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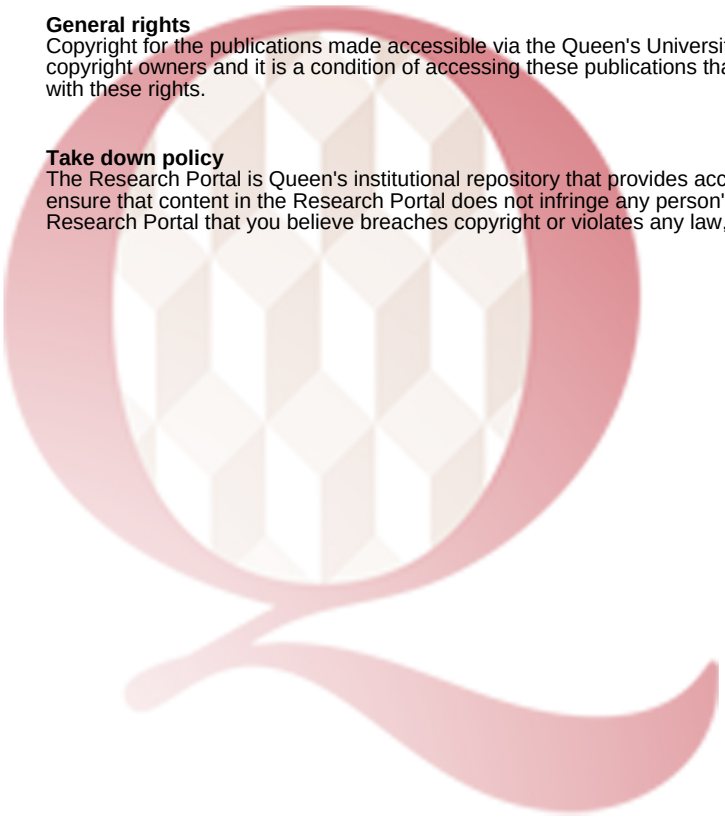

Do persistent organic pollutants interact with the stress response? Individual compounds, and their mixtures, interaction with the glucocorticoid receptor.

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Abstract

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

Key Words:

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

1. Introduction

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

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

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

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

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

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

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

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

2. Materials and methods

2.1. Chemicals

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

2.2. Mixtures

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

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

Compound	Mixture Stock Concentration (mg/ml)							Individual Stock Concentration (mg/ml)
Perfluorinated compounds (PFCs)	**Total**	**PFC**	**Br**	**Cl**	**PFC+Br**	**PFC+Cl**	**Br+Cl**	
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								
p,p'-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

2.3. GR RA cell culture and method

Recombinant U2OS cells stably expressing the human GR (U2OS-GR) were routinely cultured in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were grown in 75 cm^2 flasks in Dulbecco's modified eagle medium (DMEM) media supplemented with 10% foetal bovine serum (FBS), 2mM L-Glutamine, 1% penicillin-streptomycin and 0.5 mg/ml G418. TrypLE™ Express trypsin was used to disperse the cells from the flasks, while cell counting and viability checks prior to seeding plates were achieved by trypan blue staining and using a Countess® automated cell counter.

Cells were seeded (using DMEM supplemented with 2mM L-Glutamine, 1% Penicillin-Streptomycin, 0.5 mg/ml G418 and 10% hormone depleted FBS) at a concentration of 6 x 10^4 cells per well in 100 µl of media into black walled 96 well plates with clear flat bottoms (Grenier, Germany). The cells were incubated for 1 h at room temperature (RT) (20-25°C) to ensure that they attached evenly within each well. The cells were then incubated for 24 h at 37 °C, and subsequently exposed in assay media (DMEM supplemented with 2mM L-Glutamine and 1% Penicillin-Streptomycin) to 1/1000, 1/2000, 1/10000 and 1/20000 dilutions (0.2% DMSO in media) of the original stocks, which corresponded to 10000, 5000, 1000 and 500 times the levels in serum for the PFC, Br and PFC + Br mixtures. For the remaining mixtures (total, Cl, PFC + Cl and Br + Cl) the exposures corresponded to 1000, 500, 100 and 50 times the levels in serum. Assay media was used to dilute the stock solutions. The cortisol standard curve used covered the range of 0.02-22.7 ng/ml. A solvent control 0.2% v:v DMSO in media was also added to each plate. The cells were incubated for 48 h after which the media was discarded and the cells fixed by adding 150 µl fixing solution (10% formalin, neutral-buffered solution) per well. The plate was incubated at RT for 20 min. The fixing solution was then removed and cells washed four times with 200 µl PBS. After the last wash was removed and 100 µl of 1 µM Hoechst Staining Solution (1 µM Hoechst in PBS containing 0.5% Triton X-100) was added to each well before the plate was sealed with a black plate sealer and left at least 30 min before imaging.

2.4. High content analysis (HCA)

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

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

2.5. GR reporter gene assay (RGA)

The TGRM-Luc cell line for the detection of glucocorticoids and progestogens previously developed by Willemsen *et al.* (2004) was used. This transformed cell line was cultured in DMEM and 10% FBS, and grown in 75cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) at 37 °C with 5% CO_2 and 95% humidity**.** TrypLE™ Express trypsin was used to disperse the cells from the flasks, while cell counting and viability checks prior to seeding plates were achieved by trypan blue staining and using a Countess® automated cell counter. The RGA was carried out in assay media (DMEM supplemented with 10% hormone depleted serum).

Cells were seeded at a concentration of 4×10^5 cells/ml in 100 µl media into white walled 96 well plates with clear flat bottoms (Greiner Bio-One, Germany). The cells were incubated for 24 h and then exposed to four dilutions of each individual compound and mixture for the agonist test (cells were exposed as in section 2.3). For the individual compounds the four dilutions represent 500, 1000, 5000 and 10000 times serum levels. The cortisol standard curve covered the range of 4.5-181.2 ng/ml. A solvent control 0.2% v:v DMSO in media was also added to each plate. Antagonist tests were carried out by incubating the four dilutions of each individual compound and mixture with the positive control (90.6 ng/ml cortisol). The cells were incubated for 48 h, after which the media was discarded and the cells were washed twice with PBS. The cells were lysed with 30 µl cell culture lysis buffer (Promega, Southampton, UK) 100 µl luciferase substrate (Promega, Southampton, UK) was injected into each well and the response measured using the Mithras Multimode Reader (Berthold, Other, Germany). The response of the cell line to the various compounds was measured and compared with the solvent and positive controls.

2.6. Cell viability assay

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

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

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

2.7. Statistical analysis

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

3. Results and discussion

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

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

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

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

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

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

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

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

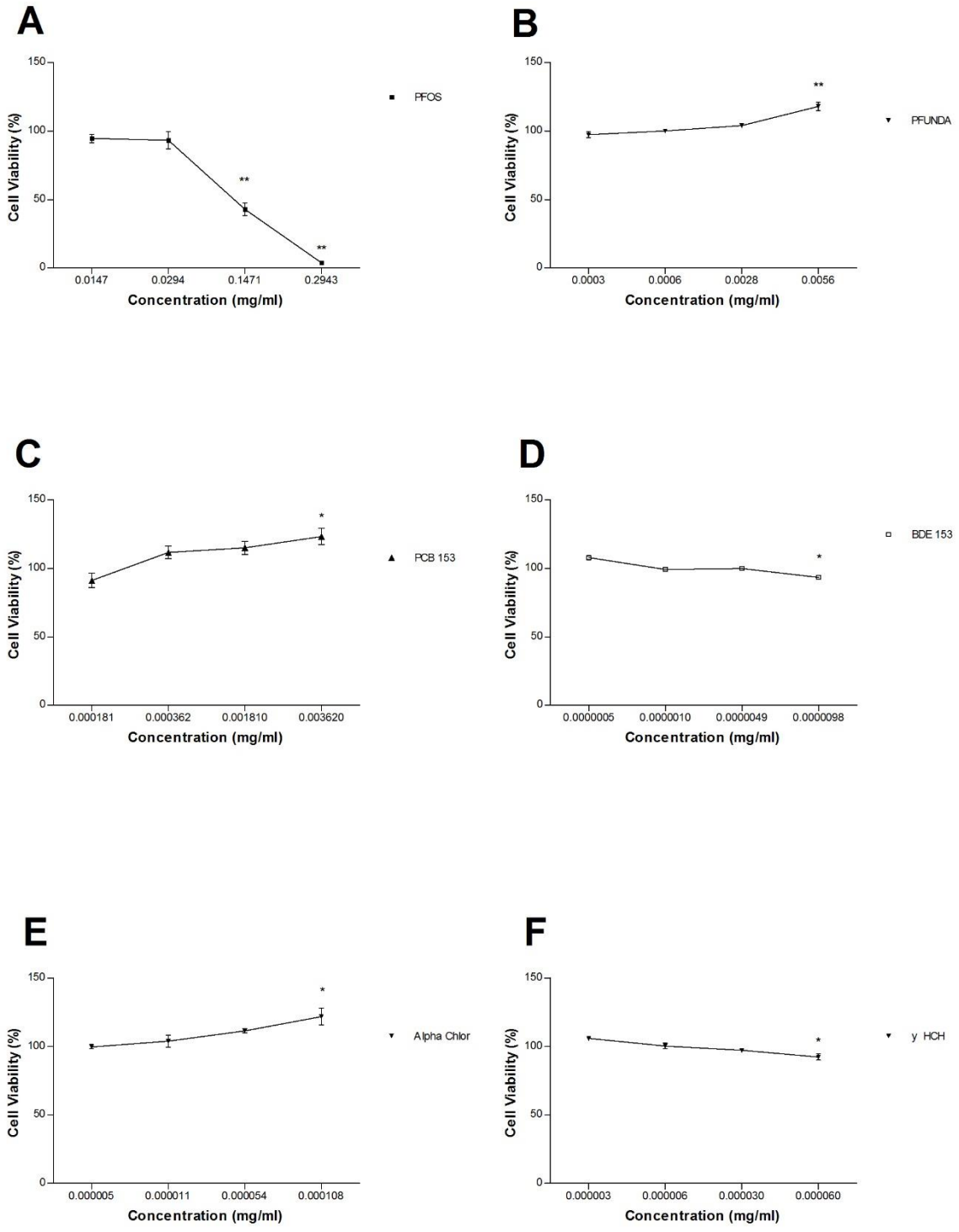

Figure 1: Viability of the TGRM-Luc cell line following exposure to individual POPs: (A) PFOS,(B) PFUNDA, (C) PCB 153, (D) BDE-153, (E) Alpha-Chlor and (F) γ-HCH; measured using the MTT Assay. Values are mean ± SEM n = 2 $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***) represent significant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

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

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

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

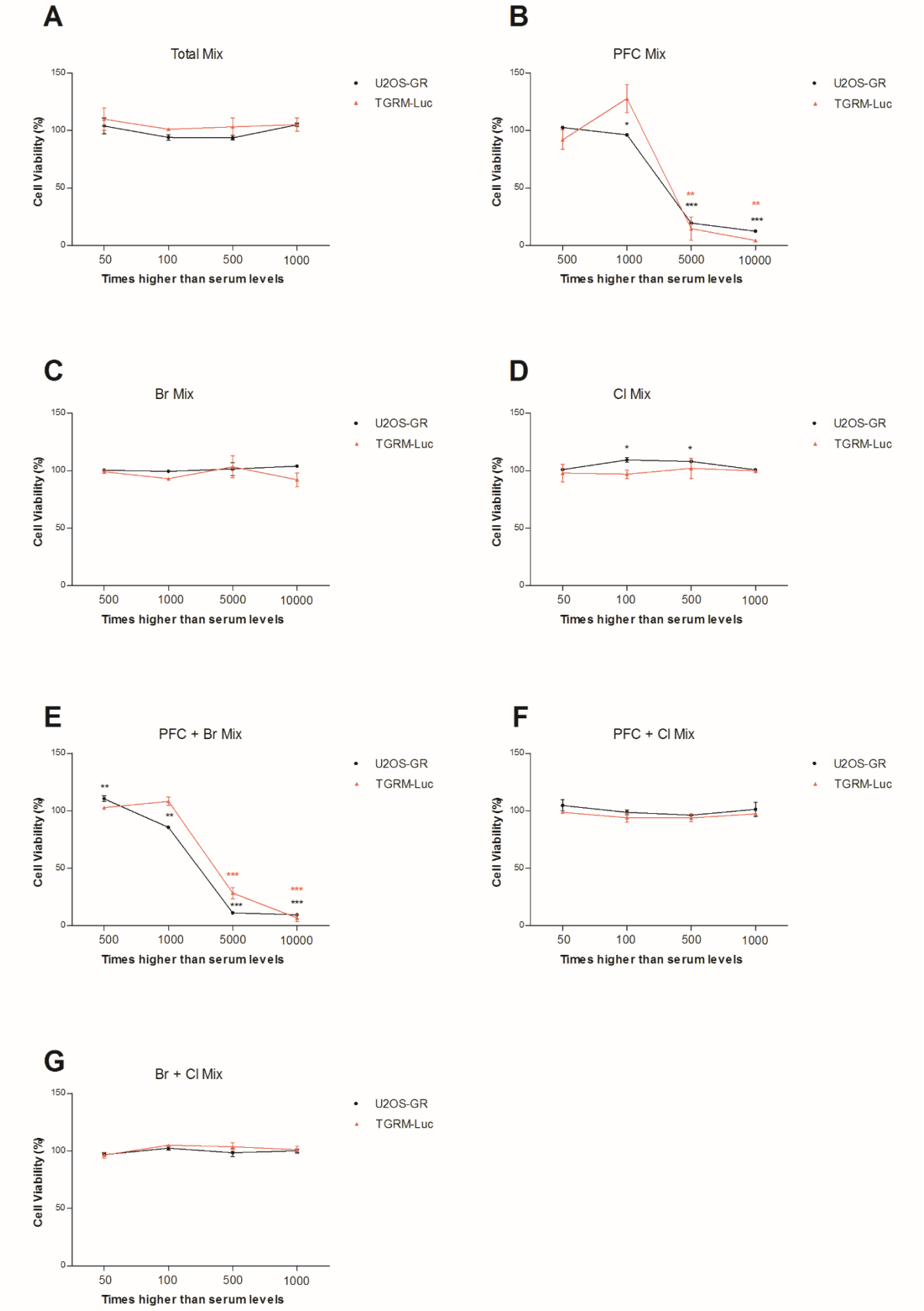

Figure 2: Viability of the TGRM-Luc and U2OS-GR cell lines following exposure to different mixtures of POPs; measured using the MTT Assay. Values are mean ± SEM n = 2 $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***) represent significant cytotoxic effects.

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

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

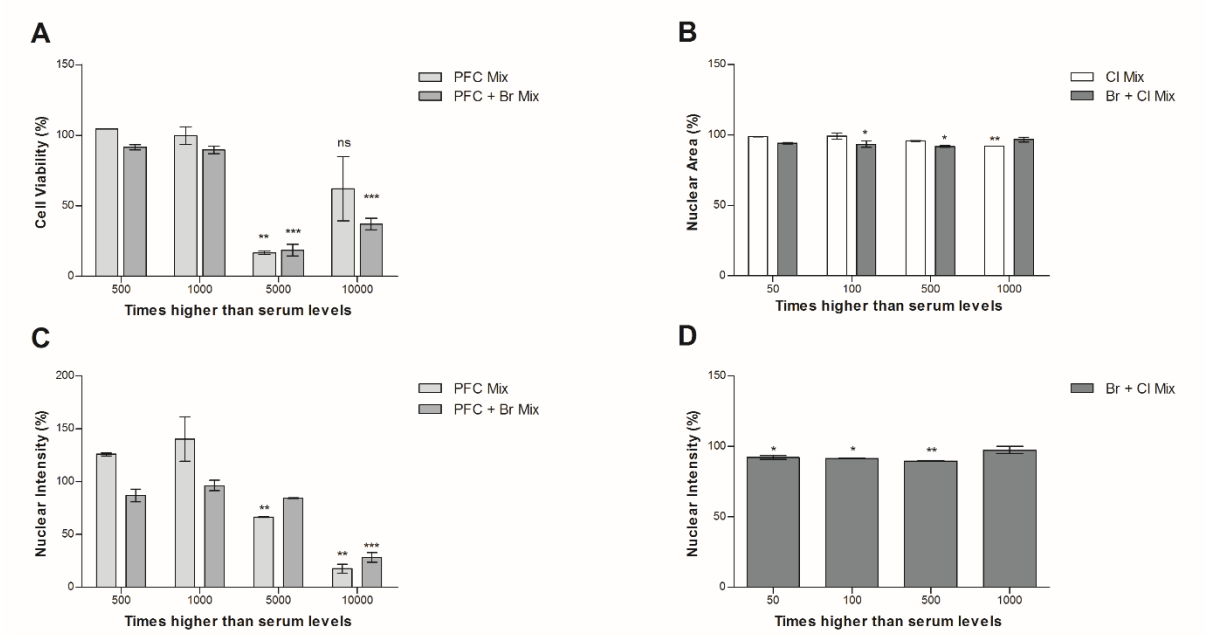

Figure 3: Nuclear changes in U2OS-GR cells measured using HCA parameters CN, NA and NI with Hoechst staining. (A) Cell viability as measured by CN for the PFC and PFC + Br mixtures. (B) Nuclear area for the Cl and Br + Cl mixtures. (C) Nuclear intensity for the PFC and PFC + Br mixtures. (D) Nuclear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

Table 2: Comparison of MTT and HCA nuclear parameter results for U2OS-GR cells. The grey shading indicates that no significant effects were found. The total, Br and PFC + Cl mixtures are not shown as toxic effects were not evident in the MTT assay or in HCA parameters. ↑ indicates increased effect e.g. for the MTT assay it shows increased cell viability. ↓ indicates decreased effect e.g. for the NA parameter it shows a decrease in nuclear size. Numbers 1-4 in the first column represent the concentration of the mixture; for PFC and PFC + Br it represents 500, 1000, 5000 and 10000 times serum levels. For the Cl and Br + Cl mixtures it represents 50, 100, 500 and 1000. Statistical significance 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					↑**											↓*
2	↓*				↓**				↑*						↓*	↓*
3	↓***	↓**			↓***	↓***			↑*						↓*	↓**
4	↓***	↓ns		↓**	↓***	↓***		↓***			↓**					

3.3. GR HCA Redistribution Assay

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

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

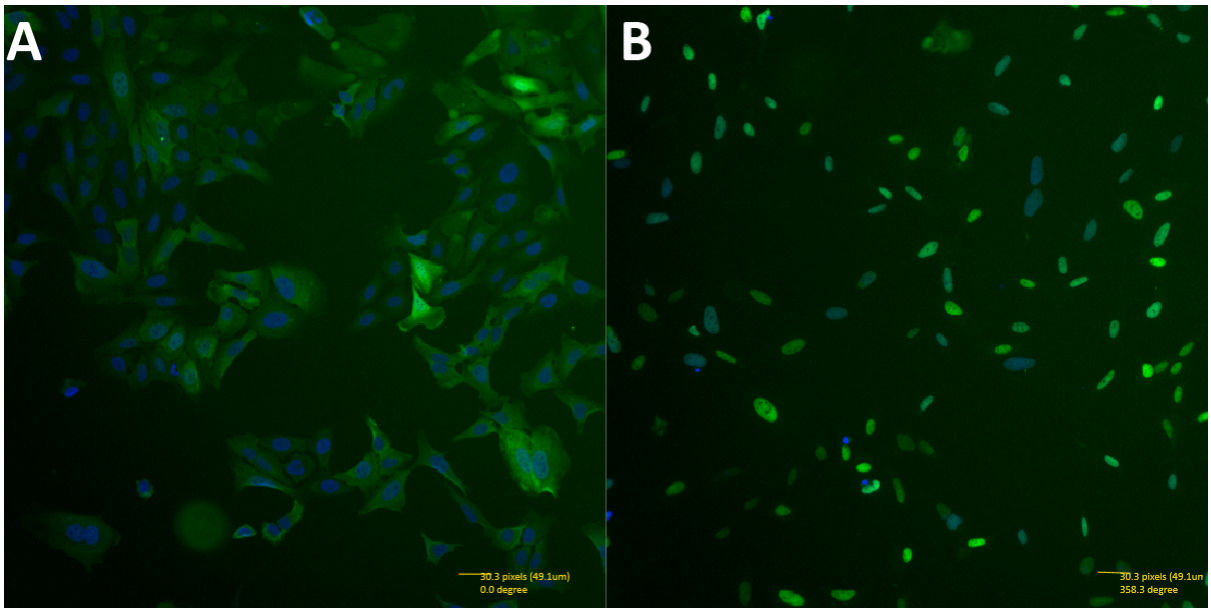

Figure 4: Example of GR RA images: (A) negative control - DMSO (B) positive control – 11.4 ng/ml cortisol. In the inactive state, the GR (images show this receptor tagged with enhanced GFP) is found in the cytoplasm in complex with heat shock proteins. Upon ligand binding, the GR translocates to the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

3.4. GR reporter gene assay

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

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

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

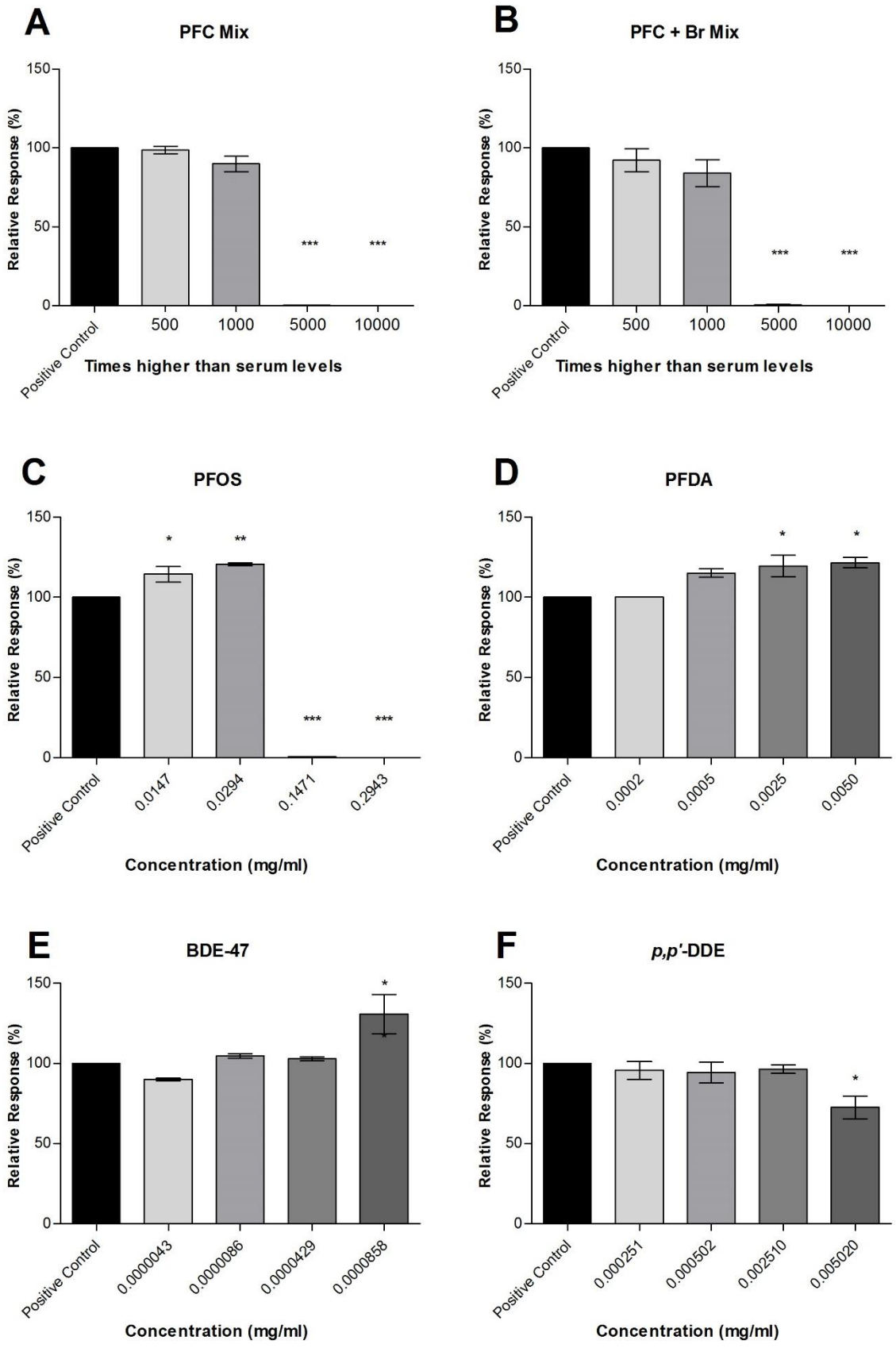

Figure 5: Antagonistic testing of mixtures and individual POPs using TGRM-Luc RGA. Positive control is 90.6 ng/ml cortisol. Response is expressed as the percentage response ± SEM for the two separate experiments (n = 2) $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***) represent significant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

4. Conclusions

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

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