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How much do PCB toxic equivalents account for PHAH toxicity in predatory birds?

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Abstract

Various diffuse polyhalogenated aromatic hydrocarbons (PHAHs) exert common toxicity through the aryl hydrocarbon receptor (AhR). Apex predators spatially and temporally integrate diffuse contamination and simultaneous exposure can cause additive toxicity. We investigated the extent to which PCBs, still amongst the most prevalent PHAHs accumulated by predators, accounted for total PHAH toxicity in raptors and fish eating birds from Britain. We analysed egg or liver extracts from six species and compared chemically determined ΣPCB-TEQs concentrations with total AhR-mediated toxicity determined using the chemical-activated luciferase gene expression bioassay (CALUX-TEQ). Dioxin-like PCB profiles in eggs and livers were dominated by congeners 118, 105 and 167. ΣPCB-TEQ and CALUX-TEQ concentrations were positively associated but not in a 1:1 relationship. ΣPCB-TEQ were broadly similar to CALUX-TEQ concentrations in eggs and livers with CALUX-TEQ concentrations >50-80 and 160-320 pg g⁻¹ lipid respectively, but were lower than CALUX-TEQ concentrations in less contaminated samples.

Keywords- Dioxin-like PCBs, TEQs, Ah receptor, CALUX-assay, sparrowhawk, kestrel, herons, gannet, merlin and peregrine falcon.
**Introduction**

Polyhalogenated aromatic hydrocarbons (PHAHs) include polychlorinated dibenzo-\(p\)-dioxins (PCDDs) and furans (PCDFs), polychlorinated biphenyls (PCBs), polychlorinated terphenyls and naphthalenes (PCNs) and polycyclic aromatic hydrocarbons (PAHs). They are ubiquitous, persistent, lipophilic and bioaccumulative compounds, characteristics that enhance the risk of exposure in a diverse array of apex predators. Many PHAHs induce effects that are mediated through a common mode of action. This involves the binding of the parent compound or their metabolites to the aryl hydrocarbon receptor (AhR) in the cytoplasm and results in a rise or fall of the transcription of specific genes via dioxin-responsive elements in the DNA (Safe, 1990; Denison and HeathPagliuso, 1998). The consequences can be adverse effects such as reproductive impairment (disruption of hormone status, embryotoxicity, teratogenicity), hepatotoxicity, immunotoxicity and neurotoxicity (Denison and HeathPagliuso, 1998; Harper et al., 2006).

PHAHs are typically found in complex mixtures in environmental matrices. The commonality in their mode of action means that the toxicity of individual PHAHs can be additive. The chemical-activated luciferase gene expression (CALUX) bioassay has been developed to quantify the overall toxicity caused by simultaneous exposure to multiple compounds that exert toxicity via the AhR. This reporter gene assay is based on AhR-mediated firefly (Photinus pyralis) luciferase expression in genetically modified cell lines (Aarts et al., 1995; Garrison et al., 1996). A vector containing the luciferase gene under transcriptional control of dioxin-responsive elements has been stably transfected into a number of cell lines, including the rat H4IIE hepatoma cell line (Aarts et al., 1995; Garrison et al., 1996; Richter et al., 1997). CALUX has been used widely for screening and quantifying the toxicity of dioxin-like compounds in blood (Van Wouwe et al., 2004; Ziccardi et al., 2000), sediments (Behnisch et al., 2002; Stronkhorst et al., 2002), various marine matrices (Tsutsumi et al., 2003; Windal et al., 2005) and bovine milk (Bovee et al., 1998).

The occurrence of PCB concentrations in the livers and eggs of many different bird species has been well documented (Harris and Elliott, 2011). Some PCB congeners (non-\(ortho\), and to a lesser extent some mono-\(ortho\) substituted congeners) have a coplanar dioxin-like configuration. These have a high affinity for the AhR and so exert similar toxic effects to other PHAHs that act via the AhR. The potencies of different dioxin-like PCB congeners are between one and five orders of magnitude lower than that of 2,3,7,8 -tetrachlorodibenzo-\(p\)-dioxin (TCDD), the most toxic dioxin (Ahlborg et al., 1990).
1992; Van den Berg et al., 1998, 2006). Because accumulation of PCBs typically exceeds that of other more potent PHAHs, such as dioxins and furans (Jemenez et al., 2007), it is often considered that PCBs are likely to be the main contributors to AhR mediated toxicity unless there are particular localised sources of other compounds. However, there is a general lack of quantitative evidence to support this contention, not is it clear how variation or future changes in PCB contamination are likely to affect AhR mediated toxicity. The aim of the present study was therefore to investigate the extent to which dioxin-like PCBs in predatory birds account for their total TEQ concentrations. This involved measuring PCB concentrations chemically in the eggs and livers of various predatory birds and comparing the resultant calculated summed toxic equivalence ($\Sigma$PCB-TEQ) with that estimated for all PHAHs as measured by CALUX assay (CALUX-TEQ).

1. Materials and Methods

1.1. Sample collection

Livers were obtained from bird carcasses sent in by members of the public to the Predatory Bird Monitoring Scheme (PBMS: http://pbms.ceh.ac.uk/), a long-term UK chemical surveillance and monitoring programme that uses birds of prey as sentinels of exposure (Walker et al., 2008). In all, 48 livers were used for the present study and were from 16 sparrowhawk (Accipiter nisus), 16 kestrel (Falco tinnunculus) and 16 heron (Ardea cinerea) carcasses that had been found at various locations from across Britain. A collection of 44 eggs, each from a separate nest and collected for the PBMS by licensed egg collectors, were also analysed. The sample was comprised of 16 addled peregrine falcon (Falco peregrinus) eggs from throughout Britain, 11 addled merlin (Falco columbarius) eggs from various locations largely in northern Britain and 16 fresh gannet (Morus bassanus) eggs, half from Ailsa Craig (West Scotland) and half from Bass Rock (East Scotland). All samples were from the PBMS tissue archive and had been collected in 2002 or 2003. Samples from these years were used because this was a period for which we had a representative selection of samples for these different species, the samples had already been characterised for PCB concentrations as part of the PBMS monitoring, and 2002-3 was a period when other major PHAHs, such as polybrominated diphenyl ethers, were also still widely accumulated by piscivorous and terrestrial predatory birds (Crosse et al., 2012a,b; 2013). Livers were excised from carcasses and kept at $-20^\circ$C until analysed. The weight, length and
breadth of collected eggs were measured and the eggs were then blown or cracked open. The eggshells were washed, air-dried and reweighed, while the egg contents were homogenised.

1.2. Analysis

1.2.1. Extraction and clean-up
A sub-sample of each egg and liver (1-2 g) was thawed, weighed accurately, ground with sand, dried with anhydrous sodium sulphate, and cold extracted in 50 ml of 1:1 acetone:hexane (v/v). The solvent in half of the extract was evaporated and the lipid content determined gravimetrically. The other half of the extract was then re-dissolved in hexane and the lipids were removed from the extract using an alumina glass column packed with pre-treated alumina (4 h at 800°C) that had been deactivated with 5% deionised water (w/w). This clean extract was then subdivided and was used for the determination of TEQ concentrations, by chemical determination of PCBs and by CALUX assay.

1.2.2. PCB analysis
The suite of the 12 dioxin-like PCB congeners (77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169, 189) was analysed using gas chromatography with electron capture detection (GC-ECD). Prior to the GC analysis, dichlobenil was added as an internal standard. A 4 µl aliquot of each extract was injected into the gas chromatograph (Agilent, Wokingham, UK) using a splitless injector, and a 50m HT8 column (0.22mm internal diameter and 0.25µm film thickness, SGE Milton Keynes, UK). The injector temperature was set at 250°C and the carrier gas was helium (2.0 ml min⁻¹). The temperature programme was: isothermal at 50°C for 2 min, 45°C min⁻¹ to 200°C, 1.5°C min⁻¹ to 240°C, 2°C min⁻¹ to 285°C, 50°C min⁻¹ to 325°C and isothermal at 325°C for 10 min. The detector temperature was set at 335°C. Residues were quantified using the internal standard method and also using calibration curves with standards of the 12 PCBs (Greyhound Ltd, Birkenhead, UK).

For quality control and assurance purposes, a blank, a sample of an uncontaminated chicken egg (or liver) and second sample of chicken egg (or liver) were spiked with a known concentration of PCB congeners with each batch of egg or liver samples, respectively. Recovery values determined from the spiked samples varied between 75% and 110%. Limits of detection (LoD), determined from the calibration curve and based on the mean weight of the sample contents that were analysed, varied between 0.776 and 4.203 ng g⁻¹ lipid.
1.2.3. **Calux assay**

The cleaned-up extract was evaporated to dryness and transported to the Wageningen University for analysis by the CALUX assay. The dried extracts were re-dissolved in 100 µl of 1:1 acetone:hexane and mixed for one minute, after which 15 or 20 µl aliquots of DMSO were added to each egg or liver extract, respectively. The acetone:hexane was then evaporated. DR CALUX® gene assay was performed as described elsewhere (Murk et al., 1998). In short, *in-vitro* cultivated rat hepatoma cells (H4IIE-luc), transfected with a stable plasmid which carries luciferase gene of fireflies (*Photinus pyralis*) as a reporter gene, were seeded on a 96-well Packard ViewPlates in medium supplemented with hormone-stripped serum. The next day, the medium was replaced with medium containing the egg or liver extracts (0.1% DMSO). After 24 hours exposure, the medium was removed and the cells were lysed. The substrate luciferin was then added to the wells to quantify the amount of luciferase produced by the cells by measuring the amount of light. Bio-luminescence was measured using a luminometer, Luminoskan RS (Thermo Life Science), equipped with two internal injectors to inject the luciferine containing FlashMix, and the NaOH for signal quenching. All samples were corrected for the solvent DMSO signal. The measured luminescence was converted into a CALUX toxic equivalent (CALUX-TEQ) value by the direct comparison of the response for a given samples to a dose –response obtained with 2,3,7,8- tetrachlorodibenzo-\(p\)-dioxin (see Supporting Information). One single global biological response is thus measured by CALUX® for all AhR ligands present in the extract. The limit of detection for the assay was 0.30 pM TEQ and all samples exceeded this.

1.3. **Data analysis**

Individual coplanar PCB congener TEQs were calculated as the product of their designated Toxic Equivalence Factor (TEF) (Van den Berg et al., 2006) and their concentration; non-detected concentrations of individual congeners were assigned a zero value. \(\Sigma PC\)B-TEQ concentrations were calculated as the sum of the TEQs for the individual congeners. \(\Sigma PC\)B-TEQ concentrations and CALUX-TEQ concentrations are expressed in pg \(\Sigma PC\)B-TEQ g\(^{-1}\) lipid. Concentrations in eggs were not adjusted for desiccation as the purpose of this study was to compare \(\Sigma PC\)B-TEQs and CALUX-TEQs, not focus on reporting absolute concentrations, and any desiccation would have affected each measurement equally.
Summary data for ΣPCB-TEQs and CALUX-TEQs concentrations are presented as medians and inter-quartile ranges, and differences between ΣPCB-TEQs and CALUX-TEQs concentrations for each species were assessed by Mann-Whitney U tests. The relationships between the ΣPCB- and CALUX-TEQs concentrations in samples were determined using linear regressions on log-transformed data; the residuals of the analyses were all normally distributed.

2. Results

Between five and eight of the 12 PCB congeners that we analysed for were detected in the livers and eggs of each species. Congeners 118, 105, 156 and 167 were found in all six species (Figure 1) and were generally the congeners detected most frequently in all samples independently of the species; PCB 118 was detected in more than 80% of the samples analysed. In contrast, PCB congeners 81, 126 and 169 were never detected. All of the congeners that were detected in livers were also found in eggs except congener 77 which was only found in some sparrowhawk livers (Figure 1).

In terms of concentrations, ΣPCB-TEQs ranged between non-detected (ND) and 248 pg g⁻¹ lipid in eggs and between ND and 2102 pg g⁻¹ lipid in livers, equivalent to wet weight (ww) concentrations of ND-11.5 pg g⁻¹ and ND-130.6 pg g⁻¹, respectively. The corresponding ranges of the measured CALUX-TEQs in eggs and livers were 7-241 pg g⁻¹ lipid and 10-467 pg g⁻¹ lipid respectively, equivalent to 0.32-12 pg g⁻¹ ww and 0.6-29 pg g⁻¹ ww. There was no significant difference between the median ΣPCB-TEQ and CALUX TEQ (Figure 2) for gannet eggs, merlin eggs or heron livers (Mann-Whitney U tests, P>0.05 in each case), but the ΣPCB-TEQ was significantly lower than the CALUX-TEQ in peregrine eggs and in kestrel and sparrowhawk livers (Mann-Whitney U ≤ 36, P<0.001 in all cases). The dispersion in the data appeared to differ between eggs and livers (Figure 2). When the variances in the data were examined, there were differences between species for both CALUX-TEQs (Levene’s test = 5.64, P<0.001) and, to a lesser extent, for ΣPCB-TEQs (Levene’s test = 2.03, P=0.083). These differences may have been more related to matrix type than species as variances in both ΣPCB-TEQs and CALUX-TEQs were generally greater in livers than eggs.

When individual ΣPCB-TEQ and corresponding CALUX-TEQ measurements were compared, the relationship between the two was always statistically significant in the eggs (Figure 3). The gradient of the relationship was significantly less than one (upper 95% confidence limit: 0.329) with the result that ΣPCB-TEQ concentrations were similar to or exceeded CALUX-TEQs in more heavily contaminated
eggs but CALUX-TEQs exceeded ΣPCB-TEQs in eggs with lower levels of contamination. For example, ΣPCB-TEQs were some 63% (median value) higher than their corresponding CALUX-TEQs concentrations in gannet eggs that were relatively contaminated (log ΣPCB-TEQ >1), but made up only 10-14% of CALUX-TEQs in less contaminated samples (Figure 3). There was a similar but less marked pattern in merlin eggs, although ΣPCB-TEQs made up on average (median value) 82% of the CALUX-TEQ. PCB concentrations in the peregrine falcon eggs were lower than those in the eggs of the other two species (Figure 3) and, as with the less contaminated gannet eggs, ΣPCB-TEQs in the peregrine eggs comprised only a small proportion (median: 7%) of the CALUX-TEQ concentration. When the data for all the eggs were pooled and analysed together with species included in the model as a factor, there was a highly significant positive relationship between ΣPCB- and CALUX-TEQ concentrations (F(1,39) = 17.1, P<0.001) but species was not a significant factor in the model (F(2,39) = 2.18, P=0.13). The ΣPCB-TEQ concentrations explained 66% of the variance in the CALUX-TEQ concentrations and the relationship between ΣPCB- and CALUX-TEQ concentrations was similar between species (Figure 3). Overall, ΣPCB-TEQs were lower than CALUX-TEQs in the eggs of all three species until ΣPCB-TEQ concentrations were approximately >65 pg g⁻¹ lipid.

When liver CALUX- and ΣPCB-TEQ concentrations were compared for each species separately (Figure 4), linear regression models could not be fitted to the data. However, there was a significant positive correlation between the two measurements for sparrowhawks (Spearman Rank correlation coefficient r = 0.697, P=0.027) and weaker (0.05<P<0.1) positive associations for herons and kestrels (Figure 4). The associations between CALUX- and ΣPCB-TEQ concentrations were weaker in livers than eggs, partly as a result of the relatively high number of livers (mainly sparrowhawk and kestrel) with detectable CALUX-TEQ concentrations but no detectable ΣPCB-TEQs. These livers comprised 69% and 56% of the sparrowhawk and kestrel samples respectively although only 12% of the heron sample (Figure 4). When the liver data for all three species were pooled and were analysed by ANCOVA, there was a significant relationship between the two TEQ measurements (r² = 0.294, F(1,43) = 10.6, P=0.002) and species was a significant factor (F(2,43) = 3.79, P=0.13; Figure 4). As with the eggs, the gradient of the relationship was significantly less than one (upper 95% confidence limit: 0.256) and the extent of correspondence between the ΣPCB-TEQ and CALUX-TEQ concentrations increased with the level of PCB contamination (Figure 4). ΣPCB-TEQs and CALUX-TEQs reached parity when ΣPCB-TEQ concentrations were approximately 160 pg g⁻¹ lipid (kestrel and heron) and 320 pg g⁻¹ lipid (sparrowhawk). Although the relationship between the two TEQ measurements was significant,
ΣPCB-TEQs explained only 29.4 % of the variation in CALUX-TEQs, and 47% of the livers had non-detected ΣPCB-TEQs but CALUX-TEQs that ranged up to 355 pg g\(^{-1}\) lipid.

4. Discussion

The results from our study, in terms of the congener prevalence and TEQ concentrations, are similar to those reported in other species elsewhere. Generally, non dioxin-like PCB congeners, principally 153, 138 and 180 predominate in birds from a range of habits and trophic status (Alcock et al., 2002; Borlakoglu et al., 1990; Caccamise et al., 2012; Jemenez et al., 2007; Norstrom, 1988; Pereira et al., 2009a) and this was also the case in the present samples (data not shown). The prevalence of non-ortho and mono-ortho, dioxin-like, congeners varies with source, and possibly with trophic strategy and species-specific metabolism (Harris & Elliott 2011). The prevalence of congeners 118, 105, 156 and 167 in the present study was consistent with observations in 17 bird species from Japan (Senthilkumar et al., 2002), although we did not detect the two PCB congeners (81, 126) with the highest TEFs (0.1) and only found PCB 77 (TEF: 0.05) in about 10% of the sparrowhawk livers; these three congeners have been found in bald eagles (Haliaeetus leucocephalus) (Elliott et al., 1996), in various piscivorous bird species (Bosveld et al., 1995; Kubiak et al., 1989; Yamashita et al., 1993) and in Chinese birds of prey (Chen et al., 2009). The observed ΣPCB -TEQ concentrations in the current study were of the same order of magnitude of those measured in owl livers and American kestrel (Falco sparverius) eggs in the United States (Coady et al., 2001) and in albatross chicks from Pacific (Caccamise et al., 2012). However, concentrations in the present study were lower than those reported in some piscivorous species (Guruge et al., 2000; Schmutz et al., 2009), in raptors from SE Asia (Chen et al., 2009) and in belted kingfisher (Ceryle alcyon), spotted sandpiper (Actitis macularius), and tree swallow (Tachycineta bicolor) eggs in the USA (Custer et al., 2010). The lower concentrations in the present study perhaps reflect the fact that birds did not come from locations known to be particularly contaminated and also our samples did not contain detectable levels of high TEF congeners. CALUX-TEQ concentrations in our samples were a similar order of magnitude to those in kestrel eggs and owl livers from the USA (Coady et al., 2001) and similar to TEQ concentrations (determined by chemical analysis of multiple PHAHs) in fish eating birds such as white-tailed sea eagles (Haliaeetus albicilla) from Germany (Kannan et al., 2003) and double-crested cormorants
(Phalacrocorax auritus) and caspian terns (Hydroprogne caspia) from the USA (Yamashita et al., 1993).

It would not be expected that ΣPCB-TEQ and CALUX-TEQ concentrations would exactly correspond, even in the same sample, and this was the case in the present study in which median CALUX-TEQ concentrations were significantly greater than ΣPCB-TEQs in peregrine falcon eggs and in kestrel and sparrowhawk livers (Figure 2). PHAHs other than PCBs may contribute to the CALUX-TEQ. However, there can also be differences in the relative potency of congeners to the species used to generate the cells lines in the CALUX assay and the avian TEFs assigned to PCB congeners (Windal et al., 2005a) which adds some uncertainty to direct comparisons of the two measures. Examination of how variation in ΣPCB-TEQ concentrations affected CALUX-TEQ concentrations (Figures 3 and 4) was arguably more informative than simple comparisons of mean values. When data for eggs, were pooled, we found a strong and significant relationship between PCB- and CALUX-TEQs, as has been reported in blood plasma for both individual congener and summed TEQs (Murk et al., 1997, 1998), but there was not a 1:1 correspondence between ΣPCB-TEQ and CALUX-TEQ concentrations. When egg ΣPCB-TEQ concentrations were relatively low (taken as <65 pg g\(^{-1}\) lipid) the median contribution of ΣPCB-TEQs to the CALUX-TEQ concentration was only 10% (inter-quartile range: 5-29%). This could have been because other PHAHs may have been present in the eggs and exerted AhR mediated toxicity. It is also possible that dioxin-like PCBs were present below the analytical limit of detection and additively contributed to the detected CALUX-TEQs. The most potent congeners exert a relatively large influence on the ΣPCB-TEQ concentration because of their high TEFs and so uncertainties and errors in calculation of ΣPCB-TEQs are likely to be relatively large in samples with low concentrations of the more potent PCBs. In contrast to eggs with relatively low levels of PCB contamination, CALUX-TEQs in the more contaminated eggs were almost completely attributable to dioxin-like PCBs. In fact, ΣPCB-TEQs exceeded CALUX-TEQs in eggs with CALUX-TEQs >100 pg g\(^{-1}\) lipid. CALUX-TEQs can be influenced by antagonistic, additive and synergistic interactions (Schroijen et al., 2004) and one possibility is that antagonism between different PHAHs may have reduced the measured CALUX response but would not have been accounted for in the additive model used to calculate ΣPCB-TEQs (Sanderson et al., 1996). However, it would be necessary to measure all PHAHs in the samples to determine whether such interactions may have occurred.

The pattern of the relationship between ΣPCB-TEQs and CALUX-TEQs in livers was broadly similar to that in eggs. When data for all livers were pooled, there was a significant positive
relationship between the two measurements and ΣPCB-TEQs explained a relatively low percentage of the CALUX-TEQs in less contaminated samples. The relationship between ΣPCB-TEQs and CALUX-TEQs was weaker in livers than in eggs. This may have been because, compared with eggs, livers may contain a greater concentration of other PHAHs that exert AhR mediated toxicity and/or very low levels of a range of the more potent coplanar PCBs. Although comprehensive analysis of other PHAHs would have to be done to confirm this, the fact that predicted (log_{10}) CALUX concentration in samples that contained no detectable ΣPCB-TEQ concentration was higher in livers (Figure 4, 95% Confidence Limits: 1.80-2.06) than in eggs (Figure 3, 1.28 - 1.61 pg g^{-1} lipid) is consistent with livers containing more non-PCB PHAHs than eggs. Higher non-PCB related CALUX-TEQ concentrations in livers than eggs may be due to differences between the two matrix types in accumulation and retention of different xenobiotics. It may also be because concentrations in eggs reflect a limited period of exposure and metabolism in a restricted portion of the population (adult females during laying) whereas liver concentration can be the result of exposure over a different and wider time period and reflect metabolism by individuals that may vary in nutritional state, age and sex (Wienburg and Shore, 2004). The observed greater variability in CALUX and PCB-TEQ concentrations in livers than in eggs (Figure 2) is also consistent with the concept that liver CALUX-TEQ concentrations reflect more heterogenous exposure and accumulation of PHAHs.

Overall, for the species that we examined, egg and liver CALUX-TEQ concentrations greater than approximately 50-80 and 160-320 pg g^{-1} lipid respectively (equivalent approximately 2.5-4 and 10-20 pg g^{-1} ww) could be largely attributed to dioxin-like PCBs. Lowest Observable Effect TEQ concentrations in eggs of 10 -2200 pg g^{-1} ww have been suggested (AMAP, 1998) while more recently, a threshold egg concentration of 400-1600 pg g^{-1} ww has been mooted for the sensitive endpoint of ethoxyresorufin-O-deethylase (EROD) induction in wild bird hatchlings (Cohen-Barnhouse et al., 2008; Harris and Elliott, 2011). A liver LOEL of 25,000 pg g^{-1} lipid for induction of cytochrome P450 enzymes and reduction in plasma thyroxin levels, has been described for common tern (Sterna hirundo) chicks (Bosveld et al., 2000). These concentrations are between one and two orders of magnitude higher than the concentration above which coplanar PCBs became the major contributors to CALUX-TEQs in our samples. Our data therefore suggest that, of the compounds that are assimilated and retained within tissues or eggs in predatory birds and that mediate toxicity through the AhR, it is the dioxin-like PCBs that appear to be associated with TEQ concentrations that approach toxicologically significant levels. Other studies have likewise attributed CALUX activity in birds largely to dioxin-like
PCBs (Jemenez et al., 2007). However, we recognise that exposure to more readily metabolised compounds from diffuse sources, such as PAHs, may also result in toxicity mediated through the the AhR. These compounds may largely have gone undetected in the present study because residues are poorly assimilated in liver and eggs (Malcolm and Shore 2003, Pereira et al., 2009b), and so would not have been present in the tissue and egg extracts that we analysed. Furthermore, it is also possible that contamination from point sources by PHAHs may result in effects in birds that are spatially localised and so are difficult to detect without targeting sampling for analysis. However, where such localised impacts have been found and linked to PHAHs in Britain (Thompson et al., 2006), dioxin-like PCBs were still found to account for most (77-92%) of the ∑TEQ concentrations.

In conclusion, our results are consistent with the premise that, in predatory birds sampled at a broad spatial scale across Britain, low level TEQ concentrations in eggs and livers may largely arise from exposure to non-PCB PHAHs and/or trace levels of dioxin-like PCBs that are present below analytical detection limits. However, dioxin-like PCB congeners appear to be the major contributors to TEQ concentrations in the more heavily contaminated eggs and livers. Although samples used in this study were collected in 2002 and 2003, there has been no clear decline since then in PCB contamination across Britain in many of the species we examined (Pereira et al., 2009a; Walker et al., 2011). Thus, there is no a priori reason to believe conclusion drawn from these samples do not remain valid. We conclude that measurement of dioxin-like PCBs, as part of wide-scale monitoring in Britain, is likely to be adequate for detecting toxicologically significant TEQ concentrations in bird eggs and carcasses. Such measurements are likely to underestimate TEQ concentrations in less contaminated samples however. A full suite of chemical analysis for PHAHs, coupled with bioassay measurements such as CALUX, would be necessary to determine actual exposures and their contribution to AhR mediated toxicity in such samples and in samples where there has been contamination from major point sources of unkown PHAHs.

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**Figure Legends**

Figure 1. Percentage of gannet (n=16), merlin (n=11) and peregrine falcon (n = 16) eggs and kestrel, sparrowhawk and heron livers (n=16 for each species) in which each coplanar PCBs was detected.

Figure 2. Median (±) inter-quartile range concentrations in (pg/g lipid) gannet (n=16), merlin (n=11) and peregrine falcon (n=16) eggs and in kestrel, sparrowhawk and heron livers (n=16 for each species) as determined by CALUX assay and by chemical measurement of planar PCB congeners. *** P<0.001.

Figure 3. Comparison of log (+1) ΣPCB-TEQ concentrations and log CALUX-TEQ concentrations for gannet, merlin and peregrine falcon eggs. The relationships between the two measures, as determined by linear regression models, are indicated by the regression lines and their 95% confidence limits. When data for all eggs was pooled, analysis was by ANCOVA (see text for details)
Figure 4. Comparison of log (+1) $\Sigma$PCB-TEQ concentrations and log CALUX-TEQ concentrations for sparrowhawk, kestrel and heron livers. The relationship between the two measures, as determined by linear regression models, was only statistically determined for pooled data (see text for details).
Figure 1

% of samples with detected concentrations

- Gannet eggs
- Sparrowhawk livers
- Merlin eggs
- Kestrel livers
- Peregrine eggs
- Heron livers
Figure 2

Median (± IQR) TEQ (pg g⁻¹ lipid)

- PCB-TEQ
- CALUX-TEQ

Species:
- gannet
- merlin
- peregrine
- kestrel
- sprwhwk
- heron

Significance:
- ***
Figure 3

Peregrine: $r^2 = 0.297$, $F_{(1,14)} = 5.92$, $P = 0.029$

Merlin: $r^2 = 0.404$, $F_{(1,9)} = 6.12$, $P = 0.036$

Gannet: $r^2 = 0.298$, $F_{(1,14)} = 5.92$, $P = 0.029$

Pooled data

**Log-log plot**

- Log of $\sum$PCB-TEQ + 1 (pg g$^{-1}$ lipid) vs. Log of CALUX (pg g$^{-1}$ lipid)

- PEREGRINE
- MERLIN
- GANNET
- POOLED DATA

Line of equality

- PEREGRINE
- MERLIN
- GANNET
- POOLED DATA
Figure 4

- **SPARROWHAWK**
- **KESTREL**
- **HERON**
- **POOLED DATA**

Log PCB-TEQ +1 (pg g\(^{-1}\) lipid) vs. log CALUX (pg g\(^{-1}\) lipid) for different bird species. Each species has its own line of equality indicating the expected log-log relationship. The pooled data combines all species, showing a trend close to the line of equality.
Supporting information S1

Introduction
The DR-Calux assay, for detecting dioxin (TCDD) and dioxin-like contaminants in environmental matrices, is based on rat-hepatoma cells (H4IIE) transfected with a stable plasmide which carries the luciferase gene of fireflies (Photinus pyralis) as a reporter gene. In the presence of dioxin-like compounds these transfected cells produce the enzyme luciferase. The luciferase thus formed reacts with added luciferine under production of light. The quantity of light produced can be measured in a luminometer. The luciferase production is used to calculate a TEQ value (TCDD-equivalent) with the aid of an TCDD-standard curve. The TEQ-value is based on the total amount of dioxin-like compounds, including furanes, PCB’s, PBB’s, PBDE’s, etc.

This method was developed at the Toxicology Section of Wageningen University and Research Centre, and validated for samples of whole blood, blood plasma, tissues (liver, muscle) and sediments.

Sample preparation
Samples were initially resolved in 100 µl of a 1:1 mixture of acetone and hexane in each vial. The contents was mixed well for 1 minute. After adding 15 µl DMSO for all egg-samples, 20 µl DMSO for all liver samples (except XE010-1 and XE013-1, these were dissolved in 15 µl ) and 100 µl DMSO for spiked samples, the acetone/hexane was evaporated under gentle flow of nitrogen gas.

Calux-assay
The assay used in-vitro cultivated H4IIE-luc cells, plated on 96-well Packard ViewPlates. Cells were grown on the plates for 24 hours until a confluent layer was reached. Sample dilutions were prepared in culture medium. Cells were exposed to the sample dilutions for 24 hours (0.4 % of DMSO). After cell lysis, according to assay protocol, the cells were stored at –80°C until measurement.

Luminescence measurement
Bio-luminescence was measured using a Luminoskan RS from Thermo Life Science, equipped with two internal injectors to inject the luciferine containing FlashMix, and the NaOH for signal quenching.

Calculations
All samples were corrected for the solvent DMSO signal. A standard graph (Figure S1) was used to interpolate the TEQ’s (pM) from the measured Relative Light Units (RLU’s). Each plate contained a 0 and a 10 pM calibration sample, which was used for plate-to-plate correction. The difference between the calculated EC50 from the standard graph and the validated EC50 concentration for TCDD (=10 pM) was used to correct data.

Samples were measured in two different dilutions, in calculating the final results always the dilution with RLU between the RLU’s of 1 pM and 10 pM TCDD was used. When samples were measured on more than one plate, the average of the results was used for further calculation.
Figure S1. Dose response curve used in CALUX assay to estimate TCDD equivalents in samples.

EC50 correction
10 / 20.2 = 0.495 (0.5)