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

3

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36 **Abstract**

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

49

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

52

53 Introduction

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

65 PHAHs are typically found in complex mixtures in environmental matrices. The commonality in
66 their mode of action means that the toxicity of individual PHAHs can be additive. The chemical-
67 activated luciferase gene expression (CALUX) bioassay has been developed to quantify the overall
68 toxicity caused by simultaneous exposure to multiple compounds that exert toxicity via the *AhR*. This
69 reporter gene assay is based on *AhR*-mediated firefly (*Photinus pyralis*) luciferase expression in
70 genetically modified cell lines (Aarts et al., 1995; Garrison et al., 1996). A vector containing the
71 luciferase gene under transcriptional control of dioxin-responsive elements has been stably
72 transfected into a number of cell lines, including the rat H4IIE hepatoma cell line (Aarts et al., 1995;
73 Garrison et al., 1996; Richter et al., 1997). CALUX has been used widely for screening and quantifying
74 the toxicity of dioxin-like compounds in blood (Van Wouwe et al., 2004; Ziccardi et al., 2000),
75 sediments (Behnisch et al., 2002; Stronkhorst et al., 2002), various marine matrices (Tsutsumi et al.,
76 2003; Windal et al., 2005) and bovine milk (Bovee et al., 1998).

77 The occurrence of PCB concentrations in the livers and eggs of many different bird species has
78 been well documented (Harris and Elliott, 2011). Some PCB congeners (non-*ortho*, and to a lesser
79 extent some mono-*ortho* substituted congeners) have a coplanar dioxin-like configuration. These
80 have a high affinity for the *AhR* and so exert similar toxic effects to other PHAHs that act via the *AhR*.
81 The potencies of different dioxin-like PCB congeners are between one and five orders of magnitude
82 lower than that of 2,3,7,8 -tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic dioxin (Ahlborg et al.,

83 1992; Van den Berg et al., 1998, 2006). Because accumulation of PCBs typically exceeds that of other
84 more potent PHAHs, such as dioxins and furans (Jemenez et al., 2007), it is often considered that PCBs
85 are likely to be the main contributors to *AhR* mediated toxicity unless there are particular localised
86 sources of other compounds. However, there is a general lack of quantitative evidence to support this
87 contention, not is it clear how variation or future changes in PCB contamination are likely to affect
88 *AhR* mediated toxicity. The aim of the present study was therefore to investigate the extent to which
89 dioxin-like PCBs in predatory birds account for their total TEQ concentrations. This involved measuring
90 PCB concentrations chemically in the eggs and livers of various predatory birds and comparing the
91 resultant calculated summed toxic equivalence ($\sum\text{PCB-TEQ}$) with that estimated for all PHAHs as
92 measured by CALUX assay (CALUX-TEQ)

93

94 **1. Materials and Methods**

95 **1.1. Sample collection**

96 Livers were obtained from bird carcasses sent in by members of the public to the Predatory Bird
97 Monitoring Scheme (PBMS: <http://pbms.ceh.ac.uk/>), a long-term UK chemical surveillance and
98 monitoring programme that uses birds of prey as sentinels of exposure (Walker et al., 2008). In all, 48
99 livers were used for the present study and were from 16 sparrowhawk (*Accipiter nisus*), 16 kestrel
100 (*Falco tinnunculus*) and 16 heron (*Ardea cinerea*) carcasses that had been found at various locations
101 from across Britain. A collection of 44 eggs, each from a separate nest and collected for the PBMS by
102 licensed egg collectors, were also analysed. The sample was comprised of 16 addled peregrine falcon
103 (*Falco peregrinus*) eggs from throughout Britain, 11 addled merlin (*Falco columbarius*) eggs from
104 various locations largely in northern Britain and 16 fresh gannet (*Morus bassanus*) eggs, half from
105 Ailsa Craig (West Scotland) and half from Bass Rock (East Scotland). All samples were from the PBMS
106 tissue archive and had been collected in 2002 or 2003. Samples from these years were used because
107 this was a period for which we had a representative selection of samples for these different species,
108 the samples had already been characterised for PCB concentrations as part of the PBMS monitoring,
109 and 2002-3 was a period when other major PHAHs, such as polybrominated diphenyl ethers, were
110 also still widely accumulated by piscivorous and terrestrial predatory birds (Crosse et al., 2012a,b;
111 2013). Livers were excised from carcasses and kept at -20⁰C until analysed. The weight, length and

112 breadth of collected eggs were measured and the eggs were then blown or cracked open. The
113 eggshells were washed, air-dried and reweighed, while the egg contents were homogenised.

114

115 **1.2. Analysis**

116 **1.2.1. Extraction and clean-up**

117 A sub- sample of each egg and liver (1-2 g) was thawed, weighed accurately, ground with sand, dried
118 with anhydrous sodium sulphate, and cold extracted in 50 ml of 1:1 acetone:hexane (v/v). The solvent
119 in half of the extract was evaporated and the lipid content determined gravimetrically. The other half
120 of the extract was then re-dissolved in hexane and the lipids were removed from the extract using an
121 alumina glass column packed with pre-treated alumina (4 h at 800°C) that had been deactivated with
122 5% deionised water (w/w). This clean extract was then subdivided and was used for the
123 determination of TEQ concentrations, by chemical determination of PCBs and by CALUX assay.

124

125 **1.2.2. PCB analysis**

126 The suite of the 12 dioxin-like PCB congeners (77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169,
127 189) was analysed using gas chromatography with electron capture detection (GC-ECD). Prior to the
128 GC analysis, dichlobenil was added as an internal standard. A 4 µl aliquot of each extract was injected
129 into the gas chromatograph (Agilent, Wokingham, UK) using a splitless injector, and a 50m HT8 column
130 (0.22mm internal diameter and 0.25µm film thickness, SGE Milton Keynes, UK). The injector
131 temperature was set at 250°C and the carrier gas was helium (2.0 ml min⁻¹). The temperature
132 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
133 285°C, 50°C min⁻¹ to 325°C and isothermal at 325°C for 10 min. The detector temperature was set at
134 335°C. Residues were quantified using the internal standard method and also using calibration curves
135 with standards of the 12 PCBs (Greyhound Ltd, Birkenhead, UK).

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

142

143 **1.2.3. Calux assay**

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

162

163 **1.3. Data analysis**

164 Individual coplanar PCB congener TEQs were calculated as the product of their designated Toxic
165 Equivalence Factor (TEF) (Van den Berg et al., 2006) and their concentration; non-detected
166 concentrations of individual congeners were assigned a zero value. $\Sigma\text{PCB-TEQ}$ concentrations were
167 calculated as the sum of the TEQs for the individual congeners. $\Sigma\text{PCB-TEQ}$ concentrations and CALUX-
168 TEQ concentrations are expressed in $\text{pg } \Sigma\text{PCB-TEQ g}^{-1}$ lipid. Concentrations in eggs were not adjusted
169 for desiccation as the purpose of this study was to compare $\Sigma\text{PCB-TEQs}$ and CALUX-TEQs, not focus on
170 reporting absolute concentrations, and any desiccation would have affected each measurement
171 equally.

172 Summary data for Σ PCB-TEQs and CALUX-TEQs concentrations are presented as medians and
173 inter-quartile ranges, and differences between Σ PCB-TEQs and CALUX-TEQs concentrations for each
174 species were assessed by Mann-Whitney U tests. The relationships between the Σ PCB- and CALUX-
175 TEQs concentrations in samples were determined using linear regressions on log-transformed data;
176 the residuals of the analyses were all normally distributed.

177

178 2. Results

179 Between five and eight of the 12 PCB congeners that we analysed for were detected in the
180 livers and eggs of each species. Congeners 118, 105, 156 and 167 were found in all six species (Figure
181 1) and were generally the congeners detected most frequently in all samples independently of the
182 species; PCB 118 was detected in more than 80% of the samples analysed. In contrast, PCB congeners
183 81, 126 and 169 were never detected. All of the congeners that were detected in livers were also
184 found in eggs except congener 77 which was only found in some sparrowhawk livers (Figure 1).

185 In terms of concentrations, Σ PCB-TEQs ranged between non-detected (ND) and 248 pg g^{-1} lipid
186 in eggs and between ND and 2102 pg g^{-1} lipid in livers, equivalent to wet weight (ww) concentrations
187 of ND-11.5 pg g^{-1} and ND-130.6 pg g^{-1} , respectively. The corresponding ranges of the measured
188 CALUX-TEQs in eggs and livers were 7-241 pg g^{-1} lipid and 10-467 pg g^{-1} lipid respectively, equivalent
189 to 0.32-12 pg g^{-1} ww and 0.6-29 pg g^{-1} ww. There was no significant difference between the median
190 Σ PCB-TEQ and CALUX TEQ (Figure 2) for gannet eggs, merlin eggs or heron livers (Mann-Whitney U
191 tests, $P > 0.05$ in each case), but the Σ PCB-TEQ was significantly lower than the CALUX-TEQ in peregrine
192 eggs and in kestrel and sparrowhawk livers (Mann-Whitney U ≤ 36 , $P < 0.001$ in all cases). The
193 dispersion in the data appeared to differ between eggs and livers (Figure 2). When the variances in
194 the data were examined, there were differences between species for both CALUX-TEQs (Levene's test
195 = 5.64, $P < 0.001$) and, to a lesser extent, for Σ PCB-TEQs (Levene's test = 2.03, $P = 0.083$). These
196 differences may have been more related to matrix type than species as variances in both Σ PCB-TEQs
197 and CALUX-TEQs were generally greater in livers than eggs.

198 When individual Σ PCB-TEQ and corresponding CALUX-TEQ measurements were compared, the
199 relationship between the two was always statistically significant in the eggs (Figure 3). The gradient of
200 the relationship was significantly less than one (upper 95% confidence limit: 0.329) with the result
201 that Σ PCB-TEQ concentrations were similar to or exceeded CALUX-TEQs in more heavily contaminated

202 eggs but CALUX-TEQs exceeded Σ PCB-TEQs in eggs with lower levels of contamination. For example,
203 Σ PCB-TEQs were some 63% (median value) higher than their corresponding CALUX-TEQs
204 concentrations in gannet eggs that were relatively contaminated ($\log \Sigma$ PCB-TEQ >1), but made up only
205 10-14% of CALUX-TEQs in less contaminated samples (Figure 3). There was a similar but less marked
206 pattern in merlin eggs, although Σ PCB-TEQs made up on average (median value) 82% of the CALUX-
207 TEQ. PCB concentrations in the peregrine falcon eggs were lower than those in the eggs of the other
208 two species (Figure 3) and, as with the less contaminated gannet eggs, Σ PCB-TEQs in the peregrine
209 eggs comprised only a small proportion (median: 7%) of the CALUX-TEQ concentration. When the
210 data for all the eggs were pooled and analysed together with species included in the model as a
211 factor, there was a highly significant positive relationship between Σ PCB- and CALUX-TEQ
212 concentrations ($F_{(1,39)} = 17.1$, $P < 0.001$) but species was not a significant factor in the model ($F_{(2,39)} =$
213 2.18 , $P = 0.13$). The Σ PCB-TEQ concentrations explained 66% of the variance in the CALUX-TEQ
214 concentrations and the relationship between Σ PCB- and CALUX-TEQ concentrations was similar
215 between species (Figure 3). Overall, Σ PCB-TEQs were lower than CALUX-TEQs in the eggs of all three
216 species until Σ PCB-TEQ concentrations were approximately $>65 \text{ pg g}^{-1}$ lipid.

217 When liver CALUX- and Σ PCB-TEQ concentrations were compared for each species separately
218 (Figure 4), linear regression models could not be fitted to the data. However, there was a significant
219 positive correlation between the two measurements for sparrowhawks (Spearman Rank correlation
220 coefficient $r = 0.697$, $P = 0.027$) and weaker ($0.05 < P < 0.1$) positive associations for herons and kestrels
221 (Figure 4). The associations between CALUX- and Σ PCB-TEQ concentrations were weaker in livers than
222 eggs, partly as a result of the relatively high number of livers (mainly sparrowhawk and kestrel) with
223 detectable CALUX-TEQ concentrations but no detectable Σ PCB-TEQs. These livers comprised 69% and
224 56% of the sparrowhawk and kestrel samples respectively although only 12% of the heron sample
225 (Figure 4). When the liver data for all three species were pooled and were analysed by ANCOVA,
226 there was a significant relationship between the two TEQ measurements ($r^2 = 0.294$, $F_{(1,43)} = 10.6$,
227 $P = 0.002$) and species was a significant factor ($F_{(2,43)} = 3.79$, $P = 0.13$; Figure 4). As with the eggs, the
228 gradient of the relationship was significantly less than one (upper 95% confidence limit: 0.256) and
229 the extent of correspondence between the Σ PCB-TEQ and CALUX-TEQ concentrations increased with
230 the level of PCB contamination (Figure 4). Σ PCB-TEQs and CALUX-TEQs reached parity when Σ PCB-TEQ
231 concentrations were approximately 160 pg g^{-1} lipid (kestrel and heron) and 320 pg g^{-1} lipid
232 (sparrowhawk). Although the relationship between the two TEQ measurements was significant,

233 ΣPCB-TEQs explained only 29.4 % of the variation in CALUX-TEQs, and 47% of the livers had non-
234 detected ΣPCB-TEQs but CALUX-TEQs that ranged up to 355 pg g⁻¹ lipid.

235

236 4. Discussion

237 The results from our study, in terms of the congener prevalence and TEQ concentrations, are
238 similar to those reported in other species elsewhere. Generally, non dioxin-like PCB congeners,
239 principally 153, 138 and 180 predominate in birds from a range of habits and trophic status (Alcock et
240 al., 2002; Borlakoglu et al., 1990; Caccamise et al., 2012; Jemenez et al., 2007; Norstrom, 1988;
241 Pereira et al., 2009a) and this was also the case in the present samples (data not shown). The
242 prevalence of non-*ortho* and mono-*ortho*, dioxin-like, congeners varies with source, and possibly with
243 trophic strategy and species-specific metabolism (Harris & Elliott 2011). The prevalence of congeners
244 118, 105, 156 and 167 in the present study was consistent with observations in 17 bird species from
245 Japan (Senthilkumar et al., 2002), although we did not detect the two PCB congeners (81, 126) with
246 the highest TEFs (0.1) and only found PCB 77 (TEF: 0.05) in about 10% of the sparrowhawk livers;
247 these three congeners have been found in bald eagles (*Haliaeetus leucocephalus*) (Elliott et al., 1996),
248 in various piscivorous bird species (Bosveld et al., 1995; Kubiak et al., 1989; Yamashita et al., 1993)
249 and in Chinese birds of prey (Chen et al., 2009). The observed ΣPCB -TEQ concentrations in the current
250 study were of the same order of magnitude of those measured in owl livers and American kestrel
251 (*Falco sparverius*) eggs in the United States (Coady et al., 2001) and in albatross chicks from Pacific
252 (Caccamise et al., 2012). However, concentrations in the present study were lower than those
253 reported in some piscivorous species (Guruge et al., 2000; Schmutz et al., 2009), in raptors from SE
254 Asia (Chen et al., 2009) and in belted kingfisher (*Ceryle alcyon*), spotted sandpiper (*Actitis macularius*),
255 and tree swallow (*Tachycineta bicolor*) eggs in the USA (Custer et al., 2010). The lower concentrations
256 in the present study perhaps reflect the fact that birds did not come from locations known to be
257 particularly contaminated and also our samples did not contain detectable levels of high TEF
258 congeners. CALUX-TEQ concentrations in our samples were a similar order of magnitude to those in
259 kestrel eggs and owl livers from the USA (Coady et al., 2001) and similar to TEQ concentrations
260 (determined by chemical analysis of multiple PHAHs) in fish eating birds such as white-tailed sea
261 eagles (*Haliaeetus albicilla*) from Germany (Kannan et al., 2003) and double-crested cormorants

262 (*Phalacrocorax auritus*) and caspian terns (*Hydroprogne caspia*) from the USA (Yamashita et al.,
263 1993).

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

291 The pattern of the relationship between Σ PCB-TEQs and CALUX-TEQs in livers was broadly
292 similar to that in eggs. When data for all livers were pooled, there was a significant positive

293 relationship between the two measurements and Σ PCB-TEQs explained a relatively low percentage of
294 the CALUX-TEQs in less contaminated samples. The relationship between Σ PCB-TEQs and CALUX-TEQs
295 was weaker in livers than in eggs. This may have been because, compared with eggs, livers may
296 contain a greater concentration of other PHAHs that exert AhR mediated toxicity and/or very low
297 levels of a range of the more potent coplanar PCBs. Although comprehensive analysis of other PHAHs
298 would have to be done to confirm this, the fact that predicted (\log_{10}) CALUX concentration in samples
299 that contained no detectable Σ PCB-TEQ concentration was higher in livers (Figure 4, 95% Confidence
300 Limits: 1.80-2.06) than in eggs (Figure 3, 1.28 - 1.61 pg g^{-1} lipid) is consistent with livers containing
301 more non-PCB PHAHs than eggs. Higher non-PCB related CALUX-TEQ concentrations in livers than
302 eggs may be due to differences between the two matrix types in accumulation and retention of
303 different xenobiotics. It may also be because concentrations in eggs reflect a limited period of
304 exposure and metabolism in a restricted portion of the population (adult females during laying)
305 whereas liver concentration can be the result of exposure over a different and wider time period and
306 reflect metabolism by individuals that may vary in nutritional state, age and sex (Wienburg and Shore,
307 2004). The observed greater variability in CALUX and PCB-TEQ concentrations in livers than in eggs
308 (Figure 2) is also consistent with the concept that liver CALUX-TEQ concentrations reflect more
309 heterogenous exposure and accumulation of PHAHs.

310 Overall, for the species that we examined, egg and liver CALUX-TEQ concentrations greater
311 than approximately 50-80 and 160-320 pg g^{-1} lipid respectively (equivalent approximately 2.5-4 and
312 10-20 pg g^{-1} ww) could be largely attributed to dioxin-like PCBs. Lowest Observable Effect TEQ
313 concentrations in eggs of 10 -2200 pg g^{-1} ww have been suggested (AMAP, 1998) while more recently,
314 a threshold egg concentration of 400-1600 pg g^{-1} ww has been mooted for the sensitive endpoint of
315 ethoxyresorufin-O-deethylase (EROD) induction in wild bird hatchlings (Cohen-Barnhouse et al., 2008;
316 Harris and Elliott, 2011). A liver LOEL of 25,000 pg g^{-1} lipid for induction of cytochrome P450 enzymes
317 and reduction in plasma thyroxin levels, has been described for common tern (*Sterna hirundo*) chicks
318 (Bosveld et al., 2000). These concentrations are between one and two orders of magnitude higher
319 than the concentration above which coplanar PCBs became the major contributors to CALUX-TEQs in
320 our samples. Our data therefore suggest that, of the compounds that are assimilated and retained
321 within tissues or eggs in predatory birds and that mediate toxicity through the AhR, it is the dioxin-
322 like PCBs that appear to be associated with TEQ concentrations that approach toxicologically
323 significant levels. Other studies have likewise attributed CALUX activity in birds largely to dioxin-like

324 PCBs (Jemenez et al., 2007). However, we recognise that exposure to more readily metabolised
325 compounds from diffuse sources, such as PAHs, may also result in toxicity mediated through the the
326 AhR. These compounds may largely have gone undetected in the present study because residues are
327 poorly assimilated in liver and eggs (Malcolm and Shore 2003, Pereira et al., 2009b), and so would not
328 have been present in the tissue and egg extracts that we analysed. Furthermore, it is also possible
329 that contamination from point sources by PHAHs may result in effects in birds that are spatially
330 localised and so are difficult to detect without targeting sampling for analysis. However, where such
331 localised impacts have been found and linked to PHAHs in Britain (Thompson et al., 2006), dioxin-like
332 PCBs were still found to account for most (77-92%) of the Σ TEQ concentrations.

333 In conclusion, our results are consistent with the premise that, in predatory birds sampled at a
334 broad spatial scale across Britain, low level TEQ concentrations in eggs and livers may largely arise
335 from exposure to non-PCB PHAHs and/or trace levels of dioxin-like PCBs that are present below
336 analytical detection limits. However, dioxin-like PCB congeners appear to be the major contributors
337 to TEQ concentrations in the more heavily contaminated eggs and livers. Although samples used in
338 this study were collected in 2002 and 2003, there has been no clear decline since then in PCB
339 contamination across Britain in many of the species we examined (Pereira et al., 2009a; Walker et al.,
340 2011). Thus, there is no *a priori* reason to believe conclusion drawn from these samples do not remain
341 valid. We conclude that measurement of dioxin-like PCBs, as part of wide-scale monitoring in Britain,
342 is likely to be adequate for detecting toxicologically significant TEQ concentrations in bird eggs and
343 carcasses. Such measurements are likely to underestimate TEQ concentrations in less contaminated
344 samples however. A full suite of chemical analysis for PHAHs, coupled with bioassay measurements
345 such as CALUX, would be necessary to determine actual exposures and their contribution to AhR
346 mediated toxicity in such samples and in samples where there has been contamination from major
347 point sources of unknown PHAHs.

348

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356

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523 **Figure Legends**

524

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

527

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

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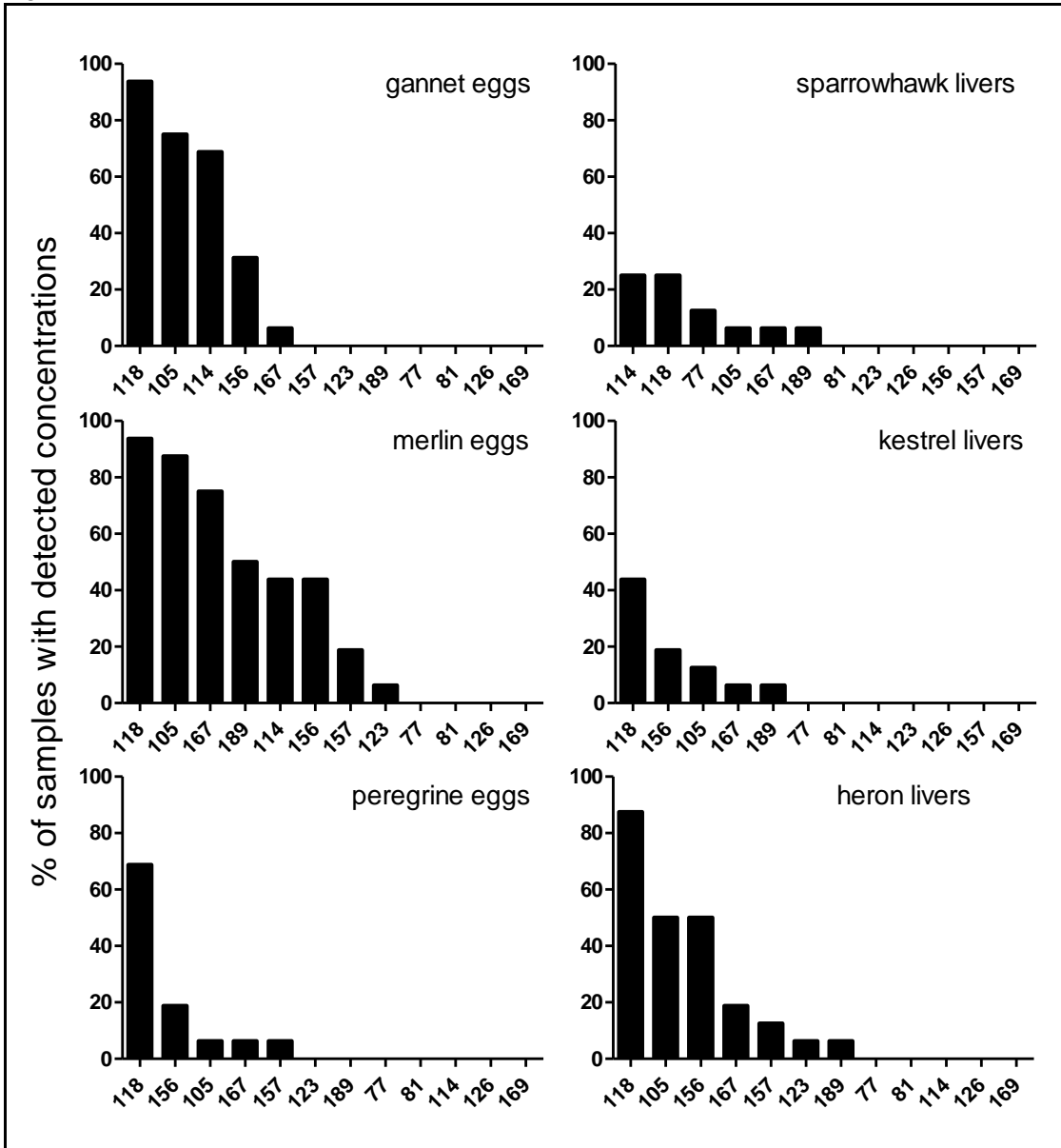
532 Figure 3. Comparison of log (+1) Σ PCB-TEQ concentrations and log CALUX-TEQ concentrations for
533 gannet, merlin and peregrine falcon eggs. The relationships between the two measures, as
534 determined by linear regression models, are indicated by the regression lines and their 95%
535 confidence limits. When data for all eggs was pooled, analysis was by ANCOVA (see text for details)

536

537 Figure 4. Comparison of log (+1) Σ PCB-TEQ concentrations and log CALUX-TEQ concentrations for
538 sparrowhawk, kestrel and heron livers. The relationship between the two measures, as determined by
539 linear regression models, was only statistically determined for pooled data (see text for details).

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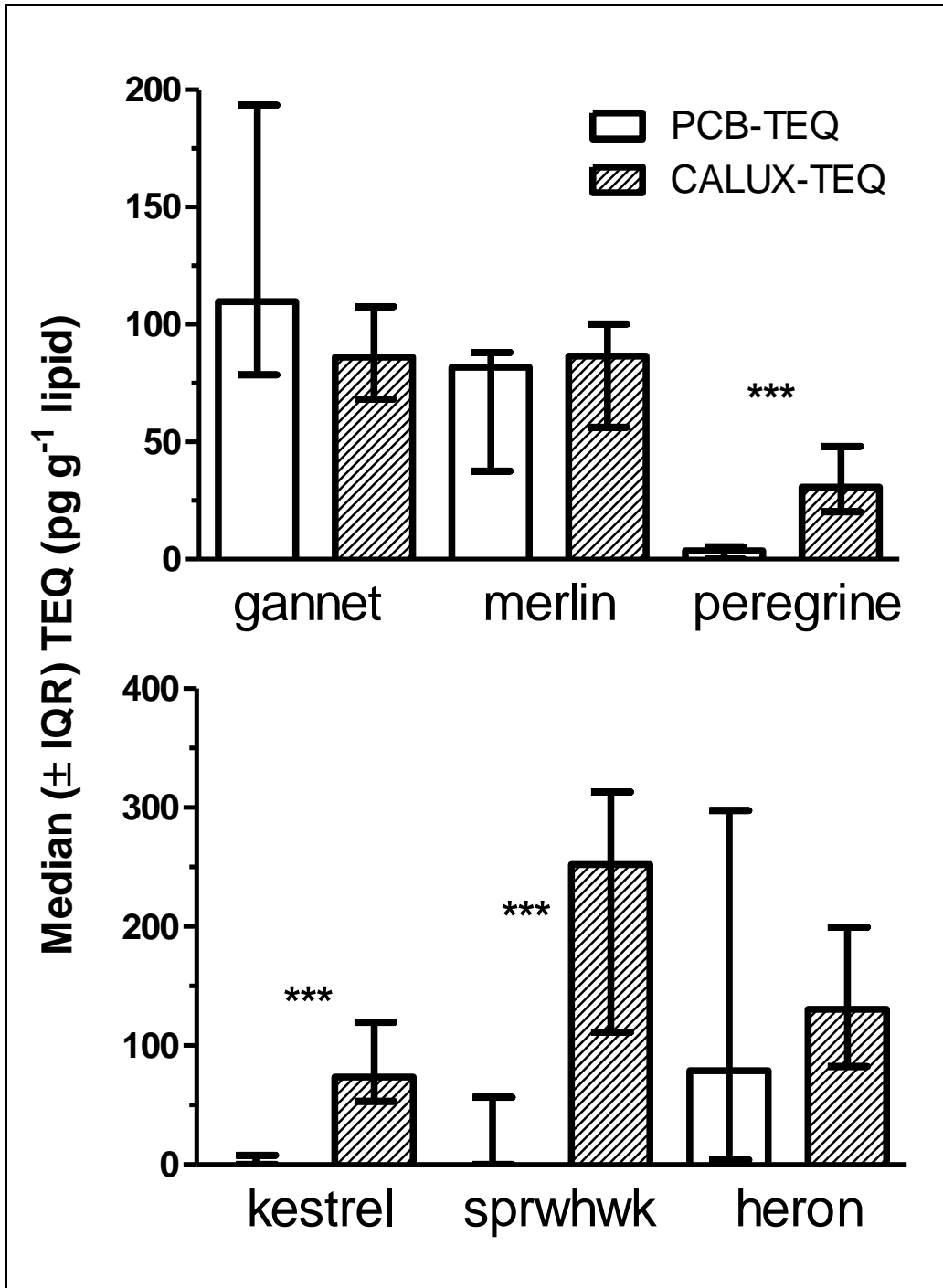
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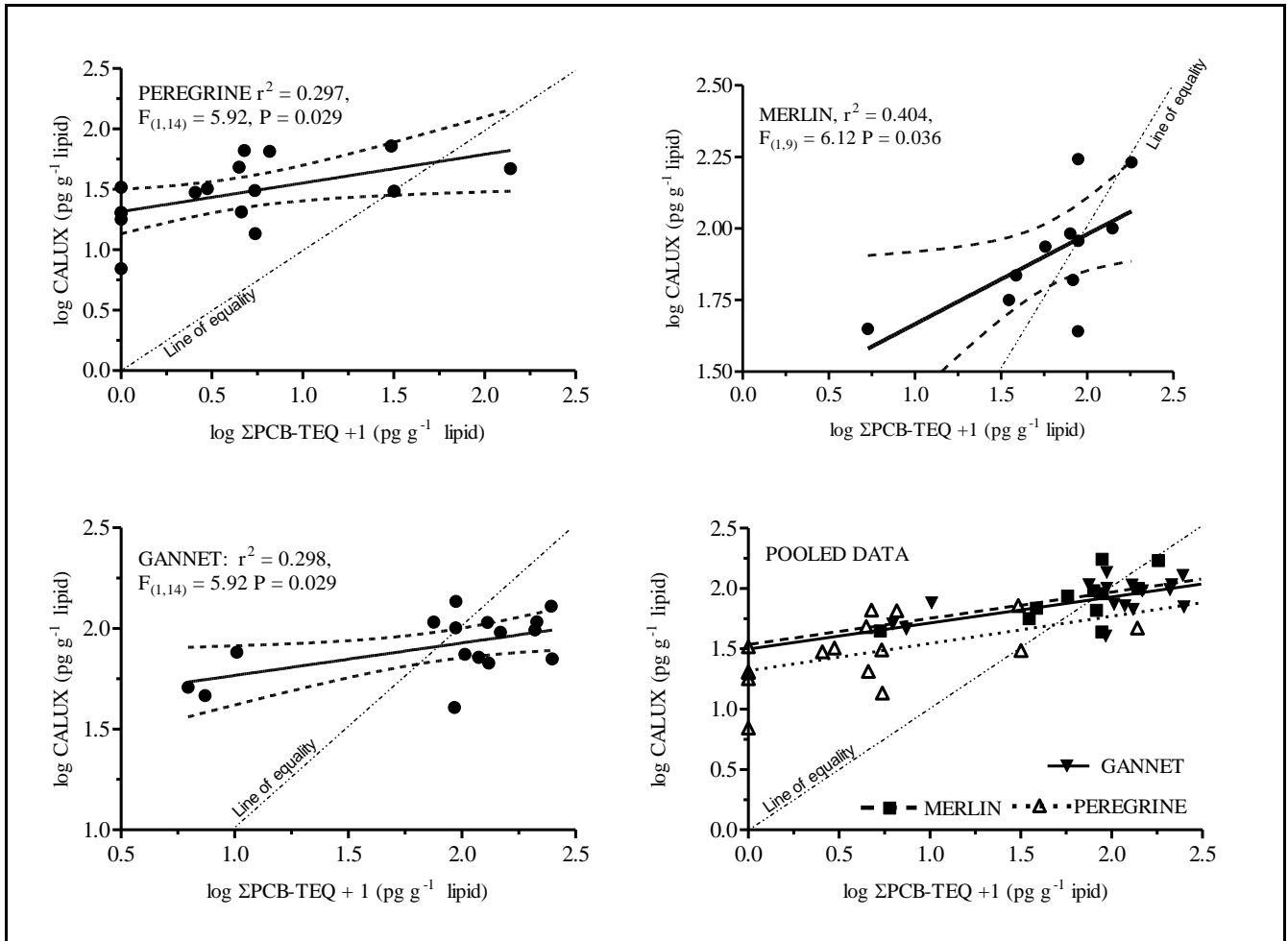
Figure 2



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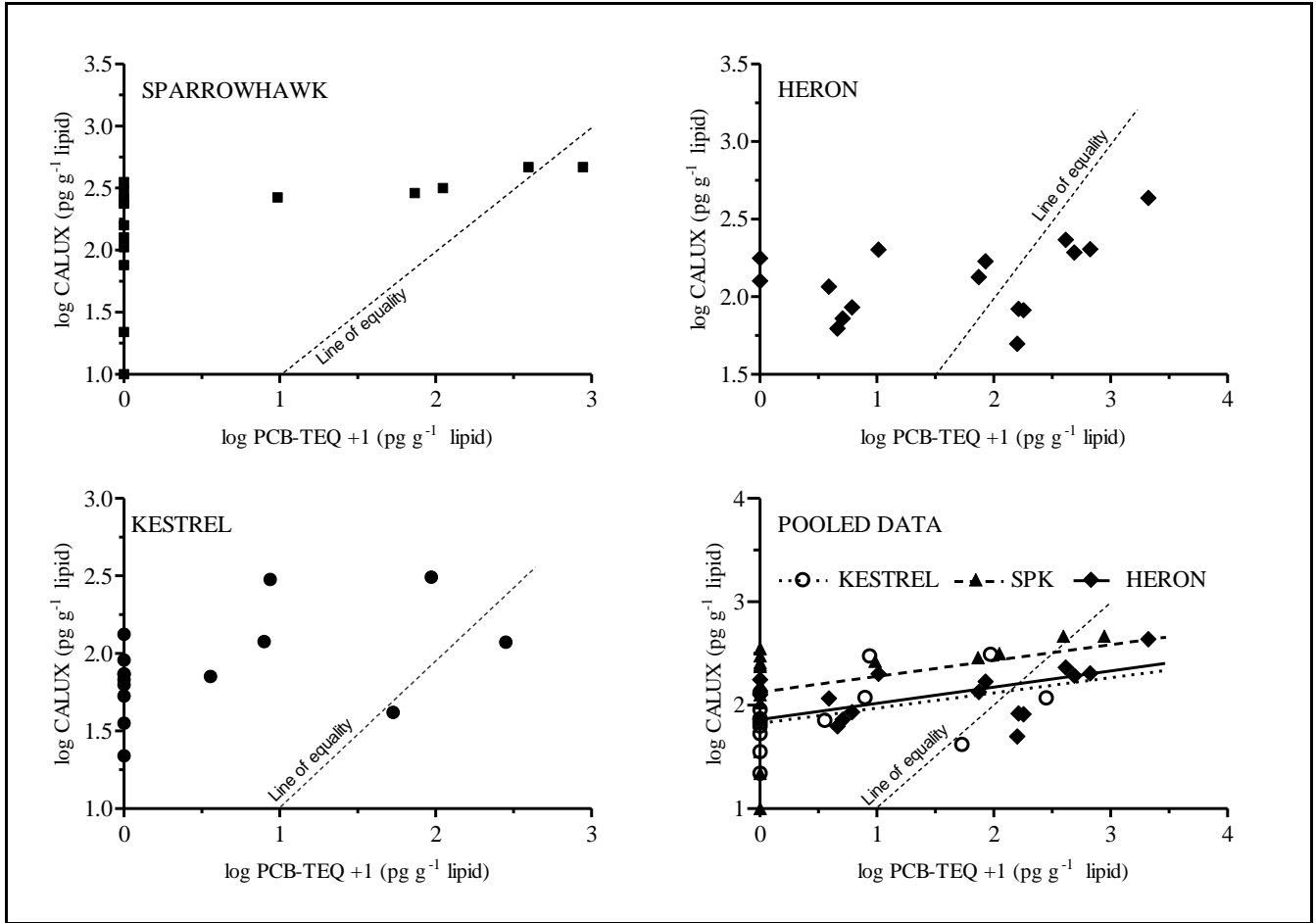
Figure 3



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Figure 4



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574 **Supporting information S1**

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577 **Introduction**

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

586

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

589

590 **Sample preparation**

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

595 **Calux-assay**

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

600 **Luminescence measurement**

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

603 **Calculations**

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

609

610 Samples were measured in two different dilutions, in calculating the final results always the dilution
611 with RLU between the RLU's of 1 pM and 10 pM TCDD was used. When samples were measured on
612 more than one plate, the average of the results was used for further calculation.

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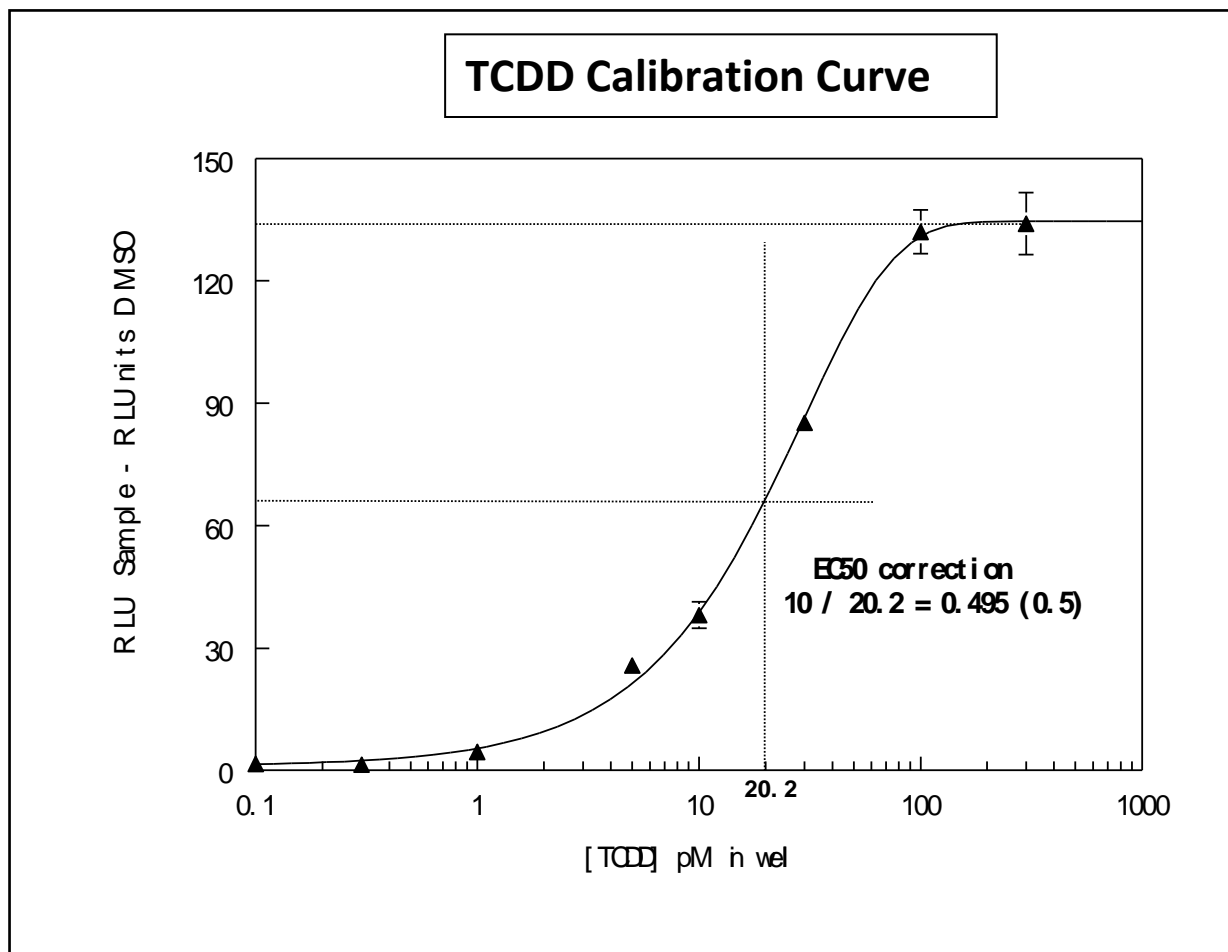


Figure S1. Dose response curve used in CALUX assay to estimate TCDD equivalents in samples