe het accumulatie-proces in elkaar zit.

can also have high levels of PFOS in their livers. The highest levels ever reported for wildlife (178000 ng/g ww) was in the liver of Wood Mice from Blokkersdijk in Belgium (Hoff et al., 2004). This study showed that small mammals could also contain high levels of PFOS if they are living in a PFC contamination hotspot. Most of the studies were performed in the Northern Hemisphere. One study determined the PFOS levels in the blood of Southern Elephant Seals (*Mirounga leoine*) from the Antarctic. These levels were much lower (<0.08 – 3.52 ng/mL) than those founding seals from the Arctic.

Waarom is geen grotere diersoort onderzocht?

1.5.2 Birds

Since the first article was published on the exposure of wildlife to PFOS, the number of articles that include birds has increased. As with mammals, most studies focus on marine birds. In Table 3 a list is presented with the number of articles in which PFC levels in birds were measured, according to family. As can be seen from the list, most studies were done on water birds. Of these water birds families, most research has been done on marine bird families, for example Laridae (gulls, terns) and Alcidae (auks),

Table 3. The number of articles that have included PFC levels of birds, arranged according per family. Whether the family consists mainly of water birds or not is also indicated.

Family	Examples	No of articles	Water birds
Spheniscidae	penguins	1	Yes
Procellaridae	fulmars, petrels	4	Yes
Phalacroracidae	cormorants	5	Yes
Gaviidae	loons	2	Yes
Sulidae	gannets	1	Yes
Diomedidae	albatrosses	3	Yes
Scolopacidae	small waders	1	Yes
Stercorariidae	skuas, jaegers	2	Yes
Pelecanidae	pelicans	3	Yes
Laridae	gulls, terns	12	Yes
Alcidae	auks	7	Yes
Anatidae	ducks, geese	3	Yes
Podicipedidae	grebes	1	Yes
Ardeidae	herons	2	Yes
Ciconiidae	storks	1	Yes
Accipitridae	eagles	5	Yes[a]
Threskiornithidae	ibisses	1	No
Paridae	tits	2	No
Corvidae	crows	2	No

a. Most studies were done on Bald Eagles, which are piscivorous and live next to rivers and lakes.

The first article published on the PFOS levels of birds were in 2001 (Giesy and Kannan, 2001). This study included the PFOS levels in plasma, egg yolk and liver of various water birds, most of them marine birds. The PFOS levels measured in the study were within the same range as those of mammals from the same study. Bald Eagles (*Haliaeetus leucocephalus*) from different areas of the USA had maximum plasma PFOS levels ranging from 2220 to 2570 ng/mL (Giesy and Kannan, 2001; Kannan et al., 2001). PFOS has even been measured in the liver of birds from remote areas such as the Barents Sea. Mean Liver PFOS levels of 13.5 ng/g ww for Black Guillemot (*Cepphus grille*) and 65.8 ng/g ww in Glaucous Gull (*Larus hyperboreus*) were measured (Haukås et al., 2007). Common Cormorants (*Phalacrocorax carbo*) from Japan contained the highest PFOS liver levels (1873 to 2249 ng/g ww) in a study that determined the PFOS levels in the liver of various bird species (Kannan et al., 2002a). The PFOA and PFHxS levels were both below the detection limit (19 and 7.5 ng/g ww, respectively) in most samples of the study, which included the Common Cormorant. PFOA was only detected in the liver of the Black-headed Gull (*Larus ridibundus*) and Black-eared Kite (*Milvus lineatus*) in the study, which included data on six bird species. PFHxS was only detected in the liver of the Black-eared Kite, which also had the second highest liver PFOS levels.

An example of the liver PFOS levels of a non-piscivorous bird, the Large-bill Crow (*Corvus macrorhynchos*), is given in the study of Senthilkumar et al. (2007). The liver PFOS levels of the Large-bill Crow was only 13 ng/g ww, much lower than the levels normally found in marine birds. Taniyasu et al. (2003) determined mean PFC levels in the liver of another crow species, the Carrion Crow (*Corvus corone*). PFOS was found in the highest levels, with a mean value of 464 ng/g ww and the PFHxS levels were below the detection limit of 5.5 ng/g ww. This study showed that even terrestrial omnivorous birds could have high levels of PFOS in their livers. The highest PFOS level in the liver of any wild bird (11359 ng/g ww) was measured in the liver of Great Tits from the Antwerp region in Belgium (Dauwe et al., 2007) However, the study area is known as a PFC hotspot since there is a fluoro-chemical plant in the vicinity. Studies in the same area showed that the PFOS liver levels in Great Tit (*Parus major*) and Blue Tit (*Parus caeruleus*) nestlings were within the same range (86 – 3322 ng/g ww) as those found in top mammal predators (Hoff et al., 2005b).

Most of the studies performed to determine the PFC levels in birds were done in the temperate and polar regions of the Northern Hemisphere. However, PFOS levels of 36.7 ng/g ww in the liver and 59.8 ng/g ww in the spleen were measured in Brown Pelican (*Pelecanus occidentalis*) samples from the north coast of Colombia. PFOS has even been measured in the blood (mean: 0.88 nm/mL) and eggs (mean: 2.5 ng/g ww) of South Polar

Skuas (*Stercorarius maccormicki*) from Antarctica (Tao et al., 2006). This indicates that PFCs could be globally distributed in birds.

Samples from museums and specimen banks have been used to determine the temporal trends in PFC levels in the environment. There was an increase in the overall PFC levels in the liver of Thick-billed Murres (*Uria lomvia*) and Northern Fulmars (*Fulmaris glacialis*) from 1975 until 2004. Holström et al. (2005) also found that the PFOS levels increased in the eggs of Common Guillemot (*Uria aalge*) from 1968 to 2003. However, there was a sharp peak in the PFOS levels in 1997 followed by decreasing levels up to 2002.

The relationship between PFCs behaviour and the behaviour of other contaminants have only been investigated for some organic compounds. There was a general difference in behaviour between the lipid-soluble chlorinated and brominated compounds and the protein-bound PFCs in the blood of Lesser Black-backed Gulls from northern Norway (Bustnes et al., 2008).

Objectives

this nature reserve in the region of antwerp
seem to be the most polluted one
known

The main objective of this study is to generate more data on PFC exposure and effects in wildlife to be able perform risk evaluation, focussing mainly on birds.

Chapter 1 investigates the relationship between the levels of PFOS in the liver, brain and feathers of a small passerine, the Zebra Finch (*Taeniopygia guttata*), under laboratory conditions. Since no method exists to extract PFOS from feathers, a new method was developed and tested. Several biochemical endpoints in the serum of the Zebra Finches were also determined as to further our knowledge of the effects of PFOS on passerines.

The influence feeding habits have on the PFC exposure of birds which share the same habitat is reported in **Chapter 2.** The PFC levels in the eggs of four different water bird species were measured to see what role feeding habits play in the exposure of birds. All the eggs were sampled in Blokkersdijk, Belgium, a nature reserve situated in the Antwerp region next to a fluoro-chemical plant. The metal levels in the eggs were also measured to investigate if there is a relationship between the levels of PFCs and metals in the eggs.

In **Chapter 3** the relationship between PFOS levels in bird eggs and the distance of the nests from the fluoro-chemical plant is investigated. Three bird species with different feeding habits were chosen. The area of investigation is also larger than in Chapter 2. Various biomarkers were used to determine the adverse effects of PFOS exposure in one of the species, the Great Tit.

In the previous chapters, the field research was performed in an area known to be PFC contaminated. **Chapter 4** reports on the usefulness of feathers to determine the exposure of birds to PFCs. To determine the influence of feeding habits and ecosystem on the PFC exposure of birds, five bird species from a non-PFC contaminated area in Belgium are studied.

Although the previous chapters focussed on birds, **Chapter 5** reports on the PFOS levels in a terrestrial ecosystem with the Wood Mouse occupying the highest trophic position.

In **Chapter 6** a risk evaluation for birds is done using the data acquired in the previous chapters. The general conclusions and some perspectives for future research are also presented.

CHAPTER 1

Feathers as indicators of Perfluorooctane Sulfonate Exposure in Zebra Finches (*Taeniopygia guttata*)

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Abstract

Perfluorooctane sulfonate (PFOS) has been measured in the liver, blood and brain of various bird species. These birds are mainly high in the food chain and piscivorous. Currently no information is available on the levels of PFOS in the feathers of birds, or on the relationship between the PFOS levels in the liver and the feathers of birds. To investigate this, we exposed Zebra Finches (*Taeniopygia guttata*) to various levels of PFOS through both food and water. We developed a new method to extract and measure PFOS in feathers. This method consists of dissolving the feathers in acid and then using solid phase extraction, followed by HPLC-MSMS to detect the PFOS. We also determined several serum biochemical endpoints to study the effects of PFOS on passerines. The highest PFOS levels were mainly found in the liver, followed by the feathers and then the brain. There was a significant positive correlation between the PFOS levels in the liver and both the brain and feathers. There was no significant correlation between the liver PFOS levels and any of the biomarkers investigated in this study.

1.1 Introduction

Recently more research has been performed on the exposure of wild birds to perfluorooctane sulfonate (PFOS). Most of the studies focused on or included fish-eating birds. These birds occupy a high position in the food chain and are thus expected to accumulate pollutants. Both blood and egg yolk samples of cormorants and gulls from the Great Lakes region in North America contained PFOS. Bald Eagles (*Haliaeetus leucocephalus*) from the same area even had PFOS plasma levels as high as 2220 ng/mL (Kannan et al., 2001). Even in more remote areas, such as the Barents Sea, mean levels of 13.5 ng/g ww for Black Guillemot (*Cepphus grille*) and 65.8 ng/g ww in Glaucous Gull (*Larus hyperboreus*) were found in liver (Haukås et al., 2007). Kannan et al. (2002a) determined the PFOS levels in the liver of various birds in Japan, with the highest values found in Common Cormorant (*Phalacrocorax carbo*). The values ranged from 1873 to 2249 ng/g ww, showing that birds can also contain high levels of PFOS.

Due to their small size and lower position in the food chain, passerines are not usually used in biomonitoring studies. The only passerines previously studied were crows. Senthilkumar et al. (2007) measured PFOS levels of 13 ng/g ww in the liver of the Large-bill Crow (*Corvus macrorhynchos*). PFOS levels as high as 1200 ng/g ww were detected in the liver of Carrion Crow (*Corvus corone*), also from Japan (Taniyasu et al., 2003). However, crows are generally larger and higher in the food chain than most passerines and therefore not the ideal examples of passerines. Only two previous studies were devoted entirely to the exposure of small passerines to PFOS. The PFOS liver levels in Great Tit (*Parus major*) and Blue Tit (*Parus caeruleus*) nestlings near a fluorochemical plant in Antwerp, Belgium, were within the same range as those found in top mammal predators. The highest concentration, 3322 ng/g ww, was from a Blue Tit nestling (Hoff et al., 2005b). Dauwe et al. (2007) described the relationship between PFOS levels in the liver and serum of Great Tits from the same polluted area. A clear relationship was found between the PFOS levels in liver and serum. The highest level of PFOS in a wild bird ever measured, namely 11359 ng/g ww, was determined in the liver of one of the Great Tits from this study. These two studies indicated that the levels of PFOS in passerines could also be very high. However, it should be taken into consideration that both studies were performed near a fluorochemical plant with a documented history of PFOS contamination in the nearby area (Hoff et al., 2005a, 2005b).

Currently the liver is used as the organ of choice to determine birds' exposure to PFOS. Alternative, less invasive techniques have also been proposed. Several studies have shown that both serum and eggs can be used as matrices for the determination of PFOS exposure (Dauwe et al., 2007; Verreault et al., 2005; Van den Brink et al., 2007; Van den Heuvel-Greve et al., 2006). As mentioned previously, Dauwe et al (2007) found a positive correlation

between the PFOS levels in the liver and serum of Great Tits from Antwerp, Belgium. PFOS levels measured in egg, liver and plasma samples of Glaucous Gulls were all within the same range, indicating that all of these matrices can be used to determine PFOS exposure (Verreault et al., 2005). Another study found no significant difference between the PFOS levels in the liver of female Common Terns (*Sterna hirundo*) and their eggs (Van den Brink et al., 2007). A high, although not significant, correlation was found between the mother bird and her clutch of eggs. Even though eggs and blood are good indicators of PFOS exposure, there are also some disadvantages in using these matrices. Blood has the disadvantage that it may be dangerous to small birds if too much is collected or if the bird is already in ill health. The collection of eggs may result in the parents abandoning the nest and the rest of the eggs. Eggs can also not be collected from scarce and vulnerable species.

Feathers are connected to the blood stream during the formation and growth period. In the blood stream, PFOS binds mainly to albumin (Jones et al., 2003). If PFOS is deposited in the feathers, they could contain PFOS, although the feathers are not connected to the blood system when fully grown. Currently no information is available on the use of feathers as reliable indicators of PFOS exposure. However, feathers have successfully been used in the biomonitoring of various other pollutants. Feathers may thus be a good alternative to using more invasive matrices for the determination of PFOS exposure in birds.

During recent years, the use of feathers in biomonitoring programmes has increased. The European Union already uses feathers as an integrated part of their marine biomonitoring programmes using Ecological Quality Objectives (ICES, 2003). In Belgium, a number of studies have been done on the use of tits as bioindicators, for both metal and organic pollution (Dauwe et al., 2002b; Eens et al., 1999; Janssens et al., 2001 Dauwe et al., 2004). Burger (1993) gave several advantages of using feathers as indicators of environmental pollution. The most important is that feathers are non-invasive and can thus be collected routinely without much stress to the birds. The same birds can also be routinely sampled, so time-trends can be investigated. Feathers from museum collections can be used to determine historical values. More scarce or vulnerable species can also be sampled.

Rats and monkeys have mainly been used in laboratory studies dealing with the effects of PFOS (Seacat et al., 2002; Seacat et al., 2003). Studies dealing with the effects of PFOS on birds have mainly been performed on Northern Bobwhites (*Colinus virginianus*) and Mallards (*Anas platyrhynchus*), although in one study chickens were used (Newsted et al., 2006; Newsted et al., 2007; Yeung et al., 2007). The little information available on the effects of PFOS on birds is thus limited to a few species. Therefore, more data are needed on both the effects and exposure to PFOS in the smaller birds. The data on biomarkers from studies on

wild birds should also be corroborated with data from controlled laboratory experiments, to ensure the effects seen in wild birds are due to PFOS exposure.

The aim of our study was to determine the relationship between the levels of PFOS in the liver, brain and feathers of a small passerine. The Zebra Finch (*Taeniopygia guttata*) was chosen as it has been the model species for passerines in various experiments, including the evaluation of the use of feathers in the biomonitoring of heavy metals and the determination the effects of heavy metals on passerines (Dauwe et al., 2002a; Gong et al., 1999; Hoogesteijn et al., 2005; Snoeijs et al., 2005). No method exists to extract PFOS from feathers; therefore, we developed and tested a new method. Several biochemical endpoints in the serum of the Zebra Finches will also be determined as to further our knowledge of the effects of PFOS on passerines.

1.2. Materials and Method

1.2.1 Animals, exposure and sampling

Sixty adult Zebra Finches were used in the experiment. Thirty male and 30 female birds were purchased from Aquaservice (Berchem, Belgium). The birds were housed in wooden breeding cages for 21 days at 25°C days before the start of the experiment. The birds were fed commercial bird feed and water ad libitum. The birds were divided into 6 groups of 10 individuals each. During acclimation, three birds died and were not replaced. One group was the control group. Two groups were exposed to respectively 1 mg/kg (F1) and 2 mg/kg (F2) PFOS in their food. Three groups were exposed to PFOS through water. The three exposure groups are 1 mg/L (W1), 2 mg/L (W2) and 5 mg/L (W5). The birds were exposed to these concentrations for 31 days. These concentrations were calculated using the LC_{50} value for Northern Bobwhites, which is 220 mg/kg (Gallagher et al., 2004). The LC_{50} value was divided by 10, as the Northern Bobwhites weighs about 10 times more than Zebra Finches. This value was then divided by 10 again to get the highest exposure concentration. The food was prepared according to the method of Seacat et al. (2003), with modifications. At the start of the exposure, the two outer most tail feathers on each side of the tail were removed. After the exposure, the birds were sacrificed by an overdose of ethyldiether and then decapitated (Friend and Franson, 1999; AVMA, 2001). The birds were weighed and the tarsus length was measured to determine the body condition index (Merilä et al., 1999). Shortly, the correlation between the body weights and tarsus lenghts were determined for each exposure group to assess the general health of the birds. Blood was collected and centrifuged (2000 rpm, 5 min) before being stored at -80°C until further analysis. The liver and brain of each bird were also dissected out, weighed and stored at -20°C until further analysis. The four re-grown outer most tail feathers of each wing were pulled out and used for the PFOS determination.

1.2.2 Biomarker assays

The serum alanine aminotransferase activity was determined by the spectrophotometric method of Bergmeyer et al. (1986a; 1986b). The cholesterol concentration was measured according to Allain et al. (1974) and the triglyceride concentration according to the method of Spayd et al. (1978). The serum protein content was determined with the Bio-Rad Protein Assay (Bio-Rad, Munich, Germany). For the determination of the haematocrit, the relative red blood cell volume was determined after centrifugation of heparinised blood in sealed glass capillaries (2000 rpm, 5 min).

1.2.3 PFOS extraction and clean up in the organs

PFOS extraction from the organs was done by solvent extraction based on the method by Berger and Haukås (2005), with adaptations. Briefly, each sample was homogenized in a polypropylene (PP) centrifuge tube using a mixer and then weighed. Internal standard (1H,1H,2H,2H-PFOS) and either 9 mL (liver samples) or 4.5 mL (brain samples) of acetonitrile were added. The PP tube was capped and the sample was thoroughly mixed using a Vortex chemical mixer. The sample was then extracted 3 times for 10 minutes in an ultrasonic bath at room temperature. Between each period of 10 minutes, the samples were thoroughly mixed. The samples were then centrifuged at 2 500 rpm for 5 minutes. One mL of the final supernatant was transferred to a micro vial containing approximately 25 mg of activated carbon and 50 µL glacial acetic acid. The sample was then mixed for 1 minute using a vortex mixer. After centrifugation (10 000 rpm, 10 minutes) 500 µL of the supernatant was transferred to a clean micro vial.

1.2.4 PFOS extraction and clean-up in feathers

The samples were weighed and then vigorously washed in acetonitrile to remove any external contamination. After having dried at room temperature, the feathers were cut into small pieces and put into a PP tube. To this 5 mL HNO_3 (69%) and 20 µl ISTD (^{13}C-PFOS) were added and left for 24 hours at room temperature. The samples were then neutralised by adding NaOH. To remove any particles the samples were filtrated under vacuum through glass fibre filter (142 mm). The samples were then extracted by solid phase extraction using Oasis HLB Plus SPE cartridges. The whole system was rinsed with 20 ml acetonitrile. Extraction took place at a flowrate of approximately 2 drops/sec. The SPE cartridge was rinsed with 2 ml acetonitrile/water (40/60) and eluted with 8 ml acetonitrile. The extract was concentrated to 1 mL at room temperature and then transferred to a micro vial containing approximately 25 mg of activated carbon and 50 µL glacial acetic acid. The sample was then mixed for 1 minute using a vortex mixer. After centrifugation (10 000 rpm for 10 minutes) 500 µL of the supernatant was transferred to a clean micro vial.

1.2.5 Determination of PFOS concentrations

The concentrations of PFOS were measured using combined liquid chromatography-mass spectrometry according using a CapLC system (Waters, USA) connected to a Quadrupole-LIT quadrupole mass spectrometer (Applied Biosystems, UK). Aliquots of 5 µl were loaded on an Optiguard C18 pre-column (10 mm x 1 mm i.d., Alltech, USA). The analysis was performed on a Fluophase PFP column (50 mm x 1 mm i.d., Thermo, USA) at a flow rate of 40 µl/min. The mobile phase was 2 mM NH4OAc (A) / Acetonitrile (B). A gradient elution was used starting at 35 % B and going to 90 % B in 5 min. At 5 min and 6 seconds, the initial conditions were resumed. PFOS was measured under (-) electrospray ionisation using the transitions from mother to daughter ion to identify them (Table 1). The dwell time was 0.1 s. The ES-capillary voltage was set at –4.5 kV and the cone voltage -100 kV. The PFOS concentration was calculated using an unextracted calibration curve.

Table 1. The transition from mother to daughter ion used to identify PFOS and the Internal Standards.

Compound	Mother Ion	Daughter Ion
PFOS	499	80; 99
1H,1H,2H,2H-PFOS	427	81
^{13}C-PFOS	503	80; 99

1.2.6 Quality Control

Laboratory blanks were extracted along with each batch of samples, consisting of all the solvents but having no sample. Spiked chicken liver samples were also extracted along the organ samples to measure recovery. In the case of the feather extraction, spiked samples consisted of acetonitrile spiked with PFOS. Recovery rates ranged between 75 and 105%. Pure acetonitrile was injected after every 8 samples to check for memory effects. A standard was injected after every 8 samples to check the stability of the HPLC-MS/MS system. After each injection of a solvent standard or spiked sample, pure acetonitrile was also injected.

1.2.7 Data Analysis

The chromatograms were analysed with Analyst 1.4 for Windows. The statistical analysis was done with SPSS 15.0 for Windows. The parametric ANOVA test was used for the comparison of the PFOS levels in the liver, brain and feathers. For the comparison of the biomarker endpoint data of the various exposure groups, the non-parametric Man Whitney-U test was used. The correlations between the PFOS levels in the liver and the PFOS levels in the brain and feathers were determined with the Pearson test. Spearman's Rank correlation was used to determine the relationships between the liver PFOS levels and the biomarker endpoints.

1.3 Results

The PFOS levels in the liver, brain and feathers of the birds from the various exposure groups are summarized in Table 2. All significant differences (ANOVA, $p < 0.05$) between the exposure groups are also indicated in Table 2. In each exposure group the highest concentrations were found in the liver, followed by the feathers and then the brain. Only in the F2 (2 mg PFOS/kg food) group the brain PFOS levels were higher than in the feathers. There was an increase in the PFOS levels with an increase in the exposure concentration for both types of exposure routes.

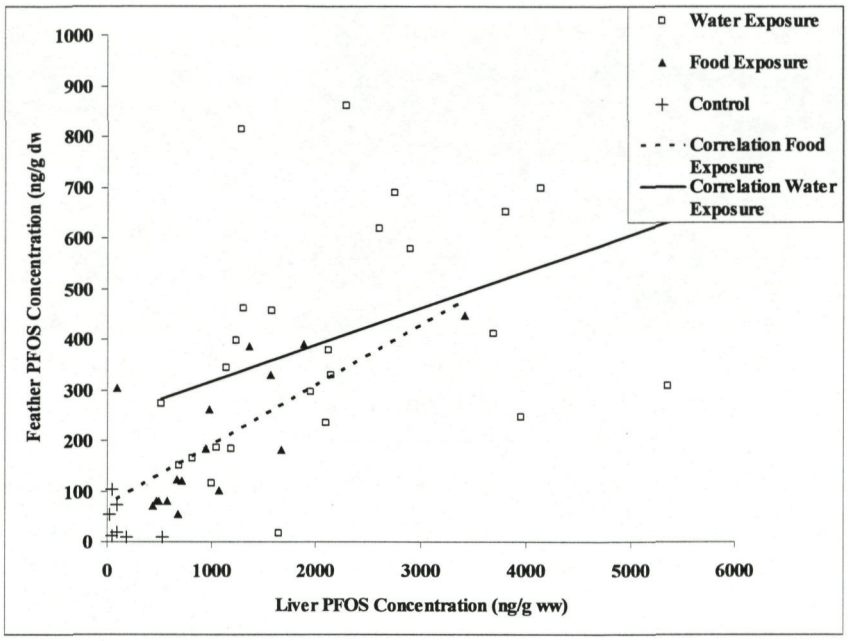
Table 2. The levels of PFOS in the liver, brain and feathers for the various exposure groups. Values are given as mean ± SEM. Significant differences ($p < 0.05$) between the exposure concentrations within either the food or water exposure group are indicated by the same letter.

Exposure Group	Liver (ng/g ww) n =45	Brain (ng/g ww) n = 39	Feathers (ng/g dw) n = 39
Control	201.6 ± 64.4 ^{a,b,e}	5.4 ± 0.7 ^{a,b,e}	39.9 ± 14.0 ^{a,b,d}
W1	1196.1 ± 156.4 ^{c}	85.1 ± 16.1 ^{c}	209.7 ± 47.0 ^{c}
W2	1997.5 ± 299.2 ^{a,d}	235.3 ± 34.2 ^{a,c,d}	434.9 ± 75.8 ^{a,c}
W5	3652.9 ± 505.5 ^{b,c,d}	440.5 ± 69.5 ^{b,c,d}	572.5 ± 55.8 ^{b,c}
F1	617.8 ± 47.2 ^{f}	82.2 ± 5.7 ^{f}	86.2 ± 9.4 ^{e}
F2	1437.7 ± 302.3 ^{e,f}	433.3 ± 38.2 ^{e,f}	288.7 ± 38.1 ^{d,e}

Exposure Groups: W1: 1 mg PFOS/L water; W2: 2 mg PFOS/L water; W5: 5 mg PFOS/L water; F1: 1 mg PFOS/kg food; F2: 2 mg PFOS/kg food.

There was a significant positive correlation between the PFOS levels in the liver and the feathers for both exposure through water (Pearson, $p < 0.001$, $R = 0.627$, $n = 33$) and food (Pearson, $p < 0.001$, $R = 0.776$, $n = 27$) (Figure 1A). Between the PFOS levels in the liver and the brain there was also a significant, positive correlation, for both the exposure through water (Pearson, $p < 0.001$, $R = 0.910$, $n = 33$) and food (Pearson, $p = 0.001$, $R = 0.633$, $n = 27$) (Figure 1B). The average ratios between the PFOS levels in the liver and feathers and between the liver and brain are summarized in Table 3. These ratios were the same for the liver versus feathers and liver versus brain in the food exposure group, but not for control or water exposure group.

A.



B.

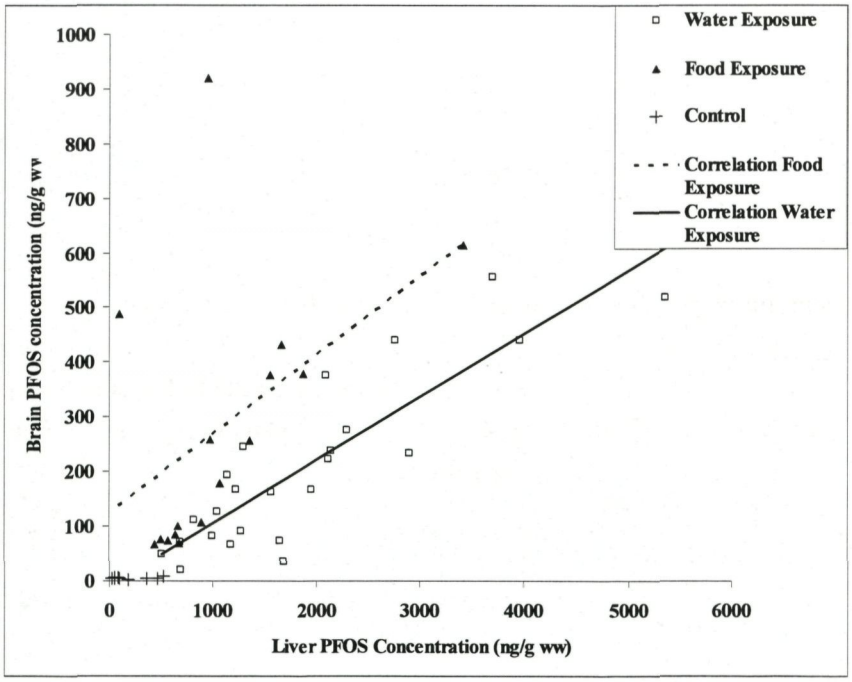


Figure 1. The correlations between the liver PFOS levels and the feather PFOS levels (A), and the brain PFOS levels (B)

Table 3. The average ratios between the PFOS levels in the liver and feathers and between the liver and brain.

Exposure route	liver:feathers	liver:brain
Control	1:13	1:43
Water	1:6	1:13
Food	1:6	1:6

The results of the various biomarkers are summarized in Tables 4 and 5. The serum cholesterol concentrations decreased as the PFOS exposure through water increased. This was not the case for the PFOS exposure through food. The serum triglyceride concentrations were lower in the exposed groups than in the control group. There was no visible trend for the response of relative liver weight, haematocrit, serum protein concentration and serum alanine aminotransferase activity. For both types of exposure groups, the body condition index increased with the increase in PFOS exposure (Figure 2). There was no significant correlation between the liver PFOS levels and any of the biomarkers.

Table 4. The results of the serum alanine aminotransferase activity, serum triglyceride concentration and serum cholesterol concentration for the various exposure groups. Values are given as mean ± SEM. Significant differences ($p < 0.05$) between the exposure concentrations within either the water or food exposure group are indicated by the same letter.

Exposure Group	Serum alanine aminotrans-ferase activity (U/L)	Serum alanine aminotrans-ferase activity (U/g protein)	Serum Triglyceride concentrations (mg/dL)	Serum cholesterol concentration (mg/dL)
Control	98.4± 12.1	$3.0 \pm 0.3^{a,b}$	421.9 ± 25.0^{a}	123.7 ± 6.8
W1	162.3 ± 79.8	2.3 ± 0.4	444.4 ± 39.5^{b}	104.5 ± 0.5
W2	110.4 ± 21.5	3.0 ± 0.6	380.8 ± 31.3^{c}	101.3 ± 9.4
W5	111.0 ± 20.4	4.8 ± 0.6^{a}	$256.0 \pm 20.4^{a,b,c}$	99.9 ± 8.0
F1	134.8 ± 12.0^{a}	$6.0 \pm 0.8^{b,c}$	302.3 ± 50.6^{d}	105.2 ± 8.6
F2	77.3 ± 13.8^{a}	2.6 ± 0.5^{c}	458.7 ± 13.8^{d}	121.1 ± 10.3

Exposure Groups: W1: 1 mg PFOS/L water; W2: 2 mg PFOS/L water; W5: 5 mg PFOS/L water; F1: 1 mg PFOS/kg food; F2: 2 mg PFOS/kg food.

Table 5. The results of the body condition index, relative liver weight, serum protein concentration and haematocrit for the various exposure groups. Values are given as mean ± SEM, except for the body condition index. Significant differences ($p < 0.05$) between the exposure concentrations within either the water or food exposure group are indicated by the same letter.

Exposure Group	Body condition index	Relative Liver Weight	Serum protein concentration (g/L)	Haematocrit (%)
Control	0.19	2.8 ± 0.2	32.7 ± 1.8[a,d]	47.9 ± 1.6
W1	0.31	2.6 ± 0.2	33.5 ± 4.7[b]	48.7 ± 2.6
W2	0.53	2. 8 ± 0.1	35.0 ± 2.9[c]	48.5 ± 1.1
W5	0.92	2.5 ± 0.1	23.3 ± 1.6[a,b,c]	50.2± 1.4
F1	0.35	3.0 ± 0.3	27.2 ± 2.0[d]	43.7 ± 1.4
F2	0.75	2.9 ± 0.1	28.2 ± 1.2	48.0 ± 2.1

Exposure Groups: W1: 1 mg PFOS/L water; W2: 2 mg PFOS/L water; W5: 5 mg PFOS/L water; F1: 1 mg PFOS/kg food; F2: 2 mg PFOS/kg food.

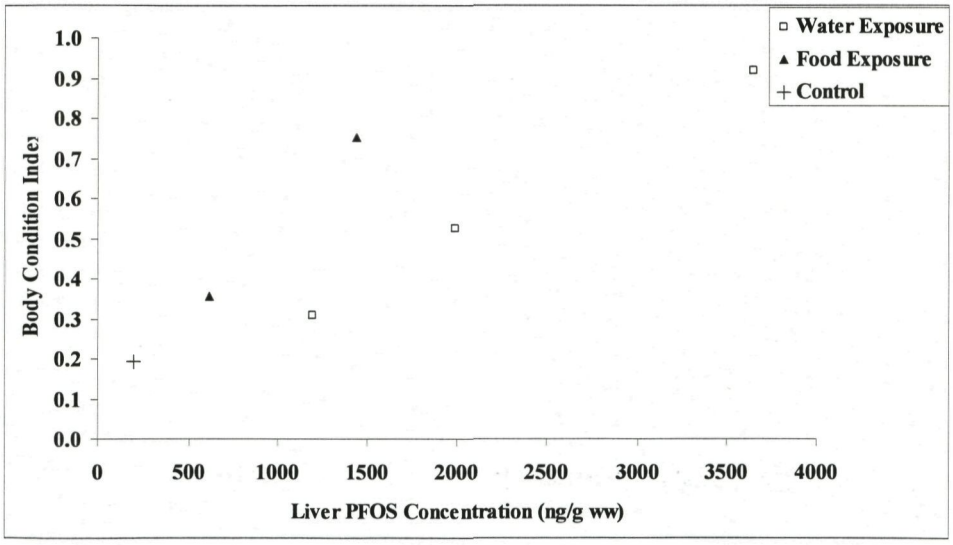


Figure 2. The correlations between the liver PFOS levels and the body condition index.

1.4 Discussion

Blood-rich organs, for example the liver and spleen, have mainly been used to determine birds' exposure to PFOS. It is known that these organs accumulate PFOS. Currently no data is available on the PFOS levels in feathers. The advantage of using feathers as indicators of PFOS exposure is that they are less invasive than other matrices.

Since high levels of PFOS were also measured in the feathers, the method we developed should be able to detect PFOS in the feathers of wild birds. Although the feather PFOS levels were lower than in the liver, they were still very high. This may be because PFOS is transported in the blood to the feathers during feather formation and growth and could then be deposited in the feathers.

The ratio between the brain and liver PFOS levels measured in the control group of our study is of the same order of magnitude as for the brain and liver PFOS level ratio (1:40) in Glaucous Gulls (Verreault et al., 2005). The ratios were smaller and more constant between liver and feathers than between liver and brain. This indicates that the ratio between feathers and liver could be less dependent on the exposure route. The significant positive correlations between the PFOS levels in the feathers and the liver from our study show that feathers can be used as indicators of liver PFOS levels.

Only two exposure points were used in the exposure through food group, which may therefore give a good correlation between the PFOS levels in the feathers and in the liver. However, even when only looking at the water exposure group, where there were three exposure concentrations, there was still a high correlation between the PFOS levels in the feathers and the liver, indicating that feathers could give a good indication of PFOS exposure in birds. Using feathers as indicators of the PFOS exposure of birds is a less invasive technique than using blood or eggs. Although we also found significant correlations between the brain and liver PFOS levels for both exposure groups, the brain should not be considered as a practical bioindicator. The birds have to be sacrificed and thus routine sampling cannot be done. In our study, the brain PFOS levels were also in general lower than those in feathers or the liver. This means that low levels of PFOS exposure might not be detected in the brain. The method to extract and measure PFOS in feathers still needs to be optimised and tested using field samples. It also shows that the route of exposure does not play a significant role in the accumulation of PFOS in the tissues. For both exposure groups the correlation was both positive and significant, although it should be noted that the food exposure group only consisted of 20 birds in total. Feathers have already been successfully used as indicators of exposure to other pollutants, such as metals and organic pollutants. International organisations also include feathers in their biomonitoring schemes (ICES, 2003). Feathers thus show promise as a non-invasive indicator of PFOS exposure in birds, which can be routinely sampled from the same individual (Burger, 1993).

Since PFOS is found in the blood, where it binds to albumin, tissues rich in blood, like the liver and spleen, would therefore have higher levels of PFOS than the brain. Verreault et al. (2005) found higher PFOS levels in the liver than in the brain of Glaucous Gulls, the same as

in our study. Except for plasma, the liver is usually the tissue with the highest level of PFOS in birds (Dauwe et al., 2007; Giesy and Kannan, 2001; Van den Brink et al., 2007; Verreault et al., 2005). The liver PFOS levels in the present study falls within the same range (86 – 11 359 ng/g ww) as those of Great Tits and Blue Tits from the Antwerp area, Belgium (Dauwe et al., 2007; Hoff et al., 2005b). Others studies on birds also found liver PFOS levels in the same range (33 – 1780 ng/g ww) (Giesy and Kannan, 2001; Kannan et al., 2001; Kannan et al., 2002a; Van den Brink et al., 2007). However, the PFOS levels in the liver and brain of this study were much higher than levels found in some studies on birds (Bossi et al., 2005; Kannan et al., 2002b; Martin et al., 2004b; Tao et al., 2006; Verreault et al., 2005). Still, the data obtained in this study could therefore be comparable to data from field studies.

This experiment shows that there is still a lot of research needed to find a biomarker that is specific for PFOS exposure. The only other study performed on passerines used mostly the same biomarkers as this study (Hoff et al., 2005b). The liver PFOS levels of the birds from both our study and the study of Hoff et al. fall within the same range (86 – 5 561 ng/g ww), thus comparison of the biomarker results is possible. The values for the serum cholesterol concentrations were lower in the current study (< 45 – 162 mg/dL) than in the study by Hoff et al. (100 – 240 mg/dL). The relative liver weight (1.9 – 4,2) and serum triglyceride concentrations (61 – 548 mg/dL) were in the same range in both studies. In this study, the haematocrit values and the serum alanine aminotransferase activity (both expressed as U/L and U/g) were higher than in the previous study. This may be to different species being used, although they are closely related. Age may also play a role, as nestlings were used in the first study and adults were used in this study. The higher the body condition index, the worse the general health of the bird is (Merilä et al., 1999). There was an increase in the body condition index with an increase in the PFOS exposure levels. This indicates that the general health of the birds worsened with an increase in PFOS exposure. The body condition index was the only biomarker that showed a clear dose-response relationship.

Hoff et al. (2005b) also found a significant negative correlation ($r = -0.30$, $p = 0.04$) between serum triglyceride concentration and liver PFOS levels in two tit species. This has also been shown in laboratory experiments with mammals (Seacat et al., 2002; Seacat et al., 2003). No biomarker used in our study showed the same correlation as in the study of Hoff et al. Since this study was a controlled laboratory exposure and the study of Hoff et al. a field study, differences are likely to occur. Other variables might have caused the response in the tits from the field study. Most of the biomarkers only indicated a decrease in the general condition of the birds with an increase in PFOS exposure. More studies are needed to find biomarkers that are specific to PFOS. Previous studies have shown that PFOS might affect the lipid metabolism (Seacat et al., 2002; Seacat et al., 2003), therefore serum triglyceride

concentration might be the most suitable biomarker to use for birds at this moment. Other biomarkers of the lipid metabolism and liver integrity should also be investigated.

1.5 Conclusions

PFOS was successfully measured in the feathers of laboratory exposed Zebra Finches. There was a significant positive correlation between the PFOS levels in the feathers and the liver of the birds. This experiment has thus shown that feathers could be used as indicators of PFOS exposure in birds.

CHAPTER 2

Role of water bird feeding habits on PFC egg levels from an industrial polluted site

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Abstract

Although many studies have been performed on the perfluorinated chemicals (PFCs) exposure in birds, only one study determined the influence of diet on the PFC exposure levels in birds. Sinclair et al. (2006) found that piscivorous ducks had a 2.5 times higher liver PFOS level than herbivorous ducks. However, not all the samples were from exactly the same location. No previous study has investigated the influence of feeding habits on the PFC exposure of birds from the same locality. In this study we determined the PFC levels in the eggs of Canada Goose (*Branta canadensis*), Common Pochard (*Aythia ferina*), Gadwall (*Anas strepera*) and Eurasian Coot (*Fulica atra*) from Blokkersdijk, Belgium, a site located next to a fluoro-chemical plant. The highest levels of PFCs in bird eggs (range 220 – 17214 ng/g ww for PFOS) were measured in this study. The Common Pochard eggs had the highest mean value for all the PFC levels and the Canada Goose eggs the lowest. The herbivorous birds had significantly lower levels of PFCs than the omnivorous birds. In addition, the birds that spend more time feeding in the water had higher levels of PFCs in their eggs. Therefore, it can be seen that even for birds that share the same ecosystem, both their feeding habit and diet might play a role in their exposure to PFCs. We also measured the metal levels to investigate if there is a relationship between the levels of PFCs and metals in the eggs. No clear correlation pattern was found between the levels of PFCs and metals in the water bird eggs.

2.1 Introduction

Birds have been shown to be particular useful bio-indicators because they are comparatively more sensitive to toxicants and are positioned high on the food chain and as such form important structural components of the ecosystem (Burger and Gochfeld, 1995). In addition, birds have drawn high public sentiments and general interest. Recently studies have shown high levels of perfluorinated chemicals (PFCs) in birds (Giesy and Kannan, 2001; Kannan et al., 2002a). PFCs have been in production and used widely for a few decades, however, their distribution in the environment has only recently been studied (Giesy and Kannan, 2002). The lack of reliable measuring techniques has been the main reason for this (van Leeuwen et al., 2007). Most studies focusing on determining the PFC levels in birds use blood or blood-rich organs (Giesy and Kannan, 2001; Kannan et al., 2001). Many studies have been performed on the levels of PFCs in fish-eating birds, since they are high in the food chain. Kannan et al. (2005) measured levels as high as 1740 ng/g ww in the liver and 1480 ng/g ww in the kidney of Bald Eagles (*Haliaeetus leucocephalus*) from Michigan in the United States of America (USA). Sinclair et al. (2006) showed that piscivorous birds had higher liver PFOS levels than non-piscivorous birds in New York State, USA. However, the birds were not all from the same location. No study has been performed to evaluate the role that feeding habit play in the PFC exposure of birds from the same locality.

It is known from laboratory studies that female birds transfer perfluorooctane sulfonate (PFOS), the PFC most commonly found in wildlife, to their eggs. The greatest portion of the PFOS burden in the eggs was associated with very low-density lipoproteins in the yolk (Newsted et al., 2007). Van den Brink et al. (2007) indicated that there was no significant difference between the PFOS levels found in the mother birds and those in the eggs in Common Tern (*Sterna hirundo*). Therefore eggs can be used as less invasive bio-indicators of PFC exposure in birds. Eggs have also been used to determine time-trends in PFC levels in Common Guillemot (*Uria aalge*) from the Baltic Sea (Holström et al., 2005).

The relationship between or the co-occurrence of PFCs and organic pollutants have previously been studied in birds. Bustnes et al. (2008) found a difference in the general behaviour of PFCs and organic pollutants in Lesser Black-backed Gulls (*Larus fuscus*). Both PFCs and metals have been measured in high levels in bird eggs (Gochfeld et al., 1998; Holström et al., 2005; Mora et al., 2008; Van den Brink et al., 2007). Currently no research has been performed to establish whether there is a relationship between PFC and metal tissue concentrations. Such a relationship is important as it can provide information about possible co-exposure routes or similar bioaccumulation patterns of these chemicals in birds.

In this study we investigated if feeding habits have an influence on the PFC exposure of birds which share the same habitat. Four water bird species with different feeding habits were chosen. The Canada Goose (*Branta canadensis*) is a grazer feeding mainly on grass and other plants close to water (del Hoyo et al., 1992). The Common Pochard (*Aythia ferina*) and Gadwall (*Anas strepera*) are duck species. The Common Pochard is a diving duck and feeds on plants and small animals found at the bottom of ponds and lakes, while the Gadwall is an herbivorous dabbling duck, feeding on aquatic plants from on or just below the water surface (del Hoyo et al., 1992). The Eurasian Coot (*Fulica atra*) is an omnivorous rail that feeds both in water and on land (del Hoyo et al., 1996). The levels of PFCs in the eggs were measured to see what role feeding habit play in the exposure of birds. All the eggs were sampled in Blokkersdijk, Belgium. Blokkersdijk is a nature reserve situated in the Antwerp region next to a fluoro-chemical plant, which produced PFOS until 2002 when production started to be phased out. PFOS levels measured in European Eel (*Anguilla anguilla*), Common Carp (*Cyprinus carpio*), Wood Mice (*Apodemus sylvaticus*), Great Tit and Blue Tit from this area have been some of the highest in the world (Dauwe et al, 2007; Hoff et al., 2005a, 2005b). We also measured the metal levels to investigate if there is a relationship between the levels of PFCs and metals in the eggs. Blokkersdijk is also located in the proximity of the city and port of Antwerp, increasing the risk of pollution from various contaminants including metals.

2.2 Materials and Methods

2.2.1 Sample collection and storage

The following water birds were chosen for the study: Eurasian Coot, Common Pochard, Canada Goose and Gadwall. Eggs were collected in the spring of 2004 in Blokkersdijk, Belgium. In total 35 eggs were collected from different nests of the four species. The eggs were stored at -20°C until analysis.

2.2.2 PFC extraction and measurement

PFC extraction from the eggs was done by solvent extraction based on the method by Berger et al. (2005) with adaptations. Briefly, initially about 1 g of each sample was homogenized in a polypropylene (PP) centrifuge tube using a mixer. The exact weights of the homogenate were then recorded. Internal standard (^{13}C-PFOS and ^{13}C-PFOA) and 4.5 mL of acetonitrile were added in each tube. The tubes were then capped and the samples were thoroughly mixed using a Vortex chemical mixer. The samples were then extracted 3 times for 10 minutes in an ultrasonic bath at room temperature. Between each period of 10 minutes, the samples were again thoroughly mixed. The samples were then centrifuged at 2500 rpm for 5 minutes. One mL of the final supernatant was transferred to a micro vial containing approximately 25 mg of activated carbon and 50 µL glacial acetic acid. The sample was then mixed for 1 minute

using a vortex mixer. After centrifugation (10000 rpm, 10 minutes) 500 µL of the supernatant was transferred to a clean micro vial.

The concentrations of PFC were then measured using combined liquid chromatography-mass spectrometry accordingly using a CapLC system (Waters, USA) connected to a Quadrupole-LIT quadrupole mass spectrometer (Applied Biosystems, UK). Aliquots of 5 µl were loaded on an Optiguard C18 pre-column (10 mm x 1 mm i.d., Alltech, USA). The analysis was performed on a Fluophase PFP column (50 mm x 1 mm i.d., Thermo, USA) at a flow rate of 40 µl/min. The mobile phase was 2 mM NH4OAc (A) / Acetonitrile (B). A gradient elution was used starting at 35 % B and going to 90 % B in 5 min. At 5 min and 6 seconds the initial conditions were resumed. The PFCs were measured under (-) electrospray ionisation using the transitions from mother to daughter ion to identify them (Table 1). The PFCs measured were PFOS, perfluorohexane sulfonate (PFHxS), perfluorooctanoic acid (PFOA) and perfluorononanoic acid (PFNA). The dwell time was 0.1 s. The ES-capillary voltage was set at –4.5 kV and the cone voltage -100 kV. The PFC concentration was calculated using an unextracted calibration curve. The chromatograms were analysed with Analyst 1.4 for Windows.

Table 1. The transition from mother to daughter ion used to identify the various PFCs and the Internal Standards.

Compound	Mother Ion	Daughter Ion
PFHxS	399	80; 99
PFOS	499	80; 99
^{13}C-PFOS	503	80; 99
PFOA	413	369
^{13}C-PFOA	417	372
PFNA	469	419

2.2.3 Metal determination

Sample preparation and acid digestion was done according to the methods of Blust et al. (1988). Briefly, about 0.1 g of wet samples were weighted and put in polypropylene tubes. The samples were dried to constant weight at 60 °C and then cooled down in a desiccator and the dry weights were determined. For each tube, 200 µL of highly purified concentrated nitric acid were added. The digestion was completed by heating in a microwave oven. The samples were initially digested 3 times for 2 minutes at each of the following power levels: 100 W, 150 W and 250 W. Finally, 50 µl of high purity hydrogen peroxide was added and the samples were then further digested at 350 W in 3 steps of 2 minutes each. The solutions were then diluted to 2 mL with Milli-Q water. Three blanks and five reference samples of skim

milk powder (BCR 151, Institute of Reference Materials and Measurements, European Commission, Geel, Belgium) were included and followed the sample process as the egg samples.

Metal concentrations in the diluted solutions were measured with Inductively Coupled Plasma Mass Spectrometer (ICP-MS, 810-MS, Varian, Australia). Analytical interference resulting from sample matrix were corrected by means of an internal standard method using yttrium for the lighter masses (Cr, Mn, Fe, Co, Ni, Cu, Zn, As) and indium for heavier masses (Cd, Pb) (Taylor et al., 1997; Mubiana et al., 2005). After analysis, metal concentrations in the eggs were calculated and expressed on dry weight basis (i.e. μg metal/g dry weight of egg).

2.2.4 Quality Control

For the PFC analysis, laboratory blanks were extracted along with each batch of samples, consisting of all the solvents but having no sample. Spiked chicken egg samples were also extracted along the water birds egg samples to measure recovery. Recovery rates varied between 86 and 114 %. Pure acetonitrile was injected after every eight samples to check for memory effects. A standard was injected after every eight samples to check the stability of the HPLC-MS/MS system. After each injection of a solvent standard or spiked sample, pure acetonitrile was also injected. The limit of detection (LOD) was based on a signal-to-noise ratio of three in the chromatogram of extracts of spiked chicken egg samples that did not contain any detectable amounts of PFCs. This incorporates all the dilution factors occurring during the extraction method. The LOD was 2.7 ng/g for PFOS, 3.1 ng/g for PFHxS, 6.9 ng/g for PFOA and 7.8 ng/g for PFNA.

For metal determinations, quality control was ensured based on the good recoveries achieved in the reference samples. Measured concentrations of certified (Cd, Co, Fe, Pb) and indicative (Co, Mn, Ni, Zn) analytes in the reference material BCR 151, were consistently between 95 – 105 % of the certified values. Furthermore, the use of internal standards during analysis with ICP-MS ensured accurate quantitative determination of metal concentrations in the solutions.

2.2.5 Data Analysis

The statistical analysis was done with SPSS 15.0 for Windows. The PFC data were log-transformed to meet the assumptions of normality. A parametric one-way ANOVA test was used for the comparison of the PFC levels in the different species. The non-parametric Mann-Whitney test was used to determine if there were any significant differences in the metal content between the species. The correlations between the PFC levels and the metals levels were determined with Spearman Rank analysis.

2.3 Results

The PFC levels in the eggs are summarised in Table 2. PFOS and PFHxS were measured in all the samples, whereas PFOA was below the detection limit in two of the Canada Goose samples. PFNA, however, was only detected in 23% of the samples. There was a general trend in the levels of PFCs in the eggs of the different bird species. The Common Pochard always had the highest levels, followed by the Eurasian Coot and then the Gadwall. The Canada Goose consistently showed the lowest PFC levels. The PFOS levels between the four species were significantly different from one another (One-way Anova, $p < 0.05$).

Table 2. The levels of PFCs (ng/g ww) in the eggs of four water bird species from Blokkersdijk, Belgium. The significant differences (one-way Anova, $p < 0.05$) between the species for each PFC are indicated by the same letters.

	Common Pochard n = 6	**Eurasian Coot** n = 20	**Gadwall** n = 5	**Canada Goose** n = 4
PFOS	9158.4 ± 2469.8^{a,b,c}	4049.0 ± 340.0^{a,d,e}	1841.6 ± 221.1^{b,d,f}	388.9 ± 93.9^{c,e,f}
PFHxS	785.8 ± 267.9^{a,b,c}	203.8 ± 24^{a,d}	98.3 ± 8.6^{b,e}	14.2 ± 2.6^{c,d,e}
PFOA	105.9 ± 39.8^{a}	70.5 ± 4.6^{b}	46.2 ± 5.9^{c}	17.6 ± 3.0^{a,b,c}
PFNA	17.0 ± 1.5	11.0 ± 1.4	8.0 ± 0.3	<7.8

All the metals measured in the study were above the method detection limit in all the samples (Table 3). However, no clear trends were observed for metal content in the eggs among the four different bird species. The highest levels in the eggs for Ag, As, Cu, Fe and Zn were observed in the Gadwall, Cd, Co, Cr and Pb in the Eurasian Coot while the highest values of Ni and Mn were seen in the Canada Goose. There were also no clear trends in the distribution of metals in bird eggs based on whether the element was biologically essential or non-essential.

Table 3. The levels of metals (mean ± SEM; ng/g dw) in the eggs of four water bird species from Blokkersdijk, Belgium. The significant differences (Mann-Whitney Test, $p < 0.05$) between the species for each metal are indicated by the same letters.

	Common Pochard n = 6	**Eurasian Coot** n = 20	**Gadwall** n = 5	**Canada Goose** n = 4
Ag	20.8 ± 5.4[a]	15.9 ± 2.1[b]	21.2 ± 1.9[c]	2.5 ± 0.4[a,b,c]
As	180.0 ± 24.0[a,b,c]	355.5 ± 27.8[a,d,e]	701.4 ± 82.5[b,d,f]	68.1 ± 13.3[c,e,f]
Cd	6.8 ± 1.4[a,b]	8.0 ± 2.5[c]	3.8 ± 0.4[a]	2.9 ± 0.2[b,c]
Co	42.0 ± 12.1	52.9 ± 14.3	38.2 ± 1.9[a]	18.8 ± 0.8[a]
Cr	5426.4 ± 1436.9	6938.5 ± 1861.8[a,b]	2434.5 ± 333.5[a]	1817.9 ± 215.4[b]
Cu	1559.8 ± 146.8[a,b]	2231.9 ± 157.3[a,c]	2684.7 ± 167.3[b,c,d]	1832.5 ± 101.0[d]
Fe	110863.5 ± 10357.5[a]	77701.8 ± 9516.0[b,c]	167321.1 ± 11086.8[a,b,d]	118122.0 ± 6407.8[c,d]
Mn	4.2 ± 0.6[a,b,c]	2.8 ± 0.4[a,d,e]	8.1 ± 0.4[b,d]	12.1 ± 3.3[c,e]
Ni	45.2 ± 2.6[a,b]	38.0 ± 2.7[c,d]	65.9 ± 2.9[a,c]	68.0 ± 7.3[b,d]
Pb	61.0 ± 12.8[a,b]	265.1 ± 30.9[a,c]	247.1 ± 24.0[b,d]	60.2 ± 17.3[c,d]
Zn	29065.6 ± 2864.4[a,b]	35611.7 ± 2248.5[c]	57530.8 ± 3677.2[a,c,d]	41396.0 ± 1895.2[b,d]

Since PFNA and PFOA were not detected in all the samples, only the correlations between PFOS and PFHxS and the metals were determined. When all the data of the four species were analysed together, PFOS and PFHxS showed the same correlation pattern with the metals (Table 4). PFOS and PFHxS had a significant positive correlation with Cr and Cd and a significant negative correlation with Mn and Ni. The significant correlations between both PFOS and PHFxS and the metals were different when the data for each species were analysed

separately (Table 5). No trends were observed in the correlations between PFOS and PFHxS and the metal levels.

Table 4. The correlations between the levels of PFCs and metals (ng/g ww) in the eggs of four water bird species from Blokkersdijk, Belgium. Only the r-value of the significant correlations is given (Spearman's Rank correlation, $p < 0.05$).

	Cr	Mn	Ni	Cd
PFOS	0.518	-0.339	-0.410	0.453
PFHxS	0.438	-0.349	-0.364	0.388

Table 5. The correlations between the levels of PFCs and metals (ng/g ww) in the eggs of four water bird species from Blokkersdijk, Belgium. Only the r-value of the significant correlations is given (Spearman's Rank correlation, $p < 0.05$). — indicates no significant correlation.

	Pb	Ag	Co	Fe	Zn
Canada Goose					
PFOS	1.000	-	-	-	-
Common Pochard					
PFHxS	-	-0.829	-	-	-
Gadwall					
PFHxS	-0.900	-	-1.000	-	-
Eurasian Coot					
PFOS	-	-	-	0.471	-
PFHxS	0.463	-	-1.000	-	0.492

2.4 Discussion

Although much research has been performed on the PFC exposure levels of birds, only one study investigated the role that feeding plays in birds' exposure to PFCs. This study showed that fish-eating birds had higher liver PFOS levels than herbivorous birds (Sinclair et al., 2006). However, the influence of feeding habits has not been determined from closely related bird species from the same locality. Using the eggs of four water bird species that have different feeding habits, we investigated the role feeding habit play in the exposure of birds. The PFC levels found in the eggs from this study were the highest ever measured in bird eggs (Table 6). The PFOS levels were higher than most levels in the time-trend study of PFOS in Common Guillemot eggs (Holström et al., 2005). However, the egg PFOS levels of the Canada Goose in our study were lower than the values obtained in the eggs of the Common Guillemots.

Table 6. PFC levels in the eggs of birds. The range of the values for each species is given in ng/g ww.

Species	Country	PFOS	PFHxS	PFOA	PFNA	Source
Canada Goose	Belgium	222.0 – 637.6	10.8 – 22.0	< 3.2 – 21.9	< 7.6	Current study
Gadwall	Belgium	1373.8 – 2683.3	70.2 – 119.5	29.7 – 60.1	< 7.6 – 8.3	Current study
Eurasian Coot	Belgium	1861.0 – 7635.7	76.8 – 488.1	28.1 – 104.1	< 7.6 – 14.4	Current study
Common Pochard	Belgium	3204.4 – 17214.0	235.4 – 1967.9	25.6 – 260.4	< 7.6 – 18.5	Current study
Double Crested Cormorant	USA	21 - 220	nm	nm	nm	Kannan et al., 2001
	USA	130 - 320	nm	nm	nm	Giesy et al., 2001
Ring-billed Gull	USA	<35 - 150	nm	nm	nm	Giesy et al., 2001
	USA	30 - 126	nm	nm	nm	Kannan et al., 2001
Laysan Albatross	Northern Pacific	3.17	< 1	< 0.5	< 1	Tao et al., 2006
Black-footed Albatross	Northern Pacific	10.9	< 1	< 0.5	< 1	Tao et al., 2006
Common Tern	Netherlands	409 - 618	nm	nm	nm	Van den Brink et al., 2007
Glaucous Gull	Norway	51.7 - 196	< 0.27 – 1.23	< 0.70	< 2.33	Verreault et al., 2005
Common Guillemot	Norway	25 - 1324	nm	< 3	nm	Holsröm et al., 2005
	Iceland	5.2 - 22	nm	< 5.8	< 32	Löfstrand et al., 2008
	Faroe Islands	6.0 - 34	nm	< 5.8	< 32	Löfstrand et al., 2008
	Norway	3.2 - 210	nm	< 5.8	< 32	Löfstrand et al., 2008
	Sweden	200 - 760	nm	< 5.8	< 32	Löfstrand et al., 2008

nm: PFC was not measured in the study.

Still, even the highest PFOS levels (1324 ng/g ww) in the Common Guillemot eggs of the time-trend study were lower than those found in the other three bird species in our study (lowest value 1373 ng/g ww). When compared to the PFOS levels in the eggs of Common Terns (409 – 618 ng/g ww) from two sites in the Western Scheldt, 30 and 55 km from Blokkersdijk, our values were much higher, except for the PFOS levels in the Canada Goose eggs (Van den Brink et al., 2007). The PFOS levels in the eggs of the Common Pochard were about 20 times higher than those of the Common Tern eggs. Also, when compared to the PFOS levels in the eggs of marine species, such as the Glaucous Gull (*Larus hyperboreus*), Ring-billed Gull (*Larus delawarensis*) and Double-crested Cormorant (*Phalacrocorax auritus*), the PFOS levels in the eggs in our study were on average about 300 times higher (Giesy and Kannan, 2001, Kannan et al., 2001, Verreault et al., 2005).

Not much research has been performed on the levels of PFCs other than PFOS in bird eggs. The PFHxS levels in the eggs from the current study were between 12 to 600 times higher than those found in Glaucous Gulls from the Norwegian Arctic (Verreault et al., 2005). In that study, PFHxS were only detected in 30% of the egg samples, whereas in our study it was detected in all the samples. In most other studies the levels of PFOA in bird eggs were below the detection limit, even in marine bird eggs which contained high levels of PFOS (Holström et al., 2005, Verreault, et al., 2005; Tao et al., 2006). However, in our study, PFOA was detected in 94% of the egg samples. As with other studies, the PFNA levels in the eggs from the current study were mainly below the detection limit (Verreault et al., 2005). PFNA were only detected in 23% of our samples.

It must be kept in mind that PFOS bioaccumulates through the food chain, therefore the eggs of carnivorous birds are expected to contain the highest PFC levels (Haukås et al., 2007; De Vos et al., 2008). This was not the case when we compare the PFC levels from our study to previous research, as done above. This shows that the Antwerp area, and in particular Blokkersdijk, is a hotspot for PFC contamination.

To determine the risk of PFCs to birds, laboratory toxicity data are needed. Very few toxicity studies have been performed on birds, and then only on two bird species, namely the Mallard (*Anas platyrhynchos*) and the Northern Bobwhite (*Colinus virginianus*) (Giesy and Jones, 2004; Newsted et al., 2005; 2007). Since three out of the four birds that were studied are ducks, we will only compare our data with the data of another duck species, the Mallard. All the levels were lower than the Lowest Observed Adverse Effect Levels (LOAEL) for eggs (53000 ng/mL) calculated for Mallards (Giesy

↳ based on what indicator?

and Jones, 2004). However, the highest level in our study, from a Common Pochard egg, was only about 3 times lower than the LOAEL for Mallards. This means that these birds may be at some risk. However, care should be taken when using these toxicity reference values to determine at what risk wild birds are. Although the laboratory exposure to determine the LOAEL was a chronic 21-week exposure, the risks of chronic exposure of birds at these high levels in nature are still unknown. Moreover, the exposure route was via food intake only. Wild birds are exposed to PFCs through various exposure routes, including food, water (both through drinking and swimming) and even atmospheric exposure.

Only one previous study investigated the influence of diet on the PFC exposure in birds that share the same ecosystem type. Sinclair et al. (2006) determined that piscivorous ducks had liver PFOS levels greater by a factor of 2.5 than those of herbivorous ducks. However, the samples were collected from different areas and feeding habits were not taken into account. No previous study looked at birds from exactly the same area. In our study, a clear pattern was observed in the PFC levels in the eggs of the four bird species. Although all four bird species in the current study were water birds, they have different feeding habits. The Common Pochard, a bottom-feeding omnivore, had significantly higher levels of all four PFCs studied. The Eurasian Coot, an omnivore that feeds mainly in the water, had the second highest levels of PFCs in their eggs. The third lowest levels of PFCs were in the eggs of the Gadwall, a herbivore. The Gadwall is a dabbling duck, meaning it feeds on various food items from the water surface or from just below the water surface. Moreover, the Canada Goose, whose main food source is grass, had the lowest levels of all PFCs in their eggs. The water birds that are herbivorous had significantly lower levels of PFCs than the birds that are omnivorous. Feeding habit might also play a role, as the birds that dive and spend more time in the water had higher levels of PFCs in their eggs. The level of PFOS as high as 1.5 μg/L has been reported for the lake at Blokkersdijk (Arcadis, 2006). Thus, birds that feed on aquatic plants and invertebrates might be exposed to higher levels of PFOS. Although the PFC levels in the sediment is known, this might also play a role in the birds exposure to PFCs. The diving ducks feed on benthic organisms; therefore, if the sediments contain high levels of PFCs, the ducks could be exposed to higher levels of PFCs. In addition, when the birds dive, the feathers of their whole body come in contact with the water. Then when the birds use their beaks to preen, they might be exposed to higher PFC levels than birds that do not dive. Therefore, it can clearly be seen that even for birds that share the same ecosystem, both their feeding habit and diet play a role in their exposure to PFCs. Further study is needed to characterise the exposure routes, either through food or during preening, of PFCs in birds. Other

factors, such as migration or difference in body size, that could also influence the birds expsoure to PFCs, should also be investigated. All these studies were performed in the same area?

No clear trend could be seen in the metal levels determined in the eggs of the four water birds species studied. Most metals in the eggs of this study were in the same range as the metal levels determined in previous studies on bird eggs (Burger, 2002; Custer et al., 2007; Gochfeld et al., 1998; Henny et al., 2008; Mora et al., 2008; Zhang et al., 2006). However, a previous study has shown that food chain differences affect the metal levels in marine birds (Burger, 2002). Even when the metals were divided into essential and non-essential elements, no clear patterns were visible. Lack of clear patterns on the metal data may be due to the many factors that have significant influences on birds' exposure to metals, such as differences among bird species, metals, bioavailability and exposure scenario (Burger, 1993). Differences in ecologies of the studied bird species may be playing an important role in determining which metals are bioavailable and taken up by the birds. It is known that in aquatic ecosystems, different metals tend to partition differently among different environmental compartments. Some metals will be more associated with living organic matter while other metals may have higher affinity for non- living organic materials or with inorganic particles (Luoma and Rainbow, 2005). Consequently, in cases where feeding is an important uptake route for birds, the type and metal content of different bird diets will result in different metals being accumulated differently among bird species. It should be noted that the differences in the metal levels in the eggs between the bird species could probably not distinguishable due to the low background levels of the metals.

Among the metals studied, only four metals (Cr, Mn, Ni and Cd) had significant correlations with both PFOS and PFHxS when all the data was analysed together (Table 4). There is no obvious trend among these four metals as the list includes well-known essential and non-essential metals Mn and Cd, respectively. While Ni and Cr represent borderline metals. When the correlation analysis was repeated with the data of each of the four bird species analysed separately, different significant correlations were found. Again, no pattern was observed relating to whether the metals are essential or non-essential. Generally, essential and non-essential metals accumulate in organisms differently. In the case of essential metals such as Zn, Cu and Mn, it is known that up to a certain point their body levels are physiologically regulated and therefore tend to bio accumulate in a different manner compared to non-essential metals such as Cd and Pb, whose concentrations in the body directly reflects environmental exposure levels (Newman and Jagoe, 1996; Luoma and Rainbow, 2005). With respect to correlations with two other studied contaminants, no clear trends or patterns could be found even

Cd = marinade.

among the various significant correlations observed between some metals and PFOS or PFHxS. This lack of explainable trends seems to highlight the importance of the differences among bird species and among the different chemical contaminants. It is also possible that the small sample sizes in the cases of the Canada Goose and Gadwall, may have substantially weakened the correlation analysis, especially when bird species-specific data were used.

2.5 Conclusions

The PFC levels in this study were the highest ever measured in the eggs of birds. There was a significant difference in the PFC levels in the eggs of four bird species that share the same ecosystem. The omnivorous birds had significantly higher PFC levels in their eggs than the herbivorous birds. The birds that dive for their food also had higher PFC levels in their eggs. The diet and feeding habit seems to play a role in the PFC exposure in birds. The study also showed that there is a complex (or no clear) relationship between the accumulation patterns of PFCs and trace metals including the highly toxic metals such as cadmium and lead.

CHAPTER 3

Perfluorooctane sulfonate exposure in three bird species from the Antwerp harbour region, Belgium

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Abstract

Perfluorooctane sulfonate (PFOS) is the perfluorinated chemical (PFC) found in the highest levels in wildlife. Blood and blood-rich organs, like liver or spleen, have mainly been used in the determination of PFOS levels in birds, but recently eggs have also been used. The aim of our study was to measure the effects of and the exposure to PFOS in bird eggs and blood in the Antwerp harbour, Belgium, using three bird species: the Great Tit (*Parus major*), the Northern Lapwing (*Vanellus vanellus*) and the Mediterranean Gull (*Larus melanocephalus*). Eggs collected at a similar distance from the fluoro-chemical plant were significantly higher in the Mediterranean Gull than in the two other species, suggesting that feeding habits may play an important role in the PFOS exposure in birds. There was a clear negative correlation between the PFOS levels in the Northern Lapwing eggs and the distance of the nest site from the fluoro-chemical plant. Great Tit eggs from the site closest to the fluoro-chemical plant also had significantly higher PFOS levels than eggs collected 1700 m further away. When comparing the PFOS levels in the eggs of the three bird species in our study to previous studies, the PFOS levels were much higher. Our study included some of the highest PFOS levels ever measured in wildlife. All this indicates that the Antwerp harbour area is a PFOS hotspot and high levels of PFOS occur even in eggs from breeding colonies a few kilometres away. There were no clear trends in the biomarker responses. Most of the PFOS levels in the eggs of the Northern Lapwing and the Mediterranean Gull were within the same range as various toxicity reference values for birds, indicating the birds close to the fluoro-chemical plant may be at risk.

3.1 Introduction

Perfluorinated chemicals (PFCs) have been produced for over 50 years and have many industrial uses (Giesy and Kannan, 2002). However, reliable measurement techniques have only become available in the last decade (van Leeuwen et al., 2007). Recently increasing attention has been given to the measurement of perfluorooctane sulfonate (PFOS) levels in wildlife (Giesy and Kannan, 2001; Kannan et al., 2002b; Olivero-Verbel et al., 2006). PFOS, the PFC that has been found in the highest levels in the environment, has even been measured in wildlife from remote areas such as the Arctic and the Antarctic (Martin et al., 2004b; Tao et al., 2006).

Although the number of studies performed on the exposure of wild birds to PFOS has risen in the last few years, most studies focussed on piscivorous birds from a high trophic level (Kannan et al., 2001; Bossi et al., 2005; Butt et al., 2007). Research has shown that the level of PFOS is higher in the birds than in their prey, suggesting the bioaccumulation of PFOS thus occurs (Tomy et al., 2004; Haukås et al., 2007). Levels of up to 2570 ng/mL were measured in the plasma of Bald Eagles (*Haliaeetus leucocephalus*) from Midwestern United States of America, showing that high levels of PFOS could be found in birds (Giesy and Kannan, 2001). Few studies have included non-piscivorous birds. As an example, Senthilkumar et al. (2007) determined the liver PFOS levels in the Large-bill Crow (*Corvus macrorhynchos*) from Japan. These PFOS levels were however about 10 to more than 100 times lower than those found in other birds.

Blood-rich organs, for example the liver, have usually been the target organ when determining the PFOS levels in birds. Recently, eggs and blood have also been used (Verreault et al., 2005; Holström et al., 2005). In the blood, PFOS is known to bind to the albumin (Jones et al., 2003). Dauwe et al. (2007) found a clear relationship between the PFOS levels in liver and serum of Great Tits (*Parus major*) from the Antwerp region in Belgium. There was no significant difference between the levels of PFOS in the eggs and liver of Common Tern (*Sterna hirundo*) females from the Western Scheldt (Van Den Brink et al., 2007). Laboratory studies have shown that female birds have lower levels of PFOS in the liver than males, due to the transfer of PFOS to the eggs (Newsted et al., 2007). Using eggs is also less invasive than using organs, thus decreasing the threat to the bird populations being studied. Another advantage of using eggs is that the same individuals or breeding pairs can be monitored over a period of time. In addition, specimens from museum or species sample banks can also be used to investigate the occurrence of time trends, if they have been well preserved (Holström et al., 2005).

Studies on the toxicological effects of PFOS have also been increasing, although most of these have been on mammals under laboratory conditions (Seacat et al., 2002; Seacat et al., 2003). Laboratory studies have been performed on Mallards (*Anas platyrhynchos*) and Northern Bobwhites (*Colinus virginianus)* to assess the effects of PFOS on birds. Effects included increased liver weight, decrease in body weight and a decrease in testes length in males (Newsted et al., 2006; Newsted et al; 2007). In field studies, Hoff et al., (2005b) indicated that there is a significant increase in serum alanine aminotransferase activity (ALT), which is a biomarker of liver damage, with an increase in liver PFOS levels in wild tits. They also found a decrease in serum cholesterol and triglyceride levels with an increase in PFOS levels, which suggests that PFOS influences the lipid metabolism of exposed organisms.

The area around the Antwerp harbour has been the primary European production site for PFOS until its phasing out in 2002. Previous research on the PFOS levels in European Eel (*Anguilla anguilla*), Common Carp (*Cyprinus carpio*), Wood Mice (*Apodemus sylvaticus*), Great Tit and Blue Tit (*Cyanistes caeruleus*) have shown this area to be a hotspot for PFOS contamination (Dauwe et al, 2007; Hoff et al., 2005a, 2005b). Levels found in these organisms have been some of the highest in the world. The highest level of PFOS in a wild bird ever measured, namely 11359 ng/g ww, was determined in the liver of one of the Great Tits from this study (Dauwe et al., 2007).

The aim of the present study was to measure PFOS exposure in avian eggs and blood in the Antwerp harbour, Belgium. We selected three bird species: the Great Tit, the Northern Lapwing (*Vanellus vanellus*) and the Mediterranean Gull (*Larus melanocephalus*), all of them breeding near a fluoro-chemical plant. The Great Tit is a resident species and feeds mainly on caterpillars during the summer and seeds and berries during the winter (del Hoyo et al., 2007). Although territorial during the breeding season, Northern Lapwings form huge flocks in the winter. They mainly feed on small insects and worms, which they find while foraging in grassy areas (del Hoyo et al., 1996). The Mediterranean Gull (*Larus melanocephalus*) breeds in the Antwerp harbour region and then migrates to the Caspian Sea during the winter. They feed on terrestrial and aquatic insects, gastropods, small fish and even rodents and form huge colonies during the breeding season (del Hoyo et al., 1996). We investigated if there was a difference in the exposure of these birds to PFOS and if there was a correlation between the PFOS levels in the eggs and the distance of the nests from the fluoro-chemical plant. Various biomarkers were used to determine the adverse effects of PFOS exposure in one of the species, the Great Tit.

3.2 Materials and Methods

3.2.1 Sampling

The study area is located on the western shores of the Scheldt river, west of Antwerp, Belgium (Figure 1). The area is made up of various habitats, including wooded areas, sandy shores and grassland, but various chemical industries are located there. A nature reserve and a Bird Habitat area (covered by the EU Birds Directive 79/409/EEC), Blokkersdijk, is found in the area. Great Tit eggs (n = 18) and blood (n = 31) were collected from two sites in the region. The first site, Vlietbos, is located about 1200 m from a former fluoro-chemical production plant, thus it is supposed that this site is more contaminated with PFCs. Burchtse Weel, the second site, is some 1750 m further to the south from the first site and therefore presumably less contaminated. One egg per nest was collected between April 15th and 30th 2006. Additionally blood samples of adult Great Tits were collected between January 25th and February 2nd 2007. Great tits were sexed and aged (one-year old and older birds, following Svensson, 1992). The birds were caught using mist nets. The Northern Lapwing eggs were collected along a distance gradient from the fluoro-chemical plant during the egg-laying season (March 25th and April 5th, 2006). From 14 nests one egg per nest were sampled. The closest nest was 90 m away from the fluoro-chemical plant and the one the furthest away about 15000 m. The blood and egg samples of the Mediterranean Gull were collected on 13 and 14 May 2006 from the colony at Zandvliet, a site just north of the fluoro-chemical plant. In total 6 eggs and 29 blood samples were collected.

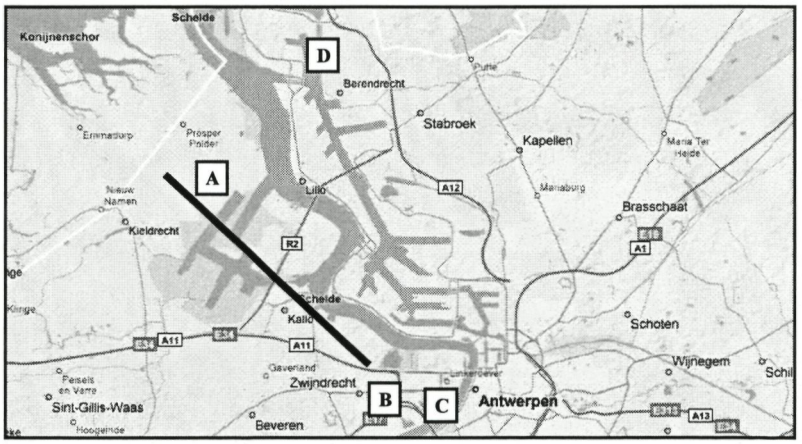

Figure 1. A map of the study sampling sites: A = Line along which Northern Lapwing eggs were collected; B = Vlietbos; C = Burchtse Weel; D = Zandvliet

3.2.2 PFOS extraction and clean up

PFOS extraction from the organs was done by solvent extraction based on the method by Berger et al. (2005) with adaptations. Briefly, each sample was homogenized in a polypropylene (PP) centrifuge tube using an Ultra-turrax T 8 mixer (IKA-WERKE, Steufen, Germany) and then weighed. Internal standard (^{13}C-PFOS and ^{13}C-PFOA) and 4.5 mL of acetonitrile were added. The PP tube was capped and the sample was thoroughly mixed using a Vortex chemical mixer. The sample was then extracted 3 times for 10 minutes in an ultrasonic bath at room temperature. Between each period of 10 minutes, the samples were thoroughly mixed. The samples were then centrifuged at 2500 rpm for 5 minutes. One mL of the final supernatant was transferred to a micro vial containing approximately 25 mg of activated carbon and 50 µL glacial acetic acid. The sample was then mixed for 1 minute using a vortex mixer. After centrifugation (10000 rpm, 10 minutes) 500 µL of the supernatant was transferred to a clean micro vial.

3.2.3 Determination of PFOS concentrations

The concentrations of PFOS were measured using combined liquid chromatography-mass spectrometry according using a CapLC system (Waters, USA) connected to a Quadrupole-LIT quadrupole mass spectrometer (Applied Biosystems, UK). Aliquots of 5 µl were loaded on an Optiguard C18 pre-column (10 mm x 1 mm i.d., Alltech, USA). The analysis was performed on a Fluophase PFP column (50 mm x 1 mm i.d., Thermo, USA) at a flow rate of 40 µl/min. The mobile phase was 2 mM NH4OAc (A) / Acetonitrile (B). A gradient elution was used starting at 35 % B and going to 90 % B in 5 min. At 5 min and 6 seconds the initial conditions were resumed. PFOS was measured under (-) electrospray ionisation using the transitions from mother to daughter ion (499 → 80/99) to identify them. The dwell time was 0.1 s. The ES-capillary voltage was set at –4.5 kV and the cone voltage -100 kV. The PFOS concentration was calculated using an unextracted calibration curve. The limit of detection (LOD) was 0.9 ng/mL and 0.15 ng/g ww for blood and eggs respectively.

3.2.4 Quality Control

Laboratory blanks were extracted along with each batch of samples, consisting of all the solvents but containing any sample. Spiked chicken egg samples were also extracted along with samples to determine recovery rates. Recovery rates were between 98 to 125%. Pure acetonitrile was injected after every 8 samples to check for memory effects. A standard solution was injected after every 8 samples to check the stability of the HPLC-MS/MS system. After each injection of a standard solution or spiked sample, pure acetonitrile was also injected.

3.2.5 Biomarkers of condition

Blood was sampled using haematocrit tubes. These tubes were centrifuged for 10 minutes at 10 000 rpm within 12 hours of sampling. The samples were stored at – 80°C until further analysis. After defrosting, the samples were diluted four times with deionised water. Total protein content, cholesterol concentration, triglyceride concentration and uric acid concentrations were determined using commercial assay kits from Horiba ABX.

3.2.6 Statistical Analysis

All statistics were performed using SPSS 5.0 for Windows. Data were log-transformed to meet assumptions of normality. We compared PFOS concentrations in Great Tit eggs between two sites with a student's t-test. Interspecific differences among the three bird species were tested with a one-way ANOVA. Because there could be a significant effect of distance to the fluoro-chemical plant, we only used egg samples collected at a similar distance (> 2000 m from the plant). The relationship between the distance to the pollution source and PFOS concentrations was tested with a parametric Pearson correlation. Total protein, cholesterol, triglyceride, uric acid and albumin concentrations were analysed with a three-way ANOVA with study site, sex and age as variables. Only main effects and two-way interactions were included in the statistical model. The level of significance for all tests was set at $\alpha = 0.05$.

3.3 Results

PFOS was detected in all the Great Tit eggs from both study sites (Figure 2). Great Tit eggs from the site closest to the fluoro-chemical plant had significantly higher PFOS levels than eggs collected further away (t-test, $t = 2.66$, $p = 0.021$).

There was a clear negative correlation between the PFOS levels in the Northern Lapwing eggs and the distance of the nestsite from the fluoro-chemical plant (Pearson correlation, $r = -0.81$, $p < 0.001$). The closer the nest was to the plant, the higher the levels of PFOS in the eggs (Figure 3). The range of the measured values was 142.7 to 46181.8 ng/g ww with a mean of 9200.3 ± 4045.5 ng/g ww. However, the mean is influenced by the three eggs taken from the nests closest to the fluoro-chemical plant. These values (31057, 42747 and 46182 ng/g ww) were about 50 times higher than the levels measured in the other Northern Lapwing eggs.

PFOS was also detected in all the samples of egg and blood samples of the Mediterranean Gull. PFOS levels in Mediterranean Gull blood ranged from 118 to 943 ng/mL and from 150 to 916 ng/g ww in eggs.

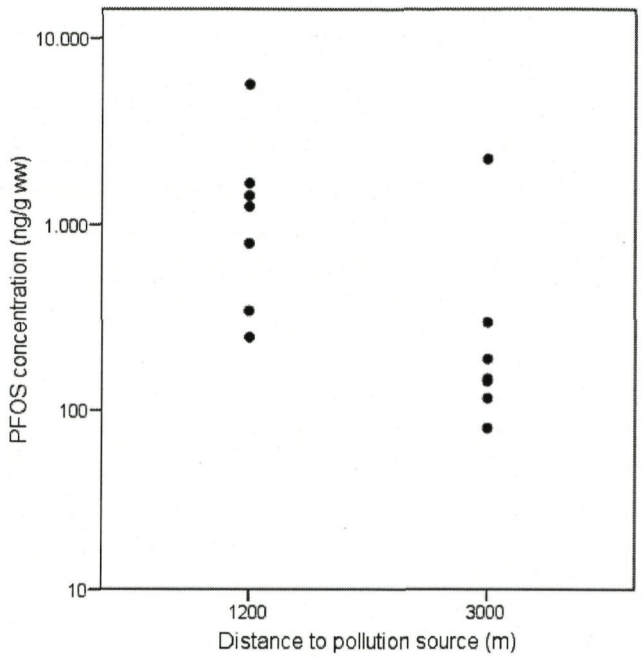

Figure 2. PFOS concentrations in Great Tit eggs from Vlietbos (left) and Burchtse Weel (right).

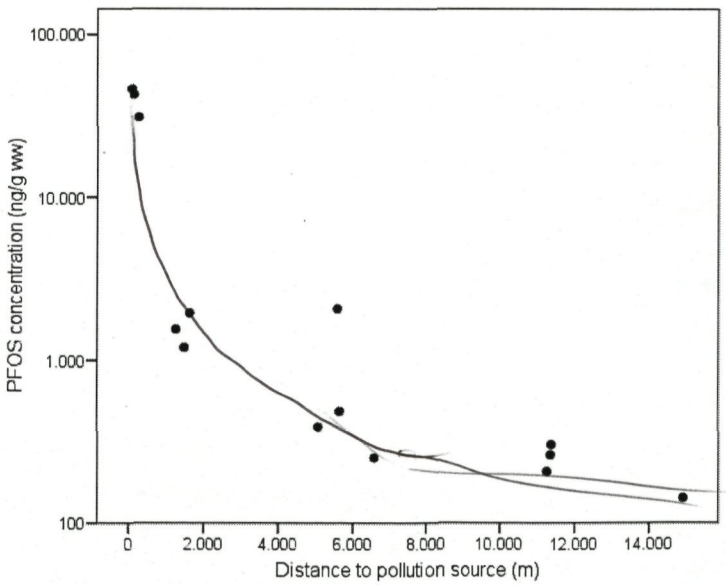

Figure 3. The PFOS concentration (ng/g ww) in Northern Lapwing eggs along a distance gradient from the previous fluoro-chemical plant.

We investigated inter-specific differences in PFOS levels among the three species in eggs collected at a similar distance from the fluoro-chemical plant (> 2000 m, Figure 4). PFOS levels did not differ significantly among the three species (one-way ANOVA, $F = 0.68$, $p = 0.5$). However, in both the Northern Lapwing as well as in the Great Tit there was one egg with a relatively high PFOS level, which could be considered an outlier. If these two samples were excluded from the analysis, PFOS levels did differ among the three species (one-way ANOVA, $F = 4.77$, $p = 0.024$), with PFOS levels being significantly higher in Mediterranean Gull than in Great Tit eggs (Tukey post-hoc test, $p = 0.021$).

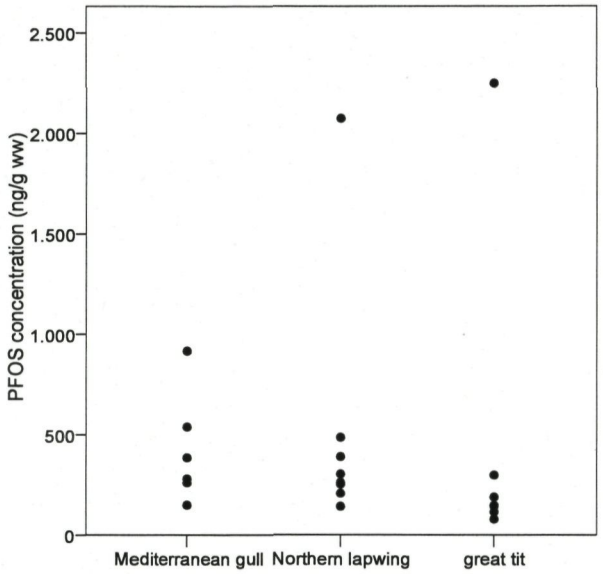

Figure 4. PFOS concentrations (ng/g ww) in eggs from Great Tit, Northern Lapwing and Mediterranean Gull sampled from the same distance (plus minus ≥ 2 000 m) from the fluoro-chemical plant.

The results of the biomarker analysis in plasma from the Great Tits are summarised in Table 1. For albumin, uric acid and triglyceride concentrations, all main effects (site, sex and age) and all two-way interactions were non-significant ($p > 0.1$, in all cases). For both cholesterol and total protein concentrations, the only significant term in the statistical model was the interaction between age and study site (total protein, $F = 4.36$, $p = 0.048$; cholesterol, $F = 6.40$, $p = 0.018$). One-year old birds from the most polluted site had lower mean plasma protein and cholesterol concentrations than older individuals from the same site and Great Tits from the less polluted site.

Table 1. Biomarker results from the Great Tits at Vlietbos and Burchtse Weel. Values are given as mean ± SEM. Significant differences ($p < 0.05$) between groups are indicated with the same letter.

	1200 m		3000 m	
	one-year old	**older**	**one-year old**	**older**
n	7	9	5	10
Total protein (g/L)	$28.7 \pm 0.9^{a,b,c}$	39.9 ± 2.2^{a}	37.8 ± 4.5^{b}	34.0 ± 3.3^{c}
Albumin (g/L)	13.4 ± 0.7	19.0 ± 0.9	17.5 ± 1.9	16.4 ± 1.6
Triglyceride (mg/dL)	98 ± 7	181 ± 29	187 ± 32	163 ± 27
Uric acid (mg/dL)	177 ± 14	103± 15	130 ± 17	163 ± 33
Cholesterol (mg/dL)	$194 \pm 11^{a,b,c}$	277 ± 17^{a}	267 ± 24^{b}	228 ± 24^{c}

3.4 Discussion

Previous studies have indicated that PFOS is found in high levels in different species of wildlife. However, most of the research focussed on marine or freshwater animals, which also occupy high trophic levels. Very few studies directly compared the influence of feeding habits on animals' exposure to PFOS, especially birds. Few studies have also reported on the influence of distance from a known point of PFOS contamination on the PFOS exposure of birds. To investigate if there was a difference in the exposure of the three bird species to PFOS and to determine the relationship between the PFOS exposure and the distance of the nests from the fluoro-chemical plant, we looked at levels of PFOS in the eggs of the three different bird species. Even though the PFOS levels in this study were determined in eggs, the values for both sites were in the same range as those in liver of tits in previous studies in the same area (see overview in Table 2). For both Vlietbos and Burchtse Weel, the PFOS levels in the eggs were on average three times lower than the PFOS liver levels determined by Dauwe et al. (2007). This is to be expected as PFOS levels tend to be lower in eggs than in liver (Van den Brink et al., 2007; Verreault et al., 2005). However, the PFOS levels in the eggs were 1.5 times higher than the liver PFOS levels from Great Tits in Blokkersdijk, which is a few hundred metres away from Vlietbos (Hoff et al., 2005b). This may be because the livers of nestlings were used in the Blokkersdijk study and eggs were used in our study. There might be some matrix effects influencing the extraction and measurement of PFOS in the two matrices.

Table 2. Comparison between PFOS levels in egg and liver of Great Tits from the Antwerp region. All values are ng/g ww.

Study	Matrix	Lowest value	Highest Value	Average
Vlietbos				
Present study	egg	247	5635	1622
Dauwe et al. (2007)	liver	2034	11358	5073
Blokkersdijk				
Hoff et al. (2005)	liver	86	2788	994
Burchtse Weel				
Present study	egg	79	2248	460
Dauwe et al. (2007)	liver	629	1775	1125
Fort IV				
Hoff et al. (2005)	liver	17	206	146

The PFOS levels in eggs of Common Terns from the Western Scheldt estuary were within the same range (225 – 1219 ng/g ww) as those from in this study for Mediterranean Gull (Van den Heuvel-Greve et al., 2006). The Common Tern colonies were located 30 and 55 km away from the fluoro-chemical plant. However, van den Brink et al., (2007) measured lower PFOS levels in both eggs and liver of Common Terns from the same colonies in the Western Scheldt. These values (208 – 618 ng/g ww) still fall within the lower range of the PFOS levels (118 – 942 ng/g ww) determined in our study. This indicates that there are still high levels of PFOS a few kilometres away from the fluoro-chemical plant. The PFOS levels found in both the eggs and blood of the Mediterranean Gull were four times higher than those found in the same matrices of Glaucous Gulls (*Larus hyperboreus*) from the Arctic (Verreault, 2005). The PFOS levels in the eggs and blood of the Mediterranean Gull determined in our study were generally in the same range or higher than the PFOS levels found in various fish-eating birds from the United States and Japan (Table 3, Giesy and Kannan, 2001; Kannan et al., 2001). Thus, when comparing the PFOS levels of the Mediterranean Gull eggs and blood to PFOS levels measured in other water birds, it can be seen that the Antwerp harbour region is a site with extremely high PFOS contamination.

The PFOS levels measured in the three eggs samples closest to the fluoro-chemical plant are among the highest ever measured in wildlife. The only PFOS levels that have been reported higher in literature are liver PFOS levels measured in wood mice from the same area (Hoff et al., 2004). The highest PFOS level in the wood mouse liver was 3.8

times higher than the highest PFOS level measured in the Northern Lapwing eggs of our study. Newsted et al., (2005) calculated the Toxicity Reference Values (TRV) for PFOS based on the characteristics of a top avian predator.

Table 3. Comparison between PFOS levels in egg and blood of various water birds. All values are given as ng/g ww for egg and ng/mL for blood.

Species	Country	Matrix	Range	Source
Mediterranean Gull	Belgium	Egg	149 - 916	Current Study
		Blood	118 - 942	Curent Study
Double-crested Cormorant	USA	Egg	21 – 220	Kannan et al., 2001
			130 - 320	Giesy et al., 2001
		Blood	34 - 243	Kannan et al., 2001
			110 – 430	Giesy et al., 2001
Ring-billed Gull	USA	Egg	30 - 126	Kannan et al., 2001
			<35 – 150	Giesy et al., 2001
Herring Gull	USA	Blood	239 - 391	Kannan et al., 2001
			66 - 450	Giesy et al., 2001

TRVs are used as guidelines in the protection of wildlife and are based on acute and chronic laboratory exposure data. The toxicological endpoints used to derive the TRV include mortality, growth, feed consumption and histopathology. Most of the PFOS levels in the eggs of the Northern Lapwing and the Mediterranean Gull were very close to the TRV for eggs (1000 ng/mL ww). The three eggs of the Northern Lapwing closest to the fluoro-chemical plant are between 30 and 45 times higher than the TRV for eggs. However, the PFOS levels of these three samples are within the same range as the No Observed Adverse Effect Levels (NOAEL) for Mallard (53000 ng/mL) and Northern Bobwhites (33000 ng/mL) (Newsted et al., 2005). This may indicate that the birds close to the fluoro-chemical plant are at risk, as adverse effects may occur. However, already at 1250 m from the fluoro-chemical plant, the PFOS levels in the eggs are about 45 times lower than the NOAEL for the Mallard and at 6000 m from the fluoro-chemical

plant all PFOS levels in the Northern Lapwing eggs are below the TRV for eggs. This indicates that the risk to the birds decreases with distance from the fluoro-chemical plant.

The analysis of Great Tit and Northern Lapwing eggs suggest a significant pollution gradient of PFOS emanating from the former fluoro-chemical plant. PFOS levels in Great Tit eggs differed significantly between two sites at different distances from this plant, although the sites were merely 1700 meters apart. In Northern Lapwing eggs we found a significant negative correlation between PFOS levels and the distance from the nest to the fluoro-chemical plant. Previously, Hoff et al. (2005b) could not find a significant correlation between the liver PFOS levels of Great Tit and Blue Tit nestlings and the distance from the fluoro-chemical plant. However, nestlings were sampled within 1000 m from the pollution source, which might explain the lack of a clear gradient (Hoff et al., 2005b). Also looking at data from the Western Scheldt estuary (Van den Brink, 2007; van den Heuvel-Greve, 2006), which is 30 and 55 km away, PFOS seems to be found in high levels throughout the region.

No significant inter-specific difference was found between the PFOS levels in the eggs of the three species from the same distance from the fluoro-chemical plant. However, after removal of two outliers, the Mediterranean Gull eggs had higher PFOS levels than the two other species. Mediterranean Gulls are known to forage in a wide area around the harbour, often several tens of kilometres from the breeding colony, whereas the other two species are known to forage more closely to their nest site. As previously suggested by Sinclair et al. (2006), fish-eating birds have 2.5 times higher levels of PFOS in their livers than herbivorous birds. The Mediterranean Gull is known to not only eat insects, but also gastropods, small fish and even rodents (del Hoyo et al., 1996). This indicates that food plays a role in the PFOS exposure in birds. As previously mentioned, high levels of PFOS were also detected in the livers and eggs of Common Terns from colonies 30 and 55 km away from the fluoro-chemical plant (de Heuvel-Greve et al., 2006; Van den Brink et al., 2007).

The serum triglyceride concentrations were in the lower part of the range of values (110 – 150 mg/dL) reported for Great Tits and Blue Tits from the same area (Hoff et al., 2005b). Hoff et al. (2005b) found that there was a significant negative correlation between the liver PFOS levels and the serum triglyceride concentration. In the current study, there was no significant difference between the serum triglyceride levels of neither the age groups from both localities. Hoff et al. (2005b) found a significant negative correlation between the liver PFOS levels and the serum cholesterol

concentration in both tit species. In our study, there was a significant difference between the serum cholesterol concentration of the juvenile birds from Burchtse Weel and the adults from Burchtse Weel and both the juveniles and adults from Vlietbos. The levels of serum cholesterol concentrations were in the same range (125 – 200 mg/dL) as values found by Hoff et al. (2005b) for both Great Tits and Blue Tits from the same area. No clear pattern was observed in the serum cholesterol, serum albumin and uric acids concentrations. The lack of clear trends is surprising, as there is a significant difference between the PFOS levels in the eggs from the two different sites in our study. More research is needed to develop biomarkers that can be linked to an increase in PFOS exposure in birds. It should be noted that in our study rather generic biomarkers were used and that more specific markers might be useful.

3.5 Conclusions

When comparing the PFOS levels in the eggs of the three bird species in our study to previous studies, the PFOS levels were much higher. Our study included some of the highest PFOS levels ever measured in wildlife. Since most of the PFOS levels in the eggs of the Northern Lapwing and the Mediterranean Gull were very close to the TRV for eggs and even some of the PFOS levels measured were within the same range as the NOAEL for Mallards and Northern Bobwhites, the birds close to the fluoro-chemical plant may be at risk. There was also a significant correlation between the PFOS levels in the eggs and the distance from the nest site to the fluoro-chemical plant. The PFOS levels were higher in the eggs of the Mediterranean Gull than in the eggs of the other two species from the same distance from the fluoro-chemical plant. This indicates that food plays a role in the PFOS exposure in birds. All this indicates that the Antwerp harbour area is a PFOS hotspot and high levels of PFOS occur even in eggs from breeding colonies a few kilometres away. Although there was a significant difference between the PFOS levels in the eggs of the Great Tits from Vlietbos and Burchtse Weel, there are no clear trends in the biomarker responses. Further research is needed to develop biomarkers that can be linked to an increase in PFOS exposure in birds.

CHAPTER 4

The relationship between PFC levels in the feathers and livers of birds from different trophic levels in Belgium

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Abstract

Currently there is no data available on the use of feathers as indicators of perfluorinated chemical (PFC) exposure in birds. Feathers are less invasive than other biomonitoring techniques. Previous research found that fish-eating birds had higher levels of PFOS in their livers than herbivorous birds. Until now, no study has evaluated the PFC levels in birds with different feeding habits and different positions in the food chain from different habitats. We investigated the PFC exposure of five different bird species from the same geographic region in Belgium. The highest mean liver PFOS levels were found in the Grey Heron (476 ng/g ww) followed by the Herring Gull (292 ng/g ww), Eurasian Sparrowhawk (236 ng/g ww), Eurasian Magpie (17 ng/g ww), whereas the Eurasian Collared Dove (12 ng/g ww) had the lowest levels. The PFOS levels in the feathers showed a different pattern. The Grey Heron had the highest levels (247 ng/g dw), the Eurasian Sparrowhawk (102 ng/g dw) had the second highest feather PFOS levels followed by the Herring Gull (79 ng/g dw), the Eurasian Collared Dove (48 ng/g dw) and the lowest levels were in the Eurasian Magpie (31 ng/g dw). Overall, there was a significant positive correlation between the feather and liver PFOS levels, indicating that feathers could be an alternative bioindicator for PFOS exposure in birds. However, caution should be taken as there was no significant correlation between the PFOS levels in the feathers and liver of the individual species. In general, birds from a higher trophic level had higher PFC levels in their tissues. This indicates that diet plays a role in PFC exposure in birds and confirms the bioaccumulation potential of PFC.

4.1 Introduction

Since reliable measuring techniques for perfluorinated chemicals (PFCs) have recently become available, there has been an increase of PFC exposure studies of biota, including both man and wildlife. Studies have shown that the various PFCs are found globally in wildlife, even in remote areas such as the Arctic and the Antarctic (Martin et al., 2004b; Tao et al., 2006). Furthermore, they are also known to bio-accumulate in the food chain (Haukås et al., 2007).

There is a large variation in the occurrence and levels of PFCs in the environment. Perfluorooctane sulfonate (PFOS) is normally found at the highest levels in wildlife. This may be because a large number of perfluorinated substances degrade to PFOS as a final metabolite (Giesy and Kannan, 2002). Another perfluorinated sulfonate, perfluorohexane sulfonate (PFHxS), has also been measured increasingly in various studies. The PFHxS levels were either below the detection limit (Bossi et al., 2005; Holström et al., 2005; Kannan et al., 2002b) or in levels much lower than those of PFOS (Haukås et al., 2007; Kannan et al., 2002a; Senthilkumar et al., 2007). Next to the sulfonates, another important group of PFCs are the perfluoro-carboxylic acids. Similar to PFHxS, perfluorooctanoic acid (PFOA) and perfluorononanoic acid (PFNA) were either below the detection limit in biota samples or found at relatively low levels.

The first articles published on PFC exposure in wildlife included bird species. Some of the levels in birds corresponded to those found in mammalian predators. PFOS levels in Bald Eagle (*Haliaeetus leucocephalus*) plasma were as high as 2 570 ng/mL (Giesy and Kannan, 2001). However, most of the birds studied were fish-eating and occupied a trophic position high in the food chain. Moreover, most of the species studied were marine birds. Only two previous studies focussed on small passerines. Both Hoff et al. (2005b) and Dauwe et al. (2007) studied Great Tits (*Parus major*) and Blue Tits (*Cyanistes caeruleus*) from the Antwerp region in Belgium. PFOS levels of up to 11359 ng/g ww were measured in the liver of Great Tits. These studies showed that even small birds that are low on the food chain could accumulate high levels of PFCs. It should however be taken into account that this area is a hotspot for PFOS contamination. Sinclair et al. (2006) found that the liver PFOS levels of fish-eating ducks were twice as high as those of herbivorous ducks, indicating that food plays an important role in PFOS exposure. Although most bird studies included many different species, so far no comparative study has focussed on birds with different feeding habits and different positions in the food chain from different habitats.

PFCs have been measured preferably in blood and blood-rich organs, like liver and spleen. The biggest drawback when using these organs is that the birds need to be sacrificed. As alternative sampling techniques, eggs and serum have successfully been used as less invasive methods to measure PFC exposure in birds (Dauwe et al., 2007; Van Den Brink et al., 2007). Sampling of these matrices may still lead to stress in birds, thus making it less suitable for rare or endangered bird species. As an alternative, feathers have several advantages as indicators of environmental pollution (Burger, 1993). For example, heavy metals and some organic pollutants have successfully been measured in feathers (Dauwe et al., 2002; Dauwe et al., 2005; Eens et al., 1999; Jaspers et al., 2006; Jaspers et al., 2007). Being non-invasive, feathers can be collected routinely without much stress to the birds. Scarce or vulnerable species can also be sampled or feathers can be collected at the nest. Moreover, time-trends can be investigated since the same individuals can be sampled repeatedly over time.

In the present study we investigated PFC exposure in five different bird species from the same geographic region in Belgium. The different species were chosen on grounds of their feeding habits and the ecosystem in which they occur. The species investigated include: the Grey Heron (*Ardea cinerea*), a freshwater carnivore, feeding mainly on fish (del Hoyo et al., 1992). The Herring Gull (*Larus argentatus*), a marine omnivore, feeding on various food items, including waste from fishing trawlers and rubbish dumps (del Hoyo et al., 1996). The Eurasian Sparrowhawk (*Accipiter nisus*) is a terrestrial carnivore, whose prey is made up of mainly small birds (del Hoyo et al., 1994). Found in many urban areas, the Eurasian Magpie (*Pica pica*), a terrestrial omnivore, has a diet that includes, worms, insects and human food waste (Perrins and Brooks, 1994). The Eurasian Collared Dove (*Streptopelia decaocta*) is a terrestrial herbivore, eating mainly seeds (del Hoyo et al., 1997). From these bird species, both liver and feathers samples were analysed to determine if feathers could successfully be used to determine the exposure of birds to PFCs. The influence of feeding habit and habitat type in the exposure levels of birds was also investigated.

4.2 Materials and Methods

4.2.1 Sample collection and preparation

Five species were used for this study: the Grey Heron, a freshwater carnivore, the Herring Gull, a marine omnivore, the Eurasian Sparrowhawk, a terrestrial carnivore, the Eurasian Magpie, a terrestrial omnivore and the Eurasian Collared Dove, a terrestrial herbivore. All of the birds were obtained from Wildlife Rescue centres in the western part of Flanders, Belgium. The livers and two outer-most tail feathers, as well as the

spleen of the Grey Heron, were collected. Ten birds per species were sampled. For each sample, about 1 g of tissue (both organs and feathers) was analysed.

4.2.2 PFC extraction in the liver and spleen

The method of Berger and Haukås (2005) was used with adaptations. Each sample was weighed and then homogenized in a polypropylene (PP) centrifuge tube using a mixer. ^{13}C-PFOS and ^{13}C-PFOA were added as internal standards together with 4.5 mL of acetonitrile. The samples were thoroughly mixed using a Vortex mixer. The samples were extracted for 10 minutes in an ultrasonic bath and then mixed using a Vortex mixer. This procedure was repeated twice. After centrifugation (2500 rpm for 5 minutes), 1 mL of the final supernatant was transferred to a micro vial for clean-up.

4.2.3 PFC extraction in feathers

The samples were weighed and any external contamination was removed by washing the feathers in acetonitrile. The feathers were dried at room temperature, cut into small pieces and put into a PP tube. To digest the feathers, 10 mL HNO_3 (69%) and 20 µl of each internal standard (^{13}C-PFOS and ^{13}C-PFOA) were added and left for 48 hours at room temperature. To increase the pH of the mixture, 40 mL NaOH was added. The samples were filtrated under vacuum through glass fibre filter (142 mm) to remove any particles. The samples were then extracted by solid phase extraction using Oasis HLB Plus SPE cartridges. Briefly, the whole system was rinsed with 20 ml acetonitrile. The samples were extracted at a constant flow rate of about 2 drops/sec. The SPE cartridge was rinsed with a mixture of 2 ml acetonitrile/water (40/60) and eluted with 4 ml acetonitrile. The eluted extract was concentrated to 1 mL at room temperature.

4.2.4 Extract clean-up

The 1 mL extracts from both the organ and feather samples were transferred to a microvial containing approximately 25 mg of activated carbon and 50 µL glacial acetic acid. The sample was then mixed for 1 minute using a vortex mixer. After centrifugation (10000 rpm, 10 minutes), 500 µL of the supernatant was transferred to a clean micro vial.

4.2.4 Determination of PFC concentrations

The PFC levels were measured using combined liquid chromatography-mass spectrometry. The system consisted of a CapLC system (Waters, USA) connected to a Quadrupole-LIT quadrupole mass spectrometer (Applied Biosystems, UK). Aliquots of 5 µl were loaded on an Optiguard C18 pre-column (10 mm x 1 mm i.d., Alltech, USA). The analysis was performed on a Fluophase PFP column (50 mm x 1 mm i.d., Thermo,

USA) at a flow rate of 40 µl/min. The mobile phase was 2 mM NH4OAc (A) / Acetonitrile (B). A gradient elution was used starting at 35 % B and going to 90 % B in 5 min. At 5 min and 6 seconds the initial conditions were resumed. The various PFCs were measured under (-) electrospray ionisation using the transitions from mother to daughter ion to identify them (Table 1). The dwell time was 0.1 s. The ES-capillary voltage was set at –4.5 kV and the cone voltage -100 kV. The PFC concentrations were calculated using an unextracted calibration curve. ^{13}C-PFOS was used as internal standard for the perfluoro-sulfonates and ^{13}C-PFOA for the perfluoro-carboxylates.

Table 1. The transition from mother to daughter ion used to identify the various PFCs and the Internal Standards.

Compound	Mother Ion	Daughter Ion
PFHxS	399	80; 99
PFOS	499	80; 99
^{13}C-PFOS	503	80; 99
PFOA	413	369
^{13}C-PFOA	417	372
PFNA	469	419

4.2.5 Quality Control

Laboratory blanks were extracted along with each batch of samples, consisting of all the solvents but having no sample. Spiked chicken liver samples were also extracted along the organ samples to measure recovery. Recovery rates ranged between 77 to 108%. In the case of the feather extraction, spiked samples consisted of acetonitrile spiked with the various PFCs. Pure acetonitrile was injected after every 8 samples to check for memory effects. A standard was injected after every 8 samples to check the stability of the HPLC-MS/MS system. After each injection of a solvent standard or spiked sample, pure acetonitrile was also injected. The limit of detection (LOD) was based on a signal-to-noise ratio of three in the chromatogram of extracts of spiked samples that did not contain any detectable amounts of PFCs. This incorporates all the dilution factors occurring during the extraction method.

4.2.6 Data Analysis

The chromatograms were analysed with Analyst 1.4 for Windows. The statistical analysis was done with SPSS 15.0 for Windows. Data were log-transformed to meet the assumptions of normality. A parametric one-way ANOVA test was used for the comparison of the PFOS levels in the various tissues of the five species, as well as inter-

species comparison. The correlations between the liver PFC levels and the PFC levels in the feathers were determined with the Pearson test.

4.3 Results

The results for the PFOS levels in organs and feathers are summarised in Figure 1, with the significant differences summarised in table 2. PFOS was detected in all the liver, feathers and spleen samples. The highest mean liver PFOS levels were found in the Grey Heron followed by the Herring Gull, Eurasian Sparrowhawk, Eurasian Magpie whereas the Eurasian Collared Dove had the lowest levels.

This pattern was different for the feather PFOS levels. Although the highest mean feather PFOS levels were also found in the Grey Heron, the Eurasian Sparrowhawk had the second highest feather PFOS levels followed by the Herring Gull. In addition, the Eurasian Collared Dove had higher levels than the Eurasian Magpie. Moreover, average levels in feathers were higher than average levels in liver for the Eurasian Collared Dove and Eurasian Magpie, while this was not the case for the other three species

PFHxS was present in 62% of the liver samples (Table 3). However, PFHxS was only detected in 40% of the Grey Heron, Eurasian Sparrowhawk and Herring Gull feather samples. It was not detected in any of the feather samples of the Eurasian Magpie and the Eurasian Collared Dove. PFOA was only detected in half of the Eurasian Sparrowhawk liver samples and in a single Herring Gull liver sample (Table 4). It was not detected in any of the feather samples. PFNA was detected in 55% of the Grey Heron and Herring Gull liver samples and in two of the Eurasian Sparrowhawk samples, but not in any of the Eurasian Magpie and Eurasian Collared Dove samples (Table 5). PFNA was not detected in any of the feather samples.

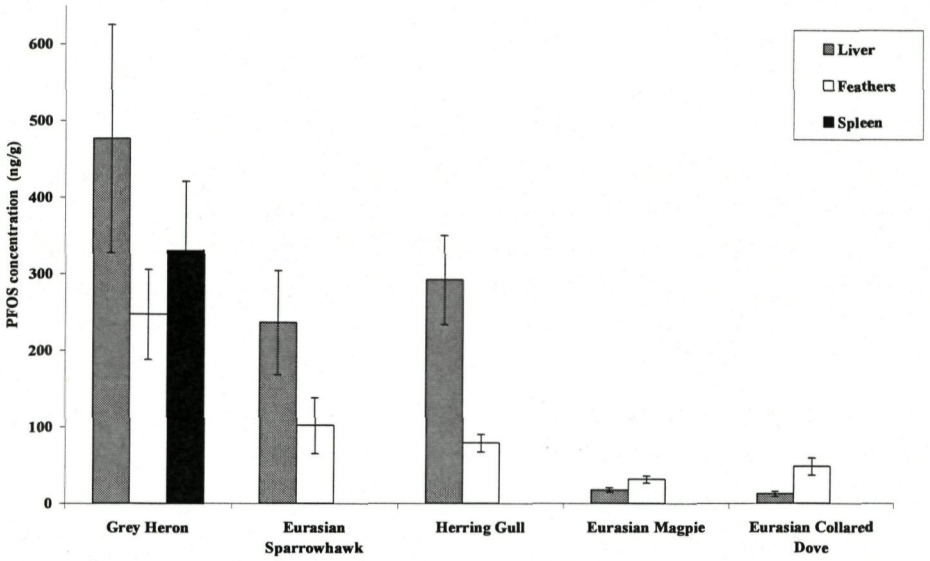

Figure 1. The levels of PFOS (Mean ± SEM ng/g ww for liver and spleen and dw for feathers) in the various tissues of the different bird species. n = 10 for each species.

Table 2. The significant differences (Anova, $p < 0.05$) between the five species for each tissue are indicated by the same latter.

Tissue	Species	Significant differences ($p < 0.05$)
Liver	Grey Heron	a,b
	Eurasian Sparrowhawk	c,d,m
	Herring Gull	e,f,n
	Eurasian Magpie	a,c,e,o
	Eurasian Collared Dove	b,d,f,p
Feathers	Grey Heron	g,h,i,j
	Eurasian Sparrowhawk	g,k,m
	Herring Gull	h,l,n
	Eurasian Magpie	i,k,l,o
	Eurasian Collared Dove	j,p

Table 3. The levels of PFHxS (Mean ± SEM ng/g ww for liver and spleen and dw for feathers) in the various tissues of the different bird species. n = 10 for each species.

Tissue	Species	% Detected (LOD = 3.2 ng/g)	mean ± SEM (ng/g ww)	Range of values above LOD (ng/g ww)
Spleen	Grey Heron	60	33.6 ± 19.5	3.4 – 120.7
Liver	Grey Heron	80	50.5 ± 17.2	4.5 – 122.2
	Eurasian Sparrowhawk	80	13.1 ± 4.0	6.3 – 40.6
	Herring Gull	50	7.8 ± 0.5	6.2 – 9.0
	Eurasian Magpie	50	5.4 ± 0.5	4.2 – 6.7
	Eurasian Collared Dove	50	4.7 ± 0.4	3.5 – 6.1
Feathers	Grey Heron	70	20.8 ± 4.4	6.2 – 37.3
	Eurasian Sparrowhawk	20	20.2 ± 12.3	7.9 – 32.5
	Herring Gull	30	31.6 ± 3.8	25.7 – 38.8
	Eurasian Magpie	0	-	-
	Eurasian Collared Dove	0	-	-

Table 4. The levels of PFOA (Mean ± SEM ng/g ww for liver and spleen and dw for feathers) in the various tissues of the different bird species. n = 10 for each species.

Tissue	Species	% Detected (LOD = 7.3 ng/g)	mean ± SEM (ng/g ww)	Range of values above LOD (ng/g ww)
Spleen	Grey Heron	0	-	-
Liver	Grey Heron	0	-	-
	Eurasian Sparrowhawk	50	11.9 ± 1.1	9.4 – 15.9
	Herring Gull	10	28.2	-
	Eurasian Magpie	0	-	-
	Eurasian Collared Dove	0	-	-
Feathers	Grey Heron	0	-	-
	Eurasian Sparrowhawk	0	-	-
	Herring Gull	0	-	-
	Eurasian Magpie	0	-	-
	Eurasian Collared Dove	0	-	-

Table 5. The levels of PFNA (Mean ± SEM ng/g ww for liver and spleen and dw for feathers) in the various tissues of the different bird species. n = 10 for each species.

Tissue	Species	% Detected (LOD = 7.6 ng/g)	Mean ± SEM (ng/g ww)	Range of values above LOD (ng/g ww)
Spleen	Grey Heron	0	-	-
Liver	Grey Heron	50	12.4 ± 1.5	9.1 – 17.5
	Eurasian Sparrowhawk	20	8.2 ± 0.3	7.9 – 8.5
	Herring Gull	60	11.6 ± 1.6	7.6 – 16.9
	Eurasian Magpie	0	-	-
	Eurasian Collared Dove	0	-	-
Feathers	Grey Heron	0	-	-
	Eurasian Sparrowhawk	0	-	-
	Herring Gull	0	-	-
	Eurasian Magpie	0	-	-
	Eurasian Collared Dove	0	-	-

PFHxS was detected in six spleen samples and PFOS in all of the spleen samples of the Grey Heron. However, neither PFOA nor PFNA were detected in the spleen. Although PFOS levels were lower in spleen than in liver and higher in spleen than in feathers, no significant differences were found among the PFOS levels in liver, spleen and feathers of the Grey Heron when comparing pared individual PFOS levels.

Table 6. The R-values and the p-values for the Pearson test used to determine if there is a correlation between the PFOS levels in the feathers and livers of the five birds species. n = 10 for each species.

Species	R-value	p-value
Grey Heron	0.124	0.733
Herring Gull	-0.006	0.986
Eurasian Sparrowhawk	0.333	0.348
Eurasian Magpie	0.258	0.472
Eurasian Collared Dove	0.470	0.170

The average PFOS levels in the liver were higher than in the feathers for the Grey Heron, Eurasian Sparrowhawk and the Herring Gull. The opposite was the case for the Eurasian Magpie and the Eurasian Collared Dove. There was no significant correlation between the liver PFOS levels and the feather PFOS levels in any one of the species when paired individual data were used (Table 6).

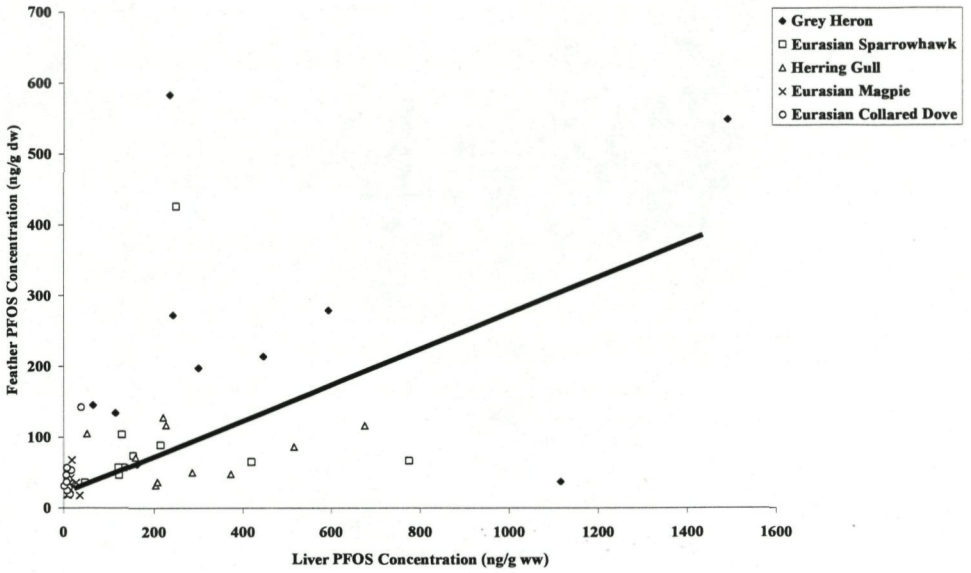

Figure 2. The correlation between the feather PFOS levels and the liver PFOS levels when all the data for the five species are analysed together (Pearson, $R = 0.622$, $p < 0.01$).

However, when the data of the five species were analysed together, there was a significant positive correlation between the levels of PFOS in the liver and in the feathers (Pearson, $R = 0.622$, $p < 0.01$; Figure 2).

4.4 Discussion

No data is available on the levels of PFCs in the feathers of wild birds; therefore, it is not known whether feathers could be used as indicators of PFC exposure in wild birds. Liver has mainly been the organ of choice when determining birds' exposure to PFCs. To investigate the usefulness of feathers as indicators of PFC exposure, we determined the PFC levels in both the feathers and livers of five bird species. The PFC levels in both liver and feathers were also compared between the species, as to determine the influence feeding habit and habitat have on birds' exposure to PFCs. From all PFCs analysed, PFOS was present at the highest levels in all analysed tissues for all investigated bird species. We will therefore mainly discuss the PFOS data.

The PFC levels found in liver in our study were mainly above the levels of other studies, except for the levels found in Bald Eagle in the United States of America (USA) (Table 7). Kannan et al. (2002a) detected PFOS levels in the liver of Grey Heron from Japan at 10 times lower levels than in our study. The levels of PFOA and PFHxS were below the detection limit (19 and 7.5 ng/g ww respectively) in their study, the same as PFOA (LOD = 7.3 ng/g ww) in our study.

Table 7. Comparison between the levels of PFCs in the liver of various bird species. The range of the values obtained in each study is given as ng/g ww. nm indicates that the particular PFC was not measured in the study.

Species	Country	PFOS	PFHxS	PFOA	PFNA	Study
Grey Heron	Belgium	66.2 – 1489.1	< 3.2 – 120.7	< 7.3	< 7.6 – 17.5	Current study
Herring Gull	Belgium	52.3 – 676.6	< 3.2 – 9.0	< 7.3 – 28.2	< 7.6 – 16.9	Current study
Eurasian Sparrowhawk	Belgium	47.6 – 775.0	< 3.2 – 40.6	< 7.3 – 15.9	< 7.6 – 8.5	Current study
Eurasian Magpie	Belgium	8.5 – 37.1	< 3.2 – 6.7	< 7.3	< 7.6	Current study
Eurasian Collared Dove	Belgium	< 2.5 – 39.5	< 3.2 – 6.1	< 7.3	< 7.6	Current study
Common Cormorant	Italy	32 – 150	< 7	29 – 450	nm	Kannan et al., 2002a
Bald Eagle	USA	26.5 - 1740	<38	< 38	nm	Kannan et al., 2005
Common Loon	Canada	11 – 26	nm	< 2.0	< 0.5	Martin et al., 2004b
Northern Fulmar	Canada	1.0 – 1.5	nm	< 2.0	< 0.5	Martin et al., 2004b

However, PFHxS was detected in 80% of liver samples of the Grey Heron in our study. PFNA and PFOA were only detected in respectively 26% and 18% of the liver samples of all the bird species in our study. Martin et al. (2004b) measured various PFCs in Northern Fulmar (*Fulmarus glacialis*) and Black Guillemot (*Cepphus grylle*). PFOA and PFNA were mainly below the limit of detection. The same was found for the levels of PFOA and PFNA in eggs of two albatross species from the North Pacific Ocean (Tao et al., 2006). PFNA and PFOA could not be quantified in any of the feather samples of the current study. The PFC levels n the liver samples from our study indicates that even about 100 km away from a known PFC hotspot, the liver PFC levels are still higher than most other studies.

There was no significant difference between the PFOS levels in the liver and spleen of the Grey Heron. The liver had higher PFOS levels than the spleen. In comparison, Olivero-Verbel et al. (2006) found higher levels of PFOS in the spleen than in the liver of Brown Pelicans (*Pelicanus occidentalis*). Higher PFOS levels were also measured in the spleen of Harbour Seals (*Phoca vitulina*) from the Dutch Wadden Sea compared to their liver tissue (Van de Vijver et al., 2005). However, there was also no significant difference between the PFOS levels in these two organs. The spleen acts as a reservoir for oxygenated blood and mainly as a producer of B- and T-cells involved in immune responses (Robinson et al., 2008). Like the liver, the spleen is thus also a blood-rich organ. More research is therefore needed to see how PFOS and the other PFCs are distributed within the body of birds.

When the relationship between the feather and liver PFOS levels was calculated for each species individually, no significant correlations were found. The small sample size ($n = 10$ for each species) may be the reason for this. When the sample size was increased to 50 by analysing the data of all the birds together, there was a significant positive correlation between the feather PFOS levels and the liver PFOS levels. This correlation between the feather and liver PFOS levels indicates that feathers could be an alternative to measure birds' exposure to PFOS. However, it was interesting to note that there were significantly higher levels of PFOS in the feathers than in the liver of the Eurasian Magpie and the Eurasian Collared Dove (Figure 3). Although the highest mean PFOS levels were found in the Grey Heron, 40% of the Grey Herons in our study had a higher PFOS level in the feathers than in the blood, whereas for the Eurasian Collared Dove almost 100 % had a ratio < 1. External contamination is unlikely to have played an important role, since the feathers were washed with acetonitrile before extraction to remove PFOS from the surface of the feathers.

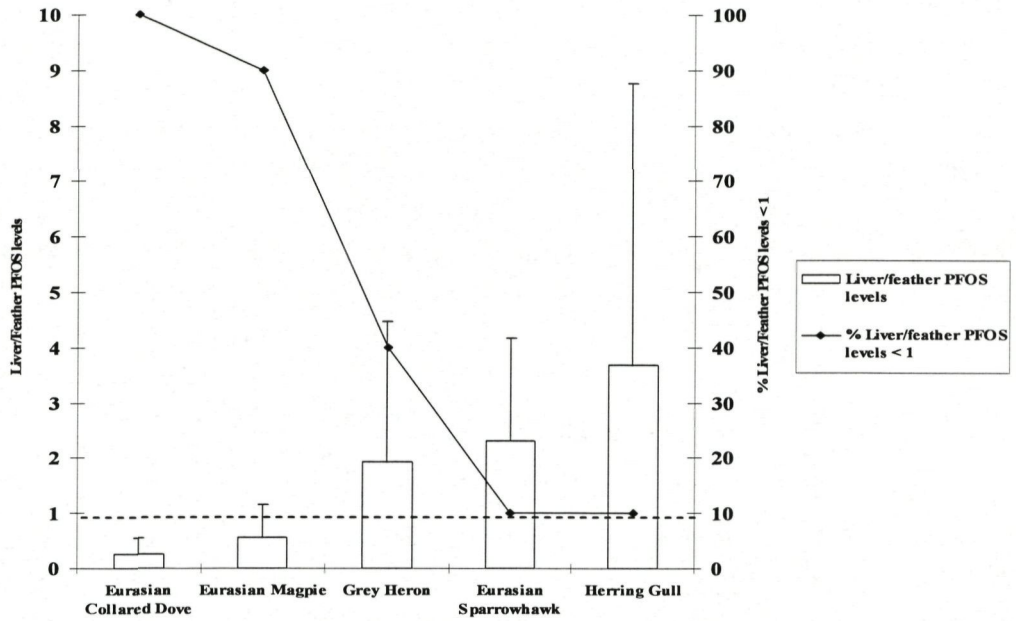

Figure 3. The relationship between the PFOS levels in the liver and feathers of the five bird species. The dashed line indicates a liver/feather PFOS level ratio of 1. The solid line indicates the percentage of birds per species with a liver/feather PFOS ratio less than 1.

Depositing PFOS in feathers might be an elimination route for PFOS in birds. Laboratory studies have shown female birds to have lower levels of PFOS in their livers than males, due to the deposition of PFOS into the eggs (Newsted et al., 2007). At low exposure concentrations, relatively more PFOS might be excreted in the feathers than in the liver. However, when a threshold value is reached, accumulation might then occur in the liver. In other words, the feathers might have a limited storage capacity for PFOS, mainly since they are only connected to the blood stream during their growth period. Since no other data exists on the PFOS levels in feathers, more research is needed to investigate whether birds with a low PFOS burden may have relative higher levels in their feathers than in liver. It should also be investigated if the liver/feather PFOS levels are species dependent or not. Feathers could however already be used as an indication of PFOS exposure in a population, but not necessarily of each individual.

No previous study on birds has compared the role of both diet and habitat in the birds' exposure to the various PFCs. Sinclair et al. (2006) found that piscivorous ducks had a 2.5 times higher PFOS level in their livers than herbivorous ducks. This showed that diet and the trophic level plays and important part in the exposure to PFCs. Only the role of diet was investigated, not the role that habitat plays since all the birds were ducks. In our study, the

liver PFOS levels were significantly higher in the Grey Heron, Herring Gull and the Eurasian Sparrowhawk than in the Eurasian Magpie and the Eurasian Collared Dove. This indicates that diet and habitat do play a role in the exposure to PFOS in birds. The birds with higher liver PFOS levels were either carnivorous or marine omnivores. The same observation was made in the study of Senthilkumar et al. (2007), where cormorants, a piscivorous marine species, had the highest liver PFOS levels in a group of birds that were studied. Many other studies also indicated that birds that are marine or carnivorous have high PFOS levels (Giesy and Kannan, 2001; Holström et al., 2005; Van den Brink et al., 2007; Van den Heuvel-Greve et al., 2006). However, studies from the Antwerp region (Belgium) near a fluoro-chemical plant indicated that small passerines, which feed on seeds and insects, could also contain high levels of PFOS. Some of the highest levels of PFOS in birds were measured in the liver and blood of Great Tits and Blue Tits from this area (Dauwe et al., 2007; Hoff et al., 2005b). It has to be kept in mind though, that research on other organisms indicated that this region is a hotspot for PFOS contamination. Some of the highest levels of PFOS in wildlife have been measured in this particular region (Hoff et al., 2004; Hoff et al., 2005b). There is no data available on the PFOS levels in birds that are higher in the food chain from this area to make a comparison with our study.

Three different groups can be distinguished in the bird feathers that were analysed: the Grey Heron (highest), then the Herring Gull, Eurasian Sparrowhawk and Eurasian Collared Dove, followed by the Eurasian Magpie. The feather PFOS levels of the Grey Heron were significantly higher compared to all the other species. However, the feather PFOS levels of the Herring Gull, Eurasian Sparrowhawk and Eurasian Collared Dove were also significantly higher than the Eurasian Magpie's. As with the liver PFOS levels, this suggests that diet and habitat seem to play a role in the exposure of the birds to PFOS. Although the differences among the feather PFOS levels of the different species were less obvious, they still showed that piscivorous birds tend to have higher levels of PFOS feather levels than other birds. Birds that occupy a higher trophic level tend to have higher levels of PFOS in their feathers. Aquatic birds also have higher PFOS levels in their livers and feathers than terrestrial birds. Various studies found that PFOS accumulates and even biomagnifies through the food chain (de Vos et al., 2008; Haukås et al., 2007). As mentioned before, feathers show a promise as an alternative, less invasive matrix to measure PFCs in, but more research is still needed.

To determine the risk of the PFOS to the birds, we looked at the available toxicity data of PFOS on birds. Very few toxicology studies are available and the longest exposures were only 21 weeks. All of the liver PFOS levels measured were below the laboratory-based NOAEL (No Observed Adverse Effects Level) and LOAEL (Lowest Observed Adverse Effects Level) derived for both Northern Bobwhites (*Colinus virginianus*) and Mallards (*Anas*

platyrhynchos) (Table 8; Giesy and Jones, 2004). However, 17% of the liver PFOS levels of the Grey Heron, Eurasian Sparrowhawk and Herring Gull were higher than the Toxicological Reference value (TRV) calculated for top avian predators (600 ng/g ww (Newsted et al., 2005). The TRV are calculated as exposure threshold values for birds in nature. The PFOS and PFHxS liver levels in the Grey Heron are also higher than the levels measured in Grey Heron from Japan (Kannan et al., 2002a). It should be noted that only two samples were analysed in the study from Japan, which could be the reason for the difference between the two studies. The few PFHxS, PFOA and PFNA levels that were above the limit of detection in our study were generally higher than in any previous study (Haukås et al., 2007; Martin et al., 2004b; Tao et al., 2006; Verreault et al., 2005). Although the levels were higher than many previous studies, more research is needed to see if the birds are at risk from exposure to these three PFCs. Since 10% of the liver PFOS values are above the TRV, they might be at risk from at least PFOS exposure.

Table 8. The NOAEL (No Observed Adverse Effects Level) and LOAEL (Lowest Observed Adverse Effects Level) in liver for both Northern Bobwhites (*Colinus virginianus*) and Mallards (*Anas platyrhynchos*) (Giesy and Jones, 2004; Newsted, 2005). All values are in ng/g ww.

Species	NOAEL		LOAEL	
	Male	Female	Male	Female
Mallard	61000	11000		
Northern Bobwhite	88000	-	86000	4900

4.5 Conclusions

PFOS was the PFC present in all samples and at the highest levels. The significant positive correlation between the feather and liver PFOS levels indicates that feathers could be an alternative bioindicator for PFOS exposure in birds. However, in some of species the levels of PFOS in the feathers were higher than in the liver. It should therefore be investigated what factors influence the ratio between the PFOS levels in the liver and feathers in birds. Nevertheless, feathers could already be used as an indication of general PFOS exposure in a bird population. When looking at the levels of PFCs in both the liver and feathers, it can be seen that species from a higher trophic level have higher PFC levels. Herbivorous, terrestrial birds had the lowest PFC levels in their liver and feathers. Therefore, both diet and habitat seem to play a role in the PFC exposure in birds.

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CHAPTER 5

Characterisation of perfluorooctane sulfonate in a terrestrial ecosystem

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Abstract

No previous study has investigated the bioaccumulation of PFOS in a terrestrial ecosystem. We collected Wood Mice (*Apodemys sylvaticus*), Bank Vole (*Clethrionomys glareolus*), various invertebrates and fruit from two sites in Belgium. PFOS was detected in almost all of the samples from both the contaminated site, Blokkersdijk, and the control site, Galgenweel. The highest mean level of PFOS at both sites was found in the liver, which was significantly higher in Blokkersdijk than in Galgenweel. At Galgenweel the highest PFOS levels were in millipedes, followed by woodlice, then slugs and earthworms containing the lowest levels of PFOS. The pattern was different for Blokkersdijk, where the highest PFOS levels measured was in earthworms, followed by slugs and millipedes. The lowest PFOS levels were in woodlice. The PFOS levels in the fruit of the European Elder were higher than in the Common Blackberry at both sites. There was no significant difference between the liver PFOS levels of the Bank Vole and the Wood Mouse from Blokkersdijk. The liver PFOS levels of the Wood Mice at Blokkersdijk were on average 2.4 times lower in 2006 than 2002, although not significantly lower. The diet weighted bioaccumulation factors (BAFs) were almost the same for Galgenweel (18.4) and Blokkersdijk (17.8).

5.1 Introduction

In the last few years much attention has been given to the levels of perfluorinated chemicals (PFCs) in wildlife. Early research into the exposure of wild animals to the various PFCs showed that PFOS is the PFC occurring in the highest levels in animals (Kannan et al., 2002a, 2002b). The mean of liver PFOS levels of Polar Bears (*Ursus maritimus*), a top predator, from East Greenland were as high as 2470 ng/g ww (Smithwick et al., 2005). Even in smaller predators, for example the American Mink (*Mustela vison*), levels of up to 3680 ng/g ww have been measured (Giesy and Kannan, 2001). Many studies have also been performed on the biomagnification and bioaccumulation of PFOS in food chains. Using stable isotope ratios, van den Vijver et al. (2003) showed that marine mammals with the highest trophic position contained the highest PFOS levels. Bioaccumulation was also shown to take place in a food web consisting of invertebrates and various fish species of Lake Ontario in Canada (Martin et al., 2004b). Houde et al. (2006) reported trophic magnification factors up to 156 in the food web of the Bottlenose Dolphin (*Tursiops truncatus*) from the United States of America (USA). However, all of these studies were performed in either marine or freshwater ecosystems. No previous study has characterised a food web that is located in a terrestrial ecosystem.

Various studies have looked at the temporal distribution of PFOS in wildlife. Most of the studies investigated time trends in the PFOS levels of bird eggs from museums. Using Common Guillemot (*Uria aalge*) eggs, Holström et al. (2005) showed there was a sharp peak in PFOS levels in 1997, followed by a decrease in PFOS levels until the end of the study in 2003. PFC levels were increasing in the livers of Thick-billed Murres (*Uria lomvia*) and Northern Fulmars (*Fulmaris glacialis*) between 1975 and 2004 (Butt et al., 2007). However, no study has been performed to determine the PFOS levels in terrestrial mammals over a period of time.

In a previous study, the PFOS exposure of Wood Mice (*Apodemus sylvaticus*) living near a perfluoro-chemical production plant in Belgium was investigated (Hoff et al., 2004). Although these animals were low in the food chain, they contained high levels of PFOS in their livers. The highest level of PFOS ever found in wildlife (178550 ng/g ww) was measured in the liver of one of the Wood Mice from this study. Other studies have also shown that the Antwerp region is a hotspot for PFOS contamination (Dauwe et al, 2007; Hoff et al., 2005a, 2005b). Although the study of Hoff et al (2004) investigated the PFOS exposure in Wood Mice, they did not determine the PFOS levels in the potential food items of the Wood Mice.

Most of the laboratory studies on the effects of PFOS on animals have been performed on rodents. Data on effect studies in the field are limited. Hoff et al. (2004) examined various biochemical endpoints in the liver and serum of Wood Mice from a PFOS polluted area. The liver weight, relative liver weight and the liver microsomal lipid peroxidation level increased significantly with the liver PFOS levels. The liver PFOS levels were also negatively related to the serum alanine aminotransferase activity, which is an indicator of liver damage.

The aim of this study was to characterise the PFOS levels in a terrestrial food chain. Wood Mice were chosen as the animal occupying the top trophic position in the food chain. Wood Mice and various invertebrates, as well as fruit of the Common Blackberry (*Rubus fruticosus*) and European Elder (*Sambucus nigra*), were collected from two sites in the Antwerp region. The results of the liver PFOS levels of the Wood Mice were also compared with a previous study in the area (Hoff et al., 2004) to determine if there has been a change in the PFOS levels of the Wood Mice over time. Serum biomarkers were also analysed and compared with the results of the previous study by Hoff et al (2004).

5.2 Materials and Methods

5.2.1 Sample collection

Sampling took place from September to November 2006 at two localities near Antwerp, Belgium (Figure 1). The first locality, Blokkersdijk, is located near of a fluoro-chemical plant and Galgenweel, the control site, is three kilometers further away. Wood Mice were captured alive in both Blokkersdijk (n = 20) and Galgenweel (n = 21) using Shermann Live traps. At Blokkersdijk six Bank Voles (*Clethrionomys glareolus*) were also captured. In the laboratory, the Wood Mice were anaesthetised using di-ethyl ether and blood was taken with the retro-orbital puncture method. The blood samples were centrifuged at room temperature for 5 minutes at 4000 rpm. The serum was used for the biomarker assays. Both the Wood Mice and the Bank Voles were sacrificed by an over-dose of di-ethyl ether. The animals were then sexed and the weight of the body and liver was determined. The liver, pancreas, lungs, kidneys and spleen were collected from each individual Wood Mouse. Only the liver was collected from each Bank Vole. The eye lenses of the Wood Mice were collected and fixed in 10% formaldehyde. Plant and invertebrate samples were also collected from each site. Invertebrate samples included slugs (order Stylommatophora), millipedes (class Diplopoda), woodlice (order Isopoda) and earthworms (order Haplotaxida), all of which were identified up to order or at least class level. The plant samples included the fruit of Common Blackberry and European Elder. Samples were frozen at -20°C until analysis.

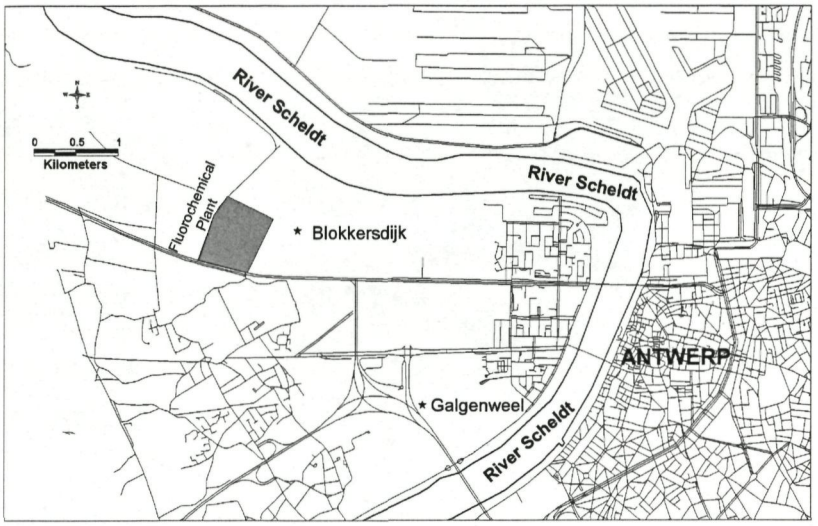

Figure 1. The location of the two study sites, Blokkersdijk and Galgenweel, near Antwerp, Belgium (taken from Hoff et al., 2004).

5.2.2 Age Determination

The age of the Wood Mice was determined by the method of Vandorpe et al., 1979. Briefly, the eye lenses were dried at 80°C for 24 hours and then weighed with an accuracy of 0.1 mg. The age of the animals in days was calculated using the equation: exp((weight of both lenses in mg+15.213)/6.568).

5.2.3 PFOS extraction and measurement

PFOS extraction from all the samples was done by solvent extraction based on the method by Berger and Haukås (2005) with adaptations, as describe earlier in Chapter 4 for eggs. Only the sample preparation for the PFOS extraction differed for each type of sample. Between 0.5 and 1 g of the liver and kidney samples of the Wood Mouse and the liver samples of the Bank Vole was homogenised in a polypropylene (PP) centrifuge tube using a mixer and then weighed. Due to the small size of the other organs, the organ samples were pooled to create two pooled samples. These pooled samples were also homogenised in a PP centrifuge tube and then weighed. The invertebrate and plant samples were pooled and then divided into five pooled samples of about 1 g each. Each pooled sample was first crushed with a mortar and pestle before being homogenised in a PP centrifuge tube. All the samples were then weighed before extraction.

5.2.4 Bioaccumulation Factors

The bioaccumulation factors (BAFs) were determined as follows:

$$BAF = \frac{[PFOS]liver}{[PFOS]food\ item}$$

To take the diet of the Wood Mouse into account, the diet weighted BAF was calculated as follows:

$$BAF = \frac{[PFOS]liver}{[PFOS]weighted\ diet}$$

where: [PFOS]weighted diet = ([PFOS]plant x 0.85) + ([PFOS]animal x 0.15)

and ([PFOS]plant) = ([PFOS]elder x 0.5) + ([PFOS]blackberry x 0.5)

and ([PFOS]animal) = ([PFOS]earthworm x 0.75) + ([PFOS]millipedes x 0.0833) + ([PFOS]woodlice x 0.0833) + ([PFOS]slugs x 0.0833)

5.2.5 Serum Biomarker assays

The serum alanine aminotransferase activity was determined by the spectrophotometric method of Bergmeyer et al. (1986a; 1986b). The cholesterol concentration was measured according to Allain et al. (1974) and the triglyceride concentration according to the method of Spayd et al. (1978). The serum protein content was determined with the Bio-Rad Protein Assay (Bio-Rad, Munich, Germany).

5.2.6 Quality Control

Laboratory blanks were extracted along with each batch of samples, consisting of all the solvents but having no sample. Spiked pork liver samples were also extracted along the samples to measure recovery. Pure acetonitrile was injected after every 8 samples to check for memory effects. A standard was injected after every 8 samples to check the stability of the HPLC-MS/MS system. After each injection of a solvent standard or spiked sample, pure acetonitrile was also injected.

5.2.7 Statistical Analysis

The statistical analysis was done with SPSS 15.0 for Windows. The non-parametric Mann-Whitney test was used to determine any significant differences between PFOS levels in the different matrices at each site, as well as any significant differences between the PFOS levels at each site for each matrix. The correlations between the PFOS levels and the biomarker levels were determined with Spearman Rank analysis.

5.3 Results

PFOS was detected in almost all of the samples from both the contaminated site, Blokkersdijk, and the control site, Galgenweel. PFOS was only below the detection limit in the Common Blackberry fruit from Galgenweel. The PFOS levels in the various organs are shown in Figure 2. The highest mean level of PFOS at Blokkersdijk was found in the liver, followed by the pancreas, lungs and kidneys, with the spleen having the lowest levels. At Galgenweel the pattern was the same, except that the pancreas had higher levels than the liver. Significant differences between the two sites were only determined for the PFOS levels in the liver and the kidney, as the sample size of the other organs were too small ($n = 2$). There was a significant difference between the liver PFOS levels of Galgenweel and Blokkersdijk as well as a significant difference between the kidney PFOS levels of the two sites. There was no significant difference between the liver PFOS levels in males and females from both sites (Mann Whitney test, $p < 0.05$).

The PFOS levels in the various invertebrates are shown in Figure 3. At Galgenweel the highest PFOS levels were in millipedes, followed by woodlice, then slugs and earthworms containing the lowest levels of PFOS. However, it should be noted that only one pooled sample of millipedes were analysed at Galgenweel.

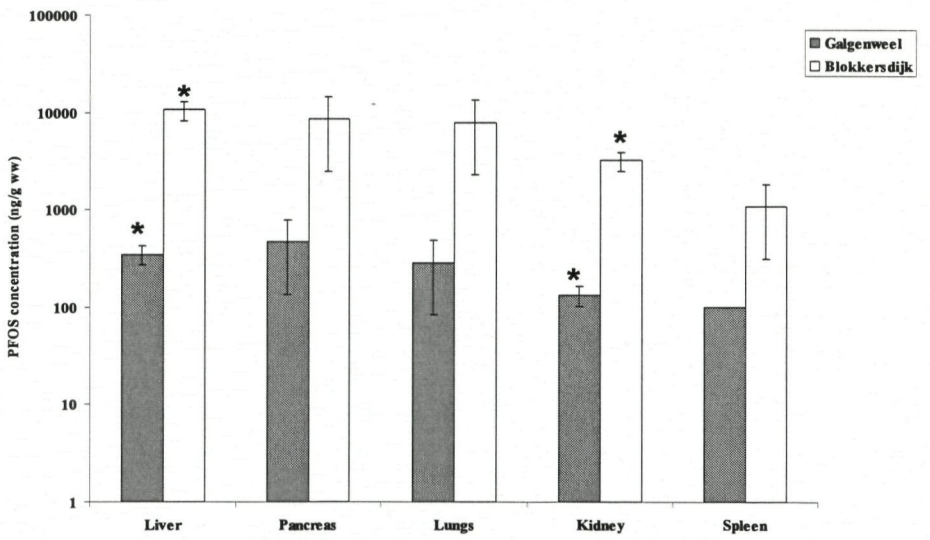

Figure 2. The PFOS levels (ng/g ww) of the various organs of the Wood Mouse from both Galgenweel and Bokkersdijk. Significant differences (Mann- Whitney test, $p < 0.05$) in the PFOS levels in the livers and kidneys between sites are indicated by *. $n = 21$ for liver and kidney for Galgenweel and $n = 20$ for Blokkersdijk. $n = 2$ for the other organs, except for spleen at Galgenweel where $n = 1$.

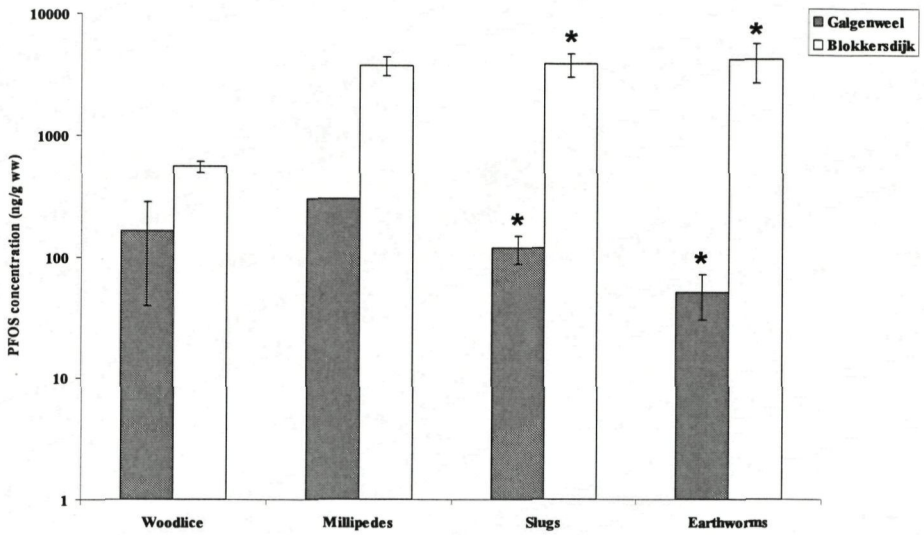

Figure 3. The PFOS levels (ng/g ww) of the various invertebrates from both Galgenweel and Bokkersdijk. Significant differences (Mann- Whitney test, $p < 0.05$) in each matrix between sites are indicated by *. n = 5, except for millipedes at Galgenweel where n = 1.

The pattern was different for Blokkersdijk, where the highest PFOS levels measured was in the earthworms, followed by the slugs and millipedes. The lowest PFOS levels were in the woodlice. There were significant differences between the PFOS levels of Galgenweel and Blokkersdijk for the slugs and earthworms.

The PFOS levels in the fruit of the Common Blackberry and European Elder at the two sites are shown in Figure 4. The PFOS levels in the fruit of the European Elder were higher than in the Common Blackberry at both sites. There was also a significant difference in the PFOS levels in the fruit of the European Elder between the two sites. The PFOS levels in the fruit of the Common Blackberry at Galgenweel were below the detection limits.

The liver PFOS levels were higher than all the other matrices measured at both sites. The liver PFOS levels of the Wood Mice were on average 2.5 times higher than the PFOS levels in the earthworms at Blokkersdijk. The earthworms were the invertebrates with the highest levels of PFOS at Blokkersdijk. The PFOS levels in the woodlice from Blokkersdijk, which had the lowest PFOS levels of the invertebrates, were about 19 times lower than the liver PFOS concentrations of the Wood Mice. At Galgenweel the liver PFOS levels of the Wood Mice were almost the same as the PFOS levels in the millipedes, which was the invertebrate that had the highest PFOS levels at Galgenweel. However, it should be noted that only one sample was analysed. Even the invertebrates with the lowest PFOS levels at Galgenweel, the earthworms, had PFOS levels of only 7 times less than the liver PFOS levels of the Wood

Mice. When comparing the PFOS levels in the fruit of the European Elder and the Wood Mice liver, the PFOS level in the liver was 25 times higher at Galgenweel and 230 times higher at Blokkersdijk.

The bioaccumulation factors (BAFs) for the Wood Mouse and its various food items are summarised in Table 1. The highest BAF was between the Wood Mouse and the Common Blackberry (839.8) at Blokkersdijk. The BAF of the weighted diet of the Wood Mouse was slightly higher at Galgenweel (18.4) than in Blokkersdijk (17.8).

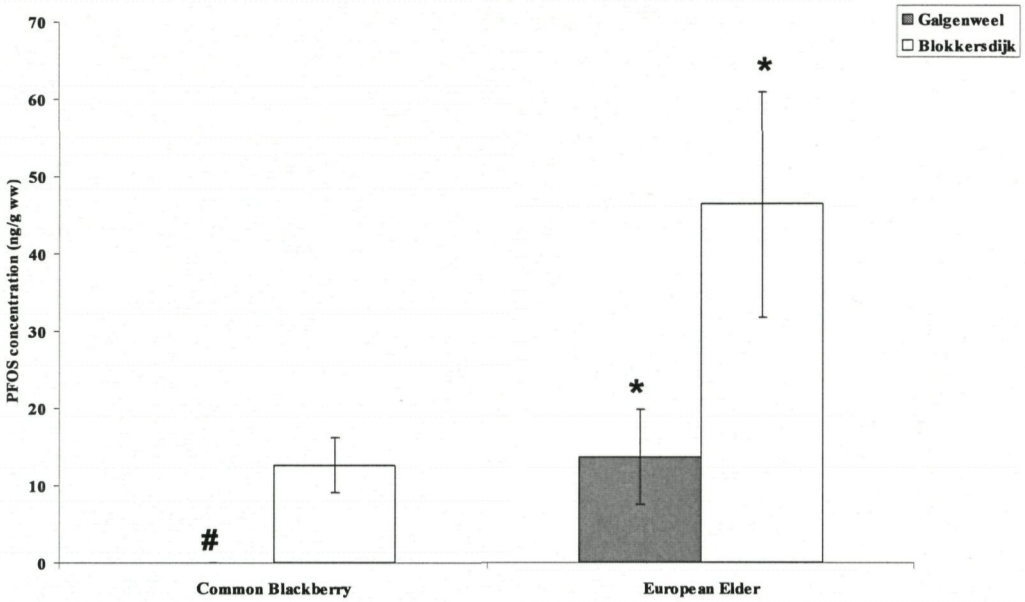

Figure 4. The PFOS levels (ng/g ww) of the fruit of two plants from both Galgenweel and Bokkersdijk. Significant differences (Mann- Whitney test, $p < 0.05$) in each matrix between sites are indicated by *. # indicates that the PFOS levels were below the LOD. $n = 5$ for both species.

There was no significant difference (Man-Whitney, $p < 0.05$) between the liver PFOS levels of the Bank Vole ($n = 6$) and the Wood Mouse ($n = 20$) from Blokkersdijk (Figure 5).

Table 1. The bioaccumulation factors (BAFs) for the Wood Mouse and each of its food items. In the bottom row the diet weighted BAF is given that is calculated according to the diet of the Wood Mouse (weighted diet = 85% plant + 15% animal food items, see Materials and Method 5.2.4).

	BAF	
Food Item	**Galgenweel**	**Blokkersdijk**
Earthworms	6.8	2.5
Slugs	2.9	2.8
Millipedes	1.2	2.8
Woodlice	2.2	19.3
European Elder	25.4	229.6
Common Blackberry	-	839.8
Weighted Diet	18.4	17.8

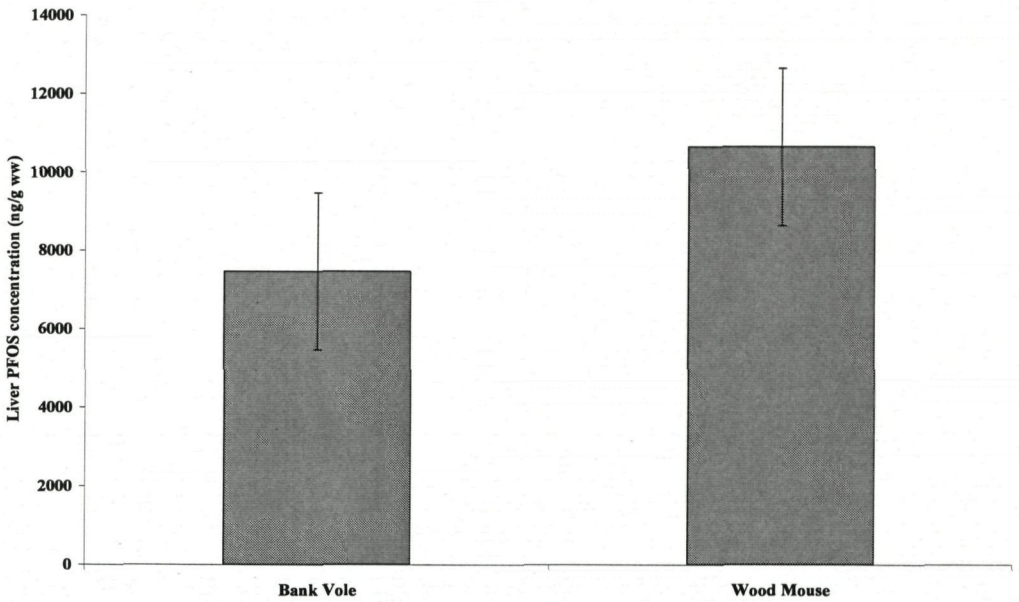

Figure 5. The liver PFOS levels (ng/g ww) of the Bank Vole and Wood Mouse at Blokkersdijk. n = 6 for Bank Vole and n = 20 for Wood Mouse.

The significant differences between the different matrices at each site are given in Table 2 (Galgenweel) and Table 3 (Blokkersdijk).

Table 2. The p-values of all the significant differences between the different matrices from Galgenweel. – indicates there was no significant difference.

	Kidney	Woodlice	Millipedes	Slugs	Earthworms	Common Blackberry	European Elder
Liver	0.000	0.029	-	0.012	0.001	0.001	0.001
Kidney		-	-	-	0.021	0.001	0.001
Woodlice			-	-	-	0.005	0.049
Millipedes				0.047	-	0.005	-
Slugs					0.047	0.005	0.009
Earthworms						0.005	-
Common Blackberry							0.019

Table 3. The p-values of all the significant differences between the different matrices from Blokkersdijk. – indicates there was no significant difference.

	Kidney	Woodlice	Millipedes	Slugs	Earthworms	Common Blackberry	European Elder
Liver	0.001	0.001	-	-	-	0.001	0.001
Kidney		0.025	-	-	-	0.001	0.001
Woodlice			0.009	0.009	0.009	0.009	0.009
Millipedes				-	-	0.009	0.009
Slugs					-	0.009	0.009
Earthworms						0.009	0.009
Common Blackberry							0.016

A comparison of the liver PFOS levels at both sites between our study and a previous study (Hoff et al., 2004) are shown in Figure 6. The liver PFOS levels at Blokkersdijk were 2.4 times lower in 2006 than 2002, although the difference was not statistically significant.

The results of the various serum biomarkers are given in Table 4. There was a significant difference in the age, Liver Mass Index (LMI) and Serum Alanine Aminotransferase activity (both U/L and U/g prot) between the sites.

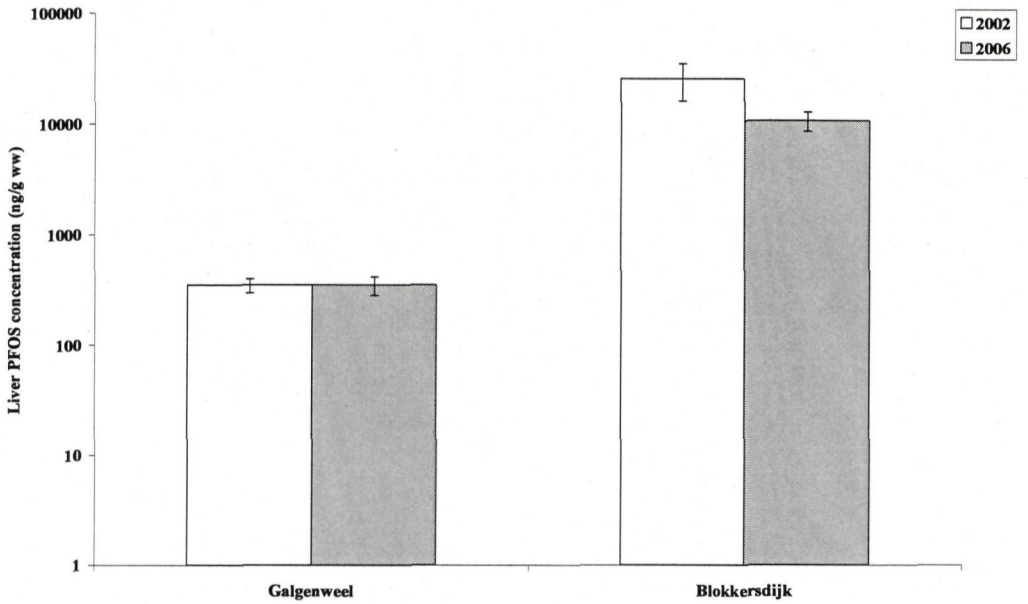

Figure 6. The liver PFOS levels (ng/g ww) at Galgenweel and Blokkersdijk in 2004 and 2006

Table 4. The results of the serum biomarkers for Wood Mice from Galgenweel and Blokkersdijk. All significant differences in the level of the biomarkers between the two sites are indicated by *. Data from Hoff et al. (2004) are also given for comparison.

	Current Study		Hoff et al., 2004	
	Galgenweel	**Blokkersdijk**	**Galgenweel**	**Blokkersdijk**
Age	173*	95*	71	70
	(31 – 521)	(40 – 211)		
Liver Mass Index	0.05*	0.06*	0.04	0.05
	(0.04 – 0.08)	(0.04 – 0.07)		
Triglyceride concentration (mg/dL)	151	74	102	106
	(50 – 421)	(51 – 99)		
Cholesterol concentration (mg/dl)	164	104	143	132
	(71 – 741)	(55 – 161)		
Alanine aminotransferase activity (U/L)	163*	122*	142	125
	(114 – 248)	(83 – 160)		
Alanine aminotransferase activity (U/g prot)	2.6	1.9	2.2	1.9
	(1.5 – 4.2)	(1.1 – 2.6)		
Protein concentration (g/L)	64*	65*		
	(49 – 79)	(55 – 78)		

There was a positive significant correlation (Spearman Rank correlation, r = 0.516, p = 0.02) at Blokkersdijk and a significant negative correlation at Galgenweel (Spearman Rank correlation, r = -0.435, p = 0.049) between age and liver PFOS levels (Figure 7). There was a positive significant correlation between the liver PFOS levels and the LMI at Galgenweel (Spearman Rank; r = 0.483, p = 0.031), but not at Blokkersdijk (Figure 8). There were no other significant correlations between the liver PFOS levels and the biomarkers.

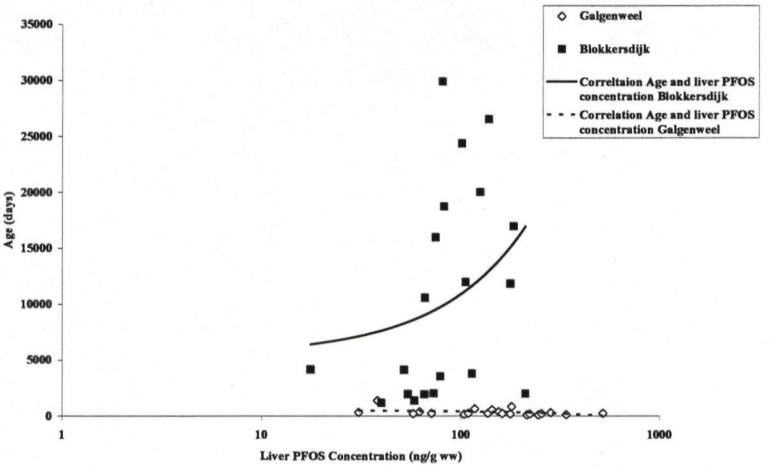

Figure 7. The relationship between age (days) and the liver PFOS levels (ng/g ww) in the Wood Mice from Galgenweel and Blokkersdijk. Significant correlations ($p < 0.05$) are indicated by the lines.

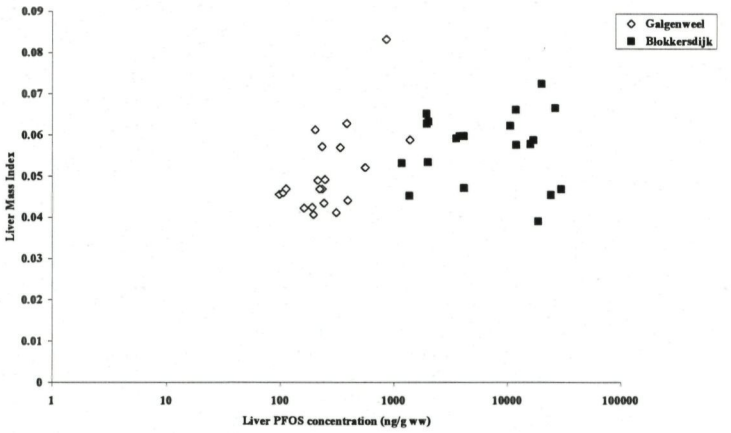

Figure 8. The relationship between the Liver Mass Index and the liver PFOS levels (ng/g ww) in the Wood Mice from Galgenweel and Blokkersdijk. Significant correlations ($p < 0.05$) are indicated by the lines.

5.4 Discussion

Although much attention has been given to the levels of PFOS in wildlife, most studies have been performed on marine or freshwater organisms. Very little data exists on the PFOS exposure of terrestrial animals. Various studies have shown that PFOS biomagnifies and bioaccumulate in wildlife. Again, these studies all focussed on the marine or freshwater environment (van den Vijver et al., 2003; Martin et al., 2004b; Houde et al., 2006). No previous study has investigated a food web that is located in a terrestrial ecosystem. In our study, we investigated the PFOS levels in various organisms in a terrestrial ecosystem, with the Wood Mouse occupying the highest trophic level.

PFOS was detected in all the samples from both the contaminated site Blokkersdijk, and the control site, Galgenweel, except for the fruit of the common Blackberry, which was below the detection limit at Galgenweel. This shows that PFOS is present throughout the whole ecosystem in the Antwerp region, and not only at the contaminated site. Due to lack of sufficient amount of sample, the pancreas, lungs and spleen samples were pooled and then divided into two pooled samples. No statistics were done on these samples, but they are included in the discussion to give an idea of the distribution of PFOS in the body of the Wood Mice. The highest PFOS levels at both sites were in the liver and the lowest in the spleen. No other study has looked at the organ distribution of PFOS in rodents; therefore, a comparison has to be made with other mammals. Houde et al. (2006) also found the highest levels of PFOS in the liver of Bottlenose Dolphins, as well as higher levels of PFOS in the lungs than in the kidney, the same as in our study. The mean PFOS levels in the liver of various marine mammals were also higher than the mean PFOS levels in their kidneys (Van den Vijver et al., 2003). There was no significant difference between the levels of PFOS in the livers of the male and female Wood Mice at both sites. Hoff et al. (2004) also did not find any significant difference between the liver PFOS between the sexes of Wood Mice at the same study sites.

As a representation of the PFOS exposure of the Wood Mice, the PFOS levels in their livers were used. Wood Mice have a varied diet, which is time and habitat dependent (Abt et al., 1998). Therefore, some assumptions were made on the composition of the diet of the Wood Mouse to calculate a weighted diet BAF. A weighted diet BAF takes the food preference of the organism into account and gives a more realistic BAF than when only using one food item to calculate the BAF. In a study on the transfer and accumulation of metals from the food items to the Wood Mouse in the same area, Rogival et al. (2006) used a diet composition that consisted of 85% of plants and 15% of animals, which was also used in this study. Although acorns are a major food item of Wood Mice, the determination of PFOS levels in acorns could practically not be determined due to problems of sample preparation. Therefore to determine

the BAF for the weighted diet of the Wood Mouse, the fruit of the Common Blackberry and European Elder were taken as the main plant items eaten by the Wood Mouse, in a 1:1 ratio. The main animal food item of Wood Mice are earthworms, therefore they comprised 75% of the animal food items in our BF calculation, with the other invertebrates making up the other 25% in equal amounts. The weighted BAF for Galgenweel (18.4) was slightly higher than the weighted BAF for Blokkersdijk (17.8). Although the liver PFOS levels in Blokkersdijk were significantly higher than the liver PFOS levels at Galgenweel, the BAFs are almost the same. This may be because of the relatively high levels of PFOS in the earthworms (4173 ng/g ww) at Blokkersdijk. The earthworm PFOS levels were 81.4 times higher at Blokkersdijk than at Galgenweel, whereas the PFOS levels in the plants were only 4.3 times higher at Blokkersdijk than in Galgenweel. Since plants make up the major part of the diet of Wood Mice, it is expected that the difference between the BAFs from the two sites would not be that large, since the difference in the PFOS levels in the plants of the two sites was not that large. This shows that the uptake and elimination of PFOS by the individual food items are important, as this influence the BAF between sites with different levels of PFOS exposure. Since earthworms are the food item with the highest levels of PFOS at Blokkersdijk, they may be a very important source for PFOS uptake in Wood Mice, whereas at Galgenweel earthworms contained the lowest PFOS levels and therefore did not influence the BAF as much as at Blokkersdijk. The diet weighted BAFs in our study (18.4 and 17.8) are about 6 times higher than the diet weighted BAF (2.9) for Lake Trout in Lake Ontario (Martin et al., 2004b). Although Houde et al. (2006) did not calculate a diet weighted BAF for Bottlenose Dolphins; the BAFs for each individual prey (1.8 to 13) were also lower than the diet weighted BAFs for both Galgenweel and Blokkersdijk. More research is needed to determine whether these differences between the BAFs are influenced more by the ecosystem type (marine and freshwater vs. terrestrial) or the composition of the diet.

Although the liver PFOS levels of the Bank Vole were lower than those of the Wood Mouse in Blokkersdijk, this difference was not significant. There is some degree of overlap in the diet of Bank Vole and Wood Mice, depending on the season. In the autumn, when our study took place, Bank Voles eat more berries and fungi, whereas the diet of the Wood Mouse includes more acorns and animal material (Abt et al., 1998). This may explain why there is a difference in the liver PFOS levels between Bank Voles and Wood Mice. The invertebrates in our study had higher levels of PFOS than the fruit of the plants; therefore, Wood Mice are expected to have higher PFOS levels.

Due to the short half-life of PFOS in rodents, they can be important in monitoring the temporal change in exposure to PFOS in animals. No data is available on the half-life of PFOS in Wood Mice, but the half-life of PFOS in laboratory-exposed rats was about 100 days

(Butenhoff et al., 2006). The liver PFOS levels of the Wood Mice from our study were compared to the results from a previous study four years earlier at the same sites to determine any time trends. There was a decrease, although not significant, in the liver PFOS levels of the Wood Mice at Blokkersdijk from 2002 to 2006. There was no significant difference between the levels of PFOS from the two time periods at Galgenweel. This indicates that the levels of PFOS exposure through diffusion in Galgenweel did not change in the fours years between the sampling periods. The decrease at Blokkersdijk may be because of the phasing out of the production of PFOS that started in 2000. The PFOS levels from 2002 at Blokkersdijk may therefore be peak levels, which are now starting to decrease. Since the half-life of PFOS in rats are only 100 days, the PFOS levels should have already been at the 2006 levels at about 100 days after the previous study, if the Wood Mice's exposure to PFOS completely stopped. However, this is not the case, since the Levels at Blokkersdijk only decreased by about 2.4 times in the four years between the two studies. This indicates that PFOS is persistent in the environment and the Wood Mice are still exposed to high levels of PFOS. More studies are needed in the future to see if this trend persists or not.

The Liver Mass Index was significantly higher in Blokkersdijk than in Galgenweel, whereas the Alanine amino transferase activity (ALT; both U/L and U/g prot) were significantly lower in Blokkersdijk than in Galgenweel. The same results were found by Hoff et al. (2004) for the Serum ALT. These biomarkers indicate liver damage. Hoff et al (2004) also found a significant difference between the Liver Mass Index between the two sites, indicating that the livers of the Wood Mice are relatively larger at Blokkersdijk. An enlarged liver has been shown to be an effect of PFOS exposure. Except for a significant positive correlation between the liver PFOS levels and the LMI at Galgenweel, there were no other significant correlations between liver PFOS levels and the biomarkers. All the biomarker results were in the same range as the biomarker results from a previous study in the same area (Table 4). This may be because there is no significant difference in the liver PFOS levels from the two studies. The was a significant negative correlation between age and liver PFOS levels in Galgenweel, but a significant positive correlation between age and liver PFOS levels at Blokkersdijk. Hoff et al. (2004) found significant positive correlations between age and liver PFOS levels at both sites. The Wood Mice caught at Blokkersdijk were significantly younger than the ones caught at Galgenweel in our study. This may indicate that PFOS accumulation is age depended. The biomarker results may also indicate that the effects of PFOS are dose-dependent, meaning there are different effects depending on the level of PFOS expsoure.

5.5 Conclusions

With this study, we showed that PFOS bioaccumulation also occurs in a terrestrial food chain. Liver seems to be the organ that accumulates the highest levels of PFOS in Wood Mice.

There were higher PFOS levels in the Wood Mouse livers than in any of the invertebrates. The fruits of the plants had the lowest PFOS levels of all the groups of animals analysed. The BAFs for the two sites were almost the same, indicating that bioaccumulation occurs, independent of the PFOS exposure level. There was a decline in the liver PFOS levels at Blokkersdijk over time, although the decline was not significant. At Galgenweel the liver PFOS levels were the same in 2006 as in 2002. This all indicates that PFOS is persistent in the ecosystem.

CHAPTER 6

General Conclusions and Perspectives for Future Research

6.1 General Conclusions

To be able to perform a risk evaluation for a chemical or group of chemicals, it is necessary to have a large amount of data on both the exposure and the effects of these chemicals. However, when a risk assessment is performed for a specific chemical, the predicted environmental concentration (PEC) and the predicted no effect concentration (PNEC) is calculated from existing exposure and effects data that are relevant to the chemical and the specific environment of concern. The PEC will be calculated from exposure data and the PNEC from effects data. Although PFCs have been produced for decades, analytical methods to determine the PFC levels in the environment have only been available since the end of the last century. Since then, an increasing amount of articles has been published on the exposure of wildlife to PFCs, and in particular PFOS. PFOS is the PFC that occurs in the highest levels in wildlife. Most data available on the exposure of wildlife are on marine or freshwater animals that occupy the higher trophic levels. Data on the effects of PFCs, mainly from laboratory studies, are available for mostly rodents and monkeys. Only two bird species, the Mallard and the Northern Bobwhite, have been used to generate basic toxicity data on PFCs. Very little data is available on the effects of PFCs on wildlife.

The main aim of this thesis was to generate more data on both the exposure and effects of PFCs on wildlife, focussing on birds. By generating new data the ultimate goal of the thesis is to perform a risk evaluation for birds using this new data. To accomplish this, several objectives were formulated:

1. Test whether feathers can be used as indicators of PFC exposure
2. Investigate the role feeding habit and habitat play in PFC exposure
3. Determine the relationship between PFC exposure and the distance from a fluoro-chemical plant
4. Evaluate several biochemical endpoints for their usefulness as biomarkers of PFC exposure.

No data is available on the levels of PFCs in feathers. Since PFOS is found in the blood, it might be deposited in the feathers during feather formation. Various other contaminants have successfully been measured in feathers. To be able to measure the PFOS levels in feathers, we developed a new PFOS extraction method for this matrix. In Chapter 1 PFOS was successfully measured in the feathers of laboratory exposed Zebra Finches. This was the first time that the PFOS levels in feathers could successfully be measured. There was a significant positive correlation between the liver PFOS levels and the feather PFOS levels of the birds. This indicates that feather may give a good indication of PFOS exposure in birds. The advantage of using feathers as indicators of PFOS exposure is that they are less invasive that

using other matrices, for example liver or eggs. The new method developed to measure PFCs in feathers was then tested with samples from the field in Chapter 4. Again, there is no data available on the PFC levels in the feathers of wild birds. The feathers and livers of 10 individual birds from five species were analysed for PFOS, PFHxS, PFOA and PFNA. The five species were chosen to represent different feeding habits and habitat types and included the Grey Heron (freshwater carnivore), Eurasian Sparrowhawk (terrestrial carnivore), Herring Gull (marine omnivore), Eurasian Magpie (terrestrial omnivore) and Eurasian Collared Dove (terrestrial herbivore). Only PFOS was detected in all the feather samples. There was a significant positive correlation between the feather and liver PFOS levels when all the paired data of the five different species were analysed together. However, in two species the levels of PFOS in the feathers were higher than in the liver. Depositing PFOS in feathers might be an elimination route for PFOS in birds, therefore at low exposure concentrations, more PFOS might be excreted in the feathers than in the liver. However, when a threshold value is reached, accumulation might then occur in the liver. Nevertheless, this indicates that feathers could be an alternative indicator for PFOS exposure in birds, possibly as an indication of general PFOS exposure in a bird population.

Only one previous study has focussed on the influence of feeding habits on the PFOS exposure in birds. This study showed that piscivorous birds have higher liver PFOS levels than herbivorous birds. To determine the role that feeding habit plays in a bird's exposure to PFCs, we first examined the levels of PFCs in the eggs of water birds from the same ecosystem in Chapter 2. The four species that were chosen were all water birds, but had different diets and feeding habits. The Canada Goose is a grazer, eating mainly grass found next to water. The Gadwall is a herbivorous dabbling duck, eating plant material from the water surface. The Common Pochard is an omnivorous diving duck, gathering food by diving. The Eurasian Coot is an omnivorous rail, feeding both in the water and on land. There was a significant difference in the PFC levels in the eggs of the four bird species that share the same ecosystem. The herbivorous birds had significantly lower PFC levels in their eggs than the omnivorous birds. Birds that spent more time on and especially under the water searching for food had higher PFC levels in their eggs. Thus, according to our data, not only does diet play a significant role in the PFOS exposure of bird, but that feeding habits might also play a role. In Chapter 3 we investigated the PFOS levels in the eggs of three bird species (Great Tit, Mediterranean Gull and Northern Lapwing) from the same area, but with different feeding habits. The PFOS levels were higher in the eggs of the Mediterranean Gull than in the eggs of the other two species from the same distance from the fluoro-chemical plant. Although all three species feed on insects, the diet of the Mediterranean Gull also includes gastropods, small fish and even rodents. To investigate the role both feeding habits and habitat play in the PFC exposure of birds, we examined five different bird species (Grey

Heron, Eurasian Sparrowhawk, Herring Gull, Eurasian Magpie and Eurasian Collared Dove) from the same region, but from different ecosystems in Chapter 4. Higher PFC levels were measured in both the liver and feathers of the species from the higher trophic levels. Herbivorous, terrestrial birds had the lowest PFC levels in their liver and feathers. Therefore, it is clear that both diet and habitat play a role in the PFC exposure in birds. This was the first time that the role of both habitat and feeding were investigated in bird species with so many different feeding habits.

Although there are studies that investigated the PFOS levels in birds from a PFOS contaminated site and compared it to control sites, no study has determined if there is a correlation between PFOS exposure and distance from a point source of PFOS contamination. In Chapter 3 we investigated the relationship between the PFOS levels in bird eggs and the distance of the nest site from a fluoro-chemical plant. There was a significant correlation between the PFOS levels in the eggs of the Northern Lapwing and the distance of the nest site from the fluoro-chemical plant. Although Vlietbos and Burchtse Weel are only 1250 m apart from each other, there was a significant difference between the PFOS levels in the eggs of the Great Tits from the two sites. This indicates that the distance from the fluoro-chemical plant highly influences the exposure of birds to PFOS.

To evaluate the usefulness of various biochemical endpoints as biomarkers for PFOS exposure, Zebra Finches were exposed to PFOS under laboratory conditions (Chapter 1). However, there was no significant correlation between the liver PFOS levels and any of the biomarkers investigated in the study. In Chapter 3, although there was a significant difference between the PFOS levels in the eggs of the Great Tits from Vlietbos and Burchtse Weel, there are no clear trends in the biomarker responses.

The bioaccumulation factors (BAFs) for PFOS have been determined in marine and freshwater ecosystems, but not for a terrestrial ecosystem. In Chapter 5 we investigated the levels of PFOS in the various compartments of a terrestrial ecosystem, with the Wood Mouse occupying the highest trophic level. The highest PFOS levels were measured in the Wood Mouse livers, then in the various invertebrates, with the lowest PFOS levels in the fruit of two plants. The BAFs were almost the same for the control site, Galgenweel (18.4), than for the contaminated site, Blokkersdijk (17.8). The BAFs were also higher than those for marine or freshwater ecosystems. No previous study has looked at the levels of PFOS exposure in a terrestrial ecosystem over time. We therefore compared our data from Blokkersdijk to data from a study four years earlier, also in Blokkersdijk. There was a decline in the liver PFOS levels at Blokkersdijk over time, although the decline was not significant. At Galgenweel the

liver PFOS levels were the same in 2006 as in 2002. This all indicates that PFOS is persistent in the ecosystem.

The main goal of gathering exposure and effects data is to perform either a risk evaluation or a risk assessment. To perform a risk evaluation of a certain chemical compound for a specific organism, the levels of that compound in the organism from the field is compared to existing toxicity data. To evaluate the risk birds might have from PFC exposure, we compared the PFOS levels in both eggs and liver from the birds in our study with toxicological threshold values available for birds. The distance from the fluoro-chemical plant in Antwerp was also taken into account. The various toxicity threshold values available for birds are shown in Table 1, as well as the percentage of samples above the threshold value for both liver and eggs. The mean distance of the sampling point from the fluoro-chemical plant in Antwerp for all the samples above the threshold value are also given in Table 1.

Table 1. The various toxicity threshold values (ng/g ww for liver, ng/mL for egg) for birds and the percentage of samples above the threshold value for both liver and eggs. The mean distance from the fluoro-chemical plant in Antwerp for all the samples above the threshold value are given in meters.

	Threshold Value (Newsted et al., 2005)		% of samples above threshold value	Mean Distance from fluorochemical plant
Mallard	**LOAEL Liver Male**	61000	0	-
	LOAEL Liver Female	11000	1	1271
	NOAEL Egg	53000	0	-
Northern Bobwhite	**LOAEL Liver Male**	88000	0	-
	LOAEL Liver Female	4900	3	1000
	LOAEL Egg	62000	0	-
	NOAEL Egg	33000	4	173
Top Avian Predator	**TRV Liver**	600	28	12735
	TRV Egg	1700	51	676
	PNEC Liver	350	40	7473
	PNEC Egg	1000	59	997

Data from two previous studies in the area (Dauwe et al., 2007; Van den Brink et al., 2007) are also included in the evaluation. In total, the data of 75 egg samples and 72 livers samples were evaluated. Although the toxicity threshold values for eggs are given in ng/mL, and the PFOS levels from both our study and the study of Van den Brink (2007) are given in ng/g ww, approximate comparison is still possible since about 1 ml was used in the extraction process for all samples. Most of the PFOS levels found in the liver and eggs were below the No Observed Adverse Effects Level (NOAEL) and the Lowest Observed Adverse Effects Level (LOAEL) for both Mallards and Northern Bobwhites. The few values that were above the NAOEL and LAOEL were less than 1500 m from the fluoro-chemical plant. However, when the Toxicity Reference Values (TRV; Newsted 2005) for Top Avian predators were used, the amount of PFOS levels measured above these TRVs increased. Although the TRV for liver (600 ng/g) was relatively lower than the TRV for eggs (1700 ng/mL), 51% of the samples were above the TRV for eggs compared to 28% of the samples that were above the TRV for liver. The mean distance from the fluoro-chemical plant of the of the egg samples that were above the TRV for eggs (676 m) was also smaller than the same distance for the liver samples above the TRV for liver (12735 m). The same trend was seen when comparing the PNEC values derived using the European Commission methodology (Newsted et al., 2005) for liver and egg PFOS levels. The PNEC for eggs were relatively higher (1000 ng/mL) than the PNEC for liver (350 ng/g ww), but more egg samples (59%) were above the PNEC than liver samples (40%) and the mean distance from the fluoro-chemical plant were bigger for the liver samples (7473 m) than for the egg samples (997 m). This all indicates that eggs may be more sensitive when determining the risk of PFOS to birds. When looking at the data, even at a mean distance of 12735 m from the fluoro-chemical plant 28% of the birds are at risk when looking at the PFOS levels in their livers. However, several points need to be taken into consideration. First, there might be other sources of PFC contamination in the area other than the fluoro-chemical plant in Antwerp, although this is unlikely, since Antwerp was the primary production site of PFCs in Europe. Second, there are only very little toxicity data of PFCs, even for PFOS, for birds. Only two species were used in toxicity testing when the various threshold values were calculated. Therefore, inter-species differences might play a big role in the risk evaluation using these toxicity data. Third, the role of diet and habitat were not taken into account in the risk evaluation. This thesis has shown that these two factors do indeed play a role in the birds' exposure to PFCs and should therefore be accounted for in the risk evaluation. To take the role of diet into account, the TRVs determined for top avian predators by Newsted et al. (2005) were adjusted for birds with a lower trophic level and different diet.

The TRVs were calculated as follows by Newsted et al. (2005):

$$\text{Top Avian TRV} = \frac{\text{LOAEL of toxicity study}}{\text{Overall Uncertainty Factor (UF)}}$$

where $UF = UF_A \times UF_L \times UF_S$

and UF_A = intertaxon extrapolation (6 for top avian predator)

UF_L = toxicological endpoint (Used a LOAEL, therefore 2)

UF_S = exposure duration (3 for a chronic laboratory test)

The selection of uncertainty factors were based on the USEPA Great Lakes Initiative. We used the same UF_L and UF_S for our analysis, but we changed the UF_A according to the diet of the birds. The birds were divided into three feeding classes, each with its own UF_A (Table 2). Since the Northern Bobwhites sued during the study are mainly herbivores, a UF_A of 1 was used. For omnivores, the UF_A was 3 and for carnivores the UF_A was 6, the same value used for the top avian predators.

Table 2. The different bird species according to their feeding class.

Carnivores $UF_A = 6$	**Omnivores** $UF_A = 3$	**Herbivores** $UF_A = 1$
Common Tern	Common Pochard	Eurasian Collared Dove
Eurasian Sparrowhawk	Eurasian Coot	Canada Goose
Grey Heron	Great Tit	Gadwall
	Mediterranean Gull	
	Northern Lapwing	
	Eurasian Magpie	
	Herring Gull	

When we do take the role of diet into account the percentage of birds at risk decreases (Table 3). This again stresses the importance of diet in birds' exposure to PFCs. The results of the evaluation are shown in Figure 1 for liver and Figure 2 for eggs. Figure 3 shows the zones where birds might be at risk from PFC exposure, when their diet is also taken into account.

Table 3. The various toxicity reference values (TRVs) (ng/g ww for liver, ng/mL for egg) for birds and the percentage of samples per feeding type above the threshold value for both liver and eggs. The mean distance from the fluoro-chemical plant in Antwerp for all the samples above the TRV are given in meters.

	Threshold Value		% of samples above threshold value	Mean Distance from fluorochemical plant
Avian Carnivore	**TRV Liver**	600	4	117000
	TRV Egg	1700	0	-
Avian Omnivore	**TRV Liver**	1200	15	1708
	TRV Egg	3400	25	392
Avian Herbivore	**TRV Liver**	3600	0	0
	TRV Egg	10200	0	0

The herbivorous birds seem to be at no risk, with none of the PFOS levels in the liver of eggs of any of the herbivorous birds being above the TRV. Only 4 % of the liver PFOS levels were above the TRV for carnivore, whereas none of the PFOS levels of the eggs was above the TRV for carnivores. Omnivores seemed the most at risk, with 15% of the liver samples being above the TRV and 25% of the egg samples being above the TRV. When taking the diet into account, less birds seem to be at risk. This is very important, as

At the end of this thesis, more data on both the exposure and effects of PFCs on wildlife have been generated, especially for birds, filling much needed gaps in the information on PFC exposure of and effects on wildlife. The risk evaluation to PFOS for wild birds was also performed, indicating that birds close to the fluoro-chemical plant are at risk.

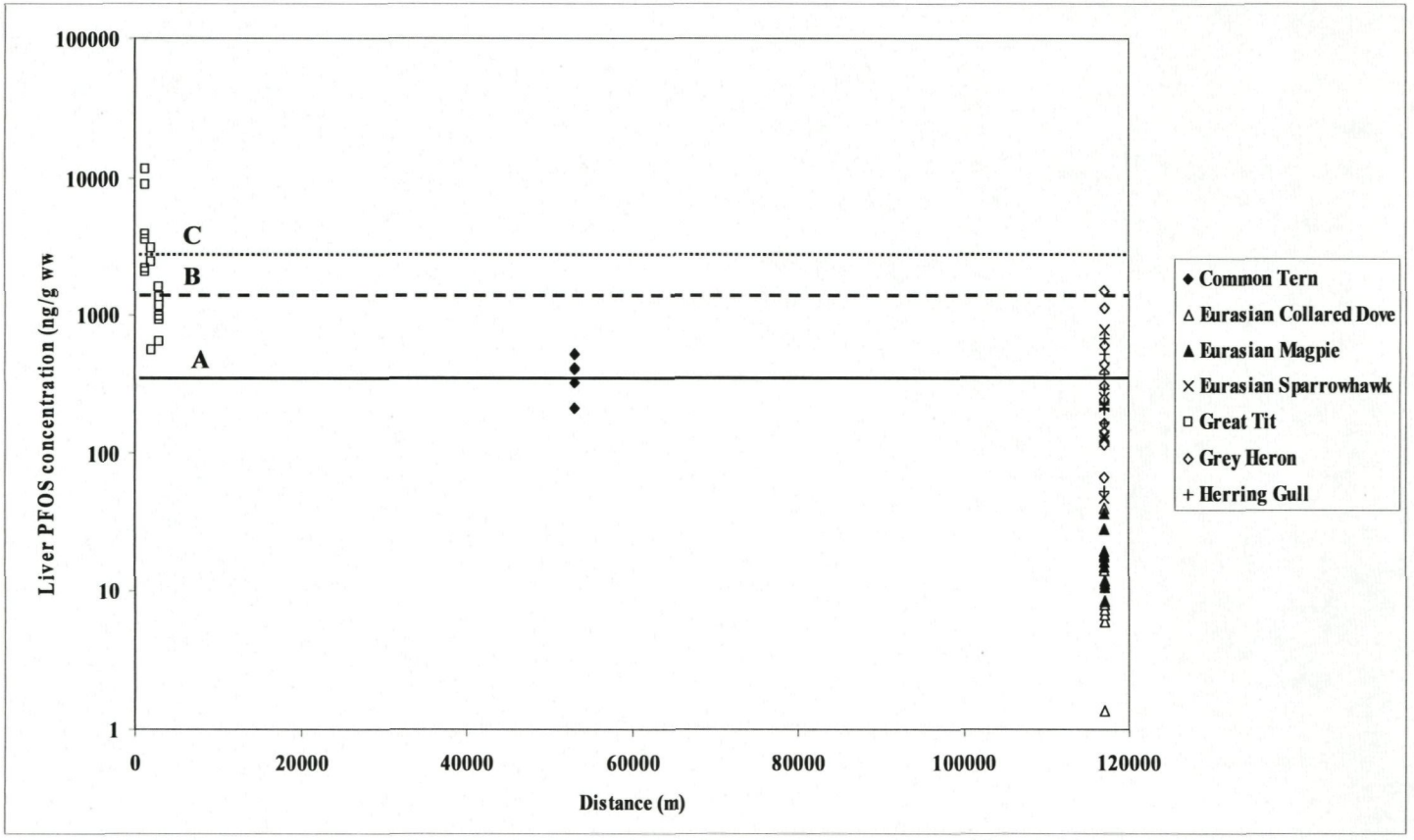

Figure 1. The influence of distance from the fluoro-chemical plant in Antwerp on the liver PFOS levels of the bird species from this study (Eurasian Collared Dove, Eurasian Magpie, Eurasian Sparrowhawk, Great Tit, Grey Heron and Herring Gull) and previous studies from the same area (Common Tern, van den Brink et al., 2007; Great Tit, Dauwe et al., 2007). The lines indicate the various TRV values in liver for the three feeding types: A = Avian Carnivore TRV; B = Avian Omnivore TRV; C = Avian Herbivore TRV.

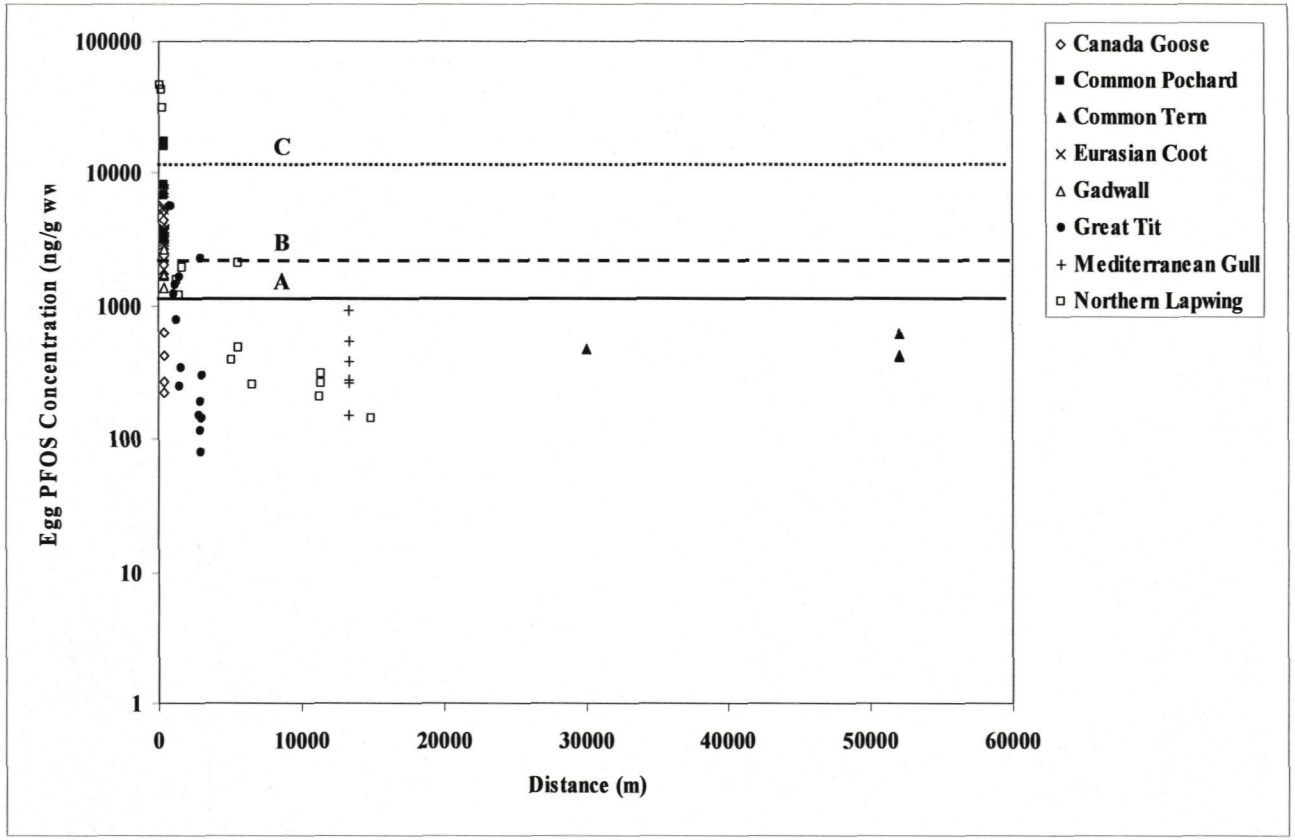

Figure 2. The influence of distance from the fluoro-chemical plant in Antwerp on the egg PFOS levels of the bird species from this study (Canada Goose, Common Pochard, Eurasian Coot, Gadwall, Great Tit, Mediterranean Gull and Northern Lapwing) and a previous study from the same area (Common Tern, van den Brink et al., 2007). The lines indicate the various TRV values in liver for the three feeding types: A = Avian Carnivore TRV; B = Avian Omnivore TRV; C = Avian Herbivore TRV.

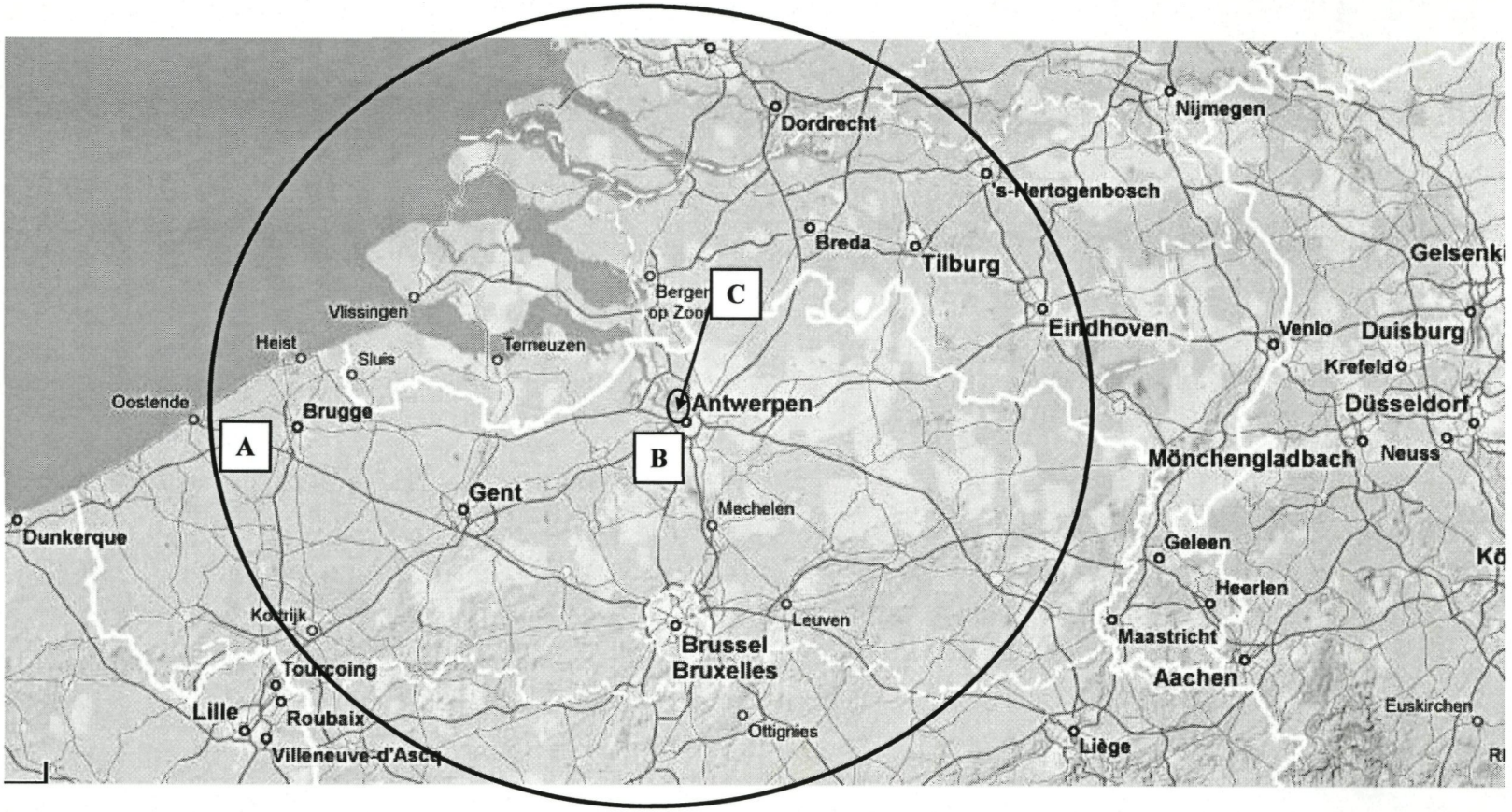

Figure 3. A map showing the areas where birds might be at risk based on the avian TRVs determined for the tree feeding types of birds. A = radius based on the liver TRV for carnivores (117 km from fluoro-chemical plant); B = radius based on the liver TRV for omnivores (1,7 km from fluoro-chemical plant); C = radius based on the egg TRV for omnivores (0.39 km from fluoro-chemical plant).

6.2 Perspectives for Future Research

Although the amount of research on PFCs has increased in the last decade, there are still many unanswered questions. Most of the research has focussed on PFOS and PFOA, since these are the PFCs found in the highest levels in the environment. However, since PFOS is now being replaced by new chemicals, more research on these replacement chemicals, such as perfluorobutane sulfonate (PFBS), is needed.

Our research has shown that PFCs could be measured in the feathers of birds. Feathers have the potential to be used as indicators of PFC exposure in birds, but it is necessary to determine if the ratio between the PFC levels in the liver and feathers is species dependent or not. In our study, we only looked at the PFC levels in the feathers of 50 individual birds. To determine the relationship between the PFC levels in feathers and liver, the liver/feather ratio of PFCs should be determined in a large amount of individuals from the same population. The uptake and storage of PFCs in feathers should also be investigated, to determine the role feathers play in the elimination of PFCs. The distribution of PFCs in the different parts of the feather should also be determined, as this will help to optimise the extraction method and could increase the limit of detection of PFCs in feathers. PFOA and PFNA levels were below the detection limits in feathers in our study. Therefore, it should be investigated whether all PFCs accumulate in the feathers, or just certain groups.

The distribution of PFCs in the different organs and tissues in birds are not well known. Most studies only measured PFCs in the liver, blood or egg. The few studies that have included other matrices have shown that other blood-rich organs, such as the spleen, also contain PFCs at the same level or lower than the liver. Whether the distribution of PFCs in the body of birds is species dependent or not should be investigated as to further our knowledge on the behaviour of PFCs in the body of birds.

We have shown that feeding and habitat plays an important role in the exposure of birds to PFCs. However, further research is needed on the PFC levels in the various compartments of the ecosystems in which the birds live. The PFC levels in the food items of the various bird species needs to be investigated to determine the importance of food in the PFC exposure of birds. It is also important not only to focus on birds of prey or piscivorous birds, but also to include birds with various diets. The bioaccumulation factors for birds from various habitats should also be determined, to determine if certain groups of birds are more likely to be exposed to PFCs. The PFC levels in the abiotic compartments of the ecosystem should also be measured, as this will give an indication of the biomagnification of PFCs in the ecosystems where birds

live. Most of the research on the distribution of PFCs in ecosystems has been performed on aquatic ecosystems. Very little is known about the PFC levels in terrestrial ecosystems. In our study we showed that the BAFs for animals in a terrestrial ecosystem is the same or higher than those for aquatic ecosystems. Therefore, more research is needed on the PFC levels in a terrestrial food chain, which includes mammalian top predators.

Most laboratory studies on the effects of PFCs on animals have been performed on rats and monkeys. Toxicity data and effects studies should be performed on a wider range of species, including wild animals. Toxicity data of only two bird species are currently available. PFC toxicity data on more bird species should be generated, do determine the inter-species differences. The results of laboratory studies on the effects of PFCs should also be compared to data from field studies. This would shed more light on the influence of various other factors on the toxic mode of action of PFCs. To be able to perform biomonitoring of PFC exposure in birds, biomarkers of PFC exposure should be developed. Currently the biomarkers that are used in most studies only indicate general condition or are not specific for PFCs. Further research is needed to develop biomarkers that can be linked to an increase in PFC exposure in birds. The uptake and elimination of PFCs by birds should be investigated, as to understand the accumulation and distribution of PFCs in birds better. Using the toxicity data available, various TRVs should be developed for specific animal species or at least groups of animals. Currently the few TRVs that are available are not used as guidelines by authorities. TRVs should therefore be determined that could be used in the biomonitoring of PFCs in the environment.

CHAPTER 7

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Summary in English

This study can be divided into two parts. In Chapters 1 to 5 more data on perfluorinated chemicals (PFC) exposure and effects in wildlife were generated. In Chapter 6 this data was used to perform a risk evaluation, focussing mainly on birds.

In Chapter 1 the relationships between the levels of perfluorooctane sulfonate (PFOS) in the liver, brain and feathers of the Zebra Finch (*Taeniopygia guttata*) were investigated under laboratory conditions. Currently there is no information available on the levels of PFOS in the feathers of birds, therefore we developed a method to extract PFOS from feathers. The liver of the Zebra Finches contained the highest PFOS levels, followed by the feathers and the lowest levels were mainly found in the brain. There was a significant positive correlation between the PFOS levels in the liver and both the brain and feathers. Several biochemical endpoints were also studied to determine the effects of PFOS on passerines. There was no significant correlation between the liver PFOS levels and any of the biomarkers investigated.

Previous studies found that piscivorous ducks had higher levels of PFOS in their livers than herbivorous ducks. Therefore, in Chapter 2, we investigated whether the feeding habits of the birds will influence their exposure to PFCs. Eggs from four water bird species were sampled in Blokkersdijk, Belgium, a nature reserve situated in the Antwerp region next to a fluoro-chemical plant. Both the PFC and metal levels in the eggs were measured to determine if there is a relationship between the levels of PFCs and metals in the eggs. The PFC and metal levels were determined in the eggs of Canada Goose (*Branta canadensis*), Common Pochard (*Aythia ferina*), Gadwall (*Anas strepera*) and Eurasian Coot (*Fulica atra*), which all have different feeding habits. The highest PFC levels were measured in the eggs of the Common Pochard, whereas the Canada Goose eggs contained the lowest PFC levels. The herbivorous birds had significantly lower levels of PFCs than the omnivorous birds. In addition, the birds that spend more time feeding in the water had higher levels of PFCs in their eggs. No clear correlation pattern was found between the levels of PFCs and metals in the water bird eggs.

Three bird species, the Great Tit (*Parus major*), the Northern Lapwing (*Vanellus vanellus*) and the Mediterranean Gull (*Larus melanocephalus*), were used in Chapter 3 to determine the relationship between PFOS levels in bird eggs and the distance of the nests from the fluoro-chemical plant. The three bird species have different feeding and migration habits. The Mediterranean Gull eggs contained the highest levels of PFOS.

There was a clear decrease with an increase indistance of the nest site from the fluoro-chemical plant in the PFOS levels in the Northern Lapwing eggs. This trend was also seen in the eggs of the Great Tit, where eggs from the site closest to the fluoro-chemical plant had significantly higher PFOS levels than eggs collected 1700 m further away. The PFOS levels in the eggs of the three bird species were some of the highest PFOS levels ever measured in wildlife. Various biomarkers were used to determine the adverse effects of PFOS exposure in one of the species, the Great Tit, although no clear trends were found.

The usefulness of feathers to determine the PFC exposure of wild birds was investigated in Chapter 4. The PFC levels in the liver and feathers of five bird species from a non-PFC contaminated area in Belgium were measured. The highest mean liver PFOS levels were found in the Grey Heron (*Ardea cinerea,* 476 ng/g ww) followed by the Herring Gull (*Larus argentatus,* 292 ng/g ww), Eurasian Sparrowhawk (*Accipiter nisus,* 236 ng/g ww), Eurasian Magpie (*Pica pica,* 17 ng/g ww), whereas the Eurasian Collared Dove (*Streptopelia decaocta,* 12 ng/g ww) had the lowest levels. The PFOS levels in the feathers showed a different pattern. The Grey Heron had the highest levels (247 ng/g dw), the Eurasian Sparrowhawk (102 ng/g dw) had the second highest feather PFOS levels followed by the Herring Gull (79 ng/g dw), the Eurasian Collared Dove (48 ng/g dw) and the lowest levels were in the Eurasian Magpie (31 ng/g dw). No correlation was found between the PFC levels in the feathers and livers of the individual species. However, there was a significant correlation when the data of all five species were combined. In general, birds from a higher trophic level had higher PFC levels in their tissues. This indicates that diet may play a role in PFC exposure in birds.

In Chapter 5 we determined the PFOS levels in a terrestrial ecosystem with the Wood Mouse (*Apodemys sylvaticus*), occupying the highest trophic position. Wood Mice, various invertebrates and fruit from two sites in Belgium were collected. PFOS was detected in almost all of the samples from both the contaminated site, Blokkersdijk, and the control site, Galgenweel. For each sample type, higher PFOS levels were found in Blokkersdijk than in Galgenweel. At both sites, the highest mean level of PFOS at both sites was found in the liver of the Wood Mouse. At Galgenweel the highest PFOS levels in the invertebrates were in millipedes, followed by woodlice, then slugs and earthworms. A different pattern was found at Blokkersdijk, where the highest PFOS levels measured was in earthworms, followed by slugs and millipedes and then the lowest in woodlice. The fruit of the European Elder contained higher PFOS levels than the fruit of the Common Blackberry at both sites. The liver PFOS levels of the Wood Mice at Blokkersdijk were on average 2.4 times lower in 2006 than 2002, although not

significantly lower. The diet weighted bioaccumulation factors (BAFs) were almost the same for Galgenweel (18.4) and Blokkersdijk (17.8).

Using the data from the previous chapters, a risk evaluation for birds was done in Chapter 6. We first used the Toxicity Reference Values (TRVs) for Top Avian Predators to determine what percentage of birds in our study might be at risk. A high percentage of birds were at risk when using this method. However, we also calculated the TRVs for omnivorous and herbivorous birds, using the TRV for Top Avian Predators published in literature. TRVs for both the levels of PFCs in eggs and liver were calculated. When applying these TRVs to our data, the percentage of birds at risk was lower. The distance from the fluoro-chemical plant where birds might be at risk differed for the different feeding types of birds: carnivorous birds were at risk in an area around the fluoro-chemical with a radius of 117 km, whereas the omnivorous birds were at risk in an area with a radius of 0.39 km from the fluoro-chemical plant).

More data on both the exposure and effects of PFCs on wildlife, especially birds, have been generated. After the risk evaluation for wild birds to PFOS was performed, it indicated that birds close to the fluoro-chemical plant were at risk.

Samenvatting in het Nederlands

Deze studie bestaat uit twee delen. In hoofdstukken 1 tot 5 werden meer data gegenereerd over de blootstelling van wilde dieren aan PFC en de effecten ervan. In hoofdstuk 6 werden deze data gebruikt om een risico-evaluatie uit te voeren, voornamelijk gericht op vogels.

In hoofdstuk 1 werden de relaties tussen de concentraties van perfluoroctaan sulfonaat (PFOS) in de lever, hersenen en veren van zebravinken (*Taeniopygia guttata*) onderzocht onder laboratoriumcondities. Momenteel is er geen informatie beschikbaar over de concentraties van PFOS in de veren van vogels, daarom hebben we een nieuwe methode moeten ontwikkelen om PFOS uit veren te extraheren. De lever van de zebravinken bevatte de hoogste PFOS-concentraties, gevolgd door de veren, en de laagste concentraties waren in de hersenen. Er was een significante positieve correlatie tussen de PFOS-concentraties in de lever, en die in de hersenen en veren. Verscheidene biochemische eindpunten werden ook bestudeerd om de effecten van PFOS op zangvogels te bepalen. Er was geen significante correlatie tussen de PFOS-concentraties in de lever en in de bestudeerde biomarkers.

Vorige studies hebben aangetoond dat visetende eenden hogere PFOS-concentraties in hun levers hadden als herbivore eenden. Daarom onderzochten we in hoofdstuk 2 of de voedingsgewoonten van vogels hun blootstelling aan perfluorochemicaliën (PFCs) beïnvloeden. Eieren van vier watervogels werden verzameld in Blokkersdijk (België), een natuurpark in de regio van Antwerpen naast een fluor-chemische fabriek. Beide PFC- en metaalconcentraties werden gemeten in de eieren om te bepalen of er een correlatie bestond tussen de concentraties van PFC en metaal in de eiren. De PFC- en metaalconcentraties werden bepaald in de eieren van Canadese gans (*Branta canadensis*), tafeleend (*Aythia ferina*), krakeend (*Anas strepera*) en meerkoet (*Fulica atra*), allemaal vogels met verschillende voedingsgewoonten. De hoogste PFC-concentraties werden gedetecteerd in de eieren van de tafeleenden en de laagste in de eieren van Canadese ganzen. De herbivore vogels hadden significant lagere concentraties van PFCs in hun eieren als die van de omnivore vogels. Ook waren er hogere concentraties van PFCs in de eieren van vogels die langer in het water voeden. Er werd geen duidelijke correlatie gevonden tussen de concentraties van PFCs en metalen in de eieren van de watervogels.

Drie vogelsoorten, de koolmees (*Parus major*), de kievit (*Vanellus vanellus*) en de zwartkopmeeuw (*Larus melanocephalus*), werden gekozen om in hoofdstuk 3 de relatie

te bepalen tussen, aan de ene kant, de PFOS-concentraties in vogeleieren en, aan de andere kant, de afstand tussen het nest en de fluor-chemische fabriek. De drie vogelsoorten hebben verschillende voedings- en migratiegewoonten. De zwartkopmeeuw-eieren bevatten de hoogste PFOS-concentraties. Er was een duidelijke afname in de PFOS-concentraties in de kieviteieren naarmate de afstand tussen het nest en de fluor-chemische fabriek groter werd. Deze trend was ook aanwezig bij de eieren van de koolmees. De koolmeeseieren van het gebied het dichtst bij de fluor-chemische fabriek hadden significant hogere PFOS-vlakken als eieren die 1700 m verder weg verzameld werden. De PFOS-concentraties in de eieren van de drie vogelsoorten waren van de hoogste ooit gemeten in wilde dieren. Verschillende biomerkers werden ook gebruikt om de effecten van PFOS in de koolmees te bepalen. Geen duidelijke trends werden gevonden.

Het gebruik van veren om de PFC-blootstelling in vogels te bepalen is onderzocht in hoofdstuk 4. De PFC-concentraties werden gemeten in de lever en veren van vijf vogelsoorten van een gebied in België die niet PFC-vervuild is. De hoogste gemiddelde PFOS-concentraties in de lever werden gemeten in de blauwe reiger (*Ardea cinerea,* 476 ng/g ww), gevolgd door de zilvermeeuw (*Larus argentatus,* 292 ng/g ww), de sperwer (*Accipiter nisus,* 236 ng/g ww), de ekster (*Pica pica,* 17 ng/g ww) en de laagste concentraties bij de turkse tortel (*Streptopelia decaocta,* 12 ng/g ww). De PFOS-concentyraties in de veren vertoonden een ander patroon. De blauwe reiger had de hoogste concentraties (247 ng/g dw), daarna kwamen de sperwer (102 ng/g dw) en de zilvermeeuw (79 ng/g dw), gevolgd door de turkse tortel (48 ng/g dw) en de laagste concentraties waren bij de ekster (31 ng/g dw). Geen correlatie werd gevonden tussen de PFC-concentraties in de veren en levers van de individuelen soorten. Er was echter een significante correlatie tussen de PFOS-concentraties in de veren en lever als alle data van de vijf soorten samengevoegd werden. Over het algemeen hadden vogels van een hoger trofisch niveau hogere PFC-concentraties in hun weefsel. Dat duidt aan dat voeding een rol kan spelen bij PFC-blootstelling bij vogels.

In hoofdstuk 5 hebben we de PFOS-concentraties onderzocht in een terrestrisch ecosysteem, met de bosmuis (*Apodemys sylvaticus*) op het hoogste trofische niveau. Er werden stalen van bosmuizen, verschillende invertebraten en fruit uit twee gebieden in België verzameld. PFOS was boven de detectielimit in de meeste stalen van beide het gecontamineerde gebied, Blokkersdijk, en het controolgebied, Galgenweel. In elk van de verschillende matrices werden de PFOS-concentraties hoger in Blokkersdijk als in Galgenweel. In beide gebieden werden de hoogste gemiddelde PFOS-concentraties gemeten in de levers van de bosmuizen. In Galgenweel werden de hoogste PFOS-

concentraties bij invertebraten gemeten in de miljoenpoten, gevolgd door pissebedden en dan naaktslakken en regenwormen. Een ander patroon werd gevonden in Blokkersdijk, waar de hoogste PFOS-concentraties werden gemeten bij regenwormen, gevolgd door naaktslakken en miljoenpoten en uiteindelijk werd de laagste bij pissebedden gemeten. Het fruit van de vlier bevatte hogere PFOS-concentraties als het fruit van de gewone braam in beide gebieden. De PFOS-concentraties in de lever van de bosmuis in Blokkersdijk waren ongeveer 2,4 keer lager in 2006 dan in 2002, alhoewel niet significant lager. De bioaccumulatie factoren (BAFs), die gecorrigeerd zijn in functie van het dieet van de bosmuizen, waren bijna dezelfde in Galgenweel (18.4) en Blokkersdijk (17.8).

In hoofdstuk 6 werd een risico-evaluatie voor vogels uitgevoerd door gebruik te maken van de data van de vorige hoofdstukken. We hebben eerst de toxiciteitsreferentiewaarden (TRVs) voor de top vogelpredators gebruikt om te bepalen welk percentage van de vogels uit onze studie word bedreigd. Een hoog percentage vogels blijkt bedreigd te zijn als we deze methode gebruiken. We hebben echter ook de TRVs berekend voor omnivore en herbivore vogels door gebruik te maken van de TRV voor top vogelpredators die gepubliceerd zijn in de literatuur. TRVs werden berekend voor de PFC-concentraties in beide de eieren en de levers. Als deze TRVs toegepast worden op onze data, blijkt het percentage vogels dat bedreigd is lager te zijn. De oppervlakte van het gebied waar de vogels bedreigd worden verschilt in functie van het type vogel: omnivore vogels worden bedreigd in een straal van 390m rond de fluor-chemische fabriek, terwijl voor carnivore vogels dit het geval is in een straal van 117 km rond de fabriek.

Meer data over beide de blootstelling en effecten van PFCs op wilde dieren, voornamelijk vogels, zijn gegenereerd. De risico-evaluatie van PFOS voor wilde vogels heeft aangetoond dat vogels die dichtbij de fluor-chemische fabriek voorkomen worden bedreigd.

Opsomming in Afrikaans

Hierdie studie bestaan uit twee dele. In Hoofstukke 1 tot 5 is meer data oor die blootstelling en effekte van geperfluoreerde chemikalië (PFC's) in wilde diere gegenereer. In Hoofstuk 6 is hierdie data gebruik om 'n risiko-evaluasie uit te voer wat fokus op voëls.

In Hoofstuk 1 is die verhouding tussen die vlakke van perfluoro-oktaansulfonaat (PFOS) in die lewer, brein en vere van Sebravinke (*Taeniopygia guttata*) ondersoek onder laboratoriumtoestande. Tans is geen inligting beskikbaar nie oor die vlakke van PFOS in die vere van voëls nie, dus moes ons 'n nuwe metode ontwikkel om PFOS te ekstraëer vanuit vere. Die lewer van die Sebravinke het die hoogste PFOS-vlakke bevat, gevolg deur die vere en die laagste vlakke was in die brein. Daar was 'n beduidende positiewe korrelasie tussen die PFOS-vlakke in die lewer en beide die brein en vere. Verskeie biochemiese eindpunte was ook gebruik om die effekte van PFOS op sangvoëls te bepaal. Daar was geen beduidende korrelasie tussen die lewer PFOS-vlakke en enige van die biomerkers nie.

Vorige studies het aangetoon dat vis-etende eende hoër PFOS-vlakke in hulle lewers het as herbivoriese eende. Dus, in Hoofstuk 2, het ons ondersoek of die voedingsgewoontes van voëls hulle blootstelling aan PFC's beïnvloed. Die eiers van vier watervoëls was versamel in Blokkersdijk, België, 'n natuurreservaat in die Antwerpengebied langs 'n fluoro-chemiese fabriek. Beide PFC- en metaalvlakke was gemeet in die eiers. Die PFC- en metaalvlakke was gemeet in die eiers van Kanadese Gans (*Branta canadensis*), Tafeleend (*Aythia ferina*), Krakeend (*Anas strepera*) and Eurasiese Bleshoender (*Fulica atra*), almal voëls met verskillende voedingsgewoontes. Die hoogste PFC-vlakke was gemeet in die eiers van die Tafeleende en die laagste in die eiers van die Kanadese Ganse. Die herbivoriese voëls het beduidende laer vlakke van PFC's in hulle eiers gehad as dié van die omnivoriese voëls. Daar was ook hoër vlakke van PFC's in die eiers van voëls wat langer in die water na voedsel soek. Geen duidelike korrelasie was gevind tussen die vlakke van PFC's en metale in die eiers van die watervoëls nie.

Drie voëlspesies, die Grootmees (*Parus major*), die Noordelike Kiewiet (*Vanellus vanellus*) en die Mediterreense Meeu (*Larus melanocephalus*), was gekies om in Hoofstuk 3 die verhouding te bepaal tussen die PFOS-vlakke in voëleiers en die afstand van die nes vanaf die fluoro-chemiese fabriek. Die drie voëlspesies het verskillende voedings- en migrasiegewoontes. Die Mediterreense Meeu-eiers het die hoogste PFOS-vlakke bevat. Daar was 'n duidelike afname in die PFOS-vlakke in die Noordelike

Kiewieteiers met 'n toename in die afstand van die nes van die fluoro-chemiese fabriek. Hierdie tendens was ook teenwoordig by die eiers van die Grootmees. Die Grootmeeseiers van die gebied naaste aan die fluoro-chemiese fabriek het beduidende hoër PFOS-vlakke bevat as eiers wat 1700 m verder weg versamel is. Die PFOS-vlakke in die eiers van die drie voëlspesies was van die hoogste wat ooit gemeet is in wilde diere. Verskillende biomerkers was ook gebruik om die effekte van PFOS op die Grootmees te bepaal. Geen duidelike tendense was gevind nie.

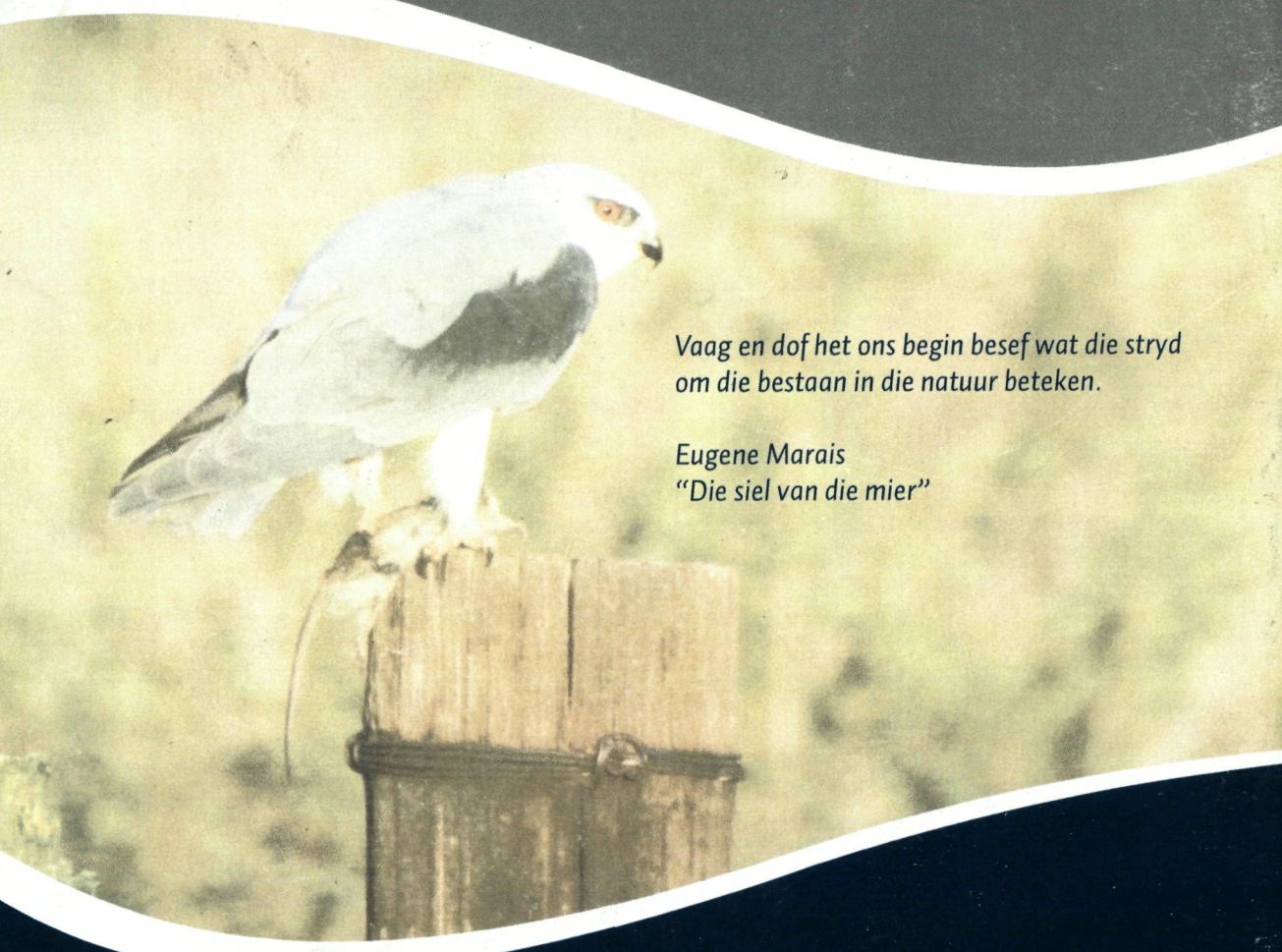
Die gebruik van vere om die PFC-blootstelling in voëls te bepaal was ondersoek in Hoofstuk 4. Die PFC-vlakke in die lewer en vere van vyf voëlspesies van 'n gebied in België, wat nie PFC-besoedel is nie, was gemeet. Die hoogste gemiddelde lewer PFOS-vlakke was gemeet in die Bloureier (*Ardea cinerea,* 476 ng/g ww), gevolg deur die Haringmeeu (*Larus argentatus,* 292 ng/g ww), Eurasiese Sperwer (*Accipiter nisus,* 236 ng/g ww), Gewone Ekster (*Pica pica,* 17 ng/g ww) en die laagste vlakke in die Turkse Tortelduif (*Streptopelia decaocta,* 12 ng/g ww). Die PFOS-vlakke in die vere het 'n ander patroon gevolg. Die Bloureier het die hoogste vlakke (247 ng/g dw) gehad, dan die Eurasiese Sperwer (102 ng/g dw) en Haringmeeu (79 ng/g dw), gevolg deur die Turkse Tortelduif (48 ng/g dw) en die laagste vlakke in die Gewone Ekster (31 ng/g dw). Geen korrelasie is gevind tussen die PFC-vlakke in die vere en lewers van de individuele spesies. Daar was egter 'n beduidende korrelasie tussen die PFOS-vlakke in die vere en lewer as al die data van die vyf spesies saam gevoeg word. In die algemeen het die voëls van 'n hoër trofiese vlak hoër PFC-vlakke in hulle weefsel. Dit dui aan dat voeding 'n rol kan speel by PFC-blootstelling in voëls.

In Hoofstuk 5 het ons die PFOS-vlakke in 'n terrestriële ekosisteem, waar die Bosmuis (*Apodemys sylvaticus*) die hoogste trofiese vlak beklee, ondersoek. Monsters van Bosmuise, verskillende invertebrate en vrugte van twee gebiede in België is versamel. PFOS was bo die deteksielimiet in die meeste van die monsters van beide die besoedelde gebied, Blokkersdijk, en die kontrole gebied, Galgenweel. In elk van die verschillende matrikse was hoër PFOS-vlakke gemeet in Blokkersdijk as in Galgenweel. In beide gebiede was die hoogste gemiddelde PFOS-vlakke gemeet in die lewers van die Bosmuise. Die hoogste PFOS-vlakke gemeet in die invertebrate in Galgenweel was in die duisendpote, gevolg deur die houtluise en dan die naakslakke en erdwurms. 'n Ander patroon is gevind in Blokkersdijk, waar die hoogste PFOS-vlakken gemeet was in die erdwurms, gevolg deur die naakslakke en die duisendpote en die laagste in houtluise. Die vrugte van die Europese Vlier het hoër PFOS-vlakke bevat as die vrugte van die Gewone Braam in beide gebiede. Die lewer PFOS-vlakke van die Bosmuis in Blokkersdijk was ongeveer 2.4 keer laer in 2006 as in 2002, alhoewel nie

beduidend nie. Die bioakkumulasie faktore (BAF's), wat aangepas is aan die dieet van die Bosmuise, was byna dieselfde in Galgenweel (18.4) en Blokkersdijk (17.8).

'n Risiko-evaluasie is uitgevoer vir voëls in Hoofstuk 6 deur gebruik te maak van die data van die vorige hoofstukke. Ons het eerste die Toksisiteitsverwysingswaardes (TRV's) vir die Top Voëlpredatore gebruik om te bepaal watter persentasie van die voëls in ons studie bedreig is. 'n Hoë persentasie van voëls was bedreig as ons hierdie metode gebruik het. Ons het egter ook die TRV's bereken vir omnivoriese en herbivoriese voëls deur gebruik te maak van die TRV vir Top Voëlpredatore gepubliseer in die literatuur. TRV's is bereken vir beide die PFC-vlakke in eiers en lewers. As hierdie TRV's toegepas word op ons data, was die persentasie van voëls wat bedreig is laer. Die afstand van die fluoro-chemiese fabriek waar die voëls bedreig kan wees was verskillend vir die verskillende tipes voëls: karnivoriese voëls in 'n gebied met 'n radius van 117 km vanaf die fluoro-chemiese fabriek was bedreig, vir omnivoriese voëls was die radius 0.39 km vanaf die fluoro-chemiese fabriek.

Meer data oor beide die blootstelling en effekte van PFC's op wilde diere, veral voëls, was gegenereer. Die risiko-evaluasie van PFOS vir wilde voëls het aangetoon dat voëls wat naby die fluoro-chemiese fabriek voorkom bedreig was.



Vaag en dof het ons begin besef wat die stryd om die bestaan in die natuur beteken.

Eugene Marais
"Die siel van die mier"