Endocrine, biotransformation and oxidative stress responses in salmon hepatocytes exposed to chemically induced hypoxia and perfluorooctane sulfonamide (PFOSA), given singly or in combination

Marianne Olufsen and Augustine Arukwe*

Department of Biology, Norwegian University of Science and Technology (NTNU),
Høyskoleringen 5, 7491 Trondheim, Norway

***Corresponding author:** arukwe@bio.ntnu.no

Abstract. The effects of hypoxia and PFOSA, given singly and also in combination on endocrine, biotransformation and oxidative stress responses were investigated in primary culture of salmon hepatocytes. Hypoxia was induced chemically using cobalt chloride ($CoCl_2$) or deferroxamine (DFO). Primary culture of salmon hepatocytes were exposed to either $CoCl_2$ (150 μM) or DFO (100 μM), in the presence or absence of PFOSA at 0, 25 and 50 μM for 24 and 48 h. Changes in transcript levels were analysed by quantitative (real-time) PCR using gene-specific primers. CYP, catalase, GST and SOD activities were analyzed spectrophotometrically. The *hif-1α* mRNA was used to validate cellular hypoxic condition, showing significantly induced transcription after 48 h exposure to DFO and $CoCl_2$. Our data show that transcript levels for endocrine (*ERα, Vtg and Zrp*), biotransformation (*cyp*1a, *cyp*3a, *gst* and *udpgt*) and oxidative stress responses (catalase (*cat*), glutathione peroxidase *(gpx) and* glutathione reductase *(gr)*) were differentially modulated by PFOSA and hypoxia alone, and these effects were dependent on the response parameters and time of exposure. In combined exposure scenarios, the observed effects were apparently hypoxia-dependent. However, the observed effects at transcript levels were not concomitant with those at functional protein levels, further emphasizing the potential differences that may exist between these biological levels. Biplot of principal component analysis (PCA) showed grouping of response variables after 48 h of exposure. The distribution of observations and variables indicate that PFOSA had little effect on most response variables, while clustering show a unique association between a given hypoxia condition (i.e. $CoCl_2$ or DFO) in combination with PFOSA and transcripts, proteins or enzyme activities.

Keywords: Hypoxia, PFOSA, Endocrine system, Oxidative responses, Physiology, Biotransformation, Climate change

Introduction

Per- and polyfluoroalkyl substances (PFASs) have been shown to pose potential threat to aquatic biota. These compounds are considered relatively persistent to degradation (Cui et al. 2010), and the environmental implications of this is apparent when global monitoring shows relatively high levels of PFASs in biota and no decreasing trend in levels (Houde et al. 2011; Prevedouros et al. 2006) even though phasing out production started in 1997 (Giesy and Kannan 2001; Kannan et al. 2004). The toxicity of PFASs vary widely between isomers, and perfluorooctane sulfonic acid (PFOS), perfluorooctanoic acid (PFOA) and perfluorooctane sulfonamide (PFOSA) have been associated with endocrine alterations, oxidative stress and biotransformation effects in several species (Arukwe and Mortensen 2011; Cheng et al. 2012; Du et al. 2013; Hagenaars et al. 2008; Krovel et al. 2008; Slotkin et al. 2008; Wågbø et al. 2012).

Increases in biological productivity can produce drastic changes in the levels of dissolved oxygen in coastal waters and estuaries that are generally influenced by anthropogenic inputs of organic contaminants. Environmental hypoxia is a phenomenon characterized by the depletion of available dissolved oxygen and release of carbon dioxide (hypercapnia) into the water, which further produce a decrease in pH (Cochran and Burnett 1996). Thus, increased CO_2 (hypercapnia) in the aquatic environment affects the O_2/CO_2 balance in fish, and external hypercapnia is suggested to cause internal hypoxia (Michaelidis et al. 2007). Hypoxia-inducible factor-1α (*hif-1α*) is stabilized during cellular hypoxia, and heterodimerizes with *hif*-1β (or aryl hydrocarbon receptor nuclear translocator: *arnt*) to form the transcription factor - *hif*-1 that modulates the expression of several genes (Wenger 2002). The *arnt* is also the heterodimerization partner to the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor (Gu et al. 2000). The AhR-*arnt* complex activates mRNA transcription of genes containing XRE (xenobiotic responsive elements) in their upstream regions, including cytochrome P450s, gst and udpgt. Thus, *hif-1α* and AhR compete for arnt, and consequently, hypoxia has been shown to decrease the expression of cytochrome P450s (Khan et al. 2007; Zhang and Walker 2007), which are involved in steroidogenesis (both in metabolism and synthesis), xenobiotic biotransformation and affect the endocrine systems (Anzenbacher and Anzenbacherová 2001; Nebert and Gonzalez 1987).

The endocrine system is tightly regulated and is important for normal development and maintenance of optimal reproductive properties. Endocrine-disrupting chemicals (EDCs) are widely

distributed and thoroughly investigated, linking exposure to effect (Kang et al. 2007; Swedenborg et al. 2010; Tiemann 2008). In fish, induction of vitellogenin (Vtg) and zona radiata protein (Zr-proteins) gene transcription and protein levels through activation of estrogen receptor (ERα), are used as endocrine-disruptor biomarker responses (Arukwe et al. 1997). PFOS produce an antagonistic effect on androgen receptor (AR), and agonistic effects on the ER (Kjeldsen and Bonefeld-Jorgensen 2013), ultimately causing physiological changes that alter sex steroid hormone levels (Du et al. 2013; Jo et al. 2014; Liu et al. 2007). The relative importance of environmental hypoxia on organismal adaptive abilities to chemical insult is not well understood. Oxygen availability is essential for normal physiological function, and hypoxia has been associated with effects on hormonal and biotransformation systems (Shang et al. 2006; Wu 2009; Wu et al. 2003).

Activation of CYP enzymes is associated with chemicals that interact with nuclear receptor, namely the AhR and pregnane X receptor (*pxr*) (Gu et al. 2000). The cytochrome *P*450 (CYP) enzymes play a central role in the oxidative metabolism of a wide range of exogenous and endogenous compounds, many of which are not identified yet (Nelson et al. 1996). Glucuronidation and conjugation by uridine diphosphate glucuronosyltransferase (UDPGT) and glutathione *S*-transferase (GST) are major pathways for the inactivation and elimination of lipophilic compounds (Leaver et al. 1992). The expression of *cyp1a1*, *udpgt* and *gst* are regulated by the ligand-dependent AhR through which ligands produce altered gene expression and toxicity (Bradshaw et al. 2002; Nelson et al. 1996). Because of their role in the detoxification and activation of foreign compounds, alterations of hepatic phase I and -II enzymes is important from a toxicological standpoint (Williams et al. 1998). In mammalian systems, PFOSA is metabolically degraded to PFOS at a slow rate, but also undergoes enterohepatic circulation and mediate oxidative stress responses (Slotkin et al. 2008; Xu et al. 2004). Sustained hypoxia is known to cause oxidative stress, due to increased formation of ROS, produced by metabolism and the electron transport chain (Gnaiger et al. 1995; Mansfield et al. 2005; Nathan and Cunningham-Bussel 2013).

In the present study, our aim was to investigate the interactive effects of hypoxia and PFOSA on endocrine and biotransformation processes, as well as oxidative stress responses. Exposure to hypoxia was achieved using cobalt chloride ($CoCl_2$) and deferoxamine mesylate (DFO), two chemicals that has been widely used in animal experiments for this purpose, and confirmed by their ability to increase *hif*-1α and vascular endothelial growth factor (VEGF)

expression levels (Guo et al. 2006; Liu et al. 1999; Miller 1989). Our hypothesis is that exposure of salmon hepatocytes to $CoCl_2$ and DFO will produce cellular hypoxia, and that interaction between hypoxia and PFOSA will affect AhR and ER signalling, leading to transcriptional changes in downstream gene responses in phase I and II pathways, the endocrine system and oxidative stress responses.

Materials and methods

Chemicals and reagents

Highly pure (>98%) linear perfluorooctane sulfonamide (PFOSA; $CF_3(CF_2)_7SO_2NH_2$) isomer was purchased from Wellington Laboratories (Guelph, ON, Canada). iScript cDNA Synthesis Kit and iTaq SYBR Green Supermix with ROX were supplied by BioRad Laboratories (Hercules, CA, USA). The original TA Cloning Kit PCR 2.1 vector, INVαF' cells, Trizol and Dulbecco's Modified Eagle Medium (DMEM) with non-essential amino acid and without phenol red, fetal bovine serum (FBS), 0.4% trypan blue and L-glutamine were purchased from Gibco-Invitrogen Life Technologies (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO), penicillin-streptomycin-neomycin solution, collagenase (C0130-1G), bovine serum albumin (BSA), N-[2-Hydroxyethyl]piperazine-N'-[2-Ethane Sulfonic Acid] (HEPES), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), ethyleneglycol bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), polyunsaturated fatty acid 1 and 2 (PUFA1 and PUFA2) were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). Tricaine methane sulphonate (MS-222) was purchased from Norsk Medisinal depot AS (Oslo Norway). GelRed Nucleic Acid Gel Stain was purchased from Biothium (Hayward, CA, USA). The ZR Plasmid Miniprep Classic was purchased from Zymo Research (Orange, CA, USA).

Animals, exposure and sampling

Atlantic salmon (*Salmo salar*) were purchased from Lundamo Hatcheries (hatch and rearing centre located at Lundamo). Fish were kept at the animal-holding facilities for Department of Biology (Sealab, NTNU) in 100-liter tanks with continuously running fresh water at 10 ^{o}C and flow rate of 40 L/h and natural photoperiod. Fish were acclimatized for two weeks and starved three days prior to liver perfusion.

Collagenase perfusion, isolation and culture of hepatocytes

Prior to liver perfusion, all glassware and instruments were autoclaved and solutions were filtration sterilized by using 0.22 μm Millipore filter (Millipore AS, Oslo, Norway). Fish were deeply anesthetised using MS-222 (70 g/L) administered 15 minutes prior to perfusion and euthanized after in accordance with regulations for animal research and approved by Norwegian Food Safety Authority (FOTS). Hepatocytes were isolated by a two-step perfusion technique with modifications as previously described (Mortensen and Arukwe 2007). The cell suspension was filtered through a 150 μM nylon monofilament filter and centrifuged at 70 x g for 5 min. Cells were washed three times with serum-containing medium and finally resuspended in complete medium. Following collagenase perfusion and isolation of hepatocytes, viability of cells was determined by the trypan blue exclusion method. A cell viability value of >90% was a criterion for further use of the cells. Cells were plated on 35 mm TPP Tissue Culture Plates (Techno Plastic Products AG, Switzerland) at monolayer density of $2.1x10^6$ cells in 3 ml DMEM medium (without phenol red) containing 0.5% (v/v) FBS, 1% (v/v) L-glutamine, 15 mM HEPES and 1% (v/v) antibiotic-antimycotic.

Plating of cells and exposure

Medium was added to plate prior to the cells, avoiding sedimentation of cells by rotating the tube every second plate. Cells were cultured at 10^oC in a sterile incubator for 24 h prior to exposure. After 24 h pre-culture, growth medium was removed and quickly replaced with exposure medium (n=20); 0.1% DMSO (control), 150 μM $CoCl_2$ or 100 μM DFO in combination with PFOSA (0, 25 and 50 μM). This gave a total of 9 exposure groups. Media and cells were harvested separately, ten wells for each exposure group after 24 and 48 h exposure time, and snap-frozen immediately in liquid nitrogen. Cells for RNA analysis were lysed in Trizol reagent for total RNA isolation according to the manufacturer's protocol (Invitrogen).

Assessment of cell viability

Resazurin assay was performed to detect viability in hepatocyte cultures exposed to different concentration (10, 50, 100, 150 and 200 μM) of $CoCl_2$ or DFO. Cells were exposed for 24 and 48 h in 96-well plates ($2.1x10^5$ cells in 300 μl). After addition of rezasurin solution (10% of medium volume), cells were incubated for 6 h at 10 ^oC on a gyratory shaker. Samples were measured

spectrophotometrically at 600 nm every 20 minutes, and 4 h was considered optimal incubation time. After selecting $CoCl_2$ (150 μM) and DFO (100 μM) concentration, viability was performed the exposure regimes described above.

Quantitative (real-time) PCR

Hepatocytes were thawed on ice and homogenized and total RNA was isolated using the protocol for Trizol reagent (Gibco-Invitrogen Life Technologies). Total cDNA for quantitative real-time polymerase chain reaction (q-PCR) analysis was generated from 1 μg total RNA from all samples using a combination of poly-T and random primers from iScript cDNA synthesis kit as described by the manufacturer (Bio-rad). Quantitative real-time PCR was used for evaluating gene expression profiles for HIF-1α, ERα, Vtg, Zrp, cyp1a, cyp3a, pxr, gst, udpgt, CAT, GPx and GR. For each treatment, expression of individual gene targets was analyzed using the Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA, USA). Each 25 μl qPCR reaction contained - 12.5 μl of iTAQ TM SYBR Green Supermix with ROX (Bio-Rad), 1 μl of cDNA (not applied in negative control), 200 nM of each forward and reverse primers and remaining volume was autoclaved MQ-H_2O. The three-step real-time PCR program included an enzyme activation step at 95^{o}C (5 min) and 40 cycles of 95^{o}C (30 s), 55–65^{o}C (15 s) (depending on the primers used; see Table 1), and 72^{o}C (15 s). Controls lacking a cDNA template were included to determine the specificity of target cDNA amplification. Cycle threshold (Ct) values obtained were converted into mRNA copy number using standard plots of Ct-value versus log copy number. The criterion for using the standard curve is based on equal amplification efficiency (usually 90%) with unknown samples and this is checked prior to extrapolating unknown samples to the standard curve. The standard plots were generated for each target sequence using known amounts of plasmid containing the amplicon of interest, as described previously by (Arukwe 2006). Data from each exposure group were averaged and expressed as percentage of control (i.e. minus hypoxia and PFOSA).

Biochemical analysis

Cells for biochemical analysis were washed in 1 ml of 0.1 M sodium phosphate buffer (0.15 M KCl, 1 mM EDTA, 1mM DTT and 10% glycerol at pH 7.4), then homogenized in 150 μl of buffer using a Potter-Elvehjelm Teflon-glass homogenizer (Glas-Col®, Terre Haute, USA). Homogenate

was centrifuged at 12,000 x g for 30 s at 4°C, pellet was discarded and 10 µl supernatant was added to a well in 96-well black fluorometric (blank bottom) plate containing 140 µl NADPH (mg/ml) and incubated for 10 min at 37°C. Total cellular protein content was determined by the method of Bradford (Bradford 1976), using a BSA standard. The ethoxyresorufin- and methoxyresorufin O-deethylase activities were measured using ethoxyresorufin or methoxyresorufin dissolved in DMSO as substrates. The reaction was started by adding 1 µl of 250 µM substrate and fluorescence was measured for 20 min (excitation 535 nm; emission 590 nm).

Glutathione S-transferase (GST) activity in post-mitochondrial supernatant (PMS) samples was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate as described by Habig et al (1974). PMS were prepared from hepatocytes by centrifugation as previously described (Pesonen and Andersson 1987).

Catalase and superoxide dismutase (SOD) activities were measured in PMS fractions as described in kit protocol provided by the producer (Arbor Assays). Optical density (absorbance) was read at 450 nm to detect SOD, and fluorescence was read (emission 585±5 nm: excitation 575±5 nm). Absorbance and fluorescence values were converted to enzyme activity by a standard curve for either SOD or catalase, and data were normalized against total protein content in each sample and expressed as (U/ml) / mg protein. All biochemical assays were run with a negative control (two parallels) containing the sample solvent specific for each assay.

Immunochemical protein detection

Semi-quantitative vitellogenin (Vtg) was essentially performed as described previously (Arukwe et al. 1997) using polyclonal rabbit anti-salmon Vtg antibodiy, diluted 1:3000. 50 µl supernatant was applied to each 96-well ELISA plate. Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (GAR-HRP, Bio-Rad) diluted 1:3000 and the substrate H_2O_2/*o*-phenylenediamine dihydrochloride (OPD) were used for detection of product at 492 nm using a Synergy HT microplate reader from Bio-Tek Instruments, Inc (Winnoski, VT).

Statistics

Data are presented as mean percentage of control (i.e. minus hypoxia and PFOSA) within the same exposure duration/time ± standard error of mean (SEM). Normal distribution was assessed using

Shapiro-Wilks test and homogeneity of variance was tested with Levene's test. Comparison of different concentrations of PFOSA treatment, singly or in combination with $CoCl_2$ or DFO, groups and control group was done using One-way ANOVA with post-hoc (Tukey) using SPSS. We used Simca-P 12 to perform multivariate analysis making principal component analysis (PCA) plots. All observations and variables were used to produce PCA-plots. Observations (exposure groups) must be independent when investigated applying PCA, so our data was separated based on exposure duration (24 and 48 h). PCA-plots presented herein were produced by first component (PC1) and second component (PC2), and percent of variation (R2X) is shown for each plot. Linear correlation between variables clustered in PCA-plot was analyzed using Pearson r-test, and correlation value is presented as p-value in text.

Results

Cell viability and experimental validation

Exposure to DFO or $CoCl_2$ concentration above 100 and 150 μM respectively, reduced cell viability noticeably in salmon primary hepatocytes (Data not shown). Cell viability in the different exposure scenarios was comparable between treatments and satisfactory for further analysis showing a survival rate above 65% (data not shown). The *hif-1α* expression was used as a validator for cellular hypoxic response in hepatocytes exposed to DFO and $CoCl_2$, both singly and in combination with PFOSA after 48 h (Olufsen et al. 2014).

Oxidative stress responses

Oxidative stress responses were generally not affected after 24 h exposure, while consistent increases were observed at 48 h after exposure hypoxic condition alone and in combination with PFOSA and these effects were mostly significant with DFO exposure (Fig. 1 and 2). Overall, exposure to PFOSA alone (25 and 50 μM) produced non-significant increases in *cat*, *gpx* and *gr* mRNA levels at 24 and 48 h, although an apparent, but non-significant time-related increases were observed for *cat* and *gpx* (Fig. 1). Exposure to PFOSA concentrations alone did not affect *cat* transcripts, and hypoxic conditions (i.e. $CoCl_2$ and DFO) increased *cat* mRNA, whose effects were sustained in combined exposure with PFOSA concentrations (Fig. 1A). For *gpx,* while $CoCl_2$ alone did not produce significant changes *gpx* mRNA, combined exposure with PFOSA concentrations

produced significant increases at 48 h. On the other hand, DFO exposure alone produced significant increase of *gpx* mRNA at 48 h, and this effect was sustained in the presence of PFOSA concentrations, albeit slightly decreased compared with DFO exposure alone (Fig. 1B). Hepatocytes exposed to $CoCl_2$ showed a slight increase of GR expression at 48 h, and the presence of PFOSA concentrations elevated this effect (albeit not significant), but more so for 25 μM PFOSA, compared to 50 μM (Fig. 1C). Exposure to DFO alone or in combination with PFOSA concentrations produced a non-significant increase of *gr* mRNA expression after 48 h (Fig. 1C). Due to small sample size, enzyme activity was only measured for of catalase, showing a general increase at 48 h, both with PFOSA concentrations alone and in combination with hypoxic conditions, significantly so in $CoCl_2$ exposure alone and in combination with 25 μM PFOSA (Fig. 2). $CoCl_2$ alone and in combination with PFOSA increased catalase activity significantly (Fig. 2), while DFO did not affect enzyme activity.

Biotransformation responses

The *cyp1a, cyp3a and pxr* transcripts were not significantly affected by PFOSA exposure either 24 h or 48 h after exposure. However, *cyp1a* was increased, albeit not significant, by $CoCl_2$ exposure alone at 24 h and the presence of PFOSA concentrations decreased this effect at the same exposure time (Fig. 3A). At 48 h, $CoCl_2$ non-significantly reduced *cyp1a* expression compared with 24 h, and the combined exposure with PFOSA increased this effect (significant only at combined exposure with 25 μM PFOSA). On the other hand, DFO significantly increased *cyp1a* mRNA at 48 h only, but not effects was observed when DFO was given in combination with PFOSA concentrations at 48 h (Fig. 3A). For *cyp3a,* a significant increase was observed by hypoxic conditions, with and without PFOSA concentrations at 48 h (Fig. 3B). Hypoxia (i.e. $CoCl_2$ and DFO) produced non-significant increase of *pxr* expression at both 24 and 48 h, while combined exposure with PFOSA concentrations sustained these increases of *pxr* expression at 48 h and reduced it at 24 h (albeit not significant: Fig. 3C). EROD activity was not affected at 24 h exposure to either PFOSA concentrations or hypoxic conditions (i.e. $CoCl_2$ or DFO; Fig. 4). There is a general decreased in EROD activity at 48 h in all exposure conditions, compared to 24 h, except at combined 50 μM PFOSA and DFO, where no effect was observed (Fig. 4A). MROD activity was not altered by exposure to either hypoxia or PFOSA alone, while combined $CoCl_2$ and PFOSA concentrations

significantly increased MROD activity at 24 h, and these effects were reduced at 48 h of exposure (Fig. 4B).

The *gst* and *udpgt* mRNA expressions were non-significantly increased by PFOSA concentrations at 48 h exposure, compared to control or 24 h exposure (Fig. 5A and B, respectively). Exposure of cells to $CoCl_2$ or DFO produced increases of *gst* mRNA expression at 48 h and these effects were significantly maintained in the presence of PFOSA concentrations at the same time interval (Fig. 5A). For *udpgt,* $CoCl_2$ produced an increase in *udpgt* at 48 h of exposure, and the effect was sustained in the presence of PFOSA concentrations at the same time interval. DFO on the other hand produced a significant increase of *udpgt* expression at 48h, and this effect as maintained by PFOSA in an apparent concentration dependent manner (Fig. 5B). Exposure to PFOSA alone produced non-significant increase of GST activity at 48 h of exposure (Fig. 5C). GST enzyme activity was increase by $CoCl_2$ alone, whose effect was further increased in the presence of PFOSA in an apparent concentration dependent manner (Fig. 5C). Exposure of cells to DFO alone did not produce any effects at 24 h, but combined exposure with 50 μM PFOSA resulted to a significant elevation of GST activity at 24 h (Fig. 5C). At 48h, DFO exposure alone produced a significant increase of GST activity, and the presence of PFOSA concentrations further elevated this effect, more so for 25 μM PFOSA (albeit not significant, Fig. 5C).

Endocrine responses

Alteration of gene transcription occurred only at 48 h of exposure (Fig. 6). PFOSA exposure alone and in combination with hypoxia (i.e. $CoCl_2$ and DFO) produced a concentration dependent increase of *ERα* mRNA expression at 48 h (Fig. 6A). $CoCl_2$ exposure alone did not produce a significant increase of *ERα*. For DFO, no time related changes in *ERα* was observed and the presence of PFOSA produced an apparent concentration dependent increase of *ERα* at 48 h (Fig. 6A). For *Vtg* and *Zrp* transcripts, exposure to PFOSA alone produced minor, but non-significant changes at both 24 and 48 h, compared to control (Fig. 6B and C, respectively). $CoCl_2$ exposure alone produced increases of *Vtg* and *Zrp*, and the presence of PFOSA further increased the *Vtg* and *Zrp* transcript levels. DFO exposure alone produced a significant increase of *Vtg* and *Zrp* expression at 48 h and the presence of PFOSA maintained these effects at the same time interval (Fig. 6B and C, respectively). Cellular levels of Vtg protein levels showed that 50 μM PFOSA produced a non-

significant increase of Vtg protein levels at 24 and 48 h of exposure (Fig. 7). On the hand, exposure to CoC2 and DFO alone, significantly increased Vtg protein levels at 24 h exposure, and these effects were reduced in the presence of PFOSA concentrations, at the same time interval (24h) and no effects were observed at 48 h of exposure (Fig. 7).

Multivariate analysis

Multivariate principal component analysis (PCA) after 24 h exposure did not show grouping of any variables or observations, which were located closely around neutral point [0.0]. Distribution of PCA in biplot after 48 h exposure is presented in Figure 8, showing a unique grouping of both observations and variables. Most gene transcripts are clustered and well explained by PC1, with the exception of *ERα* and *gr* that are not well explained by the plot and located close to [0.0]. Vtg and Zr-protein were located relatively close to EROD and MROD, suggesting a possible interaction between CYP activity and endocrine responses, but the grouping may also be coincidental due to their close proximity to [0.0]. CAT, SOD and GST enzyme activities clustered together and were explained by PC2. Analysis of correlation between variables was performed and will be presented below. There is a positive correlation between GST and CAT activity ($p<0.01$) and between gene trascripts involved in oxidative stress (*gst*, *gpx*, *hif-1α*, *cat:* $p<0.01$). There was no significant correlation between enzyme activity and transcripts for oxidative stress parameters.

Transcriptional changes of *hif-1α, cyp1a*, *cyp3a*, *gst* and *udpgt* are positively correlated ($p<0.01$). EROD, MROD, Vtg and Zr-protein are grouped in the PCA (Fig. 8), but data are not well explained by the model. Transcription of *Zrp*, *Vtg* and *hif-1α* are positively correlated ($p<0.01$). Transcripts of *cyp3a*, *Zrp* and *Vtg* clustered very closely and Pearson's test show a positive correlation ($p<0.01$). *ERα* is not located with the other gene transcripts in PCA and no significant correlation was observed.

Discussion

Hypoxic conditions in the aquatic environment reflect the level of dissolved oxygen and may arise from several sources. There is a growing concern about the effects of global warming leading to reduction in oxygen content in several aquatic environments (Obenour et al. 2012; Scavia et al. 2013; Thomas and Rahman 2012). Previously, the alteration of biotransformation and hormonal

systems by hypoxia has been reported in both in vivo and in vitro studies (du Souich and Fradette 2011; Shang et al. 2006; Wu et al. 2003). In addition, hypoxia has been shown to produce cellular oxidative stress responses in several fish species (Clotfelter et al. 2013; Gnaiger et al. 1995; Mansfield et al. 2005; Olsvik et al. 2013). In the present study, our data show that transcript levels for endocrine (*ERα, Vtg and Zrp*), biotransformation (*cyp*1a, *cyp*3a, *gst* and *udpgt*) and oxidative stress (*cat, gpx and gr*) responses were differentially modulated by PFOSA and hypoxia alone or in combination, and these effects were mostly hypoxia-dependent and affected by exposure duration.

Oxidative stress responses

The formation of ROS during hypoxic conditions is extensively studied (Blokhina et al. 2003; Clotfelter et al. 2013; Cooper et al. 2002; Fan et al. 2008; Kotake-Nara and Saida 2007), but less is known about the interaction between hypoxia and exposure to environmental contaminants. Previously, *in vitro* studies have shown that $CoCl_2$ can induce CAT and trigger neurite outgrowth, a process regulated by hypoxia (Kotake-Nara and Saida 2007) and associated with increased hepatic glutathione levels (Chengelis 1988). On the other hand, hypoxia decreased the level of antioxidant enzymes compared to normoxia in CD34+ cells *ex vivo* (Fan et al. 2008). Herein, we showed that hypoxia increased oxidative stress responses in salmon hepatocytes. In accordance with the present study, previous studies have reported increased oxidative stress response after exposure to PFASs (Arukwe and Mortensen 2011; Slotkin et al. 2008, Wågbø et al. 2012; Xu et al. 2004), most likely caused by xenobiotic metabolism and/or increased peroxisomal proliferation (Olufsen et al. 2014; Wågbø et al. 2012).

The toxicological potential of ROS is dependent on levels exceeding the scavenging capacity of antioxidant machinery (Livingstone 2001). Cytoprotective enzymes such as CAT, SOD, GPx, GR, GST and UDPGT are encoded through the antioxidant responsive element (ARE), forming a network of protective machinery against oxidative stress (Giuliani et al. 2013; Osburn and Kensler 2008; Regoli and Giuliani 2014). Changes in the antioxidant responses are generally difficult to predict, since induction, inhibition, biphasic or temporary changes, experimental conditions, intensity and duration of exposure, species, tissues, metabolic status are all confounding factors that may affect the observed response (Giuliani et al. 2013; Regoli and Giuliani 2014; Regoli et al. 2014). In the present study, we showed that CAT activity was increased by $CoCl_2$

alone and in combination with PFOSA, in the same way as *GPx* and *GR* mRNA was increased by combined exposure. It is possible that the hypoxic conditions increased H_2O_2 formation, requiring CAT for cellular protection. Overall, data from the present study suggest that oxidative stress responses observed in combined exposure scenarios were mainly due to a hypoxic response. These finding are in accordance with previous studies showing the modulation of hypoxic and physiological responses in fish (Lushchak and Bagnyukova 2006, 2007; Lushchak et al. 2005; Tripathi et al. 2013). Exposure of *Clarias batrachus* to experimental hypoxia (0.98 ± 0.1 mg/L dissolved oxygen: DO) resulted in the development of oxidative stress and physiological adaptation to enhance oxygen transport capacity (Tripathi et al. 2013).

Biotransformation responses

Xenobiotic biotransformation is an important mechanism for organisms to handle chemical stress through detoxification and excretion of compounds with toxic potential. Biotransformation of xenobiotics in fish liver is generally associated with the CYP1, 2 and 3 families (Monostory et al. 1996). PFOSA is metabolized in fish liver producing PFOS, most likely by CYP activity (Tomy et al. 2003), and marine medaka embryos exposed to PFOS showed a time-dependent induction of *arnt* and *cyp1a* transcription (Fang et al. 2012). Herein, we observed time and exposure related changes of *cyp1a, cyp3a and pxr* mRNA, albeit PFOSA alone generally did not produce any change. Recent studies showed that AhR activation and *cyp1a1* expression induced by PCB126 were significantly inhibited by hypoxia, suggesting a possible important role of hypoxia in xenobiotic metabolism (Vorrink et al. 2014). Furthermore, biological processes regulated by *hif*-1α were negatively affected by PCB126, potentially affecting adaptive responses and cell survival in hypoxic environments (Vorrink et al. 2014). In the present study, gene expression was increased after 48h while enzyme activities were affected after 24 h, showing time-related difference between mRNA and enzyme activity. Elsewhere, exposure to hypoxia (dissolved oxygen, DO: 1.7 mg/L for 2 to 4 weeks) produced significant decreases in hepatic *cyp1a* mRNA and protein levels (du Souich and Fradette 2011; Rahman and Thomas 2012). Some studies have suggested post-transcriptional inhibition of CYP1A activity (Fradette et al. 2007). It should be acknowledged that gene expression analysis may not directly be translated into enzyme activity. For example, gene expression analysis of several *cyp3a* isoforms exposed to hypoxic conditions showed an increase of *cyp3a* expression

independent of *pxr* modulation (Fradette and du Souich 2003). This is in accordance with our data showing that significant increase in *cyp3a* independent of non-significant increases in *pxr* transcription after hypoxia exposure, singly and in combination with PFOSA.

We have previously reported the induction of *hif-1α* by $CoCl_2$ and DFO using the same experimental materials as in the present report (Olufsen et al. 2014). Cells adapt to reduction in oxygen levels by shifting energy production from mitochondrial fatty acid β-oxidation to glycolysis during periods of cellular hypoxia (Liu et al. 2014). Elsewhere, *in vivo* mammalian studies using rabbit, and rodent *in vitro* hepatocyte studies reported decreases in *cyp1a* and *cyp*1a2 mRNA and protein expression by hypoxia, whose effects are mediated through HIF-1α, cellular cytokines and ROS (Fradette et al. 2007; Fradette and du Souich 2004). While these studies may show some discrepancies with our findings, there are direct relevance of the effects of hypoxia observed in the present study showing persistent increase of *cyp1a, cyp3a* and *pxr* mRNA. The modulation of xenobiotic-mediated alterations in *cyp1a* and *cyp3a* expression combined with hypoxia is not surprising, given that *hif-1α* response to hypoxia and the AhR-*cyp1a* response to xenobiotics are both mediated through arnt. Our findings and other studies showing decreases of CYPs suggest potential interaction sites between these two signalling pathways (Bugel et al. 2010; Fleming et al. 2009).

GST mRNA and enzyme activity induction is higher in combined exposure regimes compared to single exposure treatments. GST is known to have multifunctional properties, acting as part of the antioxidant system in removal of secondary oxidation products and H_2O_2 and in xenobiotic metabolism (Hayes and McLellan 1999; Livingstone 2001; Wallace 1989). Our data show that hypoxia increased oxidative stress responses more than PFOSA and it is possible that increased ROS formation is a results of hypoxic stress, rather than increased xenobiotic biotransformation (Blokhina et al. 2003). Hypoxia also increased transcription of *cat* and *gpx,* suggesting an increased H_2O_2 level and supporting hypoxia altered metabolic modifications to produce ROS. The effects of $CoCl_2$ at transcription and enzyme levels paralleled each other, while effects of DFO were evident only at transcriptional level. Interestingly, our data show that GST and CAT activity are correlated, supports the link between GST induction and ROS formation and the relevance of exposure treatments to activity levels. In a different report, we showed changes in lipid peroxidation in hepatocytes exposed to combined hypoxia and PFOSA (Olufsen et al. 2014),

supporting the role of GST in the adaptation to secondary oxidation products due to interaction between ROS and cellular components (Livingstone 2001; Storey 1996; Wallace 1989).

Furthermore, we observed that exposure of hepatocytes to hypoxia, produced elevated effects on *udpgt* transcription that paralleled *gst* transcripts. There is no other studies known to us that have studied the effects of hypoxia on *udpgt* mRNA in any fish species or lower vertebrate. The *udpgt* is multigene enzyme family that plays significant roles in the excretion of both endogenous and xenobiotic compounds (Clarke et al. 1992), in addition to cytoprotective roles (Regoli and Giuliani 2014). In fish, several *udpgt* gene isoforms have been described, with prototypical substrates such as bilirubin, testosterone, and phenolic xenobiotic (Clarke et al. 1992). Therefore, the possible formation of glucuronides in hepatocytes exposed to hypoxia may provide a physiological means for controlling reduction in oxygen levels through their excretion and removal (George and Taylor 2002) or cellular transport in a receptor-inactive form.

Effects on hormonal responses

Suspicions that PFASs affects the endocrine system initiated the MAMA study, showing that serum levels of PFOSA and MePFOSA were positively correlated with human serum and milk estradiol (E2) levels, while perfluorononanoic acid (PFNA) (a 9-carbon perfluoralkyl acid (PFAA)) was negatively associated with milk E2 (von Ehrenstein et al. 2009; White et al. 2011). Investigations of endocrine responses in fish exposed to PFOS have produced ambiguous results, showing both increase (Cheng et al. 2012) and decrease (Hagenaars et al. 2008) of Vtg levels. Several PFASs, including PFOS and PFOA, have previously been shown to exert a weak estrogenic effect in juvenile rainbow trout (Benninghoff et al. 2011) and there are suggestions that these effects are mediated through ERα and androgen receptor (AR) (Kjeldsen and Bonefeld-Jorgensen 2013). Our data show that exposure to PFOSA alone triggered an increase of *ERα* transcription, which according to other studies may be directly through interaction with ERα or induction that could be triggered indirectly through alteration of CYP activity leading to a change in steroid hormone composition. Interestingly though, we observed that hypoxia was the main regulator of estrogenic responses related to ERα activation. Hormonal status of fish is strongly dependent on the physical parameters in their surroundings (Arukwe and Goksoyr 2003), and hypoxic conditions can ultimately affect hormonal status in fish through interference with steroidogenic enzymes (Cheek et al. 2009).

Changes in ambient oxygen levels are considered a stressor in fish (Cech and Crocker 2002; Fivelstad et al. 1999), and stress is generally associated with a decrease in both sex steroid hormones and impaired reproductive responses (Clearwater and Pankhurst 1997; Haddy and Pankhurst 1999; Schreck et al. 2001; Wu 2009). The response of fish endocrine systems to stressors may be biphasic (Schreck 2010) and stress has been reported to accelerate reproduction processes in fish, apparently dependent on the fish species, maturational stage and the severity of the stress and stressor (Schreck et al. 2001). In this study, a relationship between the endocrine, oxidative and biotransformation responses due to hypoxia exposure was confirmed by PCA plot showing that most gene transcripts are clustered and well explained by the model, with the exception of *ERα* and *gr*.

A direct comparison between the aforementioned hypoxia studies and the present study is inevitable to make, as cellular and systemic hypoxia was chemically induced using $CoCl_2$ and DFO in the present study. The induction of hypoxia is supported by the fact that *hif-1α* expression was observed in $CoCl_2$ and DFO exposed hepatocytes at 48 h of exposure, either singly or in combination with PFOSA (Olufsen et al. 2014). However, the time-dependent effects that occurred mostly at 48 h and long lag-time from hypoxia exposure until *hif-1α* transcriptional response suggest a possible secondary rather than primary response to altered cellular oxygen saturation reflecting the mechanisms of $CoCl_2$ and DFO induction of hypoxic condition. Regardless, the exact impact of internal hypoxia on *hif-1α* transcription compared to regulation at the protein levels is not well understood (Dery et al. 2005; Rimoldi et al. 2012; Rissanen et al. 2006; Soitamo et al. 2001; Terova et al. 2008). Nevertheless, there is incoherence between mRNA expression and protein levels for Vtg and Zr-protein in the present study, representing the potential shortcomings of using *in vitro* systems in investigating hormonal systems, since the mechanisms are very complex and often orchestrated by several organs. Furthermore, the endocrine systems are regulated through feedback mechanisms to prevent estrogenic responses during suboptimal physiological conditions (Pankhurst and Munday 2011; Schreck et al. 2001).

Broader implication of climate change

It should be acknowledged that the present study was performed using cultured salmon hepatocytes and does not directly reflect effects on a whole organism due to the absence of the complete metabolic machinery in an intact organism. But, it does provide some mechanistic overview of the

potential effects of climate change on the endocrine, oxidative and biotransformation pathways of organisms, thereby contributing to the growing burden of evidence indicating that teleosts – despite their superior acid-base regulatory capacity – are more susceptible towards hypoxia than previously assumed (Ishimatsu et al. 2005; Munday et al. 2012). PFOSA exposure produced minimal estrogenic, oxidative and biotransformation effects, as most of the observed effects in the present study were hypoxia dependent. Although, an altered or untimely induction of genes involved in the gametogenesis or overt physiology might cause both excessive energy costs and, in worst case scenario, reproductive or developmental failure (Arukwe and Goksoyr 2003), such consequences cannot be predicted solely based on low- or high abundance mRNA measurements reported in the present study. However, exposure to combined hypoxia and PFOSA provided some indications of interactive effects at the level of gene transcription. Regardless, these findings should evoke a general concern for possible combined effects of near-future climate change scenarios and various emerging contaminants present in the aquatic environments. Given that hypoxia does, in fact, possess the potential for affecting oxidative, biotransformation, physiological and reproductive functions in fish, as suggested in the present study, the interactive physiological and endocrine disruptive effects of the numerous emerging contaminants in the environment and climate change require further and integrated investigations.

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Figure Captions

Figure 1. Transcriptional changes of *cat* (A), *gpx* (B) and *gr* (C) in salmon hepatocytes exposed to $CoCl_2$ (150 μM) or DFO (100 μM), given singly and in combination with PFOSA (0 (control), 25 and 50 μM). Cells were harvested after 24 and 48 h and analyzed by qPCR. Values are presented as mean (n=5) percent of control ± SEM. Significant difference between control and exposure groups at the different time intervals were analyzed by Tukey's test ($p<0.05$), and denoted with asteriks (*).

Figure 2. Alterations in CAT activity in salmon hepatocytes exposed to $CoCl_2$ (150 μM) or DFO (100 μM), given singly and in combination with PFOSA (0 (control), 25 and 50 μM) for 24 and 48 h. Values are presented as (units/ml)/mg protein ± SEM. Significant difference between control and exposure groups at the different time intervals were analyzed by Tukey's test ($p<0.05$), and denoted with asteriks (*).

Figure 3. Modulation of *cyp1a* (A), *cyp3a* (B) and *pxr* (C), mRNA level in salmon hepatocytes exposed to $CoCl_2$ (150 μM) or DFO (100 μM), given singly and in combination with PFOSA (0 (control), 25 and 50 μM). Cells were harvested after 24 and 48 h and analyzed by qPCR. Values are presented as mean (n=5) percent of control ± SEM. Significant difference between control and exposure groups at the different time intervals were analyzed by Tukey's test ($p<0.05$), and denoted with asteriks (*).

Figure 4. Ethoxyresorufin O-deethylase (EROD: A) and methoxyresorufin O-deethylase (MROD: B) activities in salmon hepatocytes exposed to $CoCl_2$ (150 μM) or DFO (100 μM), given singly and in combination with PFOSA (0 (control), 25 and 50 μM) for 24 and 48 h. Values are presented as average (n=5) nmol resorufin/mg protein/min ± SEM. Significant difference between control and exposure groups at the different time intervals were analyzed by Tukey's test ($p<0.05$), and denoted with asteriks (*).

Figure 5. Gene expression of *gst* (A) and *udpgt* (B) was analyzed by Q-PCR and GST activity (C) was analyzed spectrophotometrically. Salmon hepatocytes were exposed to $CoCl_2$ (150 μM) or

DFO (100 μM) singly and in combination with PFOSA (0 (control), 25 and 50 μM) and harvested after 24 and 48 h. Q-PCR data are presented as mean (n=5) percent of control (same harvest time) ± SEM and GST activity is presented as pmol/min/mg protein ± SEM. Significant difference between control and exposure groups at the different time intervals were analyzed by Tukey's test ($p<0.05$), and denoted with asteriks (*).

Figure 6. Transcriptional changes of *ER-α* (A), *Vtg* (B) and *Zrp* (C) in salmon hepatocytes exposed to $CoCl_2$ (150 μM) or DFO (100 μM) singly and in combination with PFOSA (0 (control), 25 and 50 μM). Cells were harvested after 24 and 48 h and analyzed by qPCR. Values are presented as mean (n=5) percent of control ± SEM. Significant difference between control and exposure groups at the different time intervals were analyzed by Tukey's test ($p<0.05$), and denoted with asteriks (*).

Figure 7. Alterations of vitellogenin level in salmon hepatocytes exposed to $CoCl_2$ (150 μM) or DFO (100 μM), given singly and in combination with PFOSA (0 (control), 25 and 50 μM) for 24 and 48 h. Values are presented as absorbance at 492 nm ± SEM. Significant difference between control and exposure groups at the different time intervals were analyzed by Tukey's test ($p<0.05$), and denoted with asteriks (*).

Figure 8. Biplot showing the distribution of PCA values for mRNA expression (gCAT, gCYP1A1, gCYP3A27, gER-a, gGPx, gGR, gGST, gHIF-1a, gPXR, gUDPGT, gVTG and gZRP), protein levels (Vitellogenin and Zr-protein) and enzyme activity (EROD, MROD, CAT, SOD and GST). Salmon hepatocytes were exposed to $CoCl_2$ (150 μM) or DFO (100 μM) singly and in combination with PFOSA (25 and 50 μM) and harvested after 48 h. All groups were compared and variation between variables is explained by PC1 = 44.0 % and PC2 = 11.9 %.

Table 1. Primer pair sequences, accession numbers, amplicon size and annealing temperature conditions for genes of interest used for real-time PCR.

Target Gene	Primer sequence*		Amplicon size (nucleotides)	Annealing temperature (°C)	GenBank Accession number
	Forward	Reverse			
acox	AATGTTCCTGCCCACCTTGC	GCCAAACTTGGGTCCTATGTCC	89	60	GU138074
cat	CATCCAGAAACGTTGGGTTC	GAGGCACCTCTACGGGTGTA	129	60	DQ367886
cyp1a	GAGTTTGGGCAGGTGGTG	TGGTGCGGTTTGGTAGGT	76	60	AF364076
cyp3a	ACTAGAGAGGGTCGCCAAGA	TACTGAACCGCTCTGGTTTG	146	55	DQ361036
ERα	TCCAGGAGCTGTCTCTCCAT	GATCTCAGCCATACCCTCCA	173	55	DQ009007
gpx	GTGGGGAGTGGAAATCATGT	ATTTGTTGAATGGGGAGCTG	131	60	NM_001140889
gr	AGGAAGCTGGCACACAGACT	CTTCTCCTCTTTGCCCACAC	248	60	BT045539
Pxr	CAAAGGAGGCTGTGGAGAAG	AGTGGGAAAAGGTGATGTCG	147	60	EF517132
udpgt	ATAAGGACCGTCCCATCGAG	ATCCAGTTGAGGTCGTGAGC	113	55	DY802180
gst	CGCATTGACATGATGTGTGA	TGTCGAGGTGGTTAGGAAGG	121	57	DQ367889
Vtg	AAGCCACCTCCAATGTCATC	GGGAGTCTGTCCCAAGACAA	391	57	DY802177
Zr-protein	TGACGAAGGTCCTCAGGG	AGGGTTTGGGGTTGTGGT	113	55	AF407574

*Sequences are given in the 5´- 3´order.

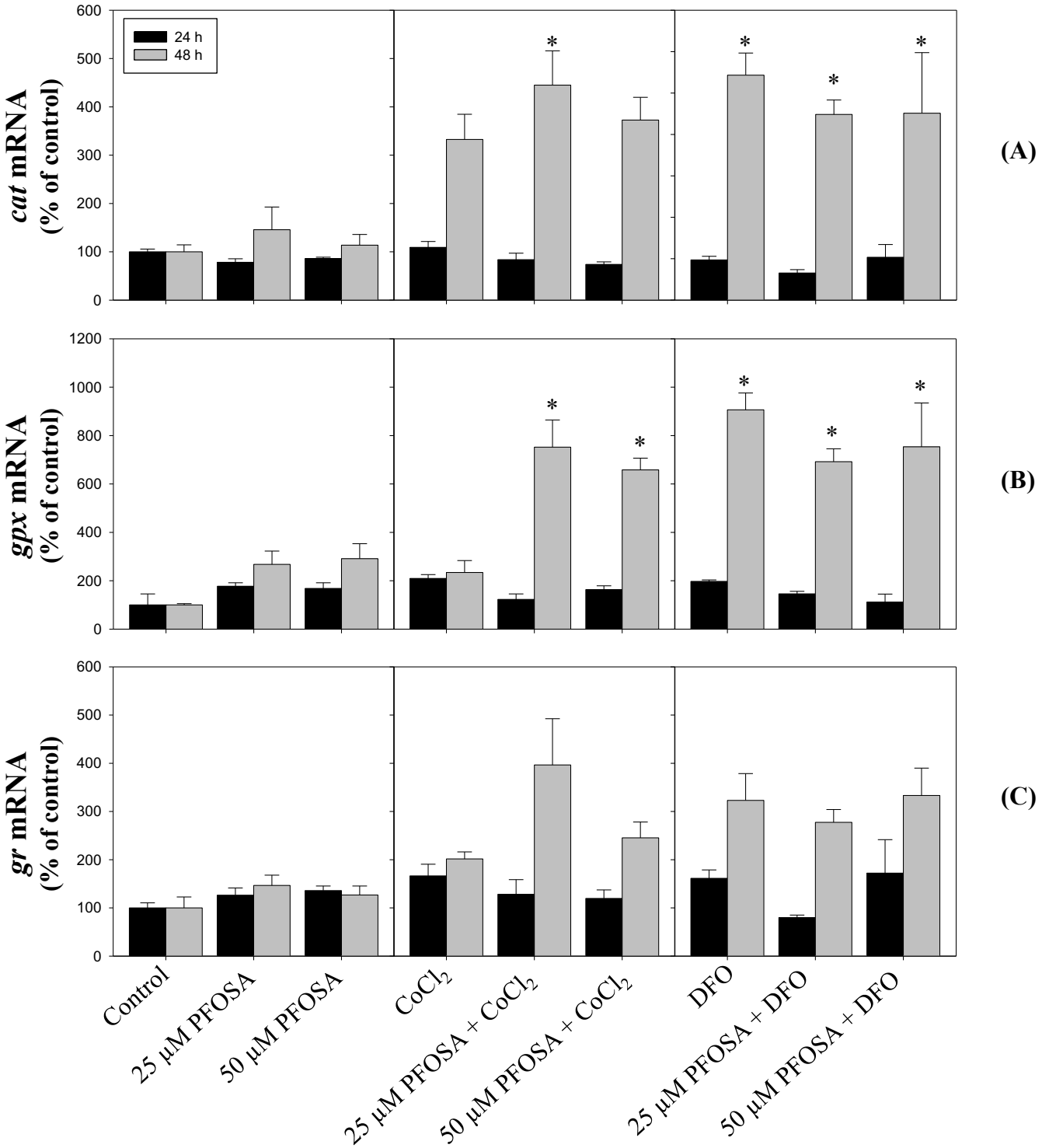
24 h
48 h
cat mRNA (% of control)
gpx mRNA (% of control)
gr mRNA (% of control)
(A)
(B)
(C)
Control
25 μM PFOSA
50 μM PFOSA
CoCl2
25 μM PFOSA + CoCl2
50 μM PFOSA + CoCl2
DFO
25 μM PFOSA + DFO
50 μM PFOSA + DFO

Fig. 1

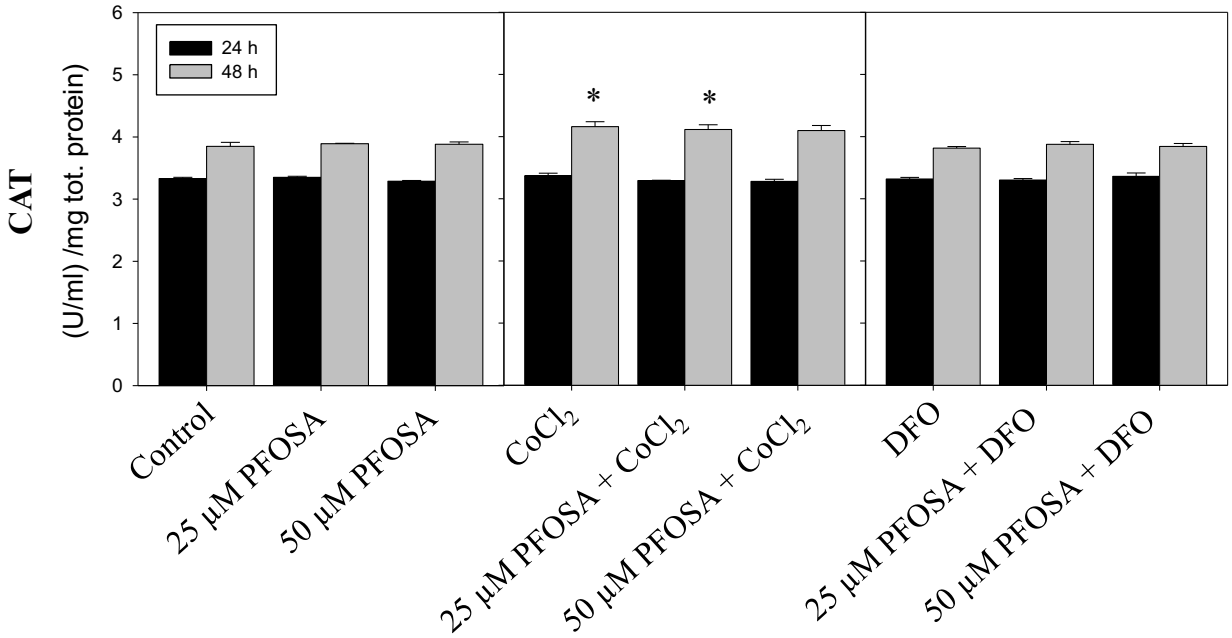
CAT
(U/ml) /mg tot. protein)
24 h
48 h
0
1
2
3
4
5
6
*
*
Control
25 µM PFOSA
50 µM PFOSA
CoCl2
25 µM PFOSA + CoCl2
50 µM PFOSA + CoCl2
DFO
25 µM PFOSA + DFO
50 µM PFOSA + DFO

Fig. 2

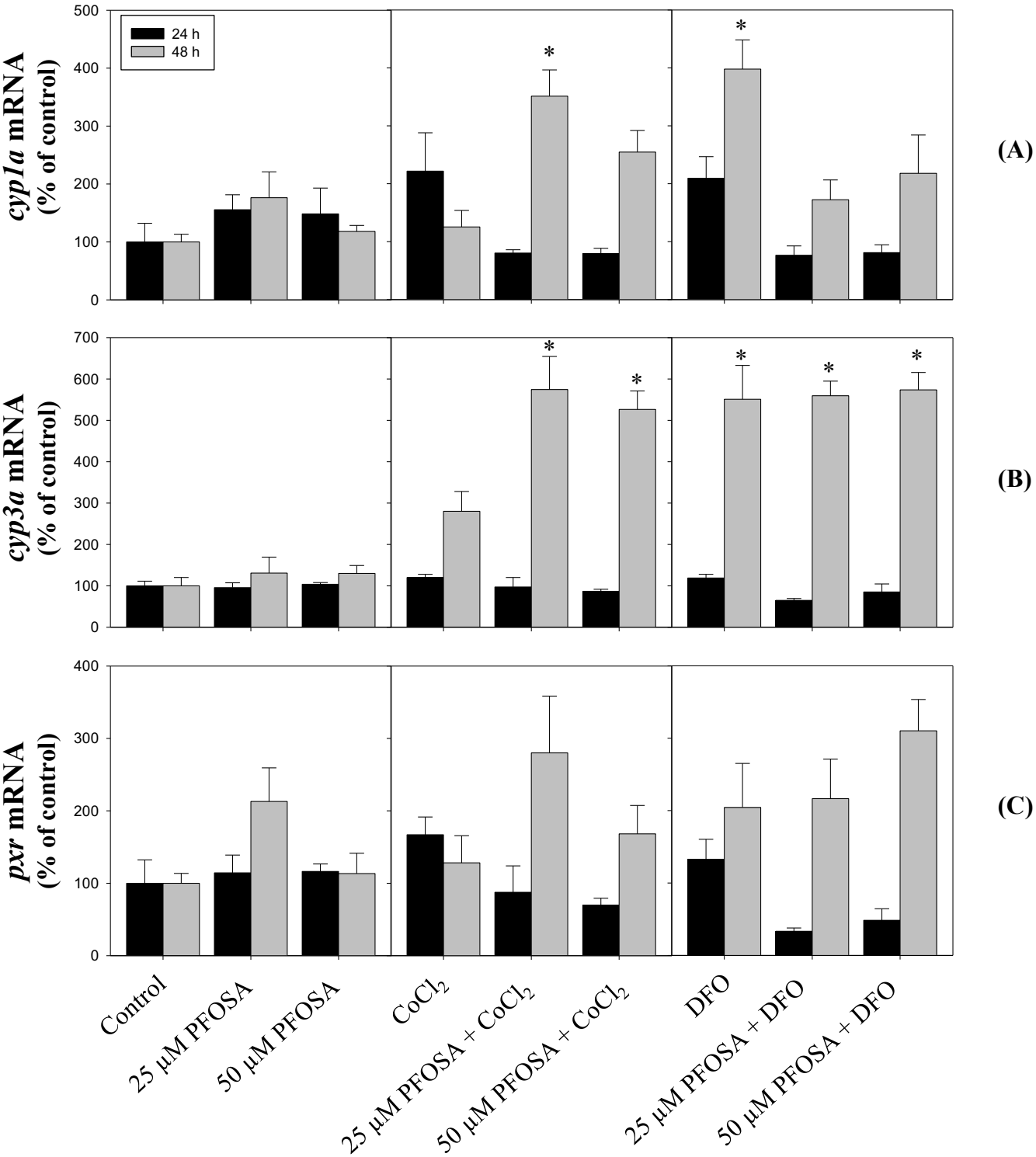
24 h
48 h
cyp1a mRNA (% of control)
cyp3a mRNA (% of control)
pxr mRNA (% of control)
(A)
(B)
(C)
Control
25 μM PFOSA
50 μM PFOSA
CoCl2
25 μM PFOSA + CoCl2
50 μM PFOSA + CoCl2
DFO
25 μM PFOSA + DFO
50 μM PFOSA + DFO

Fig. 3

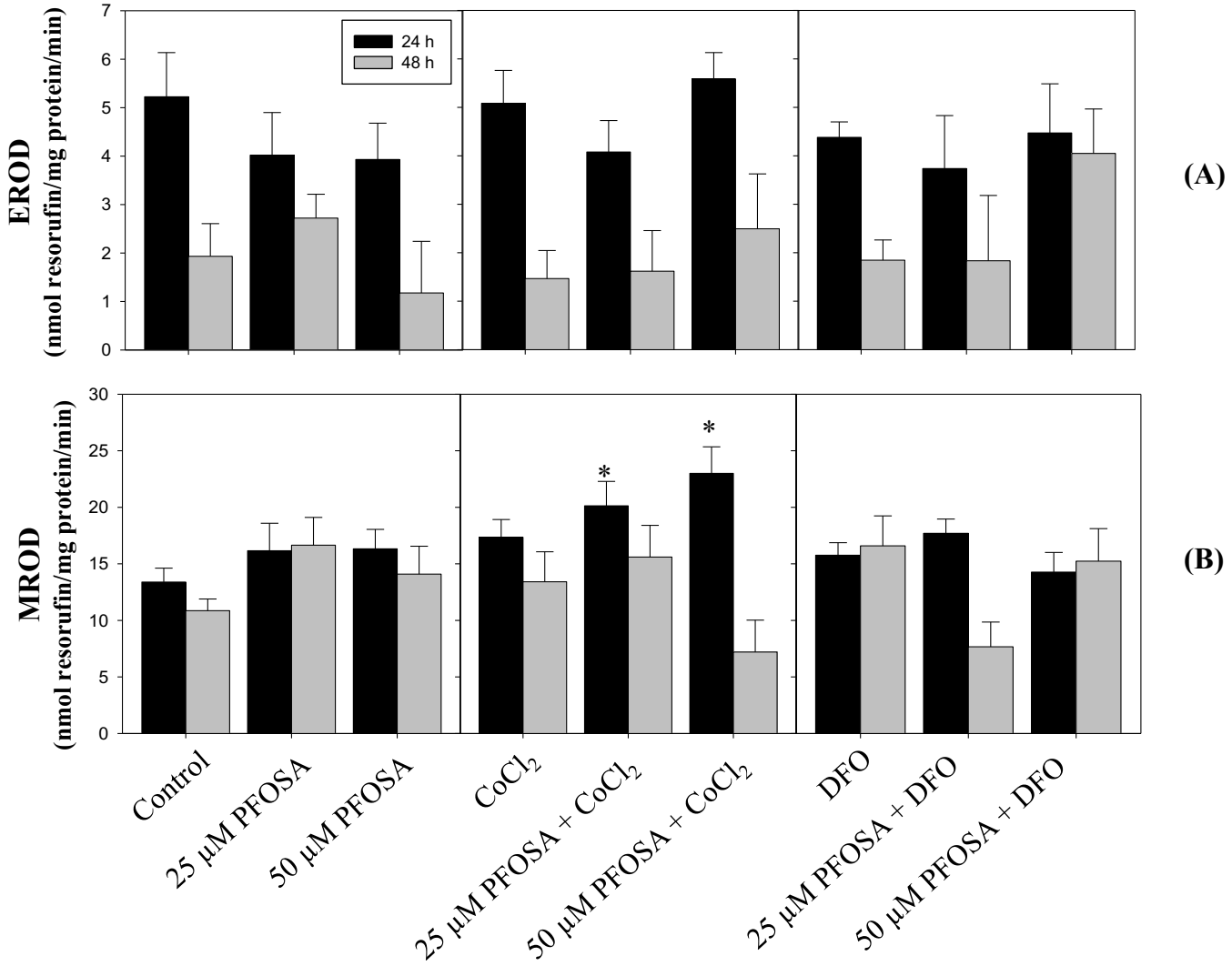
EROD
(nmol resorufin/mg protein/min)
24 h
48 h
(A)
MROD
(nmol resorufin/mg protein/min)
(B)
Control
25 μM PFOSA
50 μM PFOSA
CoCl2
25 μM PFOSA + CoCl2
50 μM PFOSA + CoCl2
DFO
25 μM PFOSA + DFO
50 μM PFOSA + DFO

Fig. 4

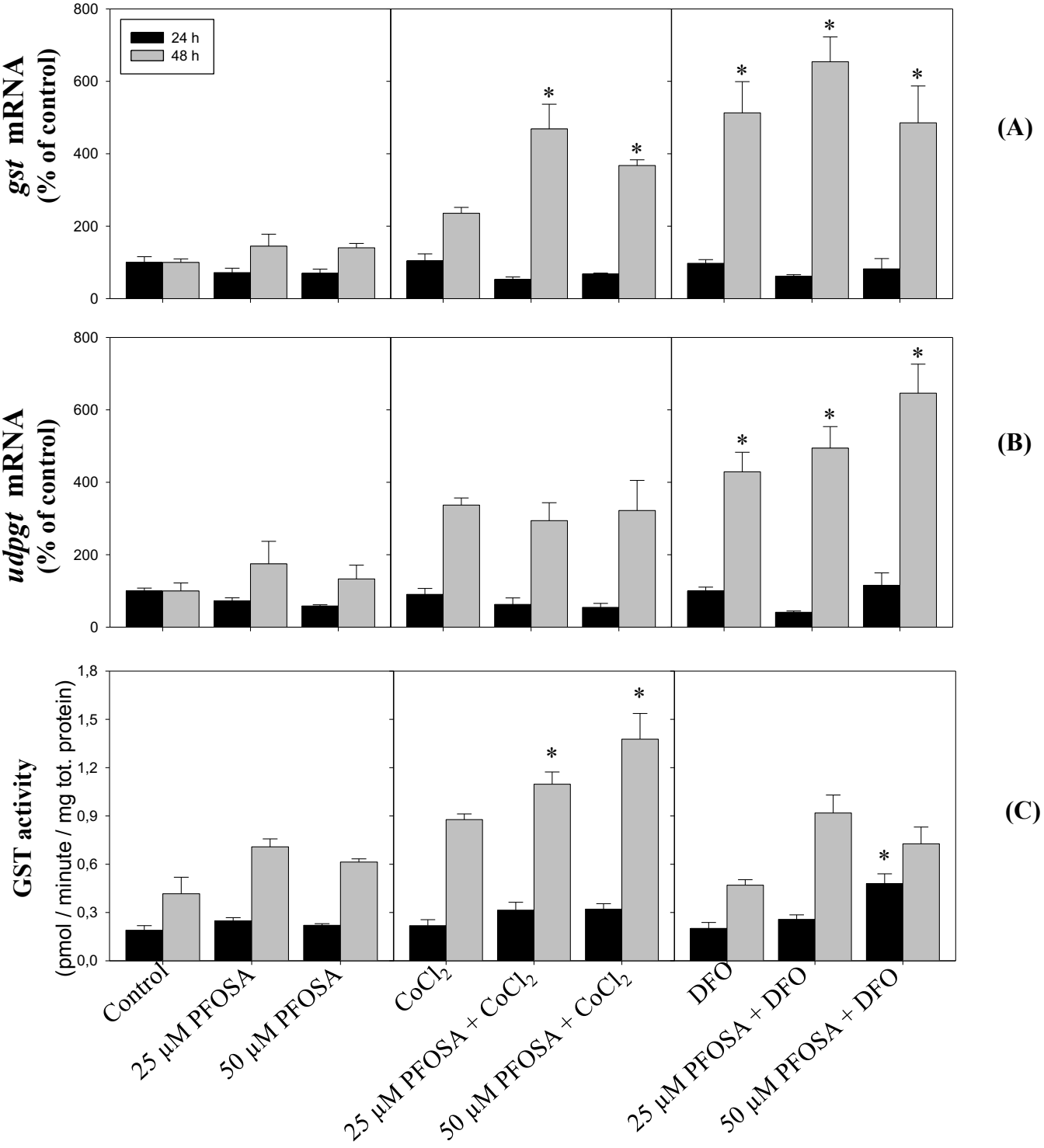
gst mRNA (% of control)
udpgt mRNA (% of control)
GST activity (pmol / minute / mg tot. protein)
24 h
48 h
(A)
(B)
(C)
Control
25 µM PFOSA
50 µM PFOSA
CoCl2
25 µM PFOSA + CoCl2
50 µM PFOSA + CoCl2
DFO
25 µM PFOSA + DFO
50 µM PFOSA + DFO

Fig. 5

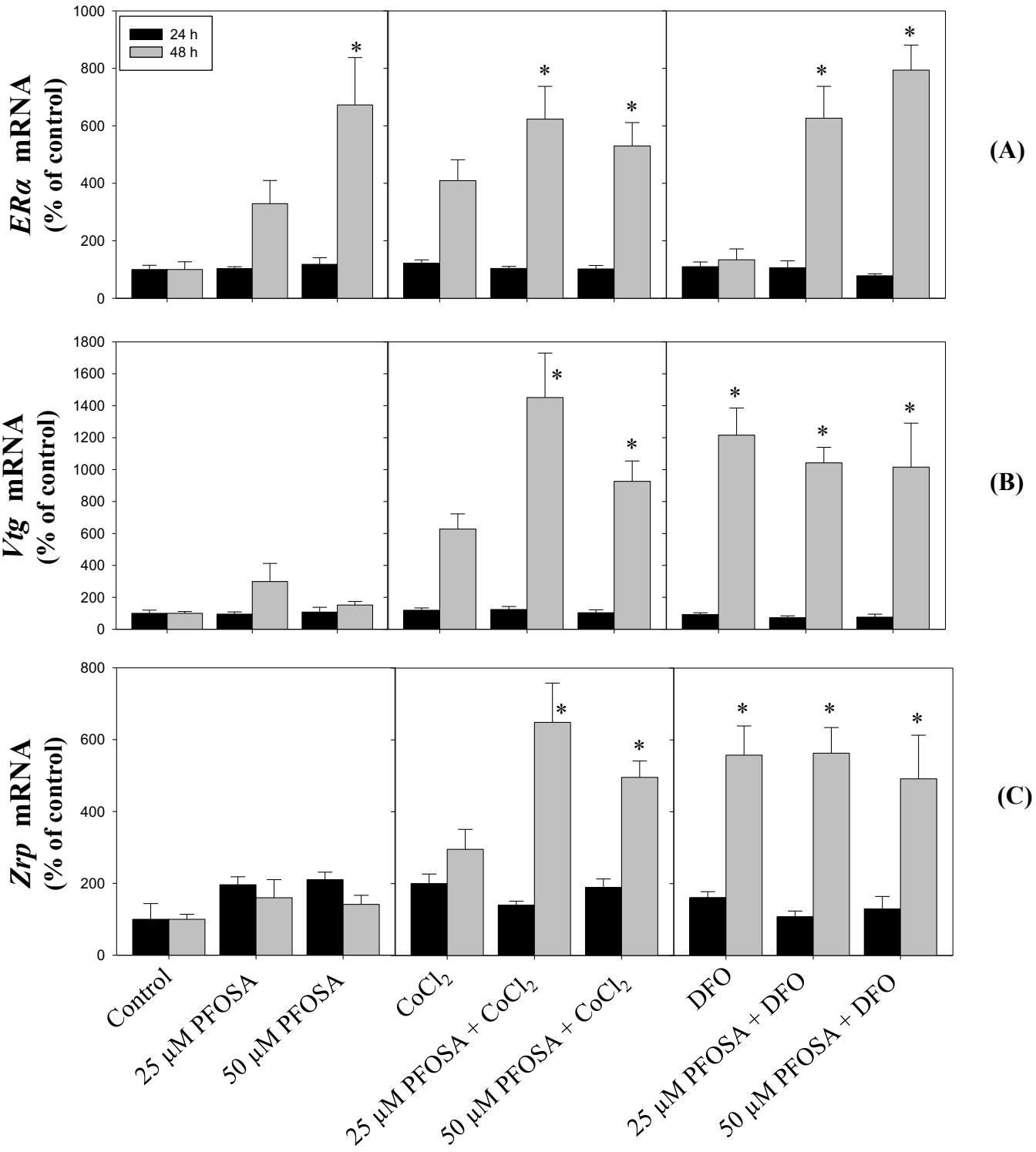
ERα mRNA (% of control)
Vtg mRNA (% of control)
Zrp mRNA (% of control)
24 h
48 h
(A)
(B)
(C)
Control
25 μM PFOSA
50 μM PFOSA
CoCl2
25 μM PFOSA + CoCl2
50 μM PFOSA + CoCl2
DFO
25 μM PFOSA + DFO
50 μM PFOSA + DFO

Fig. 6

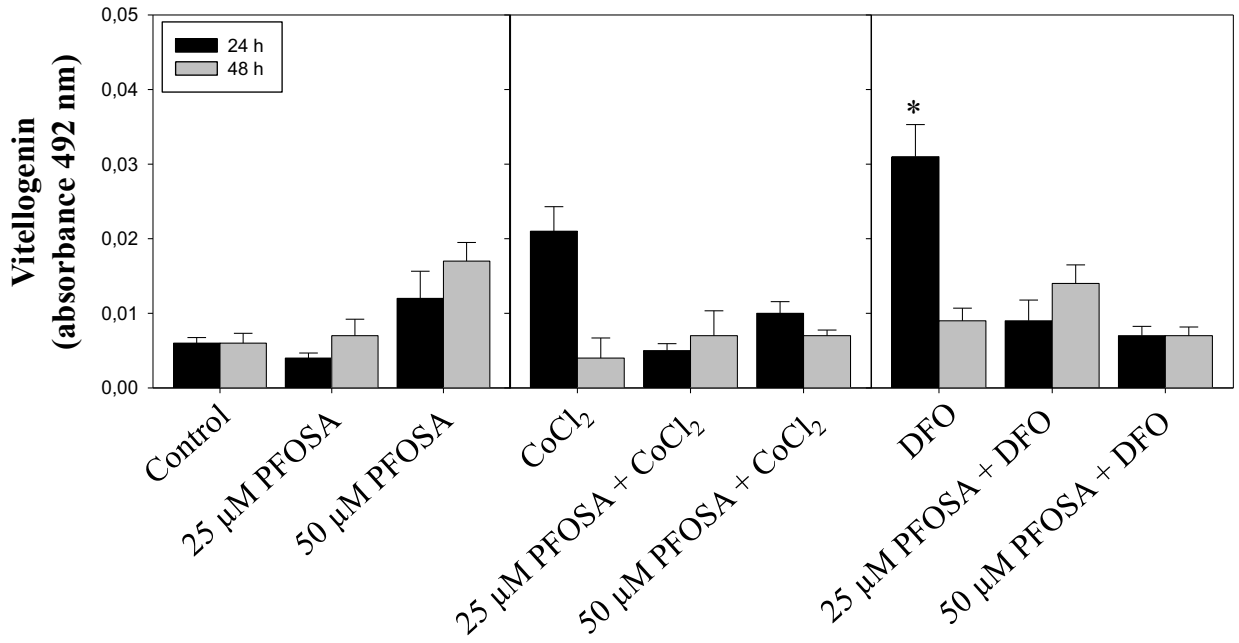
Vitellogenin
(absorbance 492 nm)
0,05
0,04
0,03
0,02
0,01
0,00
24 h
48 h
*
Control
25 µM PFOSA
50 µM PFOSA
CoCl2
25 µM PFOSA + CoCl2
50 µM PFOSA + CoCl2
DFO
25 µM PFOSA + DFO
50 µM PFOSA + DFO

Fig. 7

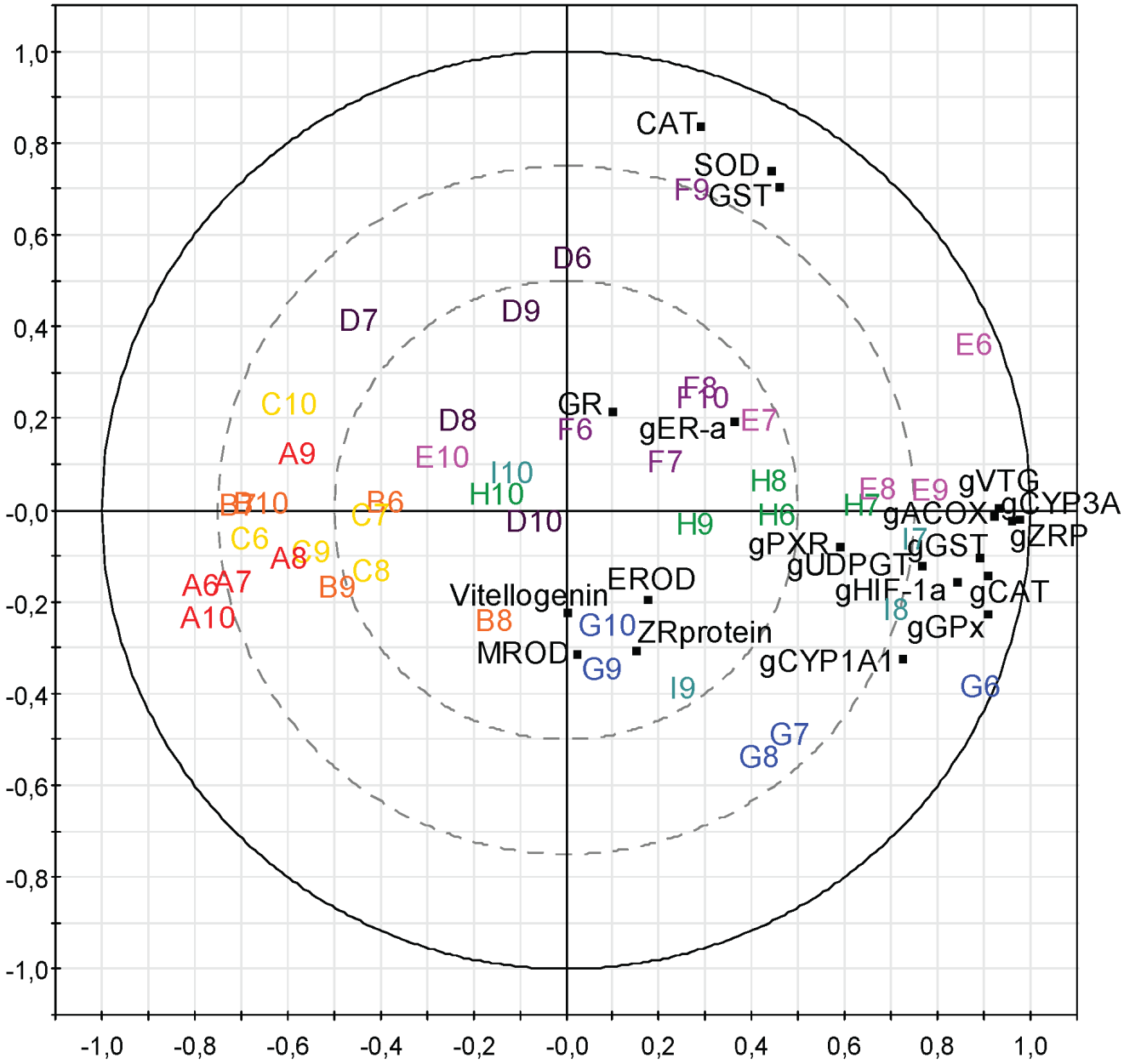
1,0
0,8
0,6
0,4
0,2
-0,0
-0,2
-0,4
-0,6
-0,8
-1,0
-1,0
-0,8
-0,6
-0,4
-0,2
-0,0
0,2
0,4
0,6
0,8
1,0
CAT
SOD
F9
GST
D6
D7
D9
E6
C10
GR
F8
F10
D8
F6
gER-a
E7
A9
E10
I10
F7
H8
E8
E9
gVTG
H10
H7
gACOX
gCYP3A
B10
B6
C7
D10
H9
H6
gPXR
gZRP
C6
C9
A8
I7
gGST
gUDPGT
A6
A7
B9
C8
EROD
gHIF-1a
gCAT
Vitellogenin
A10
B8
G10
ZRprotein
I8
gGPx
MROD
G9
gCYP1A1
I9
G6
G7
G8

Fig. 8