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

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ABSTRACT

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

The PFSA concentrations measured at the site of the fluorochemical plant were among the highest ever reported in eggs with median concentrations of 10380 ng/g (extrapolated), 99.3 ng/g and 47.7 ng/g for PFOS, PFHxS and PFDS respectively. Furthermore, the median concentration of 19.8 ng/g for PFOA was also among the highest ever reported in bird eggs. Although these concentrations decreased sharply with distance from the fluorochemical plant, levels found in the adjacent sites were still high compared to what has been reported in literature. Moreover, based on what is known in literature, it is likely that these concentrations may cause toxicological effects. PFOS was the dominant contributor to the PFSA and PFAAs (63.4–97.6%) profile at each site, whereas for PFCAs this was PFOA at the plant site and the nearest locations (41.0–52.8%) but PFDoA (37.7%) at the farthest location.

Although there is some evidence that PFAAs concentrations close to the plant site are decreasing in comparison with earlier measurements, which may be due to the phase out of PFOS, more research is necessary to understand the extent of the toxicological effects in the vicinity of this PFAAs hotspot.

1. Introduction

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

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

PFOS, PFOA and related compounds have been phased out by

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

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

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

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

2. Materials and methods

2.1. Study species and sample collection

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

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

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

2.2. Chemical analysis

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

2.3. Sample extraction

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

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

2.4. UPLC-TQD analysis

We analyzed PFAAs by UPLC coupled tandem ES(-) mass spectrometry (ACQUITY, TQD, Waters, Milford, MA, USA) using an ACQUITY BEH C18 column (2.1 × 50 mm; 1.7 μm, Waters, USA), mobile phase: 0.1% formic acid in water(A), 0.1% formic acid in acetonitrile(B), solvent gradient: from 65% A to 0% A in 3.4 min and back to 65%A at 4.7 min, flow rate: 450 μL/min, injection volume: 10 μl. To retain any PFAA contamination originating from the system, we inserted an ACQUITY BEH C18 pre-column (2.1 × 30 mm; 1.7 μm, Waters, USA) between the solvent mixer and the injector. Identification and quantification was based on multiple reaction monitoring (MRM) of the following diagnostic transitions: 213 → 169 (PFBA), 313 → 296 (PFHxA), 315 → 270 ($^{13}C_2$-PFHxA), 413 → 369 (PFOA), 421 → 376 ($^{13}C_8$-PFOA), 463 → 419 (PFNA), 472 → 427 ($^{13}C_9$-PFNA), 513 → 469 (PFDA), 519 → 474 ($^{13}C_6$-PFDA), 613 → 569 (PFDoA), 613 → 319 (PFDoA), 615 → 169 ($^{13}C_7$PFDoA),

615 → 570 ($^{13}C_7$PFDoA), 570 → 525 ($^{13}C_7$PFUdA), 663 → 619 (PFTrA), 713 → 669 (PFTeA), 713 → 369 (PFTeA), 299 → 99 (PFBS), 399 → 99 (PFHxS), 403 → 103 ($^{18}O_2$-PFHxS), 599 → 80 (PFDS), 499 → 80 (PFOS), 499 → 99 (PFOS) and 507 → 80 ($^{13}C_8$-PFOS).

2.5. Calibration

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

2.6. Quality assurance

One procedural blank per 10 samples was analyzed as quality control. Minor levels of contamination (<0.4 pg/μL) of PFOA and PFOS were subtracted from the correspondent concentrations found in the samples. For PFOA and PFOS, the quality of the applied method was evaluated by 3 laboratories on spiked egg samples; a triplicate analysis of a sample, spiked with linear (61.7 ng/g and 63.2 ng/g for PFOA and PFOS respectively) or branched (32.2 ng/g and 32.0 ng/g for PFOA and PFOS respectively) isomers of PFOS and PFOA, was performed in each laboratory (Table S1). No significant differences were detected between the laboratories. For the spiked samples, an accuracy of 93–107% was achieved. The precision of the applied method varied between 2 and 4% (Table S1). The limit of quantifications (LOQs), corresponding to a signal-to-noise ratio 10, ranged from 0.02 ng/g to 1 ng/g for PFBS, PFHxS, PFOS, PFDS, PFOA, PFDoA and PFTrA. Due to some high noise levels the LOQs for PFBA, PFHxA, PFNA, PFDA and PFTeA are considerably higher and ranged from 1.4 ng/g to 4.3 ng/g. Individual LOQs are displayed in Table 1. For all samples, of which concentrations were within the linear range of the calibration curve, recoveries of the ISTDs were calculated. The samples were corrected for recoveries, which were between 92% and 110%. At two locations, some PFOS concentrations were outside the linear range of the calibration curve and therefore the samples were 10–800 times diluted. As a consequence, the internal standards were no longer visible and therefore a correction based on the recoveries was extrapolated.

2.7. Statistical analysis

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

Differences in concentrations between the different sampling locations were evaluated in two ways. First of all, we performed a one way ANOVA using Least Significant Difference (LSD) test for Post-hoc analysis for PFAAs found in all samples, i.e. PFOS and PFOA. Secondly, for PFAAs with at least one value above the LOQ (i.e. PFDoA, PFTrA, PFHxS and PFDS), we used a reverse Kaplan Meier (KM) analysis and a Mantel-Cox test for pairwise comparisons among sampling sites. This analysis is commonly used for survival analysis of left censored data (Gillespie et al., 2010) and has been proven useful to cope with levels below the LOQ (Jaspers et al., 2013). Details about how to perform this analysis with SPSS are provided in Gillespie et al. (2010). As reverse KM is a nonparametric analysis we used untransformed data to perform the analysis.

To study correlations between the individual compound concentrations, and between the ∑PFSAs and ∑PFCAs in each study site Spearman rank correlation analyses were performed.

For each site the composition profiles were determined by calculating the proportions of individual compounds to the total concentrations of PFAAs, PFSAs and PFCAs in each egg and then averaging the percentages for all the eggs at a site. For this calculation, values below the LOQ were replaced with a value of LOQ/2 (Bervoets et al., 2004; Custer et al., 2000).

3. Results

3.1. PFAAs levels

An overview of median levels, ranges and detection frequencies of PFAAs in the eggs is given in Table 1. Some PFOS levels at 3 M and Vlietbos exceeded the linear range of the calibration curve and

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

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

were thus higher than 16 ng/mL. Although these levels were already very high, the extrapolated levels have been used in this study.

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

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

Figure 1 shows the PFAAs concentrations in function of the distance from the pollution source the center of the plant site is considered to be the pollution source (0 m).

3.2. PFAAs profile

For all the sampling sites PFOS was the dominant contributor to the ∑PFSAs (Fig. S1) and to the ∑PFAAs as its contribution to the ∑PFAAs ranged between 97.6 ± 0.3% (mean ± SE) at the plant site and 63.4 ± 6.4% at Tessenderlo. For ∑PFCAs, the major compound was PFOA at the plant site (43.2 ± 6.5%), Vlietbos (52.8 ± 4.3%) and Rot (41 ± 5.6%), but not at Tessenderlo where it accounted for the 27.0 ± 7.5% and where PFDoA and PFTrA represented 37.7 ± 6.5% and 35.2 ± 5.2% respectively (Fig. 2).

3.3. Correlations

All correlations found among PFAAs at the different sampling sites are summarized in Table 2. Significant correlations were observed mostly at the plant site (14 significant correlations), followed by Vlietbos (13), Rot (3) and Tessenderlo (where no significant correlations were observed). All significant correlations were positive. At the plant site PFOS, PFDS, PFDoA and PFTrA were all

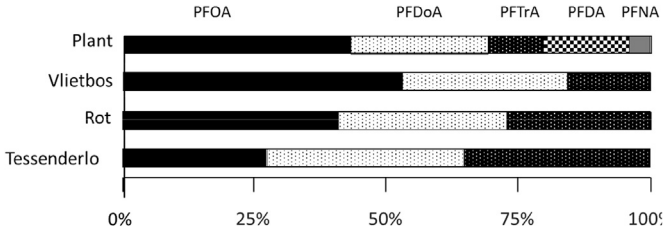

Fig. 2. Composition profile of PFCAs in eggs of great tit at the four sampling sites; a fluorochemical plant and at three sites with increasing distance from the plant site: 1 km (Vlietbos), 2.3 km (Rot) and 70 km (Tessenderlo, reference site).

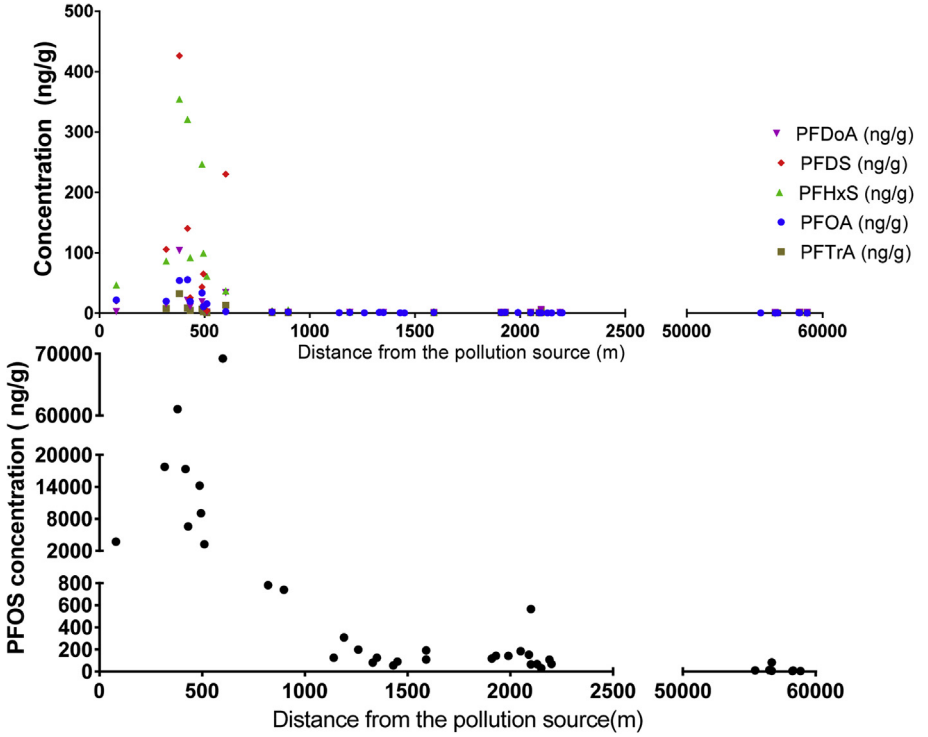

Fig. 1. a) PFAAs concentrations along the distance gradient from the pollution source. The center of the fluorochemical plant is considered to be the pollution source (0 m). b) PFOS concentrations along the distance gradient from the pollution source.

Table 2
Correlations found between different PFAAs in the different sampling sites. Values in bold are significant correlations.

		Plant site (n = 11)		Vlietbos (n = 11)		Rot (n = 11)		Tessenderlo (n = 8)	
		p-value	Rho	p-value	Rho	p-value	Rho	p-value	Rho
PFOS	PFOA	0.654	0.155	**0.016**	0.702	0.818	0.082	0.233	−0.7
	PFHxS	0.451	0.255	**0.008**	0.745	/	/	/	/
	PFDS	**<0.001**	0.891	**0.005**	0.776	/	/	/	/
	PFNA	0.296	0.347	/	/	/	/	/	/
	PFDA	0.614	0.172	/	/	/	/	/	/
	PFDoA	**<0.001**	0.927	**<0.001**	0.898	**0.009**	0.739	0.858	−0.112
	PFTrA	**<0.001**	0.900	**0.015**	0.707	0.097	0.525	0.614	0.308
PFOA	PFHxS	**0.031**	0.664	**0.038**	0.630	/	/	/	/
	PFDS	0.435	0.264	**0.024**	0.671	/	/	/	/
	PFNA	**0.013**	0.719	/	/	/	/	/	/
	PFDA	**0.031**	0.648	/	/	/	/	/	/
	PFDoA	0.341	0.318	**0.015**	0.706	0.620	0.169	0.718	−0.224
	PFTrA	0.755	0.109	0.062	0.579	0.481	0.238	0.219	−0.667
PFHxS	PFDS	0.503	0.227	**0.009**	0.744	/	/	/	/
	PFNA	**0.007**	0.758	/	/	/	/	/	/
	PFDA	**0.022**	0.677	/	/	/	/	/	/
	PFDoA	0.214	0.409	**0.038**	0.629	/	/	/	/
	PFTrA	0.341	0.318	0.052	0.597	/	/	/	/
PFDS	PFNA	0.197	0.421	/	/	/	/	/	/
	PFDA	0.427	0.267	/	/	/	/	/	/
	PFDoA	**0.003**	0.827	**0.002**	0.825	/	/	/	/
	PFTrA	**0.010**	0.755	0.089	0.536	/	/	/	/
PFNA	PFDA	**<0.001**	0.858	/	/	/	/	/	/
	PFDoA	**0.038**	0.630	/	/	/	/	/	/
	PFTrA	0.113	0.506	/	/	/	/	/	/
PFDA	PFDoA	0.210	0.410	/	/	/	/	/	/
	PFTrA	0.462	0.248	/	/	/	/	/	/
PFDoA	PFTrA	**<0.001**	0.945	**0.009**	0.740	**0.038**	0.641	0.199	0.688
∑PFSA	∑PFCA	**0.034**	0.6	**<0.001**	0.882	**0.006**	0.791	0.683	0.3

correlated with each other, whereas PFOA, PFHxS, PFNA and PFDA were also correlated with each other. However, PFNA was also correlated with PFDoA. At Vlietbos PFOS levels were correlated with levels of all other compounds. Although many of these compounds were also related with each other, no correlations were observed between PFTrA and PFOA, PFHxS and PFDS. At Rot PFOS was only correlated with PFDoA, which was also correlated with PFTrA. Overall PFCA levels (∑PFCA) were correlated with overall PFSA levels (∑PFSA) at the plant site, Vlietbos and Rot (Fig. 3).

4. Discussion

4.1. PFAAs levels

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

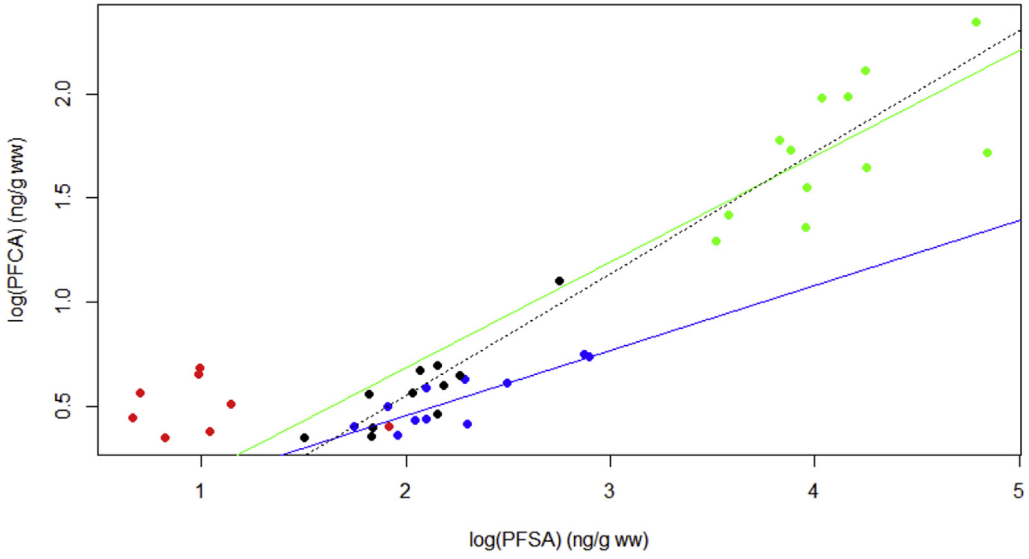

Fig. 3. Correlations between ∑PFSA and ∑PFCA concentrations amongst sites. Green = 3 M, Blue = Vlietbos, Black = Middenvijver-Rot and Red = Tessenderlo.

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

To be able to make comparisons among species, some examples of PFAAs concentrations found previously in terrestrial and marine bird eggs are shown in Table 3.

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

The highest mean PFOS concentration detected at the plant site in the present study is more than 18 times higher than the highest mean concentration reported in Great Blue Heron (*Ardea herodias*) eggs (1014 ng/g) in 1993 in the Mississippi river, in a colony located approximately 20 km from a 3 M fluorochemical plant site (Custer et al., 2010). In the same study, concentrations of the other reported PFAAs (PFDS, PFHxS, PFDA, PFNA, and PFOA) were also, at least two times, lower than the ones we measured in the present study. It must be considered that PFOS was still produced in the plant when the Great Blue Heron eggs were collected. Mean concentrations found in the same Great Blue Heron colony in 2010 (465 ng/g; Custer et al., 2013) remain among the highest ever found, however they are 41 times lower than the one reported at the fluorochemical plant in the present study. Mean PFOS (109 ng/g), PFHxS (0.52 ng/g) and PFOA (0.9 ng/g) concentrations found in free range chicken eggs (*Gallus gallus*) collected at a distance of less than 500 m from a fluorochemical manufacturing plant in China (Wang et al., 2010), were lower than those found in the present study in Vlietbos and very similar to the ones found in Rot, located at 1 and 2.3 km from the plant site respectively. In fact, PFOS and PFOA levels found 1 km away from the fluorochemical plant in China (9.8 and 0.15 ng/g respectively) were lower than those found in our reference site, Tessenderlo, 70 km away from the plant site. We must consider that when the study of Wang et al. (2010) was performed the plant in China still produced PFOS and related compounds. A study on PFOS concentrations in chicken eggs in Belgium also showed high concentrations of PFOS (highest mean concentration of 3500 ng/g) in the vicinity of the same fluorochemical plant as in the present study (D'Hollander et al., 2011). However, this mean concentration is similar to the lower PFOS levels at the plant site in the present study.

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

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

Within each site, the variability between the concentrations of the individual PFAAs of the different nestboxes was considerably high. The highest variability was observed at the plant site where the minimum and maximum concentrations varied up to 20 times. The variability of the PFSA concentrations was higher compared to those observed for the PFCAs. These differences could be indicating variations in concentrations in the laying females. For example,

Table 3
Median PFAAs concentrations in bird eggs (ng/g ww) published in the literature. All studies on levels in terrestrial birds and four studies with higher levels in sea birds have been included. *Geometric means; ** median levels; *** range; NP = levels were measured but were not provided; NA = not assessed; § Levels found in active fluorochemical plant † Levels found in a fluorochemical plant not used since 2002.

Species	Country	Year	PFHxS	PFOS	PFDS	PFOA	PFNA	PFDA	PFDoA	PFTrA	PFTeA	Publication
*Corvus frugilegus***	Germany	2009	<LOQ	5.3	NA	0.5	2.1	0.8	NA	NA	NA	Rüdel et al., 2011
Paradoxornis webbiana	Korea	2006	1.3	314.1	1.1	0.8	40	114.2	25.6	NA	NA	Yoo et al., 2008
*Strix aluco**	Norway	1986–2009	0.05	10.9	0.06	<LOQ	<LOQ	0.20	0.12	0.36	NA	Ahrens et al., 2011
Falco peregrinus	Sweden	2006	0.80	83	0.66	<LOD	1.6	3.1	3.2	7.3	2.7	Holmström et al., 2010
Tachycineta bicolor	Minnesota (USA)	2008–2009	NP	141	NA	<LOD	NP	5.51	NP	NA	NA	Custer et al., 2012
*Tachycineta bicolor**	Minnesota (USA)	2007–2011	0.95	270	NA	18.7	3.10	5.47	1.96	NA	NA	Custer et al., 2014
Gallus gallus §	China	2009	0.52	109	NA	0.9	<LOD	<LOD	<LOD	NA	NA	Wang et al., 2010
Gallus gallus†	China	2009	0.24	85.2	NA	0.76	<LOD	<LOD	<LOD	NA	NA	Wang et al., 2010
*Ardea herodias**	Minnesota (USA)	1993	1.5	940	33	<LOD	0.9	3.6	3.7	NA	NA	Custer et al., 2010
*Ardea herodias**	Minnesota (USA)	2010–2011	0.65	342	8	0.6	2.55	22	12.9	NA	NA	Custer et al., 2013
Phalacrocorax auritus	San Francisco (USA)	2009	LOD-25.2	483.7	NA	ND-24.3	13.4	13.8	7.08	NA	NA	Sedlak and Greig 2012
*Phalacrocorax carbo***	Sweden	2007–2009	2.5	552	2.06	4.05	20.7	44.8	23.9	23.7	4.08	Nordén et al., 2013
*Phalacrocorax carbo***	Germany	2009	2.8	400	NA	1.1	2.7	10.4	1.0	NA	NA	Rüdel et al., 2011
*Parus major****	Belgium	2006	NA	19–5635	NA	NA	NA	NA	NA	NA	NA	Lopez Antia et al., 2017
*Vanellus Vanellus****	Belgium	2006	NA	143–46182	NA	NA	NA	NA	NA	NA	NA	Lopez Antia et al., 2017
*Ichthyaetus melanocephalus****	Belgium	2006	NA	150–916	NA	NA	NA	NA	NA	NA	NA	Lopez Antia et al., 2017

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

4.2. PFAAs profile

Our results showed that PFOS is the major contributor to the total PFAAs. This is in agreement with the literature on PFAAs in bird eggs (e.g., Ahrens et al., 2011; Custer et al., 2012; Nordén et al., 2013; Rüdel et al., 2011). Regarding the PFCAs composition profile, PFOA, followed by PFDoA, is the major contributor at the plant site, Vlietbos and Rot whereas at PFDoA and PFTrA are the major contributors at Tessenderlo. Moreover, a trend can be observed for PFOA to reduce and PFTrA to increase their relative concentrations with the increase in distance (Fig. 2). In the plant site and surrounding areas the PFCAs composition profile could be explained by the influence of a direct contamination source, where PFOA is the main product (Prevedouros et al., 2006), whereas 70 km away from the plant site, in Tessenderlo, the composition profile could be explained by the atmospheric and biological degradation of the volatile polyfluorinated precursor compounds (fluorotelomer alcohols; FTOH), and the fact that long chain fluorinated compounds (PFTrA and PFDoA) are more bioaccumulative than shorter chain ones (PFOA) (Armitage et al., 2009; Conder et al., 2008; Ellis et al., 2004; Houde et al., 2006). The composition profile found in Tessenderlo is similar to the ones found in eggs of tawny owl in Norway (Ahrens et al., 2011) and peregrine falcon in Sweden (Holmström et al., 2010), where PFTrA and PFUndA were the major contributors to $\sum$PFCAs.

4.3. Toxicological implications

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

Regarding PFOS, both laboratory and field studies are present. Molina et al. (2006) indicated that PFOS caused a significant reduced hatchability of the chicken embryo after in *ovo* exposure at a dose as low as 0.1 μg/g egg. Pathological changes in the liver, including bile duct hyperplasia, periportal inflammation and necrosis were observed at a dose of 1.0 μg/g after *in ovo* exposure. Peden-Adams et al. (2009) observed increased spleen mass, increased lysozyme activity, suppressed total sheep red blood cell -specific immunoglobulin production, shorter right wings and increased frequency and severity of brain symmetry in chickens at in *ovo* exposure level of 1 μg/g. Newsted et al. (2005) derived a predicted-no-effect concentration of 1 μg/ml PFOS in egg yolk based on chronic and acute dietary exposures of northern bobwhite quail (*Colinus virginianus*) and mallard (*Anas platyrhynchos*). According to these values, the PFOS concentrations observed at the plant site may cause physiological and neurological effects on great tits if we assume equal sensitivity between species. Moreover, in a PFAAs contaminated area in east central Minnesota, USA, reduced hatching success was associated with PFOS concentrations as low as 150 ng/g in eggs of tree swallow (*Tachycineta bicolor*) (Custer et al., 2012, 2014). If great tit have the same sensitivity as tree swallow, the current PFOS contamination at all the sampling locations, except Tessenderlo, would result in reduced hatchability.

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

Reduced hatching success and a decrease in tarsus length and embryo mass have been observed in chickens that were exposed *in ovo* to PFHxS concentrations up to 38 μg/g (Cassone et al., 2012). Furthermore, a reduction in plasma thyroid hormone levels was observed at concentrations up to 0.89 μg/g (Cassone et al., 2012), a level about 5 times higher than the one found at the plant site in the present study.

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

5. Conclusion

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

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

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Appendix A. Supplementary data

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