

1 **Developmental toxicity of perfluorooctane sulfonate (PFOS) and its**  
2 **chlorinated polyfluoroalkyl ether sulfonate alternative F-53B in the domestic**  
3 **chicken**

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10 **Abstract**

11 The chlorinated polyfluoroalkyl ether sulfonate F-53B is used as a mist suppressant in the  
12 Chinese electroplating industry. Due to the regulations on perfluorooctane sulfonate (PFOS),  
13 its use is expected to increase. Until now, F-53B toxicity data have been scarce and are, to our  
14 knowledge, lacking for birds. This study therefore investigated the effects of PFOS and F-53B,  
15 separately and as mixtures, on the development of the chicken (*Gallus gallus domesticus*).  
16 Compounds were injected *in ovo*, before incubation, at 150 and 1500 ng/g egg. At embryonic  
17 day 20, a significantly lower heart rate was observed in all treated groups compared to the  
18 control group and hatchlings exposed to the high dose of F-53B had a significantly enlarged  
19 liver (8 %). Embryonic survival was not affected and no significant effects on hatchling body  
20 mass or oxidative stress parameters were found. Our results suggest that these compounds  
21 likely have different toxicity thresholds for the investigated endpoints, and/or different modes  
22 of action. This study thereby underlines the potential developmental toxicity of PFOS and F-  
23 53B at environmentally relevant concentrations. Assessment of PFOS alternatives should  
24 therefore continue, preferably prior to their large scale use, as they should be ensured to be less  
25 harmful than PFOS itself.

## 26 **Introduction**

27 Perfluorooctane sulfonate (PFOS) was in production for approximately 40 years before its  
28 ubiquity in the environment became apparent by the turn of the century.<sup>1</sup> A voluntary phase-  
29 out of PFOS by 3M, the major manufacturer, followed from 2000 to 2002, which started a  
30 series of measures to restrict the production and use of the compound. In 2009, PFOS and its  
31 salts were listed in Annex B of the Stockholm Convention.<sup>2</sup> However, due to a lack of readily  
32 available alternatives, one of the exemptions of the Convention for the production and use of  
33 PFOS is the electroplating industry.<sup>2</sup> To avoid the formation of highly toxic chromium vapors  
34 during the electroplating process, mist suppressants are added to the metal bath, which  
35 increases safety for the workers. These mist suppressants are commonly based on PFOS and  
36 its salts.<sup>3</sup>

37 In China, where the electroplating industry is well developed, the chlorinated polyfluoroalkyl  
38 ether sulfonate 6:2 Cl-PFESA (trade name F-53B) has been used as a mist suppressant since  
39 the 1970s. Similar to PFOS, the environmental presence and potential hazard of F-53B have  
40 been overlooked for decades,<sup>3</sup> and it is likely that due to the reduction in the use of PFOS, the  
41 demand for fluorinated alternatives, such as F-53B, will increase. Until now, F-53B has been  
42 unregulated and toxicity data are scarce and scattered,<sup>4</sup> however, literature on the topic seems  
43 to be quickly expanding. F-53B was first reported in Chinese river water in 2013<sup>3</sup> and was  
44 subsequently found in sediment,<sup>5</sup> aquatic organisms from China,<sup>6,7</sup> and even in human serum  
45 and placenta.<sup>8,9</sup> A worrisome observation was made by Gebbink et al. (2016) when F-53B was  
46 detected in liver of polar bears (*Ursus maritimus*), killer whales (*Orcinus orca*) and ringed seals  
47 (*Pusa hispida*) from Greenland, although in relatively low concentrations, indicating long-  
48 range transport.<sup>10</sup> Potential for long-range transport similar to PFOS has now been estimated,  
49 suggesting that F-53B can potentially reach remote regions and distribute on a global scale.<sup>11</sup>  
50 Like PFOS, F-53B was also found in the liver of killer whale fetuses,<sup>10</sup> showing similar

51 maternal transfer rates for both compounds. The esterification of the fluorocarbon chain of F-  
52 53B was thought to make the molecule more degradable than the environmentally persistent  
53 PFOS. Nonetheless, current literature suggests similar persistence<sup>3</sup> and even higher  
54 bioaccumulative potential of F-53B in comparison to PFOS.<sup>6,12</sup>

55 Due to its high persistence, PFOS is still one of the most dominating poly- and perfluoroalkyl  
56 substances (PFASs) detected in environmental samples. It is commonly found in bird eggs, as  
57 a high load of PFOS can be deposited into the egg by the mother.<sup>13-16</sup> The embryonic stage is  
58 highly sensitive to xenobiotic compounds and prenatal exposure can affect the birds, even later  
59 in life.<sup>17</sup> PFOS has been found to affect survival, morphometrics and immunology, as well as  
60 brain asymmetry and alterations in cognitive behavior of avian embryos among others (see  
61 Table 1 for an overview of the available literature). Although few studies are available, F-53B  
62 has already been shown to exert an effect on the nervous system of rodents,<sup>18,19</sup> and besides its  
63 detection in human placenta and killer whale fetuses, it has also been suggested to be maternally  
64 transferred in frogs,<sup>12</sup> increasing concerns about its developmental toxicity. These concerns  
65 have recently been confirmed in zebrafish (*Danio rerio*) embryos, where malformations and  
66 cardiac malfunction were observed,<sup>20</sup> and in mouse embryonic stem cells where neural  
67 differentiation was disrupted.<sup>19</sup>

68 To our knowledge, the toxicity of F-53B in birds has not yet been assessed. Birds are key  
69 organisms in many ecosystems, they can occupy multiple trophic levels within the food chain  
70 and contribute to a variety of ecosystem services. Therefore, they are a valuable tool in  
71 ecotoxicological studies and risk assessment.<sup>21</sup> In addition, due to the accessibility and the  
72 relative isolation of the avian embryo, they can be used to investigate the embryotoxicity of  
73 environmental contaminants.<sup>22</sup>

74 The aim of this experiment was therefore to investigate the avian developmental toxicity of the  
75 largely uninvestigated compound F-53B in relation to, and in combination with, exposure to  
76 the well-investigated PFOS. By exposing fertilized chicken eggs to environmentally relevant  
77 concentrations prior to incubation, maternal transfer was mimicked and developmental effects  
78 of these two compounds on survival, heart rate, liver mass, body mass and oxidative stress  
79 parameters in the hatchlings were investigated.

80 **Table 1:** Overview of the available studies on *in ovo* exposure to PFOS in birds and comparison of the applied concentrations, injection method  
 81 and endpoints. For clarity, non-significant trends are not mentioned in this table.

Authors	Species	Doses	Injection method	Endpoints	Day	Effects
Molina et al. 2006 <sup>23</sup>	Chicken	0.1, 1.0, 10, 20 µg/g egg	Air cell at ED0	Hatching success	1	Reduced in all treatments (dose dep) No effect From 1.0 µg/g egg 0.1 µg/g egg
				Body and organ weight	7	
				Liver histopathology	7	
				LOAEL	1	
O'Brien et al. 2009 <sup>24</sup>	Chicken	0.1, 5.0, 100 µg/g egg	Air cell at ED0	Pipping success	Pipping	Reduced at 100 µg/g egg (dose dep) No effect (PPARα-regulated genes) 93 µg/g egg
				Gene expression	1	
				LD50	1	
Peden-Adams et al. 2009 <sup>25</sup>	Chicken	1, 2.5, 5 µg/g egg	Air cell at ED0	Hatching success	1	No effect Increased spleen mass in all treatments Increased liver mass from 2.5 µg/g egg No effect Increased at 5 µg/g egg Right wing shorter in all treatments Increased frequency in all treatments No effect ALT, LDH, CK decreased Total SRBC-specific Ig decreased in all treatments Increased plasma lysozyme activity in all treatments
				Organ mass	14	
				Body mass (change)	1,7,14	
				Body length	14	
				Limb measurements	14	
				Brain asymmetry	14	
				WBC counts	14	
				Blood chemistry	14	
Immune function	14					
Pinkas et al. 2010 <sup>26</sup>	Chicken	5, 10 µg/g egg	Chorioallantois end at ED0	Embryo survival	ED19	Reduced
				Hatching success	1	Reduced
				Body weight	1	No effect
				Morphological and functional score	1	No effect
				Imprinting behavior	1	Reduced
				Protein kinase C	1	Reduced
Strömqvist et al. 2012 <sup>27</sup>	Chicken	20, 100 µg/g egg	Air cell at ED15	Embryo survival	ED18	No effect No effect (PPARα-regulated genes)
				Gene expression		

82 **Table 1 continued:** Overview of the available studies on *in ovo* exposure to PFOS in birds and comparison of the applied concentrations, injection  
 83 methods and endpoints. For clarity, non-significant trends are not mentioned in this table.

Authors	Species	Doses	Injection method	Endpoints	Day	Effects
Nordén et al. 2012 <sup>28</sup>	Chicken	0.03, 0.1, 0.3, 1.0, 2.0 µg/g egg	Air cell at ED4	Embryo survival Liver mass Palmitic acid β-oxidation LOEL	ED10	No effect No effect Induced from 0.1 µg/g egg 0.1 µg/g
Nordén et al. 2016 <sup>29</sup>	Chicken Great cormorant (GC) Herring gull (HG)	0.1, 0.3, 1, 3, 10 µg/g egg	Air cell at ED4 Corresponding stage for GC and HG	Embryo survival Body and organ weight LD50 NOEL LOEL BMD <sub>10</sub> BMDL <sub>10</sub>	ED19 (chicken) Pipping (GC, HC)	Chicken: reduced at 10 µg/g egg GC: no effect HG: reduced at 10 µg/g egg Chicken: No effect GC: increased liver mass at 10 µg/g egg HG: increased body mass at 10 µg/g egg 8.5 µg/g egg 2.7 µg/g egg 0.9 µg/g egg 1.3 µg/g egg 0.4 µg/g egg
Parolini et al. 2016 <sup>30</sup>	Yellow-legged gull	0.1, 0.2 µg/g egg	Albumen at ED1	Pipping success Body mass (change) Tarsus length Liver and brain mass Total antioxidant capacity Total oxidant status Protein carbonyl content DNA fragmentation	Pipping	No effect
This study	Chicken	0.15, 1.5 µg/g egg	Yolk sac at ED0	Pipping success Hatching success Embryonic heart rate Body mass Liver mass Enzyme activity Oxidative damage Gene expression	Pipping 1 ED14,17,20 1 1 1 1 1	No effect No effect Reduced at 0.15 µg/g egg (ED20) No effect No effect No effect No effect No effect

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## 85 **Material and methods**

86 This study was approved by the Norwegian Food Safety Authority (Mattilsynet; FOTS ID  
87 9134) and was conducted in the animal laboratory facilities at the Department of Biology at  
88 NTNU, Norway, according to the appropriate regulations and protocols.

89 Potassium perfluorooctane sulfonate (PFOS; CAS no. 2795-39) was purchased from Sigma-  
90 Aldrich (St. Louis, MO, USA), with a stated purity of  $\geq 98$  %. However, our analysis showed  
91 a purity of 90 %, with PFHpS (7.6 %) and PFNS (1.4 %) as main impurities. Potassium 2-[(6-  
92 chloro-1,1,2,2,3,3,4,4,5,5,6,6-dodecafluorohexyl)oxyl]-1,1,2,2-tetrafluoroethanesulfonate  
93 (6:2 Cl-PFESA or F-53B; CAS no. 73606-19-6) was kindly donated by Dr. Thanh Wang  
94 (Örebro University) and had a stated purity of  $\geq 98$  %.<sup>31</sup>

### 95 *Vehicle preparation*

96 An emulsion of peanut oil and water, using lecithin as an emulsifier, was used as a vehicle to  
97 transfer the compounds into the egg yolk, as the majority of PFOS in bird eggs is found in the  
98 yolk, and not in the albumen.<sup>14</sup> The emulsion was prepared in accordance to Brunström and  
99 Örberg (1982) by dissolving lecithin (L- $\alpha$ -phosphatidylcholine from egg yolk, Sigma-Aldrich,  
100 St. Louis, MO, USA) in dichloromethane (DCM; Merck, Darmstadt, Germany) and peanut oil  
101 (Sigma-Aldrich, St. Louis, MO, USA).<sup>32</sup> Then, DCM was evaporated at 35 °C and 300 mbar  
102 using a rotary evaporator (RV 10 digital, IKA). The compounds, dissolved in ethanol, were  
103 then added to the lecithin/peanut oil mixture and the ethanol was evaporated at 40 °C and 175  
104 mbar. After autoclave sterilization (20 min, 121 °C), two parts (1.6 mL) of the mixture were  
105 added to three parts (2.4 mL) of sterile distilled water to form an emulsion.<sup>32</sup> All emulsions  
106 were sonicated (ultrasonic processor GEX 400 connected to a four-element probe) for 30  
107 seconds prior to injection. For each compound separately, an emulsion was prepared in a low  
108 and high concentration of 75 and 750 ng/ $\mu$ L to achieve final exposure doses of 150 and 1500



109 ng/g egg, respectively. In addition, mixtures of the two compounds in all four possible  
110 combinations of the two concentrations were prepared in order to achieve a 3 x 3 full factorial  
111 study design (Fig.1). Both nominal and actual doses in this study reflect the PFOS anion only,  
112 not the salt. The exposure doses of 150 and 1500 ng/g egg correspond to 0.30 and 3.0 nmol/g  
113 egg for PFOS and to 0.28 and 2.8 nmol/g egg for F-53B, respectively and represent the wide  
114 range of PFOS concentrations found in wild bird eggs.

#### 115 *Embryonic exposure and chick sampling*

116 Broiler chicken (*Gallus gallus domesticus*) eggs from the hybrid breed ‘Ross 308’ were kindly  
117 donated by a local hatchery (Soknedal, Norway) and were kept at 18 °C until injection (two to  
118 three days). Out of 160 eggs, 122 eggs were randomly divided over eight treatment groups and  
119 38 were divided over three control groups to investigate the potential effect of the injection  
120 (needle puncture and/or emulsion; Fig. 1). Fifteen eggs were selected as untreated control (not  
121 injected, not punctured) and another fifteen eggs were injected with a control emulsion without  
122 the compounds (vehicle control). In addition, eight eggs were only punctured with a needle,  
123 without being injected (Fig. 1).

124 The injected volume was 2 µL/g egg, hence the total injected volume was adjusted according  
125 to the individual egg mass (mean ± SD: 62.3 ± 3.4 g). Eggs were injected in the yolk sac at  
126 embryonic day (ED) zero, before the start of incubation. The entire injection procedure was  
127 conducted in a laminar flow cabinet. Prior to injection, the blunt segment of each egg was  
128 cleaned with ethanol (70 % v/v). Using a round shaped dentist drill bit mounted on an electrical  
129 drill (Robust 140W 9922 (GS), Hong Kong, P.R. China), a 0.6 mm hole was made in the  
130 eggshell until visibility of the inner shell membrane. The solution was injected using a  
131 Hamilton syringe (250 µL) and disposable needles (BD Microlance™ 3, 25 G, 0.5 × 16 mm)  
132 and the hole was sealed with paraffin. The eggs were divided over three incubators (type 180,

133 America A/S, Thisted, Denmark and J. Hemel, Verl, Germany) in which they were placed  
134 horizontally and kept at 37.5 - 38 °C and 60 % humidity). Eggs in incubator 2 and 3 were  
135 turned continuously (90° per hour) by an automatic egg-turning device, but due to a failure in  
136 the turning mechanism, eggs in incubator 1 required manual turning twice per day. To avoid  
137 effects related to the incubators such as variations in temperature, humidity or egg rotation,  
138 eggs were randomly divided between the three incubators, as well as between the shelves  
139 within the incubators, following a randomized block design with incubator as a blocking factor.

140 In the initial stage of incubation, eggs were regularly candled to monitor the embryonic  
141 development. The heart rate of embryos was determined using a digital egg monitor (Buddy,  
142 Avitronics, Cornwall, UK) at multiple time points, with systematic measurements on ED14, 17  
143 and 20. Eggs that showed no or an arrested development were removed from the incubator and  
144 frozen at -20 °C. These eggs were opened later to determine embryonic viability and  
145 developmental status.

146 The last three to five days before hatching (ED16 - 18), eggs were transferred to hatching boxes  
147 at the bottom of the same incubator and relative humidity was increased to 70 - 80 %. After 20  
148 days of incubation, the first eggs started to hatch. When dry, chicks were taken out of the  
149 incubator, weighed, and then euthanized by decapitation using scissors. The liver was  
150 dissected, weighed, divided for chemical and oxidative stress analyses, and snap frozen at -80  
151 °C. Pipping and hatching success was determined by calculating the percentage of fertile eggs  
152 that were able to pip (externally) and hatch, respectively. External pipping is defined as the  
153 stage at which the chick breaks the outer egg shell membrane to commence hatching and a  
154 pipping star appears.

155 *Chemical analysis*

156 Analysis of the targeted compounds, PFOS and F-53B, was performed at the Norwegian  
157 Institute for Air Research (NILU) in Tromsø, Norway. The internal and recovery standards for  
158 chemical analysis consisted of <sup>13</sup>C-labelled PFOS in MeOH and perfluoro-3,7-  
159 dimethyloctanoic acid in MeOH, respectively (Wellington Laboratories, Guelph, Canada). The  
160 extraction and clean-up procedure was based on the Powley method,<sup>33</sup> and was adapted from  
161 Herzke et al. (2012)<sup>34</sup> for emulsions, and from Ahrens et al. (2011)<sup>16</sup> for livers. Detailed  
162 information on these procedures is given in section 1 of the Supporting Information (SI), and  
163 results of the emulsion and liver analyses are shown in Table S1 and S2, respectively.  
164 Compounds were analyzed by ultrahigh pressure liquid chromatography triple-quadrupole  
165 mass-spectrometry (UHPLC-MS/MS). Analysis was performed as described in detail by  
166 Hanssen et al. (2013) (also described in the SI section 1.2).<sup>35</sup> Method blanks and standard  
167 reference material (SRM; Pike perch sample QM03-2, QUASIMEME) samples were  
168 processed with every batch of ten samples. No blank contamination was encountered. The  
169 measured concentrations of the SRM were within the acceptable range, except for PFNA with  
170 an average recovery of 185 % due to levels close to the limit of detection. Recoveries of the  
171 internal standards varied in all samples between 73 and 113 % with the exception of 6:2 FTS  
172 and PFTeA with average recoveries of 128 and 140 %, respectively.

173 *Oxidative stress*

174 Expression of a suite of genes involved in the oxidative stress pathway was investigated in  
175 chicken liver samples using real-time quantitative PCR (qPCR). Target genes were catalase  
176 (CAT), glutathione reductase (GR), superoxide dismutase (SOD) 2, glutathione S-transferase  
177 (GST) α2, glutathione peroxidase (GPx) 4, nuclear factor erythroid 2 like 2 (NFE2L2),  
178 glutamate-cysteine ligase catalytic subunit (GCLC) and glutamate-cysteine ligase modifier  
179 subunit (GCLM). Target and reference genes, including their primer details, can be found in

180 Table S3. Details on primer design, sample preparation, qPCR protocol, quality control and  
181 data treatment can be found in section 2 of the SI.

182 Oxidative damage and enzymatic activity were investigated in chicken liver samples using a  
183 spectrophotometer. All reagents for the CAT, GPx and GR assays and kits for the determination  
184 of GST activity, lipid peroxidation (malondialdehyde; MDA) and protein carbonyl content  
185 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Detailed information on the  
186 sample preparation, analytical methods (excluding assays using kits) and quality control can  
187 be found in the SI section 3 and Table S5.

### 188 *Statistical analysis*

189 Statistical analysis was performed using R (version 3.4.0).<sup>36</sup> Details of the performed  
190 calculations and statistical tests can be found in section 4 of the SI. In brief, to test the effect of  
191 the compounds on the parameters of interest, a two-way analysis of variance (ANOVA),  
192 followed by Tukey's or Dunnett's post-hoc test, was performed on a linear model that included  
193 both compounds separately and in interactions (corresponding to the mixtures). To test the  
194 effect of all treatment groups separately, a one-way ANOVA was performed, followed by a  
195 Dunnett's post-hoc test. To account for possible variation caused by the incubators, 'incubator'  
196 was added as a blocking factor to the model. When the residuals of the model were not normally  
197 distributed, a Kruskal-Wallis test was performed instead, followed by a Dunn's post-hoc test.  
198 Survival curves were plotted and tested for effects of the treatment using the Tarone-Ware test.  
199 Binomial generalized linear models were used to investigate effects of the compounds on  
200 pipping and hatching success. Body mass was normalized for egg mass, and the hepatosomatic  
201 index (HSI) was calculated to obtain a liver mass normalized for body mass. The hepatic  
202 fraction (%) of the total injected concentrations was calculated as described in the SI (section  
203 4) and was used to estimate the distribution of the compounds to the liver.

204 Based on the statistical analysis between the three types of control groups (details in SI, section  
205 5), the injected vehicle control was found to be the most relevant for comparison with the  
206 treated groups. Therefore, in all analyses, treated groups were only compared with the injected  
207 vehicle control.

## 208 **Results and discussion**

### 209 *Concentrations of F-53B and PFOS in the liver of the hatchlings*

210 Actual concentrations detected in the liver can be found in Table S2 and ranged from 1.08 –  
211 2.17 mg/g, 11.6 – 28.4 mg/g, 1.04 – 2.20 mg/g and 7.87 – 23.4 mg/g, for PFOS LD, PFOS HD,  
212 F-53B LD and F-53B HD, respectively. The percentage of the total injected amount (based on  
213 the nominal concentration) found in the liver ranged from 15 - 19 % and 10 - 16 % for PFOS  
214 and F-53B, respectively (Table S2), indicating that chicks distribute both PFOS and F-53B to  
215 the liver. The liver can therefore be considered a target organ for these compounds in birds.

216 When comparing the isomeric composition in the liver with that in the PFOS standard (37 %  
217 branched, 63 % linear), chicken embryos from this study preferentially accumulated the linear  
218 isomer (75 %) over the branched isomer (25 %) in the liver. This is in agreement with the  
219 findings of O'Brien et al. (2011), who also found an enrichment of the linear isomer relative to  
220 the *in ovo* injected technical mixture.<sup>37</sup>

### 221 *Effect on embryonic heart rate*

222 Although eggs and treatments were randomized between and within the three incubators  
223 (randomized block design), the heart rate of the embryos measured at ED20 was affected by  
224 the incubator they developed in ( $F_{2,98} = 4.77$ ,  $p = 0.011$ ). A post-hoc Tukey test showed that  
225 the heart rate of embryos from incubator 1 was significantly lower than those of incubator 2 ( $p$   
226 = 0.04) and 3 ( $p = 0.01$ ), potentially due to the manual rotation of the eggs. It is also known  
227 that the embryonic heart rate is sensitive to changes in relative humidity and temperature within

228 the incubator.<sup>38</sup> However, these parameters were monitored twice daily and no aberrant values  
229 were observed. To focus on the effect of the compounds, only incubator 2 and 3 were  
230 considered for further statistical investigation. No effect of the incubator on heart rate was  
231 found at ED14 or 17.

232 While no effect of the compounds was found on the heart rate measured at ED14 or 17, a  
233 decreased heart rate was detected at ED20, the day before hatching. The heart rate of control  
234 embryos at ED20 was  $298 \pm 23$  beats per minute (bpm), while the heart rate in exposed embryos  
235 was lower, ranging from  $247 \pm 19$  bpm in the PFOS low dose group, to  $279 \pm 39$  bpm in the F-  
236 53B low dose group (Table 2). Compared to the control group, all exposed groups had a  
237 significantly lower heart rate (one-way ANOVA:  $F_{8,61} = 2.06$ ,  $p = 0.054$ ), especially F-53B HD  
238 (Dunnett's test:  $t = 3.11$ ,  $p = 0.017$ ) and PFOS LD (Dunnett's test:  $t = 3.06$ ,  $p = 0.019$ ).  
239 Moreover, a significant interaction was observed between the two compounds in mixture (two-  
240 way ANOVA:  $F_{4,61} = 3.54$ ,  $p = 0.012$ ). This interaction shows that F-53B HD and PFOS LD  
241 and HD lower the heart rate more when occurring alone, than when occurring as mixtures (i.e.  
242 PFOS LD + F-53B HD and PFOS HD + F-53B HD; Table 2). This suggests a possible  
243 antagonistic reaction between the two compounds at these concentrations.

244 A decreased heart rate could be related to a decreased body mass (e.g. due to a developmental  
245 delay) and metabolism. In the current study, body mass at hatching was not correlated with  
246 heart rate (Spearman's  $\rho = 0.11$ ,  $p = 0.30$ ). Reductions in heart rate can also be related to  
247 morphological alterations that can occur during cardiogenesis. Data on this are lacking for  
248 birds, but cardiac defects were reported in exposed rodent fetuses,<sup>39</sup> and heart malformations  
249 and altered heart rate were observed in zebrafish and medaka (*Oryzias melastigma*) embryos  
250 exposed to PFOS.<sup>40-42</sup> Similar to our results in chicken embryos, PFOS only altered the heart  
251 rate of medaka embryos in the late developmental stages.<sup>41</sup> In zebrafish embryos, F-53B  
252 induced cardiac toxicity by decreasing the heart rate, in combination with the incidence of

253 pericardial edema (also seen as an effect of PFOS) and downregulation of several genes related  
254 to cardiac development.<sup>20</sup> Cardiac development is a critical phase in embryogenesis and  
255 disruptions might ultimately affect survival, warranting further investigation in birds.

#### 256 *Survival, pipping and hatching success*

257 Pipping and hatching success of the treated groups ranged between 75 – 93 % and 64 – 93 %,  
258 respectively (Table 2). No statistical differences in pipping success ( $\chi^2_{10} = 5.4$ ,  $p = 0.86$ ),  
259 hatching success ( $\chi^2_{10} = 7.9$ ,  $p = 0.64$ ), nor in embryo survival (Tarone-Ware test,  $p = 0.92$ ;  
260 Figure S2) were found between treatments. Interestingly, all chicks that pipped in the present  
261 study also subsequently hatched, except for three out of eleven chicks exposed to a low dose  
262 of PFOS, who pipped externally and did not survive the hatching process. Although not  
263 statistically significant, this observation could be biologically relevant, indicating the potential  
264 of PFOS to affect hatchability at low doses.

265 The lowest PFOS dose that has previously been reported to affect hatching success after *in ovo*  
266 injection was 0.1  $\mu\text{g/g}$  egg.<sup>23</sup> This result could not be confirmed by other similar studies  
267 (including the current study) and has therefore been thought to be due to the vertical placement  
268 of the egg in the incubator. It has been shown that a vertical egg orientation increases the  
269 toxicity of the compound, as opposed to a more natural, horizontal position.<sup>43</sup> Another study  
270 on chickens observed reduced embryo survival and pipping success at 10  $\mu\text{g/g}$  egg when PFOS  
271 was injected in the air cell at ED4.<sup>29</sup> Critical developmental phases (such as organogenesis)  
272 occur within the first four days of incubation, and it was found that, compared to injection at  
273 ED4, embryos exposed prior to incubation exhibited a higher sensitivity to the investigated  
274 endpoints.<sup>44</sup> Finally, O'Brien et al. (2009) injected PFOS prior to incubation, positioned the  
275 eggs horizontally in the incubator and observed reduced pipping success at 100  $\mu\text{g/g}$  egg.<sup>24</sup> In  
276 contrast to the present study, PFOS in the study of O'Brien et al. (2009) was injected in the air

277 cell instead of in the yolk sac. Even though a higher sensitivity of chicken embryos to the  
278 compound can be expected when exposed via the yolk,<sup>45</sup> the effect concentration found by  
279 O'Brien et al. (2009) is much higher than the environmentally relevant doses used in our study.

280 In contrast to the lack of effects in experimental studies, Custer et al. (2014) found a negative  
281 association between hatching success and PFOS concentration in eggs of free-living tree  
282 swallows (*Tachycineta bicolor*), with decreasing hatching success from 150 – 200 ng/g egg.  
283 These levels corresponded to the low PFOS dose in the current study.<sup>46</sup> It is therefore important  
284 to consider the discrepancy between laboratory studies and field studies at all times, as many  
285 extrinsic factors can contribute to xenobiotic toxicity in biota.

286 Similar to PFOS, F-53B did not affect pipping, hatching or survival of the chicken embryos at  
287 the investigated concentrations. In zebrafish, F-53B did not affect the hatching success of  
288 exposed embryos either, but it did significantly delay their hatching.<sup>20</sup> F-53B also decreased  
289 the zebrafish embryos' survival, be it at higher concentrations than found in the environment.

290 In the present study, the exact time of hatching was not recorded, however, this could be  
291 assessed in future experimental work on the effects of F-53B.



292 **Table 2:** The amount of viable eggs, pipping and hatching success and embryonic heart rate (mean  $\pm$  SD) at ED14, 17 and 20 per treatment group.

Treatment group		Total viable eggs ( <i>n</i> )	Pipping success (%)	Hatching success (%)	Heart rate at ED14 (bpm)	Heart rate at ED17 (bpm)	Heart rate at ED20 (bpm) all inc	Heart rate at ED20 (bpm) inc 2 + 3
Controls	Non-injected	14	93	93	249 $\pm$ 28	253 $\pm$ 14	258 $\pm$ 31	269 $\pm$ 21
	Punctured	7	86	86	263 $\pm$ 9	257 $\pm$ 12	276 $\pm$ 23	284 $\pm$ 18
	Vehicle-injected	9	78	78	259 $\pm$ 16	263 $\pm$ 25	290 $\pm$ 30	298 $\pm$ 23
PFOS	LD	11	91	64	242 $\pm$ 28	244 $\pm$ 20	248 $\pm$ 22	247 $\pm$ 19
	HD	13	85	85	248 $\pm$ 22	253 $\pm$ 19	250 $\pm$ 34	267 $\pm$ 14
F-53B	LD	12	75	73 ( <i>n</i> =11)*	251 $\pm$ 23	246 $\pm$ 20	263 $\pm$ 48	279 $\pm$ 39
	HD	11	91	91	253 $\pm$ 23	266 $\pm$ 17	257 $\pm$ 33	248 $\pm$ 37
Mixtures	PFOS LD + F-53B LD	14	93	93	258 $\pm$ 23	256 $\pm$ 19	265 $\pm$ 30	267 $\pm$ 20
	PFOS HD + F53B HD	16*	81	80 ( <i>n</i> =15)	241 $\pm$ 44	256 $\pm$ 21	272 $\pm$ 30	278 $\pm$ 28
	PFOS LD + F-53B HD	11	82	82	261 $\pm$ 21	256 $\pm$ 20	265 $\pm$ 26	266 $\pm$ 28
	PFOS HD+ F-53B LD	12	83	83	234 $\pm$ 35	251 $\pm$ 23	249 $\pm$ 41	261 $\pm$ 26

293 \* one egg was opened after pipping and was not included when calculating hatching success

294 *Effect on body mass*

295 A significant effect of the incubator on hatchling body mass was found (type II ANOVA for  
296 unbalanced data:  $F_{2,84} = 7.45$ ,  $p = 0.001$ ). Further, no significant effect of the compounds on  
297 the hatchling body mass was found.

298 In concordance with our study, no effect on body mass has been found in other avian *in ovo*  
299 studies on PFOS (Table 1). Only in herring gulls was an increased body mass observed at  
300 exposure concentrations of 10  $\mu\text{g/g}$  egg.<sup>29</sup> Studies on mammals have found postnatal growth  
301 delay in rat pups following *in utero* exposure to PFOS, which was associated with  
302 hypothyroxinemia,<sup>47</sup> and a decreased thyroid hormone level by PFOS has been found in adult  
303 rats, mice and monkeys.<sup>39,48,49</sup> Thyroid hormones are known to be important for growth and  
304 development, and it has been hypothesized that PFOS competes for target binding to the  
305 hormone receptor. However, the PFOS concentrations in the present study did not exert an  
306 effect on the body mass of newly hatched birds at the investigated concentrations.

307 Limited data exist on the effects of F-53B. However, a recent study on zebrafish larvae showed  
308 that F-53B, at environmentally relevant concentrations, caused a significant decrease in the  
309 body weight of the larvae, which was also related to a disruption in the thyroid hormone  
310 system.<sup>50</sup> In contrast, no effect of F-53B on the body mass of the hatchlings was found in the  
311 current study, which likely reflects interspecific and experimental differences.

312 *Effect on the hepatosomatic index (HSI)*

313 Chicks exposed to a high dose of F-53B (including mixture groups) showed a significant 8 %  
314 increase in their HSI ( $H = 5.9$ ,  $df = 2$ ,  $p = 0.053$ ; Dunn's post-hoc test:  $z = 2.4$ ;  $p = 0.026$ )  
315 compared to chicks not exposed to this compound (Fig. 2). No such increase was observed in  
316 chicks exposed to PFOS. A high dose of F-53B and PFOS (including single and mixture

317 treatments) corresponded to an actual concentration range of 7.87 – 23.4 mg/g and 11.6 – 28.4  
318 mg/g detected in the liver, respectively (Table S2).

319 The HSI is considered a measure of the body condition of an animal, as it reflects both the  
320 metabolic energy demands and the short-term nutritional status.<sup>51</sup> It is sensitive to  
321 environmental contaminants, and hepatic enlargement is therefore a common symptom in  
322 exposure studies. The effect of F-53B on the HSI has, to our knowledge, not been investigated  
323 yet. However, in similar avian exposure studies, PFOS has been found to increase the liver  
324 mass of chicken hatchlings at exposure concentrations from 2.5 µg/g egg.<sup>25</sup> Further, the HSI in  
325 great cormorants (*Phalacrocorax carbo sinensis*) increased by 18 % following exposure to 10  
326 µg PFOS/g egg.<sup>29</sup> Also in northern bobwhite quails (*Colinus virginianus*), an increase in the  
327 HSI was observed after dietary PFOS exposure.<sup>52</sup> Considering the comparatively low doses  
328 used in our study, it is not surprising that PFOS did not exert an effect on HSI. The finding that  
329 F-53B, in contrast to PFOS, did have an effect at 1500 ng/g egg may therefore indicate a higher  
330 hepatotoxicity potential in birds compared to PFOS.

331 Hepatotoxicity of PFOS has been mostly linked to PPAR $\alpha$ -mediated pathways, as PFOS  
332 structurally resembles fatty acids, the endogenous PPAR ligands. Interestingly, it was recently  
333 shown that F-53B has an even higher binding affinity to PPARs than PFOS.<sup>53</sup> An increased  
334 HSI could be related to changes in lipid metabolism (PPAR-dependent), as well as to  
335 histopathological changes and hepatic injuries as found in PFOS-treated rats.<sup>54</sup> This highlights  
336 the need for further investigations on these endpoints in birds. Finally, together with the  
337 concentrations detected in the liver of the hatched chicks, the increased HSI confirms that the  
338 liver is a target organ for F-53B exposure.

339 *Effects on the oxidative stress response*

340 Exposure to certain environmental pollutants is known to increase production of reactive  
341 oxygen species (ROS) and hence trigger an oxidative stress response in birds,<sup>55</sup> potentially  
342 leading to a detrimental effect on the embryonic development.<sup>56</sup> In this study, a significant  
343 interaction effect of PFOS and F-53B was found on the gene expression of SOD2 ( $F_{4,45} = 3.34$ ,  
344  $p = 0.018$ ) and GR ( $F_{4,44} = 2.70$ ,  $p = 0.043$ ), as shown in Figure S1 and Table S4. Both  
345 compounds when exposed separately, in both low and high dose, triggered a mild increase  
346 (1.43 - 1.85 fold) in SOD2 expression compared to the control group. This indicates an  
347 oxidative stress response by increasing the production of the SOD2 enzyme. However, when  
348 the compounds occurred in a mixture of different doses (PFOS HD + F-53B LD and PFOS LD  
349 + F-53B HD), the expression was similar to the control group (Figure S1A and B and Table  
350 S4). This result shows an antagonistic effect for the latter mixtures. For GR on the other hand,  
351 no effect was observed in chicks exposed to a low dose of PFOS or a high dose of F-53B. Yet,  
352 when the compounds occurred in a mixture and in the respective doses, GR expression  
353 decreased twofold (Figure S1C and Table S4). Although not significant, a corresponding trend  
354 was observed in the enzyme activity of GR in chicks exposed to that mixture (PFOS LD + F-  
355 53B HD). GR is an essential enzyme in the oxidative stress pathway, catalyzing the reduction  
356 of glutathione,<sup>57</sup> hereby providing the substrate for several anti-oxidative stress enzymes (e.g.  
357 glutathione-S-transferase and glutathione peroxidase). A decrease in the expression and  
358 activity of this enzyme could therefore cause a hampered defense against ROS.

359 Activity and expression of other investigated enzymes and genes, respectively, were not  
360 significantly affected by the treatments. In concordance with the lack of an oxidative stress  
361 response, no lipid or protein damage, potentially related to oxidative stress, could be detected.  
362 Therefore, it can be assumed that embryonic exposure of chickens to PFOS and F-53B at  
363 environmentally relevant concentrations did not trigger any major changes in the oxidative

364 stress response and did not cause oxidative damage to lipids or proteins. Other pathways could  
365 therefore be more relevant to the toxic mode of action of these compounds.

366 In conclusion, environmentally relevant concentrations of PFOS and its alternative F-53B were  
367 found to significantly decrease the heart rate of avian embryos immediately before hatching.  
368 Further, F-53B significantly increased the liver mass of the hatchlings. Further investigations  
369 on liver metabolizing enzymes and - histopathology might therefore elucidate the mechanisms  
370 of this compound. Based on our results, the oxidative stress pathway is unlikely to be a target  
371 pathway at these concentrations. Although no effects were observed on survival,  
372 pipping/hatching success or body mass, the sublethal effects observed could potentially lead to  
373 fitness consequences later in life,<sup>17</sup> especially in the context of multiple stressors. Therefore,  
374 potential effects on survival cannot be ruled out, as e.g. hatching delay and postnatal survival  
375 were not assessed in the current study, and further investigations are necessary. F-53B is  
376 increasingly detected in the environment, and interest in the toxicity of this compound is  
377 therefore also increasing. The current study contributes to the knowledge gap that exists  
378 regarding the toxicity of F-53B in birds, and biota in general. The results of this study show  
379 that there is potential for this alternative compound to exceed the toxicity of PFOS at  
380 environmentally relevant concentrations. In combination with other available toxicity data, the  
381 current study highlights that the chlorinated polyfluoroalkyl ether sulfonate F-53B can be a  
382 compound for future concern and should not be overlooked.

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### 390 **Supporting information**

391 Details on extraction and clean-up (chemical analysis); details on quantification (chemical  
392 analysis); concentrations in emulsions (Table S1); hepatic concentrations (Table S2); primer  
393 design; sample preparation for qPCR analysis; qPCR protocol; quality control of the qPCR  
394 analysis; qPCR data treatment; primer sequences (Table S3); interaction effects on gene  
395 expression (Figure S1 and Table S4); homogenization procedure for oxidative stress assays;  
396 CAT assay protocol; GR assay protocol; GPx assay protocol; normalization of assay results;  
397 intra- and interplate variation for oxidative stress assays (Table S5); statistical analysis;  
398 comparison of control groups; survival plot (Figure S2).

### 399 **References**

- 400 (1) Giesy, J. P.; Kannan, K. Global distribution of perfluorooctane sulfonate in wildlife.  
401 *Environ. Sci. Technol.* **2001**, *35* (7), 1339–1342.
- 402 (2) United Nations Environment Program (UNEP). SC-4 / 17 : Listing of perfluorooctane  
403 sulfonic acid, its salts and perfluorooctane sulfonyl fluoride. **2009**, 15–18.
- 404 (3) Wang, S.; Huang, J.; Yang, Y.; Hui, Y.; Ge, Y.; Larssen, T.; Yu, G.; Deng, S.; Wang,  
405 B.; Harman, C. First report of a Chinese PFOS alternative overlooked for 30 years: its  
406 toxicity, persistence, and presence in the environment. *Environ. Sci. Technol.* **2013**, *47*  
407 (18), 10163–10170.
- 408 (4) Wang, Z.; Dewitt, J. C.; Higgins, C. P.; Cousins, I. T. A never-ending story of per-and  
409 polyfluoroalkyl substances (PFASs)? *Environ. Sci. Technol.* **2017**, *51* (5), 2508–2518.
- 410 (5) Lin, Y.; Ruan, T.; Liu, A.; Jiang, G. Identification of novel hydrogen-substituted

- 411 polyfluoroalkyl ether sulfonates in environmental matrices near metal-plating facilities.  
412 *Environ. Sci. Technol.* **2017**, *51* (20), 11588–11596.
- 413 (6) Shi, Y.; Vestergren, R.; Zhou, Z.; Song, X.; Xu, L.; Liang, Y.; Cai, Y. Tissue distribution  
414 and whole body burden of the chlorinated polyfluoroalkyl ether sulfonic acid F-53B in  
415 crucian carp (*Carassius carassius*): evidence for a highly bioaccumulative contaminant  
416 of emerging concern. *Environ. Sci. Technol.* **2015**, *49* (24), 14156–14165.
- 417 (7) Liu, Y.; Ruan, T.; Lin, Y.; Liu, A.; Yu, M.; Liu, R.; Meng, M.; Wang, Y.; Liu, J.; Jiang,  
418 G. Chlorinated polyfluoroalkyl ether sulfonic acids in marine organisms from Bohai  
419 Sea, China: occurrence, temporal variations, and trophic transfer behavior. *Environ. Sci.*  
420 *Technol.* **2017**, *51* (8), 4407–4414.
- 421 (8) Shi, Y.; Vestergren, R.; Xu, L.; Zhou, Z.; Li, C.; Liang, Y.; Cai, Y. Human exposure  
422 and elimination kinetics of chlorinated polyfluoroalkyl ether sulfonic acids (Cl-  
423 PFESAs). *Environ. Sci. Technol.* **2016**, *50* (5), 2396–2404.
- 424 (9) Chen, F.; Yin, S.; Kelly, B. C.; Liu, W. Chlorinated polyfluoroalkyl ether sulfonic acids  
425 in matched maternal, cord, and placenta samples: a study of transplacental transfer.  
426 *Environ. Sci. Technol.* **2017**, *51* (11), 6387–6394.
- 427 (10) Gebbink, W. A.; Bossi, R.; Rigét, F. F.; Rosing-Asvid, A.; Sonne, C.; Dietz, R.  
428 Observation of emerging per- and polyfluoroalkyl substances (PFASs) in Greenland  
429 marine mammals. *Chemosphere* **2016**, *144*, 2384–2391.
- 430 (11) Gomis, M. I.; Wang, Z.; Scheringer, M.; Cousins, I. T. A modeling assessment of the  
431 physicochemical properties and environmental fate of emerging and novel per- and  
432 polyfluoroalkyl substances. *Sci. Total Environ.* **2015**, *505*, 981–991.
- 433 (12) Cui, Q.; Pan, Y.; Zhang, H.; Sheng, N.; Wang, J.; Guo, Y.; Dai, J. Occurrence and tissue

- 434 distribution of novel perfluoroether carboxylic and sulfonic acids and legacy  
435 per/polyfluoroalkyl substances in black-spotted frog (*Pelophylax nigromaculatus*).  
436 *Environ. Sci. Technol.* **2018**, *52* (3), 982–990.
- 437 (13) Holmström, K. E.; Berger, U. Tissue distribution of perfluorinated surfactants in  
438 common guillemot (*Uria aalge*) from the Baltic Sea. *Environ. Sci. Technol.* **2008**, *42*  
439 (16), 5879–5884.
- 440 (14) Gebbink, W. A.; Letcher, R. J. Comparative tissue and body compartment accumulation  
441 and maternal transfer to eggs of perfluoroalkyl sulfonates and carboxylates in Great  
442 Lakes herring gulls. *Environ. Pollut.* **2012**, *162*, 40–47.
- 443 (15) Groffen, T.; Lopez-Antia, A.; Prinsen, E.; Eens, M.; Bervoets, L. Perfluoroalkylated  
444 acids in the eggs of great tits (*Parus major*) near a fluorochemical plant in Flanders,  
445 Belgium. *Environ. Pollut.* **2017**, *228*, 140–148.
- 446 (16) Ahrens, L.; Herzke, D.; Huber, S.; Bustnes, J. O.; Bangjord, G.; Ebinghaus, R. Temporal  
447 trends and pattern of polyfluoroalkyl compounds in tawny owl (*Strix aluco*) eggs from  
448 Norway. *Environ. Sci. Technol.* **2011**, *45* (19), 8090–8097.
- 449 (17) Martinson, S. C.; Bird, D. M.; Shutt, J. L.; Letcher, R. J.; Ritchie, I. J.; Fernie, K. J.  
450 Multi-generational effects of polybrominated diphenylethers exposure: embryonic  
451 exposure of male American kestrels (*Falco sparverius*) to DE-71 alters reproductive  
452 success and behaviors. *Environ. Toxicol. Chem.* **2010**, *29* (8), 1740–1747.
- 453 (18) Zhang, Q.; Liu, W.; Niu, Q.; Wang, Y.; Zhao, H. M.; Zhang, H. F.; Song, J.; Tsuda, S.;  
454 Saito, N. Effects of perfluorooctane sulfonate and its alternatives on long-term  
455 potentiation in the hippocampus CA1 region of adult rats *in vivo*. *Toxicol. Res.* **2016**, *5*  
456 (2), 539–546.



- 457 (19) Yin, N.; Yang, R.; Liang, S.; Liang, S.; Hu, B.; Ruan, T.; Faiola, F. Evaluation of the  
458 early developmental neural toxicity of F-53B, as compared to PFOS, with an *in vitro*  
459 mouse stem cell differentiation model. *Chemosphere* **2018**, *204*, 109–118.
- 460 (20) Shi, G.; Cui, Q.; Pan, Y.; Sheng, N.; Sun, S.; Guo, Y.; Dai, J. 6:2 Chlorinated  
461 polyfluorinated ether sulfonate, a PFOS alternative, induces embryotoxicity and disrupts  
462 cardiac development in zebrafish embryos. *Aquat. Toxicol.* **2017**, *185*, 67–75.
- 463 (21) Smits, J. E. G.; Fernie, K. J. Avian wildlife as sentinels of ecosystem health. *Comp.*  
464 *Immunol. Microbiol. Infect. Dis.* **2013**, *36* (3), 333–342.
- 465 (22) Ottinger, M. A.; Abdelnabi, M.; Quinn, M.; Golden, N.; Wu, J.; Thompson, N.  
466 Reproductive consequences of EDCs in birds: what do laboratory effects mean in field  
467 species? *Neurotoxicol. Teratol.* **2002**, *24* (1), 17–28.
- 468 (23) Molina, E. D.; Balander, R.; Fitzgerald, S. D.; Giesy, J. P.; Kannan, K.; Mitchell, R.;  
469 Bursian, S. J. Effects of air cell injection of perfluorooctane sulfonate before incubation  
470 on development of the white leghorn chicken (*Gallus domesticus*) embryo. *Environ.*  
471 *Toxicol. Chem.* **2006**, *25* (1), 227–232.
- 472 (24) O'Brien, J. M.; Carew, A. C.; Chu, S.; Letcher, R. J.; Kennedy, S. W. Perfluorooctane  
473 sulfonate (PFOS) toxicity in domestic chicken (*Gallus gallus domesticus*) embryos in  
474 the absence of effects on peroxisome proliferator activated receptor alpha (PPAR $\alpha$ )-  
475 regulated genes. *Comp. Biochem. Physiol. - C Toxicol. Pharmacol.* **2009**, *149* (4), 524–  
476 530.
- 477 (25) Peden-Adams, M. M.; Stuckey, J. E.; Gaworecki, K. M.; Berger-Ritchie, J.; Bryant, K.;  
478 Jodice, P. G.; Scott, T. R.; Ferrario, J. B.; Guan, B.; Vigo, C.; Boone, J. S.; McGuinn,  
479 W. D.; DeWitt, J. C.; Keil, D. E. Developmental toxicity in white leghorn chickens  
480 following *in ovo* exposure to perfluorooctane sulfonate (PFOS). *Reprod. Toxicol.* **2009**,

- 481 27 (3–4), 307–318.
- 482 (26) Pinkas, A.; Slotkin, T. A.; Brick-Turin, Y.; Van der Zee, E. A.; Yanai, J.  
483 Neurobehavioral teratogenicity of perfluorinated alkyls in an avian model. *Neurotoxicol.*  
484 *Teratol.* **2010**, 32 (2), 182–186.
- 485 (27) Strömqvist, M.; Olsson, J. A.; Kärrman, A.; Brunström, B. Transcription of genes  
486 involved in fat metabolism in chicken embryos exposed to the peroxisome proliferator-  
487 activated receptor alpha (PPAR $\alpha$ ) agonist GW7647 or to perfluorooctane sulfonate  
488 (PFOS) or perfluorooctanoic acid (PFOA). *Comp. Biochem. Physiol. - C Toxicol.*  
489 *Pharmacol.* **2012**, 156 (1), 29–36.
- 490 (28) Nordén, M.; Westman, O.; Venizelos, N.; Engwall, M. Perfluorooctane sulfonate  
491 increases  $\beta$ -oxidation of palmitic acid in chicken liver. *Environ. Sci. Pollut. Res.* **2012**,  
492 19 (5), 1859–1863.
- 493 (29) Nordén, M.; Berger, U.; Engwall, M. Developmental toxicity of PFOS and PFOA in  
494 great cormorant (*Phalacrocorax carbo sinensis*), herring gull (*Larus argentatus*) and  
495 chicken (*Gallus gallus domesticus*). *Environ. Sci. Pollut. Res.* **2016**, 23 (11), 10855–  
496 10862.
- 497 (30) Parolini, M.; Colombo, G.; Valsecchi, S.; Mazzoni, M.; Possenti, C. D.; Caprioli, M.;  
498 Dalle-Donne, I.; Milzani, A.; Saino, N.; Rubolini, D. Potential toxicity of  
499 environmentally relevant perfluorooctane sulfonate (PFOS) concentrations to yellow-  
500 legged gull *Larus michahellis* embryos. *Environ. Sci. Pollut. Res.* **2016**, 23 (1), 426–  
501 437.
- 502 (31) Wang, T.; Vestergren, R.; Herzke, D.; Yu, J.; Cousins, I. T. Levels, isomer profiles, and  
503 estimated riverine mass discharges of perfluoroalkyl acids and fluorinated alternatives  
504 at the mouths of Chinese rivers. *Environ. Sci. Technol.* **2016**, 50 (21), 11584–11592.

- 505 (32) Brunström, B.; Örberg, J. A method for studying embryotoxicity of lipophilic substances  
506 experimentally introduced into hens' eggs. *Ambio* **1982**, *11* (4), 209–211.
- 507 (33) Powley, C. R.; George, S. W.; Ryan, T. W.; Buck, R. C. Matrix effect-free analytical  
508 methods for determination of perfluorinated carboxylic acids in environmental matrixes.  
509 *Anal. Chem.* **2005**, *77* (19), 6353–6358.
- 510 (34) Herzke, D.; Olsson, E.; Posner, S. Perfluoroalkyl and polyfluoroalkyl substances  
511 (PFASs) in consumer products in Norway – A pilot study. *Chemosphere* **2012**, *88* (8),  
512 980–987.
- 513 (35) Hanssen, L.; Dudarev, A. A.; Huber, S.; Odland, J. Ø.; Nieboer, E.; Sandanger, T. M.  
514 Partition of perfluoroalkyl substances (PFASs) in whole blood and plasma, assessed in  
515 maternal and umbilical cord samples from inhabitants of arctic Russia and Uzbekistan.  
516 *Sci. Total Environ.* **2013**, *447*, 430–437.
- 517 (36) R Core Team. R: a language and environment for statistical computing. Vienna, Austria  
518 2017.
- 519 (37) O'Brien, J. M.; Kennedy, S. W.; Chu, S.; Letcher, R. J. Isomer-specific accumulation of  
520 perfluorooctane sulfonate in the liver of chicken embryos exposed *in ovo* to a technical  
521 mixture. *Environ. Toxicol. Chem.* **2011**, *30* (1), 226–231.
- 522 (38) Noiva, R. M.; Menezes, A. C.; Peleteiro, M. C. Influence of temperature and humidity  
523 manipulation on chicken embryonic development. *BMC Vet. Res.* **2014**, *10*, 234.
- 524 (39) Thibodeaux, J. R.; Hanson, R. G.; Rogers, J. M.; Grey, B. E.; Barbee, B. D.; Richards,  
525 J. H.; Butenhoff, J. L.; Stevenson, L. A.; Lau, C. Exposure to perfluorooctane sulfonate  
526 during pregnancy in rat and mouse. I: Maternal and prenatal evaluations. *Toxicol. Sci.*  
527 **2003**, *74* (2), 369–381.

- 528 (40) Shi, X.; Du, Y.; Lam, P. K. S.; Wu, R. S. S.; Zhou, B. Developmental toxicity and  
529 alteration of gene expression in zebrafish embryos exposed to PFOS. *Toxicol. Appl.*  
530 *Pharmacol.* **2008**, *230* (1), 23–32.
- 531 (41) Huang, Q.; Fang, C.; Wu, X.; Fan, J.; Dong, S. Perfluorooctane sulfonate impairs the  
532 cardiac development of a marine medaka (*Oryzias melastigma*). *Aquat. Toxicol.* **2011**,  
533 *105* (1–2), 71–77.
- 534 (42) Huang, H.; Huang, C.; Wang, L.; Ye, X.; Bai, C.; Simonich, M. T.; Tanguay, R. L.;  
535 Dong, Q. Toxicity, uptake kinetics and behavior assessment in zebrafish embryos  
536 following exposure to perfluorooctanesulphonicacid (PFOS). *Aquat. Toxicol.* **2010**, *98*  
537 (2), 139–147.
- 538 (43) McKernan, M. A.; Rattner, B. A.; Hale, R. C.; Ottinger, M. A. Egg incubation position  
539 affects toxicity of air cell administered polychlorinated biphenyl 126 (3,3',4,4',5-  
540 pentachlorobiphenyl) in chicken (*Gallus gallus*) embryos. *Environ. Toxicol. Chem.*  
541 **2007**, *26* (12), 2724.
- 542 (44) DeWitt, J. C.; Meyer, E. B.; Henshel, D. S. Environmental toxicity studies using  
543 chickens as surrogates for wildlife: effects of injection day. *Arch. Environ. Contam.*  
544 *Toxicol.* **2005**, *48* (2), 270–277.
- 545 (45) Henshel, D. S.; Hein, B.; Wagey, R.; Vo, M.; Steeves, J. D. The relative sensitivity of  
546 chicken embryos to yolk- or air-cell injected 2,3,7,8-tetrachlorodibenzo-p-dioxin.  
547 *Environ. Toxicol. Chem.* **1997**, *16* (4), 725–732.
- 548 (46) Custer, C. M.; Custer, T. W.; Dummer, P. M.; Etterson, M. A.; Thogmartin, W. E.; Wu,  
549 Q.; Kannan, K.; Trowbridge, A.; McKann, P. C. Exposure and effects of perfluoroalkyl  
550 substances in tree swallows nesting in Minnesota and Wisconsin, USA. *Arch. Environ.*  
551 *Contam. Toxicol.* **2014**, *66* (1), 120–138.

- 552 (47) Lau, C.; Thibodeaux, J. R.; Hanson, R. G.; Rogers, J. M.; Grey, B. E.; Stanton, M. E.;  
553 Butenhoff, J. L.; Stevenson, L. A. Exposure to perfluorooctane sulfonate during  
554 pregnancy in rat and mouse. II: Postnatal evaluation. *Toxicol. Sci.* **2003**, *74* (2), 382–  
555 392.
- 556 (48) Seacat, A. M.; Thomford, P. J.; Hansen, K. J.; Olsen, G. W.; Case, M. T.; Butenhoff, J.  
557 L. Subchronic toxicity studies on perfluorooctanesulfonate potassium salt in  
558 cynomolgus monkeys. *Toxicol. Sci.* **2002**, *68* (1), 249–264.
- 559 (49) Chang, S.-C.; Thibodeaux, J. R.; Eastvold, M. L.; Ehresman, D. J.; Bjork, J. A.;  
560 Froehlich, J. W.; Lau, C.; Singh, R. J.; Wallace, K. B.; Butenhoff, J. L. Thyroid hormone  
561 status and pituitary function in adult rats given oral doses of perfluorooctanesulfonate  
562 (PFOS). *Toxicology* **2008**, *243* (3), 330–339.
- 563 (50) Deng, M.; Wu, Y.; Xu, C.; Jin, Y.; He, X.; Wan, J.; Yu, X.; Rao, H.; Tu, W. Multiple  
564 approaches to assess the effects of F-53B, a Chinese PFOS alternative, on thyroid  
565 endocrine disruption at environmentally relevant concentrations. *Sci. Total Environ.*  
566 **2018**, *624*, 215–224.
- 567 (51) Adams, S. M.; Shugart, L. R.; Southworth, G. R.; Hinton, D. E. Application of  
568 bioindicators in assessing the health of fish populations experiencing contaminant stress.  
569 In *Biomarkers of environmental contamination*; McCarthy, J. F., Shugart, L. R., Eds.;  
570 CRC Press, 1990; pp 333–353.
- 571 (52) Newsted, J. L.; Coady, K. K.; Beach, S. A.; Butenhoff, J. L.; Gallagher, S.; Giesy, J. P.  
572 Effects of perfluorooctane sulfonate on mallard and northern bobwhite quail exposed  
573 chronically via the diet. *Environ. Toxicol. Pharmacol.* **2007**, *23* (1), 1–9.
- 574 (53) Li, C.-H.; Ren, X.-M.; Ruan, T.; Cao, L.-Y.; Xin, Y.; Guo, L.-H.; Jiang, G. Chlorinated  
575 polyfluorinated ether sulfonates exhibit higher activity toward peroxisome proliferator-

576 activated receptors signaling pathways than perfluorooctanesulfonate. *Environ. Sci.*  
577 *Technol.* **2018**, *52* (5), 3232–3239.

578 (54) Cui, L.; Zhou, Q. F.; Liao, C. Y.; Fu, J. J.; Jiang, G. Bin. Studies on the toxicological  
579 effects of PFOA and PFOS on rats using histological observation and chemical analysis.  
580 *Arch. Environ. Contam. Toxicol.* **2009**, *56* (2), 338–349.

581 (55) Marasco, V.; Costantini, D. Signaling in a polluted world: oxidative stress as an  
582 overlooked mechanism linking contaminants to animal communication. *Front. Ecol.*  
583 *Evol.* **2016**, *4*, 95.

584 (56) Dennery, P. A. Effects of oxidative stress on embryonic development. *Birth Defects Res.*  
585 *Part C - Embryo Today Rev.* **2007**, *81* (3), 155–162.

586 (57) Halliwell, B.; Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*, Fourth.;  
587 Oxford University Press, 2007.

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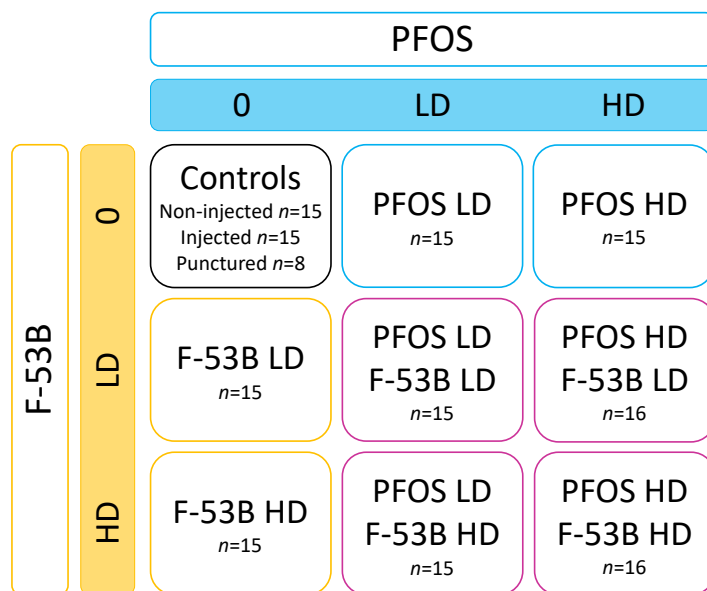
## 590 **Figure captions**

591 **Figure 1:** The 3 x 3 full factorial design of this study. All control and treatment groups are  
592 represented with their respective sample size. LD: low dose (150 ng/g egg) and HD: high dose  
593 (1500 ng/g egg).

594 **Figure 2:** The effect of F-53B on the mean hepatosomatic index (HSI; liver mass / body mass)  
595 of the hatchlings. A high dose of F-53B increases the liver mass with 8 % compared to groups  
596 (including mixtures) not exposed to F-53B ( $p = 0.026$ , as signified by the asterisk). Error bars  
597 represent the standard error of the mean.

598 **Figures**

599 Figure 1



600

601 Figure 2

