Environment International 52 (2013) 9–16

Contents lists available at SciVerse ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Perfluoroalkyl substances in soft tissues and tail feathers of Belgian barn owls (*Tyto alba*) using statistical methods for left-censored data to handle non-detects

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ARTICLE INFO

Article history:
Received 29 June 2012
Received in revised form 30 October 2012
Accepted 2 November 2012
Available online 23 December 2012

Keywords:
PFASs
Feathers
External contamination
Non-detects
Reverse Kaplan–Meier
Survival analysis

ABSTRACT

Perfluoroalkyl substances (PFASs) were investigated in tail feathers and soft tissues (liver, muscle, preen gland and adipose tissue) of barn owl (*Tyto alba*) road-kill victims (n = 15) collected in the province of Antwerp (Belgium). A major PFAS producing facility is located in the Antwerp area and levels of PFASs in biota from that region have been found to be very high in previous studies. We aimed to investigate for the first time the main sources of PFASs in feathers of a terrestrial bird species. Throughout this study, we have used statistical methods for left-censored data to cope with levels below the limit of detection (LOD), instead of traditional, potentially biased, substitution methods.

Perfluorooctane sulfonate (PFOS) was detected in all tissues (range: 11 ng/g ww in muscle–1208 ng/g ww in preen oil) and in tail feathers (<2.2–56.6 ng/g ww). Perfluorooctanoate (PFOA) was measured at high levels in feathers (<14–670 ng/g ww), but not in tissues (more than 50%<LOD). Perfluorohexane sulfonate (PFHxS) could only be quantified in liver and preen oil, while other PFASs were sporadically detected in liver. PFOS levels in feathers and liver were highly correlated ($r = 0.78$, $p < 0.01$), in contrast to PFOA ($r = -0.11$, $p = 0.78$). Combined with high PFOA levels in feathers this suggests that PFOA may be present on the external surface of feathers, due to external contamination originating from the air in the vicinity of point sources. Therefore the possibility of using feathers as a passive air sampler for high PFOA levels should be investigated in the future.

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1. Introduction

Increasing scientific interest in perfluoroalkyl and polyfluoroalkyl substances (PFASs; Buck et al., 2011) is due to their global distribution and environmental persistence (Giesy and Kannan, 2001; Yamashita et al., 2005). PFASs have been associated with immuno-, hepato- and developmental toxicity (Lau et al., 2007). The most common PFASs are perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA). In recent years, PFOS and PFOA have been detected in top predators, such as marine mammals and birds of prey. Since the manufacture of PFOS was ceased by 3M (the major producer) in 2002, global production of PFOS has dropped, while the production of other PFASs continued or even increased (Lau et al., 2007). Although the volatility of PFOS and PFOA is relatively low, their precursors, such as fluorotelomer alcohols or fluorinated sulphonamides, are volatile at atmospheric temperature and pressure, and may contribute to the atmospheric transport of PFASs (Lau et al., 2007). Some studies have found indications for bioaccumulation and biomagnification of PFASs in fish-eating birds of prey (reviewed by Lau et al., 2007), but limited data are available in terrestrial birds of prey (Ahrens et al., 2011; Meyer et al., 2009; Senthilkumar et al., 2007; Shlosberg et al., 2011).

Birds of prey have played a prominent role in the documentation of anthropogenic pollution with organic compounds (Furness, 1993; Ratcliffe, 1967). Located at the top of the food chain, they are particularly susceptible to the accumulation and biomagnification of organic pollutants (Furness, 1993). As birds of prey are often protected species, the validation of non-destructive biomonitoring techniques is needed. Feathers have valuable characteristics for non-destructive biomonitoring purposes: sampling can potentially be independent of season, age or sex; molted feathers can be collected at the nest; and feathers can be stored at room temperature and transported without special precautions. Previously, feathers have proven useful for non-destructive biomonitoring of heavy metal contamination (Burger, 1993) and organic pollutants (Jaspers et al., 2006a, 2007). One study to date has measured PFASs in feathers (Meyer et al., 2009) but due to several uncertainties the authors could not conclude on the reliability of feathers for monitoring PFASs. In particular, the possibility of external contamination with PFASs on the feather surface was not investigated.

In the current study, we investigate PFASs in feathers and tissues of Belgian barn owls (*Tyto alba*), collected in the province of Antwerp. The barn owl, a terrestrial bird of prey monitored in Belgium by the

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0160-4120/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.envint.2012.11.002

barn owl study group (Kerkuilwerkgroep vzw), is resident year round and mostly feeds on small mammals, such as mice, small voles and shrews (Snow and Perrins, 1998). Previous studies (Dauwe et al., 2007; Hoff et al., 2005) have found very high concentrations of PFASs in biota in the vicinity of Antwerp (Belgium) near a fluoro-chemical plant (3M). Since barn owls are potentially predating on highly contaminated prey around the Antwerp region, we expect to find quantifiable PFAS levels in their feathers, and that these levels will correlate to concentrations in their tissues.

In this study, we aim to investigate for the first time the main sources for PFASs in feathers as levels can both originate from internal sources (via the blood connection during feather growth) as well as external contamination (via e.g. air, dust and water). Therefore we estimate correlations between concentrations in tail feathers and soft tissues. We use statistical methods for left-censored data throughout this study to cope with levels below the limit of detection (LOD) and we discuss the benefit of using these methods in comparison with substitution methods. Finally, we will discuss the biomagnification potential of PFOS in the barn owl as the top predator of a terrestrial ecosystem, using data on its prey in the vicinity of Antwerp.

2. Materials and methods

2.1. Sampling

A major PFAS chemical plant (3M) is located in the close vicinity of the city of Antwerp in Belgium (Fig. SI-1, see Supplementary data online). For this reason we have chosen to study levels in barn owls from the province of Antwerp. In 2008 and 2009, 15 barn owl carcasses (10 females, 3 males, and 2 unknown gender) were collected around Antwerp (Fig. SI-1) by the barn owl study group (Kerkuilwerkgroep vzw). Cause of death was road kill. The carcasses were stored in the freezer at −18 °C. Before analysis the carcasses were partly thawed and the birds were dissected for muscle, liver, preen gland and adipose tissue (when available), using disposable scalpel knives and a clean pair of tweezers for each bird. Preen oil and adipose tissue could only be sampled in sufficient amount for analysis from seven individuals. The tissues were individually stored in clean polystyrene containers and kept frozen until analysis. Tail feathers were pulled and stored in low density polyethylene zip lock bags at room temperature until analysis. Samples were shipped with CITES permission to the Norwegian Institute for Air Research (NILU, FRAM Centre, Tromsø, Norway) for PFAS analysis.

2.2. Chemical analysis

Approximately 1 g of each soft tissue sample or 300 mg of preen oil was accurately weighed and homogenized prior to analysis. We followed the analytical method of Powley et al. (2005) for PFASs in biological matrices. The homogenized sample was spiked with internal standard (^{13}C-PFOA/PFOS) and thoroughly mixed with 10 mL of methanol. After centrifugation (2000 rpm, 5 min), the supernatant was concentrated to 1.5 mL in a Rapidvap and cleaned up with ENVI-carb and glacial acetic acid. Samples were analyzed with liquid chromatography–mass spectrometry (LC–MS/TOF).

Feathers were washed twice prior to analysis. First, distilled water and a pair of tweezers were used to assure efficient washing between the barbs. The feathers were subsequently dried at room temperature and cut into small pieces (1 mm). For the second wash, the feathers were immersed in 20 mL hexane and set in an ultrasonic bath for 10 min. The hexane was decanted and the feathers were left to dry in the fume hood. For the analysis of PFASs in feathers we modified the analytical method for PFASs in biological matrices (Powley et al., 2005) by including a digestion step with a KOH/MeOH mixture to resolve bound PFASs from the feathers. Approximately 200–300 mg of homogenized feather sample was spiked with internal standard (^{13}C-PFOA/PFOS) and 2 mL 200 mM KOH in methanol was added. After 60 min, 10 mL methanol was added and samples were extracted three times in an ultrasonic bath for 10 min with vortex in between. Samples were left to soak overnight. In the morning 200 μL 2 M HCl in methanol was added and the ultrasonic bath-vortex cycle was repeated once more. After centrifugation (2000 rpm, 5 min), the supernatant was concentrated to 1.5 mL in a Rapidvap and cleaned up with ENVI-carb and glacial acetic acid.

All equipment used for instrumental analysis was from Waters (Milford, MA), unless otherwise specified. Analytical measurements were performed according to Huber et al. (2011). The sample extracts (injection volume 50 μL) were analyzed on a HPLC consisting of a 1525 μ pump and a 2777 autosampler coupled to a quadrupole time-of-flight (QTOF) mass spectrometer (model: qtof micro) through an electrospray ionization (ESI) probe, operated in negative mode at −3 kV. The analytes were separated on an ACE C_{18} column (150×2.1 mm, 3 μm particle diameter d_p) equipped with a guard-column (5×2.1 mm, d_p 3 μm) from ACT (Aberdeen, UK) using a gradient of 200 μL min^{-1} consisting of methanol and water (both containing 2 mM NH_4OAc). The initial mobile phase was 50:50 methanol/water, followed by a 5 min ramp increase to 85:15, a 5 min hold at 85:15, a 0.5 min ramp to 99:1, and hold for 15 min until reverting to initial conditions. The mobile phase was degassed by sonication for 20 min prior to analysis. Full scan (*m/z* 100–1200) high-resolution mass spectra were recorded throughout the chromatographic run. Alternating cone voltages of 20, 35, and 50 V were applied, and the cone temperature was 120 °C. Nitrogen was used as nebulizing (maximum flow) and desolvation gas (600 L h^{-1}, 350 °C). The HPLC–MS system was controlled by MassLynx 4.0 software.

Quantification was done using the internal standard method with [$^{13}C_4$]-PFOS and [$^{13}C_4$]-PFOA. The following PFASs were targeted for analysis: perfluorooctane sulfonamide (PFOSA), perfluorobutanoate (PFBA), perfluoropentanoate (PFPA), perfluorohexanoate (PFHxA), PFOA, perfluorononanoate (PFNA), perfluorodecanoate (PFDcA), perfluoroundecanoate (PFUnA), perfluorododecanoate (PFDoA), perfluorotridecanoate (PFTriA), perfluorotretradecanoate (PFTeA), perfluorohexane sulfonate (PFHxS), PFOS and perfluorodecane sulfonate (PFDcS). Concentrations are presented on a wet weight basis (ww).

2.3. Quality assurance and quality control

The analytical quality of the laboratory has been approved in inter-laboratory studies (Van Leeuwen et al., 2009). As standard procedure, laboratory blanks, LODs, recoveries, and standard reference materials (SRMs) were examined. For each sample, a high resolution full scan spectrum was used to control positive detections (typical mass tolerance 50 ppm). The LODs were calculated as 3 times signal to noise ratios for each compound in the samples (MassLynx 4.0, QuanLynx). An appropriate range of noise in the chromatogram was used in cases of non-detects. The LODs for the PFASs in the different tissue samples are presented in Table SI-1 (SI). No laboratory contamination for any of the analyzed compounds was detected.

Average recoveries for ^{13}C-PFOS and ^{13}C-PFOA were 67% for both in liver, 27% and 57% in preen oil, 90% and 40% in adipose tissue, 88% and 108% in muscle, and 88% and 48% in feathers, respectively. During the project more ^{13}C-labeled PFASs became commercially available and the method was tested analyzing feathers from golden eagle (*Aquila chrysaetos*), a species formerly tested for very low PFAS contamination (unpublished data). To ten feather homogenates the whole range of analyzed PFASs and a suite of 9 ^{13}C labeled PFAS was added (10 ng/g). The precision of the analytical method (RSD of the ten parallel feather analyses) varied between 12 and 32%, and the accuracy of the spiked amount ranged between 47% and 100% for all PFASs with the exception of PFBA, PFPA, PFTrA and PFTeA and PFDcS; no results for these compounds will be presented. The performance of the analytical method applied for soft tissue samples was monitored using

a fish tissue from an intercalibration exercise carried out within the EU project PERFORCE, resulting in an accuracy between 86 and 135%.

2.4. Statistics

Data below LOD were treated using methods of survival analyses for left-censored data (Gillespie et al., 2010; Helsel, 2005, 2006). The distributions of PFAS concentrations in barn owl feathers and soft tissues were estimated using the reverse Kaplan–Meier (KM) method (Gillespie et al., 2010). The cumulative distributions were plotted for feathers and each tissue type for PFOS, PFOA, and PFHxS when at least one value above LOD was available (Fig. 1a–c). Median concentrations and other quantiles can be read from the graphs (e.g., the median concentration is where the graph crosses y = 0.5). For each distribution function, the LOD and the proportion below LOD can be seen as the (x,y) coordinates, respectively, of the left-most point on the graph; if no values are below LOD, then the left-most point is at the origin. This method of presenting the distribution gives more information than substituting values below LOD with LOD divided by 2 or its square root, and it does not obscure the uncertainty inherent in values below detection. The KM estimator is nonparametric, meaning that it does not assume any distributional shape such as the lognormal. It is a cumulative step function, with a step up at each exact value. In the presence of outliers, such as a few extremely large concentrations, the right-most steps of the function will be far to the right of the other steps, but the rest of the distribution will be not be affected. Thus, outliers will affect the highest percentiles, but not the median or other percentiles.

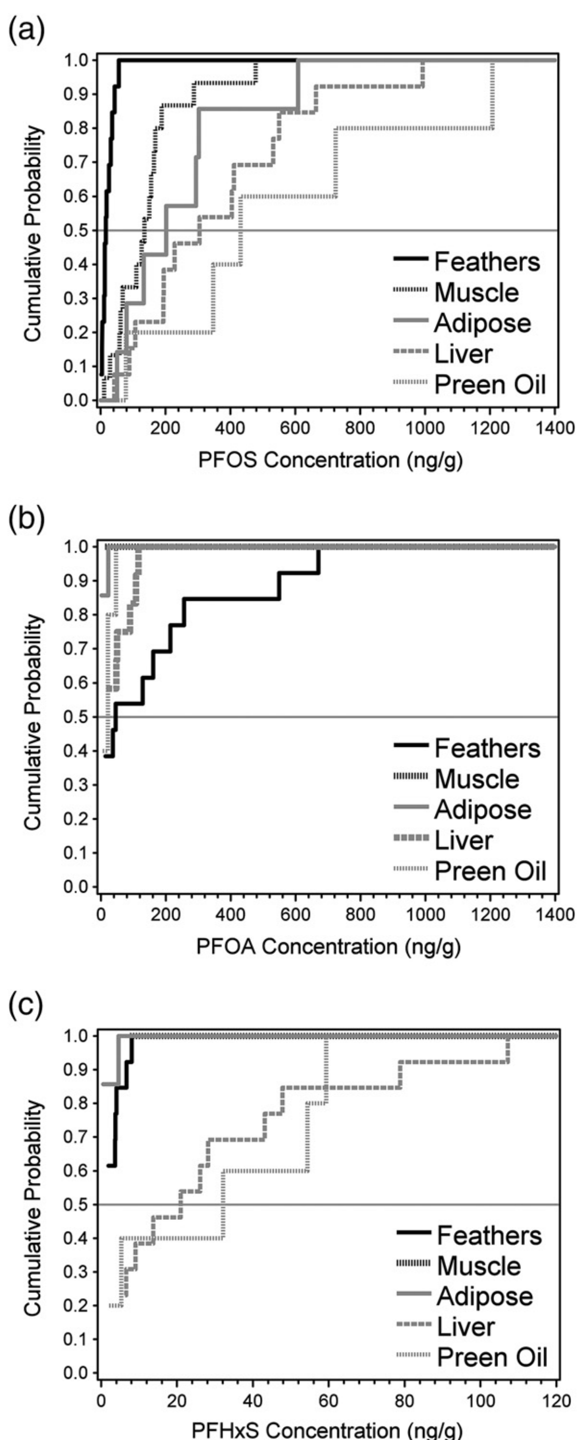

Fig. 1. Cumulative probability plot of PFOS (a), PFOA (b) and PFHxS (c) concentration distributions in soft tissues and tail feathers of the barn owl (*Tyto alba*). The reference line at probability 0.5 crosses each curve at its median concentration. All concentrations are in ng/g wet weight. In Figure 1(b), all PFOA concentrations in muscle were below the LOD of 13.3 ng/g.

We used 13 reported concentrations that were between the LOD and the limit of quantification (LOQ) as exact values because they were all small (all estimated values below LOQ were less than 13.7 ng/g, except one (37.1 ng/g)) and any error would have had little impact on the distribution function estimates. The "lifereg" procedure in SAS 9.2 for Windows (SAS Institute Inc., Cary, NC, USA) was used to estimate the cumulative distribution function of concentration levels for each compound (see Gillespie et al. 2010 for details). Since the sample was composed of 10 females, 3 males and two birds of unknown sex, gender differences could not be investigated.

For each bird, PFAS profiles per tissue were calculated using mean proportions of individual PFAS compounds to the total concentration of sum PFASs and then averaging the percentages for all birds. For this calculation, values below LOD were replaced by the LOD itself or an estimated value between LOD and LOQ when available.

Correlation coefficients were estimated to assess the strength of the linear relationship between congener levels in feathers and levels in soft tissues, and also between the latter ones; maximum likelihood estimates of the correlation coefficients were based on the bivariate normal distribution (after logarithmic transformation), and assuming that the raw data follow log-normal distributions. For complete data sets (PFOS concentrations in soft tissues), estimates were obtained using the "corr" procedure in SAS. For left-censored data, a maximum likelihood estimation (MLE) program was implemented using the R statistical software (R Development Core Team, 2011). More details on this MLE program are provided in SI.

3. Results

3.1. PFAS levels and cumulative distribution functions

For three PFAS compounds (PFOS, PFOA and PFHxS) we had sufficient data above LOD to estimate the concentration distributions using the reverse Kaplan–Meier calculation (Fig. 1). Overall, the magnitude of the PFOS values was highest, followed by PFOA and then PFHxS.

PFOS could be detected in all soft tissues and feathers analyzed in this study (Table 1). Fig. 1(a) presents the distribution for PFOS by tissue type, and shows that the highest PFOS concentrations were in preen oil, followed by liver, adipose tissue, muscle, and tail feathers. The median values for each tissue type can be read on the graph at the points where the horizontal line at a cumulative probability of 0.5 crosses each curve. For PFOS, these median values were 431.2 ng/g ww in preen oil, 266.1 ng/g ww in liver, 202.7 ng/g ww in adipose tissue, 134.6 ng/g ww in muscle, and 16.9 ng/g ww in feathers. When looking at patterns in individual owls, the ordering of the tissues was almost always consistent, even though the sample sizes were fairly small:

the PFOS concentration in muscle was higher than in feathers in 14/14 owls, adipose tissue was higher than muscle in 7/7 owls, liver was higher than adipose in 5/6 owls, while preen oil was higher than liver in only 2/4 owls. Among all PFOS measurements (53 in total for soft tissues and feathers), only 1 was below LOD in feathers.

For PFOA, a different pattern is seen in Fig. 1(b). Tail feathers had by far the highest concentrations, followed in order by liver, preen oil, adipose tissue and muscle. Apart from the tail feathers, the order of tissue concentrations is similar to PFOS, considering that liver and preen oil are quite close. Among all the PFOA measurements, 37/55 were below LOD, including all 16 values for muscle, all but one for adipose tissue, and 8/13 for liver. The percentage below LOD for each tissue type can be read from Fig. 1(b) at the point where the distribution function ends on the left side of the plot. For example, for preen oil 40% (2/5) were below LOD and for muscle 100% of the samples were below LOD. The median values were 85.9 ng/g ww in tail feathers, <16.2 ng/g ww in liver, 21.5 ng/g ww in preen oil, <2.3 ng/g ww in adipose tissue, and <13.3 ng/g ww in muscle. Considering pair-wise comparisons of tissues in the same owl excluding indeterminate cases (e.g., where both values were below LOD), the concentration in adipose tissue was higher than in muscle in 1/1 owl, preen oil was higher than adipose tissue in 2/2, liver was higher than preen oil in 1/4, and feathers were higher than liver in 8/10 owls.

PFHxS values were highest in liver and preen oil (Fig. 1(c)), comparable to PFOA levels in the same tissues. Like PFOS, tail feather levels of PFHxS were quite low, with levels comparable to adipose tissue and muscle. Among all the PFHxS measurements, 35/55 were below LOD, including all 16 values for muscle, all but one for adipose tissue, and 8/14 for feathers. The median values were <1.9 ng/g ww in feathers, 17.4 ng/g ww in liver, 32.1 ng/g ww in preen oil, <0.6 ng/g ww in adipose tissue and <7.6 ng/g ww in muscle. Considering pair-wise comparisons of tissues in the same owl excluding indeterminate cases, the concentration in tail feathers was higher than in adipose tissue in 3/4 owls, liver was higher than tail feathers in 9/9 owls, and preen oil was higher than liver in 2/4 owls. Muscle could not be compared to either tail feathers or adipose tissue due to its high LOD (Table SI-1 in SI).

Sporadically, other PFAS compounds were detected as well, mostly in liver samples: PFHxA was found above LOD in one feather sample (128.8 ng/g ww), PFNA in two muscle samples (111 and 158 ng/g ww), PFDcA in five liver samples (7.8–47.8 ng/g ww), PFUnA in one liver sample (35.5 ng/g ww), PFDoA in two liver samples (32.7 and 53.4 ng/g ww), PFTriA in one liver sample (44.4 ng/g ww) and in one muscle sample (5.7 ng/g ww). PFOSA, a precursor of PFOS, was not detected in any of the samples (LOD ranged from 0.5 ng/g ww in adipose tissue to 45.2 ng/g ww in liver) (Table SI-1).

Table 1
Concentrations (ng/g ww; median, range, and fraction below the limit of detection (LOD)) of PFASs in tail feathers and soft tissues from Belgian barn owls. When the median value falls below the LOD, it is given as <LOD-value.

	Tail feathers (n = 13)	Muscle (n = 15)	Liver (n = 13)[a]	Preen oil (n = 5)[b]	Adipose tissue (n = 7)
PFOS	15.8	135.2	304.5	431.2	202.7
	(<2.2–56.6)	(11.1–477)	(42–992)	(78–1208)	(51–609)
	1/13<LOD	0/15<LOD	0/13<LOD	0/5<LOD	0/7<LOD
PFOA	37.1	<13.3	<16.2	21.5	<2.3
	(<14.1–670)	(–)	(<16.2–116)	(<5.2–46.6)	(<2.3–22.6)
	5/13<LOD	15/15<LOD	7/12<LOD	2/5<LOD	6/7<LOD
PFHxS	<1.9	<7.6	21.0	32.1	<0.6
	(<1.9–8.1)	(–)	(<6.3–107)	(<2.1–59.3)	(<0.6–4.6)
	8/13<LOD	15/15<LOD	3/13<LOD	1/5<LOD	6/7<LOD

[a] Except n = 12 for PFOA.
[b] Results are only available for 5 preen oil samples due to low amount of sample.

3.2. PFAS profile

The profile in soft tissues was clearly dominated by PFOS: the contribution of PFOS to the total sum of PFASs was 79% in liver and 89% in preen oil, while PFOS was the only compound detected above LOD in adipose tissue and muscle. On the other hand, PFOA was the main compound in feathers, contributing 76% to the sum PFASs, while PFOS represented the remaining 23%.

3.3. Correlations

Table 2 presents correlations for PFOS between feathers and soft tissues. PFOS levels in tail feathers and liver were highly correlated ($r = 0.78$; 95% Confidence Interval (CI): 0.38, 0.94; $p < 0.01$), but this was not the case for PFOS levels between tail feathers and muscle ($r = 0.19$; 95% CI: −0.40, 0.67; $p = 0.53$) or between muscle and liver ($r = 0.46$; 95% CI: −0.12, 0.81; $p = 0.10$).

PFOA levels in tail feathers and liver were not significantly correlated ($r = -0.11$, 95% CI: −0.70, 0.63; $p = 0.78$). For PFHxS, correlations between tail feathers and liver showed a strong relationship ($r = 0.86$, 95% CI: 0.47, 0.97; $p < 0.01$). Only one other correlation could be estimated for PFHxS, between liver and preen oil, which was found to be not significant ($r = 0.16$, 95% CI: −0.71, 0.83, $p = 0.77$).

Because paired data above LOD for both feathers and preen oil were only available for three individuals, correlations between PFAS levels in tail feathers and preen oil could not be calculated.

Strong correlations were also found between some PFAS compounds within the same tissue (Table 3). For tail feathers, PFOS and PFOA were significantly correlated ($r = 0.67$, $p = 0.01$), with a similarly high but non-significant correlation between PFOA and PFHxS ($r = 0.52$, $p = 0.13$). PFOS and PFHxS were highly and significantly correlated in both liver ($r = 0.77$, $p < 0.01$) and preen oil ($r = 0.86$, $p = 0.01$).

4. Discussion

As described above, the PFOS, PFOA and PFHxS concentration distributions could effectively be estimated using the reverse Kaplan–Meier calculation. We found that PFOS could be quantified in all tissues, including tail feathers, and PFOS was the predominating compound in the soft tissues, while PFOA was the main compound in tail feathers. Further, PFOS levels in feathers and liver were highly correlated, while PFOA levels in feathers and liver were not significantly correlated. First, we discuss the use of the Kaplan–Meier method to handle data below LOD. Next, the results on levels, profiles and correlations will be discussed separately in the following subsections. Finally we discuss the biomagnification and potential hazards of PFOS in the barn owl from the Antwerp region.

4.1. Use of the reverse Kaplan–Meier estimator to handle non-detects

The most common method to deal with data below the LOD is to substitute them with a fraction of the LOD or the LOD itself. Yet, substitution methods can create bias and may even obscure patterns in the data, especially as the fraction of substituted values increases (Gillespie et al., 2010; Helsel, 2006; Leith et al., 2010). Substitution methods are still common practice because of their easy application. However, substitution may become increasingly problematic as monitored contaminants become less prevalent and a larger proportion of data will have values below the LOD (Leith et al., 2010). Further, recent developments in analytical techniques for PFAS have led to dramatic decreases of LOQs and LODs. For example, where earlier LODs of 1 ng/g PFOS were quite common, now 5 to 10 pg/g is achievable when applying state-of-the-art instrumentation. When the percentage below LOD is high, substitution methods can make it look like exposures are decreasing over time when actually the change is an artifact of a decrease of LODs. Therefore the use of methods developed for censored data (below or above a certain threshold) is strongly

Table 2
Correlations between tail feathers and soft tissues for PFOS (correlation coefficient, 95% confidence interval, p-value and sample size). Significant correlations are indicated in bold.

	Muscle	Adipose tissue	Liver	Preen Oil
Tail feathers	r = 0.19 (−0.40, 0.67) p = 0.53 n = 13	r = 0.59 (−0.29, 0.93) p = 0.15 n = 7	**r = 0.78** (0.38, 0.94) p<0.01 n = 12	r = −0.17 (−0.97, 0.95) p = 0.83 n = 4
Muscle	–	**r = 0.85** (0.28, 0.98) p<0.01 n = 7	r = 0.46 (−0.12, 0.81) p = 0.10 n = 13	r = 0.83 (−0.18, 0.99) p = 0.06 n = 5
Adipose Tissue		–	r = 0.63 (−0.37, 0.95) p = 0.16 n = 6	r = 0.99 – – n = 3
Liver			–	r = 0.24 (−0.94, 0.98) p = 0.77 n = 4
Preen Oil				–

recommended. Although such methods have been available since the early 1970's, they have hardly ever been used in practice for environmental sciences.

Throughout this study we have applied statistical methods for left-censored data (i.e. uncertain data at the left end of the distribution, such as non-detects) to cope with the large fraction of values below LOD for PFOA and PFHxS. If substitution methods had been applied for the present data, compounds with more than 50% of values below LOD would have been excluded and only levels of PFOS, PFOA in feathers and PFHxS in liver could have been discussed. This would have been a great loss of information in comparison to the results presented in this paper. Furthermore, additional bias could have arisen, as the value used for substitution can influence the PFAS distributions and subsequent statistical outcomes, especially at low level contamination (Leith et al., 2010).

As can be seen in Fig. 1, the cumulative concentration distributions resulting from the reverse Kaplan–Meier method give more information than substituting values below LOD and they do not obscure the uncertainty inherent in values below LOD. Furthermore, subsequent statistical analysis can be performed on the left-censored datasets using procedures in standard statistical software such as SAS, JMP or R (for more details see Gillespie et al. (2010) and SI). Therefore, the practical implementation of these methods is available and we strongly encourage their common use in future studies with low level pollutants and/or with high rates of censorship.

Table 3
Correlations (correlation coefficient, 95% confidence interval, p-value and sample size) between different PFASs in the same tissue or feathers. Significant correlations are indicated in bold.

	PFOS vs. PFOA	PFOS vs. PFHxS	PFOA vs. PFHxS
Tail Feathers	*r* = **0.67** (0.16, 0.90) *p* = 0.01 *n* = 13	–	*r* = 0.52 (−0.17, 0.89) *p* = 0.13 *n* = 13
Muscle	–	–	–
Adipose tissue	–	–	–
Liver	*r* = −0.14 (−0.63, 0.44) *p* = 0.65 *n* = 12	*r* = **0.77**, (0.41, 0.92) *p*<0.01 *n* = 13	*r* = 0.01 (−0.56, 0.59) *p* = 0.97 *n* = 12
Preen oil	*r* = 0.16 (−0.65, 0.80) *p* = 0.72 *n* = 5	*r* = **0.86** (0.34, 0.98) *p* = 0.01 *n* = 5	*r* = −0.03 (−0.76, 0.73) *p* = 0.95 *n* = 5

4.2. PFAS levels

In other bird studies from industrialized areas, PFOS was found generally the dominant PFAS (Ahrens, 2011). The concentrations in this study are comparable to the PFOS levels found in herring gull (*Larus argentatus*) and Eurasian sparrowhawk (*Accipiter nisus*) from Belgium (Meyer et al., 2009), but they are generally higher than reported in other studies with birds feeding on a high trophic level in Northern or Antarctic ecosystems (Ahrens et al., 2011; Bustnes et al., 2008; Holmström et al., 2010; Shlosberg et al., 2011). Surprisingly, the highest levels of PFOS were found in preen oil (up to 1208 ng/g ww) which is a very lipid rich substance and known to contain high levels of lipophilic substances such as PCBs (Jaspers et al., 2008; Yamashita et al., 2007). Although PFOS is not a lipophilic substance, the specific composition of preen oil, usually monoester waxes made of saturated fatty acids with different degrees of methyl branching and long-chain monohydroxy fatty alcohols (Campagna et al., 2012), may explain its high levels of PFOS. Yet, other studies have shown the accumulation of PFOS in lipid rich tissues as well. For example, PFOS was found in elevated concentrations in seal blubber and herring gull adipose tissue compared to other tissues (Gebbink and Letcher, 2012; Van de Vijver et al., 2005).

PFOA was measured at high levels in feathers, but not in soft tissues. Although the levels in feathers only originate from exposure during feather growth, while the levels in soft tissues are also influenced by recent exposure, the high values in feathers suggest an external source of contamination. PFOA may be present on the feather surface and was not washed off by our extensive washing procedures (using distilled water and hexane; see Materials and methods section). Although hexane has been shown to remove external contamination with persistent organic pollutants (POPs) originating from preening the feathers (Jaspers et al., 2008), PFOA levels in preen oil are low and therefore probably not a source of external contamination (but the sample size for preen oil in is limited (n = 5)). External contamination with PFOA is probably originating from the air (wet or dry depositions), but study on the sources of PFCAs to either air or precipitation is an area where more research is needed (Cousins et al., 2011). The use of methanol as a washing solvent may be able to remove PFOA from the feathers. However, washing with methanol will also result in the removal of all other PFASs and may not be the suitable method to distinguish between external and internal contamination.

Our high PFOA levels in feathers are in contrast with the study of Meyer et al. (2009), where the authors could not detect PFOA in the feathers of birds from Belgium (LODs not reported). Levels of PFOA in eggs of tawny owls (*Strix aluco*) from Norway and peregrine falcons (*Falco peregrinus*) from Sweden were mostly not found above the LOD (0.01–0.05 ng/g ww; Ahrens et al., 2011). Other studies on seabirds have also indicated very low levels of PFOA (Butt et al., 2010). The detection of PFOA in the current study can indicate a source near exposure, as the fluoro-chemical plant (3 M) near Antwerp is still active. Furthermore, there are studies showing that fluoropolymer plants are a source of PFCAs to the atmosphere (Cousins et al., 2011). Although PFOS has been listed in Annex B of the Stockholm Convention, there are numerous exceptions for the use of PFOS (and PFOA) in continued industrial applications. Therefore, it seems that concentrations in feathers are reflecting the environmental contamination rather than their ingestion through diet, which can also explain the discrepancy with the low levels found in the tissues (Table 1). However, the preferential transfer of PFOA from the blood to the feathers cannot be excluded, as levels in blood could not be determined in this study.

PFHxS was measured above LOD in 77% of the liver (range: 6.3–107 ng/g) and 80% of the preen oil (<2.1–59.3 ng/g) samples, but not in the other soft tissues (LOD ranged from 0.6 in adipose to 7.6 ng/g in muscle). This was comparable with 50–80% detection in liver samples previously analyzed from bird species found dead in Belgium in 2004 (Meyer et al., 2009). PFHxS could be detected in tail feathers from five barn owls of the current study (2.1–8.1 ng/g). This is in the lower range (38%) compared with the study of Meyer et al. (2009), who found 20–70% above LOD in feathers of birds of prey from Belgium (although LODs, not reported in that paper, may differ from the current study). However, the predatory bird species analyzed in that study were either aquatic (herring gull and gray heron [*Ardea cinerea*]) or feeding on passerine birds (i.e. the sparrowhawk). This is substantially different from the diet of the barn owl, which mainly consists of small mammals. The contribution of small birds in the diet has been shown very important for the accumulation of POPs in predatory birds (Jaspers et al., 2006b). Perhaps a similar story may be true for certain PFASs or the accumulation of PFHxS may be different in the terrestrial environment compared to the aquatic environment (see also Holmström et al., 2010). Reports on PFHxS in literature are scarce. PFHxS has been reported in eggs (<0.27–1.23 ng/g ww) and plasma (1.12 ± 0.15 ng/g ww) from glaucous gull (*Larus hyperboreus*) in the Norwegian Arctic and in liver samples from glaucous gull (0.26 ± 0.06 ng/g ww) and black guillemot (*Uria aalge*; 0.17 ± 0.02 ng/g ww) from the Barents Sea (Butt et al., 2010). In the study of Ahrens et al. (2011), the mean concentration of PFHxS in tawny owl eggs from Norway was only 0.05 ng/g. PFHxS could also be analyzed in peregrine falcon eggs from Sweden at levels ranging from 0.07 to 2.7 ng/g ww. In contrast with the current study, those studies were carried out in remote areas away from any PFAS production plant.

PFOSA could not be detected in this study. This is in great contrast with findings in ten tissues (blood, brain, fatty tissue, gall bladder, heart, kidney, liver, lung, muscle and spleen) from red-throated divers (*Gavia stellata*) from the German Baltic Sea, where PFOSA accounted for ~20% of the total PFAS amount in most tissues (Rubarth et al., 2011), but is in line with the low detection frequency in peregrine falcon eggs (Holmström et al., 2010) and tawny owl eggs (Ahrens et al., 2011). This discrepancy may be due to a different availability or accumulation of PFOSA in the marine and terrestrial environment or a high contamination with PFOSA in the Baltic Sea. Herzke et al. (2009) found PFOSA at minor concentrations in samples from the European shag (*Phalacrocorax aristotelis*) and common eider (*Somateria mollissima*). PFOSA has also been quantified in fish (Martin et al., 2004; Yoo et al., 2008) with the ratio of PFOS to PFOSA lower than in the birds that prey on them (Gebbink et al., 2009). Therefore it was also suggested that predatory birds may have the ability to transform or eliminate PFOSA (Gebbink et al., 2009; Holmström and Berger, 2008).

4.3. PFAS profile

Our results showed that the profile in soft tissues was dominated by PFOS, while PFOA was the main compound in feathers. This clearly indicates another potential route of exposure for PFASs in feathers. PFOA was only a minor compound in preen oil and is not preferentially bound to dust particles (Kato et al., 2009), indicating external contamination with PFOA directly from the air is the most likely pathway. Preen oil consists to a large degree of waxes based on C12–C23 alkane-1,2-diols esterified with C10–C20 acids, hydroxyl-fatty acids and fatty alcohols, varying in composition depending on species and season (Campagna et al., 2012). It is currently unknown to which degree PFASs can be excreted via the preen gland, but the results of the present study revealed high levels for PFOS and low levels for PFOA and PFHxS in preen oil from the barn owl.

Although many studies on PFASs have been performed in aquatic food webs, data on PFASs in terrestrial biota are scarce with few studies having reported PFASs in terrestrial birds. In the study of Meyer et al. (2009), PFAS levels in liver and feathers of the Eurasian sparrowhawk, magpie and collared dove were reported. The authors found PFOS as the dominating PFAS compound in both matrices for all investigated bird species. Other studies reporting PFAS concentrations in terrestrial birds have mostly focused on eggs. Holmström et al. (2010) found PFOS as the predominant compound in Swedish peregrine falcon eggs, followed by PFTRiA and PFUnA. This was also the pattern found in eggs from tawny owls (Ahrens et al., 2011): PFOS presented 83% of the total PFAS concentrations, followed by PFTriA (4.9%) and PFUnA (3.1%). PFOS was also found as the predominant compound in egg yolk of the parrot bill (*Paradoxornis webbiana*) from Lake Shiwa, Korea, but contributed less than 50% to the total PFASs, with PFUnA accounting for 25% and PFOA 1% (Yoo et al., 2008).

In comparison with aquatic birds, Herzke et al. (2009) reported PFOS as the main compound in egg, plasma and liver samples from the European shag and common eider. This was also the case in herring gull eggs from the Great Lakes (Gebbink et al., 2009), red-throated divers from the German Baltic Sea (Rubarth et al., 2011), waterbird eggs from South China (Wang et al., 2008) and tissue samples from grey heron and herring gull from Belgium (Meyer et al., 2009). Therefore it seems that PFOS is generally the predominant compound in birds, both in terrestrial and aquatic environments, which is comparable to what is generally found for other wildlife (Butt et al., 2010; Houde et al., 2006).

4.4. Correlations

PFOS levels in tail feathers and liver were highly correlated, but this was not the case with muscle. This may be due to the chemical properties of PFASs which are mainly bound to proteins in the blood, thus reducing the suitability of muscle tissue.

The result that PFOA levels in feathers and liver were not significantly correlated again suggests that PFOA may be present on the external surface of the feathers and was not washed off by our extensive washing procedures (using distilled water and hexane). Yet, PFOS and PFOA were significantly correlated in feathers, which seems in contradiction with different exposure pathways for PFOS and PFOA in feathers (internal versus external contamination). When we look into more detail, two owls showed very high levels of PFOA (Fig. 2), probably due to point sources. A third individual showed relatively high levels of PFOA, but low levels for PFOS. Exclusion of those three owls, resulted in a higher and more significant correlation ($r = 0.92$, $p = 0.0002$) between PFOS and PFOA in feathers. This suggests mostly similar exposure routes in the absence of local point sources, while the sources of PFOS are likely different from those of PFOA for the three individuals showing high levels of PFOA. Clearly, external contamination with PFOA on the feathers should be further investigated in the future (e.g. as has been done for heavy metals; Dauwe et al., 2003; Jaspers et al., 2004). It may be possible that feathers act as a passive air sampler when in contact with high PFOA air concentrations in the vicinity of point sources. This would be very interesting for biomonitoring purposes as it has been shown that concentrations in the atmosphere show fast responses to changing emissions (Cousins et al., 2011).

4.5. Biomagnification and risk evaluation in the barn owl

The diet of the barn owl consists mostly of small mammals, i.e. mice, small voles and shrews (Snow and Perrins, 1998). To study biomagnification in the barn owl, we have used PFOS data that were obtained for the wood mice (*Apodemus sylvaticus*) in the region of Antwerp in 2006 (D'Hollander et al., in preparation). We only used the data from Galgenweel (mean PFOS 153 ± 37 ng/g ww) as this was the control site in that study, reflecting background contamination with PFASs in the region of Antwerp. The biomagnification factor (BMF) for PFOS was calculated according to $BMF_{PFOS} = [PFOS_{barn\ owl}]/[PFOS_{wood\ mice}]$.

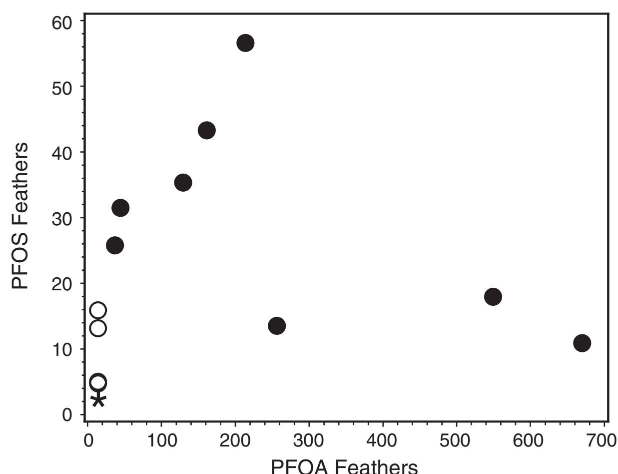

Fig. 2. Scatterplot of concentrations in Feathers for PFOS vs. PFOA. Black dots represent exact values. Open circles represent values below the limit of detection (LOD) in PFOA, plotted at the LODs. The asterisk represents values below the LOD in both PFOS and PFOA, plotted at the LODs.

The BMF that was obtained for barn owl liver relative to wood mice liver measures 1.99. No information is available on temporal trends of PFOS in biota from Belgium. Therefore the presented BMF should be employed with caution as we cannot rule out a decrease of PFOS contamination in wood mice and barn owl from 2006 to 2008/2009.

When we compare the levels in the barn owl with the toxic reference value (TRV) that was estimated for avian top predators for PFOS in liver (600 ng/g ww; Newsted et al., 2005), two barn owls showed liver concentrations greater than the TRV. This means that 15% of the birds that were included in this study may have been at risk from PFOS exposure alone. The predicted no effect concentration was estimated at 350 ng/g ww for liver tissue (Newsted et al., 2005), which was exceeded by six birds out of 13. Although only road-kill victims were included in this study, birds with high levels of PFOS are probably not more likely to end up as road kill. On the contrary, Bujoczek et al. (2011) recently reported that road-kill results in the random elimination of healthy individuals. Therefore, current levels of PFOS in the barn owl from the Antwerp region may present a health risk, especially in combination with other pollutants (e.g. OHCs). As high concentrations in other studies have been reported as well (Dauwe et al., 2007; Hoff et al., 2005; Meyer et al., 2009), continued monitoring of the concentrations in biota from the Antwerp region is warranted.

5. Conclusions

PFOS could be quantified in all tissues, including tail feathers. While PFOS was the predominating compound in the soft tissues, PFOA was the main compound in feathers. Overall, the results indicate that PFOS levels in feathers and liver are highly correlated, while PFOA levels in feathers may be originating from external contamination by wet or dry deposition caused by source near exposure. It may be possible that feathers act as a passive air sampler for PFOA in the vicinity of point sources, which should now be investigated more in depth. The current levels of PFOS in the barn owl from the Antwerp region may contribute to negative health effects, which underlines the importance to continue the follow up of PFASs in biota from Antwerp (Belgium).

Acknowledgments

The Kerkuilwerkgroep vzw is greatly acknowledged for the collection and storage of the barn owl carcasses and their help with the collection of the samples. We also thank Kerby Shedden, Ph.D., Department of Statistics, University of Michigan (USA), who wrote the R script to estimate the correlation coefficient for a bivariate normal distribution in the presence of left-censored data. We want to thank Sandra Huber, Ph.D., for developing the LC/MS methods applied in this study. Leslie Niziol, M.S., of the University of Michigan (USA) is greatly acknowledged for her help with the high-resolution graphs. Veerle Jaspers is a post-doctoral researcher funded by the Funds of Scientific Research Flanders and received a NFR YGGDRASIL mobility grant for a research stay at NILU in Tromsø (Norway). In addition, we thank the University of Antwerp for funding Igor Eulaers and Marcel Eens.

Appendix A. Supplementary data

A description of the correlation program used for left censored data, LODs for the different tissues (Table SI-1), the study area with sampling sites (Figure SI-1) and scatterplots for the different correlations (Figures SI-2 – SI-5) are uploaded as Supporting Information. Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.envint.2012.11.002.

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