MIXTURE-SPECIFIC GENE EXPRESSION IN ZEBRAFISH (DANIO RERIO) EMBRYOS EXPOSED TO PERFLUOROOCTANE SULFONIC ACID (PFOS), PERFLUOROHEXANOIC ACID (PFHxA) AND 3,3',4,4',5-PENTACHLOROBIPHENYL (PCB126).

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Abstract

Perfluorooctane sulfonic acid (PFOS) and 3,3',4,4',5-pentachlorobiphenyl (PCB126) are persistent organic pollutants of high concern because of their environmental persistence, bioaccumulation and toxic properties. Besides, the amphiphilic properties of fluorinated compounds such as PFOS and perfluorohexanoic acid (PFHxA) suggest a role in increasing cell membrane permeability and solubilizing chemicals. The present study aimed at investigating whether PFOS and PFHxA are capable of modifying the activation of PCB126 toxicity-related pathways. For this purpose, zebrafish embryos were exposed in semi-static conditions to 7.5 µg/L of PCB126 alone, in the presence of 25 mg/L of PFOS, 15.7 mg/L of PFHxA or in the presence of both PFOS and PFHxA. Quantitative PCR was performed on embryos aged from 24 hours post fertilization (hpf) to 96 hpf to investigate expression changes of genes involved in metabolism of xenobiotics (*ahr2, cyp1a*), oxidative stress (*gpx1a, tp53*), lipids metabolism (*acaa2, osbpl1a*), and epigenetic mechanisms (*dnmt1, dnmt3ba*).

Cyp1a and *ahr2* expression were significantly induced by the presence of PCB126. However, after 72 and 78 h of exposure, induction of *cyp1a* expression was significantly lower when embryos were co-exposed to PCB126+PFOS+PFHxA when compared to PCB126-exposed embryos. Significant upregulation of *gpx1a* occurred after exposure to PCB126+PFHxA and to PCB126+PFOS+PFHxA at 30 and 48 hpf. Besides, embryos appeared more sensitive to PCB126+PFOS+PFHxA at 78 hpf: *acaa2* and *osbpl1a* were significantly downregulated; *dnmt1* was significantly upregulated.

While presented as environmentally safe, PFHxA demonstrated that it could affect gene expression patterns in zebrafish embryos when combined to PFOS and PCB126, suggesting

that such mixture may increase PCB126 toxicity. This is of particular relevance since PFHxA is persistent and still being ejected into the environment. Moreover, it provides additional information as to the importance to integrate mixture effects of chemicals in risk assessment and biomonitoring frameworks.

Introduction

Per- and polyfluoroalkylated substances (PFASs) are amphiphilic molecules which are used for their surface-active properties to repel both oil and water in coating for textiles, food packages, surface treatment and fire-fighting foams (Carloni, 2009). However, they represent a group of chemicals which are extremely persistent, bioaccumulative and toxic which are ubiquitously found in aquatic environments. Particularly, the 8-carbon perfluoroalkyls, perfluorooctanoic acid and perfluorooctane sulfonic acid (PFOS), have attracted most attention as these chemicals were extensively used in industrial applications and represent final degradation products of many PFASs. Consequently, they can be found at concentrations from the ng/L up to the µg/L in the environment (Ahrens and Bundschuh, 2014; Ahrens et al., 2009; Kannan et al., 2005; Kärrman et al., 2011; Skutlarek et al., 2006). In fish, they cause abnormal development, reduced offspring survival, and endocrine disruption (Ahrens and Bundschuh, 2014). The toxicity of PFOS is related to reactive oxygen species production, causing lordosis and pericardial edemas (Shi et al., 2008; Shi and Zhou, 2010; Zheng et al., 2011). PFOS is also known to interfere with lipid metabolism in different vertebrates including several fish species (Cheng et al., 2016; Oakes et al., 2005). In addition, it also presents endocrine disrupting properties, particularly regarding sexual and thyroid hormone synthesis (Bonefeld-Jorgensen et al., 2014; Du et al., 2009; Kang et al., 2016; Oakes et al., 2005). Global regulatory efforts have led to a reduction in the emission of PFOS-related and long-chain PFASs; however, introduction of replacement chemicals results in perfluorinated end-products such as perfluorohexanoic acid (PFHxA) (Scheringer et al., 2014). To the best of our knowledge previous reports did not identify toxic effects in mammals or teleosts (Chengelis et al., 2009; Iwai and Hoberman, 2014; Loveless et al., 2009). Contrary to PFOS, PFHxA presents low bioconcentration capacities which make it difficult to detect and study in animal tissues after assimilation (Martin et al., 2003a; Martin et al., 2003b). However, it is recalcitrant against chemical, physical and biological degradation (Rahman et al., 2014). Consequently, environmental concentrations of PFHxA are expected to increase with its ongoing production which may lead to continuous exposures and adverse effects in wildlife and humans (Cousins et al., 2016). Moreover, research has demonstrated that PFASs are able

to modulate the toxicity of other chemicals (Jernbro et al., 2007) as “chemosensitizers”. The exact process remains unclear; however, it was hypothesized that they alter biological membrane fluidity (Hu et al., 2003; Liao et al., 2014; Liu et al., 2008; Matyszewska and Bilewicz, 2008) and inhibit efflux transporter systems (Fischer et al., 2013; Keiter et al., 2016). Both effects may lead to increased local concentrations of xenobiotics in the cells and therefore may increase their toxicity.

In the present study, we investigated the modulation effect of PFOS and PFHxA in zebrafish embryos regarding the activation of toxicity-related pathways of PCB126. The latter was selected as a reference chemical as it represents one of the most abundant planar polychlorobiphenyl which can be found in the ng/L range in the environment (Ge et al., 2014). Moreover, its toxicity during zebrafish development is well described (Brown et al., 2002; Grimes et al., 2008; Jonsson et al., 2007a; Jonsson et al., 2012; Liu et al., 2015; Liu et al., 2016). PCB126 is recognized as a strong inducer of biotransformation enzymes (*i.e*. Cyp1a) *via* the aryl hydrocarbon receptor (AhR) pathway (Hahn, 2001), subsequently triggering toxicity mainly via oxidative stress (Liu et al., 2015; Schlezinger et al., 2006). The present study hypothesized that the presence of PFOS and PFHxA would increase the activation of the toxicity pathways of PCB126. To assess it, several stages during zebrafish embryonic development were selected: 24, 30, 48, 54, 72, 78 and 96 hours post fertilization (hpf). Embryos were exposed to the three compounds alone, two binary mixtures (PCB126+PFOS and PCB126+PFHxA) and a mixture of all three compounds (ternary mixture; PCB126+PFOS+PFHxA). Expression of different genes known to be involved in PCB126 toxicity were studied in order to investigate whether the presence of PFASs would modify the patterns. Genes of the aryl hydrocarbon receptor (AhR) mediated pathway (*ahr2*, *cyp1a*) (Fang et al., 2012; Jonsson et al., 2007a; Jonsson et al., 2007b), the oxidative stress response (*gpx1a*) (Shi and Zhou, 2010), and a marker for apoptosis (*tp53*) (Shi et al., 2008) were studied. In order to address the possibility whether PFASs interfere with membrane integrity by altering lipid homeostasis, genes belonging to lipid metabolism (*acaa2*, *osbpl1a*) were monitored (Hu et al., 2005; Shi et al., 2009) Moreover, expression of two methyltransferases genes, *dnmt1* and *dnmt3ba*, was measured in order to highlight underlying mechanisms of toxicity (Aluru et al., 2015).

1. Material & Methods

1.1. Chemicals

Potassium perfluorooctanesulfonate (PFOS, $C_8F_{17}SO_3K$, CAS number: 2795-39-3), perfluorohexanoic acid (PFHxA, $C_6HF_{11}O_2$, CAS number: 307-24-4), 3,3´,4,4´,5-pentachlorobiphenyl 126 (PCB126, $C_{12}H_5Cl_5$, CAS number: 57465-28-8) and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (Germany). Analytical standards of native PFOS, PFHxA, PCB126 and mass labelled $^{13}C_4$ PFOS, $^{13}C_2$ PFHxA, and $^{13}C_{12}$ PCB126, were purchased from Wellington Laboratories (ON, Canada). Methanol was LC-MS grade and taken from Fisher Scientific (Ottawa, Canada). Stock solutions of PFOS (403.5 g/L) and PCB126 (13.3 µg/L) were prepared in DMSO while PFHxA stock solution (1.57 g/L) was in reconstituted water. Reconstituted water was prepared according to ISO 7346-2 (1996) (294 mg/L $CaCl_2 \cdot 2\ H_2O$, 123.3 mg/L $MgSO_4 \cdot 7\ H_2O$, 63 mg/L $NaHCO_3$, 5.5 mg/L KCl) in MilliQ (Millipore®) water. It was also used for the preparation of exposure solutions. Zebrafish embryos were exposed to 6 different solutions: PCB126, PFOS, PFHxA, PCB126+PFOS, PCB126+PFHxA and PCB126+PFOS+PFHxA. The binary mixture of PFOS and PFHxA was not investigated since the focus was made on the modulation of PCB126 toxicity pathway. Exposure to the single compounds PFOS and PFHxA is only discussed in the supplementary data since it did not trigger any significant change in expression of the investigated genes (Appendix D). Final test concentrations were selected based on a previous study (Keiter et al., 2016): 25 mg/L (50µM) of PFOS, 15.7 mg/L (50 µM) of PFHxA and 7.5 µg/L (23 nM) of PCB126. Beakers used for exposures were pre-incubated for 24 hours with the chemical solutions in order to limit loss of compounds due to glass adsorption. Highest content of DMSO was 0.014% (v/v) and a control was performed to monitor that there is no specific effect of the solvent (data not shown).

1.2. Chemical analysis

Concentrations of PFOS, PFHxA and PCB126 in water were analyzed at 0, 24 and 96 hpf to ensure the dosing levels over the course of exposure.

Analysis of PFOS and PFHxA. Water samples were sonicated for homogenization, diluted 100 times and then transferred to LC-vials, where methanol, ammonium acetate and mass-labelled standards ($^{13}C_4$ PFOS and $^{13}C_2$ PFHxA) were added to the final concentration of 5 µg/L. Separation and analysis of PFOS and PFHxA were performed by an Acquity ultra-performance liquid chromatograph coupled to a Xevo TQ-S tandem mass spectrometer (Waters Corporation, Milford, US) based on the method described in the ISO 25101 (2009) using external standards and adjusting for volume differences. Details of the instrumental analysis can be found in the Appendix A.

Analysis of PCB126. Liquid-liquid extraction using dichloromethane:n-hexane (1:4) was performed to extract PCB126 in the dosing water followed by column cleanup with 40% H_2SO_4 modified silica. Instrumental analysis was performed on an Agilent 6890N (Agilent Technologies, USA) gas chromatograph coupled to AutoSpec Ultima high resolution mass spectrometer. Details of the extraction and instrumental analysis of PCB126 can be found in the Appendix A.

1.3. Fish maintenance and sampling

The research facilities at MTM are affiliated with the Swedish Board of Agriculture, Jönköping, Sweden (# 5.2.18-861/15). Adult zebrafish (AB strain; ZFIN ID: ZDB-GENO-960809-7) were purchased from Karolinska Institute (Stockholm, Sweden) and kept on a 14 h: 10 h light: dark cycle. Maintenance of adult zebrafish and egg production were according to standard protocols (Westerfield, 2007). Fish were kept in semi-static conditions where water exchange as well as conductivity, pH and temperature were automatically controlled and regulated by a ProfiLux 3.1 controller (GHL Advanced Technology, Kaiserslautern, Germany). Carbon hardness, nitrates, nitrites (EasyTest stripes, JBL) and ammonia (Tetra) contents were monitored twice a week. Fish were fed two times a day either with flakes (TetraRubin®, Tetra) or freshly prepared artemias (Ocean Nutrition®). Spawning trays were placed in the tanks the evening before spawning.

One beaker was prepared for each studied developmental stage: 24 hpf (prim-6), 30 hpf (prim-16), 48 hpf (long-pec), 54 hpf (long-pec/pec-fin), 72 hpf (protruding mouth), 78 hpf and 96 hpf (early larvae stages) (Kimmel et al., 1995). Exposures of zebrafish embryos did not exceed 96 hpf to comply with any national animal welfare law, guideline and policy. Sets of 30 fertilized eggs between 0 and 2 hpf were selected visually and directly transferred into glass beakers filled up with 25 ml of either reconstituted water, solvent solution (0.014 volume % DMSO) or fresh test solutions. Every day 20 ml of each test solution were renewed. Glass beakers were covered with Parafilm® (Parafilm, Menasha, WI, USA) to prevent from evaporation, and incubated at 26 ±1 °C. At each sampling time, embryos were euthanized in a saturated ethyl 4-aminobenzoate solution, washed using Phosphate Buffer Saline (PBS) and kept in RNA*later*® solution (Ambion™) at -20°C. Each experiment was repeated three times representing independent biological replicates.

1.4. qPCR analysis

Prior to RNA extraction, embryos were disrupted in lysis buffer (provided by Macherey-Nagel NucleoSpin® RNA extraction kit) using a cordless mixer motor and pellet pestles (VWR, Germany). The following steps were according to the guidelines of the kit. Subsequently, cDNA was synthetized from 1.6 µg of RNA according to M-MuLV Reverse Transcriptase (Invitrogen®) associated protocol. All cDNA samples were diluted 10 times prior to use, and kept at -20°C until further analysis.

Primers used in qPCR analysis were designed using the online free software Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Appendix B). Specificity, efficiency and linearity ranges were established for all primer pairs using DNA electrophoresis, melting curve and standard curve analyses (Appendix C).

Each qPCR reaction was processed using 2X Power SyBR green (Applied Biosystems™), 200 nM of each primer, 2 µl of cDNA dilution completed up to 15 µl of MilliQ water. Samples were run in technical duplicates. Results were normalized to the expression of *β-actin* and *rpl13a* as they showed the highest stability among 4 investigated genes (*i.e. eef1a1*, *b2m*, *β-actin* and *rpl13a*). Finally, data were analyzed using the Pfaffl method with PCR efficiency correction (Pfaffl, 2004).

1.5. Statistical Analyses

Statistical analyses were performed on qPCR data from three independent experiments using GraphPad Prism® 7 software. Normality was controlled on log-transformed data using Shapiro Wilk's test ($p<0.05$); homoscedasticity was checked with Brown-Forsythe's test ($p<0.05$). Subsequently, one-way ANOVA on treatments followed by Sidak's multiple comparisons *post-hoc* test was performed at each developmental stage separately to identify significant differences. A $p<0.05$ was considered to be a statistically significant difference.

2. Results

2.1. Alteration of gene expression

Exposure to DMSO did not trigger any change in gene expression compared to the water control (data not shown). Exposure to PFHxA lead to an increase in the average expression of *dnmt3ba* at 78 hpf and *osbpl1a* at 96 hpf. However, these differences were not significant from controls (Appendix D). Exposure to PFOS tended to increase *cyp1a* expression and to decrease *dnmt3ba* expression at 96 hpf (not significant, Appendix D).

2.1.1. AhR pathway

AhR-related genes are encoding a pathway involved in the metabolism of xenobiotics and detoxification. The upregulation of these genes is used as a well-known biomarker for dioxin-

like compounds such as PCB126. Since our own results showed that PFOS and PFHxA don't induce the AhR-pathway, the changes in expression are due to PCB126 uptake. Therefore, it is possible to investigate whether the availability of PCB126 is modified in co-exposure. Exposure to PCB126 alone triggered a significant upregulation of *ahr2* at 48, 54 and 78 hpf (fold change ranging from 1.8 to 3.3) (fig. 1). *Ahr2* expression was significantly induced from 48 to 78 hpf after exposure to PCB126+PFOS (1.8-2.5) and PCB126+PFHxA (1.8-2.4) (fig. 1). Significant upregulation of *ahr2* expression was observed at 48 hpf (fold change of 2.82) and 54 hpf (1.96) after exposure to the ternary mixture (fig. 1). While exposures to PCB126, PCB126+PFOS and PCB126+PFHxA triggered their highest induction at 72 or 78 hpf, average induction of *ahr2* expression after exposure to the ternary mixture was going down from 30 hpf until reaching a basal level of expression at 96 hpf. Particularly, at 78 hpf, the activation of *ahr2* was significantly lowered by the presence of the three compounds (fig. 1).

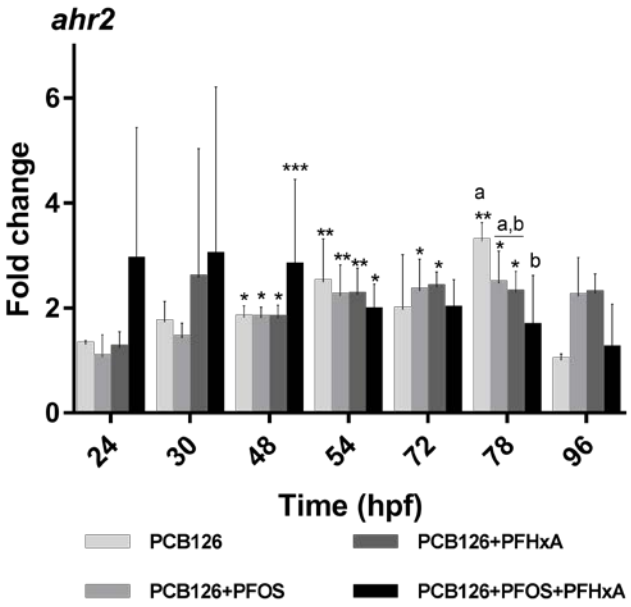

Figure 1: Normalized fold change in expression of *ahr2* across embryonic development of zebrafish after exposure to PCB126, PCB126+PFOS, PCB126+PFHxA and PCB126+PFOS+PFHxA. Significant upregulation of *ahr2* was reported between 48 hpf and 78 hpf after exposure to PCB126+PFOS and PCB126+PFHxA. PCB126 alone triggered a significant induction of *ahr2* expression at 48, 54 and 78 hpf. A significant induction was reported after exposure to the ternary mixture at 48 and 54 hpf. At 78 hpf, induction of *ahr2* expression was significantly lowered by the ternary mixture. Data are expressed as mean ± SD (n=3). ***: $p<0.001$; **: $p<0.01$; *: $p<0.05$ from control; different letters indicate a significant difference between exposures.

As expected, exposure to PCB126 caused a significant upregulation of *cyp1a* throughout the early development of zebrafish (maximum fold change at 72 hpf of 1562) (fig. 2). A peak of upregulation was reported at 72 hpf after exposure to the ternary mixture and 72-78 hpf in other cases (fig. 2). No significant difference was observed between exposure to the single compound PCB126 and binary mixtures. Similar to *ahr2* expression profile, the ternary mixture lowered the induction of *cyp1a* between 54 hpf and 96 hpf (maximum fold change at

72 hpf of 948). This effect was significant at 72 hpf and 78 hpf when compared to single and binary exposures (fig. 2).

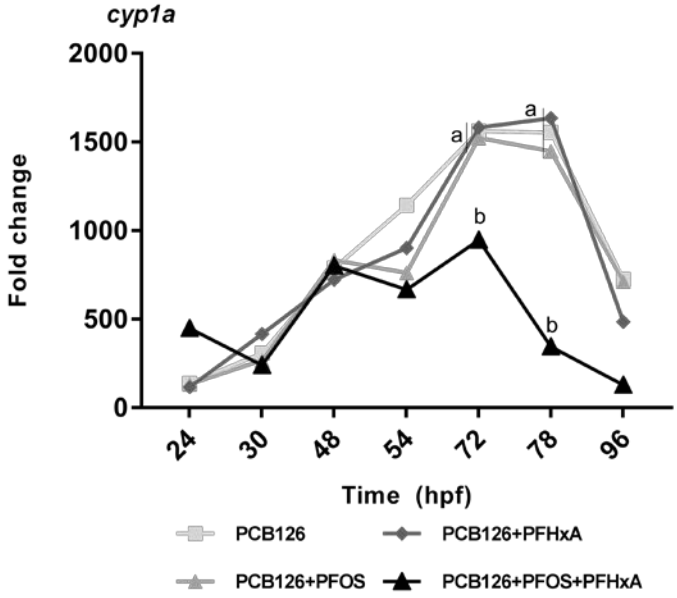

Figure 2: Normalized fold change in expression of *cyp1a* after exposure to PCB126, PCB126+PFOS, PCB126+PFHxA and PCB126+PFOS+PFHxA. Significant induction of *cyp1a* was reported from 24 hpf until 96 hpf after exposure to PCB 126 alone or in mixture with PFASs ($p<0.001$). The ternary mixture was significantly decreasing this induction both at 72 hpf ($p<0,05$) and 78 hpf ($p<0,001$) when compared to single and binary exposures. Data are expressed as mean ± SD (n=3). Different letters indicate significant differences between exposures.

2.1.2. Oxidative stress

Significant upregulation of *gpx1a* expression, coding the anti-oxidant enzyme glutathione peroxidase 1a, was observed after 30 and 48 hours of exposure to PCB126+PFHxA and to the ternary mixture (fig. 3). Moreover, this effect was significantly different from PCB126 exposure (fig. 3). No other significant effect was reported, however the ternary mixture tended to increase *gpx1a* expression at 78 hpf (not significant, Appendix E). Besides, the gene *tp53*, marker for cell apoptosis, was not regulated (Appendix F).

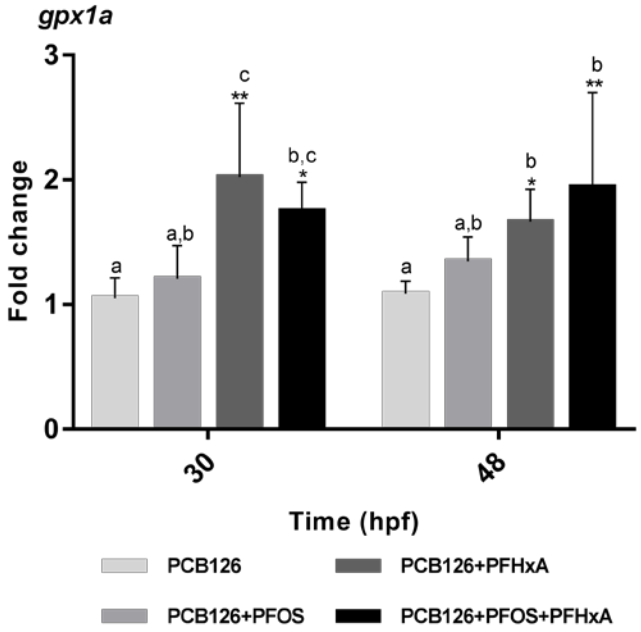

Figure 3: Normalized fold change in expression of *gpx1a* at 30 hpf and 48 hpf in zebrafish embryos after exposure to PCB126, PCB126+PFOS, PCB126+PFHxA and PCB126+PFOS+PFHxA. Exposure to PCB126+PFHxA and PCB126+PFOS+PFHxA significantly increased *gpx1a* expression at 30 and 48 hpf. Data are expressed as mean ± SD (n=3). **: p<0.01; *: p<0.05 from control. Different letters indicate significant differences between exposures at each time-point.

2.1.3. Lipids metabolism

The ternary mixture decreased the expression of *osbpl1a*, involved in cellular lipid binding and cholesterol metabolism, at 78 hpf (fig. 4a) when compared to effects underwent following PCB126, PCB126+PFHxA and PCB126+PFOS exposures. However, *osbpl1a* downregulation was not significantly different from controls. No significant effect was reported at other time points (Appendix G). The expression of *acaa2*, involved in fatty acid β-oxidation, was downregulated at 78 hpf and 96 hpf after exposure to the ternary mixture (fig. 4b). No effect was reported at any other developmental stage (Appendix H).

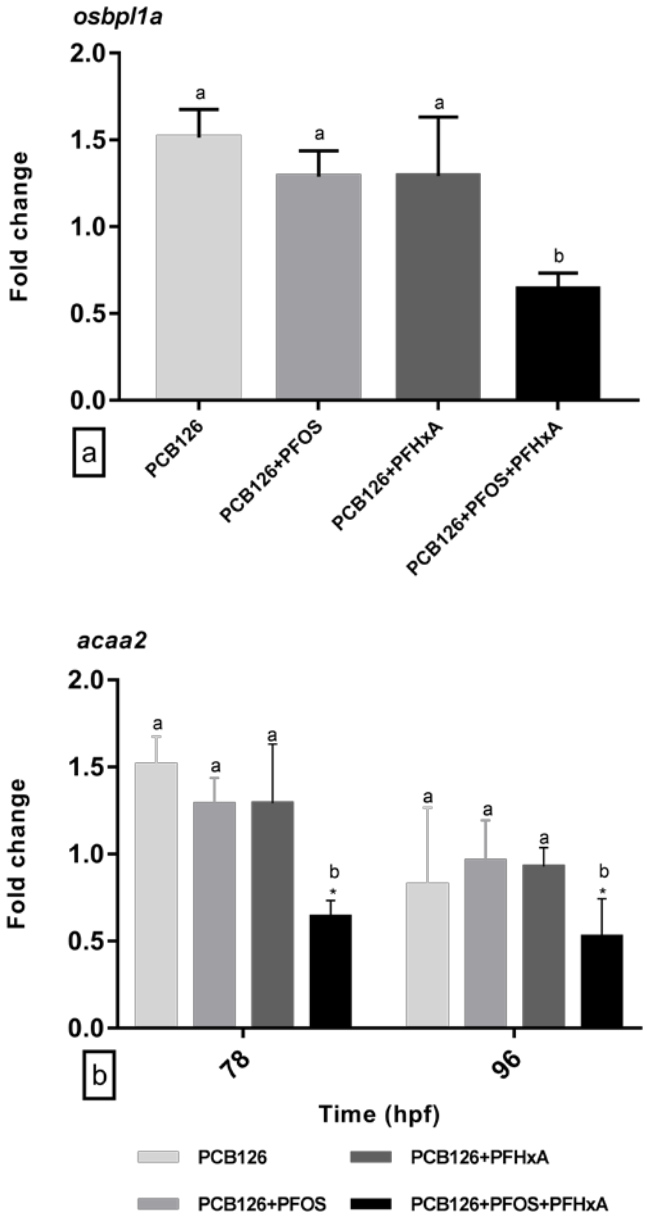

Figure 4: Normalized fold change in expression of a) *osbpl1a* at 78 hpf and b) *acaa2* expression at 78 and 96 hpf in zebrafish embryos following exposure to PCB126, PCB126+PFOS, PCB126+PFHxA and PCB126+PFOS+PFHxA. Exposure to the ternary mixture is significantly decreasing *osbpl1a* and *acaa2* expression ($p<0.05$) when compared to the other exposures. *Acaa2* expression after exposure to PCB126+PFOS+PFHxA is also significantly different from controls (*: $p<0.05$). Data are expressed as mean ± SD (n=3). Different letters indicate a significant difference between exposures.

2.1.4. Epigenetic machinery

When normalized to their expression at 24 hpf, expression of *dnmt1* and *dnmt3ba* was decreasing across development under unexposed conditions (fig. 5). When exposed to the ternary mixture, expression of *dnmt1* underwent a 3-fold increase between 48 hpf and 96 hpf (fig. 5a). Exposure to PCB126+PFOS showed a specific increase at 78 hpf (fig. 5a). Nothing similar was reported for *dnmt3ba* expression which remained very low following all exposures (fig. 5b). However, a low but significant increase in *dnmt3ba* expression was reported at 96 hpf after PCB126 exposure together with a low decrease at 30 hpf (fig. 5b).

Moreover, when normalized to control conditions, expression of *dnmt1* was significantly increased at 78 hpf after exposure to the ternary mixture when compared to controls and to PCB126-exposed embryos (fig. 6a). The average fold change of *dnmt1* after exposure to the ternary mixture at 96 hpf was higher than controls, however the difference was not significant (Appendix I). Besides, exposure to the ternary mixture tended to increase *dnmt3ba* expression at 78 hpf (not significant, fig. 6b). No significant effect was reported at other time-points (Appendix J).

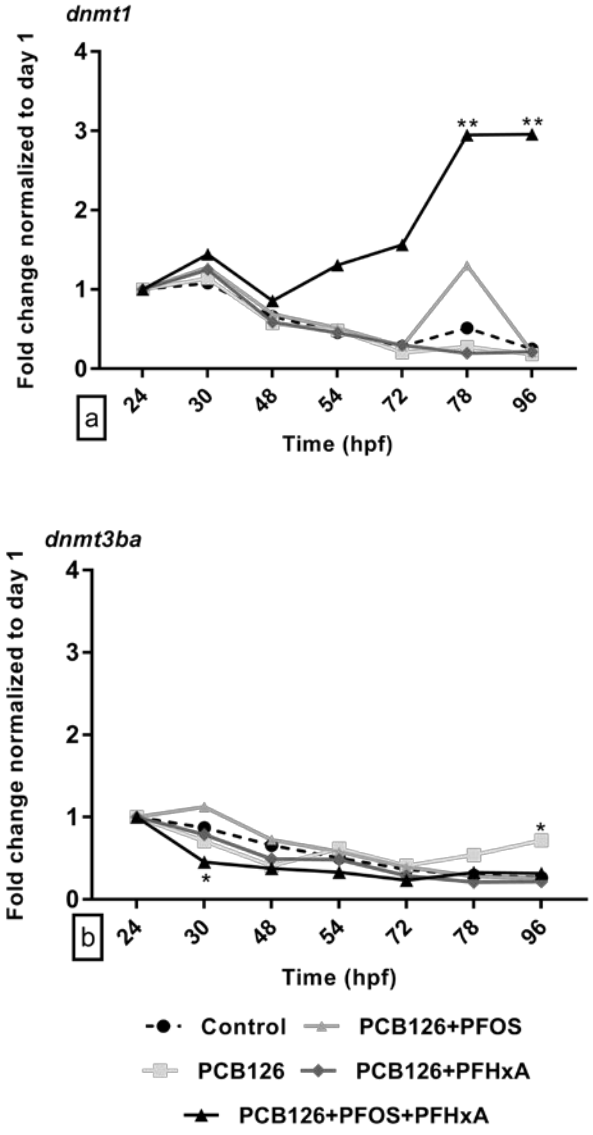

Figure 5: Time-dependent expression of a. *dnmt1* and b. *dnmt3ba* after exposure to PCB126, PCB126+PFOS, PCB126+PFHxA and PCB126+PFOS+PFHxA. Expression of *dnmt1* was induced after exposure to PCB126+PFOS+PFHxA instead of undergoing a decrease across development. Expression of *dnmt3ba* is following the decrease observed in unexposed conditions. A low but significant increase was reported after exposure to PCB126 at 96 hpf together with a low decrease at 30 hpf. Data are expressed as the mean fold change in relation to day 1 of the respective exposure (n=3). **: $p<0.01$; *: $p<0.05$ from control.

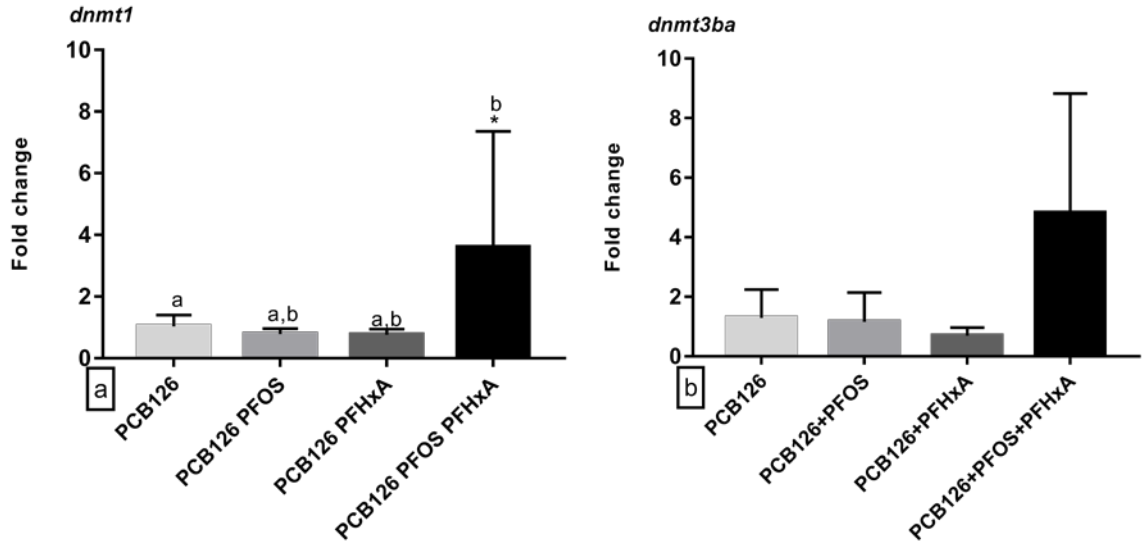

Figure 6: Normalized fold change in expression of a. *dnmt1* and b. *dnmt3ba* in zebrafish embryos of 78 hpf after exposure to PCB126, PCB126+PFOS, PCB126+PFHxA and PCB126+PFOS+PFHxA. Significant upregulation of *dnmt1* expression and tendency to upregulation of *dnmt3ba* expression were reported after exposure to the ternary mixture (not significant). Data are expressed as mean ± SD (n=3). *: $p<0.05$ from control. Different letters indicate a significant difference between exposures

2.2. Chemical analysis

Analysis of PFOS and PFHxA in water showed that measured concentrations were respectively 30% lower and 15% higher than nominal concentrations and that they were rather stable overtime (Appendix K). Concentration of PFOS was measured between 14 and 20 mg/L while PFHxA was between 16 and 18 mg/L (Appendix K). In contrast, analysis of PCB126 reported that the targeted level was not reached since the average level was 4.6% of the nominal concentration only (Appendix K). The maximal measured concentration was 0.8 µg/L while the targeted concentration was 7.5 µg/L. Measured concentrations were approx. 10 to 100 times less than nominal ones (Appendix K). However, they were neither statistically different overtime nor between the different exposures. Therefore, this did not prevent from comparing the biological effects of the different exposures across development.

3. Discussion

Zebrafish embryos were exposed to nominal concentrations of 7.5 µg/L PCB126, 25 mg/L PFOS and 15.7 mg/L PFHxA. The selected concentrations are high when compared to environmental levels: PFOS and PFHxA in surface water have been reported from contaminated sites up to the low µg/L range (Ahrens and Bundschuh, 2014; Kärrman et al., 2011; Skutlarek et al., 2006) and PCB126 in the ng/L range (Ge et al., 2014). However, the focus of the present study was to gain understanding of changes in key-pathways altered by PCB126 when it is in combination with chemosensitizers such as PFOS and PFHxA. Changes were studied using expression analysis of genes involved in toxicity mechanisms of the

investigated compounds. It was shown in previous studies that several PFASs such as PFOS are capable of altering membrane permeability (Hu et al., 2003; Keiter et al., 2016; Liao et al., 2014; Liu et al., 2008; Matyszewska and Bilewicz, 2008). Therefore, we hypothesized that the activation of the toxicity pathways of PCB126 would be increased by the presence of PFOS or/and PFHxA as a result of its higher cellular concentration.

Induction of Cyp1a is a well-described biomarker for AhR-activating chemicals such as PCB126 (Hahn, 2001). The binding of AhR-agonist molecules on AhR2 results in the translocation of the complex to the nucleus and to its binding to a regulatory sequence in *cyp1a* promoter to induce its transcription (Schmidt and Bradfield, 1996). The exacerbate activity of Cyp1a is known to increase the reactive oxygen species content and to cause oxidative stress (Schlezinger et al., 2006) which is highly toxic to the organism. In addition, it was previously suggested that the energetic costs required to metabolize and excrete contaminants cause a negative impact on growth (Rowe et al., 2001). As expected, all exposures involving PCB126 triggered *cyp1a* expression. *Cyp1a* induction after exposure to PCB126 alone and in mixture with PFHxA was overall increasing from 24 hpf until 78 hpf when reaching a plateau, and underwent a steep decrease between 78 and 96 hpf. The kinetic was slightly different when exposed to PCB126+PFOS as a slight decrease in *cyp1a* expression was reported between 48 and 54 hpf, together with a slight decrease between 72 and 78 hpf. In addition, exposure to the mixture PCB126+PFOS+PFHxA modified the kinetic of *ahr2* and *cyp1a* expression as shown in fig. 1. Expression of *cyp1a* did not reach the same level as in other conditions and showed a steep decrease after 72 hpf. Surprisingly, exposure to the ternary mixture caused a lower induction in embryos aged from 54 hpf to 96 hpf when compared to what was triggered by PCB126, PCB126+PFOS and PCB126+PFHxA. Particularly, the induction of *cyp1a* expression was decreased by around 80% at 78 hpf. Data were no more significantly different at 96 hpf, which is likely due to the observed decrease in expression under all conditions tested. This might be explained by the activation of repressive feedbacks to reduce Cyp1a activity. A previous study reported an increase in Cyp1a activity after co-exposure to PFOS and TCDD using in vitro systems (Hu et al., 2003). An effect from PFOS with PCB126 alone was not confirmed in zebrafish embryos; however, a direct comparison of the two studies is difficult since different models, concentrations, chemicals, and test methods were used. Since similar phenotypic oxidative damages were reported in all conditions involving PCB126 (data not shown), it is unlikely that the observed reduction in Cyp1a expression is reflecting a decrease in Cyp1a-induced toxicity. Another explanation might be negative feedbacks, such as *ahrrb* activation, reducing Cyp1a activity. Besides,

PFASs may act on other pathways that are able to influence the AhR-pathway *e.g. via* a cross-talk, and which were not investigated in the present study. However, physiological endpoints and effects on later stages need to be investigated to identify whether long-term consequences for the organism can be expected.

A hyperactivity of Cyp1a was reported to increase the reactive oxygen species content, thus inducing oxidative stress (Schlezinger et al., 2006; Schlezinger et al., 1999). Despite a high induction in *cyp1a* expression under all conditions involving PCB126 exposure, a low oxidative stress response (upregulation of *gpx1a*) was only reported in the presence of PCB126+PFHxA and PCB126+PFOS+PFHxA. This suggests that PFHxA is the PFAS involved in this effect. Since this chemical is not known to induce any toxicity so far, it is of particular relevance to report that it is capable of having a synergistic effect with PCB126. However, the effect was low and transient, requiring further investigation for long-term consequences. Besides, exposure to PFOS and PCB126 is known to induce oxidative stress and anti-oxidant responses (Liu et al., 2015; Shi et al., 2008; Shi and Zhou, 2010). Even though only a low oxidative stress response was reported in this present study, a preliminary test on acute toxicity showed sublethal effects on larvae exposed to PCB126 alone or in mixture (data not shown). Pericardial edemas, reduced blood flow across the cardiovascular system and lordosis were reported, as previously described in literature (Grimes et al., 2008; Jonsson et al., 2007a; Liu et al., 2015), all endpoints that may reflect oxidative stress (Teraoka et al., 2003). Therefore, a higher upregulation of *gpx1a* and *tp53* expression was expected. This discrepancy might be explained by the limited number of genetic markers that were selected as oxidative stress involves several other contributors. Moreover, related studies used different models such as *in vitro* (Hagenaars et al., 2008; Liu et al., 2007; Oakes et al., 2005) and *ex vivo* (Schlezinger et al., 2006) models. A previous study on zebrafish embryos reported induction of *gpx1a* from concentrations of 50 µg/L PCB126 and even higher concentrations were required for apoptosis induction (Liu et al., 2015), which is in accordance with the present results. However, Shi et al (2008) reported upregulation of *tp53* after exposure to 0.1-5 mg/L PFOS whereas no effect was observed in this study, suggesting that higher concentrations may have different toxicity mechanisms.

As previously described, PFOS is able to interfere with lipid metabolism and steroidogenesis in several fish species (Hu et al., 2005; Oakes et al., 2005; Shi et al., 2009). In the present study, no effect of PFOS alone on this pathway was reported. However, Oakes et al. (2005) exposed different fish species to 3 mg/L PFOS for 14-28 days and measured different endpoints such as protein activities and dosing of hormone levels. Shi et al. (2009) exposed

zebrafish embryos to 0.5 mg/L PFOS until 192 hpf and showed an increase in the osbpl1a protein content. Moreover, *acaa2* expression was only reported to be altered after 21 days of exposure to PFOS in rats (Hu et al., 2005). Altogether, it seems that alterations of lipid metabolism and steroidogenesis by PFOS requires an extended exposure time and/or a more advanced development. The present study reported the downregulation of *osbpl1a* at 78 hpf and *acaa2* at 78 and 96 hpf after exposure to PCB126+PFOS+PFHxA, however these effects were relatively low. Osbpl1a is involved in cellular lipid-binding, transport and cholesterol homeostasis while Acaa2 is part of the β-oxidation of fatty acids. It is likely that this regulation is due to non-specific effects that do not involve PCB126 but both PFASs only because of their abilities to modify properties of lipid biological membranes and interfere with lipid signaling pathways (Gorrochategui et al., 2014). However, this cannot be confirmed in this study since a mixture of PFOS and PFHxA without PCB126 was not investigated.

As methylation of DNA is one key-pathway for gene regulation, DNA methyltransferases expression was assessed. These enzymes are required in the early development as they are involved in gene expression control *via* the specific methylation of DNA (Takayama et al., 2014). We reported an increase in *dnmt1* overtime after exposure to the ternary mixture and it was significantly upregulated at 78 hpf. Alterations of epigenetic marks such as DNA methylation in germ cells by environmental factors can be stable enough to reflect later damages that may remain across generations (Skinner, 2014a; Skinner, 2014b). DNA methyltransferases are in charge of transferring a methyl group from a donor, S-adenyl-methionin, to specific cytosines on DNA. Therefore, an increase in expression of these 2 enzymes is likely to increase the methylation level of genomic DNA. Moreover, a negative correlation between methylation level and gene expression has been previously reported (Riggs, 1975). Consequently, it is expected that an increase in *dnmts* expression is decreasing expression of the genes they target. Therefore, modification of *dnmts* expression may be one mechanism involved in the specific alteration of gene expression patterns at 78 hpf after exposure to PCB1216+PFOS+PFHxA. However, the link between upregulation of *dnmts* and decreased expression of the investigated genes, *i.e. osbpl1a, acaa2, ahr2* and *cyp1a* cannot be drawn from this study and needs further investigation on methylation levels of DNA.

According to our results, PFOS and PFHxA seem to have a synergistic action regarding PCB126 toxicity, which is of particular relevance since it gives information about the potential of a “non-toxic” chemical, PFHxA, to be actively involved in mixture toxicity. However, it is not known whether this is due to (i) specific interaction between both chemicals or (ii) to the overall ∑PFASs concentration which is twice as much as in binary

mixtures. In the first case, it would mean that using twice as much as PFOS or twice as much as PFHxA would lead to similar effects to what is observed. In the second case, the specific presence of both compounds is required as they may act at different complementary levels. Moreover, developmental stages were differentially sensitive to exposure. Zebrafish embryos appeared more sensitive to PCB126+PFOS+PFHxA at 78 hpf. This might be explained by hatching which occurs around this developmental stage. Indeed, the chorion may act as a physical barrier for the embryo from its environment and the chemicals. Consequently, its removal might increase the chemicals availability and trigger a stress. The latter is suggested by the low induction of *cyp1a* expression in unexposed conditions at 78 hpf (data not shown). Another explanation might be changes in expression of the genes throughout developmental processes. Indeed, gene expression in embryos is following specific spatial and temporal expression patterns (Braunig et al., 2015; Otte et al., 2010) which will indirectly affect their sensitivity toward exposure.

Finally, a general high variation between biological replicates was reported and was the main source for lack of significance. Variations in chemical concentrations may represent one of the potential sources (Appendix K). Variability of PFOS and PFHxA concentrations was about 16% and 6.2% in average, respectively. The variability of PCB126 was about 26% in average and its concentration was measured to be only 4.6% of the nominal level. The differences between measured and nominal concentration might be explained by (i) adsorption on test vessels, (ii) loss of the compounds during solution preparation and quantification, and (iii) uptake into zebrafish embryos. However, since the nominal concentration was not reached in water samples taken at 0 hpf, the third hypothesis can be ruled out together with the first one. Thus, this is likely to be due to adsorption and loss of compound during preparation steps of solutions. Therefore, it can´t be excluded that non-statistically significant variations in PCB126 concentrations, and PFOS and PFHxA to a lower extent, have introduced variability in gene expression results. However, these variations were randomly distributed and independent from the exposure scenario. Besides, variability in qPCR results may come from slight differences between exposure start, natural variation in sensitivity toward exposure and high plasticity of developing organisms.

4. Conclusion

Distinct patterns were reported for the ternary mixture when compared to single compounds and binary mixture exposures. Particularly, exposure to PCB126+PFOS+PFHxA lowered the induction of the AhR pathway and influenced expression of several genes involved in

epigenetic mechanisms, lipids metabolism and oxidative stress at specific developmental windows. Environmental samples can contain several compounds which may act as synergistic chemosensitizers. The present study suggests that synergism occurs between PFOS and PFHxA regarding PCB126 toxicity in zebrafish embryos at the molecular level. This is of particular relevance in risk assessment since under regular environmental conditions organisms are exposed to multiple chemicals. There is a general view on chemicals with low bioconcentration factors to be environmentally safe but the present study demonstrated the ability of PFHxA to affect gene expression patterns induced by PCB126. Whether it affects the overall physiology of the organism requires further investigation since it could be subject to continuous environmental exposure due to its extreme persistence and ongoing use.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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