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1 **Do persistent organic pollutants interact with the stress response? Individual compounds, and**
2 **their mixtures, interaction with the glucocorticoid receptor.**

3
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11

12 *Abstract*

13 Persistent organic pollutants (POPs) are toxic substances, highly resistant to environmental
14 degradation, which can bio-accumulate and have long-range atmospheric transport potential (UNEP
15 2001). The majority of studies on endocrine disruption have focused on interferences on the sexual
16 steroid hormones and so have overlooked disruption to glucocorticoid hormones. Here the endocrine
17 disrupting potential of individual POPs and their mixtures has been investigated *in vitro* to identify any
18 disruption to glucocorticoid nuclear receptor transcriptional activity. POP mixtures were screened for
19 glucocorticoid receptor (GR) translocation using a GR redistribution assay (RA) on a CellInsight™ NXT
20 High Content Screening (HCS) platform. A mammalian reporter gene assay (RGA) was then used to
21 assess the individual POPs, and their mixtures, for effects on glucocorticoid nuclear receptor
22 transactivation. POP mixtures did not induce GR translocation in the GR RA or produce an agonist
23 response in the GR RGA. However, in the antagonist test, in the presence of cortisol, an individual POP,
24 *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE), was found to decrease glucocorticoid nuclear
25 receptor transcriptional activity to 72.5% (in comparison to the positive cortisol control). Enhanced
26 nuclear transcriptional activity, in the presence of cortisol, was evident for the two lowest
27 concentrations of perfluorodecanoic acid (PFOS) potassium salt (0.0147 mg/ml and 0.0294 mg/ml),
28 the two highest concentrations of perfluorodecanoic acid (PFDA) (0.0025 mg/ml and 0.005 mg/ml)
29 and the highest concentration of 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) (0.0000858 mg/ml). It
30 is important to gain a better understanding of how POPs can interact with GRs as the disruption of
31 glucocorticoid action is thought to contribute to complex diseases.
32

33 *Key Words:*

34 Persistent organic pollutants, Glucocorticoid receptor, Reporter gene assay, Mixtures, High content
35 analysis.

36 *1. Introduction*

37 Persistent organic pollutants (POPs) are toxic organic substances that are highly resistant to
38 environmental degradation, bio-accumulate and have long-range atmospheric transport potential
39 (UNEP 2001). This group of environmental chemicals have been detected in human adipose tissue,
40 serum and breast milk samples collected in Asia, Europe, North America and the Arctic (Bi *et al.* 2006;
41 Pereg *et al.* 2003; Sjödin *et al.* 1999; Sjödin *et al.* 2008) due to their lipophilic nature and resistance to
42 degradation (de Wit *et al.* 2004). The high lipid solubility of POPs enables them to pass through
43 biological barriers, such as the placental (Beesoon *et al.* 2011; Inoue *et al.* 2004; Ode *et al.* 2013) and
44 blood–brain barriers. A large number of POPs have been shown to be endocrine disrupting chemicals
45 (EDCs) in animals and humans which alters hormone-mediated responses (Birnbaum and Staskal 2004;
46 Boas *et al.* 2006; Darnerud 2003; Schantz and Widholm 2001; Zoeller 2005). The majority of studies
47 have focused on endocrine disruption of the sex steroid hormones and so have overlooked the
48 disruption to glucocorticoid hormones.

49 Induction of the hypothalamic–pituitary–adrenal (HPA) axis occurs when individuals are faced
50 with a stressful situation. The hypothalamus will secrete corticotropin-releasing hormone (CRH),
51 which causes the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland in
52 the brain to stimulate the release of cortisol from the adrenals. The glucocorticoids, cortisol in humans
53 and corticosterone in rodents, are central to the regulation of many physiological processes including
54 the control of energy metabolism and the modulation of the immune system (Charmandari *et al.* 2005;
55 Sapolsky *et al.* 2000). The release of glucocorticoids alters the individuals physiological state in
56 response to environmental conditions (Ricklefs and Wikelski 2002; Wingfield and Sapolsky 2003).
57 Physiological changes shift energy investment away from reproduction and redirect it towards survival
58 (Wingfield and Sapolsky 2003). Glucocorticoids are therefore extremely important to survival and have
59 been strongly associated with fitness traits such as breeding success and individual quality (Angelier
60 *et al.* 2009, Angelier *et al.* 2010; Bókony *et al.* 2009; Goutte *et al.* 2011). Glucocorticoids, in addition,
61 also play important roles in the process of immunomodulation (Jondal *et al.* 2004). Despite the
62 importance of glucocorticoids for the regulation of physiological processes, the relationship between
63 environmental chemicals and potential disruption of the HPA axis has not been extensively studied
64 (Odermatt *et al.* 2006).

65 Glucocorticoids are lipophilic and can cross the blood–brain barrier where they bind to
66 glucocorticoid receptors (GRs). In humans, the hippocampus and frontal lobes of the brain contain
67 GRs. These are parts of the brain that are involved in cognitive functions such as memory and
68 emotional maladjustments including impulsivity. Changes in the function of the HPA-axis may lead to
69 altered stress responses and changes in cognitive functions. Glucocorticoids are responsible for
70 maturation of tissues essential for neonatal survival (Langlois *et al.* 2002), therefore disruption of

71 normal HPA axis activity may have widespread consequences. In humans, elevated cortisol and
72 aldosterone levels are associated with low birth weight (Martinez-Aguayo *et al.* 2011). Lanoix and
73 Plusquellec (2013) suggested that a disruption of the stress system could explain an association
74 between environmental contaminants and mental health, especially in children and elderly people.

75 In contrast to the human estrogen and androgen receptors that are mainly expressed in the
76 gonads, the human GR is expressed in every cell type (Akner *et al.* 1994). GR disruption has the
77 potential to affect numerous processes. In stressful situations, when levels of glucocorticoids are high,
78 GR activation is necessary for the HPA feedback regulation (de Kloet *et al.* 1998). GR deficient mice
79 have a range of abnormalities including hyper activation of the HPA axis, impaired lung function and
80 die shortly after birth (Cole *et al.* 1995). Hyper activation of the HPA axis is expected if GR signalling is
81 disrupted as the HPA axis is subject to feedback inhibition from circulating glucocorticoids which act
82 through GRs (Keller-Wood and Dallman 1984). Hyper activation of the HPA axis is associated with
83 psychiatric disorders including anorexia nervosa, obsessive-compulsive disorder and anxiety.
84 Furthermore, glucocorticoid-mediated feedback inhibition is impaired in people who suffer from
85 depression (Jurueña *et al.* 2003). Hyperactivation of the HPA axis has also been associated with
86 hyperthyroidism (Tsigos and Chrousos 2002). Patients with excessive levels of corticosteroids are at a
87 higher risk of developing cardiovascular disease (Pimenta *et al.* 2012). Disruption of glucocorticoid
88 signalling could also have implications for obesity, as this system is central to adipocyte differentiation.
89 EDCs have been found to promote adipogenesis in the 3T3-L1 cell line through the activation of the
90 GR, thus leading to obesity (Sargis *et al.* 2009).

91 POPs have been linked to GR disruption. Methylsulfonyl metabolites from PCBs have been
92 found to act as GR antagonists (Johansson *et al.* 1998). POPs can also disrupt regulation of adrenal
93 hormone secretion and function at different levels of the HPA axis. The human H295R adrenal cell
94 model highlighted that the adrenal cortex is a potential target for perfluorononanoic acid (PFNA)
95 (Kraugerud *et al.* 2011), polychlorinated biphenyls (PCBs) (Li & Wang 2005; Xu *et al.* 2006) and
96 polybrominated diphenyl ethers (PBDEs) (Song *et al.* 2008). POPs can also decrease adrenal hormone
97 production; as has been observed for the organohalogen pesticide γ -HCH (Lindane) (Oskarsson *et al.*
98 2006; Ullerås *et al.* 2008). Methylsulfonyl metabolites of dichlorodiphenyldichloroethylene (DDE)
99 caused a decrease in H295R cell viability (Asp *et al.* 2010). Furthermore reduced plasma corticosterone
100 levels were recorded *in vivo* in suckling mice following administration of these DDE metabolites to
101 their lactating mothers (Jönsson *et al.* 1993). In arctic birds, high baseline corticosterone
102 concentrations and a reduced stress response have been associated with high concentrations of
103 organochlorines, PBDEs and their metabolites in blood plasma (Verboven *et al.* 2010). Reduced

104 responsiveness of the HPA axis has been demonstrated in amphibians (Gendron *et al.* 1997) and birds
105 (Mayne *et al.* 2004) and this has been associated with exposure to POPs.

106 This study aimed to assess the interaction of individual POPs and their mixtures at the GR level
107 and to see if they disrupted this nuclear receptor's transcriptional activity. Two *in vitro* bioassays were
108 used; a high content GR redistribution assay (RA) and a GR reporter gene assay (RGA). The GR RA was
109 used as a screening method for the POP mixtures as it measures GR translocation and would therefore
110 presumably detect any GR activity, agonism or antagonism. The GR RGA uses a human mammary gland
111 cell line, with natural steroid hormone receptors for glucocorticoids and progestogens, which has been
112 transformed with a luciferase gene (Willemsen *et al.* 2004), thereby allowing endocrine disruption at
113 the level of nuclear receptor transcriptional activity to be identified. Disruption of GR activity is
114 important and can have significant implications on health however the interaction of individual POPs
115 and their mixtures with GRs has not been extensively studied.

116

117 2. Materials and methods

118

119 2.1. Chemicals

120 All PBDEs, PCBs and other organochlorines were originally purchased from Chiron As (Trondheim,
121 Norway) and all perfluorinated compounds (PFCs) were obtained from Sigma-Aldrich, St. Louis, MO,
122 USA except perfluorohexanesulfonic acid (PFHxS) which was obtained from Santa Cruz (Dallas, US).
123 Hexabromocyclododecane (HBCD), phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO),
124 thiazolyl blue tetrazolium bromide (MTT) and the steroid hormone cortisol were obtained from
125 Sigma–Aldrich (Dorset, UK). Hoechst nuclear stain was purchased from Perbio (Northumberland,
126 England). Cell culture reagents were supplied by Life Technologies (Paisley, UK) unless otherwise
127 stated. All other reagents were standard laboratory grade.

128

129 2.2. Mixtures

130 Mixtures of the test POPs were designed and premade by the Norwegian University of Life Sciences,
131 Oslo. Seven mixtures were used in the assays: (1) total mixture, containing all the test compounds, (2)
132 perfluorinated mixture (PFC), (3) brominated mixture (Br), (4) chlorinated mixture (Cl), (5)
133 perfluorinated and brominated mixture (PFC + Br), (6) perfluorinated and chlorinated mixture (PFC +
134 Cl) and (7) brominated and chlorinated mixture (Br + Cl). The chemicals included in the mixtures and
135 their respective concentrations in the stock solution are shown in Table 1 (Berntsen *et al.* 2015). The
136 concentration of the working stocks for the individual POPs is also shown in Table 1; individual
137 intermediate stocks were prepared of each POP (1/2, 1/10 and 1/20 dilutions of the working stocks).

138 The POP mixtures used in this study were based on concentrations of relevant POPs as measured in
139 human blood and breast milk, according to recent studies of the Scandinavian population (Haug *et al.*
140 2010, Knutsen *et al.* 2008; Polder *et al.* 2008; Polder *et al.* 2009; Van Oostdam *et al.* 2004) as described
141 in Berntsen *et al.* (2015). The compounds were mixed in concentration ratios relevant to human
142 exposure. The stocks of the total mixture, Cl mixture and the Cl sub-mixtures were ten times more
143 diluted compared to the PFC and the Br mixtures, and the combined PFC and Br mixture.
144

145 Table 1. The composition and concentrations of original stocks supplied by the Norwegian University
 146 of Life Sciences, Oslo. Mixtures: the estimated concentration of POPs in Total, Cl, PFC + Cl and Br + Cl
 147 stock solutions is 1000000 times estimated concentration in human serum. In comparison to PFC, Br
 148 and PFC + Br estimated concentration of POPs is 10000000 times estimated concentration in human
 149 serum. For the individual POPs the concentration of each working stock is shown. Intermediate stocks
 150 were prepared from the working stocks (1/2, 1/10 and 1/20 dilutions). The final concentrations that
 151 the cells were exposed to (0.2% DMSO in media) was 1/1000, 1/2000, 1/10000 and 1/20000 of the
 152 original working stocks. For the individual compounds the cells were exposed to 500, 1000, 5000 and
 153 10000 times serum level).
 154

Compound	Mixture Stock Concentration (mg/ml)							Individual Stock Concentration (mg/ml)
	Total	PFC	Br	Cl	PFC+Br	PFC+Cl	Br+Cl	
Perfluorinated compounds (PFCs)								
PFOA	4.523	45.225			45.225	4.523		45.225
PFOS	29.425	294.250			294.250	29.425		294.250
PFDA	0.495	4.950			4.950	0.495		4.950
PFNA	0.800	8.000			8.000	0.800		8.000
PFHxS	3.450	34.500			34.500	3.450		34.500
PFUnDA	0.560	5.600			5.600	0.560		5.600
Polybrominated diphenyl ethers (PBDEs)								
BDE-209	0.011		0.108		0.108		0.011	0.108
BDE-47	0.009		0.086		0.086		0.009	0.086
BDE-99	0.004		0.035		0.035		0.004	0.035
BDE-100	0.002		0.022		0.022		0.002	0.022
BDE-153	0.001		0.010		0.010		0.001	0.010
BDE-154	0.002		0.018		0.018		0.002	0.018
HBCD	0.025		0.246		0.246		0.025	0.246
Polychlorinated biphenyls (PCBs)								
PCB 138	0.222			0.222		0.222	0.222	2.220
PCB 153	0.362			0.362		0.362	0.362	3.620
PCB 101	0.008			0.008		0.008	0.008	0.078
PCB 180	0.194			0.194		0.194	0.194	1.940
PCB 52	0.010			0.010		0.010	0.010	0.096
PCB 28	0.013			0.013		0.013	0.013	0.128
PCB 118	0.064			0.064		0.064	0.064	0.640
Other organochlorines								
<i>p,p'</i> -DDE	0.502			0.502		0.502	0.502	5.020
HCB	0.117			0.117		0.117	0.117	1.170
α - chlordane	0.011			0.011		0.011	0.011	0.108
oxy - chlordane	0.022			0.022		0.022	0.022	0.222
trans-nonachlor	0.041			0.041		0.041	0.041	0.408
α -HCH	0.006			0.006		0.006	0.006	0.060
β -HCH	0.053			0.053		0.053	0.053	0.526
γ -HCH (Lindane)	0.006			0.006		0.006	0.006	0.060
Dieldrin	0.024			0.024		0.024	0.024	0.240

155

156 2.3. GR RA cell culture and method

157 Recombinant U2OS cells stably expressing the human GR (U2OS-GR) were routinely cultured in a
158 humidified atmosphere of 5% CO₂ at 37 °C. Cells were grown in 75 cm² flasks in Dulbecco's modified
159 eagle medium (DMEM) media supplemented with 10% foetal bovine serum (FBS), 2mM L-Glutamine,
160 1% penicillin-streptomycin and 0.5 mg/ml G418. TrypLE™ Express trypsin was used to disperse the
161 cells from the flasks, while cell counting and viability checks prior to seeding plates were achieved by
162 trypan blue staining and using a Countess® automated cell counter.

163 Cells were seeded (using DMEM supplemented with 2mM L-Glutamine, 1% Penicillin-
164 Streptomycin, 0.5 mg/ml G418 and 10% hormone depleted FBS) at a concentration of 6 x 10⁴ cells per
165 well in 100 µl of media into black walled 96 well plates with clear flat bottoms (Grenier, Germany).
166 The cells were incubated for 1 h at room temperature (RT) (20-25°C) to ensure that they attached
167 evenly within each well. The cells were then incubated for 24 h at 37 °C, and subsequently exposed in
168 assay media (DMEM supplemented with 2mM L-Glutamine and 1% Penicillin-Streptomycin) to 1/1000,
169 1/2000, 1/10000 and 1/20000 dilutions (0.2% DMSO in media) of the original stocks, which
170 corresponded to 10000, 5000, 1000 and 500 times the levels in serum for the PFC, Br and PFC + Br
171 mixtures. For the remaining mixtures (total, Cl, PFC + Cl and Br + Cl) the exposures corresponded to
172 1000, 500, 100 and 50 times the levels in serum. Assay media was used to dilute the stock solutions.
173 The cortisol standard curve used covered the range of 0.02-22.7 ng/ml. A solvent control 0.2% v:v
174 DMSO in media was also added to each plate. The cells were incubated for 48 h after which the media
175 was discarded and the cells fixed by adding 150 µl fixing solution (10% formalin, neutral-buffered
176 solution) per well. The plate was incubated at RT for 20 min. The fixing solution was then removed
177 and cells washed four times with 200 µl PBS. After the last wash was removed and 100 µl of 1 µM
178 Hoechst Staining Solution (1 µM Hoechst in PBS containing 0.5% Triton X-100) was added to each well
179 before the plate was sealed with a black plate sealer and left at least 30 min before imaging.

180

181 *2.4. High content analysis (HCA)*

182 The GR RA was imaged using a CellInsight™ NXT High Content Screening (HCS) platform (Thermo
183 Fisher Scientific, UK). This instrument analyses epifluorescence of individual cell events using an
184 automated micro-plate reader analyser interfaced with a PC (Dell precision 136 T5600 workstation).
185 Hoechst dye was used to measure nuclear morphology: cell number (CN), nuclear intensity (NI) and
186 nuclear area (NA). Data was captured for each plate at 10x objective magnification in the selected
187 excitation and emission wavelengths for Hoechst dye (Ex/Em 350/461 nm) and enhanced Green
188 Fluorescent Protein (GFP) (488/509 nm). Briefly, the U2OS-GR cell line is a recombinant cell line which
189 stably expresses the human GR fused to an enhanced GFP. The expression of the EGFP-GR is controlled
190 by a promoter and continuous expression is maintained by the addition of G418 to the culture media.

191 The primary output in the GR RA is the translocation from cytoplasm to nucleus of enhanced GFP-GR.
192 The output used was MEAN_CircRingAvgIntenDiffCh2 (difference in average fluorescence intensities
193 of nucleus and cytoplasm).

194

195 *2.5. GR reporter gene assay (RGA)*

196 The TGRM-Luc cell line for the detection of glucocorticoids and progestogens previously developed by
197 Willemsen *et al.* (2004) was used. This transformed cell line was cultured in DMEM and 10% FBS, and
198 grown in 75cm² tissue culture flasks (Nunc, Roskilde, Denmark) at 37 °C with 5% CO₂ and 95% humidity.
199 TrypLE™ Express trypsin was used to disperse the cells from the flasks, while cell counting and viability
200 checks prior to seeding plates were achieved by trypan blue staining and using a Countess® automated
201 cell counter. The RGA was carried out in assay media (DMEM supplemented with 10% hormone
202 depleted serum).

203 Cells were seeded at a concentration of 4 × 10⁵ cells/ml in 100 µl media into white walled 96
204 well plates with clear flat bottoms (Greiner Bio-One, Germany). The cells were incubated for 24 h and
205 then exposed to four dilutions of each individual compound and mixture for the agonist test (cells
206 were exposed as in section 2.3). For the individual compounds the four dilutions represent 500, 1000,
207 5000 and 10000 times serum levels. The cortisol standard curve covered the range of 4.5-181.2 ng/ml.
208 A solvent control 0.2% v:v DMSO in media was also added to each plate. Antagonist tests were carried
209 out by incubating the four dilutions of each individual compound and mixture with the positive control
210 (90.6 ng/ml cortisol). The cells were incubated for 48 h, after which the media was discarded and the
211 cells were washed twice with PBS. The cells were lysed with 30 µl cell culture lysis buffer (Promega,
212 Southampton, UK) 100 µl luciferase substrate (Promega, Southampton, UK) was injected into each
213 well and the response measured using the Mithras Multimode Reader (Berthold, Other, Germany).
214 The response of the cell line to the various compounds was measured and compared with the solvent
215 and positive controls.

216

217 *2.6. Cell viability assay*

218 As well as visual inspection of the U2OS-GR and TGRM-Luc cells under the microscope to evaluate cell
219 morphology and attachment, the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT)
220 cell viability assay was performed.

221 The cells were exposed exactly as for the GR RGA (section 2.5) after which the percentage of
222 viable cells was determined using the MTT assay. The cells were washed once with PBS before MTT
223 solution (50 µl of 2 mg/ml stock in PBS diluted 1:2.5 in assay media) was added to each well and the
224 cells incubated for 3 h. Viable cells convert the soluble yellow MTT to insoluble purple formazan by

225 the action of mitochondrial succinate dehydrogenase. The supernatant was removed and 200 µl of
226 DMSO was added to dissolve the formazan crystals. The plate was incubated at 37 °C with agitation
227 for 10 min before absorbance was measured at 570 nm with a reference filter at 630 nm using a
228 microtitre plate reader (TECAN, Switzerland). Viability was calculated as the percentage absorbance
229 of the sample when compared with the absorbance of the solvent control.

230

231 2.7. Statistical analysis

232 Exposures were carried out in triplicate wells and experiments were repeated at least twice.
233 Data was analysed using Microsoft Excel 2013 and Graphpad PRISM software version 5.01 (San Diego,
234 CA). All values shown are expressed as mean ± standard error of the mean (SEM) of the independent
235 exposures. Differences between groups were analysed by one-way ANOVA followed by Dunnett's
236 procedure for multiple comparisons. Significant effects are represented by $p \leq 0.05$ (*), $p \leq 0.01$ (**)
237 and $p \leq 0.001$ (***)).

238

239 3. Results and discussion

240

241 3.1. Cell viability of TGRM-Luc cell line after exposure to individual POPs

242

243 The MTT assay evaluates cytotoxicity by measurement of mitochondrial metabolic activity. As the
244 individual POPs were tested for GR activity using the RGA only, their toxicity was evaluated on the
245 TGRM-Luc cell line only using the MTT assay (statistically significant results are shown in Figure 1).

246 PFCs are widely reported to be cytotoxic, particularly PFOS which has been shown to affect
247 the viability of numerous cell culture systems including: human hepatocarcinoma cells (HepG2)
248 (Florentin *et al.* 2011; Shabalina *et al.* 1999), human placental syncytiotrophoblasts (Zhang *et al.* 2015),
249 neonatal Sertoli cells/gonocytes (Zhang *et al.* 2013) and neural stem cells (Wan Ibrahim *et al.* 2013).
250 These studies support the finding that PFOS in the present study (at the two highest concentrations;
251 0.147 mg/ml and 0.294 mg/ml corresponding to 5000 and 10000 times serum level respectively)
252 significantly decreased TGRM-Luc cell viability to 42.8% and 3.8% ($p \leq 0.001$) (Figure 1A). Conversely,
253 other studies have found that PFOS has no effect on cell viability in MCF-7 (Maras *et al.* 2006) and
254 human adrenocortical H295R cells (Kraugerud *et al.* 2011). As both Maras *et al.* (2006) and Kraugerud
255 *et al.* (2011) used similar concentrations of PFOS to the present study the differences observed may
256 show that PFOS is more cytotoxic to particular cell lines.

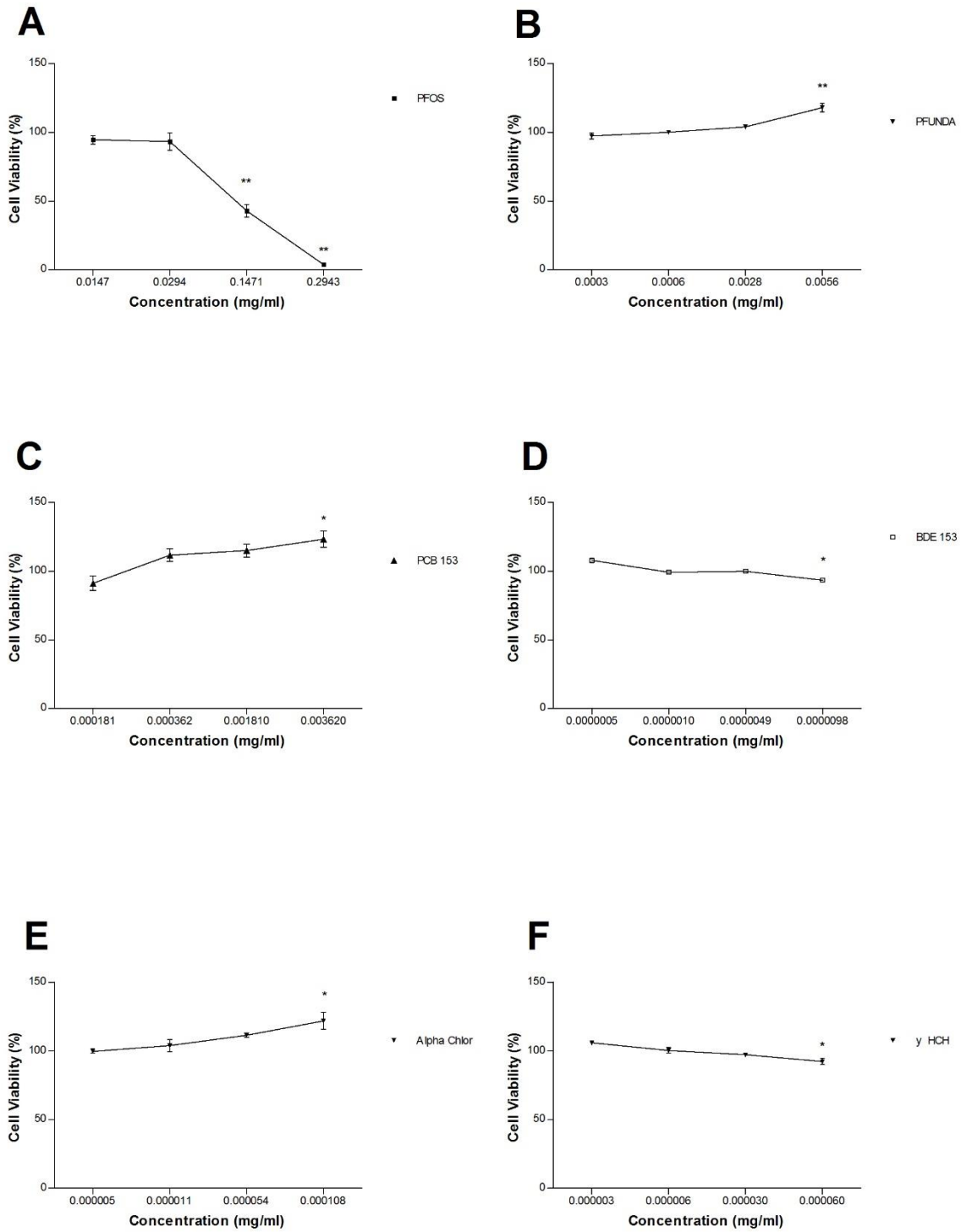
257 Perfluorooctanoic acid (PFOA) and PFOS use has been regulated worldwide.
258 Perfluoroundecanoic acid (PFUnDA), a PFOA homologue, is sometimes used as an alternative to PFOA

259 (EPA 2013). PFUnDA has been less extensively studied than PFOS or PFOA, however as PFCs with
260 longer carbon chains tend to be more persistent (Hirata-Koizumi *et al.* 2012) the toxicological potential
261 of PFUnDA could be of concern. In the current study, viability of TGRM-Luc cells significantly increased
262 after exposure to the highest concentration (0.0056mg/ml; 10000 times serum level) of PFUnDA to
263 117.9% ($p \leq 0.01$) (Figure 1B). The MTT assay relies on mitochondrial dehydrogenases of living cells to
264 cause a conversion of the coloured formazan salt (Slater *et al.* 1963). Actively proliferating cells
265 increase their metabolic activity while cells exposed to toxins may have decreased activity.
266 Alternatively, the apparent stimulatory effect seen in the MTT assay for some of the POPs, could be
267 as a consequence of cell protection or adaptive response to toxin exposure (Ruiz *et al.* 2006)

268 From the PCB group, no significant effects on cell viability were evident apart from PCB 153
269 (at the highest concentration; 0.0036 mg/ml), which significantly increased TGRM-Luc cell viability to
270 123.1% ($p \leq 0.05$) (Figure 1C). PCB-153 has previously been seen to induce cell proliferation in *in vivo*
271 experiments on rats (Lu *et al.* 2003). A commercial PCB mixture, Aroclor 1260, has been shown to
272 induce hepatocyte proliferation in rodents (Whysner and Wang 2001). In contrast, PCB-153
273 significantly induced loss of cell viability in human liver and kidney cell cultures in a concentration and
274 time-dependent manner in a study by Ghosh *et al.* (2010). Furthermore, PCB 153 has been found to
275 lower cell viability in neonatal Sertoli cell/gonocytes (Zhang *et al.* 2013). The reason for the observed
276 differences between these studies and the present study could be because Ghosh *et al.* (2010) tested
277 PCB 153 at a higher concentration (0.025 mg/ml) in comparison to the present study (the highest
278 concentration tested was 0.0036 mg/ml). Zhang *et al.* (2013) found significant decreases in cell
279 viability when using the same concentration as the present study (0.0036 mg/ml) however the cell
280 culture systems used were different.

281 The toxicity of PBDEs has been widely reported in several cell culture systems, such as rat
282 cerebellar granule cells (Reistad *et al.* 2006), human astrocytoma cells (Madia *et al.* 2004),
283 hippocampal neurons, human neuroblastoma cells, human foetal liver hematopoietic cells (He *et al.*
284 2008, He *et al.* 2009; Shao *et al.* 2008) and HepG2 cells (Hu *et al.* 2007). In the present study only one
285 PBDE, BDE-153, decreased cell viability. Exposure to BDE-153 at 0.0000098 mg/ml (the highest
286 concentration; 10000 times serum level); reduced viability to 93.3% ($p \leq 0.05$) (Figure 1D). Importantly,
287 these studies tested the PBDEs at much higher concentrations than the TGRM-Luc cell line was
288 exposed to. Schreiber *et al.* (2010) tested concentrations of BDE-47 and BDE-99 that were comparable
289 to the present study (0.1–10 μ M; BDE-47 range 48.5 – 4857.9 ng/ml; BDE-99 range 56.5 – 5646.9
290 ng/ml) in human neural progenitor cells and found no cytotoxicity. This is supported by the present
291 study which found no significant cytotoxicity for either BDE-47 or BDE-99 at any concentration tested
292 (range covered was 4.2 – 85.7 ng/ml for BDE-47 and 1.7 – 35.2 ng/ml for BDE-99).

293 From the group of other organochlorines, two compounds significantly impacted cell viability.
294 Alpha-chlordane (α -chlordane), at the highest concentration (0.000108 mg/ml), increased TGRM-Luc
295 cell viability to 121.8% ($p \leq 0.05$) (Figure 1E). This compound has been found to cause increased cell
296 proliferation in the thyroid gland and promote liver tumours in mice (Barrass *et al.* 1993). Conversely,
297 suppression of lymphocyte proliferation after α -chlordane exposure has been recorded (Chuang *et al.*
298 1992). The second compound that significantly affected TGRM-Luc cell viability in the present study
299 was γ -HCH. Exposure to the highest concentration of γ -HCH (0.00006 mg/ml) decreased cell viability
300 to 92.2% ($p \leq 0.05$) (Figure 1F). γ -HCH has similar effects on the viability of MCF-7 cells (Joseph and
301 D'Auvergne 2012) with exposure to 0.06 mg/ml significantly decreasing cell viability.



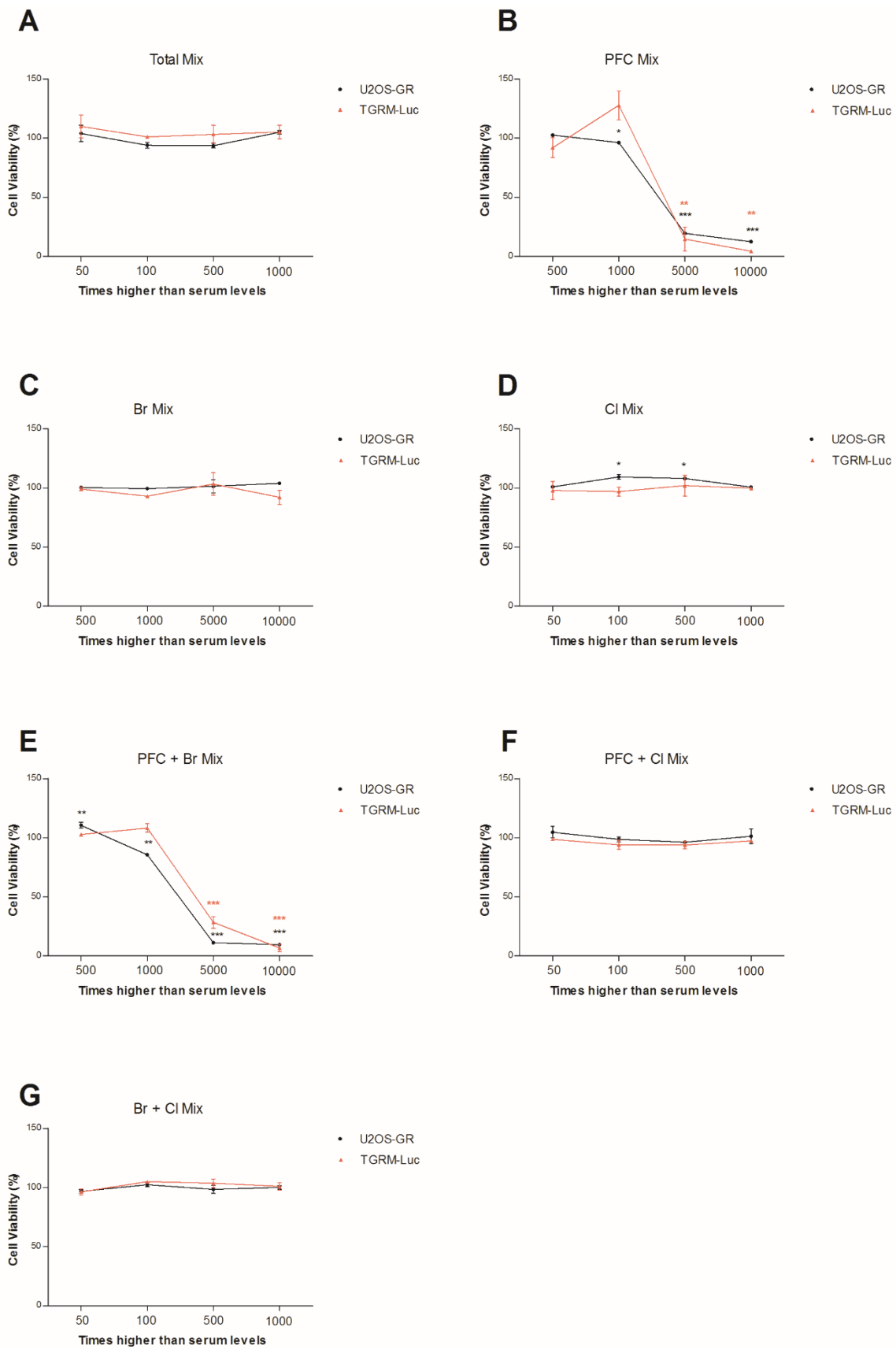
302 Figure 1: Viability of the TGRM-Luc cell line following exposure to individual POPs: (A) PFOS,(B)
 303 PFUNDA, (C) PCB 153, (D) BDE-153, (E) Alpha-Chlor and (F) γ -HCH; measured using the MTT Assay.
 304 Values are mean \pm SEM n = 2 $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***) represent significant
 305 cytotoxic effects. Only compounds which have significant results are shown. The concentrations
 306 shown are equivalent to 500, 1000, 5000 and 10000 times serum level).
 307

308 *3.2. Cell viability of TGRM-Luc and U2OS-GR cell lines after exposure to POP mixtures*

309

310 The MTT assay was also used to determine the viability of TGRM-Luc cells (used in the RGA) and the
311 U2OS-GR cells (used in the HCA assay) following exposure to different concentrations of test mixtures
312 (Figure 2).

313 The PFC mixture (at 5000 and 10000 times serum levels) decreased cell viability to 19.5% and
314 12.4% respectively (U2OS-GR cell line) and 14.6% and 4.3% respectively (TGRM-Luc cell line) (Figure
315 2B); implying that this mixture is more toxic to the TGRM-Luc cells. The toxicity in the PFC mixture is
316 likely to be from PFOS as none of the other compounds in the PFC mixture were cytotoxic to the TGRM-
317 Luc cell line (section 3.1). The PFC + Br mixture (at 5000 and 10000 times serum levels) also decreased
318 cell viability to 11.2% and 9.4% respectively (U2OS-GR) and 28.4% and 6.7% respectively (TGRM-Luc)
319 (Figure 2E). In the PFC + Br mixture the toxicity to the TGRM-Luc cell line is again likely to be from PFOS
320 (section 3.1). No significant cytotoxicity was evident for the total, Br, Cl, PFC + Cl or Br + Cl mixtures at
321 any concentration in either cell line (Figure 2A, C, D, F and G). However, exposure to the Cl mixture
322 produced small, but statistically significant increases in cell viability of 109.4% and 108.0%, at 100 and
323 500 times serum level respectively, in the U2OS-GR cell line ($p \leq 0.05$ for both) (Figure 2D).



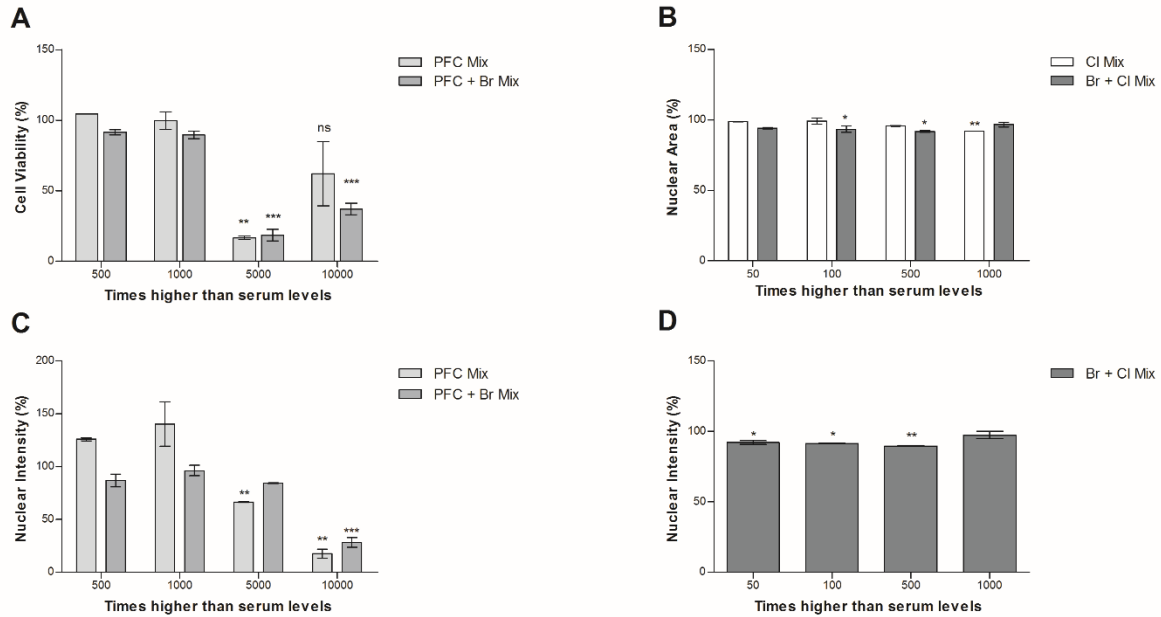
324

325 Figure 2: Viability of the TGRM-Luc and U2OS-GR cell lines following exposure to different mixtures of
 326 POPs; measured using the MTT Assay. Values are mean \pm SEM $n = 2$ $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq$
 327 0.001 (***) represent significant cytotoxic effects.

328 The HCA GR RA allows CN, NA and NI to be measured and these can be used as indicators of
329 cytotoxicity. Significant effects were evident for CN, NA and NI which indicated cytotoxicity (Figure 3).
330 CN for the second highest concentration (5000 times serum level) of the PFC mixture was significantly
331 decreased to 16.7% ($p \leq 0.01$) For the highest concentration of PFC mixture (10000 times serum level),
332 cell viability decreased to 61.9% however it was not deemed statistically significant ($p \geq 0.05$). The two
333 highest concentrations of the PFC + Br mixture (5000 and 10000 times serum level) decreased CN to
334 37.0% and 18.4% respectively ($p \leq 0.001$) (Figure 3A). NA was significantly decreased in U2OS-GR cells
335 after exposure to the highest concentration of the Cl mixture (92.0%, $p \leq 0.01$; Figure 3B). Similarly,
336 NA was decreased after exposure to the Br + Cl mixture at 100 and 500 times serum level (93.4% and
337 91.7% respectively, $p \leq 0.05$; Figure 3B). Nuclear shrinkage is a hallmark of apoptosis. However as
338 there were no significant decreases in CN for either the Cl or Br + Cl mixtures the decrease in NA did
339 not result in significant cell death. NI was decreased after exposure to the highest concentrations
340 (5000 and 10000 times serum level) of the PFC mixture (66.4% and 17.5% respectively $p \leq 0.01$ for
341 both) and the PFC + Br mixture (10000 times serum level), 28.3% ($p \leq 0.001$) (Figure 3C). The swelling
342 of nuclei is linked to compound induced necrosis and NI correlates to nuclear size with large nuclei
343 showing lower intensities (Mirochnitchenko *et al.* 1999); as NI and CN were significantly decreased for
344 both the PFC and PFC + Br mixtures it is likely that these mixtures caused the U2OS-GR cells to undergo
345 necrosis. Additionally, NI was also decreased for all concentrations of the Br + Cl mixture (apart from
346 the most concentrated), 92.0%, 91.3% and 89.5% ($p \leq 0.05$, 0.05, 0.01 respectively; Figure 3D). The Br
347 + Cl mixture may be causing the U2OS-GR cells to start to undergo necrosis however there was no
348 significant change in CN.

349 In summary, there was reasonable agreement between the MTT assay results and the CN HCA
350 parameter (Table 2) with the two highest concentrations of both the PFC and PFC + Br mixtures being
351 highlighted as decreased in both; all were statistically significant apart from CN for the PFC mixture at
352 10000 times serum level. However the MTT assay also highlighted decreased cell viability after
353 exposure to the PFC mixture (1000 times serum level), increased cell viability after exposure to the Cl
354 mixture (100 and 500 times serum levels), increased cell viability for the PFC + Br mixture (500 times
355 serum level) and decreased exposure at 1000 times serum level for the same mixture; these changes
356 were not detected in any HCA parameter. In contrast, the HCA parameters NA and NI highlighted
357 subtle changes after exposure to the Br + Cl mixture; this mixture was not deemed cytotoxic by the
358 MTT assay. In addition the NA parameter was significantly decreased after exposure to the Cl mixture
359 (1000 times serum level); this was not highlighted by the MTT assay.

360



361

362 Figure 3: Nuclear changes in U2OS-GR cells measured using HCA parameters CN, NA and NI with
 363 Hoechst staining. (A) Cell viability as measured by CN for the PFC and PFC + Br mixtures. (B) Nuclear
 364 area for the Cl and Br + Cl mixtures. (C) Nuclear intensity for the PFC and PFC + Br mixtures. (D) Nuclear
 365 intensity for Br + Cl mixture. Note: only mixtures which had at least one statistically significant effect
 366 are shown. Values are mean \pm SEM n = 2 $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***) represent
 367 significant cytotoxic effects.
 368

369 Table 2: Comparison of MTT and HCA nuclear parameter results for U2OS-GR cells. The grey shading
 370 indicates that no significant effects were found. The total, Br and PFC + Cl mixtures are not shown as
 371 toxic effects were not evident in the MTT assay or in HCA parameters. \uparrow indicates increased effect
 372 e.g. for the MTT assay it shows increased cell viability. \downarrow indicates decreased effect e.g. for the NA
 373 parameter it shows a decrease in nuclear size. Numbers 1-4 in the first column represent the
 374 concentration of the mixture; for PFC and PFC + Br it represents 500, 1000, 5000 and 10000 times
 375 serum levels. For the Cl and Br + Cl mixtures it represents 50, 100, 500 and 1000. Statistical significance
 376 is also indicated: $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***) represent significant cytotoxic effects.

	PFC				PFC + Br				Cl				Br + Cl			
	MTT	CN	NA	NI	MTT	CN	NA	NI	MTT	CN	NA	NI	MTT	CN	NA	NI
1					\uparrow^{**}											\downarrow^*
2	\downarrow^*				\downarrow^{**}				\uparrow^*						\downarrow^*	\downarrow^*
3	\downarrow^{***}	\downarrow^{**}			\downarrow^{***}	\downarrow^{***}			\uparrow^*						\downarrow^*	\downarrow^{**}
4	\downarrow^{***}	\downarrow^{ns}		\downarrow^{**}	\downarrow^{***}	\downarrow^{***}		\downarrow^{***}			\downarrow^{**}					

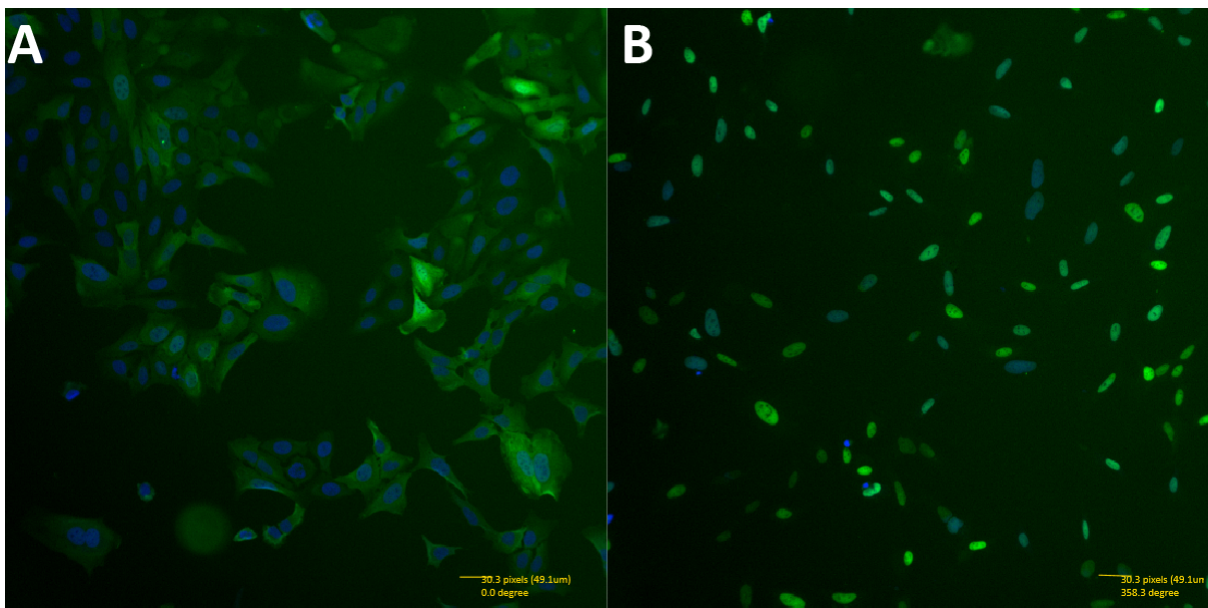
377

378 3.3. GR HCA Redistribution Assay

379

380 The GR RA was used to screen the POP mixtures for GR translocation. Glucocorticoids exert their
 381 activity through binding to GR which results in either activation or repression of a large set of

382 glucocorticoid responsive genes. In the inactive state, the GR is located in the cytoplasm (Figure 4A)
383 bound to various heat-shock proteins in a large multi-protein complex (Pratt and Toft 1997). When
384 activated by ligand binding, the GR detaches from the complex, translocates to the nucleus (Figure
385 4B) where it interacts with GR regulatory elements (GREs) to stimulate transcription and act as a
386 transcription factor to regulate the expression of its target genes (John *et al.* 2008). In the GR RA, the
387 translocation of a GFP-GR fusion protein from the cytoplasm to the nucleus is measured; both GR
388 agonists and antagonists induce nuclear translocation (Rosenfeld and Glass 2001). No significant GR
389 translocation effects were found for any of the POP mixtures at any concentration.



390

391 Figure 4: Example of GR RA images: (A) negative control - DMSO (B) positive control – 11.4 ng/ml
392 cortisol. In the inactive state, the GR (images show this receptor tagged with enhanced GFP) is found
393 in the cytoplasm in complex with heat shock proteins. Upon ligand binding, the GR translocates to the
394 nucleus, dimerizes, and acts as a transcription factor to regulate the expression of its target genes.
395 (Blue stain is Hoechst nuclear stain).

396

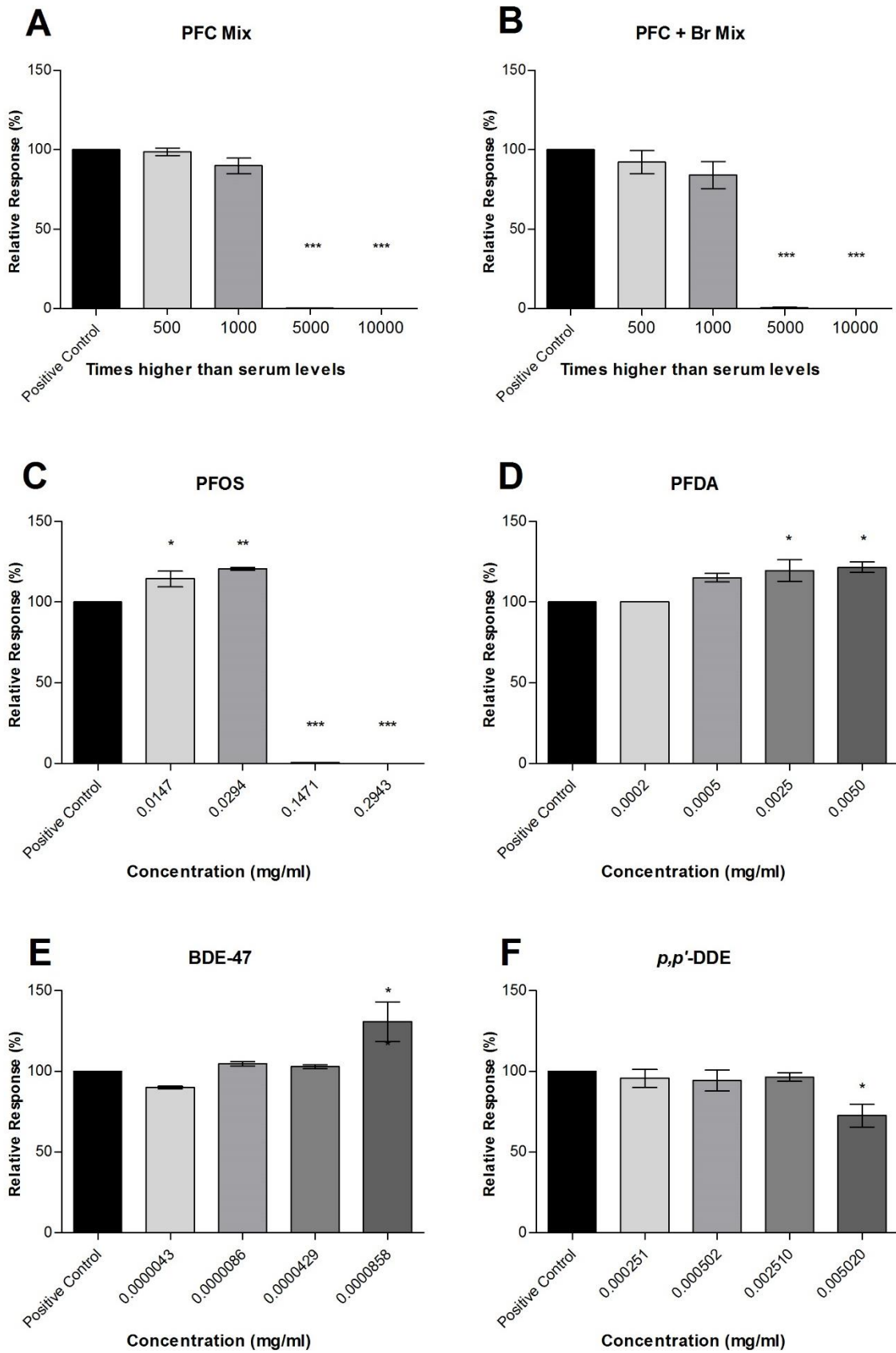
397 3.4. GR reporter gene assay

398

399 RGAs utilise the ability of steroid hormones to bind their specific receptor and to induce (or, for
400 antagonists, repress) a bioluminescent cellular signal; in this assay the cell line has been transformed
401 with the luciferase gene. The GR RGA is described in detail in Willemsen *et al.* (2004). Briefly the
402 glucocorticoid responsive TGRM-Luc cell line contains the MMTV-Luc luciferase reporter plasmid and
403 the RS-hGR α expression vector which codes for the human GR. The luciferase acts as a signalling
404 protein which is under the control of a highly regulated glucocorticoid inducible promoter resulting in
405 transcriptional activation. Therefore the RGA is useful as a measurement of transcriptional activation
406 due to the binding of a steroid hormone to its relevant receptor.

407 The mixtures and individual POPs tested in this study did not exhibit an agonistic response in
408 the TGRM-Luc cell line; the agonist activity for all compounds was below 0.13% (relative to a cortisol
409 positive control). Results from the antagonistic test appeared to indicate adverse effects on the TGRM-
410 Luc nuclear receptor transcriptional activity for the two highest concentrations of PFC and PFC + Br
411 mixtures ($p \leq 0.001$; Figure 5A and B). However, it is reasonable to suggest that the reduction in the
412 TGRM-Luc nuclear receptor transcriptional activity observed is solely as a result of the significant
413 cytotoxic effects of both mixtures on this cell line (Figure 2B and E). The same explanation could be
414 suggested for the antagonistic effects of PFOS (Figure 5C) observed at the two highest concentrations
415 as they were also cytotoxic. However for the two lowest concentrations of PFOS (0.0147 and 0.0294
416 mg/ml; 500 and 1000 times serum level), significant effects on the TGRM-Luc nuclear receptor
417 transcriptional activity were found with no significant cytotoxicity. For these concentrations,
418 transcriptional activity in the presence of cortisol increased to 114.4% and 120.7% ($p \leq 0.05$ and \leq
419 0.01) respectively. A similar effect was observed after incubation with the two highest concentrations
420 of PFDA (0.0025 and 0.005 mg/ml; 5000 and 10000 times serum level), producing a response of
421 119.6% and 121.6% ($p \leq 0.05$ both; Figure 5D) in comparison the positive control. In the PBDE group,
422 BDE 47 at the highest concentration of 0.000086 mg/ml (10000 times serum level) also produced an
423 increased response, 130.8% ($p \leq 0.05$; Figure 5E). Although not elucidated in this study upregulation
424 of the GR expression by PFOS, PFDA and BDE-47 is a possibility for the observed result, where the
425 increased levels of the receptor would provide cortisol with more of its relevant receptor target
426 resulting in the increased response seen in the RGA. High PFOS levels have been associated with higher
427 gene expression of the estrogen receptors α and β (ER α/β), the androgen receptor (AR) and the
428 pregnane X receptor (PXR) (La Rocca *et al.* 2012).

429 The primary metabolite of dichlorodiphenyltrichloroethane (DDT), *p,p'*-DDE exhibited an
430 antagonistic effect on the TGRM-Luc nuclear receptor transcriptional activity (Figure 5F). When cells
431 were exposed to the highest concentration of *p,p'*-DDE (0.005 mg/ml), transcriptional activity was
432 reduced to 72.5% compared to the positive control. This finding is supported by Zhao *et al.* (2004) who
433 found that DDE significantly depressed GR-transactivation to 60% at a concentration of 0.0032 mg/ml,
434 which is similar to the concentration where significant GR antagonism was found in the present study
435 (0.005 mg/ml). DDE is also known to be a potent AR antagonist (Kelce *et al.* 1995). In the present study,
436 *p,p'*-DDE led to significant antagonistic activity in the TGRM-Luc cell line however there was no
437 evidence that any of the mixtures containing *p,p'*-DDE (total, Cl, PFC + Cl or Br + Cl) caused GR
438 translocation in the GR RA. This could suggest that *p,p'*-DDE behaves differently in the presence of
439 other compounds in mixtures.



440

441 Figure 5: Antagonistic testing of mixtures and individual POPs using TGRM-Luc RGA. Positive control is
 442 90.6 ng/ml cortisol. Response is expressed as the percentage response \pm SEM for the two separate
 443 experiments ($n = 2$) $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***) represent significant antagonist
 444 effects. Only compounds which had significant results are shown.

445

446 *4. Conclusions*

447 This study was designed to investigate mixtures and individual POPs for their potential to disrupt GR
448 transcriptional activity. POP mixtures did not induce GR translocation in the HCA GR RA or produce an
449 agonist response in the GR RGA. However, an individual POP, *p,p'*-DDE (0.005 mg/ml), was found to
450 decrease the transcriptional activity of the glucocorticoid responsive TGRM-Luc cell line. Significant
451 increases in the TGRM-Luc nuclear receptor transcriptional activity, in the presence of cortisol, was
452 evident for the two lowest concentrations of PFOS (0.0147 and 0.0294 mg/ml), the two highest
453 concentrations of PFDA (0.0025 and 0.005 mg/ml) and the highest concentration of BDE-47 (0.000086
454 mg/ml). Upregulation of the GR expression by PFOS, PFDA and BDE-47 is a possibility for the observed
455 result, where the increased levels of the receptor would provide cortisol with more of its relevant
456 receptor target resulting in the increased response seen in the RGA however this is outside the scope
457 of this study. As the disruption of glucocorticoid synthesis and action is expected to contribute to
458 complex diseases (Odermatt and Gumy 2008) it is important to gain a better understanding of how
459 POPs may interact and affect this.

460

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465

466

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