Science of the Total Environment 478 (2014) 48–57

Contents lists available at ScienceDirect

Science of the Total Environment

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

Brominated and phosphorus flame retardants in White-tailed Eagle *Haliaeetus albicilla* nestlings: Bioaccumulation and associations with dietary proxies ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$)

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HIGHLIGHTS

- Non-PBDE BFRs and PFRs were highly detected in feathers, but poorly in plasma.
- PFR levels in feathers were up to 100-fold those of BFRs and selected OCs.
- $\delta^{15}N$ and $\delta^{13}C$ explained intra-specific variation in OC, PBDE and PFR exposure.
- $\delta^{15}N$ and $\delta^{34}S$ were respectively enriched and depleted close to an urbanised centre.

ARTICLE INFO

Article history:
Received 28 November 2013
Received in revised form 15 January 2014
Accepted 15 January 2014
Available online xxxx

Keywords:
BFR
Feather
Haliaeetus albicilla
Stable isotope
PFR

ABSTRACT

Very little is known on the exposure of high trophic level species to current-use brominated (BFRs) and phosphorus flame retardants (PFRs), although observations on their persistence, bioaccumulation potential, and toxicity have been made. We investigated the accumulation of BFRs and PFRs, and their associations with dietary proxies ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$), in plasma and feathers of White-tailed Eagle *Haliaeetus albicilla* nestlings from Trøndelag, Norway. In addition to accumulation of a wide range of polybrominated diphenyl ether (PBDE) congeners in both plasma and feathers, all non-PBDE BFRs and PFRs could be measured in feathers, while in plasma only two of six PFRs, i.e. tris-(2-chloroisopropyl) phosphate (TCIPP) and tris-(2,3-dichloropropyl) phosphate (TDCPP) were detected. PFR concentrations in feathers (0.95–3000 ng g^{-1}) were much higher than selected organochlorines (OCs), such as polychlorinated biphenyl 153 (CB 153; 2.3–15 ng g^{-1}) and dichlorodiphenyldichloroethylene (*p,p′*-DDE; 2.3–21 ng g^{-1}), PBDEs (0.03–2.3 ng g^{-1}) and non-PBDE BFRs (0.03–1.5 ng g^{-1}). Non-significant associations of PFR concentrations in feathers with those in plasma ($P \geq 0.74$), and their similarity to reported atmospheric PFR concentrations, may suggest atmospheric PFR deposition on feathers. Most OCs and PBDEs, as well as tris(chloroethyl) phosphate (TCEP), tris(phenyl) phosphate (TPHP) and tri-(2-butoxyethyl) phosphate (TBOEP) were associated to $\delta^{15}N$ and/or $\delta^{13}C$ (all $P \leq 0.02$). Besides $\delta^{15}N$ enrichment, $\delta^{34}S$ was depleted in nestlings from fjords, inherently close to an urbanised centre. As such, both may have been a spatial proxy for anthropogenic disturbance, possible confounding their use as dietary proxy.

1. Introduction

Since the 1960s, flame retardants (FRs) have been incorporated in consumption products in order to inhibit or minimise their inflammation. From an estimated total of 175 produced FRs (Alaee et al., 2003), polybrominated diphenyl ethers (PBDEs) have been studied most intensively. PBDEs were identified to be persistent, bioaccumulative and toxic (Chen and Hale, 2010; de Wit, 2002; Wiseman et al., 2011), and their use has therefore been legally regulated or voluntarily reduced on largely a worldwide scale (Directive EEC, 2003; European Court of Justice, 2008; Renner, 2004; UNEP Stockholm Convention, 2013). In order to comply with international fire regulations (Betts, 2008), Firemaster 550, containing the brominated flame retardants (BFRs) 2-ethylhexyl 2,3,4,5-tetrabromobenzoate (EH-TBB) and bis(2-

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http://dx.doi.org/10.1016/j.scitotenv.2014.01.051

ethylhexyl) tetrabromophthalate (BEH-TEBP), was introduced as alternative for the Penta-BDE mixture. At the same time, 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE) was already produced since the mid-1970s and now also replaces the Octa-BDE mixture (Covaci et al., 2011; de Wit et al., 2010). In addition, certain phosphorus flame retardants (PFRs), e.g. tris(phenyl) phosphate (TPHP), tris-(2-chloroisopropyl) phosphate (TCIPP) and tris-(2,3-dichloropropyl)-phosphate (TDCPP), existing already in complementary use, serve nowadays also as BFR alternatives (van der Veen and de Boer, 2012). Recent studies have suggested bioaccumulative properties for the above listed non-PBDE BFRs (Tomy et al., 2007; Wu et al., 2011) and PFRs (Evenset et al., 2009; Leonards et al., 2011), as well as their persistence and long-range transport (de Wit et al., 2010), and toxic properties (van der Veen and de Boer, 2012). Nonetheless, many research gaps regarding their environmental distribution and ecotoxicity remain still to be filled (Covaci et al., 2011; van der Veen and de Boer, 2012), which is of critical importance to further assess possible environmental risks.

Ever since the observed detrimental effects of certain organochlorines (OCs), among which especially dichlorodiphenyltrichloroethane (DDT) and its metabolites (Ratcliffe, 1970), birds of prey have been successful early warning systems for a variety of environmental pollutants, including flame retardants (Chen and Hale, 2010; Eulaers et al., 2014). Among other high trophic level species, the White-tailed Eagle *Haliaeetus albicilla* has been a promising model species to study spatio-temporal variation in pollutant exposure and associated health effects (Gjershaug et al., 2008; Helander et al., 2002, 2008; Korsman et al., 2012; Nordlöf et al., 2010, 2012; Nygård and Polder, 2012). The detection of a wide range of organohalogenated compounds (OHCs) in nestling White-tailed Eagles (Bustnes et al., 2013; Eulaers et al., 2011a,b) and their adverse impact on nestling health (Sonne et al., 2010, 2012) underline the toxicological relevance of sampling at the nestling stage. In addition, sampling nestlings rather than adults offers methodological advantages, such as small-scale geographical accuracy (Elliott et al., 2009; Eulaers et al., 2013; Olsson et al., 2000) and the minimisation of confounding by age-related metabolism and life-time bioaccumulation, reproductive state and migratory activity (Eulaers et al., 2011a,b; Ramos et al., 2013). Nonetheless, no study thus far has been dedicated to assess nestling exposure to current-use non-PBDE BFRs and PFRs. In fact, avian exposure to these compounds is very poorly investigated in general, with only a few studies presenting non-PBDE BFR or PFR concentrations (Table 1).

While nestling exposure is predominantly studied in blood (Bourgeon et al., 2013; Bustnes et al., 2013; Elliott et al., 2009; Eulaers et al., 2011a,b; Olsson et al., 2000), the use of nestling body feathers is a recent and promising alternative strategy that implies minimal sampling invasiveness while providing an integrated picture of dietary input (Hobson and Clark, 1992) and pollutant exposure (Eulaers et al., 2011b, 2013) over the larger part of the nestling stage. Body feathers are connected to the blood circulation upon feather growth, are metabolically inactive (Jardine et al., 2006), and contain concentrations that are highly associated with internal body burdens (Eulaers et al., 2011b). In addition, since exposure of higher trophic level species is expected to stem primarily from diet ingestion (Ruus et al., 2002), the analysis for stable isotope (SI) ratios of carbon (δ^{13}C: ^{13}C/^{12}C) and nitrogen (δ^{15}N: ^{15}N/^{14}N) has been used successfully. The ratio of heavier ^{15}N to lighter ^{14}N SIs increases systematically throughout the food chain (Jardine et al., 2006; Kelly, 2000) due to the preferential deamination of light amine groups during de- and transamination processes (Macko et al., 1986). In addition, δ^{13}C indicates the origin of a particular food chain because different photosynthesis mechanisms, e.g. C3 versus C4, result in typical δ^{13}C signatures that are largely not altered throughout the food chain (Jardine et al., 2006; Kelly, 2000). As such, δ^{15}N and δ^{13}C have become established proxies to discriminate between trophic levels and food chain origins, respectively, both in intra-specific (Bourgeon et al., 2013; Bustnes et al., 2013; Elliott et al., 2009; Eulaers et al., 2011a,b, 2013; Sørmo et al., 2011) as well as inter-specific investigations (Eulaers et al., 2011b, 2013; Guzzo et al., 2014) of exposure in nestling avian predators. Almost no attention has been paid to the suitability of sulphur SIs (δ^{34}S: ^{34}S/^{32}S) in exposure assessment studies although earlier ecological studies have successfully used δ^{34}S to discriminate between food chain components in nestlings of both marine (Moreno et al., 2010; Ramos et al., 2009) and terrestrial avian predators (Resano et al., 2011; Resano-Mayor et al., 2013). Moreover, recently, δ^{34}S was successfully used to explain trophodynamics of mercury and selenium in nestling Yellow-legged Gulls (Ramos et al., 2013) and as a proxy for the degree of urbanisation to explain OC and PBDE exposure (Morrissey et al., 2013a).

The first objective of the present study was to investigate if White-tailed Eagle nestling body feathers and plasma could be used to quantify exposure to legacy and current-use FRs, and if quantification is influenced by matrix-specific differences in metabolic activity and exposure time-frame. Under this aim, we also investigated the possibility to expand the established range of OHCs, for which feathers predict internal body burdens, to current-use FRs. Secondly, we investigated how dietary proxies (δ^{13}C, δ^{15}N and δ^{34}S) can be most informatively employed to investigate intra-specific variation in pollutant exposure. Finally, throughout the objectives, results for the FRs were benchmarked against two recalcitrant and two more volatile OCs, i.e. polychlorinated biphenyl (CB) 153 and dichlorodiphenyldichloroethylene (*p,p′*-DDE), and CB 28 and hexachlorobenzene (HCB), respectively.

2. Material and methods

2.1. Sampling

As part of a reintroduction programme of White-tailed Eagle into Ireland (Nygård et al., 2010), 21 nestlings from separate nests were captured shortly before their anticipated fledging dates during the breeding season of 2011 in Trøndelag, Norway (Fig. 1). Both sex (8 male and 13 female) and age (6 to 9 weeks of age) were estimated based upon morphometric measurements. The sampled nests were located in three distinct habitat types, i.e. fjord ($n = 3$), island/sound ($n = 8$) and skerry/open coast ($n = 10$) (Fig. 1). Prior to their transport to Ireland, all nestlings were sampled for body feathers and blood. Body feathers, gently pulled from the dorsal region, were pooled per individual and stored in low-density Ziploc bags at ambient temperature. Blood was collected in standard cryotubes through brachial venipuncture with a heparinised syringe. It is accepted that drawing a blood volume equal to 1% of the total body weight does not pose any immediate or long-term health effects on the bird (McGuill and Rowan, 1989). Extrapolated to the bird with the lowest body weight (i.e. 2950 g) it was safe to draw 14.75 mL of blood, whereas we never exceeded the maximum of a standard 10 mL syringe. All blood samples were centrifuged within 12 h of sampling. The resulting plasma was transferred into sterile Eppendorf® tubes and stored at −20 °C. All samplings were conducted ethically in accordance with the Norwegian Animal Research Authority's guidelines (http://www.fdu.no).

2.2. Pollutant analysis

The pollutant concentrations were determined in body feathers and plasma at the Toxicological Centre (University of Antwerp, Belgium), according to a modified method reported by Eulaers et al. (2014). Briefly, individual feathers were thoroughly rinsed with distilled water and dried overnight at ambient temperature. After length measurements of pooled feather samples (mean: 370 mm; range: 240–530 mm), a narrow section of one random feather per individual was cut off for SI analysis (Figure SupInfo-1). The remaining feather matrix was cut in ~1 mm pieces, weighed (mean: 0.1865 g; range: 0.0917–0.2633 g), spiked with internal standards: 500 pg μL^{-1} CB 143; 100 pg μL^{-1} BDE 77; 100 pg μL^{-1} BDE 128; 1 ng μL^{-1} triamyl phosphate (TAP); 1 ng μL^{-1} tris(propyl) phosphate (TPP-d15); 1 ng μL^{-1} tris-(2,3-dichloropropyl)-phosphate (TDCPP-d15); 1 ng μL^{-1} tri-(2-

Table 1
Concentration ranges (ng g^{-1}) of the targeted non-PBDE BFRs and PFRs reported in previous studies of avian species.

Species	Matrix	TCEP	TCIPP	TBOEP	TPHP	EHDPP	TEHP	BTBPE	EH-TBB	BEH-TEBP	Year	Location	Reference
Herring Gull (*Larus argentatus*)	Egg (ww)	<0.10–0.55	<0.20–4.1	0.16–2.2		<0.09–0.17					2010	USA	Chen et al. (2012)
Great-black-backed Gull (*Larus marinus*), European Shag (*Phalacrocorax aristotelis*),	Egg (ww)	<0.33–6.1	<6.7	<120	<20	<23	<0.02–8.7				2008–2010	N0	Leonards et al. (2011)
Common Eider (*Somateria mollissima*) and White-tailed Eagle (*Haliaeetus albicilla*)	Blood (ww)	<0.17–6.0	0.24–10	<440	<0.20–2.2	<25	<3.3				2010		
Kittiwake (*Rissa tridactyla*)	Liver (lw)	<62–190	<12–97	<69	26–120	210–650	<9.0				2008	N0	Evenset et al. (2009)
Common Eider (*Somateria mollissima*)	Liver (lw)	<100–206	<26–133	<100	40–165	257–619	<13						
Chicken (*Gallus gallus domesticus*) and duck (*Anas platyrhynchos domesticus*)	Muscle (lw)	34–160	3.9–21	48–270	<0.06–210	<0.05–22	<0.04–14				2011	CN	Ma et al. (2013)
Peregrine falcon (*Falco peregrinus*)	Egg (lw)							13–13		1.1–4.5	2007–2009	USA	Guerra et al. (2012)
	Egg (lw)							3.3–7.4		<1.0	2003–2006	ES	
Light-vented bulbul (*Pycnonotus sinensis*)	Egg (lw)							<0.74–4.3			2010–2012	CN	Sun et al. (2014)
Yellow-bellied Prinia (*Prinia flaviventris*)	Egg (lw)							<0.74–7.3					
Plain Prinia (*Prinia inornata*)	Egg (lw)							<0.74–5.6					
Dark green white-eye (*Zosterops japonicus*)	Egg (lw)							<0.74–7.5					
Northern Fulmar (*Fulmarus glacialis*)	Egg (lw)							<0.02–0.15			2003	FO	Karlsson et al. (2006)
Double-crested Cormorant (*Phalacrocorax auritus*)	Egg (lw)							<2.0	<8.0	<12	2007	USA	Klosterhaus et al. (2012)
Glaucous Gull (*Larus hyperboreus*)	Egg (lw)							<0.27–0.96			2006	N0	Verreault et al. (2007)
	Plasma (lw)							<0.20–0.26					
Chinese-pond Heron (*Ardeola bacchus*)	Muscle (lw)							<0.60			2005–2007	CN	Zhang et al. (2011)
	Kidney (lw)							<0.60					
	Liver (lw)							<0.60–20					
White-breasted Waterhen (*Amaurornis phoenicurus*)	Muscle (lw)							<0.60–1.0					
	Kidney (lw)							<0.60–4.0					
	Liver (lw)							<0.60–3.0					
Slate-breasted Rail (*Gallirallus striatus*)	Muscle (lw)							<0.60–20					
	Kidney (lw)							<0.60–27					
	Liver (lw)							<0.60–40					
Ruddy-breasted Crake (*Porzana fusca*)	Muscle (lw)							<0.60–9.3					
	Kidney (lw)							0.60–40					
	Liver (lw)							2.1–31					
Common Snipe (*Gallinago gallinago*)	Muscle (lw)							<0.60–0.90					
	Kidney (lw)							<0.60					
	Liver (lw)							<0.60–4.0					
Watercock (*Gallicrex cinerea*)	Muscle (lw)							0.07–0.39			2007	CN	Shi et al. (2009)
	Kidney (lw)							0.12–0.89					
	Liver (lw)							0.27–2.41					
													Gauthier et al. (2007)
													Gauthier et al. (2009)

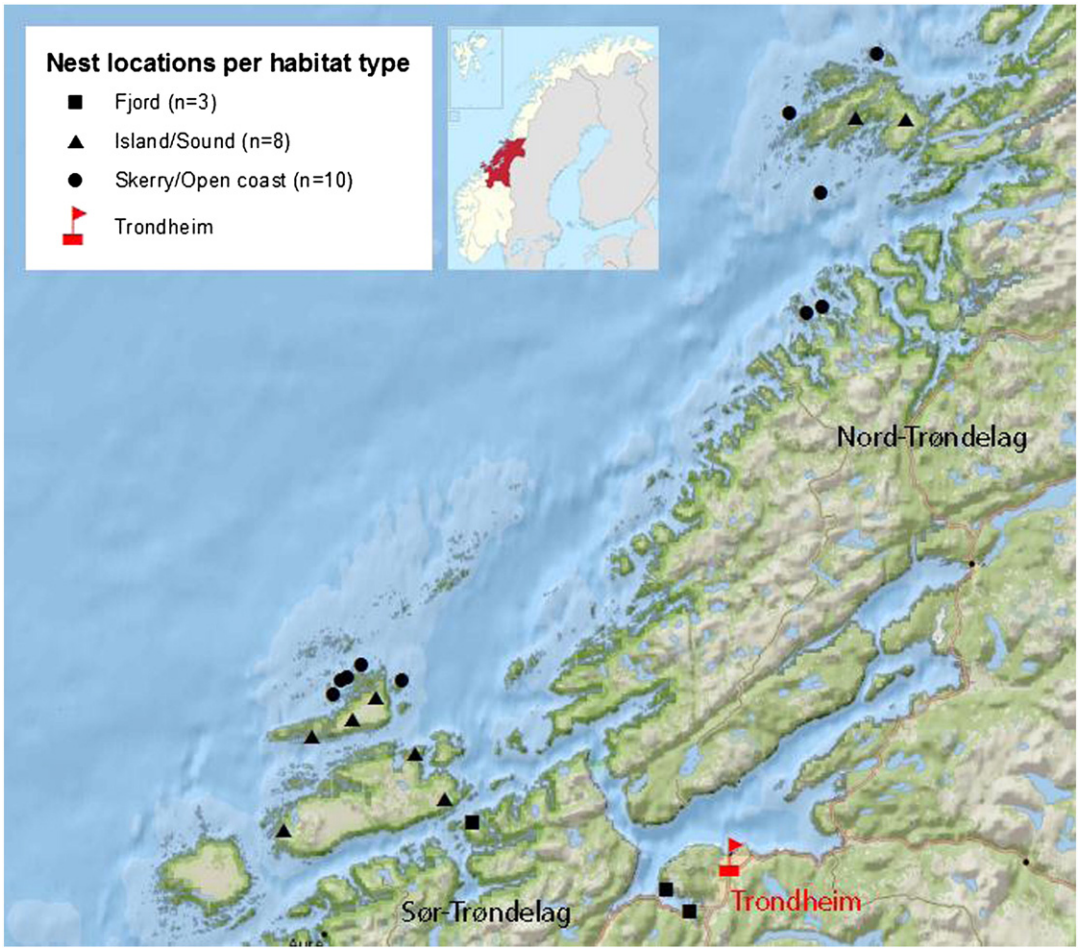

Fig. 1. Nest locations, classified per habitat type, of the sampled White-tailed Eagle nestlings.

butoxyethyl)-phosphate (TBOEP-d6); and 1 ng μL^{-1} tris(chloroethyl) phosphate (TCEP-d12). After overnight incubation at 45 °C in 8 mL of HCl (4 M) and 8 mL of hexane:dichloromethane (4:1, v:v), the feather analytes were liquid/liquid extracted using hexane:dichloromethane (4:1, v:v).

The liquid/liquid extraction of the plasma analytes, using hexane:dichloromethane (3:1, v:v), was preceded by three steps, with intermittent gentle vortexing. First, 1 mL of plasma was spiked with the above internal standards. Secondly, 0.5 mL of MilliQ water and 200 μL of formic acid (98%) were added. Lastly, 2 mL of hexane:dichloromethane (3:1, v:v) was added and gentle vortexing resulted in the separation of an organic layer. The feather and plasma extracts were fractionated on Supelclean™ ENVI™-Florisil® SPE cartridges (3 mL; 500 mg; Supelco, Bellefonte, PA, USA) topped with anhydrous Na_2SO_4 (400 mg). The first fraction (f_1) contained OCs and BFRs and was eluted with 10 mL of hexane, the second one (f_2), containing PFRs, with 10 mL of ethyl acetate. While f_2 was directly concentrated until dry with a nitrogen flow and reconstituted in *iso*-octane, f_1 was first cleaned-up on acidified silica (800 mg; 44% H_2SO_4) and eluted with 8 mL of hexane:dichloromethane (1:1, v:v).

The targeted BFRs, i.e. (MeO-)PBDEs, EH-TBB, BTBPE and BEH-TEBP, were quantified using gas chromatography (GC; Agilent GC 6890, Palo Alto, CA, USA) coupled to electron capture negative ionisation mass spectrometry (MS; Agilent MS 5973). Non-PBDE BFRs were separated on a DB-5 capillary column (15 m * 0.25 mm * 0.10 μm; J&W Scientific, Folsom, CA, USA), while a longer DB-5 capillary column (30 m * 0.25 mm * 0.25 μm) was used for OxC and (MeO-)PBDEs. CB 28, CB 153, HCB, *p,p′*-DDE and PFRs, i.e. tris(chloroethyl) phosphate (TCEP), TCIPP, tri-(2-butoxyethyl)-phosphate (TBOEP), TPHP, 2-ethylhexyl diphenyl phosphate (EHDPP) and TDCPP, were quantified using GC equipped with an HT-8 capillary column (25 m * 0.22 mm * 0.25 μm; SGE Analytical Science, Zulte, Belgium), coupled to electron impact MS.

Pesticide-grade solvents were obtained from Merck KGaA Chemicals (Darmstadt, Germany) and Acros Organics (Geel, Belgium). Every 10th sample, a procedural blank and a certified reference material (BCR 397 human hair; Institute for Reference Materials and Measurements, Geel, Belgium) were analysed. Earlier studies have shown the analytical precision of the analytical methods (Gill et al., 2004; Kucklick et al., 2009). All compounds were blank-subtracted using the average procedural blank values. The limit of quantification (LOQ) was set at 3 * SD of the procedural blanks, or, for analytes not detectable in blanks, calculated from a 10:1 signal to noise ratio. LOQs in feathers ranged from 0.05 to 5.0 ng g^{-1} dw, while those in plasma ranged from 0.02 to 1.0 ng g^{-1} ww (Table 1).

Recently, concern has been raised that conventionally expressing the amount of a pollutant in a feather as a concentration may be biased, and alternative methods, such as expressing the amount per feather length (Bortolotti, 2010) or as a deposition rate (Garcia-Fernandez et al., 2013), have been proposed. Since the masses and lengths of the analysed feathers were significantly associated ($R = 0.72$; $P < 0.01$; Figure SupInfo-2), we have chosen conventional reporting for concentrations in order to facilitate comparability among studies.

2.3. Stable isotope analysis

The analysis for SI ratios ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$) was carried out at the laboratory of Oceanology (University of Liège, Belgium). The feathers

were only washed with distilled water. Washing with an organic solvent with the purpose to remove lipids would have been futile as the feather lipid content has been shown to have no significant effect on the SI ratios (Mizutani et al., 1992). Moreover, washing with an organic solvent would have decreased the strength of the hypothesised associations between feather and plasma OHC levels (Jaspers et al., 2008). Per individual, a narrow transverse section (~0.01 g) of one washed feather was cut off in order to represent the measurement of SIs along the entire period of feather growth (Figure SupInfo-1). This section was cut in ~1 mm pieces and homogenised, and 2 mg was crimped into a tin combustion cup along with 2 mg W_2O_3 powder in order to optimise combustion. SI ratios were determined using isotopic ratio mass spectrometry (Isoprime 100, Isoprime, UK) coupled in continuous flow to an elemental analyser (Vario microtube, Elementar, Germany). The isotopic ratios of carbon, nitrogen and sulphur were conventionally expressed as δ values (‰), relative to the international standards Vienna PeeDee Belemnite (vPDB), atmospheric N_2 (AIR) and Canon Diablo Troilite (CDT), respectively. Pure gasses of CO_2, NO_2 and SO_2 were used and calibrated against certified reference materials, i.e. sucrose (IAEA C-6; $\delta^{13}C = -10.8 \pm 0.5$‰), $(NH_4)_2SO_4$ (IAEA-N2; $\delta^{15}N = 20.3 \pm 0.2$‰) and Ag_2S (IAEA-S1; $\delta^{34}S = -0.3 \pm 0.3$‰), obtained from the International Atomic Energy Agency (IAEA, Vienna, Austria). The analysis' performance was positively assessed by the included procedural blanks, duplicate samples and reference samples, i.e. sulfanilic acid. Sulfanilic acid was also used for elemental data calculation ($\%_{nitrogen} = 8.1 \pm 0.1$, $\%_{carbon} = 41.6 \pm 0.1$ and $\%_{sulphur} = 18.5 \pm 0.1$). Routine measurements were precise to within 0.1‰ for $\delta^{13}C$, 0.2‰ for $\delta^{15}N$ and 0.3‰ for $\delta^{34}S$, respectively and the obtained analytical precision was 0.1‰ SD.

2.4. Statistical analysis

The statistical analysis was performed using R 3.0.2 (R CoreTeam, 2013), rejecting the null-hypothesis at $\alpha = 0.05$. Concentrations below the LOQ were substituted per compound by DF * LOQ, with DF (detection frequency) being the proportion of samples with concentrations above LOQ (Eulaers et al., 2011a). All model data and residuals were explored for influential outliers, normality and homoscedasticity (Zuur et al., 2010). All pollutant concentrations were $\log_{10}$-transformed in order to approach a normal distribution. Per FR class, i.e. PBDEs, non-PBDE BFRs and PFRs, the accumulation profile was based upon the relative proportion of each compound to the total sum of their respective class. The associations between plasma and feather concentrations were investigated using linear regression analysis. Parameters of significant associations were Standardised Major Axis estimated while those of non-significant were determined using Ordinary Least Squares. Analysis of Variance (ANOVA) was used to investigate differences in SI ratios among habitats. Post-hoc comparisons were made using Tukey Honest Significant Differences and P values were adjusted for multiple comparisons (P_{adj}). In addition, Principle Component Analysis (PCA) of the SIs was performed in order to better visualise habitat-specific differences using a biplot of the factor scores, as well as to reduce the complexity in the SI data into fewer factors. In order to investigate how SIs (both SI ratios and PCA scores) and habitats may explain variation in exposure, quantified in body feathers, candidate models were selected using Akaike's Information Criteria adjusted for small sample size bias (AIC_c; n/K < 40). As such, both the fit and complexity of the candidate models was accounted for (Johnson and Omland, 2004). Models for which the AIC_c difference (Δ_i) < 2 compared to the most parsimonious model (lowest AIC), were accepted as competing models (Burnham & Anderson 2002). As proposed by Anderson and Burnham (2002), we reported AIC_c, Δ_i, Akaike weight (w_i), log-likelihood (log(λ)) for each model, as well as its fit (R^2).

3. Results

Low but still quantifiable concentrations in feathers were found for BDE 28 and all non-PBDE BFRs ($0.52 \leq DF \leq 1.00$), but these compounds remained largely < LOQ in plasma ($0.00 \leq DF \leq 0.12$). This is supported by negative intercepts β_0 ($-1.95 \leq \beta_0 \leq -0.14$) for the associations between plasma and feather concentrations, indicating higher concentrations in the latter (Table 2). Nonetheless, most targeted PBDE congeners were sufficiently detected in both matrices ($0.52 \leq DF \leq 1.00$), and showed significant and positive associations ($0.23 \leq R^2 \leq 0.63$; all $P \leq 0.05$). With the exception of BDE 47 ($R^2 = 0.63$; $P \leq 0.01$), the fitted associations for PBDEs ($0.23 \leq R^2 \leq 0.24$; all $P \leq 0.05$) were not as strong as those for CB 153 and p,p'-DDE ($0.56 \leq R^2 \leq 0.61$; all $P \leq 0.01$; Table 2). Associations were non-significant for HCB ($P = 0.90$), BDE 99 and 154 ($0.35 < P < 0.50$).

All targeted PFRs were highly detected in feathers ($0.52 \leq DF \leq 1.00$), while only TCIPP and TDCPP were detected in plasma ($0.59 \leq DF \leq 0.65$) with levels not being associated to those in feathers ($0.00 \leq R^2 \leq 0.01$; $0.74 \leq DF \leq 0.80$; Table 2). PFR feather concentrations (0.95–3000 ng g^{-1}) were typically one to two orders of magnitude those of CB 153 and p,p'-DDE (2.3–21 ng g^{-1}), and two to three orders of magnitude those of BFRs (0.03–2.3 ng g^{-1}; Table 2). An exceptionally high TCEP concentration (3000 ng g^{-1}) was found in the nestling closest to Trondheim. While feather concentrations of the less abundant PFRs, i.e. TBOEP, TPHP, EHDPP and TDCPP (Figure SupInfo-3), were typically still tenfold those of PBDEs, plasma concentrations of the detected PFRs, i.e. TCIPP and TDCPP, were within the same concentration range of BDE 47 (Table 2). BDE 47 (0.16–2.3 ng g^{-1}) and 99 (0.16–2.3 ng g^{-1}), BEH-TEBP (0.09–1.5 ng g^{-1}), TCEP (14–3000 ng g^{-1}) and TCIPP (14–220 ng g^{-1}) were proportionally the most bioaccumulated compounds in their respective class, i.e. PBDEs, non-PBDE BFRs and PFRs (Figure SupInfo-3).

$\delta^{13}C$ and $\delta^{15}N$ were significantly and positively associated to each other ($R^2 = 0.34$; $P < 0.01$), and were each also significantly, but negatively, correlated with $\delta^{34}S$ ($0.42 \leq R \leq 0.44$; $P < 0.01$). This is confirmed by the PCA's correlation biplot from the first and second principle components (PCs), explaining respectively 75.3 and 14.0% of the observed variation (Fig. 2a). Factor loadings indicated that PC1 discriminated between $\delta^{13}C$ and $\delta^{15}N$ on the one hand and $\delta^{34}S$ on the other hand, and that PC2 further discriminated between $\delta^{13}C$ and $\delta^{15}N$. As such, the biplot showed that fjords could be distinctively separated from the other two habitats (Fig. 2a–d). Significant differences in $\delta^{13}C$ among habitats ($F_{2,18} = 4.51$; $P = 0.03$) indicated that fjords were enriched in ^{13}C compared to islands/sounds and skerries/open coast ($0.02 \leq P_{adj} \leq 0.05$), while $\delta^{13}C$ in the latter two was not significantly different ($P_{adj} = 0.78$; Fig. 2b). Significant differences among habitats for $\delta^{15}N$ ($F_{2,18} = 6.73$; $P < 0.01$) and $\delta^{34}S$ ($F_{2,18} = 8.24$; $P < 0.01$) showed a significant enrichment of ^{15}N and a significant depletion of ^{34}S in fjords compared to islands/sounds and skerries/open coast (all $P_{adj} \leq 0.01$), while $\delta^{15}N$ and $\delta^{34}S$ in the latter two locations were not significantly different from each other ($0.92 \leq P_{adj} \leq 0.93$; Fig. 2c–d). These habitat specific differences were heavily influenced by the two nestlings closest to Trondheim, inherently in the fjord habitat, being most enriched in $\delta^{15}N$ and $\delta^{13}C$, and most depleted in $\delta^{34}S$.

The AIC_c based model selection (Table 2, see Table SupInfo-1 for all competing models) showed that $\delta^{15}N$ recurred most often as a proxy, more specifically for p,p'-DDE, tetra- and penta-BDEs, as well as TCEP and TPHP (all $P \leq 0.04$). A combination of $\delta^{15}N$ and $\delta^{13}C$ was often included in the short-list of competing models, but was only the most parsimonious for CB 153 and TBOEP. In these cases, one factor was significant (CB 153: $\delta^{15}N$ and TBOEP: $\delta^{13}C$), while the other one was marginally significant (both $P_{adj} = 0.06$). However, except for the just-mentioned TBOEP ($P < 0.01$) and for BDE 28 ($P < 0.01$), $\delta^{13}C$ never explained a significant proportion of the observed variation. With regard to models with significant parameter estimates, inclusion of $\delta^{13}C$ always increased the fit of the model when complementing

Table 2
Body feather and plasma concentrations (median and range), model parameter estimates for their association, and model parameters for the most parsimonious model explaining variation, of selected OC and FR concentrations in White-tailed Eagle nestlings.

	Body feathers ($n = 21$)		Plasma ($n = 17$)		Feather-plasma association ($n=17$)				Most parsimonious model ($n = 21$)				
	LOQ	Concentration	LOQ	Concentration	β_0	β_1	R^2	P		β_0	β_x	R^2	P
CB 28	0.50	0.50 (0.26–1.4)	0.10	<LOQ (0.00)[a]	n/a[b]				$\delta^{15}N$	−0.90	0.04	0.01	0.68
CB 153	0.20	4.1 (2.3–15)	0.05	1.8 (0.49–7.2)	−0.52	1.15	0.56	<0.01**	$\delta^{15}N + \delta^{13}C$	−7.41	0.40; −0.15	0.46	<0.01**; 0.06[T]
HCB	0.20	0.64 (0.16–1.9)	0.05	0.29 (0.19–0.72)	−0.48	0.02	0.00	0.90	$\delta^{15}N + \delta^{34}S$	6.49	−0.34; −0.14	0.23	0.30; 0.06[T]
p,p′-DDE	0.40	5.4 (2.3–21)	0.10	1.9 (0.59–13)	−0.71	1.32	0.61	<0.01**	$\delta^{15}N$	−2.41	0.25	0.32	<0.01**
BDE 28	0.05	0.05 (0.03–0.18)	0.02	<LOQ (0.12)[a]	n/a[b]				$\delta^{13}C$	2.60	0.20	0.36	<0.01**
BDE 47	0.05	0.59 (0.38–2.3)	0.02	0.17 (0.07–0.59)	−0.58	1.20	0.63	<0.01**	$\delta^{15}N + \delta^{34}S$	−3.41	0.32; −0.05	0.81	<0.01**; 0.07[T]
BDE 99	0.05	0.25 (0.16–0.89)	0.02	0.03 (0.02–0.15)	−1.24	0.32	0.03	0.50	$\delta^{15}N$	−2.79	0.17	0.26	0.02*
BDE 100	0.05	0.12 (0.07–0.27)	0.02	0.06 (0.02–0.15)	0.11	1.59	0.23	0.05*	$\delta^{15}N$	−3.77	0.22	0.45	<0.01**
BDE 153	0.05	0.09 (0.05–0.21)	0.02	0.02 (0.02–0.05)	−0.81	0.82	0.23	0.05*	$\delta^{15}N$	−2.06	0.08	0.05	0.34
BDE 154	0.05	0.07 (0.03–0.18)	0.02	0.03 (0.01–0.07)	−1.95	−0.28	0.06	0.35	$\delta^{15}N$	−3.30	0.16	0.10	0.16
6-MeO-BDE 47	0.05	0.13 (0.05–0.22)	0.02	0.08 (0.03–0.24)	0.16	1.38	0.29	0.03*	Habitat	−1.05	S/O>I/S>F[c]	0.28	0.05*
2-MeO-BDE 68	0.05	<LOQ (0.00)[a]	0.02	0.03 (0.02–0.05)	n/a[b]					n/a[b]			
EH-TBB	0.05	0.05 (0.04–0.12)	0.02	<LOQ (0.00)[a]	n/a[b]				$\delta^{13}C$	−0.61	0.03	0.02	0.51
BTBPE	0.05	0.05 (0.03–0.16)	0.05	<LOQ (0.00)[a]	n/a[b]				$\delta^{34}S$	−1.97	0.04	0.05	0.34
BEH-TEBP	0.05	0.26 (0.09–1.5)	0.02	<LOQ (0.00)[a]	n/a[b]				$\delta^{13}C$	−2.40	−0.10	0.05	0.32
TCEP	1.00	110 (14–3000)	0.20	<LOQ (0.00)[a]	n/a[b]				$\delta^{15}N$	−5.93	0.62	0.42	<0.01**
TCIPP	1.00	91 (14–220)	0.20	0.22 (0.12–0.74)	−0.73	0.06	0.00	0.80	$\delta^{13}C$	3.94	0.10	0.08	0.22
TBOEP	5.00	6.2 (3.1–22)	1.00	<LOQ (0.00)[a]	n/a[b]				$\delta^{15}N + \delta^{13}C$	−8.48	0.35; −0.24	0.41	0.06[T]; <0.01**
TPHP	1.00	15 (5.9–250)	0.20	<LOQ (0.00)[a]	n/a[b]				$\delta^{15}N$	−3.75	0.38	0.25	0.02*
EHDPP	1.00	12 (5.4–25)	0.20	<LOQ (0.12)[a]	n/a[b]				$\delta^{34}S$	1.36	−0.02	0.01	0.60
TDCPP	1.00	4.3 (0.95–21)	0.20	0.22 (0.13–0.32)	−0.67	−0.04	0.01	0.74	$\delta^{15}N$	−0.87	0.12	0.04	0.40

[T] $P < 0.10$.
* $P < 0.05$.
** $P \leq 0.01$.
[a] <LOQ (DF): compounds with DF < 0.50 were not considered for further statistics. The appropriate DF for each compound is reported between brackets.
[b] A statistical test was not possible as the compound was not detected in feathers.
[c] S/O = skerry/open coast; I/S = island/sound; F = fjord.

another factor, e.g. for CB 153, and BDE 28 and 100 (Table SupInfo-1). Also the addition of $\delta^{34}S$ to $\delta^{15}N$ increased the fit of models, e.g. for BDE 47 and TCEP. However, similarly to $\delta^{13}C$, $\delta^{34}S$ never explained a significant part of the variation. The larger part of the models with significant parameter estimates exhibited a mediocre fit ($0.20 \leq R^2 \leq 0.46$), with the model for BDE 47 exhibiting the highest fit ($R^2 = 0.81$). Habitat was only a significant factor for BDE 28 ($P = 0.01$) and 6-MeO-BDE 47 ($P = 0.05$). Finally, alternative candidate models including PCA scores never performed better than those that incorporated the individual SI ratios, and generally acquired a lower fit.

4. Discussion

The present study is, to the best of our knowledge, the first to investigate such a wide range of OHCs, including legacy OCs and PBDEs, as well as currently-used non-PBDE BFRs and PFRs in plasma and feathers of a high trophic level avian species. Selected OCs, such as CB 153, *p,p′*-DDE, and BDE 47 were treated as benchmark compounds for comparison with 'new' compounds. OC concentrations were similar to those already reported (Eulaers et al., 2011a,b), were significantly associated between plasma and feathers, and were lower than those reported for nestlings from northern Norway, sampled during 2008–2010 (Bustnes et al., 2013; Eulaers et al., 2011b,2013; Figure SupInfo-4). In addition, both the present study and Bustnes et al. (2013) indicated that plasma CB 153 and *p,p′*-DDE concentrations had decreased compared to their concentrations in whole blood in nestlings from the Baltic, sampled during 1996–1998 (Olsson et al., 2000; Figure SupInfo-4). However, spatial variation may confound such temporal comparisons. Nonetheless, nestlings sampled in 2009 in the same region as the present study also exhibited elevated concentrations of CB 153 and *p,p′*-DDE, both in plasma and feather, as well as elevated feather PBDE (Eulaers et al., 2011a; Figure SupInfo-4). These observations confirm the earlier demonstrated inter-annual variability in White-tailed Eagle nestling exposure (Eulaers et al., 2013). Furthermore, the above-described decreasing concentrations over time promote the suitability of White-tailed Eagle nestlings, in addition to adult specimens (Gjershaug et al., 2008; Helander et al., 2002, 2008; Nygård and Polder, 2012) to investigate temporal variation of these compounds. Moreover, they suggest that plasma and feathers are valid sampling matrices, and generally that the White-tailed Eagle is a suitable bio-indicator for environmental pollution with OCs and PBDEs.

To present day, only few studies have investigated avian exposure to current-use non-PBDE BFRs and PFRs (Table 1). The present study successfully quantified plasma concentrations of two PFRs, i.e. TCIPP and TDCPP, earlier suggested to have low environmental degradability (van der Veen and de Boer, 2012). Leonards et al. (2011) also reported PFRs in blood/plasma from different bird species across Norway among which White-tailed Eagle nestlings from the current study region. Unfortunately, all species and sampled regions were pooled in their report, making it therefore unclear if their reported higher TCIPP concentrations (0.24–10 ng g^{-1} ww) and seemingly wider range of detected analytes were in fact related to the analysed White-tailed Eagle nestling plasma. Generally, PFR concentrations in environmental media, such as air, surface water and sediment, were higher than those of PBDEs (Salamova et al., 2013; van der Veen and de Boer, 2012). Therefore, we assume that the low detectability of the other targeted PFRs in the present study may be tied to low bioavailability. In this sense, Leonards et al. (2011) observed preferential partitioning of PFRs in sediments rather than in biota. Low exposure may also be a result of efficient environmental biodegradation or metabolic transformation, as supported by short (1.2–49.6 h) depuration half-life ranges for TPHP (van der Veen and de Boer, 2012). Furthermore, PFRs may have limited lipid affinity, as is suggested by moderate log K_{ow} values (1.44–5.73; Bergman et al. 2012). The present study indeed shows that plasma PFR concentrations in nestling birds of prey are relatively low and reside within the same concentration range as for the legacy and current-use BFRs. Non-PBDE BFR concentrations, on their turn, were as expected typically one degree of magnitude lower than those of the recalcitrant CB 153 and *p,p′*-DDE (Table 2; Eulaers et al., 2011a, b). Low feather concentrations of non-PBDE BFRs, and their low detectability in plasma, were in agreement with earlier observations for internal tissues (Verreault et al., 2007; Shi et al., 2009; Zhang et al., 2011).

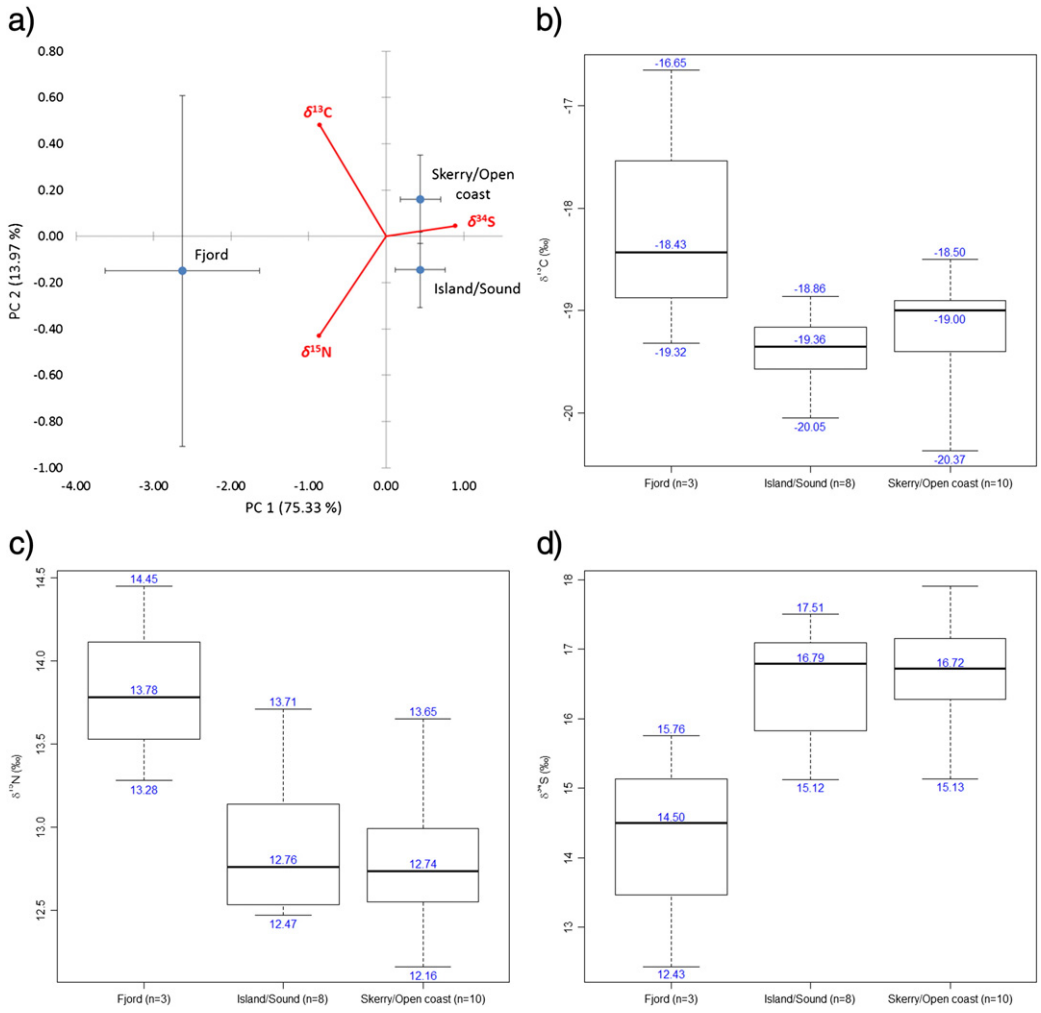

Fig. 2. Habitat-specific (a) PCA factor scores (mean ± SE) of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$, and habitat-specific values (median and range) for (b) $\delta^{13}C$, (c) $\delta^{15}N$, and (d) $\delta^{34}S$.

BTBPE seems nonetheless to be lipophilic (log K_{ow} = 8.31; Bergman et al. 2012) and to possess bioaccumulation and biomagnification potential (Tomy et al., 2007; Wu et al., 2011). Possibly, high excretion rates and low gastrointestinal absorption (Hakk et al., 2004) may be resulting in low exposure. At this point, no conclusions can be drawn for EH-TBB and BEH-TEBP, because exposure data as well as pharmacokinetic information are poorly investigated (Covaci et al., 2011).

Plasma is considered representative for the internal pollutant body burden, because it equilibrates with the internal tissues. Moreover, plasma can be relatively easily and non-destructively sampled from nestling birds of prey, which are often protected, and is therefore conventionally used as sampling matrix to study nestling exposure (Bourgeon et al., 2013; Bustnes et al., 2013; Elliott et al., 2009; Eulaers et al., 2011a,b; Olsson et al., 2000). Body feathers are connected to the blood circulation during their growth and consequently reflect plasma concentrations of OCs and PBDEs, as shown in the present study as well as previously (Eulaers et al., 2011a,b). In addition, feathers are metabolically inactive (Jardine et al., 2006) and therefore archive the nestling's exposure as well as its assimilated diet (Hobson and Clark, 1992) over a large time-frame. In the present study, this corresponds to the period from feather growth initiated around day 27 (Watson, 2010) continuing up to sampling around day 60. The feathers-specific minimal invasiveness upon sampling, lack of metabolism and longer exposure time-frame promote their use as a valuable alternative and/or complementary sampling matrix. The combination of long-term sequestration of low concentrations, too low to be quantified in plasma due to supposedly rapid biotransformation and/or excretion, and the lack of metabolism in feathers, were likely responsible for the detection of current-use FR concentrations in feathers only. This hypothesis is supported by the detection of the lower halogenated benchmark OCs and PBDEs, i.e. CB 28 and PBDE 28, as well as by generally higher concentrations in feathers compared to plasma (Table 2). This is consistently so for all compounds in the present study, as well as earlier ones (Figure SupInfo-4).

Nonetheless, concentrations of PFRs typically showed concentrations one to two orders of magnitude higher than those of CB 153 and *p,p′*-DDE and even two to three orders of magnitude higher than those of BFRs (Table 2). Unexpectedly, PFR concentrations in both matrices were non-significantly associated and were highly similar with recently reported atmospheric BFR and PFR concentrations (Salamova et al., 2013). These two observations may hint at the possibility that feather concentrations of PFRs may be primarily due to air-borne particles trapped by preen oil on the feather surface, which were likely not removed by the washing step with distilled water (Jaspers et al., 2008). As such, feathers may have acted as passive air samplers, rather than depicting bioaccumulation or biomagnification. This may be supported by the absence of significant associations between $\delta^{15}N$ (the established proxy for an individual's trophic level; Jardine et al., 2006; Kelly, 2000) and concentrations of most PFRs in feathers (Table 2). Similar observations have been made for the higher-brominated BFRs and non-PBDE BFRs, which may be due to external contamination stemming from sources within the body, e.g. preen oil. The higher brominated BDE 153 and 154 are very lipophilic ($8.6 \leq \log K_{ow} \leq 9.0$) and have been shown to accumulate preferentially in nestling preen oil (Eulaers et al., 2011a). In this sense, the targeted non-PBDE BFRs also possess high log

K_{ow} values (7.73–9.23), which may have resulted in their detection in feathers, but not in plasma. In any case, non-detection of a compound in plasma does not necessarily diminish the validity of its feather concentration, as was recently shown in a regional investigation for the current-use BFRs tetrabromobisphenol-A and hexabromocyclododecane in Barn Owl (*Tyto alba*) body feathers (Eulaers et al., 2014).

As shown in earlier studies on nestling exposure, sampling of nestlings allowed the identification of small-scale geographical variation in pollutant exposure and dietary ecology (Elliott et al., 2009; Eulaers et al., 2013; Olsson et al., 2000). For most compounds positive associations were found with $\delta^{15}N$, suggesting their bioaccumulation potential in White-tailed Eagle nestlings. This supports earlier findings on White-tailed Eagle nestlings (Bustnes et al., 2013; Eulaers et al., 2013) as well as for nestlings of other high trophic level species (Bourgeon et al., 2013; Elliott et al., 2009; Sørmo et al., 2011). In this sense, associations with $\delta^{15}N$, enriched in nestlings from fjords, may identify spatial sources, as seems to be the case for the investigated PBDEs and PFRs. Positive associations of PBDE concentrations with $\delta^{15}N$ in White-tailed Eagle seem at first to contradict negative associations in coastal Herring Gull (*Larus argentatus*; Sørmo et al., 2011). $\delta^{15}N$ in the present study was enriched in fjord habitats, inherently more inland, and $\delta^{15}N$ is relatively depleted in refuse dumps (Ramos et al., 2013), on which Gulls may feed, thus both seemingly indicating land-derived sources of PBDEs. Intuitively, $\delta^{15}N$ enrichment would indicate a larger proportion of marine derived prey, as primary producers in the marine environment are naturally enriched in ^{15}N compared to land derived primary producers (Kelly, 2000). Enriched $\delta^{15}N$ and $\delta^{13}C$ in nestlings from fjords may indeed reflect benthic/inshore feeding, compared to pelagic/offshore feeding on islands and skerries or at sounds and the open coast (Fig. 2a–d; Hobson, 1993), or may suggest local feeding on washed-up Atlantic Salmon (*Salmo salar*) specimens migrating upstream a large river feeding the fjord. However, the present study cannot rule out whether land-based run-off from agricultural areas, present at the two nests close to Trondheim, may have enriched $\delta^{15}N$ in fjords, due to the use of nitrate-based fertilisers (Choi et al., 2007; Hofmeister et al., 2013). Therefore, enriched $\delta^{15}N$ may well be a proxy for anthropogenic activity, possibly confounding its use to study bioaccumulation and biomagnification. This hypothesis may be supported by the observed depletion of $\delta^{34}S$ at those two nests. Although the use of $\delta^{34}S$ as an ecological proxy has not yet entirely been unravelled, it is known that $\delta^{34}S$ depletion is due to the disturbance of the natural biogeochemical sulphur cycle with biogenic sulphur emissions originating from the combustion of coal, oil and natural gas, that typically exhibit $\delta^{34}S$ values close to 0 (Thode, 1991). Since $\delta^{34}S = 20.3$ for seawater (Thode, 1991), depleted $\delta^{34}S$ values have been suggested to be indicative of a larger proportion of terrestrial prey compared to marine prey (Moreno et al., 2010; Ramos et al., 2009; Resano et al., 2011; Resano-Mayor et al., 2013). However, the ingestion of terrestrial prey by nestlings in fjords would have resulted in lower $\delta^{13}C$ and $\delta^{15}N$ values (Kelly, 2000). As such, the depletion of $\delta^{34}S$ in fjords is more likely due to their inherent closer vicinity to an urbanised centre, i.e. Trondheim. Previous studies have shown that industrial discharges as well as anaerobic conditions are suspected to favour the production of H_2S in soil and water, which is not assimilated in primary producers and ultimately causes $\delta^{34}S$ depletion in biota (Morrissey et al., 2013a; Tucker et al., 1999). The present study supports the observation that $\delta^{15}N$ may also serve as an indicator for anthropogenic activity (Morrissey et al., 2013b), and clearly shows that the additional analysis of $\delta^{34}S$ is advantageous to interpret values of more conventionally analysed $\delta^{13}C$ and $\delta^{15}N$ values.

Nonetheless, the present study clearly shows that $\delta^{15}N$ is an important proxy to explain variation in OC, PBDE and PFR exposure. Although $\delta^{15}N$ may indicate anthropogenic input, and thus further support atmospheric deposition of PFRs on the feather surface, these associations with $\delta^{15}N$ likely present to a large extent bioaccumulation and biomagnification. If variation in exposure was largely driven by the spatial vicinity to Trondheim, the most parsimonious models would have indicated $\delta^{34}S$ as a strong influence, rather than $\delta^{15}N$ and $\delta^{13}C$. Finally, the outcome of the model selection showed that models including PCs of the SI data never performed better than those that incorporated the individual SI ratios, and generally acquired a lower fit as well (Table SupInfo-1). Therefore, although such models may be informative (Morrissey et al., 2013a), we recommend investigation of individual SI ratios as well.

5. Conclusions

Different accumulation of non-PBDE BFRs and PFRs in feathers and plasma clearly showed that matrix-specific differences in metabolic capacity and exposure time-frame may draw different pictures of exposure. As such, although the use of feathers should always be validated against the conventionally used plasma, feathers may be useful as an alternative or complementary matrix to study nestling exposure. In such investigation, the use of benchmark compounds is most informative to study accumulation and associations with dietary proxies to less-well-known compounds, such as current-use FRs. Furthermore, the inclusion of $\delta^{34}S$ has been shown to be promising to investigate variation in OHC exposure, although its interpretation as an applied proxy needs more fundamental investigations. In this sense, further investigations on the use of $\delta^{15}N$ and $\delta^{34}S$ may be warranted in order to identify to which extent they represent spatial variation due to point sources rather than their common use as a dietary proxy. Finally, the present study shows that a dual-matrix approach is useful to study nestling exposure, and that nestlings of White-tailed Eagle are promising bio-indicators of environmental pollution with a large range of OHCs, ranging from legacy OCs and PBDEs to current-use BFRs and PFRs.

Conflict of interest statement

The authors confirm that no competing personal or financial interests exist regarding the submitted manuscript. All authors have thoroughly read the manuscript, and agreed that the work is ready for submission to Science of the Total Environment. Further, all authors accept responsibility for the manuscript's contents.

Acknowledgements

All authors thank the University of Antwerp and FWO-Flanders for funding and support. The collection of White-tailed Eagles for the Irish reintroduction was in part funded by the Irish White-tailed Eagle reintroduction programme (www.goldeneagle.ie), led by Allan Mee. The Norwegian Environment Agency granted permission to collect the birds. The collecting of birds in the field was done by Inge Dahlø, Steinar Garstad, Bertil Nyheim, Martin Pearson, Frithjof Pedersen, Livar Ramvik and Asgeir Østvik. Tom Roger Østerås took daily care of the birds.

Appendix A. Supplementary data

Competing candidate models to explain most parsimoniously the observed variation in White-tailed Eagle nestling exposure, the mass–length relationship for the analysed feathers and the procedure to subdivide them for the simultaneous analysis for pollutants and stable isotopes were uploaded as Supplementary information, and Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2014.01.051.

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