Effects of perfluoroalkyl substances (PFAS) and benzo[a]pyrene (BaP) co-exposure on phase I biotransformation in rainbow trout (Oncorhynchus mykiss)

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Research Article

Keywords: Biotransformation, perfluorooctanoic acid (PFOA), perfluorooctanesulfonic acid (PFOS), in vivo, in vitro, Rainbow trout, RTL-W1

Posted Date: April 25th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1554855/v1

Abstract

The presence of perfluoroalkyl substances (PFAS) in the environment, especially in aquatic ecosystems, continues to be a significant concern for human and environmental health. Previous studies have suggested that several PFAS do not undergo biotransformation due to their chemical stability, yet perfluorooctanesulfonic acid (PFOS)- and perfluorooctanoic acid (PFOA)-exposed organisms have presented altered activity of important biotransformation pathways. Given the fundamental role of biotransformation in biological organisms and the significant distribution of PFAS in aquatic environments, the present study investigated the influence of PFOA and PFOS on phase I biotransformation enzymes *in vitro* using the rainbow trout liver RTL-W1 cell line and *in vivo* using juvenile rainbow trout. Cells and fish were exposed and co-exposed to environmentally relevant concentrations of PFOA, PFOS, and benzo[a]pyrene (BaP), for 72 h and 10 days, respectively, prior to measurements of cytotoxicity and biotransformation ability through measurements of CYP1A1-, CYP1A2- and CYP3A4-like activities. Our results indicate that exposure to PFAS-BaP binary mixtures altered CYP1A-like activity *in vivo*; however, those alterations were not observed *in vitro*. Similarly, while BaP did not significantly induce CYP3A4, exposure to the PFAS led to significantly lower enzymatic activity relative to basal levels. These observations may have implications for organisms simultaneously exposed to PFAS and other environmental pollutants for which biotransformation is necessary, especially in detoxification mechanisms. Furthermore, the interference with biotransformation pathways could potentially increase adverse outcomes, compromising the stability of fish populations inhabiting PFOA- and PFOS-polluted environments.

Introduction

The ubiquitous presence of per- and polyfluoroalkyl substances (PFAS) in the environment has been under global surveillance, as these classes of compounds are now considered a contaminant of emerging concern (CEC) in the 21st century (Krewski et al., 2019). For the last 60 years, these compounds have been manufactured with continued production of many long-chain PFAS through 2015 within the United States (Teaf et al., 2019). Perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) are among the most popular PFAS discussed in the literature. PFAS contain strong covalent bonds between carbon and fluorine atoms responsible for their desirable oil and water-repelling properties. These compounds are industrial surfactants used for manufacturing purposes ranging from flame retardants to non-stick cookware. Due to their extremely stable structure, PFAS are known to have a long half-life in the environment and biological tissues, reminiscent of previously banned persistent organic pollutants (POPs), such as polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT). Many of the previously banned POPs are characterized as persistent, bioaccumulative, and toxic (PBT) chemicals, inciting concern among the general public and the scientific community over the potential human health and environmental risks posed by PFAS (Teaf et al., 2019).

The lack of PFAS biotransformation processes associated with the high structural stability has led to high bioaccumulation in the liver, kidney, and blood serum of humans and wildlife (De Silva et al., 2021;

Mortensen et al., 2011). Organisms residing in PFAS-contaminated environments may be at higher risk of severely bioaccumulating these substances (Ankley et al., 2021). In the case of the aquatic environment, PFAS are taken up by fish via passive diffusion through the gills, and they associate with blood proteins in the circulatory system and extracellular matrices before being eliminated via three pathways: passive diffusion via the gill, urinary and biliary excretions (Ng and Hungerbühler, 2013; Qiang et al., 2016; Zhong et al., 2019). In rainbow trout, PFOA and PFOS have been found to preferentially accumulate in the liver following both dietary and flow-through exposures (Goeritz et al., 2013; Martin et al., 2003; Vidal et al., 2019). Their interaction with key molecular pathways and potential subsequent effects in fish remain largely unclear.

Furthermore, with the ubiquitous presence of PFAS, organisms are at significant risk of being co-exposed to other legacy POPs in the aquatic environment, such as polycyclic aromatic hydrocarbons (PAHs). PAHs have been found to widely contaminate aquatic environments and have toxic effects on fish upon activation by phase I biotransformation (Franco and Lavado, 2019; Honda and Suzuki, 2020). In general, PAH biotransformation starts with the activation of the cytochrome P450 system, leading to the upregulation of, primarily, CYP1A isoforms that oxidize the compounds (Franco and Lavado, 2019; Santana et al., 2018). In this context, the potential exposure to PAHs and PFAS mixtures may alter important biological processes, resulting in increased effects for exposed biota.

Recently, Khan et al. (2019) showed that exposure to environmentally relevant mixtures of PAHs and PFAS could modulate the neuro-dopamine homeostasis in female Atlantic cod. In addition, Dale et al. (2020) showed that, while PFAS were able to induce selected biomarkers of oxidative stress (catalase and glutathione-*S*-transferase), no effect on CYP1A1 gene expression was observed in Atlantic cod exposed to mixtures of PAHs and PFAS.

In the present study, we investigated *in vitro* and *in vivo* biological responses associated with phase I biotransformation using the rainbow trout (*Oncorhynchus mykiss*) liver cell line RTL-W1 and juvenile fish after exposure to binary mixtures of a well-studied PAH, benzo(*a*)pyrene (BaP), and either PFOA or PFOS. The selected doses were in the range of the reported concentrations of PAHs and PFAS in industrialized areas, where both groups of compounds are likely to occur simultaneously (Kunacheva et al., 2012). Biotransformation was assessed by measurements of CYP1A1-like activity (ethoxyresorufin-*O*-deethylase, EROD) in intact cells and liver microsomal fractions and of CYP1A2-like activity (methoxyresorufin-*O*-demethylase, MROD) and CYP3A4-like activity (dealkylation of 7-benzyloxy-4-trifluoromethylcoumarin, BFC) in liver microsomal fractions only. The observations presented in the present study provide significant information for PFAS-related alterations at the molecular and cellular levels and highlight the need to further investigate PFAS sub-lethal effects on the integrity of aquatic organisms.

Materials And Methods

Chemicals and reagents

Leibovitz's L-15 medium, L-glutamine, penicillin G, streptomycin, phosphate-buffered saline (PBS), and trypsin–EDTA were obtained from Gibco Life Technologies (ThermoFisher Scientific, Waltham, MA). Fetal bovine serum (FBS) was obtained from Sigma-Aldrich (St. Louis, MO). Deionized water (DI water) was obtained using a Milli-Q water purification system (Millipore, Billerica, MA). Acetonitrile, 2-propanol, methanol, and ethanol were of analytical grade and were purchased from Thermo Fisher Scientific (Waltham, MA) unless otherwise specified. Perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) were purchased from Crescent Chemical Co. (Islandia, NY).

Animals and in vivo exposures

Juvenile rainbow trout (*Oncorhynchus mykiss*) of approximately 5 months old, with an average length of 16 ± 3 cm, and without discernible gonadal morphology indicative of gender, were obtained from Westover Farms (Steelville, MO). Fish were maintained in a flow-through living-stream system (Frigid Units Inc., OH) with dechlorinated, carbon-filtered municipal water at 12°C, 0.8 PSU salinity, and with a 14:10 h light cycle for two months before experimental use. Fish were fed high-protein commercial fish feed (Silver Cup, Murray, UT) once a day.

After tank acclimation, fish were transferred to 19 L aquaria containing water spiked with either a low concentration of BaP (1 µg/L), a high concentration of BaP (10 µg/L), PFOA (1 µg/L), or PFOS (1 µg/L) alone or in binary mixtures. Exposure lasted 10 days, with four animals per tank and two replicates for each experimental and control group (8 fish/treatment group, 72 animals total). The control group was exposed to water spiked with the solvent vehicle (EtOH). Each tank received a 50% water renewal with freshwater spiked with the respective treatments every two days during the exposure period. Water quality parameters (temperature, pH, %DO, ammonia concentration, and salinity) were monitored daily.

Subcellular fractionation of liver tissue

Following the exposures, liver microsomal fractions were isolated following the protocol described by Lavado et al. (2004). Fish care and handling were conducted in accordance with an approved Institutional Animal Care and Use Committee (IACUC) protocol at Baylor University. Briefly, fish were euthanized by cervical dislocation, dissected, and the liver was removed. 100 mg of liver tissue was homogenized in microcentrifuge tubes containing 200 µL of potassium phosphate buffer (100 mM, pH = 7.4) with 100 mM KCl, and 1 mM EDTA. Samples were then centrifuged at 13,000*g* for 20 min and 4°C. The resulting supernatant was collected, placed in 4 mL ultracentrifugation tubes (Beckman Coulter Life Sciences, Indianapolis, IN), and spun at 100,000*g* for 90 min at 4°C. The pellet was resuspended in 1:10 (w/v) microsomal buffer (100 mM, pH = 7.4, 100 mM KCl, 1 mM EDTA) containing 20% glycerol, and stored at -80°C until bioassays were performed. Protein content was measured in microsomes using the Coomassie Blue method (Pierce Inc., Rockford, IL) and bovine serum albumin (BSA) as standard (Bradford, 1976).

Cytotoxicity

The viability of the cells was assessed after exposure to the selected compounds by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay(Gerlier and Thomasset, 1986). The MTT assay is a colorimetric assay based on NAD(P)H-dependent cellular oxidoreductase enzymes' ability to reduce the tetrazolium dye MTT to its insoluble formazan form, which has a purple color. At the end of 72 hours, the culture media/dye was carefully removed from each well, and formazan crystals were then solubilized by adding 200 µL of a mixture of ethanol and DMSO (1:1). The resulting color was measured by absorption spectroscopy (BioTek SynergyTM H1, Winooski, VT) at 595 nm and 650 nm, and cytotoxicity was calculated as the relative proportion (%) of viable cells with respect to the control group. No decreases in viability were observed at the concentrations to be used for the subsequent *in vitro* exposures.

In vitro exposures

The RTL-W1 cell line was routinely cultured in 75-cm^2 cell culture flasks (ThermoFisher Scientific, Waltham, MA) with Leibovitz's L-15 medium supplemented with 5% FBS, 2 mM L-glutamine, and antibiotics (50 U/mL penicillin G, and 50 µg/mL streptomycin) at 19°C in a normal atmosphere. Upon reaching approximately 90% confluence in culture flasks, cells were harvested with Trypsin-EDTA, seeded in 96-well plates (5.0 x 10^4 cells/well), and allowed to grow for 24 h. The culture medium was then changed and replaced by phenol-red free medium without FBS or antibiotics, and exposed to single or binary mixtures of BaP (1.6 µM or 160nM), PFOS (1.6 µM), or PFOA (1.6 µM) for 72 h. The control wells contained only the solvent vehicle (DMSO) at 0.5%.

CYP-1A1, -1A2, and – 3A4 associated activities

CYP1A1-like activity in intact cell monolayers was evaluated through the 7-Ethoxyresorufin-*O*-deethylase (EROD) activity bioassay, measuring the deethylation of 7-ethoxyresorufin in the presence of endogenous NADPH to resorufin, and following the protocol described by (Franco et al., 2018) and (Heinrich et al., 2014) with modifications. Briefly, after the exposure, the culture medium was removed, cells were washed with PBS, and then exposed to 200 µL of 8.0 µM 7-ethoxyresorufin solution. The plates were immediately transferred to a plate reader (Synergy™ H1, BioTek, VT) and kinetic measurements were obtained every minute for 20 min to measure the formation of resorufin at excitation and emission of 537 and 583 nm, respectively.

For normalization of the resorufin production based on protein content, the protein concentrations in each well were determined with fluorescamine as described by Thibaut et al. (2009) and using BSA as standard. Briefly, the 7-ethoxyresorufin solution was removed from the wells and the cell monolayer was rinsed with PBS before adding 50 µL deionized water. The plates were then frozen at -80°C for 24 h. After thawing at room temperature, 100 µL of PBS was added to each well, followed by 50 µL of fluorescamine solution (0.3 mg/mL in acetonitrile). After 5 min of shaking in the dark, the plates were read at excitation and emission wavelengths of 360 and 460 nm, respectively. Two plates with six technical replicates for each chemical exposure type were used to determine *in vitro* enzymatic activity.

For the *in vivo* experiment, the CYP1A1-like activity was measured via the EROD bioassay using liver microsomal fractions, at an initial 7-ethoxy resorufin concentration of 10 µM. Additionally, CYP1A2- and CYP3A4-like activities were measured via the methoxyresorufin-*O*-dealkylase (MROD) activity bioassay and the dealkylation of 7-benzyloxy-4-trifluoromethylcoumarin (BFC), respectively. Enzyme substrates were evaluated at concentrations of 10 µM for MROD and at 100 µM BFC selected based on preliminary experiments and previous studies with rainbow trout (Smith and Wilson, 2010). All enzyme activities were measured kinetically by fluorescence spectroscopy (Synergy™ H1, BioTek, VT), and normalized per mg of microsomal protein, measured via the Coomassie blue (Bradford) assay (Bradford, 1976).

Instrumental analysis of PFOA, PFOS, and BaP in water

Water samples (20 mL) were collected in duplicate at the start of the experiment, after 96 h, and after 168 h of exposure for each treatment, and frozen at -80°C until UPLC determination of BaP, PFAS, and PFOA.

Water samples were processed as described in Myers et al. (2011), with minor modifications. 5 mL of water samples were mixed with 3 mL of methanol and 1.5 mL of chloroform. The tubes were stirred using a vortexer for 60 s and then centrifuged at 5,000*g* for 20 minutes. After centrifugation, the organic and aqueous layers were separated. The tube with the aqueous layer was subjected to a second extraction with the same amounts of water, methanol, and chloroform as mentioned above. The organic layer was retrieved again. Organic phases from both extractions were combined and dried under a gentle flow of nitrogen. The residue was reconstituted in 200 µL of acetonitrile, passed through 0.2 µm filters to remove particulates and a volume of 1 to 5 µL was injected onto UPLC.

Concentrations of BaP in water samples were determined as described in Franco et al. (2022) using high-performance liquid chromatography coupled with an RS fluorescence detector (HPLC-FLD; UltiMate 3000) and via the Chromeleon 7 chromatography data system (Thermo Fisher Scientific, Waltham, MA). Chromatographic separation was accomplished using a Kinetex® C-18 column (1.7 µm, 150 x 2.1 mm; Phenomenex, Torrance, CA). 80% aqueous acetonitrile was maintained at an isocratic flow rate of 0.5 mL/min and a column temperature of 40°C. BaP was detected at excitation and emission wavelengths of 295 and 403 nm, respectively.

A simple derivatization method followed by ultra-performance liquid chromatography (UPLC) to analyze PFOA and PFOS was used as described in Shan et al. (2014). Briefly, PFOA and PFOS were first derivatized with 3,4-dichloroaniline (DCA) using a carbodiimide method, and the amidate product was detected by a UV detector at a maximum absorption wavelength of 255 nm. UPLC analyses were performed on a Thermo Dionex Ultimate 3000 UHPLC system equipped with a 150 mm × 2.1 mm Vanquish C18 (2.2 µm) reverse-phase column (ThermoFisher Scientific). Separation of PFOA-DCA and PFOS-DCA amidates was done employing an UPLC isocratic system elution at a flow rate of 0.5 mL/min with a mobile phase composed of 80% methanol and 20% H_2O. Chromatographic peaks were monitored by a diode-array detector (DAD) at 255 nm. The compounds were quantified by integrating the area under the peaks. The detection limit of the method was 0.01 µg/L.

Statistical analysis

Statistical analyses were carried out using the GraphPad Prism version 8.0.0 for MacOS (GraphPad Software, San Diego, CA). Significant differences in CYP activity between treatment groups were assessed using one-way ANOVA and a *p*-value of less than 0.05 was considered statistically significant. If an overall significance was detected, Dunnett's posthoc tests were performed to determine differences from solvent controls. All data were analyzed before statistical analysis to meet the homoscedasticity and normality assumptions of parametric tests.

Results

Measured PFOA, PFOS, and BaP concentrations in water

Measured average concentrations of BaP, PFOA, and PFOS did not present major differences at the different sampling times (0, 96, and 168 h); thus, the data were combined to reflect an overall average concentration for the entire duration of the experiment (Table 1). In general, measured concentrations did not deviate significantly from the nominal concentrations, as they were within 15% of the variance. Therefore, the nominal concentrations were used to characterize the fish response to the selected compounds.

Table 1
Perfluoroalkyl substances and benzo[a]pyrene concentrations in water from the *in vivo* experiment. BaP: Benzo[a]pyrene; PFOA: perfluorooctanoate; PFOS: perfluorooctane sulfonate; BDL: below detection limit (< 0.01 µg/L).

	BaP (µg/L)		PFOA (µg/L)		PFOS (µg/L)	
Group	**Nominal**	**Measured**	**Nominal**	**Measured**	**Nominal**	**Measured**
Solvent Control	-	BDL	-	BDL	-	BDL
BaP 1 µg/L	1	0.6 ± 0.3	-	BDL	-	BDL
BaP 10 µg/L	10	6.5 ± 2.1	-	BDL	-	BDL
PFOA 1 µg/L	-	BDL	1	0.5 ± 0.1	-	BDL
PFOS 1 µg/L	-	BDL	-	BDL	1	0.8 ± 0.2
BaP 1 µg/L + PFOA 1 µg/L	1	0.8 ± 0.1	1	0.3 ± 0.2	-	BDL
BaP 1 µg/L + PFOS 1 µg/L	1	0.5 ± 0.4	-	BDL	1	0.8 ± 0.2
BaP 10 µg/L + PFOA 1 µg/L	10	7.4 ± 1.2	1	0.4 ± 0.3	-	BDL
BaP 10 µg/L + PFOS 1 µg/L	10	5.9 ± 4.1	-	BDL	1	0.6 ± 0.3

In vivo experiment

CYP1A1-like activity in the isolated juvenile rainbow trout microsomal fractions (Fig. 1A), measured as EROD activity, displayed the highest levels in fish exposed to 1 and 10 µg/L BaP, with activities of 8.4- and 31.7-fold, respectively, relative to the control group (1.28 ± 0.55 pmol/h/mg protein). CYP1A1-like activity following PFOA exposures alone (2.68 ± 1.10 pmol/h/mg protein) was not significantly different than controls, however, exposure to PFOS alone (6.10 ± 1.56 pmol/h/mg protein) resulted in increased activity, 4.8-fold higher than the control group (Table 2). Furthermore, exposure to binary mixtures consisting of a low BaP concentration (1µg/L) and an equal concentration of either PFOA or PFOS resulted in significant decreases in CYP1A1-like activity relative to the low BaP exposure alone; CYP1A1-like activities were 2.6- and 4.1-fold lower for the BaP-PFOA and the BaP-PFOS treatments, respectively.

Table 2
In vivo effects of PFOA and PFOS on CYP-like associated activities. Data are presented as average ± SD (n = 4–8) and as pmol/min/mg protein. Statistical significance is shown as * ($p < 0.05$) and ** ($p < 0.01$) (Student's *t* comparison test).

	Exposure Group		
	Control	**PFOA (1 µg/L)**	**PFOS (1 µg/L)**
CYP1A1-like associated activity (EROD)	1.28 ± 0.55	2.68 ± 1.10	6.10 ± 1.56*
CYP1A2-like associated activity (MROD)	10.86 ± 2.93	17.26 ± 8.15	53.77 ± 18.90**
CYP3A4-like associated activity (BFC)	1222.9 ± 642.9	234.9 ± 134.4*	228.2 ± 188.1**

The CYP1A1-like activity of fish co-exposed to the high BaP concentration (10 µg/L) and 1 µg/L of PFOS was ~ 3.1-fold lower than exposures to the high concentration of BaP alone. However, the co-exposures consisting of a high concentration of BaP and 1 µg/L of PFOA demonstrated reductions in EROD activity 53-fold lower than the high concentration of BaP alone. Both co-exposure treatments with the PFAS decreased the activity to levels that were not statistically different from the control group.

CYP1A2-like activity determined in the rainbow trout liver microsomal fractions (Fig. 1B), measured as MROD activity, showed similar trends as CYP1A1-like activities. However, they were significantly higher ($p < 0.05$) in the groups exposed to 1 and 10 µg/L BaP. PFOA and PFOS exposures alone also demonstrated increases in the CYP1A2-like activity (Table 2). Fish exposed to a binary mixture of 1 µg/L or 10 µg/L BaP and an equal concentration of PFOS (20.91 ± 6.08 and 82.93 ± 44.34 pmol/h/mg protein, respectively) appeared to show an increase in CYP1A2-like activity. However, these differences were not statistically different from the controls, despite the relatively high activity observed with the fish exposed to BaP alone. Exposures to binary mixtures of 1 µg/L or 10 µg/L BaP and an equal concentration of PFOA led to CYP1A2-like activity that was not deemed significantly different from the treatment groups exposed to the corresponding concentrations of BaP alone.

CYP3A4-like activity (Fig. 1C), measured as BFC activity, had no significant differences between the untreated controls (1222.9 ± 642.9 pmol/h/mg protein) and the BaP treatments. However, fish exposed to either 1µg/L PFOA and PFOS alone had CYP3A4-like activities 5-fold lower than the controls (234.9 ± 134.4 and 228.2 ± 188.1 pmol/h/mg protein, respectively). This significant decrease in activity ($p < 0.05$) was also demonstrated in the groups with binary mixtures of 1µg/L PFOA with either BaP 1 µg/L or 10 µg/L and in the fish co-exposed 1 µg/L PFOA and 10 µg/L BaP.

In vitro experiment

Exposure to the selected doses of BaP and PFOA/PFOS in individual and binary mixtures did not lead to significant cell mortality, as survival remained above 80%.

Using the RTL-W1 rainbow trout liver cell line *in vitro*, CYP1A1-mediated metabolism was also determined using EROD activity (Fig. 2). Cells exposed to 1.6 μM PFOA or PFOS alone displayed CYP1A1-like activity similar to the control wells (5.12 ± 2.12 and 4.65 ± 1.44 pmol/h/mg protein, respectively). Contrarily, cells exposed to 160 nM or 1.6 μM BaP showed CYP1A1-like activity 3.7- to 6.0-fold higher than the controls. Although the treatments with binary mixtures of BaP and each of the PFAS appeared to have differences in activity, statistical differences were unable to be distinguished from the exposure to BaP alone.

Discussion

The present study investigated *in vitro* and *in vivo* biological responses associated with phase I biotransformation using the rainbow trout (*Oncorhynchus mykiss*) liver cell line RTL-W1 and juvenile fish after exposure to binary mixtures of benzo(a)pyrene (BaP), and either PFOA or PFOS. Our results indicate that exposure to PFAS/BaP binary mixtures altered CYP-like activity *in vivo*; however, those alterations were not observed *in vitro*. Previous studies have indicated that mixtures of PFAS and PAHs have the potential to alter biological processes in fish (Dale et al., 2020; Horvli, 2020; Khan et al., 2019). For instance, Khan et al. (2019) demonstrated female Atlantic cod (*Gadus morhua*) exposed to mixtures of PFAS and PAHs *in vivo* had altered dopaminergic signaling, including the modulation of dopamine biosynthesis, catabolism, and its receptor expression. Notably, the CYP19A1B gene expression was significantly decreased in the fish exposed to both low and high PAH doses and combined exposure to low PAH/high PFAS. In addition, Dale et al. (2020) evaluated CYP1A gene and protein expression in Atlantic cod liver microsomes following intraperitoneal exposures to PFAS/PAH mixtures and observed a significant increase in CYP1A expression in the low PAH/high PFAS exposure group relative to the control groups. However, no significant changes in gene or protein expression were observed for any of the other groups in the study. Similarly, Horvli (2020) demonstrated that PFAS were able to induce activation of CYP1A1 on their own and produced apparent synergistic effects in combination with BaP through *in vitro/in vivo* exposures in Atlantic cod.

Our results from the *in vivo* approach indicate that exposure to PFOA alone does not alter the CYP1A1-like activity in trout, but PFOS may be capable of inducing minimal activity. Trout exposed to either PFOS or PFOA alone also demonstrated that CYP1A2-like activity was similar to basal levels in PFOA-exposed fish but that the activity was higher in PFOS-exposed individuals. However, these increases were much lower than the activity that resulted from exposures to BaP alone, an AhR-ligand that has been previously shown to significantly induce CYP1A1 and CYP1A2 in trout (Jönsson et al., 2004). In contrast, we observed significant decreases in CYP3A4-like activity for the PFAS-only treatment groups, suggesting that PFAS may be capable of inhibiting CYP3A4 in trout. Although in humans, these observations were also shown in (Franco et al., 2020), when PFAS effects on biotransformation, primarily CYP3A4 expression and activity, were explored using the HepaRG cell line.

Furthermore, our results indicate that exposure to PFAS-BaP binary mixtures altered CYP1A1, -1A2, and. -3A4 activity in the isolated trout liver microsomes. The activity associated with these CYP isoforms significantly decreased to similar levels to unexposed controls. Considering that the exposure to BaP

alone induced activity of two of these enzymes (CYP1A1/2), the decreases in activity following co-exposures may suggest that PFAS could impair the ability of CYP1A isoforms to function properly in exposure scenarios where biotransformation of PAHs may be necessary, leading to significant potential for PAH bioaccumulation. In addition, if the observed PFAS effects on the CYP isoforms evaluated in the present study are similar to other biotransformation pathways, aquatic organisms that require these pathways as protective mechanisms for other types of exposure (e.g., pharmaceuticals) (Connors et al., 2013) may experience impaired ecological stability if inhabiting ecosystems simultaneously contaminated with PFAS and other substances of emerging concern.

In terms of general toxicity, it has been suggested that PFOS is more toxic compared to PFOA in fish (Zheng et al., 2012) and that these effects may be attributed to the tendency of perfluorinated sulfonic acids (e.g., PFOS) to bioaccumulate to a greater extent compared to perfluorinated carboxylic acids (e.g., PFOA) (Liu et al., 2019). With the exception of the single-exposure CYP1A1-like activities, we found that, generally, PFOS and PFOA similarly altered CYP-like activities, implying that the effects we observed may not be directly associated with the functional groups and accumulation potential but rather the chain length. Several studies in fish have found that the carbon chain length may be a determinant of PFAS toxic effects (Chambers et al.; Conder et al., 2008; Gaballah et al., 2020; Hagenaars et al., 2011). In humans, Amstutz et al. (2022) demonstrated that CYP inhibition by PFAS decreases as the carbon chain length increases. Though this relationship has not yet been explored in fish, future research is needed to determine the involvement of altered CYP activity in the hepatotoxicity in fish exposed to PFAS of varying chain lengths.

Our findings using the *in vitro* approach, the RTL-W1 rainbow trout liver cell line, were not quite consistent with our findings *in vivo*. Although there is significant evidence that CYP1A expression is inducible in this cell line (Heinrich et al., 2014), it seems that RTL-W1 monolayers were not reflective of the observed effects *in vivo* in our study. However, it should be noted that RTL-W1 has enhanced hepatotypic functions and, subsequently, increased CYP1A expression and activity when cultured as spheroids (Lammel et al., 2019). Further studies utilizing RTL-W1 in alternative systems (e.g., spheroids) to assess CYP1A activity following exposure to PFAS-containing mixtures should be performed to gather further insight into potential PFAS effects in fish and to enhance the prediction of such alterations *in vivo*.

Declarations

Acknowledgments

The authors gratefully acknowledge Dr. Matteo Minghetti from Oklahoma State University for kindly providing the RTL-W1 cell line and Professor Niels C. Bols from the University of Waterloo for kindly allowing the use of this cell line in the present study.

Funding

This research was funded by the C. Gus Glasscock, Jr. Fund of Excellence in Environmental Sciences at Baylor University.

Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

Ethics approval/declarations

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material / Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability

Not applicable.

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Megan E. Solan, and Marco E. Franco. The first draft of the manuscript was written by Megan E. Solan and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

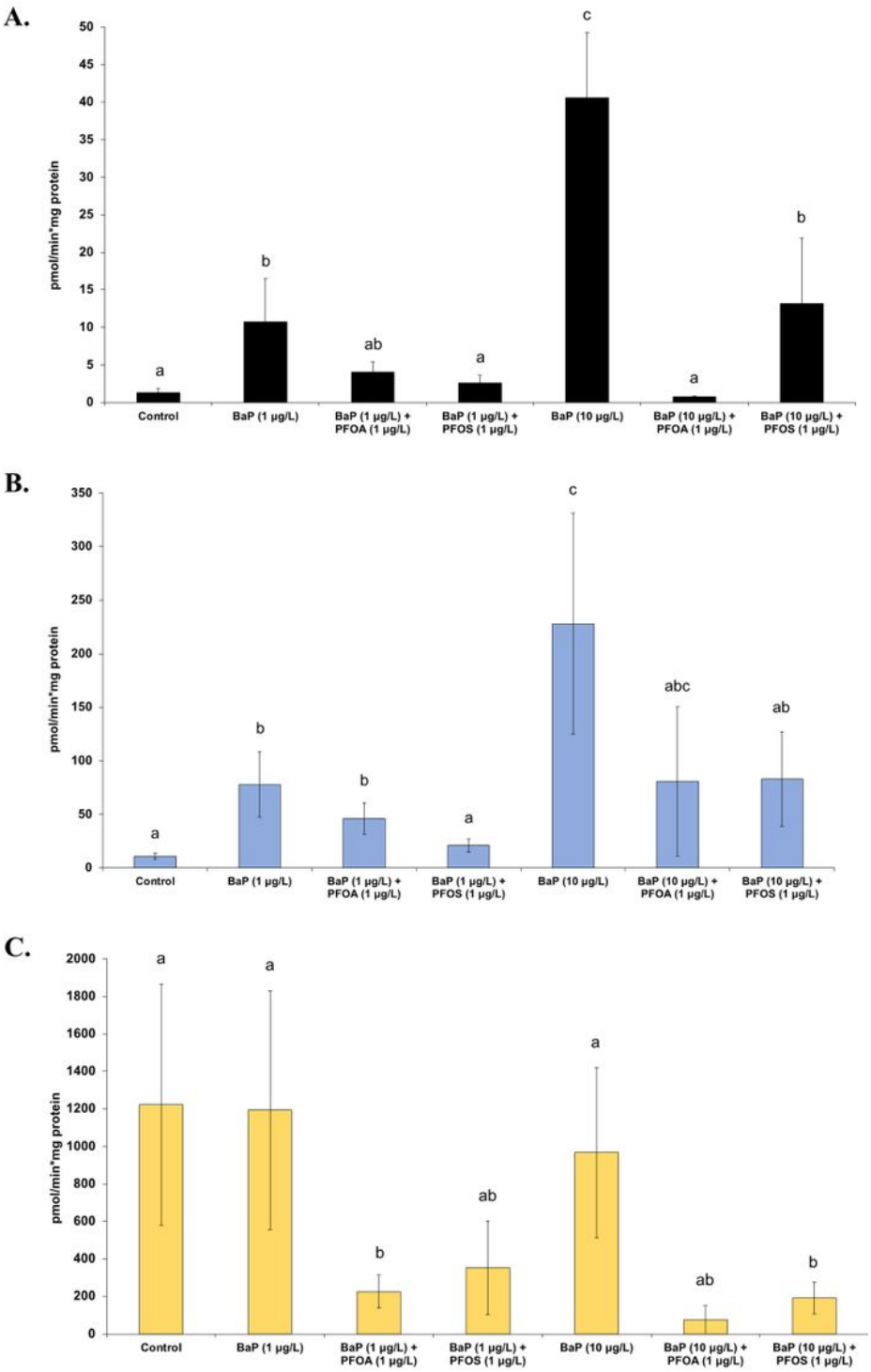

Figure 1

In vivo effects of co-exposures of BaP and PFOA, and PFOS on selected CYP-associated activities. CYP1A1-like (**A**), CYP1A2-like (B), and CYP3A4-like (**C**) activities are presented as average ± SD (n=4-8). Different letters denote significant differences between treatment groups ($p<0.05$; One-way ANOVA, Bonferroni's multiple groups comparison test).

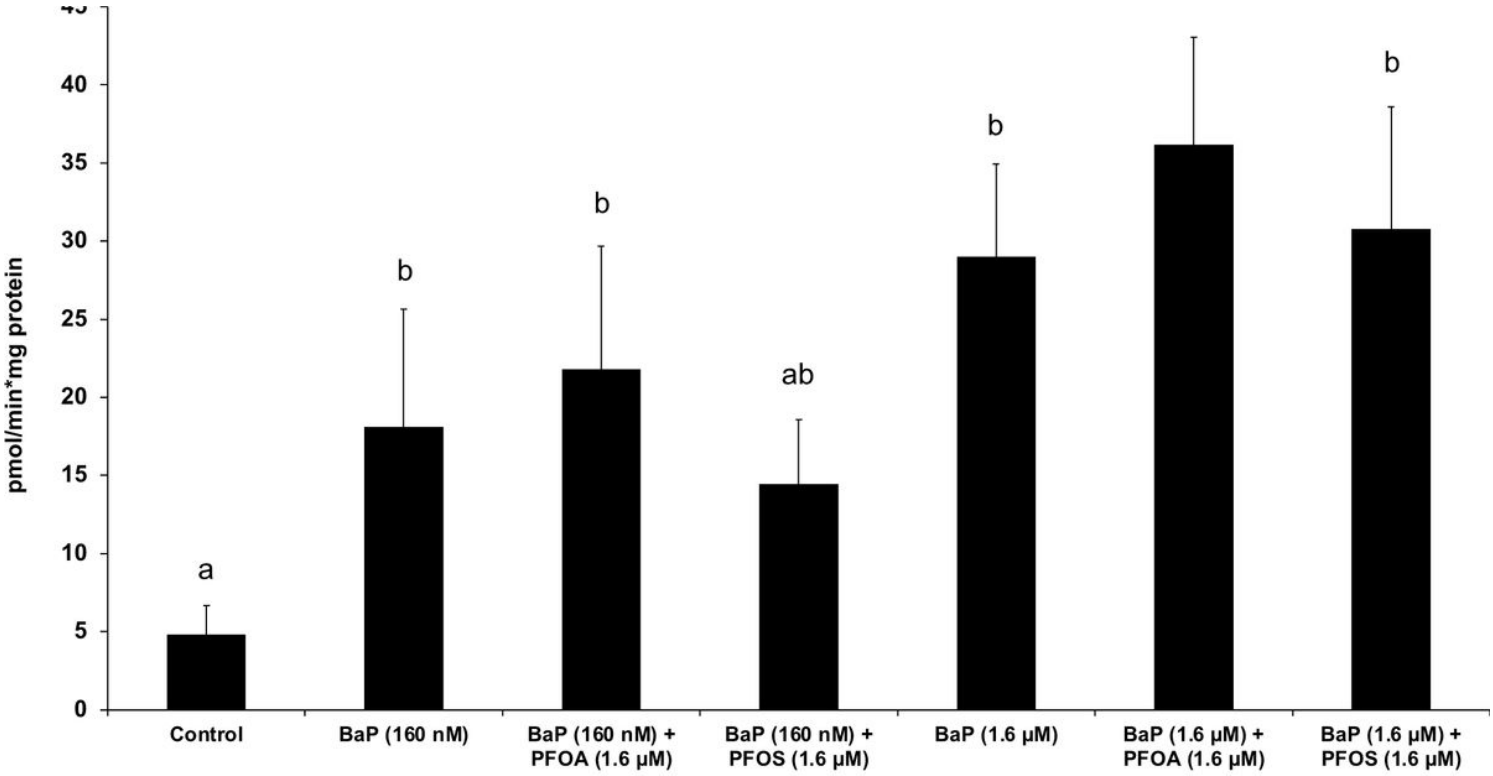

Figure 2

In vitro effects of co-exposures of BaP and PFOA and PFOS on CYP1A1-associated activity in RTL-W1 cells. The activities are presented as average ± SD (n=8-12). Different letters mean significant differences between groups ($p<0.05$; One-way ANOVA, Bonferroni's multiple groups comparison test).