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High levels of PFOS in eggs of three bird species in the neighbourhood of a fluoro-chemical plant

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ABSTRACT

We studied perfluorooctane sulfonate (PFOS) levels in the eggs of three primarily invertivorous bird species sampled in 2006 near a fluoro-chemical plant: the great tit *(Parus major)*, the northern lapwing (*Vanellus vanellus*) and the Mediterranean gull (*Larus melanocephalus)*. Our study reported some of the highest PFOS levels ever measured in wildlife to date (i.e. up to 46182 ng/g ww in lapwing eggs). A pronounced decrease in PFOS concentration in the Northern lapwing eggs with distance from the fluoro-chemical plant was found. A similar relationship was found for the great tit, with eggs being collected close to the fluoro-chemical plant having significantly higher PFOS levels than eggs collected 1700 m further away. When comparing the PFOS levels in eggs for the three species, collected between 1700 and 5500 m no significant differences were observed. In addition, when comparing PFOS levels in eggs between Northern lapwing and great tits closer to the plant (900–1700 m) no significant differences were found neither. Despite the high levels found in great tit eggs, plasmatic biochemical biomarker responses did not appear to be affected.

1. Introduction

Perfluoroalkyl acids (PFAAs) 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 two decades (van Leeuwen and de Boer, 2007). The biological monitoring of PFAAs levels in wildlife, and specially the measurement of the most abundant one, the perfluorooctane sulfonate (PFOS), has provided valuable information about the contamination sources and the environmental dynamics of these compounds (Giesy and Kannan, 2001; Houde et al., 2006; Miller et al., 2015). However, these dynamics are complex and knowledge gaps still exist about the sources and transport pathways (Liu et al., 2016; Rodriguez-Jorquera et al., 2016) and about the kinetics and the effects of these compounds on birds and mammals (Sletten et al., 2016; Tarazona et al., 2015; Wielsøe et al., 2015).

PFOS has been found in marine, fresh water and terrestrial environments and has been measured in wildlife from remote areas such as the Arctic and the Antarctic (Butt et al., 2007, 2010; Routti et al., 2015). Atmospheric and water transport can contribute to the dispersal of PFAAs (Ahrens et al., 2010; Prevedouros et al., 2006). Due to the persistence and widespread distribution of PFOS, the major global manufacturer, 3 M, phased out the production of PFOS and related compounds in 2002 (3 M Company, 2000). In addition, in 2009, PFOS was included in the Stockholm Convention on Persistent Organic Pollutants (POPs).

Birds have been frequently used for biomonitoring PFOS. Most of the studies have focused on piscivorous birds (Holmström et al., 2005; Kannan et al., 2001; Sletten et al., 2016) being top predators often showing the highest levels (Giesy and Kannan, 2001; Houde et al., 2006). On the other hand, data on terrestrial birds remain scarce and the levels found in these species are normally lower than the ones in seabirds and other waterbirds (Ahrens et al., 2011; Jaspers et al., 2013; Yoo et al., 2008). The differences in exposure between terrestrial and aquatic birds could be linked to the bioaccumulation/biomagnification of PFOS and the trophic position of prey and predator (Houde et al., 2006; Lau et al., 2007) and also to the air and water borne transport of this compound and its precursors (Holmström et al., 2010; Rüdel et al., 2011), but as mentioned before, the environmental dynamics of PFOS are complex and more information is still needed.

Blood-rich organs, for example the liver, have usually been the target organ when determining PFOS levels in birds. Recently, feathers, eggs and blood have also been used (Holmström et al., 2005; Jaspers et al., 2013; Meyer et al., 2009). In blood, PFOS is known to bind to albumin (Jones et al., 2003). Dauwe et al. (2007) found a clear

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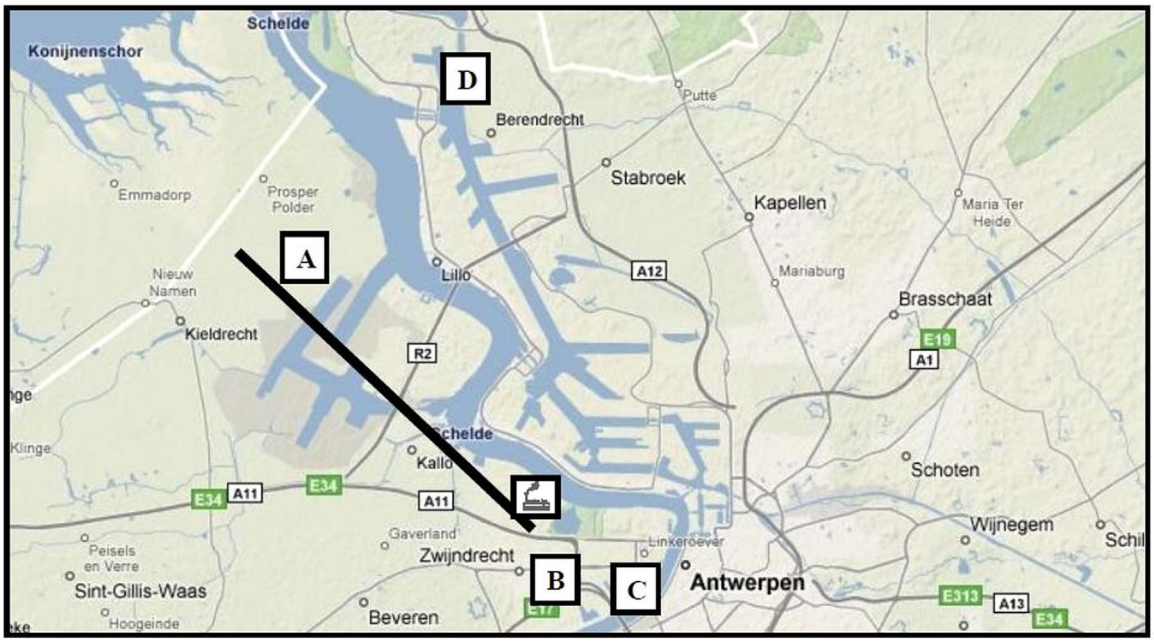

Fig. 1. A map of the study sampling sites and the fluorochemical plant: A = Line along which Northern lapwing eggs were collected; eggs and blood of great tits were collected at B = Vlietbos; and C = Burchtse Weel; eggs and blood of Mediterranean gulls were collected at D = Zandvliet.

relationship between the PFOS levels in liver and serum of great tits (*Parus major*) illustrating the potential of blood or serum as non-destructive biomonitor of PFOS accumulation. Concentrations in blood are generally low however, compared to liver (Dauwe et al., 2007). Eggs, on the contrary, have high levels of PFOS and a study on common tern (*Sterna hirundo*) females showed no significant difference between levels in eggs and liver from the Western Scheldt (Van den Brink et al., 2007). Laboratory studies confirmed that PFOS is transferred and excreted through the eggs, resulting in significantly lower liver PFOS levels in female birds than males (Newsted et al., 2007).

The ubiquity of PFOS contrasts sharply with the limited information about its effects on organisms. In birds, laboratory (Cwinn et al., 2008; Molina et al., 2006; Newsted et al., 2005; Peden-Adams et al., 2009; Yanai et al., 2008; Yeung et al., 2007) and field (Custer et al., 2012, 2014; Sletten et al., 2016) studies have been performed. Effects included altered plasma biochemistry (Hoff et al., 2005b; Peden-Adams et al., 2009), endocrine disruption (Jensen and Leffers, 2008), immune effects (Peden-Adams et al., 2009), organ dysfunction (Molina et al., 2006; Newsted et al., 2005; Peden-Adams et al., 2009) and reproductive effects (Custer et al., 2012; Molina et al., 2006; Yanai et al., 2008). In a field study, Hoff et al. (2005b) found a significant positive correlation between serum alanine aminotransferase activity (ALT), which is a biomarker of liver damage and liver PFOS levels in wild great 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*) has shown this area to be a hotspot for PFOS contamination (Dauwe et al., 2007; D'Hollander et al., 2014; Hoff et al., 2005a, 2005b). Levels found in these organisms have been among the highest ever measured in biota worldwide. The highest level of PFOS in a wild bird ever measured, namely 11,359 ng/g ww, was determined in the liver of one of the great tits from this study area (Dauwe et al., 2007).

The aim of the present study was to investigate the concentrations and the interspecific differences in egg PFOS levels in three primarily invertivorous bird species sampled near a fluoro-chemical plant: the great tit, the northern lapwing (*Vanellus vanellus*) and the Mediterranean gull (*Larus melanocephalus*). The great tit is a resident species that feeds mainly on caterpillars during the breeding season and seeds and berries during the winter (del Hoyo et al., 2007), and this species has been increasingly used as a model species in ecotoxicological studies (Eens et al., 1999). The northern lapwing is also a resident species that mainly feeds on small arthropods 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 Mediterranean during the winter, especially to the Iberian Mediterranean coast (Cama et al., 2011). They feed mainly on terrestrial and aquatic arthropods, gastropods and occasionally small fish and even rodents and form huge colonies during the breeding season (del Hoyo et al., 1996). Secondly, we investigated the suitability of eggs as bioindicator for local PFOS contamination. Eggs from the resident great tit and lapwing were collected at various distances from the pollution source and the relationship with PFOS levels was examined. Finally, for great tits various physiological plasma biomarkers were measured to study the potential adverse effects of PFOS on adult condition. The study was restricted to PFOS since at the time of study (2006) this was the only compound for which labelled standards were available.

2. Material and methods

2.1. Sampling

The study area is located on the western shores of the Scheldt river, west of Antwerp, Belgium (Fig. 1). The area is made up of various habitats, including wooded areas, sandy shores and grassland, but various chemical industries are located there. Great tit eggs (n = 18) and blood of adults (n = 31) were collected from two sites in the region. The first site, Vlietbos (B), is located about 1200 m from a fluoro-chemical production plant, thus it is supposed that this site is more contaminated with PFOS. Burchtse Weel (C), 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 during the egg laying period between April 15th and 30th 2006. Additionally blood samples of adult

great tits were collected between January 25th and February 2nd, 2007. Unfortunately sample volume was too small to measure PFOS. 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 (n = 14) 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 was sampled. The closest nest was 90 m away from the fluoro-chemical plant and the one the furthest away was about 15,000 m (A). The blood and egg samples of the Mediterranean gull were collected on 13 and 14 May 2006 from the colony at Zandvliet (D), a site 14.5 km further to the north of the fluoro-chemical plant (Fig. 1). In total 6 eggs and 29 blood samples of the Mediterranean gull were collected. To collect the blood, adult gulls were captured form the colony applying walk-in traps. Volume of the collected blood samples was too small to measure both PFOS and biomarkers and we therefore chose to measure only PFOS in total blood. The eggs from the three species were randomly collected within the clutch. All the samples were stored at −80 for later analysis.

2.2. PFOS extraction and clean up

PFOS extraction from blood and eggs was done by solvent extraction based on the method by Berger and Haukås (2005) with adaptations described in Dorneles et al. (2008). 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 4.5 mL of acetonitrile were added. The PP tube was capped and the sample was thoroughly mixed using a Vortex. The sample was then extracted 3 times for 10 min in an ultrasonic bath at room temperature. Between each period of 10 min, the samples were thoroughly mixed. The samples were then centrifuged at 2500 rpm for 5 min. 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 min using a vortex. After centrifugation (10,000 rpm, 10 min) 500 μL of the supernatant was transferred to a clean micro vial.

2.3. Determination of PFOS concentrations

The concentrations of PFOS were measured using combined liquid chromatography-mass spectrometry using a CapLC system (Waters, USA) connected to a Quadrupole-LIT quadrupole mass spectrometer (Applied Biosystems, UK) as it was described in Dorneles et al. (2008). Aliquots of 5 μL were loaded on an Optiguard C18 pre-column (10 mm × 1 mm i.d., Alltech, USA). The analysis was performed on a Fluophase PFP column (50 mm × 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 s 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 un-extracted calibration curve. The limit of detection (LOD) was 0.9 ng/mL and 0.15 ng/g ww for blood and eggs respectively. This was established on a signal to noise ratio (S/N) > 3.

2.4. Quality control

Quality control was performed as it was described in Meyer et al. (2009). Laboratory blanks were extracted along with each batch of samples, consisting of all the solvents but not containing any sample. Spiked chicken egg and blood samples were also extracted along with samples to determine recovery rates. Recovery rates were between 98 and 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.

2.5. Biomarkers of condition

Blood was sampled using haematocrit tubes. These tubes were centrifuged for 10 min at 10,000 rpm within 12 h of sampling to separate plasma from the cellular fraction. The samples were stored at –80 °C until further analysis. After defrosting, the plasma 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 (Geens et al., 2010; Van Hout et al., 2012). It should be noted that due to the small volumes of blood we were able to obtain from great tits, we could not measure, and therefore directly relate, biochemical parameters and PFOS levels in blood. Instead of this we compared the levels of the biochemical parameters between locations, knowing that significant differences exists among locations in PFOS levels.

2.6. Statistical analyses

All statistics were performed using SPSS 23 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 distance of 2900–14500 m from the plant to compare among the three species. In the neighbourhood of the plant we could compare concentrations in eggs between tits and lapwings collected at a distance of 1200–1600 m. The relationship between the distance to the pollution source and PFOS concentrations in lapwing eggs was tested on log-transformed data 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. Results and discussion

3.1. Eggs as indicators of local PFOS contamination

PFOS levels measured in the eggs of the three studied species (and in the blood of the Mediterranean gull) along with results from other studies are shown in Table 1. PFOS was detected in all great tit eggs from both study sites (Fig. 2). Great tit eggs from the site closest to the fluoro-chemical plant had significantly higher PFOS levels than eggs collected further away (Mann-Withney U *test, U = 7.00, p = 0.026)*. For both study sites, the PFOS levels in the eggs were on average three times lower than the PFOS liver levels determined by Dauwe et al. (2007) (629 – 11358 ng/g ww). Differences in PFOS levels found between these two studies could be due to several reasons. 1) Differences in tissue specific accumulation could exist between liver and eggs. Although some previous studies measured similar concentrations in eggs and livers (Holmström and Berger, 2008; Van den Brink et al., 2007; Verreault et al., 2005) of non-passerines but the analysed tissues never came from the same individuals; therefore it is difficult to know whether PFOS levels in these tissues are comparable. More research is needed in this regard. 2) Eggs were randomly collected in the present study but a variation in egg concentrations within the clutch is known to exist. In tree swallow (*Tachycineta bicolor*), a 4-fold difference within a clutch was found (Custer et al., 2012). Moreover, in Audouins' gulls it was demonstrated that PFOS concentrations

Table 1
Comparison between PFOS levels measured in the current study for the three studied species and levels measured in eggs and blood of other bird species around the world. For the comparison we selected from literature those studies which reported the highest levels.. All values are given as ng/g ww for egg and ng/mL for blood.

Matrix	Species	Country	Range	Sampling year	Source
Egg	Great tit	Belgium	19–5 635	2006	Current study
	Northern lapwing		143 – 46 182		
	Mediterranean gull		150-916		
	Common tern	Netherlands	208-1219	UNK	Van den Brink et al. (2007)
	Cormorant	Sweden	419 – 1 163	2007–2009	Nordén et al. 2013
		Germany	100 – 1 451	2009	Rüdel et al. (2011)
	Double-crested cormorant	USA	84 – 1 253	2006, 2009	Sedlak and Greig (2012)
	Blue heron	USA	171-773	2010, 2011	Custer et al. (2013)
Blood	Mediterranean gull	Belgium	118-943	2006	Current study
	Great tit	Belgium	NA – 1 625	2005	Dauwe et al. (2007)
	Double-crested cormorant	USA	110-430	90 s	Giesy and Kannan (2001)
	Blad eagle	USA	1-2570	90 s	Giesy and Kannan (2001)

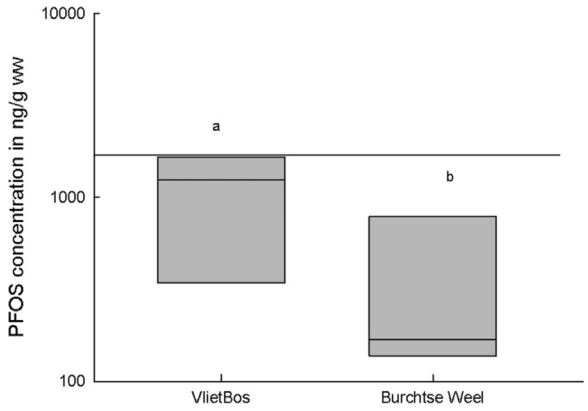

Fig. 2. PFOS concentrations in Great Tit eggs from Vlietbos and Burchtse Weel. Median, 25th and 75th percentile are reported. Significant differences ($p < 0.05$) between groups are indicated with different letters. The solid line represents the TRV (Toxicity Reference Value) for eggs (Newsted etal, 2005).

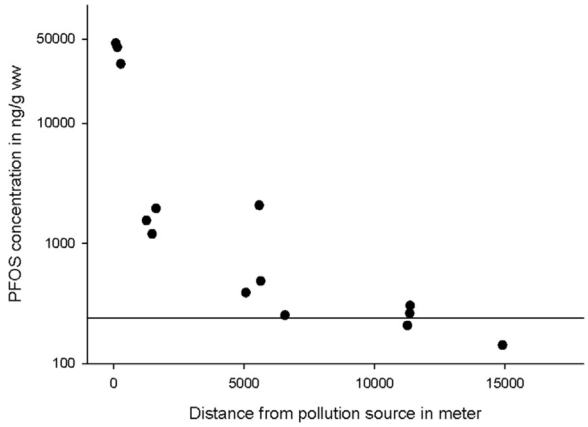

Fig. 3. PFOS concentration (ng/g ww) in Northern lapwing eggs along a distance gradient from the fluoro-chemical plant. The solid line represents the TRV (Toxicity Reference Value) for serum (Newsted etal, 2005).

decreased with the laying order of the eggs (Vicente et al., 2015). 3) Since Dauwe et al. (2007) sampled two years earlier (2004–2005), a decrease in PFOS levels is expected due to the phase out of the production of PFOS in 2002. 4) Dauwe et al. (2007) did the sampling during the winter; seasonal differences could exist in the exposure of great tits to PFOS (Yu et al., 2009; Bossi et al., 2016) (Fig. 3).

Regarding the levels found in northern lapwings' eggs, a significant negative correlation was observed between the PFOS levels and the distance from the nest to the fluoro-chemical plant (Pearson correlation on log transformed data, $r = -0.96$, $p < 0.001$; Fig. 2) The range of the measured values was 143–46182 ng/g ww with a mean of 9200 ± 4046 ng/g ww. Concentrations of PFOS were highest in three lapwing 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, and are, to the best of our knowledge, the highest PFOS concentrations ever reported in eggs (Table 1).

PFOS was detected in all the 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. The PFOS levels in eggs of common terns from the Western Scheldt estuary (colonies located 30 and 55 km away from the fluoro-chemical plant) were within the same range (208 – 1219 ng/g ww) as those from the present study for Mediterranean gull (Van den Brink et al., 2007; Van den Heuvel-Greve et al., 2006; Table 1). This despite the fact that, to the best of our knowledge, no other perfluor-related pollution source is present close to the tern colonies.

This indicates that there are still high levels of PFOS many 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 et al., 2005). The PFOS levels in the eggs and blood of the Mediterranean gull determined in our study were generally in the same range or slightly lower than the highest PFOS levels ever registered in the same matrix in other parts of Europe or the United States (Table 1). Thus, when comparing the PFOS levels of the Mediterranean gull eggs and blood to PFOS levels measured in other water birds, the Antwerp harbour region can be considered as a PFOS hotspot.

3.2. Interspecific differences in PFOS egg concentrations

We investigated inter-specific differences in PFOS levels in eggs among the three species collected furthest away from the fluoro-chemical plant (i.e. between 2900 and 14500 m, Fig. 4). PFOS levels did not differ significantly among the three species (one-way ANOVA on Ranks, $H = 3.31$, $p = 0.19$).

Despite collected further away from the pollution source, median PFOS levels were highest in the Mediterranean gull, followed by Northern lapwing, followed by levels in great tits eggs. However, differences were not statistically significant.

To be able to interpret these results correctly we have to consider some factors such as the differences in the trophic position of the species and the differences in the route of exposure (aquatic birds vs terrestrial birds), differences in distance of the nests to the fluoro-chemical plant, differences in exposure during winter or differences in the egg laying behaviour. The slightly higher levels in gull and lapwing

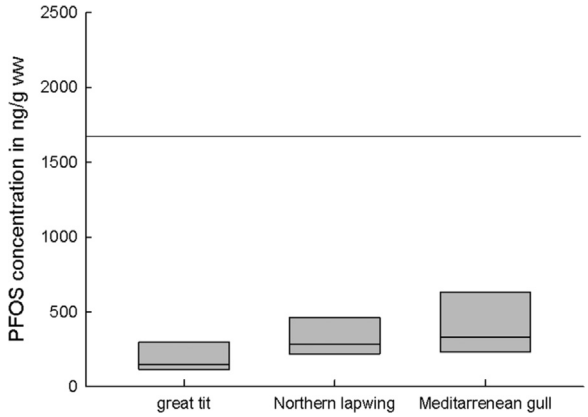

Fig. 4. PFOS concentrations (ng/g ww) in eggs from great tits, northern lapwings and Mediterranean gulls sampled from a distance between 2900 and 14500 m from the fluorochemical plant. Median, 10th, 25th, 75th and 90th percentiles are reported. The solid line represents the TRV (Toxicity Reference Value) for eggs (Newsted etal, 2005).

eggs could be explained by the higher trophic position of these species, which do not only eat insects, but also gastropods, small fish and even rodents (del Hoyo et al., 1996; Johansson and Blomquist, 1996). In addition, comparing concentrations in lapwing with gull eggs collected at 10000–14500 m, did not reveal significant differences neither (*t*-test: $t=1.34$; $p=0.216$). Sinclair et al. (2006) already showed that fish-eating birds may accumulate up to 2.5 times higher levels of PFOS in their livers compared to herbivorous birds, indicating that trophic level affects PFOS exposure and accumulation. As mentioned, high levels of PFOS were also detected in the livers and eggs of common terns, a piscivorous species, from colonies 30 and 55 km away from the fluorochemical plant (Van den Brink et al., 2007; Van den Heuvel-Greve et al., 2006). On the other hand, the Mediterranean gull migrates to the Iberian Mediterranean coast during winter (Cama et al., 2011) where additional sources of PFOS could exist. Nevertheless the possibility of a higher exposure in the wintering site is unlikely as PFOS mean concentration found in the Scheldt river, that runs near the fluorochemical plant in Antwerp, was 154 ng/L (Eschauzier et al., 2011) while PFOS concentrations found in rivers discharging in the Iberian Mediterranean sea range from 1.09 to 9.56 ng/L (Sánchez-Avila et al., 2010).

Additionally, comparing PFOS levels in the eggs of great tits and the ones found in northern lapwings' eggs collected at a distance of 1200–1600 m away from the plant did not reveal significant differences (*t*-test: $t=-1.901$; $p=0.12$). This is an unexpected result, if we consider the differences in the diet of both species; for instance great tits feed totally on terrestrial food while northern lapwings also feeds on marine and freshwater food (Johansson and Blomqvist, 1996). Unfortunately we didn't collect in the present study eggs from great tit closer to the fluoro-chemical plant.

3.3. Effect of PFOS on physiological plasma biomarkers

The results of the biomarker analysis in plasma from the great tits are summarised in Table 2. 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 apparently had lower mean plasma protein and cholesterol concentrations than older individuals from the same site and great tits from the less polluted site. 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) while the levels of serum cholesterol

Table 2
Biomarker results from the great tits at Vlietbos (=1200 m) and Burchtse Weel (=3000 m). Values are given as mean ± SEM. Significant differences ($p < 0.05$) between groups are indicated with different letters (superscript).

	1200 m		3000 m	
	One-year old	Older	One-year old	Older
n	7	9	5	10
Total protein (g/L)	28.7 ± 0.9^a	39.9 ± 2.2^b	37.8 ± 4.5^b	34.0 ± 3.3^b
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)	17.7 ± 1.4	10.3 ± 1.5	13.0 ± 1.7	16.3 ± 3.3
Cholesterol (mg/dL)	194 ± 11^a	277 ± 17^b	267 ± 24^b	228 ± 24^b

concentrations were in the same range (125–200 mg/dL) as values found by Hoff et al. (2005b). Hoff et al. (2005b) found significant negative correlations between the liver PFOS levels and the serum triglyceride and cholesterol concentrations. In the current study, no significant difference was found between the serum triglyceride levels of neither the age groups from both localities and differences between the serum cholesterol concentrations were only apparent for one year old birds. Hoff et al. (2005b) collected tits much closer to the perfluorochemical plant as in the present study probably explaining the significant relationship with the measured biomarkers. On the other hand, the levels of total proteins we found for the one-year old birds from the most polluted site seem to be in the lower part of the range for great tits (Ots et al., 1989). Hoff et al. (2005b) found no correlation between liver PFOS and protein concentration so these levels probably are rather an indication of a poor nutritional status (Lewandowski et al., 1986) in young birds in the most polluted area, not directly related with PFOS exposure. The lack of clear trends might indicate that the endpoints analysed in the current study are not very sensitive to exposure to PFOS. This is in line with the results of another study which examined the effects of PFOS in white leghorn chicks after *in ovo* exposure and also did not find significant differences in these parameters between experimental groups (Peden-Adams et al., 2009). Recently other health parameters like thyroid hormone levels (Cassone et al., 2012), immune parameters (Peden-Adams et al., 2009; Sletten et al., 2016; Smits and Nain, 2013), telomere length (Sletten et al., 2016) or oxidative stress parameters (Nakayama et al., 2008; Sletten et al., 2016) have been studied in birds in relation to PFOS and other PFAAs exposure. Although in vitro experiments and experimental exposures of laboratory animals demonstrated the effects of PFOS on some of these parameters (Jensen and Leffers, 2008; Lau et al., 2007; Nakayama et al., 2008; Peden-Adams et al., 2009; Wielsøe et al., 2015), evidence of their effects in wild populations is still scarce (Sletten et al., 2016). More research is needed to select or develop biomarkers that can be linked to an increase in PFAAs exposure in birds. It should be noted that in our study rather generic biomarkers were used and that more specific markers might be useful.

Newsted et al. (2005) calculated the Toxicity Reference Values (TRV) for PFOS based on the characteristics of an avian top predator. TRVs are used as guidelines in the protection of wildlife and are based on acute and chronic laboratory exposure data, in this case on the exposure of northern bobwhite quail (*Colinus virginianus*) and mallard (*Anas plathyrhynchos*). The toxicological and reproductive endpoints used to derive the TRV for PFOS include mortality, growth, feed consumption, histopathology, egg production, fertility, hatchability and survival and growth of offspring. For these endpoints, the lowest observable adverse effect level (LOAEL) is calculated and uncertainty factors (for the duration of the exposure, interspecific differences or LOAEL to NOAEL extrapolation) are included to calculate the TRVs. The TRVs derived by Newsted et al. (2005) for eggs are expressed as ng/mL. If we consider 1 mL equal to 1 g, for half of the northern lapwing eggs

and one third of the great tit eggs PFOS levels exceeded the TRV for eggs (1700 ng/mL ww). The three eggs of the northern lapwing closest to the fluoro-chemical plant are between 18 and 27 times higher than the TRV for eggs. This may indicate that the birds close to the fluoro-chemical plant may experience adverse effects from PFOS exposure. However, already at 6,000 m from the fluoro-chemical plant all PFOS levels in the northern lapwing eggs are below the TRV for eggs. This might indicate that the risk to birds is probably limited to a very restricted area close to the fluoro-chemical plant. The TRV for serum was 240 ng/mL. We measured in whole blood but if we compare our results of PFOS in total blood of gulls with the TRV, 20 out of 28 samples exceeded up to 4 times the TRV. Unfortunately PFOS was not measured in blood of the other species.

4. Conclusions

When comparing PFOS levels in the eggs of the three bird species in our study to previous studies, PFOS levels were much higher. Our study in the Antwerp harbour region reports some of the highest PFOS levels ever measured in wildlife. Since some eggs of the northern lapwing and the great tit presented levels of PFOS far above the TRV for eggs, and about half of the eggs of these species were close to this TRV, these birds, 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. At a distance of 2900–14500 m from the fluoro-chemical plant PFOS levels in eggs did not differ among the three species, although levels in eggs seemed to follow the gulls > lapwings > tits. This could indicate that food and/or metabolism might play a role in the PFOS exposure in birds. However, eggs of the three species were not collected at exactly the same place.

The results of the present study indicate 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. A clear gradient has been observed with distance in eggs from Northern lapwings with extremely high levels close to the fluoro-chemical plant, but a steep decrease with distance.

Further research is needed to see how PFOS levels have evolved in recent years in this highly polluted area and to detect the presence of other PFAAs compounds that are currently being produced in the fluoro-chemical plant. It is also crucial to develop specific biomarkers that can be linked to the exposure of birds to PFOS and other PFAAs compounds. Therefore, this biomarker assessment in blood along the distance gradient would be interesting.

Conflicts of interest

The authors declare that there is not conflict of interest in this study.

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