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Effects of Defined Mixtures of Persistent Organic Pollutants (POPs) on Multiple Cellular Responses in the Human Hepatocarcinoma Cell line, HepG2, using High Content Analysis Screening

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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. Most studies focus on single compound effects, however as humans are exposed to several POPs simultaneously, investigating exposure effects of real life POP mixtures on human health is necessary. A defined mixture of POPs was used, where the compound concentration reflected its contribution to the levels seen in Scandinavian human serum (total mix). Several sub mixtures representing different classes of POPs were also constructed. The perfluorinated (PFC) mixture contained six perfluorinated compounds, brominated (Br) mixture contained seven brominated compounds, chlorinated (Cl) mixture contained polychlorinated biphenyls and also *p,p'*-dichlorodiphenyldichloroethylene, hexachlorobenzene, three chlordanes, three hexachlorocyclohexanes and dieldrin. Human hepatocarcinoma (HepG2) cells were used for 2h and 48h exposures to the seven mixtures and analysis on a CellInsight™ NXT High Content Screening platform. Multiple cytotoxic endpoints were investigated: cell number, nuclear intensity and area, mitochondrial mass and membrane potential (MMP) and reactive oxygen species (ROS). Both the Br and Cl mixtures induced ROS production but did not lead to apoptosis. The PFC mixture induced ROS production and likely induced cell apoptosis accompanied by the dissipation of MMP. Synergistic effects were evident for ROS induction when cells were exposed to the PFC+Br mixture in comparison to the effects of the individual mixtures. No significant effects were detected in the Br+Cl, PFC+Cl or total mixtures, which contain the same concentrations of chlorinated compounds as the Cl mixture plus additional compounds; highlighting the need for further exploration of POP mixtures in risk assessment.

Keywords:

Persistent organic pollutants; mixtures; cytotoxicity; high content analysis.

1. Introduction

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) therefore potential effects on human health need to be investigated. POPs such as polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) are well established pollutants (de Wit *et al.* 2010; Letcher *et al.* 2010). Due to their lipophilic nature and resistance to degradation, POPs accumulate in living organisms and biomagnify in food chains (de Wit *et al.* 2004), resulting in high levels in tissues of top predators and humans (Bytingsvik *et al.* 2012; Muir and de Wit 2010). POPs are of global concern and 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).

Exposure to POPs has been associated with adverse effects in animals including neurobehavioural development disruption (Johansson *et al.* 2008), impaired memory and learning (Kuriyama *et al.* 2005; Viberg *et al.* 2003) and disruption of neural proteins involved in synapse formation and growth (Johansson *et al.* 2009). Other effects include liver hypertrophy, alteration in liver enzymes, hepatomegaly and tumours (Butenhoff *et al.* 2004), developmental problems in rodent offspring exposed *in utero* (Lindstrom *et al.* 2011) and evidence of endocrine disruption (White *et al.* 2011).

Evidence of endocrine disrupting potential, as well as other harmful effects from POPs has been reported in humans. Importantly, studies have shown that POPs can cross the placenta and accumulate in the foetus (Beesoon *et al.* 2011; Inoue *et al.* 2004; Ode *et al.* 2013). Prenatal exposure to POPs has been associated with effects on birth weight, duration of pregnancy, visual memory (Van Oostdam *et al.* 2003), impaired immune function (Heilmann *et al.* 2006) and increased risk of middle ear infections (Dewailly *et al.* 2000). Furthermore babies are exposed to POPs through breast feeding (Llorca *et al.* 2010; Waliszewski *et al.* 2009). This implies that they are exposed before birth and during the early sensitive developmental stages of their lives to POPs. The endocrine disrupting

potential of POPs in humans has also been highlighted (Bonde *et al.* 2008; Longnecker *et al.* 2007; Lyche *et al.* 2011). Exposure to POPs has been associated with decreased fertility (Harley *et al.* 2010) and infertility in women (Fei *et al.* 2009), altered sex hormone and thyroid hormone homeostasis (Ellis-Hutchings *et al.* 2006; Hallgren and Darnerud 2002; Persky *et al.* 2001), dermatological effects such as rashes and acne (Ritter *et al.* 1995) and type 2 diabetes (Grandjean *et al.* 2011).

Animals and humans are exposed to mixtures of POPs. Therefore investigating the effect of one POP in isolation is not representative of real life exposure. Instead it is more important to look at how mixtures of POPs work in combination to affect health. Only single compound exposure is currently considered by risk assessment authorities (Kortenkamp 2007), potentially leading to an ignorance of additive, synergistic or antagonistic effects and the misinterpretation of the risk of POP exposure as complex mixtures. The toxicological determination of complex mixtures has been highlighted as one of the most important challenges for modern toxicology (Fent 2003; Kortenkamp 2007; Vaiseman 2011). In the present study, the effect of complex mixtures on several cellular responses, in an *in vitro* liver model using human hepatocarcinoma (HepG2) cells, was studied. High Content Analysis (HCA) was used in conjunction with the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. HCA is a novel, high-throughput, quantitative fluorescence technique that can examine chemical induced toxicity at sub-cellular microscopic resolution. It amalgamates fluorescence microscopy with automated cell analysis software allowing the simultaneous assessment of multiple morphological and functional cell parameters (Abraham *et al.* 2004; Giuliano *et al.* 2003). Using toxicity assays, HCA can detect subtle pre-lethal changes in cell health rather than obvious lethal cytotoxicity detected by the conventional MTT assay (O'Brien 2008; O'Brien and Haskins 2007). The liver is the main metabolic organ for degrading xenobiotics. As such the liver often shows the earliest signs of injury, and therefore it is useful to model the effects of POP mixtures on a liver cell line. Furthermore, POPs such as PCBs tend to accumulate in the liver (Bachour *et al.* 1998). It may be assumed that primary human hepatocytes would be most appropriate for use in predicting human toxicity however these cells dedifferentiate over the time of

79 the assay. Furthermore as they are non-proliferative critical toxicity parameters would be lost.
80 HepG2 are considered to be one of the best single-cell models for predicting human toxicity
81 potential (O'Brien 2014). They are also highly sensitive in the recognition of effects on mitochondrial
82 DNA and mitochondrial function (Pinti *et al.* 2003). This sensitivity coupled with the ability to
83 incorporate multiple parameters suggests that HCA, using HepG2 cells, is an appropriate candidate
84 for investigating POP mixtures.

85 Assay parameters which can be optimised and measured using HCA include: cell number
86 (CN), nuclear area (NA), nuclear intensity (NI), mitochondrial membrane potential (MMP),
87 mitochondrial mass (MM) and reactive oxygen species (ROS). These markers cover cellular metabolic
88 functions and are markers of cell health (O'Brien 2008; O'Brien and Haskins 2007; Walsh *et al.* 2011).
89 O'Brien *et al.* (2006) reported that the most sensitive HCA toxicity parameters in HepG2 cells are cell
90 proliferation, mitochondrial health and NA. Enhanced biogenesis of mitochondria can increase MM
91 due to increased mitochondrial respiration and this often corresponds with reduced MMP (O'Brien
92 and Haskins 2007). Mitochondria serve as a site of regulation of programmed cell death (apoptosis).
93 Apoptosis can occur when cells are damaged by disease or noxious agents (Norbury and Hickson
94 2001). A hallmark of early apoptosis is cell shrinkage and pyknosis which is the condensation of
95 chromatin in the nucleus (Kerr *et al.* 1972). Necrosis is an alternative to apoptotic cell death by
96 which the cell passively follows an energy-independent mode of death. The cells will swell in
97 contrast to apoptotic cells which shrink. Although there are distinct differences in the mechanisms of
98 apoptosis and necrosis, there is overlap between the two processes described as the "apoptosis-
99 necrosis continuum" (Zeiss 2003). O'Brien *et al.* (2006) highlighted the need to incorporate an
100 oxidative stress biomarker in HCA screening. Excessive ROS can induce oxidative damage in cells and
101 impair cellular functions (Aims *et al.* 1993; Halliwell and Gutteridge 1999). Cellular antioxidants
102 protect against the damaging effects of ROS. However, in moderate concentrations, ROS are
103 necessary for a number of protective reactions (Halliwell and Gutteridge 1999).

This study aimed to improve the understanding of POP mixture induced toxicity by using a multi-parameter HCA cytotoxicity assay for the study of exposure of HepG2 cells to POP mixtures. It is important to assess POPs in mixtures as it reflects real life exposure and it may be possible to highlight the mechanisms by which POP mixtures induce toxicity, particularly pre-lethal toxicity. Furthermore comparison of the conventional MTT cytotoxicity assay coupled with the HCA assay will help determine if HCA can be used to better measure POP mixture toxicity. While individual POPs have been the focus of many studies, mixtures of POPs have not been extensively examined. Investigations of POP mixtures are crucial as the evidence of their health effects on humans and animals is irrefutable.

2. Materials and Methods

2.1. Chemicals

All PBDEs, PCBs and other organochlorines were originally purchased from Chiron As (Trondheim, Norway). All perfluorinated compounds (PFCs) were obtained from Sigma-Aldrich (St. Louis, MO, USA) except perfluorohexanesulfonic acid (PFHxS) which was from Santa Cruz (Dallas, US). Hexabromocyclododecane (HBCD), phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO), thiazolyl blue tetrazolium bromide (MTT) and menadione were obtained from Sigma–Aldrich (Dorset, UK). CellROX Deep Red reagent and other cell culture reagents were supplied by Life Technologies (Paisley, UK) unless otherwise stated. Mitochondrial membrane potential dye (MMPD) and Hoechst nuclear stain 33342 were purchased from Perbio (Northumberland, England). 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 cell assays (Table 1): (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.* 2016). The POP mixtures used in this study were based on concentrations of relevant POPs measured in human blood, according to recent studies of the Scandinavian population (Haug *et al*. 2010; Knutsen *et al.* 2008; Polder *et al.* 2008; Polder *et al*. 2009; Van Oostdam *et al*. 2004) as described in Berntsen *et al.* (2016). The compounds were mixed in concentration ratios relevant to human exposure. The stocks of the total mixture, Cl mixture and the Cl sub-mixtures were ten times more diluted compared to the PFC and the Br mixtures and the combined PFC + Br sub-mixture.

2.3. Cell Culture and Treatment

HepG2 cells were routinely cultured in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were grown in 75 cm^2 flasks in MEM media supplemented with 10% foetal bovine serum, 2mM L-Glutamine, 1% penicillin-streptomycin and 1mM sodium pyruvate. 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 into collagen bio-coat BD Falcon 96 well flat bottomed microtitre plates (BD Biosciences, Bedford, MA, US) at a density of 6 x 10^4 cells/ml and allowed to attach for 24 h. The cells were then exposed to 1/1000, 1/2000, 1/10000 and 1/20000 dilutions of the original stocks, which corresponded to 10000, 5000, 1000 and 500 times the levels in serum for 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. The dilutions were performed in the same media as stated above. Incubation periods were: 2 h or 48 h. A solvent control of 0.2% (v:v) DMSO in media was also included. The duration of exposure for the study was chosen to cover early cellular events/effects and the eventual consequences thereof after 48 hr exposure.

156

157 *2.4. Analysis of Multiple Cellular Parameters by High Content Analysis (HCA)*

158 Cellomics® High Content Screening reagent series multi-parameter cytotoxicity dyes were used by
159 following manufacturer's instructions. MMPD was prepared by adding 117 µl of DMSO to make a 1
160 mM stock solution. The live cell stain was prepared by adding 6.75 µl of the MMPD and 27 µl of
161 CellROX reagent (resulting in a final cell exposure concentration of 5 µM) in 1500 µl of media for
162 each assay plate. Following incubation, the plate was protected from light and 25 µl of live cell stain
163 was added to each well for 30 min at 37 °C. Cells were fixed with 10% formalin solution for 20 min at
164 room temperature (RT) and washed with PBS. Hoechst 33342 dye (at a final concentration of 1.6
165 µM) was added to each well and incubated for 10 min at RT, after which cells were washed with PBS.
166 The wells were then filled with 200 µl PBS, sealed with a plate sealer and evaluated on CellInsight™
167 NXT High Content Screening (HCS) platform (Thermo Fisher Scientific, UK). This instrument analyses
168 epifluorescence of individual cell events using an automated micro-plate reader analyser interfaced
169 with a PC (Dell precision 136 T5600 workstation).

170 Hoechst dye was used to measure parameters reflecting nuclear morphology: CN, NI and
171 NA. MMPD was used to evaluate parameters of mitochondrial function: MMP and MM. CellROX®
172 Deep Red Reagent was used to measure ROS. Data was captured for each plate at 10x objective
173 magnification in the selected excitation and emission wavelengths for Hoechst dye (Ex/Em 350/461
174 nm), MMPD (Ex/Em 554/576 nm) and CellROX reagent (Ex/Em 640/665 nm). Nine field view images
175 were acquired in each well to examine each parameter.

176

177 *2.5. MTT Assay*

178 As well as visual inspection of the HepG2 cells under the microscope to evaluate cell morphology
179 and attachment, the MTT cell viability assay was performed to act as a comparison to the HCA
180 cytotoxicity assay.

The cells were exposed exactly as in Section 2.3 after which the percentage of viable cells was determined. In the MTT assay viable cells convert the soluble yellow MTT to insoluble purple formazan by the action of mitochondrial succinate dehydrogenase. The cells were washed once with PBS. 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. The supernatant was removed and 200 µl of DMSO was added to dissolve the formazan crystals. Subsequently, the plate was incubated at 37 °C with agitation for 10 min. 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 compared to the absorbance of the solvent control.

2.6. Statistical Analysis

Exposures were carried out in triplicate wells and three independent exposures performed. The coefficient of variation (CV) was calculated for the three exposures; all parameters were below 15% except for ROS production which was below 25%. Data was analysed using Microsoft Excel 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. Data is expressed as a percentage of untreated (solvent) control for each parameter. Data was analysed by one-way ANOVA followed by Dunnett's procedure for multiple comparisons; the mean concentrations were tested for significant difference at the 95% confidence level. A p value of ≤ 0.05 was considered as significant, p ≤ 0.05 (*) ≤ 0.01 (**) ≤ 0.001 (***).

2.7. Comparison between expected and measured results

The expected values were calculated by addition of the mean value after exposure to one mixture (e.g. PFC mixture) alone with the mean value obtained after exposure to the second mixture (e.g. Br mixture) (Weber *et al.* 2005). For example:

$$\text{Mean (expected for PFC + Br)} = \text{mean (PFC)} + \text{mean (Br)} - 100\%$$

Furthermore to calculate expected SEM, the following equation was used:

$$\text{SEM (expected for PFC + Br)} = [(\text{SEM for PFC})^2 + (\text{SEM for Br})^2]^{1/2}$$

An unpaired t-test was used to calculate the significance of difference in the expected and measured values, with $p \leq 0.05$ being considered statistically significant. Interpretation of the results were as follows:

- Additive effects: measured values were not significantly higher or lower than expected values.
- Synergistic effects: measured values were significantly below expected values for parameters CN, NI and MMP and significantly above for parameters NA, MM and ROS.
- Antagonistic effects: measured values were significantly above expected values for parameters CN, NI and MMP and significantly below for parameters NA, MM and ROS.

3. Results

HCA methodology multiple parameters (CN, NA, NI, MM, MMP and ROS) representative for different cellular events and functionality were measuring using HepG2 cells exposed to defined POP mixtures. In parallel, cytotoxicity was measured using a standard MTT assay procedure.

3.1. Cytotoxicity as measured by MTT Assay

Exposure (2 h) to the highest concentrations of the PFC mixture (equivalent to 5000 and 10000 times serum levels) reduced cell viability to 82.4% and 55.5% respectively ($p \leq 0.05$, $p \leq 0.001$, Figure 1). Cell viability was also reduced after 48 h exposure however it was more pronounced 3.1 % and 1.8% cell viability respectively ($p \leq 0.001$, Figure 1). A reduction in cell viability was also evident after 2 h exposure with the highest concentration of PFC + Br mixture, 66.9% cell viability ($p \leq 0.001$, Figure 1). Reductions in cell viability were evident after 48 h exposure for the two highest concentrations of

this mixture, 1.2% and 0.9% respectively ($p \leq 0.001$, Figure 1). Total, Br, Cl, PFC + Cl and Br + Cl mixtures had no significant cytotoxic effects in the MTT assay (data is not shown for these mixtures as no significant effects were found; Br is included only to enable comparisons to PFC with and without Br).

3.2 Cytotoxicity as measured by CN, NA and NI

Exposure (2 h) to the highest concentration of the PFC mixture reduced CN to 40.9% ($p \leq 0.01$; Figure 2A). CN was also found to be significantly decreased after 48 h exposure to this mixture at the two highest concentrations, 1.7% and 0.8% cell viability respectively ($p \leq 0.001$; Figure 2A). Similarly, 48 h exposure to the PFC + Br mixture was found to lower CN at the two highest concentrations, 1.1% and 1.3% cell viability respectively ($p \leq 0.001$; Figure 2A).

NI was found to be significantly increased after 48 h exposure to the PFC mixture at the two highest concentrations, 166.3% and 156.9% nuclear intensity respectively ($p \leq 0.01$, $p \leq 0.05$; Figure 2B). The PFC + Br mixture at the two highest concentrations was also found to increase nuclear intensity, 153.7% and 142.0% respectively ($p \leq 0.001$, $p \leq 0.01$; Figure 2B). Even during the 2 h exposure the highest concentration of the PFC + Br mixture was found to significantly increase nuclear intensity to 127.3% ($p \leq 0.01$; Figure 2B).

NA was found to be significantly increased after 48 h exposure to the PFC mixture (equivalent to 5000 times serum levels), 187.2% nuclear area ($p \leq 0.001$, Figure 2C). Exposure (2 h) to the PFC + Br mixture at the highest concentration decreased nuclear area to 68.5% ($p \leq 0.05$, Figure 2C). However, after 48 h exposure, the two highest concentrations were found to increase nuclear area, 164.9% and 196.6% respectively ($p \leq 0.001$, Figure 2C).

The other mixtures (total, Br, Cl, PFC + Cl and Br + Cl) caused no significant changes to CN, NA or NA (data is not shown for these mixtures as no significant effects were found; Br is included only to enable comparisons to PFC with and without Br).

3.3 Cytotoxicity as measured by MM and MMP

All concentrations of the Cl mixture (100, 500 and 1000 times serum levels), apart from the most dilute (50 times serum levels), were found to significantly increase MM to 106.6%, 108.5% and 109.7% respectively ($p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, Figure 3) after 48 h exposure. Although the results are statistically significant, the biological significance is likely to be minor as these inductions are very low.

The only mixture to show significant changes in MM after 2 h exposure was the Br mixture (at 1000 times serum levels), increasing to 109.4% ($p \leq 0.01$, Figure 4A). However MM increased significantly after 48 h exposure to the PFC mixture at the two highest concentrations, to 160.9% and 172.5% mitochondrial mass ($p \leq 0.01$, Figure 4A). The PFC + Br mixture at the highest concentration was also found to increase MM to 206.5% ($p \leq 0.05$, Figure 4A).

MMP decreased significantly after 2 h and 48 h exposure to the PFC mixture at the two highest concentrations to 64.1% and 46.6% ($p \leq 0.001$) and 22.3% and 11.7% respectively ($p \leq 0.001$, Figure 4B). Exposure (2 h) to the PFC + Br mixture at the highest concentration was found to decrease MMP to 66.7% ($p \leq 0.05$, Figure 4B). After 48 h exposure the two highest concentrations were also found to decrease MMP to 40.0% and 25.5% respectively ($p \leq 0.001$, Figure 4B).

The total, PFC + Cl and Br + Cl mixtures caused no significant changes to MM or MMP (MM shown in Figure 3, MMP data not shown).

285 *3.4. Oxidative Stress*

286

287 Increased ROS production was detected after 2 h exposure to the highest concentration of the PFC
288 mixture, Br mixture, Cl mixture and PFC + Br mixture (190.1% $p \leq 0.001$, 149.5% $p \leq 0.05$, 137.3% $p \leq$
289 0.05 and 348.4% $p \leq 0.001$, Figure 5). The increase in red fluorescence after 2 h exposure to the
290 highest concentration of PFC + Br mixture can be seen in Figure 6. The second highest concentration
291 (500 times serum levels) of the Cl mixture also caused significant increase in ROS 135.2% ($p \leq 0.05$,
292 Figure 5).

293

294 Increased ROS production was detected after 48 h exposure to the highest concentration of PFC
295 mixture, Br mixture, Cl mixture and PFC + Br mixture (466.7% $p \leq 0.001$, 178.1% $p \leq 0.01$, 141.1% $p \leq$
296 0.01 and 525.3% $p \leq 0.001$ respectively, Figure 5).

297

298 The total, PFC + Cl and Br + Cl mixtures caused no significant changes in ROS production (data not
299 shown).

300

301 A summary of the results are shown in table 2 (2 h exposure) and table 3 (48 h exposure).

302

303

304 *3.5. Assessing the interactive effects of the PFC + Br mixture*

305 The addition of the Br mixture to the PFC mixture produced some interesting results. After 2 h
306 incubation, no significant change in CN was observed for the highest concentration of PFC + Br
307 mixture; keeping in mind that the PFC mixture alone at this concentration lowered CN to 40.9%.
308 However this observed value was not deemed statistically significant from the expected value $p >$
309 0.05. In contrast a synergistic effect was evident for this concentration for MM with a 28% ($p \leq 0.05$)
310 difference between expected and observed values. Furthermore for ROS production (2 h) exposure
311 to the PFC + Br mixture (at 5000 times serum levels) was deemed to reflect synergistic effects

between the individual mixtures as the observed ROS value (188%) was significantly higher than the expected value (113%, $p \leq 0.05$). However the ROS production induced by the highest concentration (348%, 10000 times serum levels) was not deemed to be statistically significantly different to the expected value (239%) and is therefore considered additive. Exposure for 48 h to the PFC + Br mixture again resulted in higher ROS production than expected after exposure to each of the mixtures independently. The values observed for 1000 and 5000 times serum levels were deemed to be synergistic ($p \leq 0.05$) while 500 and 10000 times serum levels were additive.

4. Discussion

Compared to single end-point cytotoxicity assays, HCA has the scope to investigate different mechanistic endpoints for cellular health and death simultaneously and therefore might better demonstrate the risk of human exposure to complex mixtures of environmental contaminants.

Cell population count or CN, followed by NA, are the most affected by cytotoxic drugs (O'Brien 2014). There was a high result concordance between using the MTT assay (Figure 1) and CN as a measure of cell viability (Figure 2A). For 48 h exposure, the same mixtures and concentrations (PFC and PFC + Br) were highlighted as statistically significant by both assays. However for 2 h exposure, the HCA parameter CN highlighted fewer mixtures and concentrations as toxic than the MTT assay. Since the MTT assay determines mitochondrial activity it is crucial to include the HCA mitochondrial parameters as a comparison. When these are considered the HCA showed MM changes in two mixtures, Cl (Figure 3) and Br mixtures (Figure 4A), that the MTT assay failed to highlight. Therefore combined HCA endpoints were found to be more sensitive at detecting cytotoxicity and revealed toxic effects where the conventional MTT endpoint showed none.

As far as the authors of this paper are aware, the combinations of compounds used in this study are unique to this collaborative group. Cellular effects of single PFCs on HepG2 cells have previously been reported for perfluorooctanoic acid (PFOA), ranging from cell cycle perturbations (starting at 50 µM), apoptosis (200–450 µM), to necrosis (400-500 µM) (Shabalina *et al.* 1999).

Florentin *et al.* (2011) observed a reduction in viability after exposure to PFOA (200 µM) or perfluorooctanesulfonic acid (PFOS) (300 µM) for 24 h. Hu and Hu (2009) reported a similar concentration-dependent decrease in cell viability for PFOA and PFOS, and pointed to additive effects in combined exposures. These concentration ranges correspond to the two highest concentrations of the PFC mixtures used in the present study, containing 54/109 µM and 273/546 µM of PFOA and PFOS respectively, where a significant reduction in cell viability (measured by MTT and CN) was observed after 48 h. However, whereas Florentin *et al.* (2011) reported no cytotoxicity after 1 h, a significant decrease in viability (measured by CN) was evident after 2 h for the two highest concentrations of the PFC mixture in our study. This may indicate that the CN parameter in the HCA is more sensitive in detecting early onset of cytotoxic alterations.

As shown by Shabalina *et al.* (1999) exposure of HepG2 cells to single PFCs can result in both apoptosis and necrosis, depending on concentration and exposure time, suggesting on overlap between the two processes described as the "apoptosis-necrosis continuum" (Zeiss 2003). Indeed, in the present study changes in specific HCA endpoints were indicative of both modes of cell death. One apoptosis-induction pathway is mediated by early mitochondrial alterations, with the opening of the mitochondrial permeability transition (MPT) pore resulting in the release of mitochondrial proteins which initiate and execute the process of cell destruction. This mitochondrial dysfunction is reflected by a decrease in MMP, generally considered an early marker for this pathway (Bernardi *et al.* 1992; Gottlieb *et al.* 2003). A decrease in MMP may be expected to accompany an increase in MM as disruption of MMP/MPT pore opening induces mitochondrial swelling (Minamikawa *et al.* 1999). A reduction in MMP was evident after 2 h incubation with the PFC mixture (64.1% at 5000, and 46.6% at 10000 times serum levels; Figure 4B), after 48 h exposure the MMP decreased further while the MM had increased. Nuclear shrinkage is a hallmark of apoptosis while the swelling of nuclei is linked to compound-induced necrosis. In contrast to the changes indicative of apoptosis observed for the PFC mixture (5000 times serum levels) at 2 and 48 h, the findings at 48 h of significantly increased NA would be indicative of nuclear swelling and necrosis (Figure 2C). NA was

not significantly changed for the higher concentration of the PFC mixture, however CN was found to be reduced significantly, suggesting that any apoptotic or necrotic cells had been lost at this point.

ROS are a known mediator of apoptosis, and the opening of the MPT pore can be initiated and augmented by elevated ROS (Atlante *et al.* 2000). In the HCA assay ROS production was measured using the cell-permeable CellROX® Deep Red Reagent, which locates to the cytoplasm, and exhibits a strong fluorescent signal upon oxidation. The induction of ROS was observed after 2h and 48 h exposure to the highest concentration of the PFC mixture (Figure 5). Similar studies with PFOA and PFOS (Hu and Hu 2009; Panaretakis *et al.* 2001) also reported induction of ROS. It is reasonable to deduce that the PFC mixture containing PFOA and PFOS potentially exerts its toxic effects on HepG2 cells through ROS-mediated cell apoptosis. It is possible that the loss of MMP and the increase in ROS are not two separate ways of inducing apoptosis but rather that they are linked. Damaged mitochondria are a major source of ROS. Excessive ROS may cause the mitochondria further damage, reducing MMP and consequently inducing apoptosis (Ricci *et al.* 2003; Simizu *et al.* 1998). This further supports the observation that the PFC mixture can induce apoptosis in HepG2 cells. However, further investigation is required to fully elucidate the mechanism.

No decrease in cell viability, measured using MTT or CN, was seen at any concentration or exposure time after exposure to the Cl mixture. However, MM was significantly increased after 48 h exposure to the Cl mixture at all concentrations tested above 50 times serum levels (Figure 3). Exposure to the Cl mixture also increased ROS production, after both 2 h exposure (500 and 1000 times serum levels), as well as after 48 h exposure (1000 times serum levels; Figure 5). Exposure to individual PCBs has been shown to lead to uncontrolled ROS accumulation and cellular oxidative stress (Liu *et al.* 2012). Mitochondria can also swell in a fully reversible manner, without inducing cell death (Minamikawa *et al.* 1999; Petronilli *et al.* 1994). Therefore the level of ROS produced by the HepG2 cells after exposure to the Cl mixture may have been sufficient to open the MPT pore and cause mitochondrial swelling but not enough to irreversibly damage the cells. This further illustrates the ability of the HCA assay to pick up early signs and/or reversible signs of cellular stress.

Incubation of HepG2 cells with the Br mixture for 2 h and 48 h did not produce any significant changes in the MTT assay or CN, NI, NA, MMP parameters in the HCA. Studies have investigated the effects of single PBDEs on different cell types. BDE-47 and BDE-99 (0.1–10 μM) proved non- cytotoxic for proliferating or differentiating human neural progenitor cells over a period of 2 weeks (Schreiber *et al.* 2010). However, cytotoxicity due to the induction of apoptosis has been reported for single PBDEs in rat cerebellar granule cells (Reistad *et al.* 2006), human astrocytoma cells (Madia *et al.* 2004), hippocampal neurons, human neuroblastoma cells, human foetal liver haematopoietic cells (He *et al.* 2008, He *et al.* 2009; Shao *et al.* 2008) and HepG2 cells (Hu *et al.* 2007). These studies tested much higher levels of the individual PBDEs than those present in the Br mixture. For example, Hu *et al.* (2007) tested 1-100 μM of BDE-209, with cytotoxicity only being evident at concentrations > 10 μM. In comparison, at the highest concentration of the Br mixture tested, BDE-209 is present at only 0.11 μM. Giordano *et al.* (2008) demonstrated that a technical mixture of PBDEs induces oxidative stress in rat cerebellar granule neurons. Time and concentration-dependent induction of ROS in HepG2 cells by BDE-209 has been observed with effects only appearing after exposure to concentrations >10 μM (Hu *et al.* 2007). They concluded that BDE-209 inhibited the proliferation of Hep G2 cells by inducing apoptosis through ROS generation. In the present study increased ROS were detected after 2 h and 48 h exposure to the highest concentration of the Br mixture (Figure 5). As the concentrations of PBDEs in the Br mixture used here are much lower in comparison to other studies, it may be possible that even at low concentrations PBDEs induce ROS generation but that it does not lead to apoptosis.

The addition of the Br mixture to the PFC mixture produced some interesting results. As the concentrations of PFCs present in the combined mixture is the same as in the PFC mixture alone, it might be expected that toxicity would stay the same or increase. Further effects were observed in HepG2 cells after 2 h exposure to the highest concentration of the PFC + Br mixture (10000 times serum levels) for NA and NI (decreased and increased respectively); there were no significant effects at this concentration for either the PFC or Br mixture alone (Figure 2). These changes in nuclear

parameters are indicative of apoptosis. Furthermore, significant synergistic effects ($p \leq 0.05$) were evident for the PFC + Br for MM (at 10000 times serum levels) and ROS (5000 times serum levels). In addition, 48 h exposure to this combined mixture resulted in synergistic effects observed for ROS production at two concentrations (1000 and 5000 times serum levels), while additive effects occurred at 500 and 10000 times serum levels. As PBDEs and PFCs are known to induce oxidative stress the synergy observed for MM and ROS is plausible.

Understanding the complexity of POP mixtures is crucial to the assessment of risks to human health. It is not a simple scenario of the more POPs present, the more risk to health; the issue is more complex. In addition to what has previously been discussed for ROS production, this is highlighted by comparing all mixtures at 1000 and 500 times the levels found in human serum. MM was increased after 48 h exposure to the Cl mixture. However no such increase was evident after 48 h exposure to the PFC + Cl mixture. Similarly 2 h exposure to the Br mixture alone, at 1000 times serum levels, caused MM to increase. However 2 h exposure to the Br + Cl mixture had no significant effects. Furthermore the total mixture, which contains all of the individual POPs, had no cytotoxic effects at any concentration. This highlights the complexity of investigating mixtures. It is also understandable that determining the toxicity of complex mixtures is considered one of the most important challenges for modern toxicology (Fent 2003; Kortenkamp 2007; Vaiseman 2011). All of the studies discussed thus far have used conventional methods to assess cell health, while the present study used HCA as a comparable but novel approach for investigating the toxicity of POP mixtures via multiple and pre-lethal toxicity markers.

Due to the requirement for a proliferating cell model in predictive cytotoxicity studies, the suitability of the HepG2 cell line in this role is supported by other studies. However as the results from HCA have now been deemed as comparable to other studies, a cell line with metabolic competence more akin to the *in vivo* state would likely further enhance the predictivity of this assay. An immortalised hepatic cell line (such as THLE2 or THLE3), which is capable of metabolic bio-activation, would enhance the biological significance of further POP studies by HCA.

Although the concentrations investigated in this study were tested above the reported concentrations of POPs in Scandinavian human serum, exposure to POPs is individualistic in nature and certain groups of people may thus have higher levels of specific compounds in their blood. Elderly people often have higher concentrations of POPs present in their serum (Salihovic *et al.* 2012) as POPs tend to bioaccumulate and their concentrations increase with age. Furthermore, people in countries who consume "traditional" foods such as whale blubber may have higher levels of POPs present in their serum (Van Oostdam *et al.* 2005; Weihe *et al.* 1996; Weihe *et al.* 2008). Finally, levels of certain compounds may be higher in specific groups of people due to occupational exposure. These differences emphasise that the present study is useful for investigating the mechanisms by which POP mixtures can exert their effects, and may, especially for any higher risk groups highlight possible health risks.

5. Conclusions

The present study is focused on developing an improved understanding of POP mixture induced toxicity. The combination of different markers allows stronger inferences to be made about the action of the POP mixtures on HepG2 cells. The Br and Cl mixtures were able to induce ROS generation but it did not lead to apoptosis. The PFC mixture induced the production of ROS and likely induced cell apoptosis accompanied by the dissipation of MMP. Comparison of the PFC + Br mixture to each independent mixture showed that for some concentrations MM and ROS induction were synergistic. These toxicological findings show that the POP mixtures can increase ROS induction and impact mitochondrial health, which could result in apoptosis. HCA has an advantage over conventional toxicity assays such as the MTT assay by simultaneously measuring multiple parameters. Consequently, HCA is more predictive because a wider spectrum of effects is assessed. Furthermore the HCA assay was able to detect early, reversible signs of cellular stress after exposure to the Cl mixture, which conventional assays would have missed. Further study on mixtures, relevant

467 to real life exposure, their toxicity and the mechanisms behind any toxic effects are important areas
468 of research.

469

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741

742

743 **Tables:**

744 Table 1. The composition and concentrations of original stocks supplied by the Norwegian University of Life
745 Sciences, Oslo. The estimated concentration of POPs in the Total, Cl, PFC + Cl and Br + Cl stock solutions are
746 1,000,000 times the estimated concentrations in human serum, in comparison with the PFC, Br and PFC + Br
747 stock solutions where the estimated concentration of POPs are 10,000,000 times the estimated concentrations
748 in human serum.
749

Compound	Mixture 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	
PFOS	29.425	294.250			294.250	29.425	
PFDA	0.495	4.950			4.950	0.495	
PFNA	0.800	8.000			8.000	0.800	
PFHxS	3.450	34.500			34.500	3.450	
PFUnDA	0.560	5.600			5.600	0.560	
Polybrominated diphenyl ethers (PBDEs)							
BDE-209	0.011		0.108		0.108		0.011
BDE-47	0.009		0.086		0.086		0.009
BDE-99	0.004		0.035		0.035		0.004
BDE-100	0.002		0.022		0.022		0.002
BDE-153	0.001		0.010		0.010		0.001
BDE-154	0.002		0.018		0.018		0.002
HBCD	0.025		0.246		0.246		0.025
Polychlorinated biphenyls (PCBs)							
PCB 138	0.222			0.222		0.222	0.222
PCB 153	0.362			0.362		0.362	0.362
PCB 101	0.008			0.008		0.008	0.008
PCB 180	0.194			0.194		0.194	0.194
PCB 52	0.010			0.010		0.010	0.010
PCB 28	0.013			0.013		0.013	0.013
PCB 118	0.064			0.064		0.064	0.064
Other organochlorines							
p,p'-DDE	0.502			0.502		0.502	0.502
HCB	0.117			0.117		0.117	0.117
α - chlordane	0.011			0.011		0.011	0.011
oxy - chlordane	0.022			0.022		0.022	0.022
trans-nonachlor	0.041			0.041		0.041	0.041
α-HCH	0.006			0.006		0.006	0.006
β-HCH	0.053			0.053		0.053	0.053
γ-HCH (Lindane)	0.006			0.006		0.006	0.006
Dieldrin	0.024			0.024		0.024	0.024

750

751 Table 2. Summary results for all HCA parameters and the MTT assay for HepG2 cells after 2 h
752 exposure to POP mixtures. The PFC, Br and PFC + Br mixtures were tested at concentrations
753 equivalent to 500, 1000, 5000 and 10,000 times serum levels. The Cl mixture was tested at
754 concentrations equivalent to 50, 100, 500 and 1000 times serum levels. Note: Total, Br + Cl and PFC

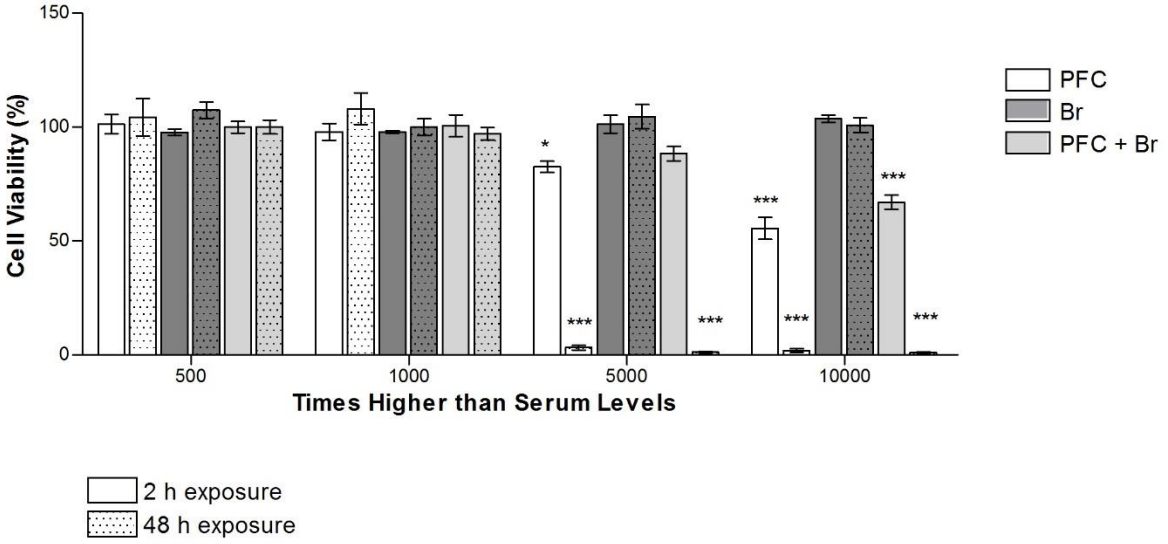
+ Cl mixture results are not shown as no significant effects were observed at any concentration. Grey indicates no significant effects were highlighted while cells with arrows indicate significant changes in that parameter. ↑ shows an increase in the parameter while ↓ shows a decrease.

Cell Health	PFC Mix				Br Mix				Cl Mix				PFC + Br Mix			
Parameter	500	1000	5000	10000	500	1000	5000	10000	50	100	500	1000	500	1000	5000	10000
CN				↓												
NI																↑
NA																↓
MM						↑										
MMP			↓	↓												↓
ROS				↑				↑			↑	↑				↑
MTT			↓	↓												↓

Table 3. Summary results for all HCA parameters and the MTT assay for HepG2 cells after 48 h exposure to POP mixtures. The PFC, Br and PFC + Br mixtures were tested at concentrations equivalent to 500, 1000, 5000 and 10,000 times serum levels. The Cl mixture was tested at concentrations equivalent to 50, 100, 500 and 1000 times serum levels. Note: Total, Br + Cl and PFC + Cl mixture results are not shown as no significant effects were observed at any concentration. Grey indicates no significant effects were highlighted while cells with arrows indicate significant changes in that parameter. ↑ shows an increase in the parameter while ↓ shows a decrease.

Cell Health	PFC Mix				Br Mix				Cl Mix				PFC + Br Mix			
Parameter	500	1000	5000	10000	500	1000	5000	10000	50	100	500	1000	500	1000	5000	10000
CN			↓	↓											↓	↓
NI			↑	↑											↑	↑
NA			↑												↑	↑
MM			↑	↑						↑	↑	↑				↑
MMP			↓	↓											↓	↓
ROS				↑				↑				↑				↑
MTT			↓	↓											↓	↓

Figures:



771

772 Figure 1. Cell viability of HepG2 cells after exposure to three POP mixtures, PFC, Br and PFC + Br. Cells were
773 exposed to concentrations of POPs (500, 1000, 5000 and 10000 times serum levels) for 2 h and 48 h and
774 cytotoxicity measured by conventional MTT. Data is expressed as a percentage of untreated control for each
775 parameter; mean ± SEM, n=3. $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***) represent significant cytotoxic
776 effects.
777

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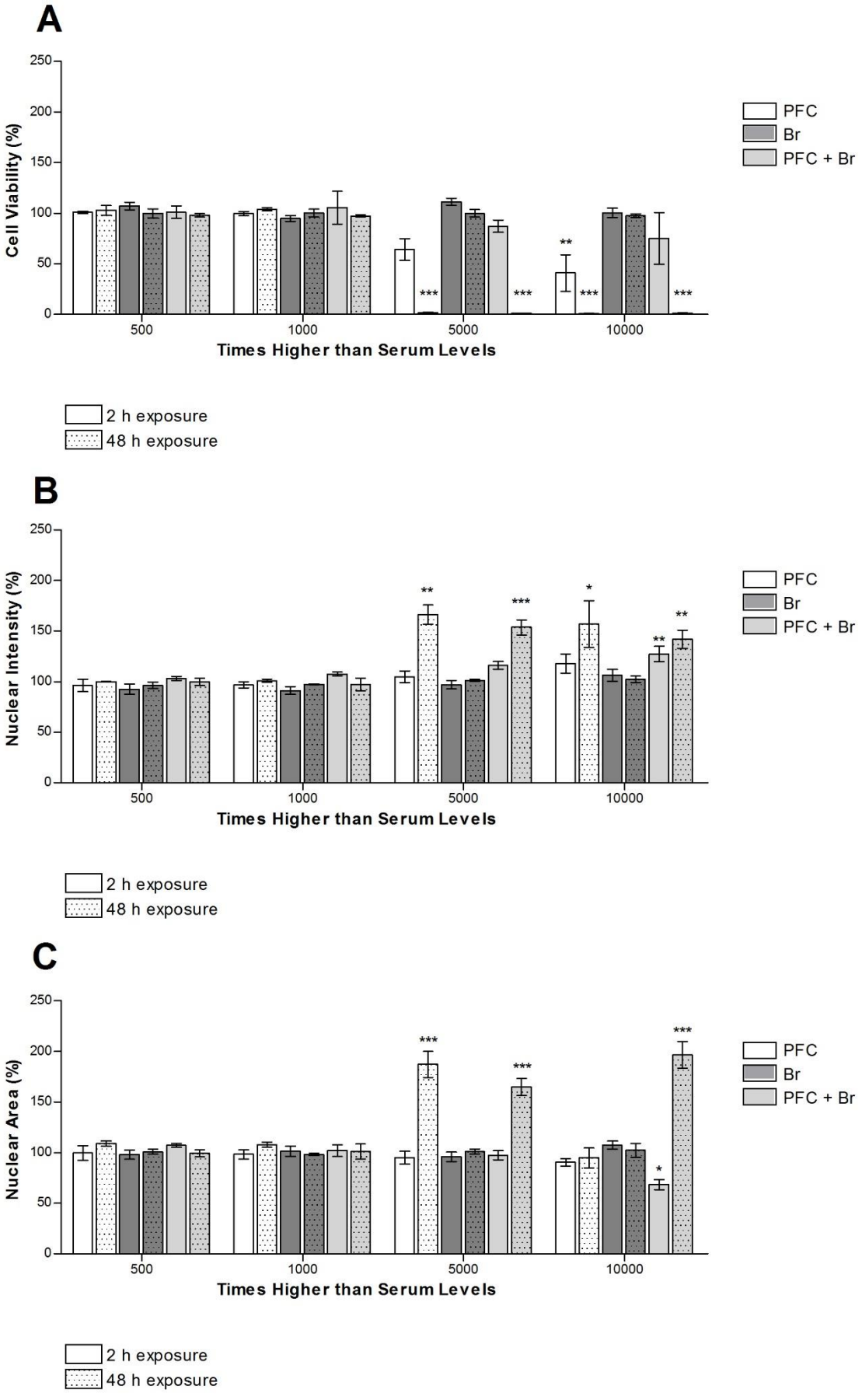
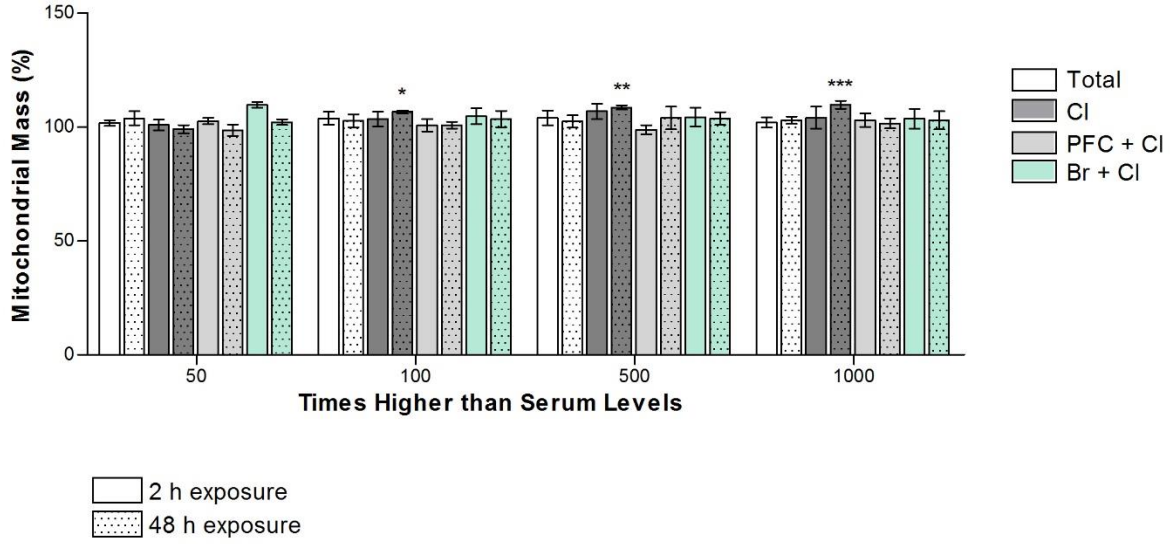

A
Cell Viability (%)
250
200
150
100
50
0
500
1000
5000
10000
Times Higher than Serum Levels
PFC
Br
PFC + Br
2 h exposure
48 h exposure
B
Nuclear Intensity (%)
250
200
150
100
50
0
500
1000
5000
10000
Times Higher than Serum Levels
PFC
Br
PFC + Br
2 h exposure
48 h exposure
C
Nuclear Area (%)
250
200
150
100
50
0
500
1000
5000
10000
Times Higher than Serum Levels
PFC
Br
PFC + Br
2 h exposure
48 h exposure

779

780 Figure 2. Nuclear changes in HepG2 cells after exposure to POP mixtures, PFC, Br and PFC + Br. Cells were
781 exposed to concentrations of POPs (500, 1000, 5000 and 10000 times serum levels) for 2 h and 48 h and
782 cytotoxicity measured by multi-parameter HCA endpoints CN (A), NI (B) and NA (C). Data is expressed as a
783 percentage of untreated control for each parameter; mean ± SEM, n=3. $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$
784 (***) represent significant cytotoxic effects.
785



786

787 Figure 3. Mitochondrial mass changes after exposure to four POP mixtures, Total, Cl, PFC + Cl and Br + Cl, in
788 HepG2 cells. Cells were exposed to concentrations of POPs (50, 100, 500 and 1000 times serum levels) for 2 h
789 and 48 h and cytotoxicity measured by multi-parameter HCA endpoint MM. Data is expressed as a percentage
790 of untreated control for each parameter; mean ± SEM, n=3. $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***)
791 represent significant cytotoxic effects.
792

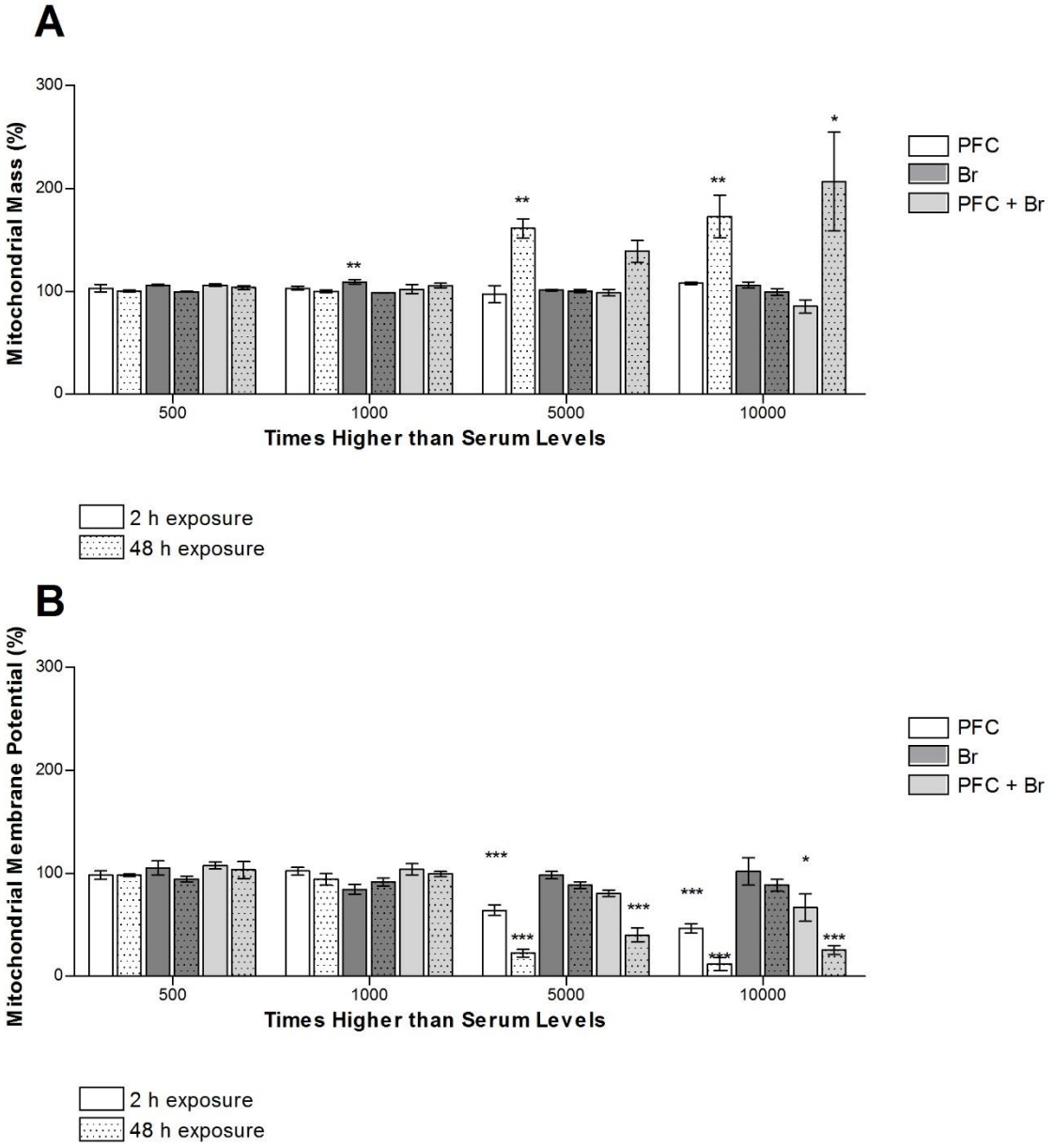


Figure 4. Mitochondrial changes after exposure to three POP mixtures, PFC, Br and PFC + Br, in HepG2 cells. Cells were exposed to concentrations of POPs (500, 1000, 5000 and 10000 times serum levels) for 2 h and 48 h and cytotoxicity measured by multi-parameter HCA endpoints MM (A) and MMP (B). Data is expressed as a percentage of untreated control for each parameter; mean ± SEM, n=3. $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***) represent significant cytotoxic effects.

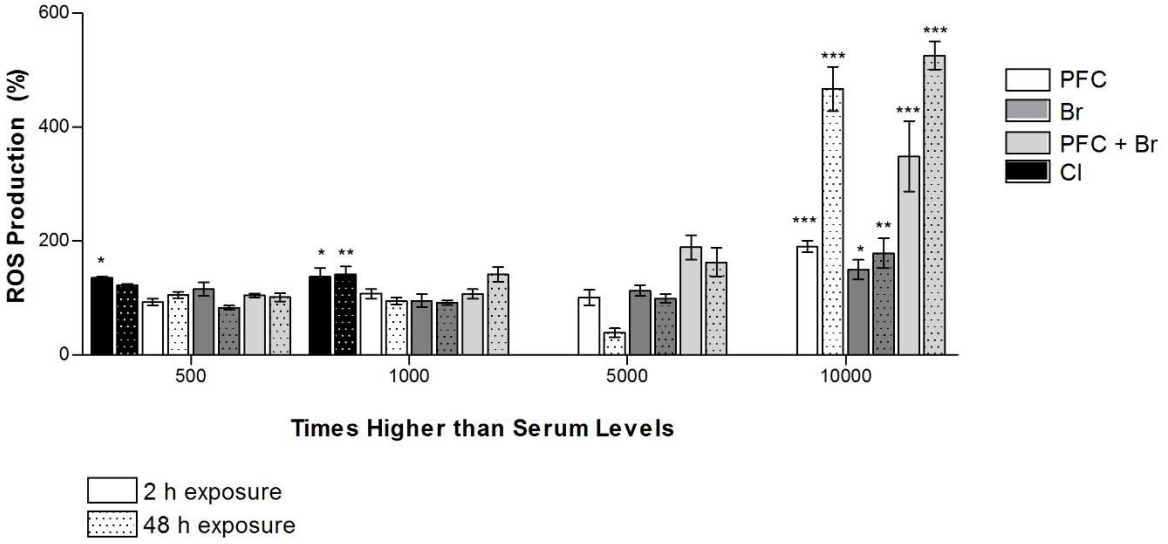


Figure 5. ROS production in HepG2 cells after exposure to four POP mixtures, PFC, Br, PFC + Br and Cl. Cells were exposed to concentrations of POPs (500, 1000, 5000 and 10000 times serum levels (NOTE – Cl mixture was not tested at 5000 or 10000 times serum levels) for 2 h and 48 h and cytotoxicity measured by multi-parameter HCA endpoint ROS. Data is expressed as a percentage of untreated control for each parameter; mean ± SEM, n=3. $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***) represent significant cytotoxic effects.

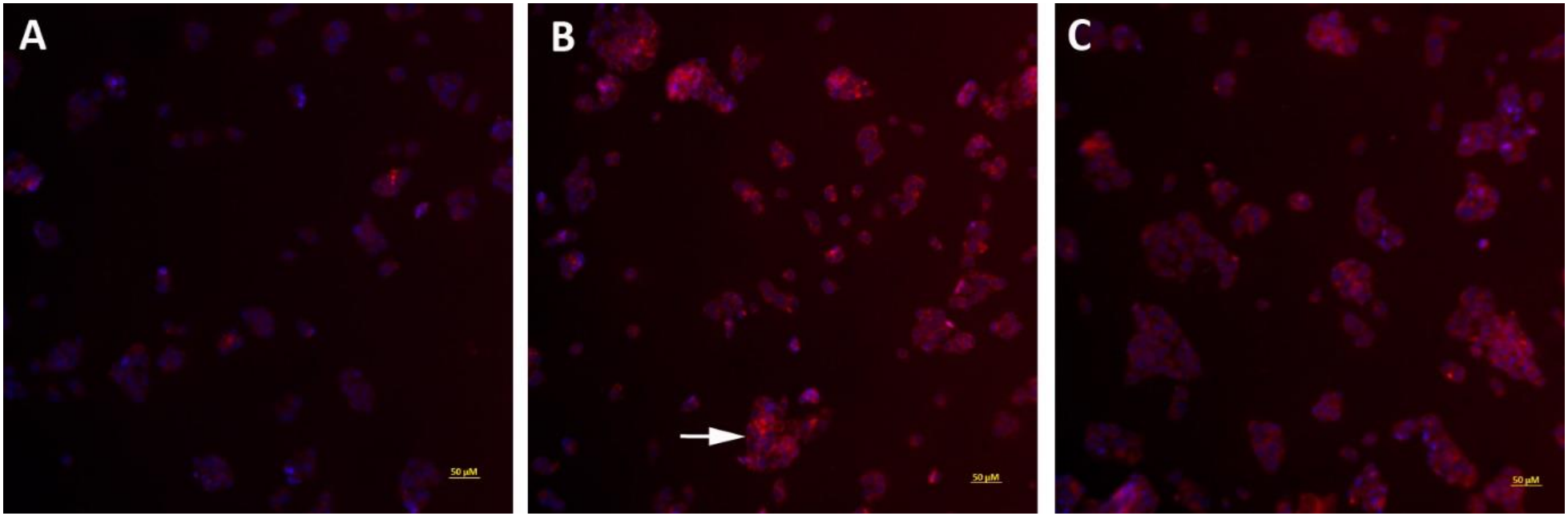


Figure 6. HCA images for (A) negative control (DMSO), (B) positive control (100 µM menadione), (C) example of mix - PFC + Br (10000 times serum level) – 2 h exposure. Each image was acquired at 10 × objective magnification using Hoechst dye (blue; nuclear staining) and CellROX dye (red; ROS). Arrow indicates an area of increased red fluorescence due to increased ROS production.