Multiple Environmental Stressors

Biological Interactions Between Parameters of Climate Change and Perfluorinated Alkyl Substances in Fish
Marianne Opsahl Olufsen

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Biological Interactions Between Parameters of Climate Change and Perfluorinated Alkyl Substances in Fish

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Norwegian University of Science and Technology
Faculty of Natural Sciences and Technology
Department of Biology
Scientific environment

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Abstract

Aquatic organisms continuously acclimatize to fluctuations in environment parameters and contaminant insult. The aim of this thesis was to investigate effects of combined exposure to quantifiable measures of climate change (hypercapnia and hypoxia) and perfluorinated alkyl substances (PFASs) on oxidative stress, lipid homeostasis and endocrine disruption in fish. The thesis consists of four papers (Paper I-IV). In Paper I and II, Atlantic cod (Gadus morhua) juveniles was exposed to perfluorooctane sulfonic acid (PFOS) (0, 100 or 200 μg/L) 1 H/day for 5 days, followed by exposure to different concentrations of carbon dioxide (CO2) (848, 2735 or 7963 ppm). Sampling was performed 3, 6 and 9 days after initiated CO2 exposure. We observed interactions between PFOS and CO2 exposure on transcription of gill glutathione peroxidase 1 (GPx1), gill manganese superoxide dismutase (MnSOD) and liver phosphatidylethanolamine N-methyltransferase (PEMT) (Paper I). Peroxisome proliferator-activated receptor β (PPAR-β) was increased in cod liver by CO2, and the response was higher in the presence of PFOS (Paper I). In Paper II, PCA-biplots showed clustering of samples based on CO2 concentration, and the distribution of response parameters indicated that hypercapnia was the main driver of hormone responses. Changes in estradiol-17β (E2), testosterone (T) and 11-ketotestosterone (11-KT) levels and E2-responsive genes were increased by hypercapnia alone at day 3 and 9, and by combined exposure scenarios (Paper II). Hypercapnia increased cytochrome P450 1A (CYP1A) mRNA alone and in combination with PFOS (Paper II). In Paper III and IV, primary Atlantic salmon (Salmo salar) hepatocytes were exposed to perfluorooctane sulfonamide (PFOSA) (0, 25 and 50 μM) singly, and in combination with hypoxia-inducible compounds (cobalt chloride: CoCl2 and deferoxamine: DFO) for 24 and 48 h. We observed that combined exposure generally altered the transcription of antioxidant responses and lipid regulation, showing higher effect of hypoxia, compared to PFOSA (PCA-plots Paper III-IV). Hypoxic condition alone, and in combination with PFOSA, increased transcription of E2-responsive genes, CYP1A and CYP3A responses (Paper III). Transcription of PPAR-α, -β and γ, and the ω6:ω3 ratio was increased by DFO and these responses were modulated in the presence of PFOSA (Paper IV). In addition, we observed a parallel increase of hypoxia-inducible factor 1α (HIF-1α), acyl coenzyme A oxidase (ACOX) and PPAR-γ, indicating a link between lipid metabolism and hypoxic responses (Paper IV). Changes in PPAR system and FA β-oxidation (ACOD and ACOX) suggests complex changes in the regulation of lipid homeostasis and FA metabolism, where PFASs modulated the responses produced by hypercapnia or hypoxia alone (Paper I and IV). Changes in the composition of ω6 and ω3 FAs in salmon hepatocytes and reduced PEMT transcription in cod liver were observed (Paper I and IV), indicating that climate change variables alone and in combination with PFASs may potentially alter membrane lipid composition and possibly produce overt physiological consequences. These studies indicate that parameters of climate change and PFASs affect sex steroids, E2-responsive genes and the CYP system, and that combined exposure generally produced higher response than single exposure (Paper II and III). These findings show that processes involved in endocrine signaling and biotransformation are very complex and hard to predict due to biphasic responses and possibly interaction between stressors. This thesis provides new and valuable insight on the combined effects of quantifiable parameters of climate change and environmental pollutants. Potential interaction between environmental stressors and the relevance of considering exposure duration were revealed, emphasizing the importance of investigating combined and chronic exposure scenarios.
List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
<th>Description</th>
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<tbody>
<tr>
<td>ω-3</td>
<td>omega 3 fatty acids</td>
<td>H₂CO₃ Carbonic acid</td>
</tr>
<tr>
<td>ω-6</td>
<td>omega 6 fatty acids</td>
<td>H₂O₂ Hydrogen peroxide</td>
</tr>
<tr>
<td>11-KT</td>
<td>11-ketotestosterone</td>
<td>HCO₃⁻ Bicarbonate</td>
</tr>
<tr>
<td>ACOD</td>
<td>Acyl coenzyme A dehydrogenase</td>
<td>HIF-1α Hypoxia-inducible factor-1α</td>
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<tr>
<td>ACOX</td>
<td>Acyl coenzyme A oxidase</td>
<td>HRE Hypoxic responsive element</td>
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<tr>
<td>AhR</td>
<td>Aryl hydrocarbon receptor</td>
<td>LA Linolelaidic acid</td>
</tr>
<tr>
<td>ALA</td>
<td>A-linoleic acid</td>
<td>mRNA Messenger ribonucleic acid</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
<td>PC Phosphatidylcholine</td>
</tr>
<tr>
<td>ARA</td>
<td>Arachidonic acid</td>
<td>PCA Principal component analysis</td>
</tr>
<tr>
<td>ARNT</td>
<td>Aryl hydrocarbon nuclear translocator</td>
<td>PE Phosphatidylethanolamine</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
<td>PEMT Phosphatidylethanolamine N-methyltransferase</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
<td>PFAS Poly- and perfluorinated alkyl substances</td>
</tr>
<tr>
<td>CO₃⁻</td>
<td>Carbonate</td>
<td>PFOS Perfluorooctane sulfonic acid</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>Cobalt chloride</td>
<td>PFOSA Perfluorooctane sulfonamide</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
<td>POP Persistent organic pollutant</td>
</tr>
<tr>
<td>DFO</td>
<td>Deferoxamine</td>
<td>PPAR Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
<td>ppm Parts per million</td>
</tr>
<tr>
<td>DPA</td>
<td>Docosapentaenoic acid</td>
<td>PUFA Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>E</td>
<td>Estrogen</td>
<td>ppm Parts per million</td>
</tr>
<tr>
<td>E₂</td>
<td>Estradiol-17β</td>
<td>PPARA Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>EDC</td>
<td>Endocrine-disrupting chemicals</td>
<td>ROS Reactive oxygen species</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
<td>SOD Superoxide dismutase</td>
</tr>
<tr>
<td>ERα</td>
<td>Estrogen receptor</td>
<td>T Testosterone</td>
</tr>
<tr>
<td>ERE</td>
<td>Estrogen responsive element</td>
<td>UDPGT Uridine diphosphate glucuronosyltransferase</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
<td>VEGF Vascular endothelial growth factor</td>
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<tr>
<td>FAD</td>
<td>Fatty acid desaturase</td>
<td>Vtg Vitellogenin</td>
</tr>
<tr>
<td>FAE</td>
<td>Fatty acid elongase</td>
<td>XRE Xenobiotic responsive element</td>
</tr>
<tr>
<td>GLA</td>
<td>γ-linoleic acid</td>
<td>ZP Zona pellucida protein</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
<td>ZRP Zona radiata protein</td>
</tr>
<tr>
<td>GR</td>
<td>Glutathione reductase</td>
<td></td>
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<tr>
<td>GSH</td>
<td>Monomeric glutathione</td>
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</tr>
<tr>
<td>GSSG</td>
<td>Glutathione disulfide</td>
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</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
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Paper I
Oxidative stress and lipid regulation in cod liver and gills after exposure to carbon dioxide (CO₂) and perfluorooctane sulfonic acid (PFOS), given singly or in combination
Manuscript
Marianne Olufsen, Sindre Andre Pedersen, Augustine Arukwe

Paper II
Effects of elevated dissolved carbon dioxide and perfluorooctane sulfonic acid, given singly and in combination, on steroidogenic and biotransformation pathways of Atlantic cod
Aquatic toxicology, July 2014
Gunnhild Preus-Olsen, Marianne Olufsen, Sindre Andre Pedersen, Robert J. Letcher, Augustine Arukwe

Paper III
Endocrine, biotransformation and oxidative stress responses in salmon hepatocytes exposed to chemically induced hypoxia and perfluoroctane sulfonamide (PFOSA), given singly or in combination
Environmental Science and Pollution Research, December 2014
Marianne Olufsen, Augustine Arukwe

Paper IV
Modulation of membrane lipid composition and homeostasis in salmon hepatocytes exposed to hypoxia and perfluoroctane sulfonamide (PFOSA), given singly or in combination
PLOSone, Volume 9, Issue 7, July 2014
Marianne Olufsen, Maria V. Cangialosi, Augustine Arukwe
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Introduction

Climate change

Parameters in the environment are continuously fluctuating, but some parameters have been shown to change beyond the level considered normal, including increased temperature and carbon dioxide (CO$_2$) concentration (Hardy, 2003). During the last decade, extensive research has focused on effects of climate change in the environment and biota. Ocean acidification is caused by increases in the release of CO$_2$ into the atmosphere, mainly formed by burning of fossil fuels and deforestation (Oreskes, 2004). Exchange of CO$_2$ between atmosphere and upper layers of water is constantly driven by concentration equilibrium constant. CO$_2$ reacts with water to form carbonic acid (H$_2$CO$_3$), which further dissociates to bicarbonate ($\text{HCO}_3^-$) or carbonate ($\text{CO}_3^{2-}$) ions and hydrogen ions ($\text{H}^+$) (equation 1), and this process lowers oceanic pH (Kikkawa et al., 2003).

Equation 1. \[
\begin{align*}
\text{CO}_2 + \text{H}_2\text{O} & \leftrightarrow \text{H}_2\text{CO}_3 \\
& \leftrightarrow \text{HCO}_3^- + \text{H}^+ \\
& \leftrightarrow \text{CO}_3^{2-} + \text{H}^+
\end{align*}
\]

Carbon \hspace{1cm} Carbonic acid \hspace{1cm} Bicarbonate \hspace{1cm} Carbonate

Contribution of CO$_2$ to the carbon cycle from natural processes (i.e. volcanoes, wildfires, biotic respiration, etc.) is in steady balance, while anthropogenic emissions arise from stable carbon deposits/reservoirs, thus increasing global available CO$_2$. Since the industrial revolution, atmospheric CO$_2$ concentration has risen from 280 to 380 parts per million (ppm) (Turley et al., 2006), and approximately 40 % of this CO$_2$ is taken up by the oceans, causing a reduction in oceanic pH by 0.1 units (global average of 8.17 to 8.07) (Cao et al., 2007; Zeebe et al., 2008). Future scenarios predict a reduction in oceanic pH of 0.2-0.4 units by the end of this century and 0.4-0.9 units within year 2300 (Caldeira and Wickett, 2003, 2005). Species are adapted to acclimatize within a specific range of temperature and pH that is considered normal within their habitat (Munday, 2014). Permanent changes, outside the species acclimatization range, force adaptation and may have major impacts on ecosystems and populations. Furthermore, Noyes and Lema (2015) reviewed how changes in temperature and pH, can affect the fate of environmental pollutants, altering bioavailability and toxicity. Wildlife acclimatizes to fluctuations in their environment, while at the same time deal with a multitude of environmental pollutants. The additional stress from pollutants may produce detrimental harm in biota by affecting the ability to further acclimatize or adapt to changes in the environment. On the other hand, organisms may have reduced ability to handle pollutant toxicity during acclimatization to environmental fluctuations (Noyes and Lema, 2015). However, little information is available on the combined effects of multiple environmental stressors in aquatic organisms, especially simultaneous exposure to alterations in environmental parameters and chemical pollutants.

Environmental pollution

Vast surveillance studies have investigated the occurrence of persistent organic pollutants (POPs), such as perfluorinated alkyl substances (PFASs) and carboxylic acids (PFCAs), and testing for their adverse effects in aquatic animals. PFASs and PFCAs have been manufactured since 1950s, as perfluorooctane sulfonyl fluoride (POSF) (Buck et al., 2012; Prevedouros et al., 2006), but remained undetected in biota and environment until recent years. The global extent of PFOS contamination in wildlife was first demonstrated by Giesy and Kannan (2001) and in humans by Kannan et al. (2004). PFASs are prone to long-range transportation (Zhao et al., 2012), because of their high persistence.
and low degradation in the environment and biota, respectively (Cui et al., 2010). During production, the hydrolysis of POSF yields perfluorooctane sulfonic acid (PFOS) and its salts. Reaction of POSF with methyl or ethylamines yields the alkyl substituted sulfonamides: N-methyl perfluorooctane sulfonamide (NMeFOSA) and N-ethyl perfluorooctane sulfonamide (NEtFOSA), respectively, which can be dealkylated and generate the ultimate PFOS precursor namely - perfluorooctane sulfonamide (PFOSA). One of the most abundant PFAS species detected in environment and biota is PFOS (Kannan, 2011). Most PFASs are readily taken up by fish through diet or directly from the surrounding environment, but are not easily excreted. Due to the unique chemistry of PFOS, being neither lipophilic nor hydrophilic, it undergoes enterohepatic circulation and bioaccumulate effectively in organisms (Johnson et al., 1984; Slotkin et al., 2008), predominantly in liver or in complex with transporter proteins in blood (albumin) (Jones et al., 2003). The mechanism of toxicity by PFOS and PFOSA is not well documented, but they have been shown to produce effects on development, hormonal disruption, immunotoxicity, fatty acid (FA) regulation and hepatotoxicity (Lau et al., 2007; Lau et al., 2004; Wågbø et al., 2012), increased oxidative stress and lipid peroxidation possibly mediated by interruption of PPAR system (Liu et al., 2009; Takacs and Abbott, 2007; Vanden Heuvel et al., 2006) (Figure 1).

Oxidative stress responses

Information of altered physiological responses is important in evaluating toxicity risks of contaminants or environmental changes. Alterations in oxidative stress responses provide information on changes in reactive oxygen species (ROS) generation or disruption of antioxidant regulation, which is potentially harmful for the organism (Sies, 1991). ROS is continuously generated by the mitochondrial electron transport chain, cytochrome P450 (CYP) activity in endoplasmatic reticulum and oxidase reactions in peroxisomes and cytosol (Halliwell and Gutteridge, 2007;
A network of cytoprotective enzymes protecting cells against ROS, namely the antioxidant machinery, are encoded in antioxidants responsive element (ARE) and include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (Osburn and Kensler, 2008; Regoli and Giuliani, 2014). Antioxidants are induced by ROS and these enzymes remove ROS or oxidized cellular components efficiently (i.e. ROS scavenging). There are several isoforms of SOD, varying in their choice of cofactor (example, CuZnSOD and MnSOD), that catalyze dismutation of superoxide (O$_2^-$) creating hydrogen peroxide (H$_2$O$_2$) and oxygen (Figure 3A). Glutathione peroxidase (GPx) catalyzes the transformation of H$_2$O$_2$ to water through oxidation of monomeric glutathione (GSH) into glutathione disulfide (GSSG). This pathway is dependent on conversion of GSSG to GSH by glutathione reductase (GR) (Figure 3B). Decomposition of H$_2$O$_2$ to water and oxygen is catalyzed by catalase (CAT) (Kirkman and Gaetani, 1984) (Figure 3C).

These antioxidants are important in ROS scavenging to avoid detrimental harm. ROS attacks and oxidizes cellular components, such as DNA, lipids and proteins, when levels exceed the scavenging capacity of the cell and this is called oxidative stress (Sies, 1991). Increased transcription and activity of antioxidants is directly related to increased ROS generation. Furthermore, a reduction in antioxidant levels may indicate that the stressor is affecting mechanisms regulating oxidative stress responses or the antioxidants themselves. Both of these scenarios may lead to oxidative stress and are therefore potentially harmful (Sies, 1991). Combined exposure of stressors that act upon this pathway of toxicity are more likely to produce adverse effects, since exceeding the antioxidant capacity is more likely to occur. Hypercapnia and hypoxia are known to increase ROS formation (Dean, 2010; Fan et al., 2008; Lushchak, 2011) and several studies have showed increased ROS formation or oxidative stress responses after exposure to PFOS or PFOSA (Arukwe and Mortensen, 2011; Liu et al., 2009; Wågbø et al., 2012). Increased metabolism and respiration from acclimatization or xenobiotic biotransformation increase ROS generation, creating a potential for oxidative stress. Altered metabolism acquiring energy release from FAs, may promote peroxisome
proliferation through activation of PPAR system, which may potentially produce oxidative stress (Devchand et al., 2004; Jansen et al., 2009). Analyzing lipid profiles or enzymes regulating FA homeostasis is a good way to detect alterations in FA composition. It has been reported that PFOSA produce changes in lipid composition, specifically ω-FAs (Wågbø et al., 2012).

**Lipid homeostasis and PPARs**

Lipids constitute a major component of cellular membranes and serve as important signaling molecules, energy storage and are directly linked to the immune system (Sargent et al., 1999; Sheridan, 1988; Tocher, 2003). Regulation of phospholipids in cellular membranes is tightly controlled and composition is specific for different membranes, specifying the stability and permeation (Wolfgang and Lane, 2006). The essential FAs, ω-3 (or n-3) and ω-6 (or n-6), are important components of cellular membranes. Transformation of these FAs is performed by several FA desaturases (FAD4, FAD5 and FAD6) and FA elongase (FAE) altering the level of saturation and length of the carbon chain, respectively (Figure 4). α-linolenic acid (ALA, 18:3n3) and linoleic acid (LA, 18:2n6) are essential FAs that must be obtained from diet. ALA function as an energy source, but can be transformed to eicosapentanoic acid (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n3), which have anti-inflammatory functions and are important components of cellular membranes (Wall et al., 2010). LA is transformed to γ-linoleic acid (GLA, 18:3n6), arachidonic acid (ARA, 20:4n6) and docosapentaenoic acid (DPA, 22:5n6) that have inflammatory functions. The ω-3 and ω-6 FAs competitively bind to transformation enzymes, where ω-6 binds to FADs and FAE stronger than n ω-3. Increased ω-6:ω-3 ratio is associated with inflammatory responses and adverse health effects. Components of ω-3 family are associated with proper receptor-mediation in membranes (Kogel et al., 2008). These FAs also serve as storage for energy released as NADPH and ATP. Changes in energetic demand, such as during environmental acclimatization, can affect ω-3 and ω-6 composition.

Another important component of cellular membranes are phospholipids where the ratio of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) is essential for membrane integrity (Li et al., 2006). PC is predominantly produced by the CDP-choline pathway (Kennedy pathway), but in liver 30% of PC is produced by PE N-methyltransferase (PEMT) converting PE to PC (Reo et al., 2002; Vance, 2013). Stressor-induced alteration in PEMT activity affecting PC:PE ratio could produce harmful effects to membrane stability and hence liver function. Overall, the ability to regulate FA pools is essential for normal homeostasis, and peroxisome proliferator-activated receptors (PPARs)
are known to be critical regulators of lipid homeostasis by controlling the balance between burning and storage of long FAs (Shi et al., 2002).

PPARs (α, β and γ) are important ligand-dependent nuclear receptors, regulating lipid composition, peroxisome proliferation, apoptosis, cell differentiation and cell cycle control (Blanquart et al., 2003; Dreyer et al., 1992; Shi et al., 2002). Many lines of evidence indicate that PPAR-α regulates lipid homeostasis by stimulating peroxisomal FA β-oxidation (Varga et al., 2011). In the liver, PPARα activation leads to upregulation of FA transporter protein and long-chain acyl CoA synthetase gene. This stimulates energy production and shortens long-chain FAs, thus preventing lipid accumulation and toxicity. Natural ligands for PPARα include LA, dodecahexanoic acid (ω-3) and EPA (Li and Glass, 2004). PPAR-β have important roles in lipid metabolism and energy homeostasis and natural ligands are thought to be free FA (Li and Glass, 2004). PPAR-γ regulates glucose homeostasis and promotes adipocyte differentiation that is essential for the development of adipocyte tissue, and endogenous ligands for PPAR-γ include oxidized LA and 15-deoxy-Δ12,14-prostagladin J2 (Li and Glass, 2004). Acyl coenzyme A oxidase (ACOX) and acyl coenzyme A dehydrogenase (ACOD) catalyzes the first step in FA β-oxidation in peroxisomes and mitochondria, respectively (Reddy and Hashimoto, 2001; Wanders et al., 2010). ACOX is involved in polyunsaturated FA (PUFA) biosynthesis and PPAR signaling pathway. Several PFASs are known to bind PPAR-α and -β agonistically, leading to further transcription of these genes, measured as messenger ribonucleic acid (mRNA), and down-stream effects (Fang et al., 2012; Shipley et al., 2004). Effects on PPAR signaling can potentially cause detrimental harm, because altered PPAR activity is associated with hepatotoxicity, adenomas and tumor formation (Guo et al., 2006; Lau et al., 2007; Tachibana et al., 2008).

**Endocrine disruption and biotransformation**

Endocrine disruptive compounds (EDCs) are chemicals that can interfere with the hormone system, and are associated with adverse effects in development, sex differentiation and reproduction. The hormone system is dependent on endogenous and exogenous signals for proper function, and during “critical stages in life”, such as juveniles and puberty, organisms are especially susceptible to EDC exposure (Arcand-Hoy and Benson, 1998; Frye et al., 2012). Changes in the environment have the potential to affect the endocrine system, and may possibly worsen the effects of EDCs (Baroiller and D'Cotta, 2001; Brown et al., 2015; Jenssen, 2006; Schreck et al., 2001). Hormones are signaling molecules important in a multitude of functions and consist of eicosanoids, amino acid derivatives and steroids, where the sex steroids include - estrogens (E), testosterone (T) and 11-ketotestosterone (11-KT). Estrogens initiate estrogenic responses through activation of estrogen receptor α (ERα), which then form a homodimer-complex that can enter the nucleus, bind to estrogen response element (ERE) and activate estrogen responsive genes (Figure 5). Estrogen-responsive genes include vitellogenin (Vtg), zona pellucida protein (ZP) or zona radiata protein (ZRP), which are commonly used as biomarker responses of endocrine disruption in fish (Arukwe and Goksoyr, 2003; Arukwe et al., 1997). PFOS has been shown to affect estrogen parameters, sexual development and reproduction in fish (Ankley et al., 2005; Fang et al., 2012; Mortensen et al., 2011; Wang et al., 2011), possibly through interaction with ER and androgen receptor (AR) (Benninghoff et al., 2011; Kjeldsen and Bonefeld-Jorgensen, 2013). Several biological pathways that include – the metabolism and synthesis by the cytochrome P450 (CYP) superfamily orchestrate the level of sex steroid in fish. CYP1 and CYP3 are known to oxidize E and T, respectively, a process necessary for further degradation by other enzymes (Young et al., 2005). Because CYPs have important functions
in hormone regulation, it is suggested that alterations in CYP activity by xenobiotic exposure may affect the endocrine system by affecting the level of sex steroids (Monostory and Dvorak, 2011).

However, CYPs have an important role in metabolism of xenobiotics, performing the first step in phase I biotransformation (Ortiz de Montellano, 2005). CYPs are regulated by a ligand-dependent transcription factor, namely - aryl hydrocarbon receptor (AhR) (Ortiz de Montellano, 2005). In the cytosol, AhR is in a complex with other protein chaperones, including the heat shock protein (hsp) and upon ligand binding, AhR breaks free of this complex, translocate into the nucleus and heterodimerize with ARNT, binds to the xenobiotic responsive element (XRE) and that induces transcription of XRE responsive genes (Figure 5) (Gu et al., 2000). AhR is a promiscuous receptor that is activated by a multitude of xenobiotic chemicals and endogenous chemicals (Denison and Nagy, 2003). It is suggested that CYP can transform PFOSA to PFOS in vivo (Tomy et al., 2003; Xu et al., 2004), but less is known about any possible interaction with AhR (Chen et al., 2015). Biotransformation of xenobiotics is important to escalate the excretion rate and reduce toxicity of contaminants. Phase II biotransformation enzymes attach a hydrophilic group to their substrate making them more easily excreted, including glucuronidation by uridine diphosphate glucuronosyltransferase (UDPGT) or glutathione conjugation by glutathione-S-transferase (GST), which are also regulated through AhR (Dietrich and Kaina, 2010).

EDCs and AhR-activating xenobiotics may produce interactive effects, possibly through crosstalk between ERs and AhR. Ligand-activated AhR can inhibit estrogen responsive gene expression, and it has been proposed that ERα may bind to AhR and increase transcription of xenobiotic responsive genes (Matthews and Gustafsson, 2006). Hypoxia has been shown to inhibit CYP1A1 mRNA and activity and the mechanism for this effect has been proposed to arise from competition between
hypoxia-inducible factor 1 alpha (HIF-1α) and AhR for their common co-factors - ARNT (Khan et al., 2007; Shan et al., 1992; Zhang and Walker, 2007). During hypoxia, HIF-1α becomes stable and can enter the nucleus where it binds ARNT and induce transcription genes through hypoxia responsive element (HRE) (Figure 5) (Kumar and Choi, 2015). However, new research has proposed that the down-regulation of CYP1A during hypoxia is due to alterations in nitric oxide and oxidant status (Rahman and Thomas, 2012). Regardless, these reports suggest that a combination of environmental stressors that affect these pathways may potentially exert interactive effect and alter the level of sex steroid or the hormonal responses in fish.

Multiple environmental stressor and their interactions

Combined exposure scenarios are gaining increased attention, and exploring the potential of interactive effects between multiple environmental stressors is of great interest (Jenssen, 2006; Noyes and Lema, 2015; Noyes et al., 2009; Schiedek et al., 2007). Investigations of complex mixtures in experimental studies have shown that responses from multiple stressors may not be easily predicted due to potential interaction between stressors (Billick and Case, 1994). Principal component analysis (PCA) is a useful tool to interpret data that consist of many responses by several exposure conditions simultaneously. Spatial orientation of responses and individual samples in a PCA-plot show which responses are affected in a similar manner, if exposure groups display exposure specific responses and if responses are associated with certain exposure treatments. However, the most effective method to investigate interactive effects of stressors is n-way analysis of variance (ANOVA), where n is the number of stressors you are comparing. For example, we use a two-way ANOVA to investigating interaction between CO₂ and PFOS. The n-way ANOVA test the data for additivity, where a significant value (p<0.05) indicates interaction between stressors (Billick and Case, 1994). Folt et al. (1999) investigated interaction between three stressors (toxin, low food and temperature) in juveniles and adults of two species cladoceran zooplankton, Daphnia pulex and Daphnia pulicaria. They reported two-way and three-way interaction between stressors on reproduction and survival that varied between life stages and species, and that most interactions were antagonistic (Folt et al., 1999).

Some of the ways that changes in environmental parameters and contaminants may increase toxic effects

- Bioavailability
- Acclimatization
- Biotransformation
- Additivity
- Interaction

Increase concentration of contaminants at site of action
Lower ability to acclimatize in the presence of contaminants
Acclimatization affects the ability of metabolism and excretion of contaminants
Combined toxicity of stressors when they affect the same pathways
Interaction between stressors produce unpredictable responses

Figure 2. Combined exposure to multiple environmental stressors may alter toxic effects in an organism in several ways. These include increased bioavailability of contaminants, increased stress load that may affect acclimatization processes or the ability to metabolize and excrete contaminants, additivity of stressors or unpredictable effects when stressors interact.
Organisms are continuously exposed to multiple environmental stressors, and the increasing concern of effects from climate change on biota have raised a question to how organism are affected by changes in environmental parameters in the presence of chemical pollutants. Some studies have already been conducted in this field (described below), and figure 2 show some potential effects of combined exposure that alter toxic responses compared to single exposure. Heugens et al. (2001) reviewed the combined exposure to toxicants and elevated temperatures. They reported that toxicants may increase the metabolic oxygen demand, and that higher temperatures will potentiate the toxic effect, because increased temperature reduces the partial pressure of oxygen ($pO_2$) in water. They also reported that combined exposure to toxicants could increase adverse thermal effects at the thermal tolerance limit, due to reduced ability for acclimatization (Heugens et al., 2001). When multiple stressors affect the same mechanism we can expect increased toxicity from combined exposure, for examples temperature and a stressor that both produce changes in metabolic processes may combined increase effects on energy metabolism and respiration (Heugens et al., 2001). A study with marine bivalves reported that combined exposure to hypercapnia and metals (Cd and Cu) produced higher degree of oxidative stress responses than single treatment, and showed that increased toxicity was due to increased bioavailability of metals during hypercapnia (Ivanina et al., 2015). The PhD thesis by Marie Löf (2004) investigated combined effects of hypoxia and sediment contaminants in amphipod (Monopheria affinis), and reported that contaminants alone produced increased transcription of oxidative stress response, but no physiological damage. However, the combination of stressors produced physiological damage supporting the hypothesized potential of xenobiotics to alter the animals ability to cope with oxygen deficiency (Gorokhova et al., 2013). The same exposure treatments also produced reproduction dysfunction observed as embryo aberrations in amphipods (Löf, 2014). Many POPs have similar structure as endogenous hormones, and may affect the endocrine system by interaction with hormone transport proteins, affecting hormone metabolism or POPs can mimic or block the effects of endogenous hormones (Colborn et al., 1993; Jenssen, 2006). In the arctic, concentration of POPs is relatively high in marine mammals and birds and it is hypothesized that climate change pose additional stress because the endocrine system is important for acclimatization to environmental stress (Jenssen, 2006). Population viability in zebrafish (Danio rerio) exposed to elevated temperatures and pollution showed that combined exposure increases a male skew which can speed declines in zebrafish populations, and they investigated the role of CYP19 in this process, albeit clear connections were not observed (Brown et al., 2015). These findings support the concern of possible increased toxicity of combined exposure to environmental stressors. We address the importance of investigating oxidative stress responses, effects on lipid homeostasis and endocrine disruption from multiple environmental stressors in this thesis. Changes in environmental parameters and PFASs can individually affect the aforementioned toxic effects and it is therefore crucial to investigate combined effects.
Aims of the thesis

Scientific understanding on the interactions between various classes of contaminants and different environmental factors, including quantifiable measures of climate change, in aquatic organisms are not well understood. However, there is a general speculation that increasing temperature raises contaminant toxicity, and decreasing salinity may increase metal toxicity and reduce the toxicity of certain contaminants. Hypoxia has been shown to produce stress on aquatic organisms, where coastal and estuarine animals are the most vulnerable species. Overall, the potential toxicological and molecular mechanisms behind these responses are not understood and studies on how these responses may be modified by quantifiable parameters of climate change are non-existent. Therefore, the aim of this thesis was to investigate the effects of quantifiable measure of climate change (ocean acidification and hypoxia), singly and also in combination with emerging environmental contaminant group, namely - perfluorinated alkyl substances (PFASs) in fish and represents a multiple stressor exposure scenario. The study was focused on investigating the physiological effects that may reduce general health condition, impairing development and reproduction. The general aim is further explained in the individual sub-aims (I-IV), and represented by the individual research papers and hypothesis as stipulated below.

1. To study the effects of hypercapnia/hypoxia and PFASs on oxidative stress responses. Our hypothesis is that exposure of fish to quantifiable measures of climate change (elevated aquatic CO$_2$ levels or hypoxia) and PFASs, singly and also in combination, will produce changes oxidative stress parameters, and that these effects will be modulated due to interactions between these environmental stressors. This was investigated by measuring transcriptional changes and activity of antioxidants systems in an in vivo and in vitro experimental approach (paper I and III).

2. To study the effects of hypercapnia/hypoxia and PFASs on lipid homeostasis. Our hypothesis is that acclimatization to changes in environmental parameters (hypercapnia and hypoxia) will produce effect on lipid homeostasis and potentially affect lipids important in cellular membranes, and that these effects will be modulated in the presence of PFASs. This was investigated by measuring transcriptional changes of PPARs and processes in lipid regulation, and analyzing lipid profiles in vivo and in vitro in fish (Paper I and IV).

3. To study the effects of hypercapnia/hypoxia and PFASs on hormonal and biotransformation pathways of Atlantic cod and salmon hepatocytes. Our hypothesis is that in vivo and in vitro exposure of fish to hypercapnia or hypoxia will alter hormonal and biotransformation pathways, and that these effects will be potentiated in the presence of PFASs. In addition, these responses will represent valuable input in the understanding of molecular mechanisms of effect or mode of action for these multiple stressors. These effects were analyzed by measuring muscle tissue sex steroid levels and transcriptional expression of genes involved in hormonal responses, steroid- and xenobiotic metabolism and hypoxic stress (Paper II and III).
The emission of CO$_2$ and other greenhouse gases due to anthropogenic activities are thought to be the main drivers of global climate change. The effects of climate change in the aquatic environment are observed as increases in average temperature and CO$_2$ levels. An increase in water temperature produces reduction in pO$_2$, and its availability to aquatic organisms. Cellular availability of O$_2$ is crucial for cellular respiration that generates energy for metabolic and maintenance pathways, including development in aerobic organisms. In addition, it has been suggested that increasing temperatures or changes in CO$_2$ levels, due to global climate change may modulate the toxicity of some environmental contaminants. For example, temperature and possibly CO$_2$ dependent shifts in toxicity can be increased by direct interaction with metabolic pathways or through indirect processes such as changes in lipid stores during the developmental history of individuals. Therefore, this thesis has studied the interactions between hypercapnia and hypoxia (quantifiable measures of climate change) and PFASs (PFOS and PFOSA) given singly and in combination on biological processes that may produce deleterious physiological effects that may reduce general health conditions, impairing development and reproduction in fish, using Atlantic salmon (Salmo salar) and Atlantic cod (Gadus morhua) as model species. Statistical analysis indicated that exposure duration is an important factor in stressors responses and interactive effects between hypercapnia and PFOS in Paper I, where combined exposure generally produced higher responses than single exposure to either stressor. In general, both studies revealed that changes in environmental conditions (hypercapnia and hypoxia) were the main drivers of toxicological and biological responses, and PCA-biplots revealed that combined stressor treatment generally exceeded that of the most severe responses of a single stressor as shown in Paper II, III and IV. Thus, our data are in accordance the aforementioned studies that report that multiple environmental stressors generally produce higher toxicity than stressors individually (Brown et al., 2015; Gorokhova et al., 2013; Ivanina et al., 2015; Löf, 2014).

**Oxidative stress responses**

Reactive oxygen species (ROS) are important signaling molecules during hypoxia, required for stabilization of HIF-1α (Chandel et al., 1998; Chandel et al., 2000). Thus, increased ROS generation lead to increased level of stable HIF-1α which can heterodimerize with ARNT and induce transcription of genes important in hypoxic responses, such as vascular endothelial growth factor (VEGF). ROS generation by complex III (electron transport chain) in mitochondria is elevated during hypoxia, but the mechanism for sensing reduced O$_2$ is not identified (Chandel et al., 2000). Hypercapnia initiates hypoxic responses and hence an increase in CO$_2$ concentration may cause oxidative stress from elevated ROS generation. Several studies have shown increased transcription and activity of antioxidant machinery from hypercapnia and hypoxia exposure (Dean, 2010; Fan et al., 2008; Lushchak, 2011), including Paper I and III. Previous studies have reported increased oxidative stress response caused by several PFASs (Arukwe and Mortensen, 2011; Liu et al., 2009; Wågbø et al., 2012), but our data do not support these findings. We observed no oxidative stress response from single treatment of PFOS or PFOSA in Paper I and III, respectively. Increased activation of the antioxidant machinery is associated with increased levels of ROS, but does not identify the pathway of increased generation. Combined exposure regimes increased higher transcription of the antioxidant machinery than single exposure to hypercapnia in juvenile cod and...
hypoxia by CoCl₂ in salmon hepatocytes (Paper I and III, respectively), while hypoxia by DFO in salmon hepatocytes produced a similar response alone and in combination with PFOSA (Paper III). In salmon hepatocytes, combined hypoxia and PFOSA exposure produced higher transcription of CAT, GPx (by CoCl₂) and GST and activity of GST than single treatment. DFO produced similar response in transcription of CAT, GPx and GR alone and in combination with PFOSA. There is some discrepancy between the chemicals used to induce hypoxia, and this should be considered in future studies. In Paper III, PCA-biplots support the observations that the most severe responses are produced by combined exposure regimes. Statistics, in Paper I, revealed interactive effects between PFOS and CO₂ on GPx1 and MnSOD transcription in gills. Transcriptional changes of several antioxidants, GPx1 (liver), CAT, CuZnSOD (only liver) and MnSOD, showed higher response in combined exposure compared to single exposure (Paper I). These responses were clearly dependent on exposure duration, where the two tissues (gill and liver) analyzed showed increased transcription of antioxidants at different points in time during the experiment. ROS can attack cellular components, i.e. lipids, proteins and DNA, when the level exceeds antioxidant scavenging capacity (Sies, 1991). Hence, there is an increased risk of approaching harmful ROS levels when exposed to a combination of stressors compared to single exposure treatment. Several antioxidant responses investigated herein showed statistical additive effects between stressors, while a few parameters indicated statistical interaction between hypercapnia and PFOS in cod (Paper I). Reports of interaction between stressors emphasize the importance of studies investigating combined exposure regimes, and these responses should be investigated further. The mechanisms leading to increased ROS levels by hypercapnia, hypoxia, PFOS and PFOSA are not well understood and the nature of interactions between stressors is therefore difficult to predict. Lipid peroxidation is one of the major outcomes of free radical-mediated injury to tissue and peroxidation of fatty acyl groups occur mostly in membrane phospholipids (Catalá, 2009).

**PPARs and lipid homeostasis**

It is suggested that PFASs can interfere with mitochondrial metabolism to increase peroxisome proliferation in vitro (Starkov and Wallace, 2002) and that PFOS mechanism of action could be through interaction with PPARs (Viberg and Eriksson, 2011). Starkov and Wallace (2002) suggested that perfluorinated acids can act as structural mimics of FAs, thereby inhibiting mitochondrial FA β-oxidation. PPARs are ligand-dependent nuclear receptors activated by FAs. *In vitro* studies have shown that PFOS can bind PPARα agonistically and to a lesser degree PPAR-γ, while PPAR-β has not yet been tested (Takacs and Abbott, 2007; Vanden Heuvel et al., 2006). In Paper IV, we investigated the response of all PPAR isoforms to PFOSA and we observed a small but significant increase of PPAR-α transcription in salmon hepatocytes exposed to 50 μM PFOSA, while other PPAR-β and PPAR-γ were unaffected. Our data indicate that PFOSA act upon the PPAR system in the same manner that was reported of PFOS effects in the aforementioned *in vitro* studies. On the other hand, Paper I showed that PPAR-β was increased in liver from juvenile cod exposed for 9 days to 100 μg/L PFOS. However, because of the long exposure duration it is possible that this effect was due to other mechanisms than binding to PPAR. We propose that chronic *in vivo* exposure to PFOS can lead to alterations in FA composition, potentially increasing FAs that are appropriate activating ligands for PPAR-β.

Several studies have investigated the role of PPAR-α in physiological processes during hypoxic conditions (Biscetti et al., 2009), such as involvement in angiogenesis (Rizvi et al., 2013) with results
showing both inductive and inhibitory effects. Hypoxia and hypercapnia shifts energy production from FA β-oxidation to glycolysis (Goda and Kanai, 2012; Liu et al., 2014), and acute and intermittent hypoxia was shown to increase lipid accumulation (Jun et al., 2013). PPARs regulate the balance between burning and storage of FAs and can affect cellular metabolism. Lipid accumulation is increased through activation of PPAR-γ, which also stimulates the use of glucose as energy source. Salmon hepatocytes exposed to hypoxia and PFOSA show parallel increase in transcription of HIF-1α, ACOX and PPAR-γ, suggestive of increased peroxisomal FA β-oxidation related to HIF expression (Paper IV), that is in conflict with liver PPAR-δ expression in cod (Paper I). However, we also observed increased transcription of PPAR-α and PPAR-δ by the same exposure regimes in salmon hepatocytes, emphasizing the complexity of the PPAR system. Alterations in PPAR transcription caused by environmental changes (i.e. hypoxia and hypercapnia), may possibly be due to increased energy demand, that increase metabolism, thus altering the FA composition and release of stored FAs. Long-term acclimation to hypercapnia switches glycolysis from aerobic to anaerobic metabolism in red muscle and heart of S. aurata after 4 days, but also there is evidence of enhanced oxidation of FAs to support ATP production under these conditions (Michaelidis et al., 2007). In Paper I, PPAR-δ mRNA expression was reduced by combined exposure of hypercapnia and PFOS (100 μg/L) after 3 days, but increased by hypercapnia and PFOS (200 μg/L) after 6 and 9 days. We see that combined exposure causes more alterations than single hypoxia or hypercapnia that could mean that the stress of the pollutant affects the energy balance in fish and contribute to the overall stress load. Expression of PPAR-δ normally elevated to increase glycolysis and lipid accumulation, but we saw in Paper III that other PPARs involved in FA metabolism can also be activated simultaneously showing a complex system regulating energy utilization.

In juvenile cod, gill ACOD mRNA expression was unaffected by hypercapnia alone, but increased by combined exposure of hypercapnia and PFOSA at day 3 (Paper I). This suggests a possible increase in mitochondrial FA β-oxidation. Adaptation to hypercapnia and hypoxia is however very dependent on species and also the tissue investigated. Switching from FA β-oxidation to glycolysis was observed in cardiomyocytes, increasing their oxygen utilization efficiency mediated through HIF-1 activation leading to increased expression of glucose transporters and glycolytic enzymes (Kaelin, 2002; Semenza, 2013), and PPAR-α/RXR-mediated suppression of mitochondrial FA β-oxidation (Belanger et al., 2007; Huss et al., 2001). Further, mitochondrial FA oxidative capacity was reduced by hypoxia, resulting in reduced mitochondrial lipid mobilization and utilization, causing consequent accumulation of intracellular lipid (Huss et al., 2001). Paper IV showed that hypoxia increased transcription of all PPAR isoforms (α, β and γ), and gene transcripts of HIF-1α, FADS, FAD6, FAE and ACOX. Expression of PPAR-α and several PPARα target genes was decreased in rat heart during hypoxia by CoCl₂ (Razeghi et al., 2001). There is a discrepancy between PPAR responses reported in these studies and those reported in Paper IV, and cellular mechanism of altered peroxisomal FA β-oxidation in adaptation to hypoxia has been reviewed by Biscetti et al. (2009) reporting the complexity of PPAR regulation during angiogenesis. Physiological hypoxic responses were not explored in this thesis. However, we emphasize that membrane FA profile was significantly altered in salmon hepatocytes by several exposure combinations. In Paper IV, changes in FA profile and PPAR transcripts suggest a possible hypoxia mediated increase in the levels of endogenous ligands for PPAR isoforms.
Previously, histomorphological analysis revealed that peroxisomal proliferation from PFAS or PFCA exposure is accompanied by increased peroxisome-related enzyme activities such as ACOX and catalase (Pastoor et al., 1987; Permadi et al., 1992; Sohlenius et al., 1992; Vanden Heuvel, 1996) indicating increased ROS generation by FA β-oxidation in peroxisomes. Our data did not show significant responses of ACOX and CAT that parallel PPAR-α alterations in salmon hepatocytes exposed to PFOSA (Paper IV). This finding is consistent with results in cod, showing the absence of effect on ACOD and CAT by PFOS in vivo (Paper I). However, we observed an increase of PPAR-α, CAT and GPx transcription from hypoxia treatment in salmon hepatocytes (Paper IV). Interestingly, PPAR-β and CAT are increased by hypercapnia after 6 days while ACOD was increased after 3 days in cod (Paper I). It is possible that mitochondrial FA β-oxidation produced changes in FA composition during the initial exposure duration, and that prolonged exposure initiated an onset of mechanisms increasing FAs metabolism to feed the energy demand of adaptation to hypercapnia.

Lipids are the main components of cellular membranes, particularly phosphatidylcholine (PC). Alterations in PEMT activity may significantly alter membrane fluidity and structure. Steatosis was observed in PEMT deficient mice, caused by loss of membrane integrity due to decreased PC/PE ratio (Li et al., 2006). In Paper I, we observed that hypercapnia reduced transcription of PEMT in liver after 9 days of exposure, potentially affecting the PC/PE ratio. Interactive effect between CO₂ and PFOS was revealed at day 3, but was not confirmed by post hoc test. However, our data indicate that PFOS does not affect membrane fluidity through direct effect on PEMT transcription, since no effect of PFOS alone was observed. Combined exposure produced the same effect as hypercapnia alone, supportive of no interaction between stressors in this pathway (Paper I). In paper IV, we observed changes in ω-6: ω-3 ratio in salmon hepatocytes after 48 hours. There was a 3.3- and 2.7-fold increase at 25 μM PFOSA singly or in combination with CoCl₂ and 5.8- and 2.3-fold increase at DFO singly or in combination with 50 μM PFOSA. Both ω-6 and ω-3 PUFAs are important components in cellular membranes and alterations in composition may affect fluidity and permeability (Stillwell and Wassall, 2003; Yu et al., 2015). Other studies have shown that PFOS affects membrane permeability, leading to increased response of other compounds, when given in binary treatments, i.e. bisphenol A (BPA) in zebrafish (Danio rerio) (Keiter et al., 2012a), TCDD and E2 in carp leukocytes and reduced mitochondrial membrane potential (Hu et al., 2003). These studies showed indirect effects of PFOS exposure that may have ecotoxicological implications. It was suggested by Hu et al. (2003) that PFOS might act by non-specific detergent-like effects on the membrane, affecting membrane permeability and fluidity, and not by affecting specific transport protein systems. The mechanism of PFOS and PFOSA effects on membrane fluidity and permeability has not been investigated in this thesis, but is an important question for future studies.

Endocrine disruption
Alterations in environmental parameters, hypercapnia and hypoxia, is stressful for teleost species (Cech and Crocker, 2002; Fivelstad et al., 1999), and stress is generally associated with decreased sex steroid hormones and impaired reproductive responses (Clearwater and Pankhurst, 1997; Haddy and Pankhurst, 1999; Schreck et al., 2001; Wu, 2009). Physical parameters in surroundings contribute to regulation of the hormonal status in fish, giving rise to the concern that climate change may cause endocrine disruption. Paper II showed that hypercapnia alone produced a significant increase in estradiol-17β (E2) level, the most potent endogenous E, and transcription of E2-responsive genes (ER-α, Vtg-α, Vtg-β, ZP-2 and ZP-3), suggesting a CO₂ concentration dependent
response. These findings suggest that alteration in E2 levels are directly associated with potential biological effects in the hypercapnia exposed cod. The androgens analyzed in juvenile cod (Paper II), T and 11-KT, were also elevated (albeit not significant) by hypercapnia. Paper III showed that hypoxia increased the expression of E2-responsive transcripts in salmon hepatocytes similar to the effect observed by hypercapnia in juvenile cod. Production of VTG protein paralleled transcription of HIF-1α and Vtg in hypoxia exposed hepatocytes, showing an apparent relationship between transcription and biological effect. In Paper III, the effect of hypoxia was supported by the PCA-biplot showing that hypoxia exposed individuals are different compared to control and the changes are related to increased estrogenic responses, oxidative stress responses and CYP gene transcript, with the exception of Erα and GR mRNA expression. A few studies have previously reported that hypoxia can increase sex steroid levels in fish (Shang et al., 2006; Wu et al., 2003), suggesting that the response is dependent on maturational stage and exposure conditions. Some studies have in fact shown that stress can accelerate reproduction processes in fish, but that is dependent on species, maturational stage and severity of stress (Schreck et al., 2001).

PFASs are suspected endocrine disruptors, but the effect varies widely between the chemical species. Conflicting results have been reported in fish exposed to PFOS, showing increase (Cheng et al., 2012) and decrease (Hagenaars et al., 2008) of Vtg levels. Previous studies indicate that PFOS exert estrogenic effects in teleost species, observed as elevated expression of estrogen responsive genes (Du et al., 2013; Fang et al., 2012; Keiter et al., 2012b; Liu et al., 2007) and altered sex steroid levels (Ankley et al., 2005; Mortensen et al., 2011; Oakes et al., 2005). The estrogenic responses of PFASs are mediated through interaction with Erα and AR (Benninghoff et al., 2011; Kjeldsen and Bonefeld-Jorgensen, 2013). Paper III showed that PFOSA increased Erα transcription, but not the down-stream responses of Vtg and ZRP transcription in salmon hepatocytes. Paper II showed that PFOS increases transcription of several hepatic E2-inducible genes in an apparent time- and concentration-specific manner (albeit only significant alteration of Vtg-8 and ZP-2) and higher E2 levels after 9 days in juvenile cod. These findings, in vivo (Paper II) and in vitro (Paper III), are in accordance with previous studies showing that PFASs cause endocrine disruption by altering estrogenic responses (Ankley et al., 2005; Benninghoff et al., 2011; Kjeldsen and Bonefeld-Jorgensen, 2013; Mortensen et al., 2011). Aforementioned studies showed a higher estrogenic response to PFOS, but shorter exposure duration and lower concentrations applied in the studies for this thesis could explain this discrepancy. However, the concentration of PFOS in Paper II are considerably higher than levels detected in the environment (Houde et al., 2011) with the exception of PFOS burdens in fish inhabiting highly polluted areas (Delinsky et al., 2010; Moody et al., 2002). The estrogenic responses detected in this study (Paper II and III) were observed a relatively long time after PFOS and PFOSA exposure, suggesting a possible influence of a yet to be identified mechanism other than interaction with Erα. Other factors can modulate E2-regulated genes, including pituitary factors (Vaisius et al., 1991), other hormones (Ding, 2005; Mori et al., 1998; Raingeard et al., 2009) and other nuclear receptors such as PPARs and thyroid hormone receptors (TRs) (Arukwe and Mortensen, 2011; Fang et al., 2012; Shipley et al., 2004) (also shown in Paper I and III). These other factors may be affected by PFOS, and indirectly alter estrogen responsive gene expression.

Paper II showed that combined CO2 and PFOS exposure increased T and 11-KT compared to control and medium CO2, respectively. E2 levels are significantly increased by combined exposure compared to control, and by severe hypercapnia and high PFOS compared to hypercapnia alone. Taken
together, these findings showed that combined exposure produced a higher response than single exposure. Alterations in transcription of Vtg-α, Vtg-δ and ZP-2 showed that combined effect of CO₂ and PFOS was time-dependent, displaying an apparent biphasic response, shifting between increased (day 3 and 9) and decreased (day 6). Several types of stress can sometimes cause endocrine responses that are biphasic with directionality that is dependent on the severity and dose/level of the stressors, as well as duration (Schreck et al., 2001). There is a discrepancy between the in vivo and in vitro study, where Paper III showed that Vtg, ZRP and ERα are increased by combined exposure of hypoxia and PFOSA. However, in Paper III, Vtg protein is not affected by combined exposure, suggesting post-transcriptional modification of E2-responsive genes. In Paper II, we observed that at day 6, Vtg and ZP-2 mRNA was lower in combined exposure treatments compared to single exposure. However, ERα mRNA was increased by combined exposure at day 6, but we do not have data for sex steroid hormone levels at day 6. The simultaneous increase in ERα and decrease of E2-responsive genes (Vtg and ZP-2) could possibly be explained by crosstalk between ERα and AhR which has been shown to inhibit transcription of ERE (Bugel et al., 2013). In paper III, salmon hepatocytes (in vitro) showed an increase of ERα, Vtg and ZRP transcription that was similar between of hypoxia alone and in combination with PFOSA, suggesting a possible contribution of other organs in regulating the endocrine signal, observed in Paper II. It is possible that acclimatization to hypercapnia initiates hypoxic responses, observed as oxidative stress, which may affect the energy consumption in fish leading to changes in physiology. Previous studies have reported alterations in metabolism following hypercapnia exposure (Langenbuch and Portner, 2003; Lannig et al., 2010). Nuclear receptors interaction due to competition between HIF-1α and AhR for ARNT binding, may affect the level of crosstalk/hijacking of ERα by active AhR when exposed to a combination of environmental stressors involved in these pathways. Interaction between AhR and ER have been extensively studied and several hypothesis of the underlying mechanisms are proposed Matthews and Gustafsson (2006), and Matthews et al. (2005) reported in a previous study that activated AhR may recruit ERα to increase transcription of xenobiotic responsive genes. The in vivo and in vitro studies in this thesis showed a relationship between endocrine, oxidative and biotransformation responses, where quantifiable measures of climate change (hypercapnia or hypoxia) were the main drivers of effect compared to PFAAs (PFOS or PFOSA).

Biotransformation pathways

Hypoxic conditions can ultimately affect hormonal status in fish through interference with steroidogenic enzymes (Cheek et al., 2009). CYP1 and CYP3 are central enzymes in steroid hormone metabolism and synthesis, regulating estrogen and testosterone (Scornaienchi et al., 2010; Young, 2005). Elsewhere, hypoxia produced significant decrease in hepatic CYP1A mRNA, protein levels and enzyme activity (du Souich and Fradette, 2011; Rahman and Thomas, 2012) potentially created by competition between hypoxia and AhR in competition for ARNT (Fleming et al., 2009; Khan et al., 2007; Wenger, 2002; Zhang and Walker, 2007). In Paper III, we observed contradicting effect to previous findings showing that DFO exposure produced increased transcription of CYP1A1 and CYP3A in salmon hepatocytes, while CYP1A1 (EROD) and CYP1A2 (MROD) activity were unaffected. Juvenile cod displayed altered transcription of CYPs where CYP1A was increased throughout the experiment, while CYP3A only increased (albeit non-significant) after 6 days during hypercapnia. The discrepancy between transcription and enzyme activity is acknowledged and it is suggested that reduced CYP1A activity may be a post-transcriptional effect (Fradette et al., 2007). Regardless, transcriptional
alterations of CYPs indicate an effect related to AhR binding and activation. However, CYP3A is thought to be involved in regulation of lipid metabolism through production of 25-hydroxycholesterol, the rate-limiting step in lipid metabolism (Honda et al., 2011) and a potent activator of ERα (Lappano et al., 2011). CYP3A activity was not analyzed in Paper III, but increased transcription of CYP3A by hypoxia indicated a possible pathway that increases the observed estrogenic responses. Several CYP3A isoforms exposed to hypoxic conditions showed increased CYP3A transcript levels independent of pregnane–X receptor (PXR) modulation (Fradette and du Souich, 2003), which is in accordance with Paper III showing that PXR is increased at a lower level than CYP3A.

CYP1, 2 and 3 are important enzymes for xenobiotic biotransformation in fish liver (Monostory et al., 1996) and previous studies have observed increase of CYP transcription by PFAS (Hickey et al., 2009; Yeung et al., 2007). PFOSA can be converted to PFOS in fish liver and this reaction is suggested to be performed by CYP enzymes (Tomy et al., 2003). In Paper II, CYP1A transcription is slightly elevated at day 3 (200μg/L) and 6 (100μg/L). Paper III did not show effects of PFOSA in CYP transcription or activity in salmon hepatocytes. Marine medaka embryos exposed to PFOS showed a time-dependent increase of ARNT and CYP1A transcription (Fang et al., 2012), while a study with carp leukocytes reported that PFOS does not interact with CYP1A1 (Hu et al., 2003). These studies and our data suggest that regulation and activation of CYPs by xenobiotic compounds are time and concentration dependent and hard to predict due to confounding exogenous and endogenous variables. The effects of hypercapnia on biotransformation pathways are not well studied. However, HIF-1α and CYP1A are used as biomarkers for environmental exposure to hypoxia and POPs where the mode of action is through the AhR, respectively (Rahman and Thomas, 2012). A recent study investigating interactive effects between PCB-126 and hypoxia suggested that hypoxia play a possible role in xenobiotic metabolism, where AhR activation and CYP1A1 transcription was inhibited by combined exposure (Vorrink et al., 2014). The same study showed that biological processes regulated by HIF-1α were inhibited by PCB-126, potentially affecting adaptive responses during hypoxic conditions (Vorrink et al., 2014). We showed, in paper III, that CYP1A transcription was unaltered in combined treatment of hypoxia and PFOSA compared to control, suggesting that PFOS reduced the response of hypoxia in salmon hepatocytes. The same response in CYP1A mRNA expression was observed in juvenile cod (Paper II) after 3 days of exposure, while combined exposure produced similar response as hypercapnia alone after 6 and 9 days. However, CYP1A2 activity (MROD) was significantly increased by hypoxia and PFOSA together, but unaffected by single exposure to either stressor in vitro (Paper III). Our findings, in Paper III, and other studies have shown a decrease in CYPs when combining CYP activators and hypoxia compared to single exposure, suggesting potential interaction sites between these two signaling pathways and potentially between ERα as well (Bugel et al., 2010; Fleming et al., 2009). Combined treatment increased CYP3A similar to hypoxia or hypercapnia alone (Paper II and III), albeit not significant in juvenile cod, suggesting that hypoxic/hypercapnic responses in CYP3A transcription was unaffected by PFOS.
Summary

Addressing the aims;

1. Our data showed that hypercapnia and hypoxia increased the transcription of the antioxidant machinery in juvenile cod and salmon hepatocytes, respectively. Combined exposure regimes generally produced a higher response than hypercapnia and hypoxia alone. Statistics revealed interaction between CO$_2$ and PFOS on transcription of antioxidants, GpX1 and MnSOD in cod gill. Responses in gill and liver were observed at different times and the significant importance of exposure duration in antioxidant response was revealed statistically. The lack of data on physiological changes would be valuable to investigate further, such as changes in antioxidants enzyme activity, malondialdehyde (MDA) assay (marker for oxidative stress) and analyzes of TBARS (product of lipid peroxidation).

2. The measures of climate changes investigated herein produced effects on the PPAR system, observed as increased transcription of all PPAR isoforms by DFO in salmon hepatocytes and of PPAR$\beta$ by increased CO$_2$ in cod liver. The responses of hypercapnia or hypoxia were modulated variably in the presence of PFASs. PPAR isoforms were modified differently between combined exposure regimes, indicating complex changes in FA regulation. Altered FA $\beta$-oxidation from combined exposure treatment was suggested because of increased transcription of ACOX and ACOD in salmon hepatocytes and cod, respectively. Increased $\omega$-6: $\omega$-3 ratio was observed by combined and single exposure of hypoxia and PFOSA dependent on concentration in primary salmon hepatocytes. CO$_2$ reduced PEMT transcription in cod liver alone and in the presence of PFOS. These findings indicate that stressors affect composition of lipids important for proper function in cellular membranes. Measuring membrane stability and fluidity would be useful to investigate if there is a link between transcriptional responses and altered lipid profiles to physiological effects and possibly detrimental harm.

3. We observed that hypercapnia and hypoxia increased transcription of E2-responsive genes alone and in the presence of PFOS or PFOSA, respectively. E2 levels were increased by hypercapnia and combined exposure to PFOS produced higher response and also increased T and 11-KT level. Changes in CYP transcription were observed from hypercapnia and hypoxia exposure, and combined CoCl$_2$ and PFOSA increased CYP1A2 activity. These findings indicate that regulation of endocrine signaling and biotransformation is complex, and that interaction between nuclear receptors ER$\alpha$, HIF1$\alpha$ and AhR may be involved during combined exposure treatment. Changes in sex steroid levels can affect processes, such as sex differentiation, accelerate the onset of puberty in juvenile fish or impair reproduction. Our data showed that hypercapnia and hypoxia alone and in the presence of PFASs produced effects on endocrine responses that may cause adverse effects in cod and salmon. Hence, further investigation should be performed to verify potential risk of developmental and reproductive disruption with potential detrimental effects to fish from expected ocean acidification scenarios in the presence of environmental contaminants.
Potential implications and future perspectives

Changes in PPAR system leading to higher metabolism may increase generation of ROS, suggesting a potential link between these two pathways. On the other hand, hypoxic responses and dealing with oxidative stress are energy demanding processes that may affect PPAR expression and regulation. Changes in lipid homeostasis and effects of oxidative stress (lipid peroxidation) can produce detrimental effects in cellular membrane, changing the lipid composition or affecting components in the membrane. Increased stress is generally associated with detrimental effects on development and reproduction affecting sex steroid system. Increased endocrine responses, such as production of Vtg, ZP and ZRP protein demand energy and could also affect regulation of FA regulation. Interaction between hypoxic responses and xenobiotic biotransformation is thought to occur through competition between HIF-1α and AhR for ARNT binding. However, crosstalk between ERα and AhR can affects steroidogenesis through transcriptional changes of xenobiotic or estrogen responsive genes. PFASs are thought to have endocrine disruptive toxicity by interacting with ERα, and CYP enzymes metabolize PFOSA possibly activated by PFOSA binding to AhR, suggesting effects on several pathways from PFASs exposure and the potential for interaction between ER and AhR. Hypoxic responses may further complicate the understanding of potential interactions.

Combined exposure treatments generally produced a higher response than single exposure. Furthermore, parameters of climate change (hypercapnia and hypoxia) generally produce higher responses than PFASs (PFOS and PFOSA), and multivariate analysis show that exposure duration is an important factor when evaluating stressor responses. Statistical interaction between PFOS and CO₂ were revealed for oxidative stress responses and regulation of membrane lipids. These findings indicate that combined exposure scenarios may produce vast changes in fish that are difficult to predict without combined experimental studies. However, investigating changes in physiological and biological function that may follow from the observed molecular alterations observed herein is necessary for a better understanding of possible adverse effect from the stressors investigated. Our data show that exposure duration significantly affected the effects of stressors in fish, suggesting that investigating chronic exposure to ocean acidification and multiple environmental stressors could provide valuable insight of toxicity by multiple environmental stressors in aquatic organisms in a future perspective.
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Paper I
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Paper II
Effects of elevated dissolved carbon dioxide and perfluorooctane sulfonic acid, given singly and in combination, on steroidogenic and biotransformation pathways of Atlantic cod

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A B S T R A C T

In the aquatic environments, the predicted changes in water temperature, pCO2 and pCO2 could result in hypercapnic and hypoxic conditions for aquatic animals. These conditions are thought to affect several basic cellular and physiological mechanisms. Yet, possible adverse effects of elevated CO2 (hypercapnia) on teleost fish, as well as combined effects with emerging and legacy environmental contaminants are poorly investigated. In this study, juvenile Atlantic cod (Gadus morhua) were divided into groups and exposed to three different water bath PFOS exposure regimes (0 control), 100 and 200 μg L−1) for 5 days at 1 h/day, followed by three different CO2 levels (normocapnia, moderate (0.3%) and high (0.9%) to the moderate CO2 level is the predicted near future (within year 2300) level, while 0.9% represent severe hypercapnia. Tissue samples were collected at 3, 6 and 9 days after initiated CO2 exposure. Effects on the endocrine and biotransformation systems were examined by analyzing levels of sex steroid hormones (E2, T, 11-KT) and transcript expression of estrogen responsive genes (Eko, Veg-u, Veg-d, ZP2 and ZP3). In addition, transcripts for genes encoding xenobiotic metabolizing enzymes (cyp1a and cyp3a1) and hypoxia-inducible factor (HIF-1α) were analyzed. Hypercapnia alone produced increased levels of sex steroid hormones (E2, T, 11-KT) with concomitant mRNA level increase of estrogen responsive genes, while PFOS produced weak and time-dependent effects on E2-inducible gene transcription. Combined PFOS and hypercapnia exposure produced increased effects on sex steroid levels as compared to hypercapnia alone, with transcript expression patterns that are indicative of time-dependent interactive effects. Exposure to hypercapnia singly or in combination with PFOS produced modulations of the biotransformation and hypoxic responses that were apparently concentration- and time-dependent. Loading plots of principal component analysis (PAC) produced a significant grouping of individual scores according to the exposure scenarios at day 6 and 9. Overall, the PCA analysis produced a unique clustering of variables that signifies a positive correlation between exposure to high PFOS concentration and mRNA expression of E2 responsive genes. Notably, this pattern was not evident for individuals exposed to PFOS concentrations in combination with elevated CO2 scenarios. To our knowledge, the present study is the first of its kind, to evaluate such effects using combined exposure to a perfluoroalkyl sulfonate and elevated levels of CO2 saturation, representative of future oceanic climate change, in any fish species or lower vertebrate.

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1. Introduction

Aquatic organisms are exposed to several emerging environmental stressors due to anthropogenic activities that include release of emerging contaminants and increased carbon dioxide (CO2) emissions, climate change and ocean acidification (Schiedek et al., 2007). The concern for interactive effects between climate change and environmental toxicants is also gaining increased attention (Jensen, 2006; Noyes et al., 2009; Schiedek et al., 2007), yet studies of how elevated levels of dissolved CO2 (pCO2) could modulate the physiological responses of aquatic species to environment contaminants are limited or non-existent. Anthropogenic
emissions of CO₂ have increased dramatically since the industrial revolution, resulting in a rise in atmospheric CO₂ concentrations of approximately 280–380 ppm (Turley et al., 2006), and rates of CO₂ emissions are still rising (Canadell et al., 2007). Increased aquatic CO₂ saturation (environmental hypercapnia) and ocean acidification are estimated to be a result of 40–50% of post-industrial CO₂ emissions that have been taken up by the oceans (Sabine et al., 2004; Zebe et al., 2008). Compared to pre-industrial values, surface ocean pH has already decreased by about 0.1 units, from a global average level of 8.17–8.07 (Cao et al., 2007).

Considering the modeling of various future scenarios of anthropogenic CO₂ emissions, pH levels are predicted to be reduced further by 0.2–0.4 units by the end of this century and 0.4–0.9 units within years up to 2300 (Caldeira and Wickett, 2003, 2005). Studies on the consequences to calcifying marine organisms have dominated, and the knowledge regarding the consequences of ocean acidification for teleosts, and especially marine species, is more limited (Ishimatsu et al., 2008). It is hypothesized that physiological effects are mainly due to increased exposure to CO₂ rather than lower ambient pH (Ishimatsu et al., 2004). Teleost species appear to adapt well to prolonged elevation of CO₂ saturations through acid-base regulation and by increasing ventilation frequencies, thereby avoiding internal acidosis (Ishimatsu et al., 2005, 2008). However, this can alter the steady-state of ions in body fluids (Hayashi et al., 2008), as well as increase energetic costs (Ishimatsu et al., 2008). Evidence of negative consequences on fitness from exposure to near future CO₂ levels have been observed in fish (Munday et al., 2010), and early life stages may be more sensitive (Baumann et al., 2012; Forsgren et al., 2013). So far, there have been mixed results from several studies (Baumann et al., 2012; Frommel et al., 2012; Munday et al., 2011). Long-term hypercapnia exposure studies have indicated general health effects such as reduced condition and growth (Ishimatsu et al., 2005, 2008).

Among emerging persistent organic pollutants (POPs), per- and polyfluorinated alkyl substances (PFAS) have gained increased attention in recent years (Houde et al., 2011; Muir and Howard, 2008). PFAS are synthetically produced and used in numerous consumer products and for industrial purposes because of their unique physicochemical properties (Buck et al., 2012; Paul et al., 2008). They are detected globally in the environment and biota, where perfluorooctane sulfonic acid (or sulfate) (PFOS) is the most concentrated PFAS (Kamn, 2011) due to its chemical persistence and tendency to bioaccumulate and biomagnify (Conder et al., 2008). PFOS exposure has been associated with numerous adverse health effects, including endocrine disruption (Lau et al., 2007; Oakes et al., 2005). Sex steroid hormones (testosterone: T, 11-ketotestosterone: 11-KT and 17β-estradiol: E2) control fundamental processes related to sexual differentiation, gametogenesis, reproduction and behavior in teleost species (Arcand-Hoy and Benson, 1998; Young et al., 2005). For example, E2 modulates gene expression through interaction with the estrogen receptor (ER), where the ERα isomorph is the best studied subtype (Menuet et al., 2005). Although a role in male reproduction has been suggested (Bounia and Nagler, 2001), E2 is mostly associated with female sexual development, reproduction responses and behavior (Arcand-Hoy and Benson, 1998; Young et al., 2005).

Hepatic synthesis of proteins involved in oocyte development, including egg yolk precursor proteins (vitellogenins; Vtg) and egg shell proteins (zona pellucida proteins; ZP, also commonly called zona radiata proteins), are among the best understood E2-mediated responses in teleosts (Arukwe and Goksky, 2003; Menuet et al., 2005). E2 also autoregulates the expression of ER (Menuet et al., 2005). Expression of these genes has become established biomarkers for estrogenic responses (Arukwe and Goksky, 2003; Yadetie et al., 1999). Reproduction and the endocrine system of fish might be susceptible toward both endocrine disrupting chemicals (EDCs) (Arcand-Hoy and Benson, 1998), multiple climatic and environmental stressors (Baroller and D’Cotta, 2001; Schreck et al., 2001). PFOS has previously been found to affect endocrine parameters, sexual development and reproduction in fish (Ankley et al., 2005; Fang et al., 2012; Mortensen et al., 2011; Oakes et al., 2005; Wang et al., 2011). However, to our knowledge there are no studies that have examined in fish how elevated pCO₂ might modulate the response to PFOS exposure on hormonal and biotransformation systems. Interestingly, the closely related environmental state of lowered oxygen saturation (hypoxia) has been associated with such effects in fish (Shang et al., 2006; Wu, 2009; Wu et al., 2003).

External hypoxia and hypercapnia share some similarities as both initially disturb the O₂/CO₂ balance in fish, and external hypercapnia has been suggested to cause internal hypoxia (Michaeldis et al., 2007). Hypoxia produces the stabilization of hypoxia-inducible factor (HIF) and subsequent up-regulation of HIF-1α (or aryl hydrocarbon receptor nuclear translocator: arnt) to form HIF-1, a transcription factor that modulates the expression of a variety of genes (Wenger, 2002). The arnt is a heterodimerization partner to the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that belongs to the helix-loop-helix-PAS (bHLH-PAS-sim) family of gene regulatory proteins. The AhR-arnit complex translocates to the nucleus where it transactivates transcription of genes containing XRE (xenobiotic responsive elements) in their upstream regions, including increases in the expression of cytochrome P450s. Thus, both HIF-1α and AhR compete for arnt, and consequently, hypoxia has been shown to decrease the expression of cytochrome P450s (Zhang and Walker, 2007; Khan et al., 2007), which are involved in steroidsogenesis (both in metabolism and synthesis). Therefore, the aim of the present study was to investigate the potential endocrine disrupting- and xenobiotic biotransformation effects of hypercapnia and PFOS, given singly and in combination. Our hypothesis is that exposure of juvenile Atlantic cod to elevated CO₂ levels will produce alterations in the hormonal and xenobiotic biotransformation pathways, and that these effects will be potentiated by combined exposure with PFOS and be valuable in deducing molecular mechanisms of effect or mode of action. These effects were analyzed by measuring muscle tissue sex steroid levels and transcriptional expression of genes involved in estrogenic responses, steroid- and xenobiotic metabolism and hypoxic stress.

2. Materials and methods

2.1. Chemicals and reagents

Perfluorooctane sulfonate acid (PFOS; linear, technical grade) was purchased from Alfa Aesar (Karlsruhe, Germany). Tricaine mesylate (MS-222) was purchased from Norsk Medisinaldepot AS. TRizol reagent was purchased from Gibco-Invitrogen Life Technologies (Carlsbad, CA, USA). ScryptTM DNA synthesis kit, Taq DNA polymerase, dNTP mix, Taq™ SYBR® Green supermix with ROX and EZ Load 100 bp Molecular Ruler were purchased from Bio-Rad Laboratories (Hercules, CA, USA). GelRed™ Nuclear Acid Gel Stain was purchased from Biotium (Hayward, CA, USA). Enzyme immune- assays for 17β-estradiol (Cat. No. 582251), testosterone (Cat. No. 582701) and 11-ketotestosterone (Cat. No. 582751) were purchased from Cayman chemical company (Ann Arbor, MI, USA).

2.2. Animals

Juvenile Atlantic cod (length 8.8 ± 0.7 cm, weight 4.4 ± 1.1 g) were purchased from Atlantic Cod Juveniles (Rissa, Norway). Fish were kept at the animal holding facilities at the Norwegian University of Science and Technology (NTNU) Centre of Fisheries and
Aquaculture (Seabao) in circulating seawater from the Trondheim fjord with a flow-through of 0.3 L min⁻¹ kg⁻¹ fish. The fish were acclimatized to a water temperature of 10 °C and 12:12 light:dark photoperiod for 2 weeks prior to the period and received no food during the acclimatization and exposure periods.

2.3. Exposure and sampling

The fish were first exposed to nominal PFOS (i.e., 0, 100 and 200 μg PFOS/L seawater) concentrations for 1 h/day⁻¹ over a 5-day period in 3 different tanks of 120 fish per tank. After termination of the PFOS exposure, fish from each group were further exposed to three different CO₂ exposure regimes (normocapnia, moderate and severe hypercapnia) of 40 fish/CO₂ exposure groups. This was achieved by introducing gas mixtures containing, either 0 (normocapnia), 0.3% (moderate hypercapnia) or 0.9% (severe hypercapnia) CO₂ into the water. Tank water pH was measured continuously to ensure a correct and stable pH. Biological samples were collected after 3, 6 and 9 days of CO₂ exposure. Fish were anesthetized using tricaine mesylate (MS-222) prior to sampling. Length and weight were measured before organs (including liver) and carcasses were collected for further analyses. At each sampling time, 5 individuals were sampled from each exposure group for parallel analysis of PFOS burden, steroid hormone and gene expression (in here and later the phrase gene expression is used synonymously to gene transcription, although it is acknowledged that additionally, e.g., translation and protein stability regulate gene expression) levels.

2.4. Chemical analyses

Accumulated burdens of PFOS were analyzed in carcasses (head, abdomen, brain and inner organs removed) of fish from the normocapnia PFOS exposure group. Concentrations were determined by high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC/MS/MS). Data were processed using Masslynx software (v4.0). Quantitative analysis was performed using the isotope dilution method with MPPFS as internal standard, a five-point calibration curve (0–400 ng/mL) for the analyze (PFOS) and a fixed concentration (20 ng/mL) of internal standard was used. The procedure for sample work-up and HPLC–MS/MS analysis are previously described by Mortensen et al. (2011) and Chu and Letcher (2009). Water CO₂ saturation during the CO₂ exposure period was estimated based on measured pH using the CO₂calc (Robbins et al., 2010), assuming a salinity of 33.8 ‰, total alkalinity of 2223 μmol kg⁻¹, a temperature of 10 °C and atmospheric pressure of 1013 hPa.

2.5. Steroid hormone extraction and analysis

In this study, steroid hormones were measured in muscle tissue of exposed fish due to the small size of the experimental fish that made it difficult to obtain enough plasma sample for the analysis. Measurement of sex steroids in whole-body homogenate has been successfully applied in our laboratory previously (Arsköved et al., 2008). In addition, alternative and non-invasive methods of analyzing steroid hormones in holding water has been successfully applied in several fish species (Sebire et al., 2007, 2009; Scott and Ellis, 2007; Felix et al., 2013). The great interest in the measurement of steroid concentrations in water or tissue homogenates, rather than in blood plasma is based on the concept that the pattern of release of the steroid matched its pattern of secretion in the plasma (Sebire et al., 2007) and it has been shown that male and female fish of many species release a wide range of sex- and stress-related steroids in free, glucuronidated and sulphated forms into the water (Sebire et al., 2007, 2009; Scott and Ellis, 2007; Felix et al., 2013). Estradiol-17β (E2), testosterone (T) and 11-ketotestosterone (11-KT) concentrations were measured in muscle tissue using enzyme immunoassay (EIA) kits from Cayman Chemical Company (Ann Arbor, MI, USA). Muscle tissue was homogenized in a 0.1 M sodium-phosphate–buffer (pH 7.4) in a volume ratio of 1:4, using a Glass–Col homogenizer (Glass-Col, Terre Haute, IN, USA) with a glass tube and a Teflon pistil. Homogenate was centrifuged (14,000 × g, 15 min, 4 °C). Supernatant (800 μL) was transferred to glass tubes for steroid hormone extraction with organic solvent. Briefly, the aqueous supernatant was thoroughly mixed with diethyl ether (4 mL) by vortexing, then the two phases were left to separate. The aqueous phase was frozen in an ethanol/dry ice bath, the steroid-containing ether phase decanted into new glass tubes and evaporated at 30 °C in a nitrogen atmosphere using a TurboVap LV Concentration Workstation sample concentrator (Caliper Life Sciences, Hopkinton, MA, USA). Dry extracts were re-suspended in 300 μL EIA buffer. Dissolved extracts were stored at −80 °C until analysis. E2, T and 11-KT were measured with EIA kits (Cayman) according to the manufacturer’s protocol. Absorbance was read at 405 nm using a Bio-Tek Synergy HT microplate reader (Bio-Tek instruments, Winooski, VT, USA). Standard curves were prepared in SigmaPlot, version 12.3 (Systat Software, 2012), using a 4-parameter logistic fit plotting the 3B/B₀ (sample bound/maximum bound) versus log concentrations.

2.6. Quantitative (real-time) PCR

Liver samples were homogenized in TRIzol reagent for total RNA isolation (Gibco-Invitrogen Life Technologies). Total cDNA was generated from 1 μg total RNA using a combination of oligo(dT) and random hexamer primers from iScript cDNA synthesis kit, as described by the manufacturer (Bio-Rad). Real-time PCR were performed with gene-specific primers (Table 1), using the Mx3000P real-time PCR system (Stratagene, La Jolla, CA) and MxProTM QPCR software. Each 25-μL DNA amplification reaction contained 12.5 μL iTaql SYBR® Green supermix with ROX (Bio-Rad), 0.83 μL cDNA and 200 μM of each of forward and reverse primers. The three-step real-time PCR program included an enzyme activation step at 95 °C (3 min) and 40 cycles of 95 °C (30 s), 60 °C (15 s) and 72 °C (15 s). Controls lacking cDNA template were included to determine specificity of target cDNA amplification. Cycle threshold (CT) values obtained from all target genes were converted into relative copy number using the same pre-made standard plot of Ct versus log copy number.

2.7. Statistical analyses

Statistical analyses were performed using SPSS Statistics software, v20.0 (IBM, 2012). Datasets were, if necessary, normalized using natural log (In) or square root transformations. Homoscedastic datasets were investigated using one-way ANOVA followed by Tukey’s post hoc multiple comparison test. Heteroscedastic datasets and/or datasets with missing values were examined using the robust Welch test of equality of means and Games-Howell post hoc test. The level of statistical significance was set to η = 0.05. As the combination of the high internal variation, low n lowered the statistical power to detect differences during all pairwise comparisons, borderline significance (0.10 < p < 0.05) were included (as previously discussed by Hackshaw and Kirkwood (2011)). Single measurements or deviating individual responses that were removed, as outliers from presented data were first identified using box-and-whiskers plots and further evaluated with Grubbs test. Multivariate data analysis was performed using Simca-P®, v12.0 (Umetrics AB, 2008). Principal component analysis (PCA) models were made separately for each sampling day. Gene transcript levels and steroid hormone levels, in addition to estrogen/androgen ratio and condition factor (CF = weight/length³ + 100), were included in
the models. Variables were centered and scaled to unit variance, and log transformed if necessary. Outliers were identified using the Hotelling T2 95% range and removed when the single observation appeared to cause major effects on the overall model. Explained variation (R²) and predicted variation (Q²) were calculated for each principal component (PC). Between various exposure scenarios, significant differences in individual scores along each PC were verified by variance analysis on extracted component scores.

3. Results

3.1. Experimental validation

No significant (p < 0.1) differences in survival and growth maintenance (length, weight, condition factor) between exposure groups and sampling days were observed. Fasting and exposure-related stress therefore did not appear to exert any considerable negative effect on the overall physiological condition of the experimental fish, which otherwise could have biased the hormonal responses assessed in the study. LC-ESI(−)-MS/MS analysis verified that PFOS readily accumulated during the short-time exposure regime, and that high burdens of PFOS were maintained in the fish during the post-exposure sampling period (Table 2). No significant (p < 0.1) alterations in PFOS burdens were observed throughout the CO₂ exposure period. However, the data presented in this paper will be expressed on the nominal PFOS concentration levels. At onset of the CO₂ exposure scenarios, tank water pH (Fig. 1) decreased by approximately 0.1 unit in all CO₂ scenario tanks as fish were being added, presumably due to the added release of CO₂ by respiration. During the exposure period, variation in water pH and estimated CO₂ saturation was negligible between the three water tanks of normal CO₂ saturation. Within the moderately (0.3%) and highly (0.9%) increased CO₂ scenarios, this internal variation was slightly higher. Mean pH was measured to be 7.70 (0%), 7.20 (0.3%) and 6.73 (0.9%), corresponding to a drop in pH of approximately 0.5 and 1.0 unit for the moderate and high CO₂ level scenarios, respectively.

3.2. Effects on sex steroid hormones and estrogenic responses

During steroid hormone analysis, samples from day 6 were split between two EIA well plates required to analyze each hormone, due to the high number of individuals. As there were indications of poor comparability in the exact concentrations given by the two standard curves, day 6 results were omitted from the final results. Further interpretations have focused on relative differences between exposure groups within each sampling day, rather than the absolute concentrations of steroid hormones.

Severe hypercapnia (0.9%) increased cellular E2 levels, compared to the control (i.e. no PFOS group) at day 3 and, in an apparent pCO₂-dependent manner, at day 9 (Fig. 2A). Exposure to PFOS alone had no significant effects on E2 levels, but at the combined exposure with 0.9% CO₂, the seemingly concentration-dependent effects produced by hypercapnia alone, was significantly increased at day 9 with 200 µg PFOS (Fig. 2A). Similarly, testosterone (T) levels increased in an apparent pCO₂-dependent manner during severe hypercapnia at day 3 and 9, although not statistically significant.
PFOs exposure had no significant effects on T levels, when given alone. Exposure to combined 100 µg PFOs and 0.3% pCO₂ produced a significant increase in T levels at day 3, while at day 9, T levels resembled the observed effects of severe hypercapnia (0.9%) exposure alone. Levels of 11-KT were not significantly altered by any exposure scenario at day 3, but were apparently increased by severe hypercapnia (0.9%) at day 9 (Fig. 2C). PFOs exposure alone did not produce significant effects on cellular 11-KT levels. 11-KT measured after combined exposure to increased CO₂ (both moderate and severe hypercapnia) and PFOs resembled effects of CO₂ exposure alone, with combined 0.9% CO₂ and 100 µg PFOS L⁻¹ significantly increasing 11-KT. The total estrogen-to-androgen ratio (E2 to T + 11-KT) showed comparable effects to the scenarios observed when these variables were measured individually, but were not statistically significant (Fig. 3).

Moderate hypercapnia (0.3% CO₂) increased hepatic ERe mRNA expression (Fig. 4) with borderline significance, compared to the control group at day 6, and severe (0.9%) compared to moderate hypercapnia, at day 9. PFOs exposure had no significant effects on ERe transcription during normocapnia exposure, while in the combined PFOs and hypercapnia (both moderate and severe) exposures, the ERe transcript were higher (albeit not significant) than during hypercapnia exposures alone, at day 6 (Fig. 4). The expression of Vtg-α (Fig. 5A) and Vtg-β (Fig. 5B) mRNA showed comparable expression patterns, displaying minor significant variations between the different exposure groups, except from the group exposed to 100 µg PFOS L⁻¹ alone which produced a borderline significant increase of Vtg-β transcripts at day 3. Although not statistically significant, a tendency toward reduction of transcription with increasing PFOs concentration and hypercapnia were observed for both Vtg subunit genes at sampling day 6 (Fig. 5A and B). These included increased transcript levels during elevated CO₂ saturation in the absence of PFOs, a seemingly antagonistic effect of combined PFOs and elevated CO₂ exposure at day 6, and a time-and concentration-dependent effect of PFOs during normal CO₂ saturation where 100 µg PFOS L⁻¹ increased transcript levels at day 6 and 200 µg PFOS L⁻¹ day 9 (Fig. 5).

ZP-2 mRNA levels (Fig. 6A) were significantly increased by severe hypercapnia (0.9% CO₂) and 200 µg PFOs exposure concentrations at day 3, as compared to the control group. At day 6, an apparent reduction effect between PFOs concentrations and elevated CO₂ (moderate and severe hypercapnia) were observed. At day 9, hypercapnia alone increased ZP-2 mRNA expression with borderline significance, while exposure to 200 µg PFOs alone, significantly increased ZP-2 transcript expression, compared to the control group. Combined PFOs and CO₂ exposures produced increased ZP-2 transcript levels, compared to the control, resembling the effects of hypercapnia alone. Borderline significance was observed in the 200 µg PFOs with 0.5% CO₂ exposure group. Transcriptional expression of ZP-3 (Fig. 6B) did not display any significant alterations following the exposure regimes except for day 9, where transcript levels in the 100 µg PFOS/0.9% CO₂ group exceeded the mRNA levels in groups exposed to PFOs and 0.3% CO₂ saturation combined (Fig. 6B). Overall, genes for the two ZP isoforms showed differential response patterns, with ZP2 sharing similarity with Vtg isoform genes, after exposure to PFOs singly or in combination with hypercapnia.

3.3. Effects on xenobiotic and steroid metabolizing system and hypoxic pathway

Exposure to hypercapnia alone increased cp1A1 mRNA expression in a concentration-dependent manner at both day 3, 6 and 9, compared to the control (Fig. 7A). This mRNA induction was extensive at day 3, and gradually decreased thereafter with time. Exposure to PFOs concentrations alone had no significant effects
on cyp1A transcription, and combined exposure to both PFOS concentrations and hypercapnia (moderate and severe) produced transcriptional changes that largely resembled those produced by hypercapnia exposure alone (Fig. 7A). For cyp3a mRNA expression, apparent concentration-dependent increases (albeit not significant) were observed after exposure to moderate and severe hypercapnia at day 6 (and also day 9; Fig. 7B). These effects were sustained in the presence of PFOS concentrations. The HIF-1α mRNA expression (Fig. 8) was significantly increased by moderate and severe hypercapnia in an apparent pCO2-dependent manner at day 6, as compared to the control (no PFOS, normal CO2). When CO2 and PFOS exposure were combined, this pattern was no longer observed (Fig. 8).

3.4. Multivariate data analysis

Loading plots of principal component analysis (PCA) produced significant grouping of individual scores according to the exposure scenarios at days 6 and 9. At day 6 (Fig. 9A), observations from combined PFOS + pCO2 exposure were situated opposite to the Vtg- and ZP-2 loadings along PC1. These individuals clustered according to nominal PFOS concentration, with 200 μg PFOS + CO2 scoring significantly higher than both the 100 μg PFOS + CO2 group (p = 0.001) and the remaining single exposure groups (p = 0.001). Scores from single exposure to either PFOS or hypercapnia were more scattered, but in general, located closer around the Vtg/ ZP-2 loadings. These patterns indicate that when exposures were combined, PFOS and hypercapnia interacted negatively with increasing PFOS concentration on the expression of Vtg- and ZP-2 at this particular day of sampling. The other E2-inducible genes did not cluster with Vtg/ZP-2, and ERα, cyp1a, cyp3a and HIF-1α loaded approximately at the opposite side of the bi-plot. This could indicate some kind of negative correlation between the responses of Vtg/ZP-2 and ERα, cyp1a, cyp3a after the various exposure regimes. However, ERα was not very distinctly modeled in the PCA, as seen by the moderate loading along both principal components (PCs).

At day 9 (Fig. 9B), scores from all three CO2 scenarios were significantly separated along the first PC with increasing CO2 saturation (p = 0.0000–0.029), revealing both elevated levels of sex steroids including E2 and cyp1a, and also a concomitant increase in transcription of the E2-responsive genes Vtg-α, Vtg-β and ZP-2 as CO2 saturation increased. The highest CO2 saturation group (i.e. severe hypercapnia) also scored significantly higher than both the normal (p = 0.036) and moderately elevated CO2 groups (p = 0.039) along PC3, signifying particularly high levels of E2 as well as higher expression of ERα and cyp1A. The E2 loading correlates positively with both the Vtg/ZP-2 clustered along PC1 and ERα along PC3. Within the normocapnia group, the individuals exposed to

![Fig. 3. The ratio of estrogen to androgen concentrations in the exposed juvenile Atlantic cod (Gadus morhua) based on 17β-estradiol (E2), testosterone (T) and 11-ketotestosterone (11-KT) presented in Fig. 2.](image)

![Fig. 4. Hepatic levels of mRNA of the gene encoding estrogen receptor α (ER-α) in juvenile Atlantic cod (Gadus morhua) after exposure to the various combinations of PFOS (0, 100 and 200 μg L⁻¹) and altered water CO2 saturation (0, 0.3 and 0.9% increase in CO2). Day 3, 6 and 9 corresponds to days into the CO2 exposure period. Messenger RNA (mRNA) levels were analyzed by real-time PCR. Data are presented as percentage (%) of control (i.e. no PFOS, normal CO2 group) and based on mean values (n = 5) ± SEM. Different letters indicate significant differences between exposure groups (p < 0.05). Asterisks (*) denote borderline significance (0.10 < p < 0.05).](image)
200 µg PFOS L⁻¹ scored significantly higher along PC1 (p=0.001), yet lower than the exposure groups exposed to severe CO₂ saturation (p=0.006). This clustering signifies a positive correlation between exposure to high concentrations of PFOS and the expression of E2 responsive genes. Notably, this pattern was not evident for individuals exposed to PFOS in combination with elevated CO₂ scenarios (Fig. 5B).

4. Discussion

Changes in water temperature, pO₂, and pCO₂ can induce hypoxic and hypercapnic conditions in aquatic animals. These are climatic conditions that are thought to affect several basic cellular and physiological mechanisms. Yet, possible adverse effects of elevated CO₂ (hypercapnia) in combination with other environmental stresses on teleost fish have scarcely been investigated. The continued elevation of oceanic CO₂ saturation is inevitable given the anthropogenic emissions scenarios predicted for the coming years (Caldeira and Wickett, 2003, 2005). Increased knowledge of physiological implications from environmental hypercapnia is therefore needed for all aquatic organisms. Despite current acknowledgment of possible adverse population effects of endocrine disruptors (Arcand-Hoy and Benson, 1998; Jenssen, 2006), studies on how hypercapnia may induce effects on sex steroid hormone system and possibly overt reproduction in aquatic organisms are apparently absent from current scientific literature. Furthermore, combined effects of elevated aquatic CO₂ saturations and emerging or legacy POPs, including the highly bioaccumulative PFOS, also constitute a knowledge gap in the literature. To our knowledge, the present study is the first of its kind to evaluate such effects using combined exposure to a PFAS and elevated levels of CO₂ saturation in any fish species or lower vertebrate.

4.1. Effects of hypercapnia

In the present study, pH in the normocapnia tank water was slightly below what is considered normal values for surface ocean water (pH 8.1 ± 0.3; Turley et al. (2006)). When considering relative alterations in pH, the 0.5 unit decrease in pH in the moderate hypercapnia exposure represents ocean acidification predicted to
Fig. 6. mRNA of the gene encoding zona pellucida protein 2 (ZP-2: A) and 3 (ZP-3: B) in hepatic tissue of juvenile Atlantic cod (Gadus morhua) after exposure to the various combinations of PFOS (0, 100 and 200 μg L⁻¹) and altered water CO₂ saturation (0.3 and 0.9% increase in CO₂). Day 3, 6 and 9 corresponds to days into the CO₂ exposure period. Messenger RNA (mRNA) levels were analyzed by real-time PCR. Data are presented as percentage (%) of control (i.e. the no PFOS, normal CO₂ group) and based on mean values (n=5) ± SEM. Different letters indicate significant differences between exposure groups (p=0.05). Asterisk (*) denotes borderline significance (0.10 < p=0.05).

occurred within year 2300 (further decrease of 0.4–0.9 pH units), while the 1.0 unit pH decrease in the high CO₂ scenario (severe hypercapnia) represents a more extreme level of acidification (Caldeira and Wickett, 2003, 2005). The experimental fish were expected to acclimatize to the altered CO₂ saturation in terms of internal pH by the time sampling was initiated (i.e. 3 days into the CO₂ exposure), and at least toward the end of the entire exposure period. This assumption was based on studies of acid–base regulatory capacity of several marine teleosts, including Atlantic cod, exposed to comparable levels of hypercapnia (Hayashi et al., 2004; Larsen et al., 1997). The observed effects in the present study may therefore, to a certain extent, represent environmentally relevant consequences of prolonged external hypercapnia. Hypercapnia alone produced significant elevation of cellular E2 levels in an apparent CO₂ saturation-dependent response pattern. A similar response was also apparent for the androgens analyzed, namely T and 11-KT. The increased expression of E2-responsive transcripts were in accordance with elevated levels of cellular E2, suggesting that the altered sex steroid levels may directly be associated with biological effects in the hypercapnia-exposed fish. Indeed, this effect paralleled hypercapnia-dependent modulation of cyp1a expression (cyp1a at day 6 and 9), singly and also in combination with PFOS. These patterns of effects were increasingly evident throughout the CO₂-exposure period, further emphasizing that the observed effects on the sex steroid and biotransformation systems may represent long-term, rather than transient responses to alteration in CO₂ saturation. Although the effects of hypercapnia were interesting, they were unexpected for the following reasons. Firstly, external hypercapnia is considered a stressor to fish (Cech and Crocker, 2002; Fivelstad et al., 1999), and stress responses are 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). Secondly, both field and laboratory studies have shown that hypoxia, a condition that is related to greater concentrations of CO₂, causes endocrine disruptive effects, such as decreases in concentrations of sex steroid levels (Wu, 2005). However, endocrine responses to stressors may be biphasic with directionality depending on the severity of the...
However, et studies were nia and possible secondary challenge or exposure dose (Schreck, 2010). Stress has indeed also been reported to accelerate reproduction processes in fish, apparently dependent on the fish species, maturational stage and the severity of stress (Schreck et al., 2001). A few exceptions of time- and sex-specific elevations in sex steroid levels in fish have also been reported following hypoxia exposure (Shang et al., 2006; Wu et al., 2003). However, any direct comparison between hypoxia studies and the present study will be difficult to make, as it was not possible to neither ascertain nor disprove any induction of internal hypoxia due to external hypercapnia in the present study. This assumption is supported by the fact that HIF-1α transcript levels were elevated in a CO2 saturation-dependent manner at day 6. However, the transient nature and long lag-time from hypercapnia onset until HIF-1α transcriptional response were indicative of a secondary rather than primary response to altered CO2 saturation. Furthermore, 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).

There is little research information about the response of fishes and other non-calcifying marine organisms to increases in the level of dissolved CO2 and reduced sea water pH that are predicted to occur over the coming century. In the orange clownfish, Amphiprion percula, elevated dissolved CO2 and reduced pH did not produce any effect on the maximum swimming speed of settlement-stage larvae, but there was, however, a weak positive relationship between length and swimming speed in the same fish, suggesting that levels of ocean acidification likely to be experienced in the near future might not, in isolation, produce significant growth and performance effects of larvae from benthic-spawning marine fishes (Munday et al., 2009). In another study, Forsgren et al. (2013), reported that, while elevated CO2 did not effect either the occurrence of spawning or clutch size, it did increase embryonic abnormalities, egg loss and significantly affected the phototactic response of

Fig. 7. Hepatic levels of cytochrome cyp1a (A) and cyp3a (B) mRNA in juvenile Atlantic cod (Gadus morhua) after exposure to the various combinations of PFOS (0, 100 and 200 μg L⁻¹) and altered water CO2 saturation (0, 0.1 and 0.9% increase in CO2). Day 3, 6 and 9 corresponds to days into the CO2 exposure period. Messenger RNA (mRNA) levels were analyzed by real-time PCR. Data are presented as percentage (%) of control (i.e. the no PFOS, normal CO2 group) and based on mean values (n = 5) ± SEM. Different letters indicate significant differences between exposure groups (p < 0.05). Asterisk (*) denotes borderline significance (0.10 < p < 0.05).
newly hatched larvae. On the mechanistic side, the causal relationship between external hypercapnia and elevated sex steroid levels and CYPs observed in the present study requires further investigation. Reduced steroid metabolism does not appear plausible given that transcript levels of hepatic cyp1a and cyp3A—central enzymes in steroid hormone metabolism (Scorinaienchi et al., 2010; Young et al., 2005) were either increased or remained at control levels during hypercapnia exposure. In mammalian systems, potential crosstalk indicate that hypoxia or hypoxia mimics are capable of reducing AhR activity measured as XRE reporter activity, CYP1A mRNA induction or EROD activity (Wenger, 2002; Zhang and Walker, 2007; Khan et al., 2007), which are involved in steroidogenesis (both in metabolism and synthesis). Our findings support the hypothesis for competition between HIF-1α and AhR for a shared pool of arnt and consequently interfere with the expression of AhR-regulated genes, in addition to other mechanisms of crosstalk that may also occur (Fleming et al., 2009). The cytochrome P450 system is involved in steroidogenesis (Arukwe and Goksøyr, 1997), and hypoxia may impair fish reproduction through this pathway, among other possible mechanisms of action.

Despite the classical roles in xenobiotic metabolism, the AhR is involved in several developmental processes, and functional interaction (or crosstalk) between AhR, endocrine systems and transforming growth factor β (TGF-β) (a member of TGF-β superfamily) has been reported (Gomez-Duran et al., 2005; Olufsen and Arukwe, 2011). Although we do acknowledge the potential discrepancy between mRNA expression and changes in protein and/or enzymes levels for biotransformation systems, as well as the presence of additional enzymes, an effect of hypercapnia on steroid synthesis rather than catabolism appears to be a more likely mode of action. Sex steroid hormones are synthesized in a shared pathway where T is precursor for both E2 and 11-KT (You et al., 2005). The apparent simultaneous increase in all three steroids could indicate that the effects of hypercapnia were exerted upstream of T synthesis in the steroidogenesis pathway or higher up in the hypothalamic–pituitary–gonadal (HPG)-axis. When the upper HPG-axis is considered, altered ion balance—as a result of avoiding internal acidosis during external hypercapnia—a new mechanism was suggested to interfere with normal neurotransmitter function in the telost brain (Hamilton et al., 2014; Nilsson et al., 2012). Altered function of γ-aminobutyric acid type A receptors (GABA, A) during near-future levels of hypercapnia have already been demonstrated and linked to altered behavior in larvae and juveniles of several teleost species (Hamilton et al., 2014; Nilsson et al., 2012). Interestingly, GABA-signaling is also involved in regulating the secretion of gonadotropin-releasing hormone (GnRH) (Zohar et al., 2010), which further regulate sex steroid synthesis through secretion of gonadotropin (Levavi-Sivan et al., 2010).

The various observed effects of GABA, both depolarizing and hyperpolarizing GnRH neurons, are however not yet fully understood (Herbston and Moenter, 2011). Although the liver is not a classical steroidogenic organ, but, there are several reports that have suggested the production of local and specialized hormones in the liver. For example, the expression of cyp19 mRNA has previously been detected in the liver of a number of teleost species including Atlantic halibut (Hippoglossus hippoglossus) (van Nes et al., 2005), goby (Trisna obesus) (Kobayashi et al., 2004), Atlantic salmon (Salmo salar) (Pavilova et al., 2010) and aromatase activity has been reported in sea bass liver (Dicentrarchus labrax) (Gonzalez and Pifferer, 2003). Hepatic aromatase expression is also well documented in rats (Purba et al., 1994; Yamaguchi et al., 2001; You et al., 2001). Hepatic cyp19 mRNA expression and enzymatic activity during peak vitellogenesis could serve as an extragonadal source of estrogen for the induction of the high Vtg levels required during that particular period of rapid oocyte growth (Pferrer and Blazquez, 2005). Thus, hepatic estrogen was suggested to act as a complement to ovarian estrogen in the stimulation of Vtg synthesis in vitellogenic organisms (Aussi et al., 2009).

4.2. Effects of PFOS

During normocapnia, PFOS exposure appeared to increase transcription of several hepatic E2-inducible genes in a time- and concentration dependent manner. Despite the general absence of statistical significance, transcripts of ERF, Vtg-α, Vtg-β and 3P-2 peaked in fish exposed to 100 μg PFOS L⁻¹ at day 6 and to 200 μg PFOS L⁻¹ at day 9. This gene induction seemed to occur independently from E2, of which levels remained unaltered by PFOS exposure. PFOS has previously been suggested to be a weak ER ligand (Benninghoff et al., 2011; Cheng et al., 2010; Liu et al., 2007), but such a direct interaction may appear as a less plausible
Fig. 9. Principal component analysis (PCA) bi-plot of sex steroid hormone levels (17β-estradiol, E2; testosterone, T; 11-ketotestosterone, 11-KT), the estrogen-to-androgen ratio (E2/T, E2/T-KT), gene transcription (ER-α, Vtg-α/β, ZP-2/F, cyp3a and cyp1a; HIF-1α) and condition factor (CF) of fish sampled at day 6 (A) and 9 (B) into the CO2 exposure period. Score letters correspond to exposure group, where A, B and C represent fish exposed to 0, 100 and 200 μg PFOS; D, E and F represent 0.3% CO2 combined with 0, 100 and 200 μg PFOS; and G, H and I represent 0.9% CO2 combined with 0, 100 and 200 μg PFOS, respectively. The total PCA model consisted of three principal components (PCs) explaining 56.4% of the total variance and with cumulative Q2 = 0.108. PC1 separated the exposure groups according to CO2 saturation, indicating higher expression of the E2-inducible genes Vtg-α/β and ZP-2 and, secondly, elevated levels of sex steroid hormones including E2 in fish exposed to 0.9% CO2 (red) compared to 0.3% (blue) and 0% (normocapnia; gray/green) respectively, regardless of PFOS exposure concentration. PC2 mainly modeled inter-group variation, while PC3 further separated the 0.9% CO2 groups (red) from the others, indicative of higher levels of ER-α, E2 and cyp1a. 200 μg PFOS alone also scored higher along PC1, indicative of estrogenic effects as seen for hypercapnia exposure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4.3. The combined effects of hypercapnia and PFOS

In combined exposure groups, steroid hormone levels appeared to be mainly determined by CO2 saturation, while estrogenic gene expression levels did not directly resemble individual exposure scenarios neither to hypercapnia nor PFOS. This was particularly evident at day 6, where an apparent antagonistic effect on Vtg- and ZP-2 mRNA was observed. No such effect was observed for ERs, of which increased mRNA levels was observed at combined PFOS and hypercapnia exposure at sampling day 6. Low-copy mRNA transcription may indeed fluctuate considerably over time (Kaufmann and van Oudenaarden, 2007). Nevertheless, the apparent inconsistency between cellular levels of E2 and detected estrogenic effects at the transcript level, as compared to hypercapnia exposure alone, might suggest altered hepatic sensitivity toward E2 in fish exposed to combined hypercapnia and PFOS. The simultaneous decrease and increase in expression of the various E2-responsive genes could perhaps be explained by crosstalk between various nuclear receptors, including AhR (Bugel et al., 2013). For example, variability in specific estrogen response element (ERE) sequences, flanking sequences and the total number of ERE-like sequences in promoters of different E2-responsive genes may produce differential affinity for ER (Gruber et al., 2004) and differential potential for crosstalk with other nuclear receptors (Scott et al., 1997). Compared to the fish exposed to PFOS alone, individuals exposed to both PFOS and hypercapnia had modulated estrogenic response, possibly yielding a different foundation for interactive effects by PFOS. The presence or activity of other nuclear receptors might also have been altered as fish had to spend energy adapting to hypercapnia, possibly altering the potential for HIF-1α and AhR crosstalk. Accumulated burdens of PFOS by the time of sampling were not analyzed in fish from the combined exposure groups. Any alterations in PFOS toxicokinetics during hypercapnia cannot be ruled out. Yet, this is not sufficient to explain the gene expression patterns observed in combined exposure scenarios.

On the biotransformation pathways, the effects of hypercapnia on organismal biotransformation pathways are not well studied. However, HIF-1α and cyp1a are used as biomarkers for environmental exposure to hypoxia and POPs whose mode of action is through the AhR (Rahman and Thomas, 2012). Exposure of Atlantic croaker (Micropogonias undulatus) to 2–4 weeks hypoxia (1.7 mg/L dissolved oxygen) was shown to produce
significant decreases in liver cytochrome P450 mRNA and protein levels compared to fish held under normoxic conditions (Rahman and Thomas, 2012). Elsewhere, hypoxia was also shown to decrease CYP1A-mediated ethoxyresorufin-O-deethylase (EROD) activity in zebrafish embryos (Fleming and Di Giulio, 2011) and cytochrome P450 mRNA levels in Atlantic cod liver (Olsvik et al., 2006). Mammalian in vivo studies using rabbit, and rodent in vitro hepatic studies also showed decreases in cytochrome P450 and cytochrome P450 mRNA and protein expression by hypoxia, whose effects on CYP1As are mediated through HIF-1α, cellular cytokines and reactive oxygen species (ROS) (Fradette et al., 2007; Fradette and Du Souich, 2004). These studies are direct opposite of the effects of hypercapnia observed in the present study showing persistent increase of cytochrome P450 mRNA in all sampling days. The modulation of xenobioc-mediated increase or decrease of cytochrome P450 expression by hypoxia is not surprising, given that HIF-1α response to hypoxia and the HIF-1α response to xenobiotics are mediated through the same nuclear dimer partner, namely the Ah receptor (Arrnt). Our findings and other studies showing decreases of CYPs suggest potential interactions sites between these two pathways (Fleming et al., 2009) and the ER pathway (Bugel et al., 2013). These interactions may have potential adverse physiological and adaptation effects to these environmental stressors, including hypercapnia and emerging contaminants.

4.4. Possible consequences on reproduction and overt physiology

In the present study, estrogenic effects were detected at the cellular level as a result of hypercapnia exposure, singly as well as in combination with PFOs, in terms of elevated levels of E2 and E2-inducible gene expression. The estrogen-to-androgen ratio were maintained without major alterations as also androgen levels (T, 11-kT) appeared to increase during hypercapnia, but a corresponding assessment of androgenic effects of elevated androgen levels is complicated by the lack of suitable biomarkers (Kloas et al., 2009). Regardless of this, sex determination and/or differentiation are, in many teleosts, suggested to be under endocrine control (Devlin and Nagahama, 2002; Guiguen et al., 2010). Early elevations in E2 are associated with female gonadal development in several species (Guiguen et al., 2010; Pihler, 2001) including, Atlantic cod (Haugen et al., 2012). Androgens also might be involved in directing oocyte development in female fish, as shown for ZP transcription and oocyte growth in Atlantic cod (Kortner et al., 2008, 2009a,b). Effects of hypercapnic exposure observed in the present study may therefore affect processes, such as sex differentiation, accelerate the onset of puberty in juvenile fish or, at later developmental stages, interfere with normal gametogenesis. As these findings could suggest potential adverse effects for fish stocks within the CO2 scenarios predicted for the upcoming years, the sex steroid disruptive effect of hypercapnia should be further investigated to verify the effects and risks associated with increased anthropogenic CO2 emissions. Until now, ocean acidification studies have mainly been concerned about consequences for invertebrates, and calciﬁying species in particular (Portner et al., 2005). The present study contributes to a growing burden of evidence indicating that teleosts – despite their superior acid–base regulatory capacity – are more susceptible toward elevated pCO2 than previously assumed (Ishimatsu et al., 2005; Munday et al., 2012).

PFOs exposure produced indications of estrogenic potential by affecting gene expression responses in a seemingly E2-independent manner. The observed transcriptional responses were however weak compared to exposure studies using well-known E2 mimics, such as nonylphenol (Meucci and Arukwe, 2006). Although an altered or untimely induction of genes involved in the gametogenesis might cause both excessive energy costs and, in worst case scenario, reproductive failure (Arukwe and Goksøyr, 2003), such consequences cannot be predicted solely based on low-abundance mRNA measurements. Interestingly, exposure to hypercapnia and PFOs in combination provided indications of interactive effects at the level of gene transcription, although the results were not sufﬁcient to significantly prove neither the presence nor absence of such effects. In either case, these novel findings indicate it is a general concern for possible combined effects of near-future hypercapnia and various POPs present in the marine environment. Given that hypercapnia does, in fact, affect physiological and reproductive functions in fish, as suggested by the present study, the potentially endocrine disruptive effects of the numerous POPs in the environ- environment require further and integrated investigations. Any kind of combination effect with environmental hypercapnia could have implications for the accuracy of current risk assessments of emerging and legacy POPs.

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Modulation of Membrane Lipid Composition and Homeostasis in Salmon Hepatocytes Exposed to Hypoxia and Perfluoroctane Sulfonamide, Given Singly or in Combination

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Abstract

The relative importance of environmental hypoxia due to global climate change on organismal ability to adapt to chemical insult and/or mechanisms of these responses is not well understood. Therefore, we have studied the effects of combined exposure to perfluoroctane sulfonamide (PFOSA) and chemically induced hypoxia on membrane lipid profile and homeostasis. Primary salmon hepatocytes were exposed to PFOSA at 0, 25 and 50 μM singly or in combination with either cobalt chloride (CoCl₂: 0 and 150 μM) or deferoxamine (DFO: 0 and 100 μM) for 24 and 48 h. CoCl₂ and DFO were used to induce cellular hypoxia because these two chemicals have been commonly used in animal experiments for this purpose and have been shown to increase hypoxia-inducible factor 1-alpha (HIF-1α) and vascular endothelial growth factor (VEGF) levels. Fatty acid (FA) profiles were determined by GC-MS, while gene expression patterns were determined by quantitative PCR. Hypoxic condition was confirmed with time-related increases of HIF-1α mRNA levels in CoCl₂ and DFO exposed cells. In general, significant alterations of genes involved in lipid homeostasis were predominantly observed after 48 h exposure. Gene expression analysis showed that biological responses related to peroxisome proliferation (peroxisome proliferator-activated receptors (PPARs) and acyl coenzyme A (ACOX)) and FA desaturation (Δ9- and Δ5-desaturases: FADS and FAD6, respectively) and elongation (FAE) were elevated slightly by single exposure (i.e. either PFOSA, CoCl₂ or DFO exposure alone), and these responses were potentiated in combined exposure conditions. Principal component analysis (PCA) showed a clustering of peroxisome proliferation responses at transcript levels and FA desaturation against membrane FAs whose changes were explained by PFOSA and chemically induced hypoxia exposures. Overall, our data show that most of the observed responses were stronger in combined stressor exposure conditions, compared to individual stressor exposure. In general, our data show that hypoxia may, singly or in combination with PFOSA produce deleterious health, physiological and developmental consequences through the alteration of membrane lipid profile in organisms.

Introduction

Anthropogenic activities leading to the emissions of carbon dioxide (CO₂) and other greenhouse gases is thought to be the main contributor to climate change [1]. In the aquatic environment, effects of climate change have already been observed as increases in temperature and CO₂ [2]. A consequence of increased water temperature is reduction in partial pressure of oxygen (pO₂), and its availability to aquatic organisms [3,4]. Oxygen is crucial for cellular respiration that generates energy for maintenance processes and development in aerobic organisms [5]. Thus, hypoxia (a quantifiable measure of climate change) may, singly or in combination with emerging pollutants such as perfluorinated compounds (PFCs) produce deleterious physiological responses that may reduce general health conditions and impaired development in organisms [6].

Emerging compounds such as poly- and perfluoralkyl substances (PFASs), organophosphate flame-retardants, detergent compounds, and several pharmaceutical substances have been linked to several biological effects in organisms and are continuously detected in the environment [7,8]. PFASs are manufactured and used in various industrial and consumer products such as fluorinated polymers, surfactants, insecticides andaerosol firefighting foams [7]. In more than 50 years, 3M Company was the major producer of perfluorooctane sulfonyle fluoride (POSF) starting from 1949, but they have voluntarily phased out production in 2002 [9]. POSF is the precursor to several PFCs, whose reaction with methyl or ethylamides yields alkyl substituted sulfonamides: N-methyl perfluorooctane sulfonamide (NMeFOSA) and N-ethyl perfluorooctane sulfonamide (NEtFOSA), respectively. Further dealkylation can generate perfluorooctane sulfonamide.

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PFOSA), which is randomly distributed in biota and has been detected worldwide in fish, mammals, birds and humans at concentrations in the range of 1-100 mg/g wet weight of tissue [10,11]. The chemical properties of PFOSA make the compound neither hydrophilic nor lipophilic and has been found to bind to carrier proteins, such as albumin, in blood [12]. PFAs can appear as both perfluorinated sulfonic (PFASs) and carboxylic acids (PFCAs) which have been shown to exert a variety of biological effects, including lipotoxicity, immunotoxicity, uncoupling of mitochondrial oxidative phosphorylation, developmental toxicity, reduction of thyroid hormone circulation, necrosis, down-regulation of hepatic transporters and tumors [13,14,15,16]. In mammalian systems, PFOSA was shown to undergo metabolic degradation at a slow rate to form PFOS, and can also undergo enterohpetic circulation, and mediate oxidative stress responses [17,18].

Energy homeostasis and its regulation is critical for normal physiology and survival, and disruption of this balance often leads to chronic disease state [19]. FAIs in fish tissues are present in different lipid classes and with different functions [20,21]. There are two classes of essential long chain polysaturated fatty acids (PUFAs): omega-3 (n-3) and omega-6 (n-6), based on the location of the first double bond in the third (n-3) or sixth (n-6) position from the methyl end of the aliphatic carbon chain [22]. Conversions of these essential fatty acids (FAIs) are orchestrated by several fatty acid desaturases (FADs) and elongase (FAE). Of the n-3 PUFAs, γ-linolenic acid (GLA: 18:3n-6) can be desaturated and elongated to form eicosapentaenoic acid (EPA: 20:5n-3) through the activity of FAD6, FAE and FAD5, further transformation involves FAD4 and FAD6 to docosahexaenoic acid (DHA: 22:6n-3) and is reversible. Whereas the n-6 PUFAs, linoleic acid (LA: 18:2n-6) can be desaturated by FAD6 to γ-linolenic acid (GLA: 18:3n-6) and elongated by FAD3 to dihomo-γ-linolenic acid (DGLA: 18:3n-6) and further desaturation by FAD5 produces arachidonic acid (ARA: 20:4n-6). ARA can thereafter through steps involving FAD4 and FAE transform into docosapentaenoic acid (DPA: 22:5n-6), and this last step is reversible. Chemically-mediated changes in the composition of lipids will affect many biological processes in the body, including lipogenesis, lipid transport, deposition and storage, peroxisome proliferation, apoptosis. Peroxisome proliferator-activated receptors (PPARs) are known to be critical and FA uptake in tissues and membrane fluidity [23]. Peroxisome proliferator-activated receptors (PPARs) are known to be critical regulators of lipid homeostasis by controlling the balance between burning and storage of long FAs [24]. PPARs are ligand-dependent transcription factors belonging to the nuclear hormone receptor superfamily [24]. The acyl coenzyme A (ACOX) catalyses the rate-limiting step in peroxisomal β-oxidation pathway of FA, and is commonly used as a biomarker for peroxisomal proliferation [24]. ACOX encoding gene in rats was regulated by PPARs through a peroxisome proliferator response element (PPRE) in the 5’ upstream region of the gene [24]. Regulation of peroxisome proliferation is controlled by PPARs and was first identified having this function in frogs (Xenopus sp.) [25]. They (PPARs) exert pleiotropic responses by regulating energy homeostasis, adipose tissue differentiation and maintenance, cell proliferation and tissue repair [26]. PPAR activities are consequently changed in accordance with a wide variety of physiological conditions, mediated through the ubiquitin-proteasome degradation system and extracellular signalling pathways and kinases that lead to receptor phosphorylation [27]. Administration of food containing PFOA induced peroxisome proliferation in Atlantic salmon (Salmo salar) [29]. In rats the same treatment has shown induced peroxisome proliferation and formation of benign liver tumors [16].

In this study, we have investigated biological pathways related to peroxisome proliferation, and lipid profile and homeostasis after exposure to chemically induced hypoxia and PFOSA, given singly and also in combination. Hypoxia was induced using cobalt chloride (CoCl2) and deferoxamine mesylate (DFO), two chemically commonly used in animal experiments for this purpose and have been shown to increase hypoxia-inducible factor 1-alpha (HIF-1α) and vascular endothelial growth factor (VEGF) levels. DFO induces hypoxia by chlorating iron for excretion and subsequently reducing the potential for oxygen transport [29] and CoCl2 is known to inhibit iron-dependent hydroxylases, resulting in an increase in HIF-1α protein accumulation, DNA binding activity, and transcription activity including VEGF induction [30,31]. Given that optimal physiological condition is required for growth and development, optimal adaptation to hypoxic stress may have detrimental consequences resulting from inability to maintain physiological processes essential for normal cellular functions. It may also produce diminished capacity to handle fluctuation of other environmental factors that could ultimately lead to reduction in general fitness [32,33,34] and increase membrane (fluidity) passage for environmental contaminants. Our hypothesis is that exposure of salmon hepatocytes to hypoxia, singly or in combination with PFOSA, will produce significant changes in membrane lipid profile and biological processes that regulate membrane lipid homeostasis, with overt health, developmental, reproductive and physiological consequences.

Materials and Methods

Chemicals and reagents

Highly pure (>98%) linear perfluoroctanoic sulfonamide (PFOSA; CF3-CF2-CO2-NH2) isomer, as well as isotopically labeled linear PFOSA14C and linear PFOSA13C were purchased from Wellington Laboratories (Guelph, ON, Canada). Script cDNA Synthesis Kit and iTaq SYBR Green Supermix with ROX were supplied by BioRad Laboratories (Hercules, CA, USA). The original TA Cloning Kit PUR 2.1 vector, INVITRO cells, TRIZol and Dabcylco’s Modified Eagle Medium (DMEM) with non-essential amino acid and without phenol red, fetal bovine serum (FBS), 0.1% trypsin blue and L-glutamine were purchased from Gibco-Invitrogen Life Technologies (Carlsbad, CA, USA). Dime-thyl sulfoxide (DMSO), penicillin-streptomycin-rose-myacin solu-tion, collagenase (C0130-1G), bovine serum albumin (BSA), N-[2-Hydroxyethyl]piperazine-N’-[2-Ethane Sulfonic Acid] (HEPES), ethylenediaminetetraacetatic acid disodium salt tiritrate (EDTA), ethyleneglycol bis-[β-aminoethyl ether]-N,N,N’,N’-tetraacetic acid (EGTA), polysaturated fatty acid 1 and 2 (PFA1 and PFA2) were purchased from Sigma-Aldrich Chemie GmbH (Munchen, Germany). Tricaine methane sulphonate (MS-222) was supplied from Biofresh (Hayward, CA, USA). The ZR Plasmid Miniprep-Classic was purchased from Zymo Research (Orange, CA, USA).

Animals, exposure and sampling

All necessary permits were obtained from the Norwegian Animal Research Authority for the described study, which complied with all relevant regulations. Atlantic salmon (Salmo salar) were purchased from Lundamo Hatcheries (batch and rearing centre located at Lundamo). Fish were kept at the university-holding facilities for Department of Biology (Nelaab, NTNU) in 100-litter tanks with continuously running fresh water at 10°C and flow rate of 40 L/h and natural photoperiod. Fish were...
acclimated 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 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 from 10 individuals by a two-step perfusion technique with modifications as previously described [35]. The cell suspension was filtered through a 150 µm nylon monofilament filter and centrifuged at 70 g for 5 min. Hepatocyte from individual fish were used across all individual exposure scenarios in such a way that all 10 fish were represented in all exposures. 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 TFF Tissue Culture Plates (Techno Plastic Products AG, Switzerland) at monolayer density of 2.1 ×10⁶ 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°C in a sterile incubator for 24 hours prior to exposure. After 24 hours pre-culture, growth medium was removed and quickly replaced with exposure medium (twenty wells for each exposure group); to 0.1% DMSO (control), 150 µM CoCl₂, 100 µM DFO, 50 µM PFOSA (singly and in combination with either 150 µM CoCl₂ or 100 µM DFO); 50 µM PFOSA (singly and in combination with either 150 µM CoCl₂ or 100 µM DFO). This gave a total of 9 different exposure groups. Media and cells were harvested separately, ten wells for each exposure group at 24 and 48 h, post-exposure and snap-frozen immediately in liquid nitrogen. Cells used for RNA analysis were lysed in Trizol reagent for total RNA isolation according to the manufacturer's protocol (Invitrogen).

Assessment of cell viability

A pilot study using different concentration (10, 50, 100, 150 and 200 µM) of CoCl₂ or DFO was performed in order to determine optimal exposure concentrations for hypoxia-inducing chemicals. Evaluation was performed using resazurin assay on cells exposed for 24 and 48 h in 96-well plates (2.1 ×10⁵ cells in 300 µl). After addition of resazurin solution (10% of medium volume), cells were incubated for 6 h at 10°C on a gyratory shaker. Samples were measured spectrophotometrically at 600 nm every 20 minutes. Viability was also investigated for all exposure groups (see below).

Quantitative (real-time) PCR

Total cDNA for quantitative real-time polymerase chain reaction (q-PCR) analysis was generated from 1 µg total RNA from all sampling using a combination of poly-T and random primers from iScript cDNA synthesis kit as described by the manufacturer (Bio-rad). RNA samples were evaluated for integrity using agarose gel electrophoresis. Quantitative real-time PCR was used for evaluating gene expression profiles for HIF1-α, FAD5, FAD6, FAE, ACOX and PPAR (α, β and γ). For each treatment, expression of individual gene targets was analyzed using the MX9000P REAL-TIME PCR SYSTEM (Stratagene, La Jolla, CA, USA). Each 25 µL qPCR reaction contained 12.5 µL of iTaq SYBR Green Supermix with ROX (Bio-Rad), 1 µL of cDNA, 200 nM of each forward and reverse primers and remaining volume was autoclaved MQ-H₂O. The three-step real-time PCR program included an enzyme activation step at 95°C (5 min) and 40 cycles of 95°C (30 s), 55-65°C (30 s) (depending on the primers used; see Table 1), and 72°C (30 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 [36]. Data from each group were averaged and expressed as percentage of control.

FA extraction and GC-MS analysis

Lipids were extracted from Atlantic salmon hepatocytes by homogenization in chloroform: methanol (2:1) solution, added with 0.01% of 2,6-di-tert-butyl-4-methylphenol (BHT) as an antioxidant, according to the method of Folch et al. [57]. FA methyl esters (FAMEs) from total lipids were prepared by acid-catalyzed transmethylation for 1 h at 100°C, using tricosanoic acid (23:0) as internal standard. Methyl esters were extracted by c-hexane, then dried by centrifugation, weighed and suspended in c-hexane (1% v/v). FAMEs analysis was performed using a Shimadzu GC-MS 2010 gas chromatograph-mass spectrometer and fitted with a fused silica capillary column (Supelco, Germany) and helium was used as carrier gas. The injector, detector and column temperatures were 250°C, 300°C and 200°C, respectively. Relative percentage of the area was obtained by using the following equation: Area% FAX = [AX/AR]×100, where: FAX = fatty acid to be quantified, AX = area of the methyl esters, X = total area of the chromatogram. Peak areas lower than 0.1% of the total area was not considered. We identified FA methyl esters by comparing retention time of samples and standards.

Statistics

Data are presented as mean percent of control with the same exposure duration ± standard error of mean (SEM). Normal distribution was assessed using Shapiro-Wilk test and homogeneity of variance was tested with Levene’s test. Comparison of different concentrations of PFOSA treatment, singly or in combination with CoCl₂ 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 of concern were investigated and based on distribution patterns and group formation we chose which groups to investigate further. Variables investigated here were Q-PCR data. Observations (exposure groups) must be independent when investigated using PCA, so data was separated on terms of exposure duration (24 and 48 h). PCA biplot presented herein were produced by first component (PC1) and second component (PC2) and percent of variation (R²X) is displayed for each plot.
Results

Evaluation of cell viability and validation of hypoxia exposure

Our pilot study showed that increasing DFO and CoCl₂ concentration above 100 and 150 μM, respectively, noticeably reduced cell viability. Cell viability in the different exposure regimes showed a cell survival rate above 65% (data not shown).

Gene expression analysis of HIF-1α was used to assess the hypoxic condition of the hepatocytes showing significant increase of mRNA expression, at 48 h compared to 24 h post-exposure, in combined exposure scenarios and by DFO alone. CoCl₂ exposure induced HIF-1α after both 24 and 48 h, albeit not significant. HIF-1α mRNA was not induced by exposure to PFOSA alone (Fig. 1).

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

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer sequence*</th>
<th>Amplicon size (basepairs)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>GCT CAG AAA GTC GGT TGT CC</td>
<td>GCC AGC TAG AAG AAC ACC TC</td>
<td>152</td>
</tr>
<tr>
<td>FADS</td>
<td>GAC CTA TAG TGC CAT TCT CC</td>
<td>TCA CTC ATG TAG AAG TAG TCC</td>
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<tr>
<td>FAE</td>
<td>CAT CTC ATT CTC ATT CCA TCT C</td>
<td>CTC TGC TCC ACT CAC ACC</td>
<td>127</td>
</tr>
<tr>
<td>PPARγ</td>
<td>GCT TCA TCA CCA GGG AGT TT</td>
<td>TCA CTC TCA TAG AAG ACC AG</td>
<td>113</td>
</tr>
<tr>
<td>PPARβ</td>
<td>CAA TGG CTC GGA TCT CAA AT</td>
<td>ACT CTA CTG GGC TGG AGC TG</td>
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</tr>
<tr>
<td>PPARα</td>
<td>CAC TGT GAT GTC CAC TGT</td>
<td>ATG GCA TCA TGT GAC ATT</td>
<td>100</td>
</tr>
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</table>

*Sequences are given in the 5′→3′ order

doi:10.1371/journal.pone.0102485.t001

Modulation of membrane FAs composition

Changes in membrane FA composition were observed after PFOSA, CoCl₂ and DFO exposures and these effects were dependent on PFOSA concentration, combined exposure with individual DFO or CoCl₂ and FA type (Table 2). After 24 h, exposure to DFO alone produced a significant reduction in ALA (18:3n-3) levels, and combined low (25 μM) PFOSA in combination with CoCl₂ or DFO increased ALA levels in salmon hepatocytes (Table 2). High PFOSA (50 μM) exposure, singly or in combination with CoCl₂ or DFO, increased membrane ALA levels at 24 h exposure (albeit not significant). Exposure of hepatocytes to PFOSA or in combination with hypoxic condition significantly reduced membrane levels of ARA (20:4n6) and EPA (20:5n-3) after 24 h (Table 2). All exposure conditions produced increases in 22:6n-3 (DHA) after 24 h. Linoleic acid (LA: 18:2n-6) was increased in all exposure groups with CoCl₂ and by 25 μM combined with DFO, while combined 50 μM PFOSA and DFO

![Figure 1. Changes in transcript levels for hypoxia-inducible factor 1α (HIF-1α) in salmon hepatocytes exposed to PFOSA (25 and 50 μM), singly or in combination with, either CoCl₂ (150 μM) or DFO (100 μM) for 24 and 48 h. Transcripts were analyzed using real-time polymerase chain reaction (qPCR) and expressed as mean percentage (%) of control ± SEM (n = 5). Asterisk (*) denotes significant difference (p<0.05) compared to control analyzed by Tukey’s test, while diamond (¤) denotes significant difference (p<0.05) with individual hypoxia treatment group (CoCl₂ or DFO) at respective time-interval. doi:10.1371/journal.pone.0102485.g001](https://www.plosone.org/figure/1.1)
### Table 2. Fatty acid (FA) profile in primary culture of Atlantic salmon hepatocytes exposed for 24h to CoCl$_2$ (150 μM) or DFO (100 μM), singly and in combination with PFOSA (25 and 50 μM).

<table>
<thead>
<tr>
<th>Lipid Name</th>
<th>Common name</th>
<th>Control</th>
<th>25 μM PFOSA</th>
<th>50 μM PFOSA</th>
<th>150 μM CoCl$_2$</th>
<th>No PFOSA</th>
<th>25 μM PFOSA</th>
<th>50 μM PFOSA</th>
<th>100 μM DFO</th>
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</thead>
<tbody>
<tr>
<td>14:0</td>
<td>Myristic Acid</td>
<td>100 ± 12</td>
<td>315.4 ± 46*</td>
<td>161.0 ± 22*</td>
<td>391.3 ± 81*</td>
<td>399.4 ± 52*</td>
<td>246.5 ± 29*</td>
<td>244.9 ± 38*</td>
<td>223.8 ± 55*</td>
</tr>
<tr>
<td>14:1</td>
<td>Myristoleic acid</td>
<td>100 ± 18</td>
<td>103.4 ± 25</td>
<td>258.7 ± 24*</td>
<td>309.7 ± 67*</td>
<td>355.8 ± 29*</td>
<td>234.3 ± 41*</td>
<td>148.6 ± 33*</td>
<td>342.3 ± 48*</td>
</tr>
<tr>
<td>15:0</td>
<td>Pentadecyl acid</td>
<td>100 ± 16</td>
<td>72.01 ± 28*</td>
<td>32.8 ± 71*</td>
<td>351.6 ± 66*</td>
<td>107.6 ± 16</td>
<td>48.1 ± 14*</td>
<td>120.7 ± 14*</td>
<td>53.3 ± 12*</td>
</tr>
<tr>
<td>15:1</td>
<td>Pentadecenoic acid</td>
<td>100 ± 22</td>
<td>209.1 ± 43*</td>
<td>324.4 ± 71*</td>
<td>311.8 ± 28*</td>
<td>391.2 ± 43*</td>
<td>217.2 ± 46*</td>
<td>309.7 ± 32*</td>
<td>376.2 ± 26*</td>
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<td>16:0</td>
<td>Palmitic acid</td>
<td>100 ± 9</td>
<td>73.7 ± 12*</td>
<td>103.4 ± 26</td>
<td>114.2 ± 15</td>
<td>11.8 ± 2*</td>
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<td>114.3 ± 15</td>
<td>45.1 ± 10*</td>
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<td>16:1</td>
<td>Palmitoleic acid</td>
<td>100 ± 21</td>
<td>158.2 ± 42*</td>
<td>418.5 ± 72*</td>
<td>349.4 ± 42*</td>
<td>201.4 ± 26*</td>
<td>376.4 ± 44*</td>
<td>184 ± 16*</td>
<td>230.2 ± 19*</td>
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<td>17:0</td>
<td>Margaric acid</td>
<td>100 ± 13</td>
<td>80.9 ± 21</td>
<td>97.3 ± 31</td>
<td>321.1 ± 31*</td>
<td>110.2 ± 14</td>
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<td>53.2 ± 17*</td>
<td>281.3 ± 24*</td>
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<tr>
<td>17:1</td>
<td>Heptadecenoic acid</td>
<td>100 ± 21</td>
<td>12.9 ± 2*</td>
<td>40.9 ± 72*</td>
<td>293.3 ± 35*</td>
<td>45.5 ± 12*</td>
<td>99.5 ± 15</td>
<td>51.9 ± 9*</td>
<td>313.8 ± 42*</td>
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<tr>
<td>18:0</td>
<td>Stearic acid</td>
<td>100 ± 7</td>
<td>107.2 ± 21</td>
<td>86.4 ± 2*</td>
<td>14.7 ± 3*</td>
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<td>13.9 ± 3*</td>
<td>255.8 ± 21*</td>
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<tr>
<td>18:1n9c=18:1n9t</td>
<td>Oleic acid-Eicosenoic acid</td>
<td>100 ± 11</td>
<td>12.5 ± 4*</td>
<td>28.3 ± 7*</td>
<td>10.9 ± 2*</td>
<td>31.5 ± 9*</td>
<td>13.9 ± 2*</td>
<td>20.4 ± 18*</td>
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<td>18:2n6c=18:2n6t</td>
<td>Linoleic acid (LA)</td>
<td>100 ± 13</td>
<td>87.1 ± 24</td>
<td>97.7 ± 12</td>
<td>181.3 ± 22</td>
<td>142.7 ± 11*</td>
<td>165.6 ± 16</td>
<td>84.3 ± 14</td>
<td>308.1 ± 32*</td>
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<td>18:3n6</td>
<td>γ-linolenic acid (GLA)</td>
<td>100 ± 26</td>
<td>82.8 ± 26</td>
<td>115.4 ± 22</td>
<td>124.6 ± 11</td>
<td>160.7 ± 23*</td>
<td>149.2 ± 11*</td>
<td>64.6 ± 19*</td>
<td>348.6 ± 62*</td>
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<td>18:3n3</td>
<td>ν-linolenic acid (ALA)</td>
<td>100 ± 21</td>
<td>113.4 ± 26</td>
<td>127.2 ± 17</td>
<td>97.3 ± 15</td>
<td>325.5 ± 59*</td>
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<tr>
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<td>Arachidic acid</td>
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<td>123.7 ± 26</td>
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<td>Eicosenoic acid</td>
<td>100 ± 21</td>
<td>141.8 ± 41*</td>
<td>117.3 ± 16</td>
<td>174.4 ± 37*</td>
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<td>208.4 ± 32*</td>
<td>17.3 ± 11*</td>
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<tr>
<td>20:2</td>
<td>Ecosadienoic acid</td>
<td>100 ± 11</td>
<td>24.8 ± 3*</td>
<td>34.2 ± 14*</td>
<td>3.3 ± 17</td>
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<tr>
<td>20:4n6</td>
<td>Arachidonic acid (ARA)</td>
<td>100 ± 8</td>
<td>48.1 ± 16*</td>
<td>48.8 ± 11*</td>
<td>80.5 ± 21</td>
<td>65.9 ± 23*</td>
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<td>65.6 ± 14*</td>
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<td>Eicosapentaenoic acid (EPA)</td>
<td>100 ± 22</td>
<td>34.9 ± 9*</td>
<td>30.5 ± 7*</td>
<td>76.6 ± 14</td>
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<td>20:1n9</td>
<td>Behenic acid</td>
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<td>66.7 ± 15*</td>
<td>206.8 ± 31*</td>
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<tr>
<td>22:0</td>
<td>Lignoceric acid</td>
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<td>94.8 ± 28</td>
<td>73.6 ± 32</td>
<td>146.3 ± 39*</td>
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<td>132.3 ± 132</td>
<td>190.6 ± 16</td>
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<td>190.5 ± 31*</td>
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<td>87.2 ± 15</td>
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<td>172.6 ± 31*</td>
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<td>Docosadienoic acid</td>
<td>100 ± 16</td>
<td>156.1 ± 41*</td>
<td>307.3 ± 62*</td>
<td>224.6 ± 51*</td>
<td>261.2 ± 21*</td>
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<td>246.9 ± 35*</td>
<td>60.2 ± 21*</td>
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<td>22:3n6</td>
<td>Docosahexaenoic acid (DHA)</td>
<td>100 ± 33</td>
<td>249.6 ± 34*</td>
<td>340.8 ± 27*</td>
<td>198.3 ± 22*</td>
<td>312.1 ± 45*</td>
<td>219.8 ± 26*</td>
<td>147.3 ± 21</td>
<td>238.6 ± 34*</td>
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<td>Lignoceric acid</td>
<td>100 ± 28</td>
<td>58.2 ± 15*</td>
<td>36.1 ± 13*</td>
<td>9.5 ± 4*</td>
<td>22.0 ± 17*</td>
<td>153.6 ± 16</td>
<td>156.6 ± 14</td>
<td>341.4 ± 41*</td>
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</table>

Data are presented as mean (n = 6) percentage of control ± (SEM). Asterisk (*) denotes exposure groups that are significantly different from control (p < 0.05). FAs that were significantly increased are shown in bold, while FAs that were significantly reduced are shown in italics.
reduced LA level after 24 h (Table 2). Membrane ALA and GLA were reduced by DFO after 24 h, but were generally increased by other exposure regimes (Table 2). Membrane FA showed different composition pattern at 24 h, compared to 48 h (Table 2 and 3). At 48 h, ARA was significantly reduced in all exposure groups, except PFOSA that produced an increase. Membrane ARA levels were significantly reduced by CoCl₂ and DFO exposure alone, compared with PFOSA exposure that increased ARA levels. Combined exposure with PFOSA and DFO or CoCl₂ sustained the hypoxic condition mediated decrease on membrane ARA levels (Table 3). Membrane EPA levels were reduced by CoCl₂ and DFO exposures at 48 h, and combined exposure with PFOSA concentrations significantly increased these effects, except combined DFO and 50 μM PFOSA, that produced a significant reduction (Table 3). While 25 μM PFOSA significantly reduced EPA levels, 50 μM PFOSA significantly increased membrane EPA levels after 48 h exposure (Table 3). Membrane LA levels were significantly increased by CoCl₂ and DFO exposures alone or in combination with PFOSA concentrations at 48 h. On the other hand, membrane ALA and GLA were significantly reduced by CoCl₂ and DFO exposure alone at 48 h, and combined exposure with 25 μM PFOSA significantly increased (DFO) and decreased (CoCl₂) ALA levels, and the opposite is true for GLA at 48 h (Table 3). All exposure conditions reduced membrane DHA levels except combined DFO and 25 μM PFOSA, and combined CoCl₂ and 50 μM PFOSA (Table 3).

Modulation of transcripts involved in fatty acid metabolism

The effects of PFOSA, given singly or in combination with CoCl₂ or DFO on FAD5, FAD6 and FAE mRNA at 48 h, while no significant effects were observed after 24 h (Fig. 2). The combined exposure of PFOSA and CoCl₂ or DFO significantly increased FAD5, FAD6 and FAE transcripts at 48 h, while no significant effects were observed after 24 h exposure (Fig. 2A, B and C, respectively). Acyl-coenzyme A oxidase (ACOX) was not significantly affected by PFOSA exposure both at 24 and 48 h (Fig. 3). No effects were observed either when CoCl₂ and DFO were given singly, or in combination with PFOSA concentrations at 24 h (Fig. 3).

Modulation of transcripts involved in lipid peroxidation

Gene expression levels of PPARγ (α, β and γ) were investigated in all exposure groups (Fig. 4) showing that exposure to PFOSA concentrations elevated PPARα mRNA levels at 48 h exposure (Fig. 4A). Exposure to CoCl₂ alone did not affect PPARα, but combined exposure with PFOSA produced significant increase after 48 h exposure (Fig. 4A). On the other hand, exposure to DFO alone significantly increased PPARα mRNA at 48 h, and combined exposure with PFOSA concentrations sustained this effect, but with reduced expression levels in combination with 25 nM PFOSA (compared DFO exposure, Fig. 4A). For PPARβ and PPARγ, no effects were observed after exposure to PFOSA concentrations either at 24 or 48 h (Fig. 4B). On the other hand, CoCl₂ and produced increases in PPARβ and PPARγ expressions, when given alone, and combined exposure with PFOSA concentrations significantly sustained these effects at 48 h (Fig. 4B and C, respectively). Otherwise, no significant PPARβ and PPARγ transcriptional changes were observed after 24 h exposure in any exposure group (Fig. 4B and C).

Principal component analysis (PCA)

A principal component analysis was used in order to explore observations and variables with correlational patterns. We chose to incorporate molecular responses (mRNA) as variables and all comparable observations. At biplot analysis after 24 h exposure, we observed that all observations were located around neutral point ([1] = 0, [2] = 0) and there is no distinct distribution pattern among or between groups. Variables were located in the right side arc of the plot, and mostly explained by principal component 1 (PC1: 49.5%), except PPARβ that is located closer to PC2 (18.6%; Fig. 5A). There was no association between observations and variables, and further evaluation of variables was not pursued. Biplot of samples exposed for 48 h showed distribution along PC1 (74.9%), where observations were clustered, although somewhat overlapping, related to separate exposure treatments (Fig. 5B). PC1 (74.9%) explained most variation in this dataset and neither the observations nor parameters were drawn particularly to PC2 (11.2%). Control, single PFOSA (25 or 50 μM) and single 150 μM CoCl₂ were located along PC1 and on the left side of PC2. Combined PFOSA (25 and 50 μM) and CoCl₂ are located further right in biplot compared to control and 25 μM PFOSA group is generally located above PC1 towards PPAR (α and γ) and ACOX. Some individual exposures are less described by the model and are located close to neutral point. All groups containing DFO were located left of PC2, showing several observations that are close to PC1 or below (Fig. 5B). Variables that were distributed in an arc along the outer ring at right side of the biplot were mainly explained by PC1. In a longer distance away from PC1, were PPARα, with PPARγ and PPARα above PC1 and PPARβ below, while FAD5, FAD6, FAE, ACOX and HIF-1α are closer to PC1. Generally, PC2 describes very little of the variation in this biplot (Fig. 5B).

Discussion

Previously, it has been shown in several studies that PFASs modulate the PPAR system and membrane FA homeostasis [38], and through these pathways induce peroxisome proliferation and oxidative stress responses [28,38,39]. Changes in the global climate are currently observed as increases in temperature and CO₂ that subsequently produce reduction in oxygen partial pressure (pO₂), and its availability to aquatic organisms. Oxygen is crucial for aerobic organisms that depend on it for cellular respiration, and because reduced environmental oxygen saturation (hypoxia) and environmental contaminants represent multiple environmental stressor. Hypoxia has been associated with effects on hormonal and biotransformation systems [40,41,42], and the relative importance of environmental hypoxia on organismal adaptive abilities responding to chemical insult are not well understood. Therefore, the present study was designed to investigate molecular and physiological effects of hypoxia and PFOSA, given singly and also in combination, on membrane FA composition and associated effects on molecular processes that regulate lipid homeostasis in fish, using a salmon hepatocyte in vitro model. Cellular hypoxia was induced using DFO and CoCl₂, two chemicals that are frequently used to induce hypoxia in in vitro models, but also have an apoptotic potential [38].

Modulation of membrane fatty acid composition

Hepatocytes adapt to reduction in oxygen levels by shifting energy production from mitochondrial fatty acid β-oxidation to
After 48 hours of exposure, Control 25 uM PFOSA 50 uM PFOSA No PFOSA 25 uM PFOSA 50 uM PFOSA No PFOSA 25 uM PFOSA 50 uM PFOSA

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Name</th>
<th>Common name</th>
<th>No PFOSA</th>
<th>25 uM PFOSA</th>
<th>50 uM PFOSA</th>
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Asterisk (*) denotes exposure groups that are significantly different from control (p < 0.05). FAs that were significantly increased are shown in bold, while FAs that were significantly reduced are shown in italics.
glycolysis during periods of cellular hypoxia [43]. As a result, the activation of the HIF complex represents an early response to hypoxia exposure. Consequently, HIF is a central adaptive change in response to hypoxia through HIF-mediated reprogramming of cellular metabolism. Thus, HIF plays an integral role in switching energetic usage from aerobic to anaerobic metabolism to generate more ATP in an oxygen independent manner, through the regulation of glucose transporter 1 and several critical glycolytic enzymes, and to inhibit mitochondrial oxidative phosphorylation [44]. Glucose metabolism under hypoxic conditions and the role of HIF has been extensively studied, but less is known on its role in lipid metabolism in response to low oxygen and possible interaction with environmental contaminants. Recently, it was shown that acute and intermittent hypoxia induced liver lipid accumulation, suggesting a prominent role for HIF in regulating hepatic membrane lipid composition and metabolism [45,46].

Figure 2. Modulation of FAD5 (A), FAD6 (B) and FAE (C) in salmon hepatocytes exposed to CoCl$_2$ (150 μM) or DFO (100 μM), singly or in combination with PFOSA (25 and 50 μM). Transcripts were analyzed using real-time polymerase chain reaction (qPCR) and expressed as mean percentage (% of control ± SEM (n = 5). Asterisk (*) denotes significant difference (p<0.05) compared to control analyzed by Tukey's test, while diamond (¤) denotes significant difference (p<0.05) with individual hypoxia treatment group (CoCl$_2$ or DFO) at respective time-interval. doi:10.1371/journal.pone.0102485.g002
In the present study, we observed significant increase in transcript levels for HIF-1α mRNA expression after hypoxia (DFO and CoCl₂) exposure, and this effect partially paralleled modifications in hepatic membrane FA composition, in the presence and absence of PFOSA. In addition, these effects did not parallel changes in transcript levels for FAD5, FAD6 and FAE. The relationship between increase in the composition of hepatic membrane FA composition and increase in FAD5, FAD6, FAE and HIF-1α expression is interesting amidst the ongoing controversy regarding the role of HIF-2 as a pro-lipogenic factor [47]. The finding showing that HIF-2α deficient mice exhibited hepatic steatosis, and the forced expression of hepatic HIF-1α, but not HIF-2α, that stimulated lipid accumulation in mice [48] suggests complicated roles of HIF-1α and HIF-2α in hepatic fat accumulation and possible mal-adaptive pathologies [43].

Previously, we showed that PFOSA produced time- and concentration-dependent alterations in the hepatic membrane content of several classes of FAs in salmon hepatocytes [39,49,50]. Herein, we show that exposure of cells to hypoxic conditions produced changes in hepatic membrane FA composition, similar to the effect of PFOSA alone, and combined exposure to hypoxia and PFOSA, further modulated the effects of hypoxic conditions alone. Note that these effects were based on hypoxia-inducing compound (DFO or CoCl₂), exposure time and PFOSA concentration. PUFAs with 20 and 22 carbons are vital components of membrane phospholipids, and represent key steps in cell signalling, and control the expression of many genes involved in lipid synthesis and metabolism, thermogenesis, and cell differentiation [51]. For example, eicosanoids, including prostaglandins, thromboxanes and leukotrienes, belong to an extensive family of oxygenated metabolites derived from 20-carbon PUFAs such as ARA and EPA [52]. In accordance with previous findings [39,49,50] and as demonstrated in the present study, salmon liver is capable of FAD6-desaturation of ALA to stearidonic acid (18:4n3) followed by elongation and FAD6 desaturation to EPA, in addition to FAD6 desaturation of Linoleic acid (18:2n-6) to γ-linolenic acid (18:3n-6) followed by elongation to Dihomo-γ-linolenic acid (DGLA, 20:3n-6) and FAD5 desaturation to ARA [54]. Our data show that hypoxic conditions reduced several n-3 PUFAs such as ALA, DHA and EPA, which were not in accordance with the increased expression of FAD5, FAD6 and FAE mRNA.

Furthermore, the availability of 20- and 22-carbon polyunsaturated FAs is highly dependent on the activity of FAD6, which mediates the rate-limiting step in the production of ARA [51]. The increase in FAD5, FAD6 and FAE mRNA expressions, three enzymes of the FA elongation pathway, paralleled the increase in membrane trans-linolelaidic acid (18:2n-6t) and DHA levels (but not ARA). This discrepancy may be explained by the fact that in fish, n-3 PUFAs are abundant and play significant roles in immune function and apoptosis [55,56]. In addition, the major regulation mechanism of FAD6 is assumed to be pre- translational [57], and our findings provide evidence that hypoxia increases the activity of the elongation machinery in order to adapt to membrane and physiological requirements due to the shortage of these FAs. Overall, the modulation of membrane FA composition observed in the present study predominantly involved an increase in FA methyl esters, indicating that hypoxia, given singly and also in combination with PFOSA, may affect lipid metabolism in Atlantic salmon. Over-production of ARA-derived eicosanoids may be responsible for a number of pathophysiological conditions in humans, such as atherosclerotic and chronic inflammation diseases [58]. Thus, our data suggest that changes in membrane FA levels compensated for lipid peroxidation through an increase by elongation and desaturation activities in order to increase membrane fluidity as a form for compensatory mechanism. This speculation is supported by the fact that the β-oxidation pathway was positively enhanced in the hepatocytes after exposure to hypoxic conditions, singly and also in combination with PFOSA concentration (see below). Elsewhere, it has been shown that PFOSA, PFOA and PFOS reduced lipid synthesis and increase β-oxidation in rat liver tissue system, with inconsistent changes in other enzymes involved in lipid metabolism [59]. The report showing that erucic acid inhibited peroxisomal β-oxidation in rats [60], provided strong support to our observed decrease of PUFAs and increase of mRNA levels for FA elongation enzymes and ACOX1 by hypoxic conditions, as it has been shown that PUFAs may repress peroxisomal β-oxidation [60]. Furthermore, it has been suggested that alteration of mRNA levels by FAs and PPAR activators is often disconnected [61]. For example, peroxisomal proliferators and essential FA deficient diets has been shown to elevate the mRNA levels for FAD5 and FAD6, while dietary

Figure 3. Transcriptional changes of ACOX mRNA in salmon hepatocytes exposed to CoCl₂ (150 μM) or DFO (100 μM), in presence and absence of PFOSA (25 and 50 μM). Transcripts were analyzed using real-time polymerase chain reaction (qPCR) and expressed as mean percentage (%) of control ± SEM (n=5). Asterisk (*) denotes significant difference (p<0.05) compared to control analyzed by Tukey’s test.

doi:10.1371/journal.pone.0102485.g003
PUFAs are known to repress these genes [57]. The peroxisomal proliferator, Wy14643, was also shown to produce delayed induction of FAD5 and FAD6 in rats, compared to the FA oxidation genes [57], prompting the authors to suggest that an induction of the desaturases could occur directly because of the degenerated direct repeat 1 (DR1) element that was reported in human FAD6 gene and binds PPARα [57].

In the present study, the hepatic membrane FA composition both decreased and decreased (depending on FA type, exposure condition and time) at 24 and 48 h, after exposure to hypoxic conditions, singly and also in combination with PFOSA concentration. Overall, the n-6:n-3 ratio was either slightly reduced (50% at 25 μM PFOSA, singly or in combination with CoCl₂) or unchanged at 24 h exposure, while at 48 h exposure a respective

![Figure 4. Modulation of PPAR-α (A), PPAR-β (B) and PPAR-γ (C) mRNA in salmon hepatocytes exposed to CoCl₂ (150 μM) or DFO (100 μM), singly and in combination with PFOSA (25 and 50 μM). Transcripts were analyzed using real-time polymerase chain reaction (qPCR) and expressed as mean percentage (%) of control ± SEM (n = 6). Asterisk (*) denotes significant difference (p<0.05) compared to control analyzed by Tukey’s test, while diamond (¤) denotes significant difference (p<0.05) with individual hypoxia treatment group (CoCl₂ or DFO) at respective time-interval.

doi:10.1371/journal.pone.0102485.g004](https://doi.org/10.1371/journal.pone.0102485.g004)
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**24 h:** PC1 = 0.495; PC2 = 0.188

- A – Solvent control
- B – 25 μM PFOSA
- C – 50 μM PFOSA
- D – 150 μM CoCl2
- E – 25 μM PFOSA + 150 μM CoCl2
- F – 50 μM PFOSA + 150 μM CoCl2
- G – 100 μM DFO
- H – 25 μM PFOSA + 100 μM DFO
- I – 50 μM PFOSA + 100 μM DFO

**48 h:** PC1 = 0.749; PC2 = 0.112
Contradictory findings have been reported regarding whether PPRs are important regulators of lipid and lipoprotein metabolism, glucose homeostasis, cellular differentiation and inflammatory responses [63,66]. Therefore, any change in FA profile may have physiological consequences for normal membrane functioning [67]. Herein, we showed that hypoxia given singly or in combination with PFOSA produced an apparent time-dependent change in the transcriptional level of PPAR isoforms. It should also be noted that these transcriptional increases paralleled increases of HIF-1α, ACOX, FAD5, FAD6 and FAE mRNA in the combined hypoxia and PFOSA exposure groups. The relationships between these variables were also confirmed by the PCA showing clustering of combined exposure groups and distribution of samples after 48 h. Different distribution pattern at 24 and 48 h in the PCA bi-plot, implies that changes in mRNA responsiveness is time-dependent.

The role of PPARs in physiological processes such during angiogenesis has been investigated under hypoxia condition [68] and reviewed by [69], showing inductive and inhibitory effects [69]. For example, mitochondrial FA oxidative capacity was reduced by hypoxia, resulting in reduced mitochondrial lipid mobilization and utilization, and consequent accumulation of intracellular neutral lipid [70]. In another study, cardiomyocytes increased oxygen utilization efficiency by switching from FA oxidation to glycolysis under hypoxic conditions, and this shift of metabolic substrate was achieved by HIF-1α-induced increase of the expression of glucose transporters and glycolytic enzymes [71,72], and PPRα/kRXR-mediated suppression of mitochondrial FA β-oxidation [70,73]. Using two different in vivo systemic hypoxia models (CoCl₂ and isovolemic hemodilution), Razeghi and co-workers [74] reported a decrease in the expression of PPRαs and several PPRα target genes including pyruvate dehydrogenase kinase 4 (PDK4), muscle carnitine palmitoyltransferase-1 (mPT1), and malonyl-CoA decarboxylase (MCD) in rat heart, and suggests a potential transcriptional mechanism for the decrease in long chain fatty acyl-CoA oxidation during hypoxia [74]. When the above mentioned reports are viewed with our data showing increased HIF-1α expression that paralleled PPAR isoforms, including PPARα− there are discrepancies as has been reported previously [69], regarding the cellular mechanism of peroxisomal β-oxidation towards hypoxia adaptation. It should be noted that we observed significant alterations of membrane FA profile towards hypoxia exposure. When the changes in membrane FA profile and PPARs data are taken together, there is a potential that hypoxia increased the level of endogenous ligands for all PPARs in salmon hepatocytes. This argument is supported by the observation showing no differences between PPAR isoforms, which were all increased by hypoxia exposure alone or in combination with PFOSA.

Modulation of peroxisome proliferation pathway

PPARs are important regulators of lipid and lipoprotein metabolism, glucose homeostasis, cellular differentiation and inflammatory responses [63,66]. Therefore, any change in FA profile may have physiological consequences for normal membrane functioning [67]. Herein, we showed that hypoxia given singly or in combination with PFOSA produced an apparent time-dependent change in the transcriptional level of PPAR isoforms. It should also be noted that these transcriptional increases paralleled increases of HIF-1α, ACOX, FAD5, FAD6 and FAE mRNA in the combined hypoxia and PFOSA exposure groups. The relationships between these variables were also confirmed by the PCA showing clustering of combined exposure groups and distribution of samples after 48 h. Different distribution pattern at 24 and 48 h in the PCA bi-plot, implies that changes in mRNA responsiveness is time-dependent.

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Combined Effects of Hypoxia and PFOSA on Lipid Homeostasis

3.3- and 2.7-fold increase at 25 μM PFOSA singly or in combination with CoCl₂, and respective 3.8- and 2.5-fold increase at DFO singly or in combination with 50 μM PFOSA, were observed. The observed selective hypoxia and PFOSA mediated increase in the n-6/n-3 PUFA ratio suggests a possible adaptive response towards acute hypoxic condition, representing a suggested mechanism for membrane defense against oxidative stress [62,63] which we are currently investigating as well (Olufsen et al. in prep). Further on a mechanistic standpoint, whether the increase of the elongation enzyme genes that did not parallel decreases in certain PUFAs in salmon hepatocytes is a direct response of hypoxic conditions and PFOSA effects in activating PPARs, or a secondary effect that was derived from altered membrane FA patterns, remains to be elucidated. Regardless, these data provide significant overview on the physiological processes that are involved in the hepatic response to hypoxic stress, given singly or in combination with environmental contaminants, and emphasizes the potential negative impact of high lipid consumption on fish tolerance to environmental hypoxia [64].

Regardless, we reported recently that PFOA, PFOS and PFOSA modulated lipid homeostasis and PPAR transcription in salmon in vivo and in vitro systems [39,50]. It has also been suggested that certain POPs can interact with transcription factors in a similar manner as FXAs, acting as a PPAR agonist [82]. Given that salmonid tissues are characterized by high concentrations of PUFAs, making them prone to oxidative damage [83] and fish are more protected from lipid peroxidation than mammals [83], the present data provide significant insight on the effects of hypoxia on cellular lipid homeostasis. Combined hypoxia and PFOSA exposures increased PPAR isoforms, suggesting that these emerging environmental stressors produced peroxisomal proliferation in salmon hepatocytes. Furthermore, PPARγ could be involved in the regulation of the peroxisomal β-oxidation pathway in Atlantic salmon [84,85]. Long-chain FAO, which are exclusively metabolized in the peroxisomes, exert an inhibitory effect on the peroxisomal β-oxidation [60]. Therefore, the activation of the elongation pathway could therefore explain the increased expression of ACOX1 that was observed after exposure to combined hypoxia and PFOSA. ACOX catalyses the rate-limiting step in peroxisomal β-oxidation pathway of FA, and is commonly used as a biomarker for peroxisomal proliferation [86]. The role of PPARγ in fat accumulation, adipocyte differentiation and immune

Figure 5. Biplot of principal component analysis (PCA) showing the scattering of HIF-1α, FAD5, FAD6, FAE, ACOX and PPARα, β and γ mRNA levels after either 24 h (A) or 48 h (B) of exposure. Salmon hepatocytes were exposed to CoCl₂ (150 μM) or DFO (100 μM) singly or in combination with PFOSA (25 and 50 μM) and gene expression was analyzed by qPCR. Letter denotes exposure treatment (A-Solvent control, B-25 μM PFOSA; C-50 μM PFOSA; D-50 μM CoCl₂; E-25 μM PFOSA+150 μM CoCl₂; F-50 μM PFOSA+150 μM CoCl₂; G-100 μM DFO; H-25 μM PFOSA+100 μM DFO; I-50 μM PFOSA+100 μM DFO) and followed by a number (1-10) denoting the individual sample. doi:10.1371/journal.pone.0102485.g005
response, lipid and carbohydrate metabolism has been reported [87]. Particularly, an antagonistic interaction between PPARs and PPARγ in the maintenance of lipid homeostasis [88] has been suggested. Contrary to previous findings by Wägbs et al. [2012] showing distinct and apparent concentration-dependent transcriptional increase of PPARγ by PFOSA exposure of salmon hepatocytes [83], the present study showed a comparable pattern of expression between PPARγ and PPARα after combined exposure to hypoxia and PFOSA.

In increased oxidative stress and lipid peroxidation in salmon fed a diet containing PFOS and PFOSA was reported [49]. ROS accumulation is a potentially harmful outcome of systemic hypoxia [5,89,90], and increased peroxisome proliferation may worsen the situation by adding to ROS load [91]. Lipid peroxidation produces alteration in membrane lipid structure that may affect membrane lipids and change in functionality [91,92]. Our data demonstrated increased PPAR transcription in combined hypoxia and PFOSA exposure, compared to single exposure, supporting the significance of multiple stressor investigations.

In summary, alteration of FAD5, FAD6 and FAE gene expression were generally more affected by hypoxia than PFOSA and combined exposure produced stronger effects than hypoxia alone. Regulation of lipid homeostasis is a very complex process with a myriad of pathways in the energetic budget and link to the immune system. Increased peroxisome proliferation may have detrimental effects due to an alteration of PPAR transcription and directly by increasing lipid peroxidation.

Our data show that PPARα, β and γ transcription were increased and these responses were stronger in hepatocytes experiencing combined hypoxia and PFOSA exposure. The combined effects of hypoxia and PFOSA on lipid homeostasis and β-oxidation in salmon hepatocytes suggest that these emerging multiple environmental stressors evoke deleterious effects with potential overt physiological consequences for development, reproduction and general health.

Author Contributions

Conceived and designed the experiments: AA. Performed the experiments: MO. Analyzed the data: MO. MVC contributed to the writing of the manuscript.

MO MVC AA. Supervised the entire experiment and was the lead author during manuscript preparation: AA. Performed lipid analysis and interpretation: MVC. Contributed in writing the manuscript: MVC.

References

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1994 Torbjørn Forseth Dr. scient Zoology Bioenergetics in ecological and life history studies of fishes.
1995 Olav Vadstein Dr. philos Botany The role of heterotrophic planktonic bacteria in the cycling of phosphorus in lakes: Phosphorus requirement, competitive ability and food web interactions
1995 Hanne Christensen Dr. scient Zoology Determinants of Otter Lutra lutra distribution in Norway: Effects of harvest, polychlorinated biphenyls (PCBs), human population density and competition with mink Mustela vision
1995 Svein Håkon Lorentsen Dr. scient Zoology Reproductive effort in the Antarctic Petrel Thalassoica antarctica; the effect of parental body size and condition
1995 Chris Jørgen Jensen Dr. scient Zoology The surface electromyographic (EMG) amplitude as an estimate of upper trapezius muscle activity
1995 Martha Kold Bakkevig Dr. scient Zoology The impact of clothing textiles and construction in a clothing system on thermoregulatory responses, sweat accumulation and heat transport
1995 Vidar Moen Dr. scient Zoology Distribution patterns and adaptations to light in newly introduced populations of Mytilus relictus and constraints on Cladoceran and Char populations
1995 Hans Haavardsholm Blom Dr. philos Botany A revision of the Schistidium apocarpum complex in Norway and Sweden
1996 Jorun Skjærmo Dr. scient Botany Microbial ecology of early stages of cultivated marine fish; impact fish-bacterial interactions on growth and survival of larvae
1996 Ola Ugedal Dr. scient Zoology Radiocesium turnover in freshwater fishes
1996 Ingrid Bjerg Einarsson Dr. scient Zoology Production of Atlantic salmon (Salmo salar) and Arctic char (Salvelinus alpinus): A study of some physiological and immunological responses to rearing routines
1996 Christina M. S. Pereira Dr. scient Zoology Glucose metabolism in salmonids: Dietary effects and hormonal regulation
1996 Jan Fredrik Børseth Dr. scient Zoology The sodium energy gradients in muscle cells of Mytilus edulis and the effects of organic xenobiotics
1996 Gunnar Henriksen Dr. scient Zoology Status of Grey seal Halichoerus grypus and Harbour seal Phoca vitulina in the Barents sea region
1997 Gunvor Øie Dr. scient Botany Evaluation of rotifer Brachionus plicatilis quality in early first feeding of turbot Scophtalmus maximus L. larvae
1997 Håkon Holien Dr. scient Botany Studies of lichens in spruce forest of Central Norway. Diversity, old growth species and the relationship to site and stand parameters
1997 Ole Reitan Dr. scient Zoology Responses of birds to habitat disturbance due to damming
1997 Jon Arne Grottum Dr. scient Zoology Physiological effects of reduced water quality on fish in aquaculture
1997 Per Gustav Thingstad Dr. scient Zoology Birds as indicators for studying natural and human-induced variations in the environment, with special emphasis on the suitability of the Pied Flycatcher
1997 Torgeir Nygård Dr. scient Zoology Temporal and spatial trends of pollutants in birds in Norway: Birds of prey and Willow Grouse used as
1997 Signe Nybo Dr. scient Zoology Impacts of long-range transported air pollution on birds with particular reference to the dipper Cinclus cinclus in southern Norway
1997 Atle Wibe Dr. scient Zoology Identification of conifer volatiles detected by receptor neurons in the pine weevil (*Hylobius abietis*), analysed by gas chromatography linked to electrophysiology and to mass spectrometry

1997 Rolv Lundheim Dr. scient Zoology Adaptive and incidental biological ice nucleators

1997 Arild Magne Landa Dr. scient Zoology Wolverines in Scandinavia: ecology, sheep depredation and conservation

1997 Kåre Magne Nielsen Dr. scient Botany An evolution of possible horizontal gene transfer from plants to sail bacteria by studies of natural transformation in *Acinetobacter calcoaceticus*

1997 Jarle Tufto Dr. scient Zoology Gene flow and genetic drift in geographically structured populations: Ecological, population genetic, and statistical models

1997 Trygve Hesthagen Dr. philos Zoology Population responses of Arctic char (*Salvelinus alpinus*) and brown trout (*Salmo trutta* L.) to acidification in Norwegian inland waters

1997 Trygve Sigholt Dr. philos Zoology Control of Parr-smolt transformation and seawater tolerance in farmed Atlantic Salmon (*Salmo salar*) Effects of photoperiod, temperature, gradual seawater acclimation, NaCl and betaine in the diet

1997 Jan Østnes Dr. scient Zoology Cold sensation in adult and neonate birds

1998 Seethaledsumy Visvalingam Dr. scient Botany Influence of environmental factors on myrosinases and myrosinase-binding proteins

1998 Thor Harald Ringsby Dr. scient Zoology Variation in space and time: The biology of a House sparrow metapopulation

1998 Erling Johan Solberg Dr. scient Zoology Variation in population dynamics and life history in a Norwegian moose (*Alces alces*) population: consequences of harvesting in a variable environment

1998 Sigurd Mjaen Saastad Dr. scient Botany Species delimitation and phylogenetic relationships between the *Sphagnum recurvum* complex (Bryophyta): genetic variation and phenotypic plasticity

1998 Bjarte Mortensen Dr. scient Botany Metabolism of volatile organic chemicals (VOCs) in a head liver S9 vial equilibration system in vitro

1998 Gunnar Austrheim Dr. scient Botany Plant biodiversity and land use in subalpine grasslands.– A conservation biological approach

1998 Bente Gunnveig Berg Dr. scient Zoology Encoding of pheromone information in two related moth species

1999 Kristian Overskaug Dr. scient Zoology Behavioural and morphological characteristics in Northern Tawny Owls *Strix aluco*: An intra- and interspecific comparative approach

1999 Hans Kristen Stenøien Dr. scient Botany Genetic studies of evolutionary processes in various populations of nonvascular plants (mosses, liverworts and hornworts)

1999 Trond Arnesen Dr. scient Botany Vegetation dynamics following trampling and burning in the outlying haylands at Solendet, Central Norway

1999 Ingvar Stenberg Dr. scient Zoology Habitat selection, reproduction and survival in the White-backed Woodpecker *Dendrocopos leucotos*

1999 Stein Olle Johansen Dr. scient Botany A study of driftwood dispersal to the Nordic Seas by dendrochronology and wood anatomical analysis

1999 Trina Falck Galloway Dr. scient Zoology Muscle development and growth in early life stages of the Atlantic cod (*Gadus morhua* L.) and Halibut (*Hippoglossus hippoglossus* L.)
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<td>Hans Martin Hansen</td>
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<td>Stein-Are Sæther</td>
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2002  Dag-Inge Øien  Dr. scient  Botany  Dynamics of plant communities and populations in boreal vegetation influenced by scything at Solendet, Central Norway
2002  Frank Rosell  Dr. scient  Zoology  The function of scent marking in beaver (Castor fiber)
2002  Janne Østvang  Dr. scient  Botany  The Role and Regulation of Phospholipase A2 in Monocytes During Atherosclerosis Development
2002  Terje Thun  Dr. philos  Biology  Dendrochronological constructions of Norwegian conifer chronologies providing dating of historical material and their role in defense, development and growth
2002  Birgit Haftfeld Borgen  Dr. scient  Biology  Effects of climatic change on the growth of dominating tree species along major environmental gradients
2002  Bård Øyvind Solberg  Dr. scient  Biology  The evolution of small GTP binding proteins in cellular organisms. Studies of RAC GT Pases in Arabidopsis thaliana and the Ral GTPase from Drosophila melanogaster
2002  Per Winge  Dr. scient  Biology  The Role and Regulation of Phospholipase A2 in Monocytes During Atherosclerosis Development
2002  Henrik Jensen  Dr. scient  Biology  Cultivation of herbs and medicinal plants in Norway – Essential oil production and quality control
2002  Jens Rohloff  Dr. philos  Biology  Behavioural effects of environmental pollution in threespine stickleback Gasterosteus aculeatus L.
2002  Åsa Maria O. Espmark Wibe  Dr. scient  Biology  Assisted recovery of disturbed arctic and alpine vegetation – an integrated approach
2002  Dagmar Hagen  Dr. scient  Biology  Reproductive strategies in Scandinavian brown bears
2003  Cyril Lebogang Taolo  Dr. scient  Biology  Population ecology, seasonal movement and habitat use of the African buffalo (Syncerus caffer) in Chobe National Park, Botswana
2003  Marit Stranden  Dr. scient  Biology  Olfactory receptor neurones specified for the same odors in three related Heliothine species (Helicoverpa armigera, Helicoverpa assulta and Heliothis virescens)
2003  Kristian Hassel  Dr. scient  Biology  Life history characteristics and genetic variation in an expanding species, Pogonatum dentatum
2003  David Alexander Rae  Dr. scient  Biology  Plant- and invertebrate–community responses to species interaction and microclimatic gradients in alpine and Artic environments
2003  Åsa A Borg  Dr. scient  Biology  Sex roles and reproductive behaviour in gobies and guppies: a female perspective
2003  Eldar Åsgard Bendiksen  Dr. scient  Biology  Environmental effects on lipid nutrition of farmed Atlantic salmon (Salmo Salar L.) parr and smolt
2004  Torkild Bakken  Dr. scient  Biology  A revision of Nereidinae (Polychaeta, Nereididae)
2004  Ingar Pareliussen  Dr. scient  Biology  Natural and Experimental Tree Establishment in a Fragmented Forest, Ambabintely Forest Reserve, Madagascar
2004  Tore Bremsen  Dr. scient  Biology  Genetic, molecular and functional studies of RAC GT Pases and the WAVE-like regulatory protein complex in Arabidopsis thaliana
2004  Liv S. Nilsen  Dr. scient  Biology  Coastal heath vegetation on central Norway; recent past, present state and future possibilities
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The effect of macronutrient composition, insulin stimulation, and genetic variation on leukocyte gene expression and possible health benefits

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Operational sexual ratio and reproductive behaviour in the two-spotted goby (*Gobiusculus flavescens*)

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Effects of complex organohalogen contaminant mixtures on thyroid hormone homeostasis in selected arctic marine mammals

Spatiotemporal variation in resource utilisation by a large herbivore, the moose
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