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Cellular Lipid Homeostasis and Oxidative Stress Responses in the Liver of Atlantic Cod (*Gadus morhua*) Exposed to a Quantifiable Measure of Climate Change (CO_2) and Perfluorooctane Sulfonate (PFOS)

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ABSTRACT

Increase in atmospheric carbon dioxide (CO_2) is generally regarded as the main cause of global climate change, and this condition has also been shown in aquatic environments. In addition, emerging contaminants are ubiquitously distributed in the aquatic environment, and interactions between CO_2 and emerging pollutants in biological systems are inevitable. The aim of this master thesis was to elucidate the combined effect of the environmental contaminant perfluorooctane sulfonate (PFOS) and increased water CO_2 concentration on oxidative stress response and lipid homeostasis in liver of juvenile Atlantic cod (*Gadus morhua*). Juvenile Atlantic cod were exposed for one hour daily to 0, 100, or 200 μg PFOS/L for a total of 5 days, and thereafter, transferred to tanks with different increases in CO_2 levels (normal, 0.3 %, and 0.9 %). Tissue samples were collected at 3, 6, and 9 days after initiated CO_2 exposure. Real-time RT-PCR was used to analyse expression of various genes involved in lipid homeostasis and oxidative stress response. The activities of selected antioxidant enzymes, and also total Glutathione and Malondialdehyde, were measured spectrophotometrically. At day 3, exposure to elevated CO_2 increased expression of *glutathione peroxidase 1* (*gpx1*), with PFOS having a small potentiating effect. Oxidative stress was likely a direct consequence of increased CO_2 level, either caused by formation of reactive oxygen and nitrogen species from nitrosoperoxycarboxylate, or by pH-dependent increase of Fenton reaction. At day 6 and 9, increased CO_2 caused an increased expression of *peroxisome proliferator-activated receptor β* (*pparβ*). A following increase in fatty acid β-oxidation and subsequent generation of hydrogen peroxide (H_2O_2) and superoxide anion ($\cdot O_2^-$) was probably the mechanism behind increased expression and activity of several antioxidant enzymes. At day 9, a 0.9 % elevation in CO_2 level caused increased Glutathione S-transferase (GST) activity. This might be due to increased need for clearance of molecules damaged by oxidative attack, which indicates that antioxidant defence system of the cell was overwhelmed. Overall, this suggests that exposure to increased CO_2 levels can induce oxidative stress in liver of Atlantic cod, and that the emerging contaminant PFOS can potentiate this effect. This might have consequences for overall fitness and survival of organisms.

SAMMENDRAG

Økning i atmosfærisk nivå av karbondioksid (CO_2) anses for å være den viktigste årsaken til globale klimaendringer. Økt CO_2-nivå observeres også i havet, og i tillegg foreligger miljøgifter i høye konsentrasjoner. Formålet med denne masteroppgaven var å avdekke den kombinerte effekten av miljøgiften perfluorooktansulfonat (PFOS) og økt nivå av CO_2 i vannet på oksidativ stressrespons og lipidhomeostase hos juvenile torsk (*Gadus morhua*). Juvenile torsk ble eksponert for 0, 100 eller 200 µg PFOS/L i en time daglig i til sammen fem dager. Deretter ble fiskene overført til tanker med ulikt CO_2-nivå i vannet (normalt sjøvann, 0,3 % økning og 0,9 % økning). Vevsprøve ble tatt på dag 3, 6 og 9 etter at CO_2-eksponeringen startet. Real-time RT-PCR ble brukt til å analysere uttrykk av gener involvert i lipidhomeostase og oksidativ stressrespons, mens aktiviteten av antioksidantenzymer og totalt nivå av glutation og malondialdehyd ble målt spektrofotometrisk. På dag 3 var det en økning i genuttrykk for *glutation peroksidase 1* (*gpx1*) som respons på økt CO_2, og PFOS hadde en svakt potensierende effekt. Oksidativt stress var sannsynligvis en direkte konsekvens av økt CO_2-nivå. På dag 6 og 9 førte en økning i CO_2-nivå til en økning i uttrykk av *peroxisome proliferator-activated receptor β* (*pparβ*). En etterfølgende økning i β-oksidering av fettsyrer og dannelse av hydrogenperoksid (H_2O_2) og superoksidanion ($\cdot O_2^-$) var sannsynligvis mekanismen bak den observerte økningen i uttrykk og aktivitet av en rekke antioksidantenzymer. På dag 9 ble det observert økt aktivitet av glutation S-transferase (GST). Dette kan ha blitt forårsaket av et økt behov for fjerning av molekyler skadet av oksidative forbindelser, som kan være en indikasjon på at cellenes forsvarssystem mot oksidativt stess var blitt overbelastet. Disse observasjonene indikerer at en kombinasjon av PFOS og økt CO_2-nivå i havet kan foårsake oksidativt stress hos juvenile torsk, og en mulig konsekvens av dette kan være nedsatt reproduksjons- og overlevelsesevne.

CONTENTS

ACRONYMS AND ABBREVIATIONS

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATPase	ATP hydrolase
cDNA	Complementary deoxyribonucleic acid
CDNB	1-Chloro-2,4-dinitrobenzene
CHP	Cumene hydroperoxide
CoA	Coenzyme A
Ct	Cycle threshold
CYP19	Cytochrome P450 19
ddH_2O	Double-distilled water
dH2O	Distilled water
DEPC	Diethylpyrocarbonate
Dil.	Dilution
DNA	Deoxyribonucleic acid
DTNB	Ellman's Reagent, 5,5'-Dithiobis-(2-Nitrobenzoic Acid)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FA	Fatty acid
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Oxidized Glutathione
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
KH_2PO_4	Potassium phosphate (K-phosphate)
MDA	Malondialdehyde
mM	millimoles L^{-1}
mRNA	Messenger ribonucleic acid
MS-222	Tricaine methanesulphonate
MQ-water	Millipore water
NaCl	Sodium Chloride
NADPH	Nicotinamide adenine dinucleotide phosphate

nm	Nano meters
$\cdot O_2^-$	Superoxide anion
OD	Optical density
·OH	Hydroxyl radical
PCR	Polymerase chain reaction
PFC	Perfluorinated compounds
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonate
PMSF	Phenylmethylsulfonyl fluoride
POP	Persistent organic pollutant
POSF	Perfluoroctanesulfonyl fluoride
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator response element
R^2	Correlation coefficient
RNA	Ribonucleic acid
RNase	RNA nuclease
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
RXR	Retinoid X receptor
SOD	Superoxide dismutase
TCA	Tricarboxylic acid cycle (citric acid cycle)
Tf	Transferrin
U	Unit
Vol.	Volume
w/v ratio	Weight/volume ratio

1 INTRODUCTION

1.1 Background

As humans invent new technology, increase energy demands, and develop new chemicals, our impact on nature gets more and more profound. Although we have utilized nature for thousands of years, our way of using it has changed dramatically during the past few centuries. Burning of fossil fuels increase atmospheric content of carbon dioxide (CO_2) and other gases such as methane (CH_4) and nitrous and sulphuric compounds (IPCC Fourth Assessment Report, 2007). Many of these gases function as climate gases and participate in the global warming we experience today, and they can also have a direct effect on living organisms (Asshoff et al., 2006; Rosenzweig et al., 2008). Much of the released CO_2 dissolve in oceans and thereby increase content of dissolved inorganic carbon and reduce pH (Haugan and Drange, 1996; Manahan, 2009). There has also been a rapid development in producing new chemicals, both for use in consumer products, industry, medicine, and in agriculture. Enormous amounts of these are released in nature without prior testing for toxicity, and many of these are persistent organic pollutants (POPs) (Stockholm Convention, 2013). Pollutants are defined as chemicals present in the environment in levels exceeding what is considered normal and they have the ability of causing environmental harm (Walker et al., 2006). POPs accumulate in organisms and food chains, and their toxicity can influence survival and reproduction, affecting overall fitness of species (Stockholm Convention, 2013). Presently we have more knowledge about the effects of our actions, and research has provided us with an understanding of how chemicals and climate changes potentially can affect biological systems (Rosenzweig et al., 2008; Wania and MacKay, 1996; WHO, 2003). The combination of climate changes and environmental pollutants can, however, have unknown and unforeseen consequences, and must therefore be investigated.

1.2 Perfluorinated compounds

Perfluorinated compounds (PFCs) are a large group emerging POPs. These are man-made substances that have been produced in large quantities during the past 60 years (3M, 1999). Because of their surface-active properties, they have been used as paper coating, refrigerants, lubricants, retardants, adhesives, insecticides, polymers, fabric protectors, and fire retardants (Giesy and Kannan, 2001; Key et al., 1997). Many of them are environmentally persistent and are distributed in human and wildlife worldwide (Giesy and Kannan, 2001; Kannan et al., 2004). Originally, the molecules were considered biologically inactive due to their recalcitrance and inertness (Key et al., 1997; Renner, 2001), but research has shown that they can exert toxicity by a wide range of mechanisms. Still, toxicity of many PFCs remains to be investigated, and there is also a need for investigation of long term effects of prenatal and early-life exposure, and also long-term eco-toxicological evaluations (Kannan, 2011). After being produced for over 50 years, many of the chemicals were phased out of production in 2000-2002 by major manufacturers in Europe and the USA, but production is maintained by some manufacturers in southeast Asia (3M, 2003; Kannan, 2011; Paul et al., 2009).

1.2.1 Biomagnification and tissue distribution

Although PFCs were discovered in human blood serum in 1968 (Taves, 1968), they received little attention before Giesy and Kannan published a report of the global distribution of PFOS in wildlife in 2001 (Braune and Letcher, 2013; Giesy and Kannan, 2001). This report was a part of a large and comprehensive program for monitoring the global distribution of PFOS. In subsequent years, thorough assessments of environmental fate and distribution of different PFCs have been performed (Braune and Letcher, 2013; Calafat et al., 2007; Houde et al., 2006; Kannan, 2011; Kannan et al., 2002; Kärrman et al., 2011; Nakayama et al., 2005; Rubarth et al., 2011; Taniyasu et al., 2003; Van de Vijver et al., 2003; Wang et al., 2008; Yamashita et al., 2005; Yeung et al., 2008) , confirming the results of Giesy and Kannan (2001). Overall, these studies show that PFOS is the major PFC species reported in human and wildlife tissues, followed by perfluorooctane sulfonic acid (PFOA). These compounds biomagnify in the food chain (3M, 2003; Giesy and Kannan, 2002; Haukas et al., 2007), and they will therefore be of environmental concern for many years to come. Highest concentrations are found in piscivorous birds and mammals, such as bald eagle, mink, otter, and polar bears, and concentrations are greatest near industrialized areas. Areas that are well investigated include the Great Lakes in USA and Baltic Sea (3M, 2003; Giesy and Kannan, 2001; Greaves et al., 2013; Martin et al., 2004).

Despite the fact that there are no emission sources for PFCs in remote areas such as the Arctic, high concentrations are found in biota and abiota (Loewen et al., 2005; Martin et al., 2004; Yamashita et al., 2005). PFOS and other PFCs have a low vapour pressure and it is unlikely that they are atmospherically transported in their vapour phase (Loewen et al., 2005). Precursor molecules with lower vapour pressure, such as fluorotelomer alcohols, can be effectively transported in the atmosphere, undergo oxidation to PFOS and PFOA, and be deposited elsewhere with rainwater (Ellis et al., 2004; Loewen et al., 2005). Long-range oceanic transport is a possible route for direct emissions from industrialized and urbanized areas to remote locations. This is a slow process, but it is nevertheless considered the far most effective method (Wania, 2007).

1.2.2 Chemistry and applications of PFOS

PFOS has the general formula ($C_8HF_{17}O_3S$) (Figure 1). The polar sulfonate group situated at the end of the fluorocarbon chain makes the compound moderately water soluble, but it also has a strong tendency to adhere to surfaces (3M, 2003). The high-energy carbon-fluorine bonds make it resistant toward hydrolysis, photolysis, microbial degradation, and metabolism by vertebrates (3M, 2003; Giesy and Kannan, 2002). It was mainly used as an additive for improving surface properties of other products, and it was also a by-product from the production process in various consumer products (Houde et al., 2006). It was used together with PFOA in fire fighting foam, and has been extensively used as a fabric and carpet protector and stain repellent, and in impregnation agents for fabric, leather and paper (Paul et al., 2009; Renner, 2001).

PFOS has become the most widespread PFC because of a broad application spectrum and high persistence. In addition, all perfluoroctanesulfonyl fluoride (POSF)-based compounds are metabolized to or broken down to PFOS (Liu et al., 2007; Yamashita et al., 2005). PFOS does not accumulate in adipose tissue like other persistent organic pollutants (POPs) – rather, it binds strongly to serum proteins and accumulates in blood, liver and brain (Jones et al., 2003; Long et al., 2013; Mortensen et al., 2011; Zhang et al., 2009).

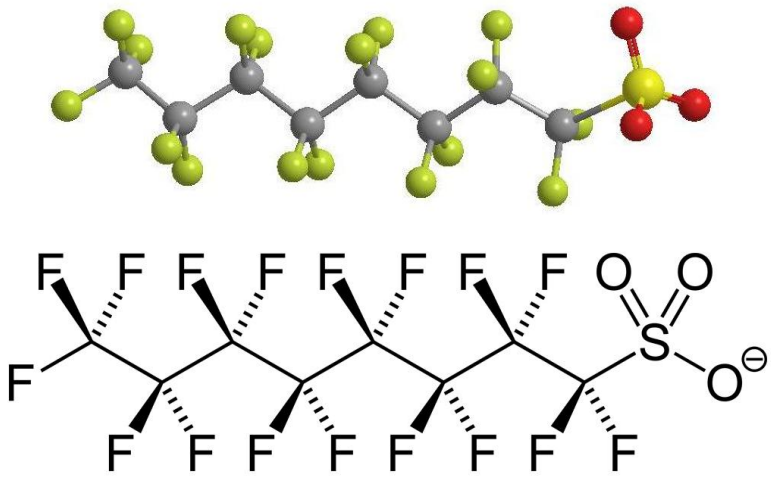

Figure 1. 3D and 2D structure of Perfluorooctane sulfonate (PFOS).

1.2.3 Biological activity of PFCs and PFOS

Toxicity of PFCs include endocrine disruption and interference of reproductive endpoints (Joensen et al., 2009; Oakes et al., 2005), interference with lipid metabolism and homeostasis by activation of peroxisome-proliferator-activated receptors (PPARs) (Arukwe et al., 2013; Kawashima et al., 1989; Wågbø et al., 2012), disruption of thyroid hormone homeostasis (Melzer et al., 2010), and they can cause oxidative damage and act as carcinogens by the secondary production of reactive oxygen species (ROS) (Bjork and Wallace, 2009; Vanden Heuvel et al., 2006). Toxicity of many PFCs have yet to be assessed (Kannan, 2011).

PFOS is hepatotoxic in rodents and monkeys, causing hepatocellular hypertrophy, lipid vacuolation, and peroxisome proliferation (Hu et al., 2002; Seacat et al., 2002; Starkov and Wallace, 2002). It has been shown to induce oxidative stress in fish, most likely by increasing β-oxidation of lipids, producing ROS as a by-product (Huang et al., 2009; Krøvel et al., 2008; Liu et al., 2007; Oakes et al., 2005; Shi et al., 2008; Shi and Zhou, 2010). It can alter the fluidity of biological membranes (Hu et al., 2003) and inhibit gap junctional intercellular communication (Hu et al., 2002). It is neurotoxic in adult and developing rodents, probably due to oxidative stress-mediated apoptosis of neuronal cells (Lee et al., 2012; Long et al., 2013). Effects on reproduction have also been revealed (Ankley et al., 2005; Oakes et al., 2005). Shi and co-workers (2008; 2009) found that oxidative damage play an important role in reproductive toxicity by PFOS, while others have suggested that the mechanism might be interference with steroid hormone metabolism (Ankley et al., 2005).

1.3 Lipid metabolism

1.3.1 Peroxisome proliferator-activated receptors

Numerous *in vivo* and *in vitro* studies have reported that PFOS and other PFCs are ligands for PPARs, both in mammals and in fish (Bjork and Wallace, 2009; Shipley et al., 2004; Takacs and Abbott, 2007; Wolf et al., 2008; Wågbø et al., 2012; Zhang et al., 2012). However, they do not activate the different receptors to the same extent as naturally occurring fatty acids (FAs) (Vanden Heuvel et al., 2006). PPARs are ligand-activated transcription factors belonging to the nuclear receptor superfamily that function as master regulators of lipid metabolism and homeostasis (Desvergne and Wahli, 1999; Laudet et al., 1992). Three isotypes of PPARs have been characterized: PPARα, PPARβ/δ (denoted PPARβ in this thesis), and PPARγ (Issemann and Green, 1990; Schmidt et al., 1992; Zhu et al., 1993). Ligand-bound PPAR forms a heterodimer with Retinoid X receptor (RXR), before binding to the peroxisome proliferator response element (PPRE) in the promoter region upstream of peroxisome proliferator target genes and thereby activates their transcription (Bardot et al., 1993; Berger and Moller, 2002) (Figure 2). Activated PPARs also activate their own transcription (Pineda Torra et al., 2002).

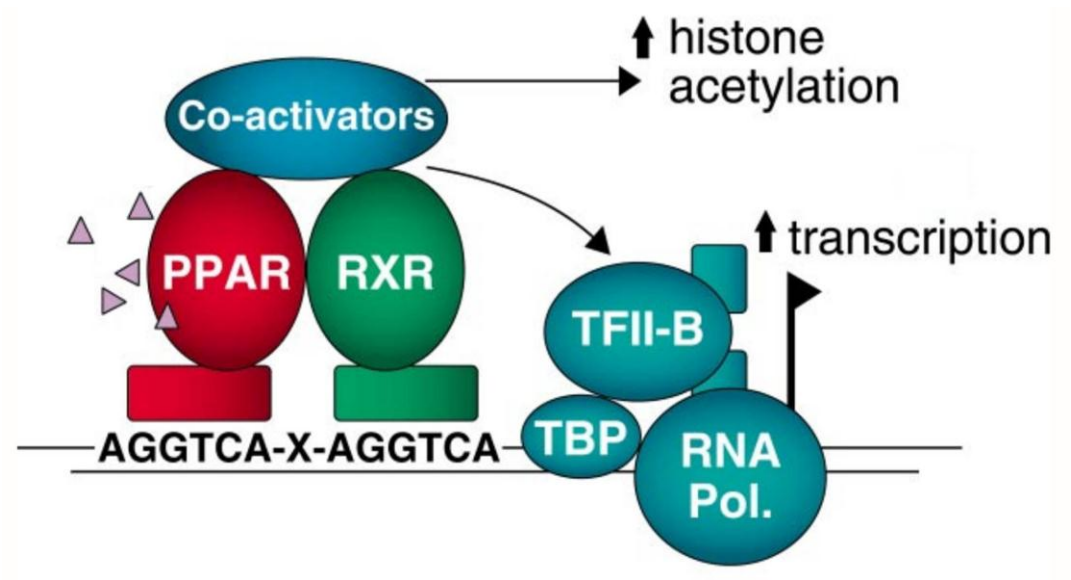

Figure 2. Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear receptor family. Ligand-bound PPARs form heterodimers with Retinoid x receptor (RXR) and binds to peroxisome proliferator response elements (PPRE) in the promoter region of target genes. Binding of co-activators is also important. Target genes are involved in lipid metabolism and homeostasis (Berger and Moller, 2002).

The different PPAR isotypes show tissue-specific expression patterns and play different roles in the organism. PPARβ have a ubiquitous expression pattern, and although the physiological roles have yet to be established, evidence shows that it enhances FA oxidation (Tanaka et al., 2003; Wang et al., 2003). PPARα is expressed in liver, kidney, heart, and muscle, and they activate genes involved in peroxisomal and mitochondrial β-oxidation of FA and lipoprotein metabolism (Tanaka et al., 2003). PPARγ is expressed in adipose tissue and intestines, where it regulates the expression of genes involved in adipogenesis (Boelsterli, 2009; Nelson and Cox, 2008; Tanaka et al., 2003).

1.3.2 Fatty acid catabolism

FAs contain chemical energy and can be used as fuels by organisms. They are catabolized in mitochondria and peroxisomes in the four-step β-oxidation process, yielding Acetyl-Coenzyme A (CoA) that subsequently enters tricarboxylic acid cycle (TCA) and thereby takes part in the oxidative ATP-generating processes in cells (Nelson and Cox, 2008). Mitochondria and peroxisomes contain different sets of enzymes having different substrate preferences (Crockett and Sidell, 1993; Kim and Miura, 2004). Peroxisomal β-oxidation is generally considered a chain shortening step, where carbon atoms of long-chain FAs are removed, rendering FAs of shorter chain length that can be degraded in mitochondria (Osmundsen et al., 1991).

The first and rate-limiting step in β-oxidation is catalysed by various Acyl-CoA dehydrogenases (ACOD) in mitochondria and by Acyl-CoA oxidases (ACOX) in peroxisomes. Both types are flavoproteins with a Flavin adenine dinucleotide (FAD) prosthetic group functioning as an electron acceptor in the oxidation of the fatty acid (Kim and Miura, 2004; Nelson and Cox, 2008). FAD in Acyl-CoA dehydrogenase is re-oxidized by electron transfer flavoprotein (ETF), which brings the electrons into the electron transport chain and thereby contribute to generation of superoxide anion ($\cdot O_2^-$). FAD in Acyl-CoA oxidase is re-oxidized by molecular oxygen in a process that generates hydrogen peroxide (H_2O_2) (Kim and Miura, 2004). Hydrogen peroxide and superoxide anion are reactive oxygen species (ROS) and can harm biological systems by attacking important macromolecules (Storey, 1996).

1.4 Oxidative stress

The toxicity of many pollutants, such as polychlorinated biphenyls (PCBs), aromatic nitro compounds, transition metals, polynuclear aromatic hydrocarbons (PAH), and also PFCs, can be mediated through oxidative damage (Livingstone, 2001; Zhou et al., 2006). These chemicals can influence the oxidative balance in cells, either by being reactive itself, or by inducing generation of reactive molecules (Livingstone, 2001; Livingstone et al., 2000). In addition, toxic compounds can cause oxidative stress by inhibiting antioxidant enzyme activity, and by inhibition of mitochondrial electron transport chain with subsequent accumulation of reduced intermediates (Reviewed by Kelly et al., 1998).

Oxidative stress is an imbalance between processes that produce radicals and pro-oxidants such as ROS (Figure 3) and reactive nitrogen species (RNS), and antioxidant enzyme activity, which is a protective cellular process (Farber, 1994; Sies, 1997). Various metabolic processes can generate ROS, including β-oxidation and oxidative phosphorylation by superoxide leakage from the electron transport chain, and ROS can also be produced through auto-oxidation of small molecules in the cell (Kelly et al., 1998; Kim and Miura, 2004; Livingstone, 2001; Storey, 1996; Turrens and Boveris, 1980). The rate of ROS generation is closely related to the amount of oxygen consumed by the cells within an organism (Storey, 1996).

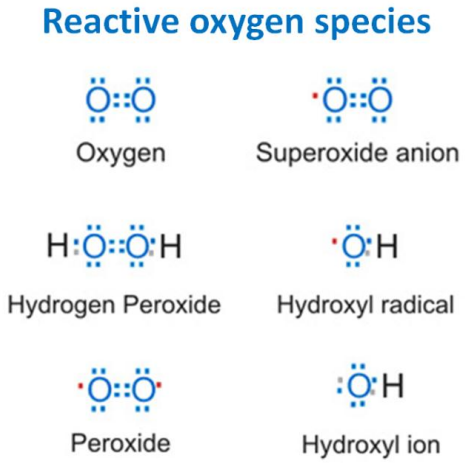

Figure 3. Various cellular processes, such as peroxisomal β-oxidation, oxidative phosphorylation, and Fenton reaction, can generate reactive oxygen species (ROS). These highly reactive molecules can interact with important biological molecules, such as DNA, proteins and lipids, causing impairment of their function. The formation of ROS might be increased by exposure to persistent organic pollutants (POPs). The figure displays various reactive oxygen species, shown with unpaired electrons.

PFOS induces oxidative stress in fish (Huang et al., 2009; Krøvel et al., 2008; Liu et al., 2007; Shi et al., 2008; Shi and Zhou, 2010). By binding to and activating PPARs, PFOS will increase transcription of *acyl-CoA oxidase* (*acod*) and *acyl CoA dehydrogenase* (*acox*) genes, and increased activity of these enzymes will cause increased levels of ROS (Bjork and Wallace, 2009; Desvergne and Wahli, 1999; Nelson and Cox, 2008). This will not have a harmful effect under normal circumstances since ROS is rapidly scavenged by antioxidant enzymes (Kim and Miura, 2004). However, when PFOS induce an increase in β-oxidation, production of hydrogen peroxide and superoxide anion can overwhelm scavenging capacity of the antioxidant enzymes, leading to oxidative stress (Green, 1995; Hu et al., 2005; Sohlenius et al., 1993).

ROS can attack and damage biological macromolecules, including DNA, proteins, and lipids, causing an impairment of their function (Alberts et al., 2008). DNA mutations or strand-breaks can be a result of oxidative damage, and might contribute to the development of cancer through interference with the cell cycle control system. Oxidative attack on functional groups in proteins can alter the protein's conformation and thereby alter or inhibit its normal function, while oxidative damage on membrane lipids might impair the integrity of the membranes and cause leakage of substances between compartments (Bindoli, 1988; Farber, 1994; Storey, 1996).

Superoxide anion ($\cdot O_2^-$) and hydroxyl radical ($\cdot OH^-$) are ROS with only one unpaired electron and will oxidize other molecules rapidly to gain an electron (Kleinveld et al., 1989; Pryor et al., 2006; Sies, 1997). Non-radical reactive oxygen species, such as hydrogen peroxide (H_2O_2), are also termed ROS. Hydrogen peroxide and superoxide anion are less reactive than hydroxyl radical, but will nevertheless oxidize and harm biological molecules (Livingstone, 2001). They can also be converted to hydroxyl radical by Haber-Weiss reaction (Equation 3), which is a two-step process catalysed by transition metals (mostly Fe^{3+}, but sometimes Cu^{3+}) that involves Fenton reaction (Equation 2), and toxicity of hydrogen peroxide and superoxide anion is highly dependent on presence of such metal ions (Livingstone, 2001; Storey, 1996).

$$Fe^{3+} + \cdot O_2^- \rightarrow Fe^{2+} + O_2 \qquad \text{Reduction of iron} \qquad (1)$$

$$Fe^{2+} + H_2H_2 \rightarrow Fe^{3+} + OH^- + \cdot OH^- \qquad \text{Fenton reaction} \qquad (2)$$

$$\cdot O_2^- + H_2O_2 \rightarrow O_2 + OH^- + \cdot OH^- \qquad \text{Haber-Weiss reaction} \qquad (3)$$

1.4.1 Antioxidant enzymes

Organisms have developed intricate protection systems in order to prevent severe damage caused by ROS and other reactive chemical species, and protection is three-levelled: 1) Prevention of ROS formation, 2) Termination of ROS by free radical scavengers such as antioxidant enzymes, and 3) Repair of damaged cell components (Sies, 1997; Storey, 1996). Gene transcription of antioxidant enzymes such as Superoxide dismutase (SOD), Glutathione peroxidase (GPx, and Catalase (Cat) are to various degrees induced by oxidative stress, in company with cofactors and co-substrates. Messenger ribonucleic acid (mRNA) levels of these genes can be used as an indicator for presence of ROS (Sen and Packer, 1996).

Superoxide dismutase

SOD is a group of key antioxidant enzymes, catalysing dismutation of superoxide anion to hydrogen peroxide and oxygen (Equation 4).

$$2\ {}^{\cdot}O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2 \qquad (4)$$

Hydrogen peroxide is further detoxified by other antioxidant enzymes (Storey, 1996). Different forms of SOD exist, either with cupper (Cu) and zinc (Zn), manganese (Mn), or iron (Fe) as cofactor metal. Mn-SOD situates in mitochondria and is induced by oxidative stress. CuZn-SOD is found in cytosol, while Fe-SOD is extracellular (Boelsterli, 2009).

Catalase

Cat is a peroxisomal enzyme responsible for degradation of hydrogen peroxide and is especially abundant in liver. Hydrogen peroxide generation in peroxisomes is high due to lipid metabolism, but high activity rate of this enzyme ensures rapid scavenging and keeps the concentration of ROS below harmful levels (Boelsterli, 2009; Hashimoto and Hayashi, 1987).

Glutathione peroxidase and Glutathione reductase

GPx is important for extra-peroxisomal inactivation of hydrogen peroxide (Raes et al., 1987). These selenium-containing enzymes use glutathione (GSH) as a substrate when converting hydrogen peroxide to water (Figure 4). In this process the cysteine thiol group of GSH is oxidized to GS· radical, which is further dismutated to glutathione disulphide (GSSG). Glutathione reductase (GR) regenerates GSH from GSSG in a NADPH-driven process, and the ratio of GSH to GSSG is therefore partly dependent on cell energy status (Boelsterli, 2009; Storey, 1996). There are several different GPx proteins, located in different cellular compartments. At least two of these are part of the antioxidant defence system (Arthur, 2000).

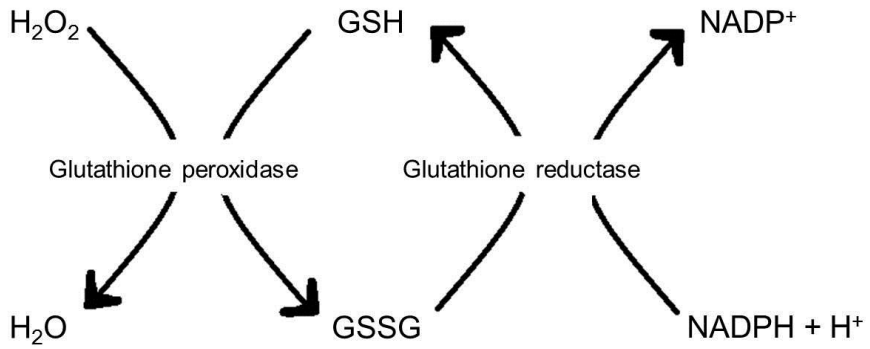

Figure 4. Overview of the Glutathione peroxidase/Glutathione reductase system. Glutathione peroxidase scavenges hydrogen peroxide (H_2O_2) and oxidizes glutathione (GSH) in the process. Glutathione reductase regenerates GSH, using NADPH as electron donor.

1.4.2 Lipid peroxidation

Oxidative degradation of membrane lipids is referred to as lipid peroxidation. Under circumstances where ROS generation is increased, membrane lipids can become oxidized by a radical, and damage will readily propagate and spread across the membrane in a chain reaction (Farber, 1994). The process is initiated when a radical, usually hydroxyl radical, removes a hydrogen atom from a methylene (-CH_2-) group in a membrane lipid. The unpaired electron on the carbon atom is stabilized by a molecular rearrangement in the molecule that generates a conjugated diene. The diene reacts with oxygen and generates a peroxy radical. The peroxy radical removes a hydrogen atom from a nearby lipid in order to become a hydroperoxide, and in this process it initiates the chain reaction (Farber, 1994; Storey, 1996).

As previously mentioned, many toxic pollutants have the ability of increasing generation of ROS, and a secondary mechanism of toxicity might therefore be peroxidation of lipids (Thomas and Wofford, 1993; Zhou et al., 2006). Irreversible cell injury can thereby be caused by various mechanisms, depending on the function of the cellular membrane where lipid peroxidation takes place (Farber, 1994). Lipid peroxidation in microsomal membranes can cause calcium release and subsequent uncontrolled activation of calcium-dependent proteases and lipases (Geeraerts et al., 1991; Orrenius et al., 1989). If mitochondrial membranes suffer from lipid peroxidation, there can be a change in membrane permeability which can disrupt energetic processes in the cell (Bindoli, 1988). Unsaturated fatty acids are susceptible to oxidative damage, since the ethylenic bonds between carbon atoms are less stable than simple covalent bonds and will be more readily oxidized by ROS (Kelly et al., 1998). Therefore, toxicants with ability of inducing lipid peroxidation can cause great harm on tissues rich in unsaturated fatty acid, such as the brain (Coyle and Puttfarcken,

1993). The final metabolites from lipid peroxidation are also reactive and can damage cells further (Boelsterli, 2009). One of these is malondialdehyde (MDA), which is mutagenic and carcinogenic (Basu and Marnett, 1983; Marnett, 1999). Presence of MDA in cells is a biomarker for lipid peroxidation (Boelsterli, 2009; Marnett, 1999).

1.4.3 Glutathione S-transferase

Glutathione S-transferase (GST) is a superfamily of enzymes with high abundance in liver cytosol, which participate in detoxification of many xenobiotics (Boelsterli, 2009). They catalyse transfer of GSH to the xenobiotic compound or a cellular component damaged by ROS attack, and thereby make it more soluble and ready for renal excretion (Storey, 1996). The substrate can be a reactive metabolite of a parent compound, and the parent compound often has the ability of inducing Glutathione S-transferase (Boelsterli, 2009). Expression of *gst* gene have been shown to increase upon exposure to PFOS (Mortensen et al., 2011).

1.5 Climate changes

Since the onset of the industrial revolution in the late 18th century there has been a marked increase in partial pressure of CO_2 (P_{CO2}) in the atmosphere that exceeds natural fluctuations (IPCC Fourth Assessment Report, 2007). The main cause of this is anthropogenic combustion of fossil fuels and land use changes such as deforestation. Humans are also responsible for emissions of other greenhouse gases, such as methane (CH_4) and nitrous oxide (N_2O). Greenhouse gases cause an increase in temperature near earth's surface, and this might influence living organisms (IPCC Fourth Assessment Report, 2007).

1.5.1 Water acidification

CO_2 is a weak acid which dissociates partially in water to produce hydrogen ion ($H^{+)}$ and bicarbonate ion (HCO_3^-), and bicarbonate is further converted to carbonate ion (CO_3^{2-}) and H^+ (Manahan, 2009) (Equation 5).

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \rightleftharpoons H^+ + CO_3^{2-} \quad (5)$$

Since equilibrium shifts to the right in slightly alkaline waters, this ionization greatly increases solubility of CO_2 in water. This is known as the carbonate system. It brings about a great buffering capacity, and this mechanism is important in reducing atmospheric content of CO_2 (Manahan, 2009). However, oceanic elevation of CO_2 level (hypercapnia) will have the potential of reducing pH in water because of H^+ generation. In the past 200 years, pH in ocean surface waters has decreased by 0.1 unit, and it will further decrease by 0.2-0.3 units during the next century (Haugan and Drange, 1996).

1.5.2 Influence on aquatic organisms

It is essential for organisms to maintain ion balance and preserve pH despite environmental fluctuations (Deigweiher et al., 2008). CO_2 enters an organism by diffusion through gill epithelia, and will equilibrate between body compartments, where it will have an acidifying effect (Pörtner et al., 2004), caused by the enzyme Carbonic anhydrase, which in red blood cells catalyse conversion of CO_2 and H_2O to H^+ and HCO_3^- (VanPutte et al., 2010). Oxygen carrying capacity of plasma respiratory proteins depends on plasma pH. In gills, excess CO_2 is released to the surrounding water and a subsequent decrease in alkalinity of plasma increases the respiratory protein's affinity for oxygen (Nelson and Cox, 2008). When respiratory proteins are faced with lower pH in tissues due to respirational CO_2 production, bound O_2 are released because of lowered affinity of the proteins. CO_2 can bind in their place and be transported out of the system (Campbell et al., 2008). However, an increase in CO_2 concentration of surrounding water might influence this intricate system in several ways:

1. More CO_2 will diffuse into the blood and reduce its pH. Reduced pH in the gills lowers oxygen carrying capacity of respiratory proteins, and can lead to hypoxia in tissues.
2. CO_2 carried from the tissues by respiratory proteins are to be released to surrounding water when it reaches the gills. If CO_2 concentration of in water is increased, the lowered concentration gradient between gills and surrounding water will reduce efficiency of this process.

Organisms must maintain pH level in all body compartments in order to survive. In fish, the initial reduction in pH in tissues (acidosis) induced by hypercapnia will gradually be compensated for by accumulation of HCO_3^-, followed by an equimolar loss of anions (Deigweiher et al., 2008; Pörtner et al., 2004; Pörtner et al., 1998), and pH in blood and extracellular fluids will return to pre-

exposure levels (Baker et al., 2009). This is achieved by up-regulation of different ionic transporters, such as the Cl^-/HCO_3^- exchanger and the Na^+/HCO_3^- co-transporter (Claiborne et al., 2002; Deigweiher et al., 2008). These protective mechanisms are energy-demanding and is paralleled with a coordinated reduction in ATP-producing and -consuming processes (Reipschläger and Pörtner, 1996), especially protein synthesis (Langenbuch and Portner, 2003).

Hypercapnia-induced oxidative stress

Hypercapnia can have a dual effect on oxidative stress response. Increased formation of ROS and RNS is a known effect of hypercapnia. Radical nitric oxide (·NO) formed in cells can react with $\cdot O_2^-$ to produce peroxynitrite ($ONOO^-$), and peroxynitrite can react with CO_2 to produce nitrosoperoxycarboxylate ($ONO_2CO_2^-$) (Denicola et al., 1996; Lymar and Hurst, 1995; Uppu et al., 1996). In a nonpolar environment, such as in biological membranes, nitrosoperoxycarboxylate can generate reactive intermediates capable of oxidation, nitration, and nitrosylation reactions (Denicola et al., 1996; Vesela and Wilhelm, 2002). Alternatively, in the aqueous environment inside the cells, nitrosoperoxycarboxylate rearranges to nitrocarbonate, which can be hydrolysed to nitrate and CO_2 or carbonate. Increased CO_2 concentration thereby participates in the scavenging of the reactive peroxynitrite and acts as a protective agent against oxidative stress (Pryor et al., 1997).

A hypercapnia-mediated reduction in pH can influence the rate of Fenton reaction. Under normal conditions, iron binds strongly to the iron binding protein Transferrin (Tf) (Garrick and Garrick, 2009). Transferrin is a single polypeptide chain with two domains, each of which can bind one ferric ion. Binding of ferric ion is, however, dependent on synergistic binding of an anion; *in vivo* this is usually carbonate or bicarbonate (Bates and Schlabach, 1975; Foley and Bates, 1988). Increased bicarbonate concentration can therefore increase ferric binding capacity of Tf. On the other hand, the binding of iron to Tf is highly dependent on pH, and a decrease in pH will increase dissociation of iron (Princiotto and Zapolski, 1975; Sipe and Murphy, 1991). An increase in free iron can lead to excessive production of hydroxyl radical in the Fenton reaction (Garrick and Garrick, 2009).

1.6 Atlantic cod as a model organism

The importance of Atlantic cod (*Gadus morhua*) in aquaculture industry is increasing and there is a political will for further development (Cohen et al., 1990; The cod genome project, 2011). Cod is considered susceptible for environmental contaminants and it is therefore commonly used in toxicology studies (Goksøyr et al., 1994; Meier et al., 2007; Olsvik et al., 2009). The resent sequencing of its genome enables us to perform gene expression studies and thereby gaining thorough knowledge of the molecular and genetic basis for toxicology in this organism (Star et al., 2011). Atlantic cod stores the majority of its lipids in the liver, which normally consists of 50-60 % fat (Holdway and Beamish, 1984; Lie et al., 1988; Love, 1970).

1.7 Objectives of this thesis

Previous studies have shown that PFOS may generate ROS-mediated oxidative damage in fish (Arukwe and Mortensen, 2011; Oakes et al., 2005), since PFOS is an agonist for PPARs (Bjork and Wallace, 2009). Anthropogenic increase in atmospheric and oceanic CO_2 concentration can cause decrease of pH in water and organisms, causing physiological effects such as decrease in protein biosynthesis rates (Langenbuch and Portner, 2003), and this again can influence organismal stress handling capacity. Additionally, hypercapnia can cause increased generation of ROS. Therefore, the objectives of this study were to investigate how oxidative stress responses and expression of genes involved in lipid metabolism is modulated by a combined exposure to PFOS and increased levels of CO_2 (a quantifiable measure of climate change). The hypothesis is that PFOS will activate PPARs causing increased expression of genes involved in FA oxidation and subsequent generation of ROS. Increased CO_2 levels will potentiate this effect by further contributing to the generation of ROS.

2 MATERIALS AND METHODS

2.1 Chemicals and reagents

PFOS (linear, technical grade) was purchased from AlfaAesar (Karlsruhe, Germany). TRIzol® reagent was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). iScript™ cDNA Synthesis Kit and iTaqTM SYBR® Green Supermix with ROX were purchased from BioRad Laboratories (Hercules, CA, USA). Dimethyl sulfoxide (DMSO) and ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). Tricaine methane sulfonate (MS-222) was procured from Norsk Medisinaldepot AS. GelRed™ nucleic acid stain was purchased from Biothium (Hayward, CA, USA). Clinical grade Isopropanol was purchased from Arcus produkter AS, Norway. Clinical grade Absolute Ethanol and Agarose were purchased from Sigma-AldrichCo., MO, USA. Chloroform was purchased from Labscan Ltd., Dublin, Ireland. Since enzyme assays and determination of MDA and GSH were performed elsewhere, information on reagents used in these procedures was not available.

2.2 Experimental design and fish exposure

Juvenile, sexually immature Atlantic cod (*Gadus morhua*) were supplied by Atlantic cod Juveniles AS, a hatchery in Fosen, Norway. The experiment was performed at Norwegian University of Science and Technology (NTNU) Centre of Fisheries and Aquaculture (Sealab) in Trondheim, Norway. Fish had a body weight of 4.5 ± 0.4 g and an average length of 8.7 ± 0.2 cm. Prior to experiment the fish were acclimatized for 9 days at 10 °C. This temperature was maintained throughout the experiment, with natural light regime for August.

2.2.1 PFOS and CO_2 exposure

Fish were equally divided into three tanks, representing the three PFOS exposure groups: No PFOS (control), 100 or 200 µg PFOS/L (Table 1). PFOS concentrations were chosen based on results from previous experiments in our laboratory (Mortensen et al., 2011). Two stock solutions of PFOS were made by solving PFOS in MQ (millipore)-water to 150 mg/L (stock solution 1) and 300 mg/L (stock solution 2). Nine 6 L tanks of sea-water were equipped with fresh air through an aquarium pump and an air stone. Three tanks received 4 mL of stock solution 1 to a total of 100 µg PFOS/L, three tanks received 4 mL of stock solution 2 to a total of 200 µg PFOS/L, and three tanks received 4 mL of MQ-water (control). The solutions were allowed to mix homogeneously aided by air bubbles. Three tanks for each exposure group were chosen to minimize stress caused by space limitation. Fish from the three large tanks were distributed into the small 6 L tanks and kept there for one hour, and thereafter transferred back to their respective large tanks. This procedure was repeated daily for a total of five days.

After PFOS exposure, the fish were transferred to nine tanks with a continuous water flow and a system that added a specified amount of CO_2 gas to the water. Fish in each PFOS exposure group were divided into three different CO_2 exposures: control (normal sea water), an increase of 0.3 % (low), and 0.9 % (high). This made a total of nine exposure regimes (Table 1). For schematic overview, see Appendix A (Table A1). pH was measured continuously. pH values were used to calculate real CO_2 exposure for verification of hypercapnia. Although pH and CO_2 in water can vary during an experiment, in the following text the different exposure regimes will be denoted 0.3 % or 0.9 % increases, for simplicity.

Table 1. The nine exposure regimes in the experiment. Fish were originally divided into three different PFOS exposure groups, and these again were divided into three different CO_2 exposure groups.

Group	*PFOS exposure*	*CO_2 exposure*
1	No (0 µg PFOS/L)	Normal (control group)
2		Low (0.3 %)
3		High (0.9 %)
4	Low (100 µg PFOS/L)	Normal
5		Low (0.3 %)
6		High (0.9 %)
7	High (200 µg PFOS/L)	Normal
8		Low (0.3 %)
9		High (0.9 %)

2.2.2 Organ harvesting

3, 6 and 9 days after initiated CO_2 exposure, 10 individuals from each exposure group were anaesthetized using tricaine methanesulfonate (MS-222) and killed by snapping the head firmly. Organs harvested from 5 fish in each exposure group were quickly embedded in 250 µL TRIzol® reagent in separate tubes to preserve RNA and snap-frozen in liquid nitrogen. Organs from remaining fish were snap-frozen directly in separate tubes for use in enzyme assays and hormonal and lipid analyses. Organs were stored at -80 °C prior to further analyses.

2.3 Gene expression analysis

Real-time reverse transcriptase (RT) PCR (also termed quantitative PCR) is commonly used for gene expression analysis. Gene expression is context-dependent, and total RNA isolated from cells or tissues represents a snap-shot telling which genes are active at that specific time (Bustin and Nolan, 2004). In toxicology, it gives a picture of whether expression is increased or decreased by specific exposures.

2.3.1 RNA isolation

Maintaining integrity of RNA is of vital importance, assuring accurate results in gene expression analyses (Holland et al., 2003; Pfaffl, 2004). Guanidium thiocyanate is a strong inhibitor of ribonucleases and has been used during RNA extraction since the 1960s (Chirgwin et al., 1979; Cox, 1968). Total RNA isolation from tissue using the guanidium thiocyanate-phenol-chloroform extraction method is described previously by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). For this purpose we used TRIzol® reagent and followed the procedure described by the manufacturer (Invitrogen). Liver samples of approximately 50 g embedded in 250 µL TRIzol were thawed on ice. 250 µL TRIzol were added, and tissue was homogenized using Polytron® PT3000 mechanical homogenizer from Kinematica AG. The homogenizer was washed in 70 % ethyl alcohol and MQ-water before use, after every second sample within an exposure group and between exposure groups. After homogenization another 500 µL TRIzol were added to a total of 1 mL and mixed by pipetting. Samples were incubated in room temperature for 5 minutes before 200 µL of chloroform were added. At this point great care was taken to do the procedure quickly, as the chloroform is highly volatile and will evaporate. Slow execution will change the amount of chloroform added and give a less uniform handling of the samples. Samples were shaken vigorously

for 15 seconds before incubation in room temperature for 3 minutes and thereafter centrifuged at 4 °C, 12,000g for 15 minutes, using an Allegra™ X-22R centrifuge from Beckman Coulter.

Because of different chemical properties of the components in the tube, three phases will form in the centrifugation procedure. Upper, aqueous phase contains RNA, while DNA and proteins are present in lipid and phenol phase (Chomczynski and Sacchi, 1987). Supernatant was transferred to a fresh tube and 0.5 mL isopropyl alcohol was added. Solution was mixed by inverting the tubes five times, followed by incubation at room temperature for 10 minutes to allow RNA to precipitate. By centrifuging at 4 °C, 15,000g for 10 minutes the RNA precipitate formed a pellet. Isopropyl alcohol was removed and the pellet was washed in 1 mL 75 % ethyl alcohol and vortexed until the pellet loosened from the bottom of the tube. Thereafter, samples were centrifuged at 4 °C, 7,500 g for 5 minutes, and ethyl alcohol was removed by vacuum suction. Pellet was suspended in 150 µL RNase-free diethylpyrocarbonate (DEPC) water, incubated at 60 °C for 10 min and stored at -80 °C.

Determination of RNA concentration and validation of RNA quality

RNA concentration was determined using NanoDrop spectrophotometry at 260 nm. Samples with concentration above 1000 µg/mL were diluted with DEPC water until below this limit, preparing them for complimentary DNA (cDNA) synthesis. Most samples had a concentration of 700-2000 ng/µL, after initial dilution of the pellet in 150 µL DEPC water.

NanoDrop spectrophotometer measures optical density (OD) at different wavelengths. OD deflection at 240 nm is specific for background absorption of various chemicals and salts used in the RNA isolation process, at 260 nm for nucleic acids and at 280 nm for proteins (BioTek Instruments Inc., 2006). 260/280 ratio is an indicator of RNA quality, and should be above 1.8 (Fleige and Pfaffl, 2006). A260/280 ratio was between 1.79 and 2.02.

In real-time polymerase chain reaction (PCR) assay, one uses a pair of specific primers for the gene to be investigated. Primers bind at opposite ends of the cDNA that represents the gene. A full-length cDNA molecule must be present, and this can be obtained only if mRNA molecules are intact. Since approximately 80 % of cell RNA content is 18S and 28S ribosomal RNA (rRNA), intact RNA will form two prominent bands on the gel (Blobel and Potter, 1967). Degraded RNA will form clouds at the far end of the gel (Mueller et al., 2004). mRNA integrity was investigated by formaldehyde gel electrophoresis, where 1 µg RNA from randomly chosen samples were separated at 75 V for 1.5 hour. The resulting gel displayed the two prominent bands of 18S and 28S rRNA (Figure 5).

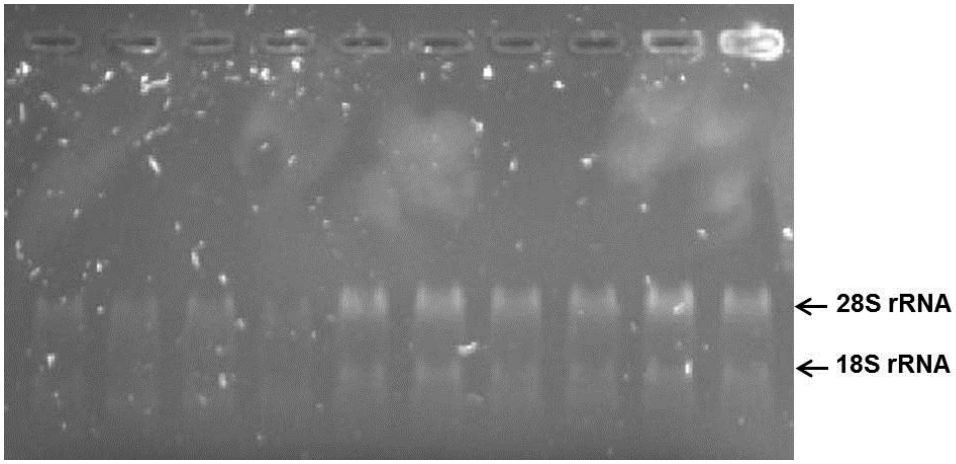

Figure 5. In order to verify integrity of isolated RNA, total RNA was separated by formaldehyde gel electrophoresis. 18S and 28S ribosomal RNA (rRNA) constitute approximately 80 % of total RNA and will therefore form two prominent bands if intact, as displayed on the gel image.

2.3.2 cDNA synthesis

Reverse transcriptase (RT) uses RNA as a template for DNA synthesis. iScript cDNA synthesis kit from BioRad was used for this purpose, and protocol was followed as described by the manufacturer. A mixture of Poly-T and random hexamer primers were used to ensure complete full-lenght conversion of all RNA molecules (Pfaffl, 2004). Poly-T primers binds to the poly-A tail present in the majority of mRNA molecules, while the random hexamer primers ensures completion of all molecules where transcription has been terminated due to secondary structures in the RNA molecules (Pfaffl, 2004).

cDNA synthesis and PCR quantification can be carried out in the same tube (one-step real-time PCR), or the two reactions can be separated into two tubes (two-step real-time PCR). By making cDNA beforehand in a separate tube, one obtains a cDNA pool that can be stored for a very long time before being used in multiple analyses (Bustin, 2000). On the other hand, increased amount of handling when setting up two reactions instead of one gives a higher risk of contamination (Bustin, 2000). Two-step real-time PCR was chosen in this experiment.

Mastermix for the separate cDNA synthesis step was made from the components described in Table 2. Volume of RNA needed for 1000 ng was calculated based on RNA concentration measurements, and additional double-distilled (dd) H_2O was added for a total of 20 µL per reaction. A negative control was included, where RNA was replaced with dd H_2O. PCR program was set at 5 min at 25 ºC, 30 min at 42 ºC and 5 min at ºC.

Table 2. Components and their respective volumes in the mix for cDNA synthesis.

Component	***Volume for one reaction***
5x iScript reaction mix	4 µL
iScript reverse transcriptase	1 µL
dd H_2O	10 µL
RNA-template (1000 ng)	X µL
dd H_2O	5-X µL
Total	***20 µL***

2.3.3 Real-Time Polymerase Chain Reaction

Real-time PCR with SYBR® green and ROX as a reference dye was used to analyse expression levels of 11 genes (Table 3). SYBR® green is a molecular probe that binds only to double-stranded DNA. It has no sequence specificity and monitors the total amount of double-stranded DNA in the sample tube. When SYBR® green binds to DNA, it emits light at 520 nm, which can be detected by the real-time PCR thermal cycler. (Clark, 2010). When the amount of DNA is doubled during the next cycle, twice as much SYBR® green will bind and fluoresce. In a perfect real-time assay, amount of fluorescence will rise exponentially until it reaches a level that is detectable over fluorescence from the reference dye. Cycle threshold (Ct) value is the number of cycles needed to reach the detectable level, and it can be used for quantitative or semi-quantitative analysis.

5 µL of total cDNA diluted 1:6 was mixed with 12.5 µL of 2x SYBR® Green PCR mix with ROX from BioRad, 6.5 µL of autoclaved water, and 0.5 µL (10 pmol/µL) of each forward and reverse primer. The real-time PCR program included a 3 minute heat shock-activation of DNA polymerase at 95 ºC, thereafter 40 cycles of 30 s at 95 ºC, 15 s at 60 ºC, and 15 s of 72 ºC. The procedure was terminated by 1 min at 95 ºC, 30 s at 65 ºC, and 30 s at 95 ºC. A final rise in temperature from 65 to 90 ºC allows the real-time PCR machine to detect melting temperature (T_m) of the DNA in the tube and generate a melting curve. Successful amplification of a single, specific cDNA will give only a single, narrow peak in the melting curve, since the T_m is sequence specific (Ririe et al., 1997). Presence of non-specific product or primer dimers will display as multiple peaks at different temperatures than the desired product. The dissociation curve in all real-time PCR analyses indicated that only one sequence had been amplified. Presence of a single product was further validated by separating the real-time PCR product from randomly chosen wells in a gel electrophoresis assay. The presence of single bonds of appropriate lengths indicated only one amplification product (Figure 6).

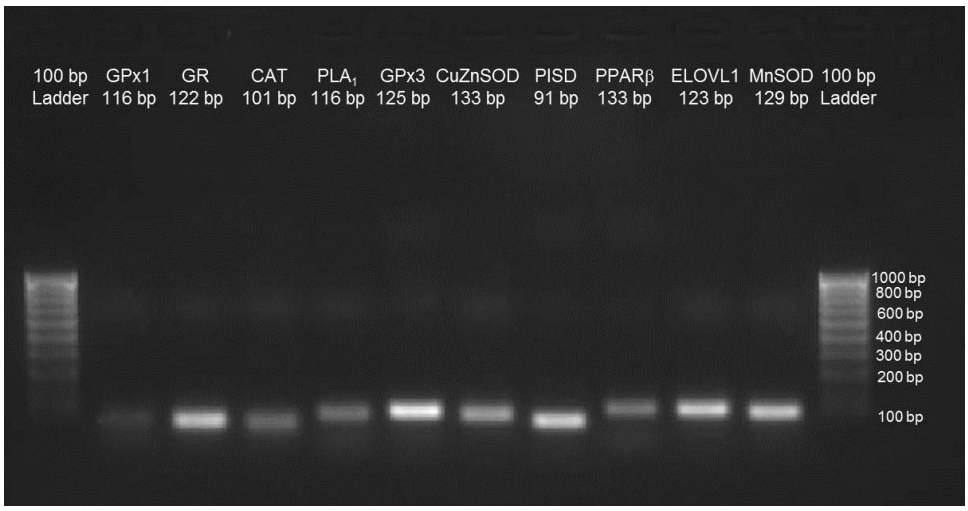

Figure 6. In order to verify specificity of real-time PCR primers, randomly chosen amplification products were separated by gel electrophoresis. Image shows the presence of a single amplification product of appropriate length in each well.

Primer design, testing and optimization

The specific cod primers used for gene expression analysis have been used previously (Kortner et al., 2011; Saele et al., 2012) (Table 3). Functionality of the primers was tested by amplification of a cod cDNA pool, and thereafter separating the amplification product using gel electrophoresis. Functional and specific primers should give one prominent band of a given length on the gel. Multiple bands or bands of incorrect length could indicate non-specific primers. If the primer contains strings of complementary bases, this could result in primer dimerization and amplification, which would be visible on the gel as a band of 30-100 bp easily distinguishable from the target sequence band (Chou et al., 1992).

Table 3. Forward and reverse primers used in real-time PCR on Atlantic cod cDNA

Gene name	Symbol	Forward Primer (5' - 3')	Reverse Primer (5' - 3')	Size (bp)
CuZn superoxide dismutase	*CuZn-sod*	CATGGCTTCCACGTCCATG	CGTTTCCCAGGTCTCCAACAT	133
Mn superoxide dismutase	*Mn-sod*	ATGTGGCCTCCTCCATTGAA	GCATCACGCCACCTATGTCA	129
Glutathione peroxidase 1	*gpx1*	GTAGGATGGCCAAAAATGTGTTTA	GGCCCCAGTCATCTGAGCTA	116
Glutathione peroxidase 3	*gpx3*	CGTTCTCGGGTTTCCCTGTA	GCTCAAACAGCGGGAACGT	125
Glutathione reductase	*gr*	TCACGCTCACCACCAAGGA	GTGTGGAGGCCAGTCGTGTT	122
Catalase	*cat*	GCCAAGTTGTTTGAGCACGTT	CTGGGATCACGCACCGTATC	101
Peroxisome proliferator-activated receptor b	*pparβ*	GGCTTCGTGGACCTCTTCCT	TCACAAATCCTTTGCCATTGG	133
Acyl-CoA dehydrogenase	*acod*	GCG TTCGTAGTGGAGAGGAG	GCGCACGTTCTCAAAGTACA	108
Phosphatidylserine decarboxylase	*pisd*	TCTGGACCTTTGGCGTCAAC	TTCAGCGGTCGTCTGAAGAA	91
Phosphatidylethanolamine N-methyltransferase	*pemt*	GGTTCTCCGTCAGGCTGAAG	CGGCGACTACTTTGGGATTC	68
CYP19 (Aromatase)	*cyp19*	GAGGAGACGCTCATCCTCAG	TAGCTGCGTGTCTTCTTCCA	167

2.3.4 Normalization of data

A standard plot was made by making a dilution series with known amounts of plasmids containing the *CYP19* (*aromatase*) gene, and amplifying it using real-time PCR. The result was a standard plot of Ct values versus the logarithm of copy number (Appendix B, Figure B1). The linear equation of this standard curve was used to normalize all gene expression data.

2.4 Enzyme assays

Activities of Catalase, Glutathione S-transferase, Glutathione reductase, Glutathione peroxidase, and Superoxide dismutase, as well as total MDA and GSH content, were determined by spectrophotometric methods. Procedures for making the reagents used are found in Appendix C (Table C1).

2.4.1 Preparation of cytosolic fraction

Cytosolic fraction of liver tissue was prepared for enzyme activity assays. pH of Tris-HCL buffer was adjusted to 7.5. Samples were homogenized (1:4 w/v ratio) in homogenizing buffer. Samples

were centrifuged at 110,000g for 1 hour and 10 minutes, and supernatants were aliquoted and stored at -80 ºC. 1 µL DTT (antioxidant) per 100 µL cytosolic fraction was added to aliquots determined for GPx activity assay.

2.4.2 Determination of enzyme activity

Determination of Catalase activity

Cat scavenges hydrogen peroxide (H_2O_2), and Cat activity was measured by the loss of absorbance due to consumption of H_2O_2 at 240 nm at a constant temperature of 18 ºC. K-phosphate buffer was adjusted to pH=7. 980 µL 100 mM K-phosphate buffer, 10 µL H_2O_2 from 1.2 M stock and 10 mL sample (cytosolic fraction) was added to a quartz spectrophotometer cuvette and absorbance at 240 nm was measured over time. Enzyme activity is expressed as a unit of activity relative to total protein, calculated by:

Cat activity (units) = Δ_{240}/0.04 * assay dil./proteins = µmol/mg proteins

Where:

Δ_{240} = change in absorbance per minute

0.04 = extinction coefficient ($M^{-1}cm^{-1}$)

Proteins = concentration of (mg/mL)

Determination of Glutathione S-transferase activity

GST conjugates GSH to electrophilic compounds. In this assay, production of a compound that absorbs light at 340 nm was measured. K-phosphate buffer was adjusted to pH=6.5. Working buffer solution (K-phosphate buffer + CDNB) was prepared by adding 1.5 mL 50 mM CDNB stock solution in 50 mL 50 mM K-phosphate buffer pH 6.5. 965 µL working buffer solution, 15 µL 100 mM GSH, and 20 mL sample (cytosolic fraction) was added to a quartz spectrophotometer cuvette, and absorbance at 340 nm was measured over time. Enzyme activity is expressed as a unit of activity relative to total protein, calculated by:

GST activity = Δ_{340}/9.6 * assay dil./proteins*1000 = µmol/mg proteins

Where:

Δ_{340} = change in absorbance per minute

9.6 = extinction coefficient ($M^{-1}cm^{-1}$)

Proteins = concentration of (mg/mL)

Determination of Glutathione reductase activity

NADPH is consumed in reduction of GSSG to GSH by GR, and GR activity can be determined by measuring consumption of NADPH at 340 nm at a constant temperature of 18 ºC. K-phosphate buffer was adjusted to pH=7. 750 µL 100 mM K-phosphate buffer pH 7, 100 µL 10 mM GSSG, 10 µL 100 mM EDTA, 100 µL 1 mg/L NADPH, and 40 mL sample (cytosolic fraction) was added to a quartz spectrophotometer cuvette, and absorbance at 340 nm was measured 5 times during 3 minutes. Enzyme activity is expressed as a unit of activity relative to total protein, calculated by:

GR activity = Δ_{340}/6.22 * assay dil./proteins*1000 = µmol/mg proteins

Where:

Δ_{340} = change in absorbance per minute

6.22 = extinction coefficient ($M^{-1}cm^{-1}$)

Proteins = concentration of (mg/mL)

Determination of Glutathione peroxidase activity

GPx catalyses oxidation of GSH and reduction of H_2O_2 or organic peroxides to H_2O or ROH (reduced organic compound). GSSH is regenerated to GSSG by GR and NADPH is consumed in the process. GPx activity can be determined by measuring consumption of NADPH at 340 nm at a constant temperature of 18 ºC. K-phosphate buffer was adjusted to pH=7.5. Stock solution of 100 U/mL GR was prepared from original stock solution (i.e. if stock solution is 1384 U/mL, take 72.25 µg/mL). 846 µL 100 mM K-phosphate buffer pH 7.5, 10 µL 100 mM EDTA, 20 µL 100 mM GSH, 10 µL 100 U/mL GR, 100 µL sample or blank solution and 10 µL 20 mg/mL NADPH was added to a plastic spectrophotometer cuvette, and absorbance at 340 nm was measured. Several blank reactions were measured before measuring samples, and $\Delta_{\text{min blank}}$ 0.10-0.15 can be considered good. Volume of NADPH was modified to get an absorbance of 0.9-1.2. 15 µL 200 mM CHP was added. Absorbance of blank reaction was subtracted from sample reaction: $\Delta_{\text{min sample}} - \Delta_{\text{min blank}} = \Delta_{\text{min final sample}}$.

Enzyme activity is expressed as a unit of activity relative to total protein, calculated by:

GPx (CHP) activity = Δ_{340}/6.22 * assay dil./proteins*1000 = µmol/mg proteins

Where:

Δ_{340} = change in absorbance per minute

6.22 = extinction coefficient ($M^{-1}cm^{-1}$)

Proteins = concentration of (mg/mL)

Determination of Superoxide dismutase activity

SOD activity was determined by monitoring the decreased reduction of cytochrome c by $\cdot O_2^-$ by the xanthine oxidase/hypoxanthine system. One unit (U) of SOD is defined as the amount of enzyme inhibiting by 50 % the reduction of cytochrome c, and different volumes of sample were used to determine 50 % inhibition of the reaction rate. K-phosphate buffer was adjusted to pH=7.8. Xanthine oxidase 6mU/mL should be prepared from available stock solution. Working buffer was made from 50 mL 100 mM K-phosphate buffer pH 7.8, 100 µL 100 mM EDTA, 0.006 g Hypoxanthine, and 0.012 g Cytochrome c.

Samples were measured at 550 nm and 18 ºC. For each sample, blank solution was measured (Δ_{blank} should be ≅ 0.08-0.1), and also three different readings with different volumes were measured (Table 4). Inhibition percentage of the blank reaction for these three volumes should be 70 % for Reading 1, 50 % for Reading 2, and 20 % for Reading 3. The results should fit on a semi-logarithmic scale, and get $y=ax^2+bx+c$ by second order regression. The x value is set out as the one that corresponds at 50 % variation of Δ_{blank}, and Volume (Vol.) necessary to reduce 50 % of blank reaction is obtained. Activity was calculated by

U.SOD/mg protein = 1000/Vol*sample dil./Proteins (mg/mL)

Table 4. Three readings at 550 nm should be performed for each sample in order to determine SOD activity.

	Blank	***Reading 1***	***Reading 2***	***Reading 3***
Working buffer (µL)	500	500	500	500
K-phosphate buffer (µL)	470	460	450	430
Sample (µL)		10	20	40
Xanthine oxidase (µL)	30	30	30	30

2.4.3 Determination of total Glutathione

Total GSH was determined by monitoring conjugation of GSH with the dye DTNB at 412 nm. GSSG produced in the reaction is converted back to GSH by GR and NADPH. Samples were homogenized (1:4 w/v ratio) in homogenizing buffer and maintained on ice for 45 minutes before centrifuging at 37,000g for 15 minutes at 4 ºC. Supernatants were aliquoted and stored at -80 ºC. K-phosphate buffer was adjusted to pH=7. 100 U/mL stock solution of GR was made (i.e. if original stock solution is 1384 U/mL, take 72.25 µL/mL to obtain 100 U/mL solution). Working buffer was prepared by adding 10 µL 100 mM EDTA to 1 mL 100 mM K-phosphate buffer pH 7.

Standards were prepared by making a stock solution of 100 mM GSH by dissolving 0.0307 g GSH in 1 mL dH_2O, and diluted as follows:

GSH 100 mM → GSH 10 mM → GSH 1 mM → GSH 100 µM

GSH 100 µM → (GSH 10 µM, GSH 20 µM, and GSH 30 µM)

100 µM of each standard were used to obtain a 1 µM, 2 µM, and 3 µM final concentrations of GSH standards.

In cuvettes for blank and standard reactions: 1 mL working buffer, 100 µL blank or standard, 5 µL 20 mM DTNB, 50 µL 4 mg/mL NADPH, and 10 µL 100 U/mL GR was added. In cuvettes for sample reactions, 1 mL working buffer, 100 µL sample, 5 µL 20 mM DTNB, 50 µL 4 mg/mL NADPH, and 10 µL 100 U/mL GR.

Δ_{min} of blank and standards were used to obtain the linear equation between absorbance and concentration (y=ax+b). Equation was used to convert Δ_{sample} to concentration in µmol/L. Following equation was used to calculate total Glutathione levels:

GSH+2GSSG (µmol/g tissue) = (concentration/1000) * sample dilution * w/v ratio * 1

2.4.4 Determination of Malondialdehyde

MDA was measured to determine changes in lipid peroxidation. Liver tissue was washed in ice-cold 0.9 % NaCl, and tissue was blotted on blotting paper. Approximately 0.1 g of tissue was weighed out and minced, before 1:3 dilution in ice-cold Tris-HCL buffer, pH 7.4. Samples were homogenized and thereafter centrifuged at 3,000g for 20 minutes. 650 µL of R1 (Table 5), 100 µL sample and 100 µH H_2O were added to microcentrifuge tubes and vortexed. 150 µL of R2 were added before vortexing and incubating at 45 ºC for 40 minutes. Samples were thereafter cooled on ice and centrifuged at 15,000g for 10 min. Absorbance at 586 nm was measured using Helma quartz cell and compared to a standard curve (Table 5).

Table 5. Reagents used for determination of malondialdehyde (MDA).

Reagent 1 (R1)	0.064 g 10.3 mM 1-methyl-2-phenylindole 30 mL acetonitrile 10 mL methanol
Reagent 2 (R2)	HCL 37 %
Standard 2 (S2)	0.0165 mL 10 mM 1,1,3,3-tetramethoxypropane 10 mL 20 mM Tris-HCL

Standard curve was generated by diluting S2 in ddH_2O (Table 6). Solution was vortexed and 150 µL of R2 was added. Solutions were vortexed again before incubation at 45 ºC for 40 minutes. Solutions were cooled on ice and centrifuged at 15,000g for 10 minutes, and absorbance at 586 nm was measured.

Table 6. Volume of S2 and ddH_2O in standard curve for MDA determination.

	Concentration µM									
	0	0.2	0.5	0.8	1.0	2.0	3.0	4.0	6.0	8.0
Volume of S2 (µL)	0	2	5	8	10	20	30	40	60	80
Volume of ddH_2O (µL)	200	198	195	192	190	180	170	160	140	120

2.5 Statistical analysis

Statistical analysis was performed using IBM SPSS® statistical software. Normality was tested using Shapiro-Wilk test and homogeneity of variance was tested using Levene's test. Normally distributed data were analysed by a one-way ANOVA followed by Tukey's test for equal sample sizes and Games-Howell for unequal sample sizes to identify results that significantly differed from control. Non-normally distributed data, and also enzyme assay data and MDA and GSH measurements, were analysed by a Kruskal-Wallis one-way analysis of variance followed by multiple t-test to identify significant differences. The α-value was set to 0.05.

3 RESULTS

3.1 Verification of hypercapnia and reduced pH

In order to verify that CO_2 added to the water dissolved and caused hypercapnia, pH was measured daily, and P_{CO2} was calculated based on these values (Figures 7 and 8). Both pH and P_{CO2} data showed a clear difference between the different exposure regimes, although there was some variation in partial pressure of CO_2 in the 0.9 % increase in CO_2 exposure groups. The tanks with normal sea water had a pH of 7.61-7.83 and a P_{CO2} of 596-1136. In the tanks representing a 0.3 % increase in CO_2 level, pH levels were between 7.08-7.44 and P_{CO2} was between 1713 and 3744. In the tanks representing a 0.9 % increase in CO_2 level, pH levels were between 6.63-6.96 and P_{CO2} was between 4844 and 11455. The pH dropped and the P_{CO2} increased when fish were added to the tanks between day 7 and 9.

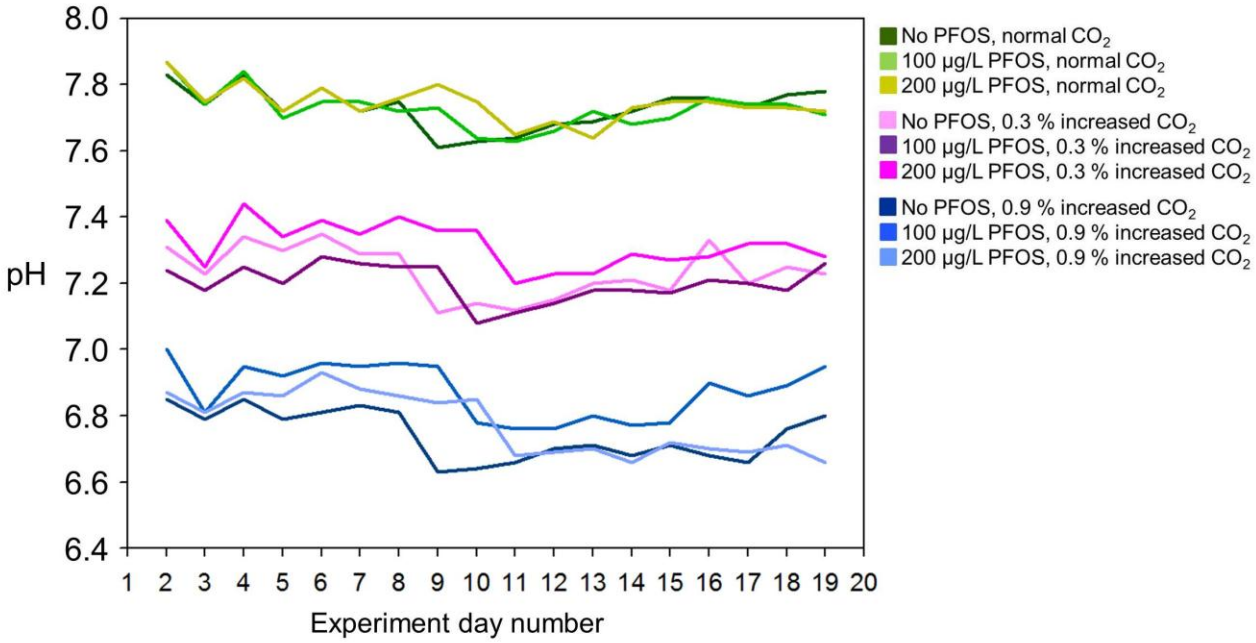

Figure 7. Measured pH in the different CO_2 exposure tanks during the experiment. Fish were added to the tanks between day 7 and 9, causing a drop in pH due to respirational CO_2.

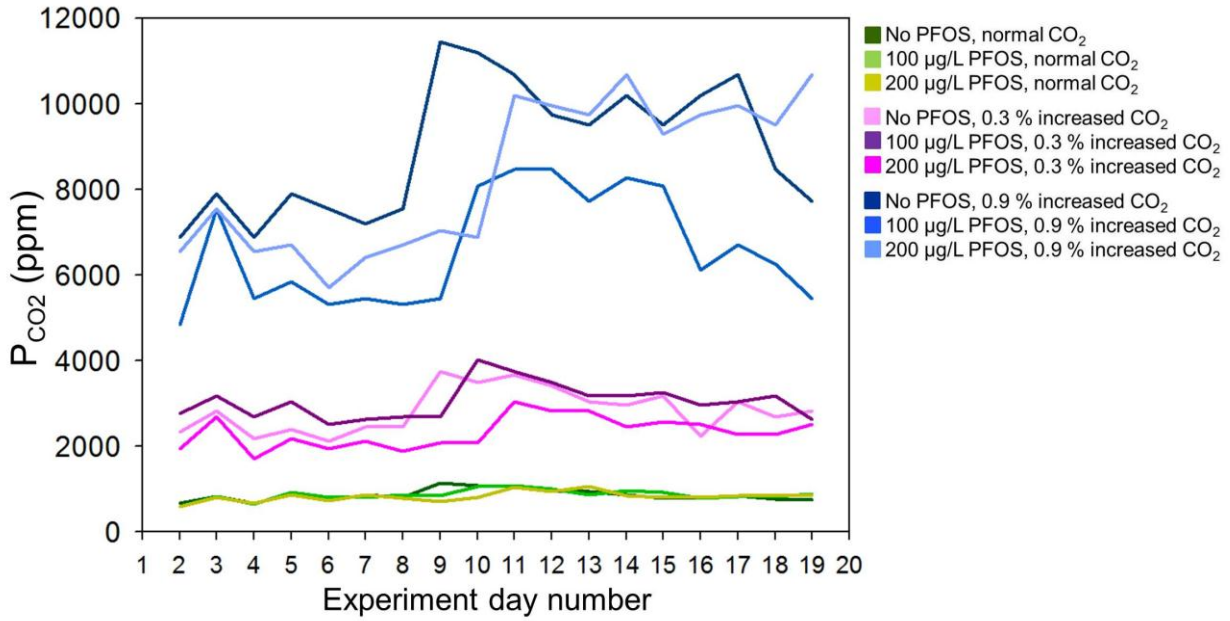

Figure 8. Calculated P_{CO2} in the different CO_2 exposure tanks during the experiment. Fish were added to the tanks between day 7 and 9, causing an increase in P_{CO2} due to respirational CO_2.

3.2 Effect on weight and length

Measurements of weight and length from day 9 after initiated CO_2 exposure showed no significant differences among the different exposure groups (Table 7). There was however, a very slight decrease in weight with increasing PFOS concentration, irrespective of CO_2 level.

Table 7. Changes in average weight and length at day 9, given in grams and cm, with SEM. Juvenile Atlantic cod were exposed to PFOS and elevated water CO_2 level, singly or in combination. Weight and length were measured after sacrifice.

	Weight (g)		**Lenght (cm)**	
	Average	***SEM***	***Average***	***SEM***
Control (No PFOS, normal CO_2)	4.51	0.32	8.85	0.17
No PFOS, 0.3 % CO_2 increase	4.30	0.25	8.73	0.14
No PFOS, 0.9 % CO_2 increase	4.41	0.30	8.76	0.16
100 µg PFOS/L, normal CO_2	4.19	0.35	8.68	0.21
100 µg PFOS/L, 0.3% CO_2 increase	4.67	0.40	8.92	0.24
100 µg PFOS/L, 0.9 % CO_2 increase	4.21	0.28	8.74	0.22
200 µg PFOS/L, normal CO_2	3.80	0.08	8.33	0.16
200 µg PFOS/L, 0.3 % CO_2 increase	4.11	0.18	8.61	0.17
200 µg PFOS/L, 0.9 % CO_2 increase	4.16	0.32	8.76	0.23

3.3 Lipid homeostasis

3.3.1 PPAR – regulation of lipid metabolism

At day 3, expression of *pparβ* was apparently reduced in fish exposed to a 0.3 % increase in CO_2 alone, and the presence of PFOS potentiated this decrease (albeit not significantly) (Figure 9). However, within the 100 and 200 µg PFOS groups, while 0.3 % CO_2 increase in combination with PFOS concentrations reduced *pparβ* below control, 0.9 % CO_2 increase either restored this effect back to control (at 100 µg PFOS) or increased the effect above control (at 200 µg PFOS/L) at day 3 (Figure 9). At day 6 and 9, *pparβ* mRNA levels showed an apparent CO_2-dependent increase, and these increases were significantly so at the 0.9 % CO_2 increase alone and in combination with 100 or 200 µg PFOS/L at day 6 (Figure 9).

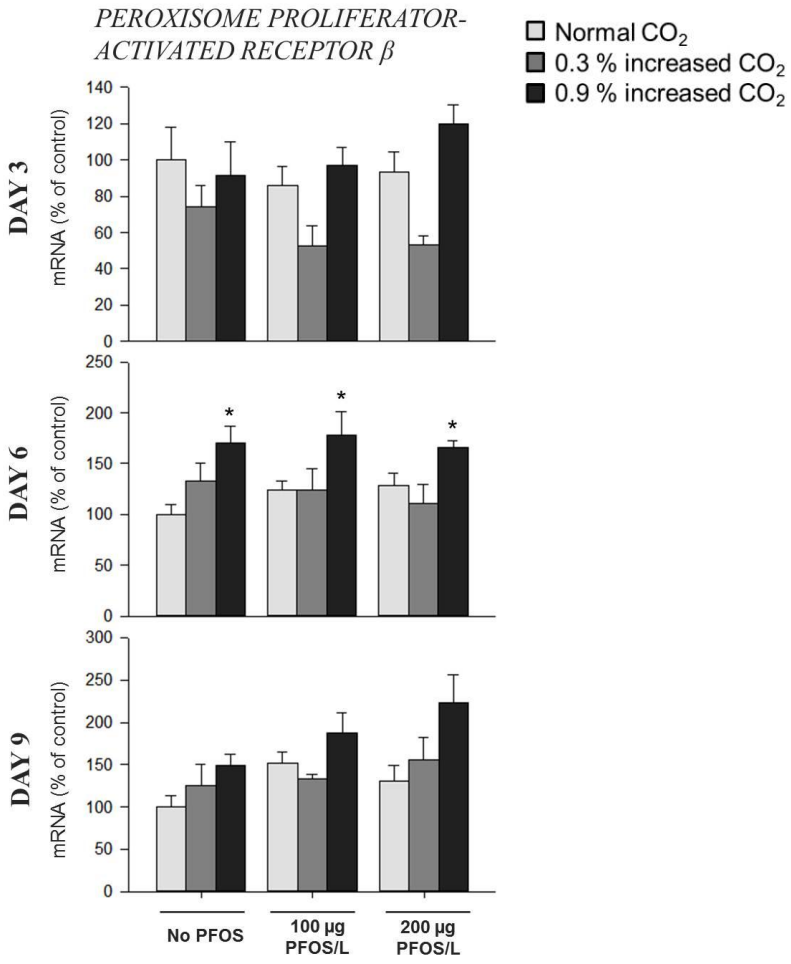

Figure 9. Changes in gene expression levels for *peroxisome proliferator-activated receptor β* (*pparβ*), expressed as percent of control, with SEM (n=4/5). Juvenile Atlantic cod were exposed to PFOS and increased water CO_2 level, singly or in combination. Changes in gene expression level in liver tissue were measured by real-time RT-PCR using specific primer pairs. Asterisks denote significant differences in gene expression compared to control (one-way ANOVA, with Games-Howell post-hoc test ($p<0.05$) for day 3+9 and Kruskal-Wallis non-parametric one-way ANOVA followed by multiple t-tests ($p<0.05$) for day 6).

3.3.2 *Expression of genes involved in lipid metabolism*

β-oxidation of fatty acids

At day 3 and 9, expression of *acod* was apparently decreased upon exposure to 0.3 % CO_2 increase, but the expression was restored back to control level by 200 µg PFOS/L at day 9 (Figure 10). There was also a slight increase in expression with increasing PFOS concentration within the 0.9 % increased CO_2 group at day 9. At day 6, there was a non-significant decrease of expression at the highest CO_2 within the PFOS exposure groups, while exposure to PFOS alone seemed to increase expression.

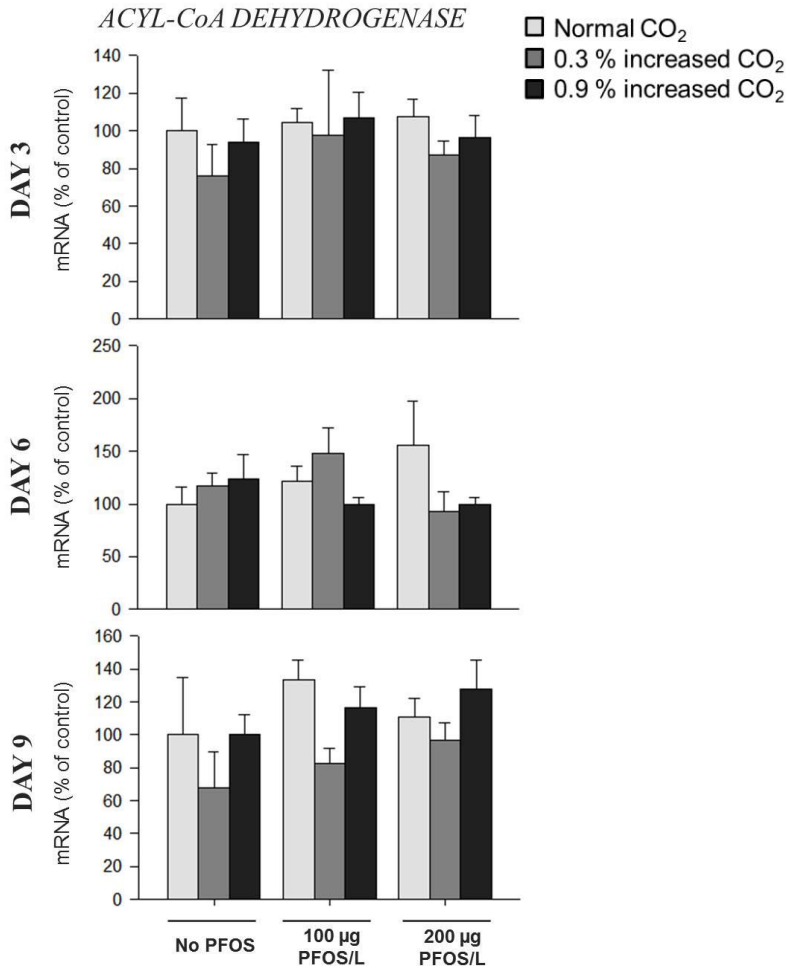

Figure 10. Changes in gene expression levels for *acyl-coa dehydrogenase (acod)*, expressed as percent of control, with SEM (n=5). Juvenile Atlantic cod were exposed to PFOS and increased water CO_2 level, singly or in combination. Changes in gene expression level in liver tissue were measured by real-time RT-PCR using specific primer pairs. Asterisks denote significant differences in gene expression compared to control (Kruskal-Wallis non-parametric one-way ANOVA, followed by multiple t-tests ($p<0.05$)).

Phospholipid head group exchange

Expression of *phosphatidyletanolamine n-methyltransferase* (*pemt*) at day 3 was significantly decreased in fish exposed to 100 µg PFOS/L and 0.3 % increased CO_2 level in combination (Figure 11A). There was also a non-significant decrease upon exposure to increased CO_2 only, and 200 µg PFOS/L combined with normal and also 0.3 % increased CO_2. Expression of *phosphatidylserine decarboxylase* (*pisd*) at day 3 was apparently decreased upon exposure to 0.3 % increased CO_2 only, and 200 µg PFOS/L only (Figure 11B).

Expression of *pemt* and *pisd* was similar at day 6 and 9. At day 6 in the no PFOS and 100 µg PFOS/L exposure groups, an increase in CO_2 seemed to increase the expression level. In the 200 µg PFOS/L exposure groups, increase in CO_2 apparently decreased expression of the two genes. An increase in PFOS concentration seemed to decrease expression within the different CO_2 exposure groups, while in the no PFOS exposure groups, expression was increased by increasing CO_2. At day 9 expressions was decreased below control level by exposure to increased CO_2 in combination with PFOS. Upon exposure to PFOS alone, expression of *pemt* apparently decreased with increasing PFOS concentration. The expression of *pemt* apparently increased upon increasing PFOS concentration within the highest CO_2 exposure group, but was not restored back to control level.

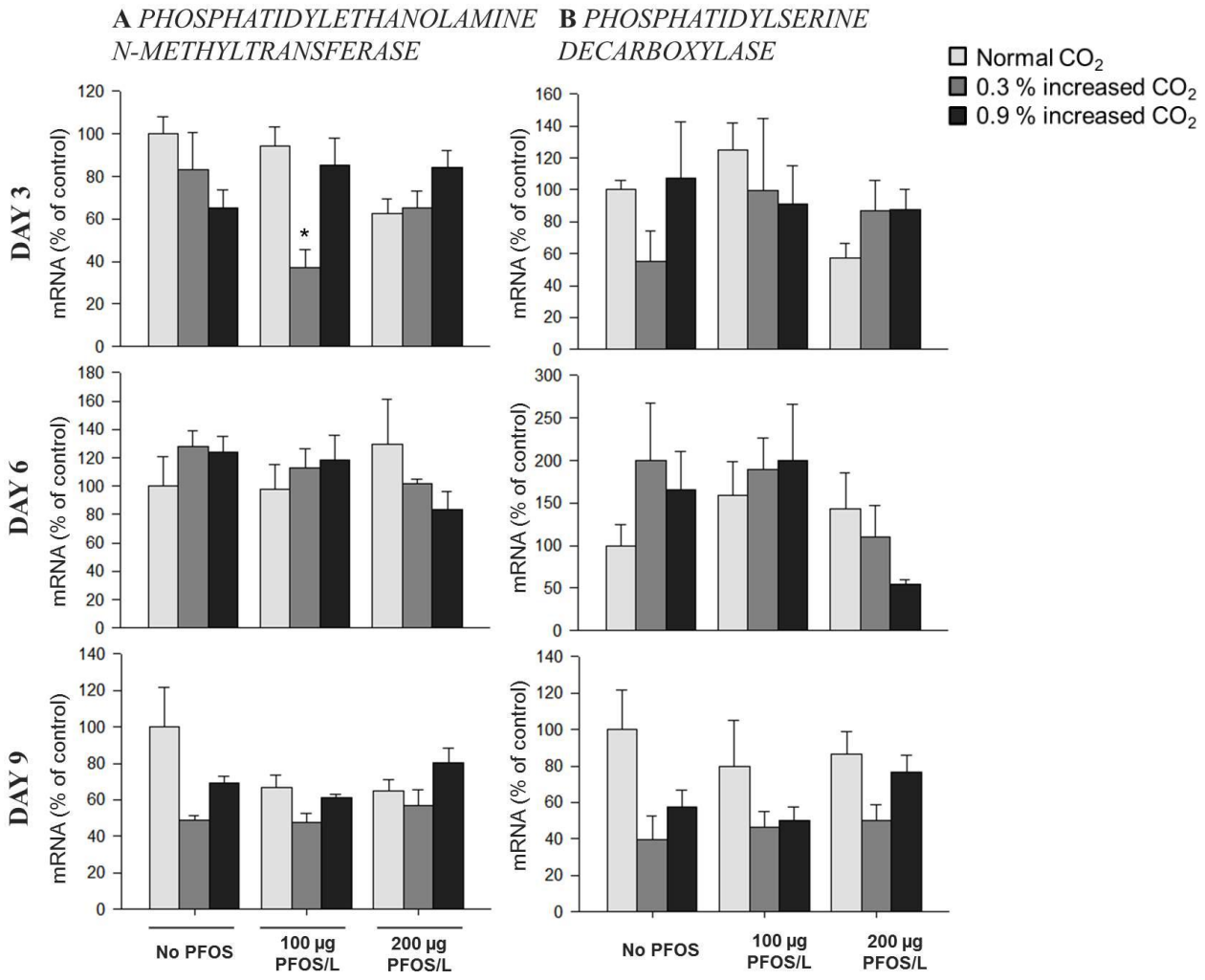

Figure 11. Changes in gene expression levels for A: *phosphatidyletanolamine n-methyltransferase* (*pemt*) and B: *phosphatidylserine decarboxylase* (*pisd*), expressed as percent of control, with SEM (n=4/5). Juvenile Atlantic cod were exposed to PFOS and increased water CO_2 level, singly or in combination. Changes in gene expression level in liver tissue were measured with real-time RT-PCR using specific primer pairs. Asterisks denote significant difference in gene expression compared to control (one-way ANOVA with Tukey's post-hoc test ($p<0.05$) for *pemt* day 6 and *pisd* day 9 and Games-Howell post-hoc test ($p<0.05$) for *pemt* day 3, and Kruskal-Wallis non-parametric one-way ANOVA followed by multiple t-tests ($p<0.05$) for *pemt* day 9 and *pisd* day 3+6).

3.4 Oxidative stress response

Because of sample pooling and consequently reduced number of observations in the enzyme assays, some results lack error bars (Figures 12, 13, 14, 15, and 17). This is also the case for the measurements of Glutathione end Malondialdehyde levels (Figures 16 and 18).

3.4.1 Superoxide dismutase

Gene expression

At day 3, expression of *CuZn-sod* was significantly decreased upon exposure to 100 and 200 µg PFOS/L combined with normal sea water or a 0.3 % CO_2 increase (Figure 12A). Exposure to 0.9 % increased CO_2 counteracted the decrease completely. For *Mn-sod* at day 3 there was a slight decrease in expression when 100 µg PFOS/L was given in combination with a 0.3 % increase in CO_2 level (Figure 12B).

At day 6, expression of *CuZn-sod* was significantly increased upon exposure to the 0.9 % increase in CO_2 at all PFOS exposure concentrations, and also upon exposure to 0.3 % increase in CO_2 alone and PFOS alone (Figure 12A). Expression of *Mn-sod* was increased upon exposure to both 0.3 and 0.9 % increase in CO_2 in all PFOS exposure regimes and significantly so in the 100 and 200 µg PFOS/L groups (Figure 12B). Expression appeared to increase concentration-dependently both to increased CO_2 within each PFOS exposure group, and also to increased PFOS concentration within CO_2 exposure groups.

At day 9, expression of both *CuZn-sod* and *Mn-sod* were non-significantly increased upon exposure to the highest CO_2 for all PFOS concentrations. Expression of *CuZn-sod* was significantly decreased by 0.3 % increased CO_2 combined with PFOS.

Enzyme activity

There was an apparent increase in SOD enzyme activity with increasing PFOS concentration, albeit this was not true for all exposures (Figure 12C). At day 6, within the group exposed to normal sea water, exposure to 100 µg PFOS/L resulted in highest activity, well above control level. At day 9, within the 0.9 % increased CO_2 exposure group, 100 µg PFOS/L resulted in lowest enzyme activity. Within the 100 µg PFOS/L exposure groups at day 6 and 9, increasing CO_2 apparently decreased activity.

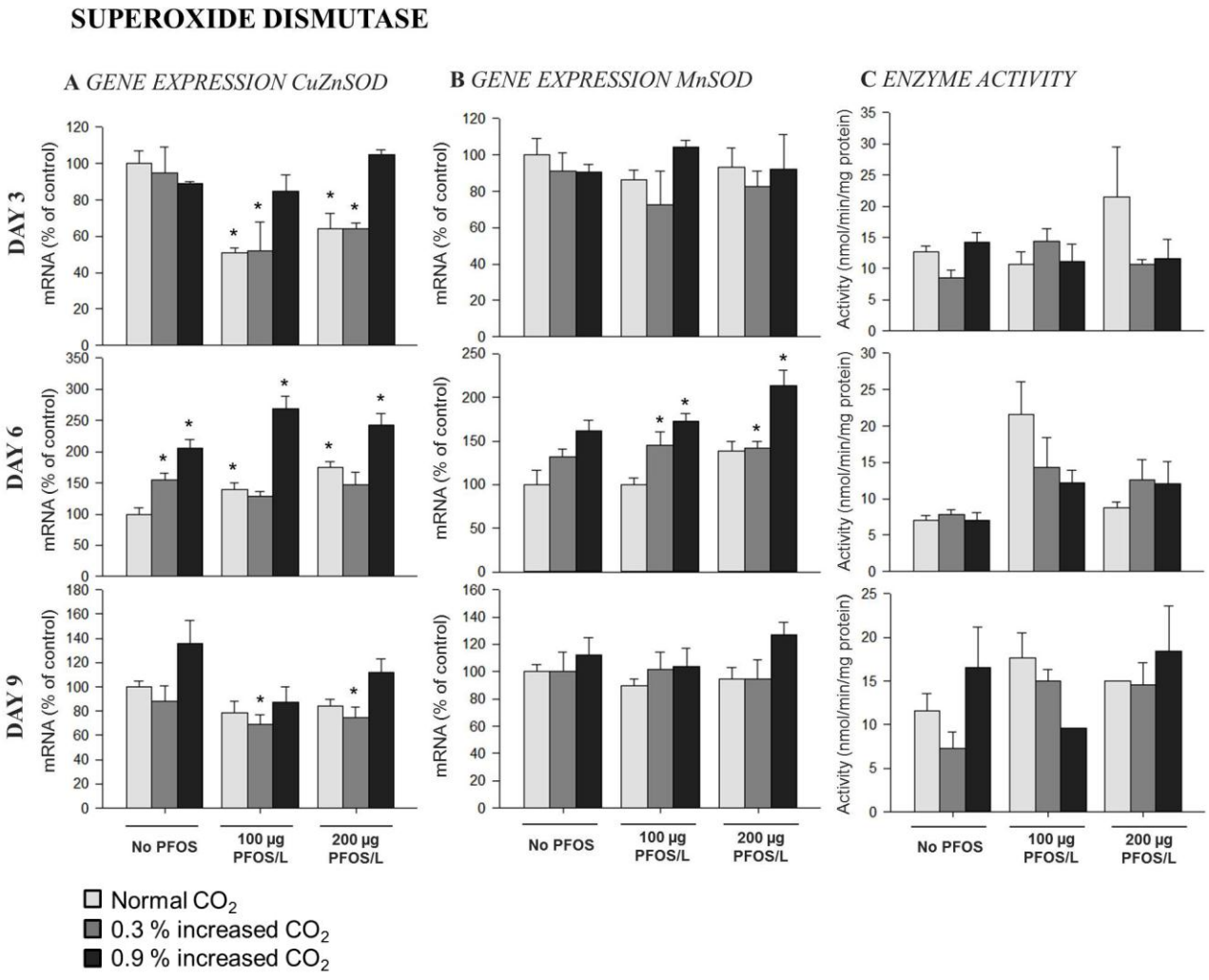

Figure 12. Changes in gene expression levels for A: *CuZn superoxide dismutase* (*CuZn-sod*) and B: *Mn superoxide dismutase* (*Mn-sod*), expressed as percent of control with SEM (n=4/5), and C: enzyme activity for SOD with SEM (n=1/2/3). Juvenile Atlantic cod were exposed to PFOS and increased water CO_2 level, singly or in combination. Changes in gene expression level in liver tissue were measured with real-time RT-PCR using specific primer pairs, and enzyme activity was measured spectrophotometrically. Asterisks denote significant difference in gene expression compared to control (Kruskal-Wallis non-parametric one-way ANOVA followed by multiple t-tests ($p<0.05$).

3.4.2 Glutathione and Glutathione-dependent enzymes

Expression of *glutathione peroxidase*

At day 3, *gpx1* was increased significantly compared to control by 0.9 % increase in CO_2 and also by exposure to PFOS alone (Figure 13A). A combination of high PFOS level and high CO_2 apparently resulted in the highest level of transcription. At day 6 within the 0.3 % increased CO_2 group PFOS apparently decreased transcription, while PFOS alone apparently increased transcription of *gpx1*. At day 9, transcription was non-significantly decreased by a 0.9 % increase in CO_2 combined with no PFOS and 100 µg PFOS/L, and by 200 µg PFOS/L only.

Expression levels for *gpx3* at day 3 were ambiguous (Figure 13B). Within the 100 µg PFOS/L exposure group, an increase in CO_2 apparently increased gene expression, while within the 200 µg PFOS/L exposure group the opposite was observed. At day 6, transcription was non-significantly increased in the 100 g PFOS/L group, but co-exposure to increased CO_2 diminished this effect. In the 200 µg PFOS/L exposure group, exposure to increased CO_2 apparently decreased expression. At day 9, expression was significantly decreased in fish exposed to both 0.3 and 0.9 % increase in CO_2 at all PFOS concentrations.

Glutathione peroxidase enzyme activity

At day 3, GPx activity was apparently increased by increasing CO_2 in fish exposed to no PFOS or 100 µg PFOS/L (Figure 13C). Within the normal CO_2 exposure group, PFOS appeared to increase activity. At day 6 and 9, within the no PFOS exposure groups, activity was non-significantly reduced upon exposure to 0.3 % increase in CO_2. At day 6 there was a slight increase in activity by 100 µg PFOS/L. At day 9, a 0.9 % increase in CO_2 seemed to decrease expression in the 100 µg PFOS/L exposure group and increase expression in the 200 µg PFOS/L exposure group. A significant decrease of expression below control level was observed by exposure to 0.3 % increased CO_2 alone.

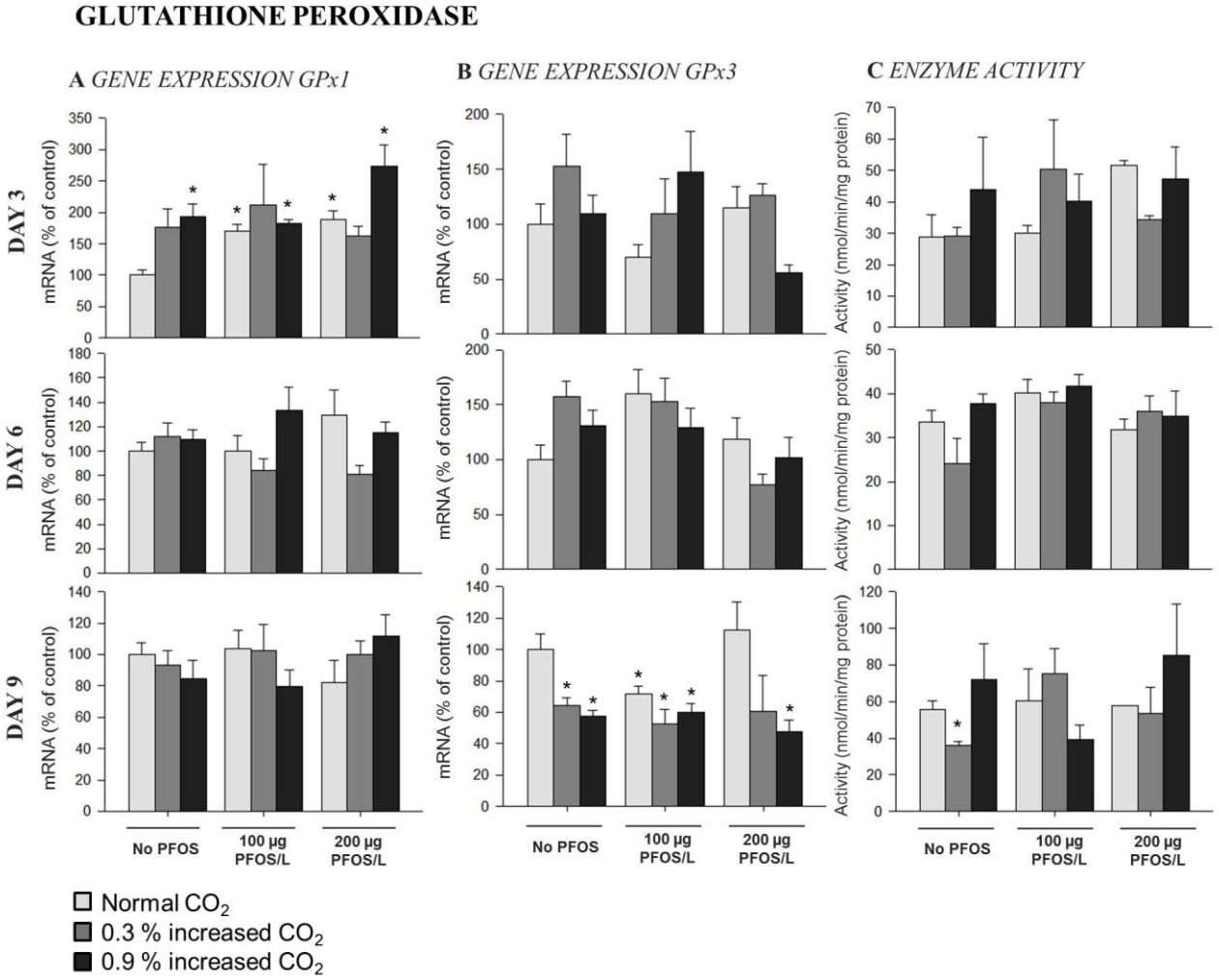

Figure 13. Changes in gene expression levels and for A: *glutathione peroxidase* (*gpx*) *1* and B: *gpx 3* expressed as percent of control with SEM (n=4/5), and C: enzyme activity for GPx, with SEM (n=1/2/3). Juvenile Atlantic cod were exposed to PFOS and increased water CO_2 level, singly or in combination. Changes in gene expression level in liver tissue were measured with real-time RT-PCR using specific primer pairs, and enzyme activity was measured spectrophotometrically. Asterisks denote significant difference in gene expression compared to control (one-way ANOVA with Tukey's post-hoc test ($p<0.05$) for *Gpx1* day 9 and *Gpx3* day 6 and Games-Howell post-hoc test ($p<0.05$) for *Gpx1* day 3+6, and Kruskal-Wallis non-parametric one-way ANOVA followed by multiple t-tests ($p<0.05$) for *Gpx3*day 6+9 and enzyme assays).

Expression of *glutathione reductase*

At day 3 within the 0.3 and 0.9 % increased CO_2 groups, transcription of *gr* was above control level in the no PFOS exposure group, and PFOS apparently decreased transcription (Figure 14A). At day 6 within the no PFOS group, increased CO_2 apparently increased expression. There was a significant increase in transcription by 100 µg PFOS/L combined with 0.9 % increase in CO_2 (Figure 14A). At day 9, expression was apparently decreased upon exposure to PFOS and 0.3 % increase in CO_2, and PFOS decreased transcription apparently within the 0.9 % increased CO_2 group.

Glutathione reductase enzyme activity

At day 3 there was an apparent decrease in enzyme activity of GR in fish exposed to 100 µg PFOS/L and normal sea water but the effect was counteracted by exposure to increased CO_2 (Figure 14B).Within the 200 µg PFOS/L group, activity seemed to decrease by increasing CO_2. At day 6 within the normal sea water and 0.9 % increased CO_2 group, PFOS increased activity non-significantly, while the opposite was true within the 0.3 % increased CO_2 group. At day 9, activity was apparently increased upon exposure to 200 µg PFOS/L only, and it seemed to decrease below control level with increasing PFOS concentration within the 0.3 % increased CO_2 group.

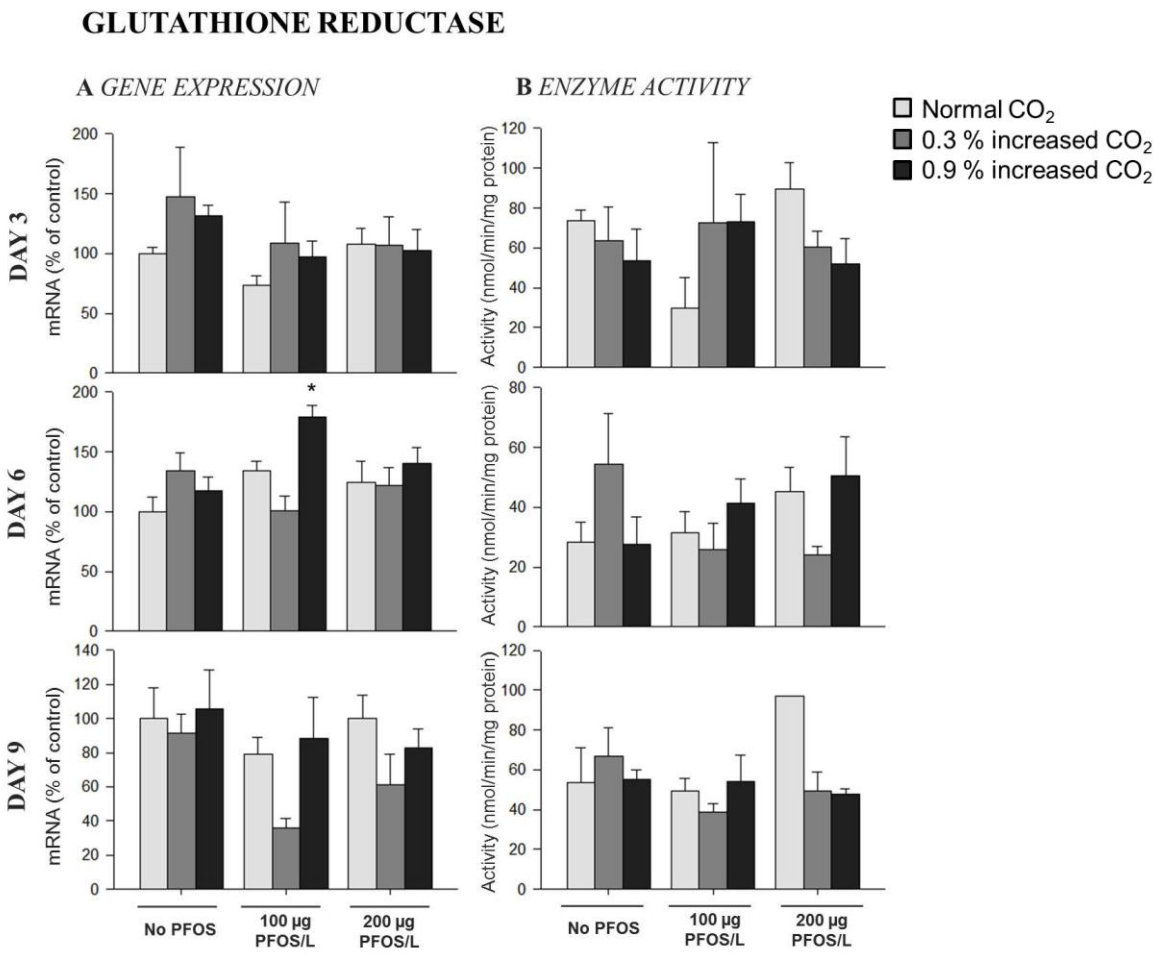

Figure 14. Changes in A: gene expression levels of *glutathione reductase* (*gr*) and B: enzyme activity for Glutathione reductase (GR), expressed as percent of control, with SEM (n=4/5). Juvenile Atlantic cod were exposed to PFOS and increased water CO_2 level, singly or in combination. Changes in gene expression level in liver tissue were measured with real-time RT-PCR using specific primer pairs, and enzyme activity was measured spectrophotometrically. Asterisks denote significant difference in gene expression compared to control (one-way ANOVA with Games-Howell post-hoc test ($p<0.05$) for *gr* day 9, and Kruskal-Wallis non-parametric one-way ANOVA followed by multiple t-tests ($p<0.05$) for *gr* day 3+6 and enzyme assays).

Glutathione S-transferase enzyme activity

At day 3, activity of GST increased non-significantly upon exposure to increased CO_2 singly, and upon exposure to 0.3 % increased CO_2 and 100 µg PFOS/L in combination (Figure 15). Activity was apparently decreased upon increased CO_2 exposure in all PFOS exposure groups at day 6. At day 9 within activity seemed to increase upon exposure to 0.9 % increase in CO_2, although activity was at the control level in the 200 µg PFOS/L exposure group.

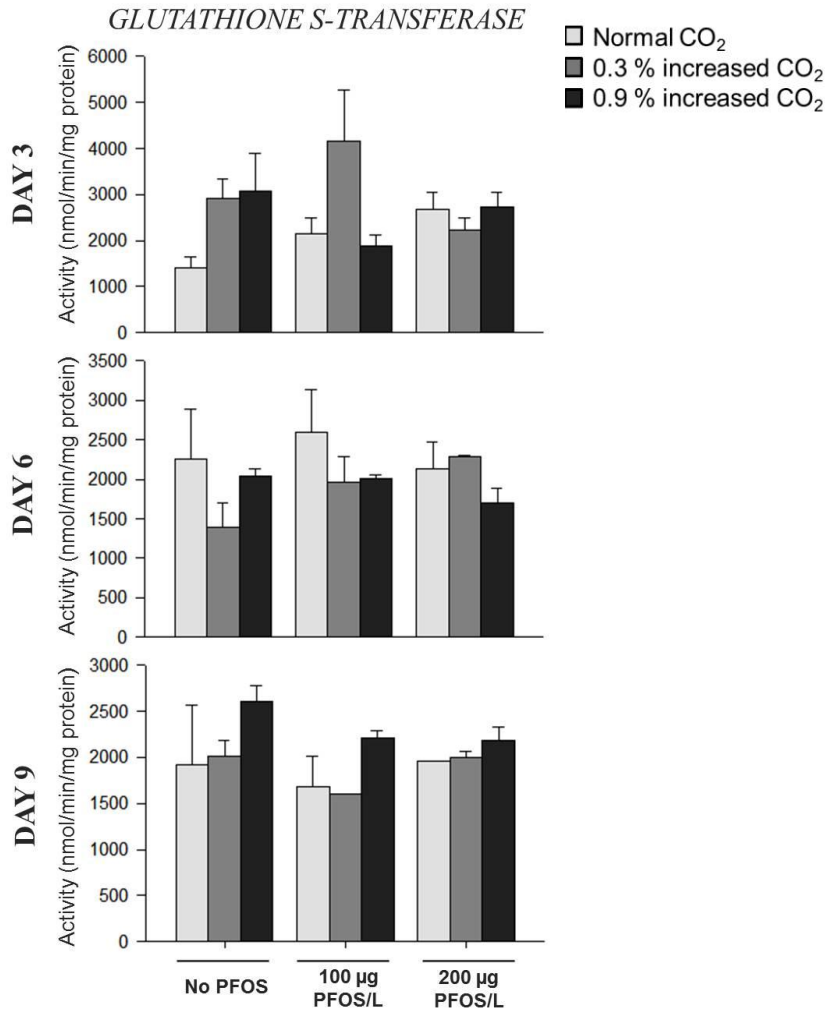

Figure 15. Changes in enzyme activity for Glutathione S-transferase (GST), expressed as nmol/min/mg protein, with SEM (n=1/2/3). Juvenile Atlantic cod were exposed to PFOS and increased water CO_2 level, singly or in combination and enzyme activity for GST was measured spectrophotometrically. Asterisks denote significant difference in gene expression compared to control (Kruskal-Wallis non-parametric one-way ANOVA, followed by multiple t-tests ($p<0.05$).

Glutathione levels

GSH levels at day 3 experienced non-significant increases by all exposure regimes, although most prominently upon exposure to 100 µg PFOS/L combined with increased CO_2. Within the 0.9 % increased CO_2 group at day 6, PFOS seemed to increase GSH level, and this was also the case at day 9 in the 0.3 and 0.9 increased CO_2 groups (Figure 16). At day 9, an increase in PFOS concentration decreased glutathione levels in the normal CO_2 group.

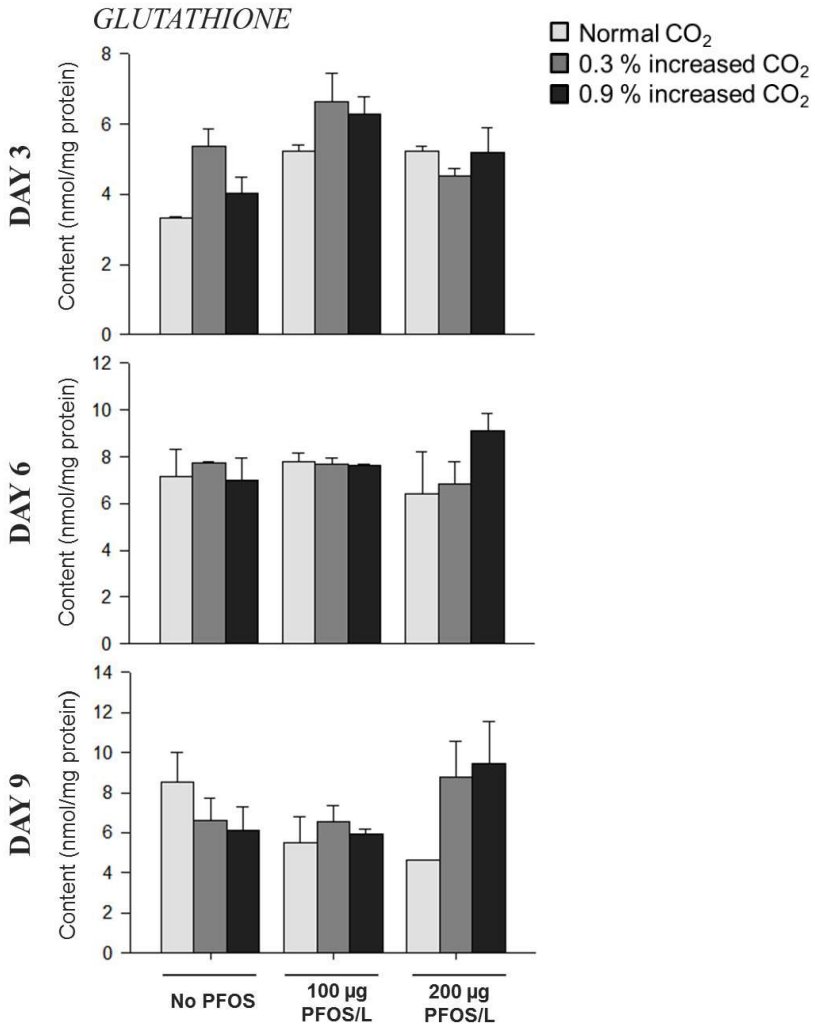

Figure 16. Changes in levels of glutathione (GSH), expressed as nmol/mg protein, with SEM (n=1/2/3). Juvenile Atlantic cod were exposed to PFOS an increased water CO_2 level, singly or in combination. Level of liver GSH was measured spectrophotometrically Asterisks denote significant difference in gene expression compared to control (Kruskal-Wallis non-parametric one-way ANOVA, followed by multiple t-tests ($p<0.05$).

3.4.3 Catalase

Gene expression

At day 3 within the 100 and 200 µg PFOS/L groups, exposure to increased CO_2 appeared to decrease expression of *cat*. At day 6, exposure to a 0.3 % increase in CO_2 apparently decreased mRNA levels, while increasing concentration of PFOS within the normal sea water group increased the expression level. At day 9, within the normal sea water and the 0.9 % increased CO_2 groups, PFOS produced a decrease of *cat* expression. Within the 100 µg PFOS/L group, *cat* expression was increased by a 0.9 % increase in CO_2 (Figure 17A).

Enzyme activity

Within the normal CO_2 group, enzyme activity of Cat was increased by 100 µg PFOS/L at day 3, 6 and 9 and by 200 µg PFOS/L at day 3 and 6, and significantly so at day 6, by 0.3 % increased CO_2 and 100 µg/L PSOS (Figure 17B). At day 3, there was a significant increase in activity upon exposure to 100 µg PFOS/L and 0.3 % increase in CO_2 in combination. 0.9 % increased CO_2 increased activity in combination with no PFOS and 200 µg PFOS/L. At day 6, activity seemed to increase upon exposure to 100 µg PFOS/L when CO_2 was normal or increased by 0.3 %. At day 9 within no PFOS and 200 µg PFOS/L groups, 0.3 and 0.9 % increased CO_2 apparently increased activity, while in the 100 µg PFOS/L group, this was true only for the 0.3 % increased CO_2.

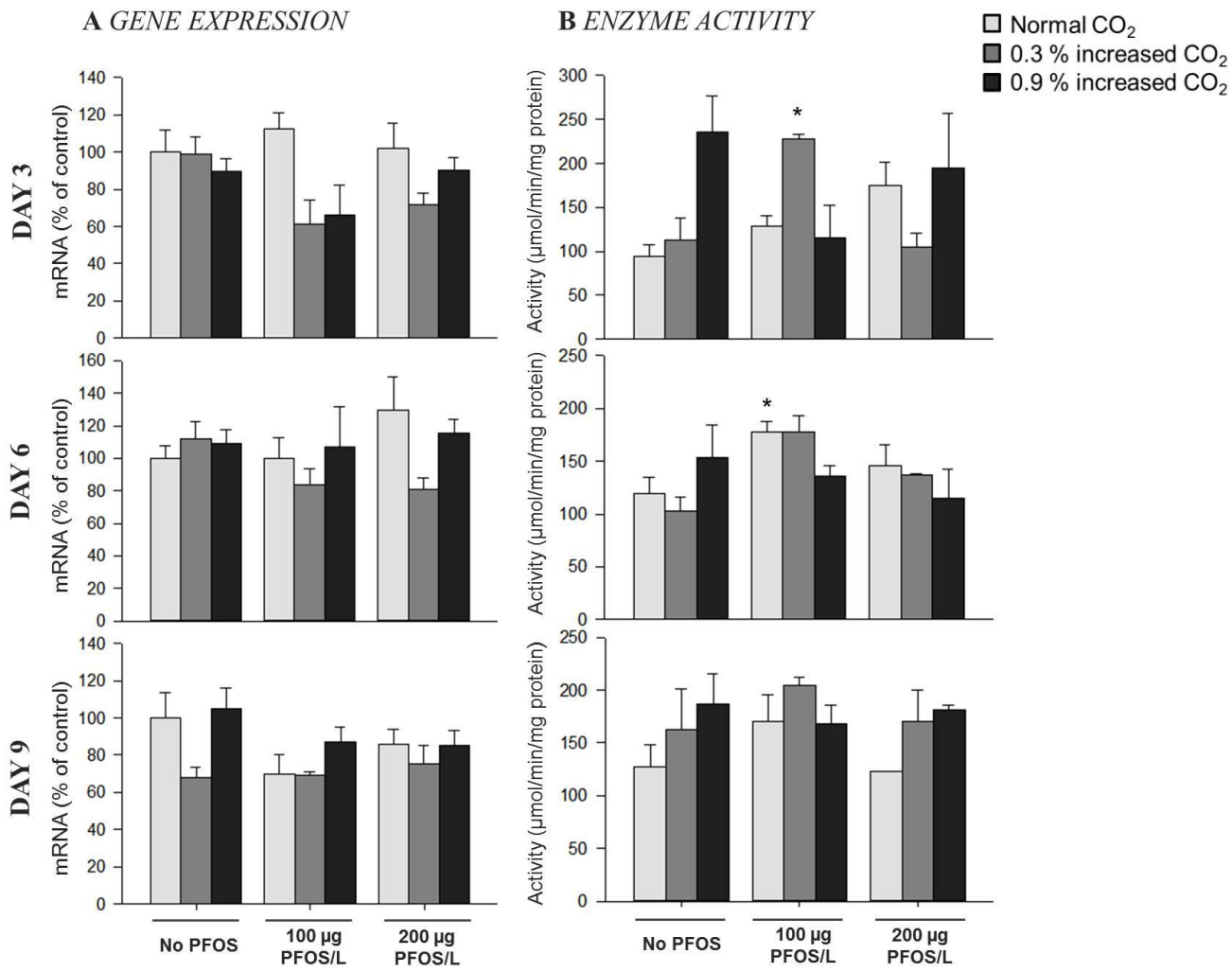

Figure 17. Changes in A: expression levels for the *catalase (cat)* gene and B: enzyme activity for Catalase (Cat), expressed as percent of control and µmol/min/mg protein, with SEM (n=/5). Juvenile Atlantic cod were exposed to PFOS and increased water CO_2 level, singly or in combination. Changes in gene expression level in liver tissue were measured with real-time RT-PCR using specific primer pairs, and enzyme activity was measured spectrophotometrically. Asterisks denote significant difference in gene expression compared to control (one-way ANOVA with Tukey's post-hoc test ($p<0.05$) for *cat* day 9 and Games-Howell post-hoc test ($p<0.05$) for *cat* day 3+6, and Kruskal-Wallis non-parametric one-way ANOVA followed by multiple t-tests ($p<0.05$) for enzyme assays).

3.4.4 Presence of lipid peroxidation metabolites

At day 3 and 6, the results were ambiguous and slightly opposite to one another. The MDA content at day 3 was reduced by all exposures, except from 0.9 % increase in CO_2 in the no PFOS group (Figure 18). At day 6, 0.3 % increase in CO_2 increased MDA levels, as did a combination of 100 µg PFOS/L and 0.9 % CO_2, and also 200 µg PFOS/L alone. At day 9, there is a subtle decrease in MDA content with increasing CO_2, and a slight elevation with increasing PFOS concentration within the 0.3 % increased CO_2 group.

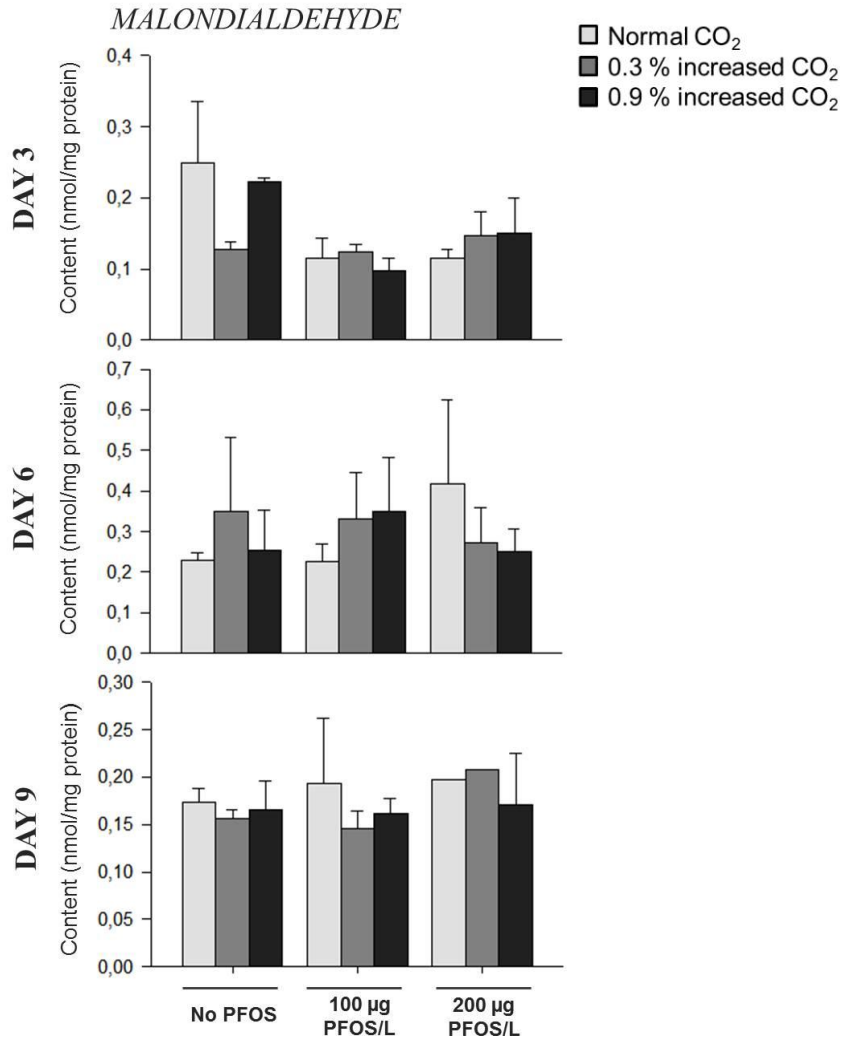

Figure 18. Changes in levels of Malondialdehyde (MDA), expressed as nmol/mg protein, with SEM (n=1/2/3). Juvenile Atlantic cod were exposed to PFOS and increased water CO_2 level, singly or in combination. Presence of MDA was measured spectrophotometrically. Asterisks denote significant difference in gene expression compared to control (Kruskal-Wallis non-parametric one-way ANOVA, followed by multiple t-tests ($p<0.05$).

4 DISCUSSION

It is well known that PFOS and other PFCs such as PFOA are inducers of oxidative stress in fish, mammals, and invertebrates (Arukwe and Mortensen, 2011; Chen et al., 2012; Lee et al., 2012; Liu et al., 2007; Oakes et al., 2005; Xu et al., 2013). Hypercapnia might cause oxidative stress and also reduced protein synthesis in fish (Langenbuch and Portner, 2003; Moran and Støttrup, 2011). Elucidating combined effects of emerging environmental contaminants and quantifiable measures of climate change are important because it increases our understanding of the detrimental impact humans have on nature. The knowledge might affect future regulations concerning CO_2 and chemical releases (Kannan, 2011).

4.1 Methods

4.1.1 Evaluation of sample quality

Maintenance of RNA integrity is vital for obtaining accurate real-time PCR results, but dissection of the fish was a possible source of contamination. Even though collection of liver tissue was performed under laboratory conditions, this kind of procedure is not 100 sterile. Stomach content or faeces was not a problem since fish were starved, however, other body fluids such as blood and bile contains enzymes and compounds that can have affected RNA quality or has the ability to inhibit PCR assays (Bustin and Nolan, 2004). This was taken into account during execution of the experiment, and measures were taken to maintain a clean working environment. Harvested organs aimed for gene expression analyses were embedded in TRIzol® reagent immediately after removal from the body and thereafter snap-frozen in liquid nitrogen in order to prevent degradation during storage.

Quality of all isolated RNA was measured using a NanoDrop spectrophotometer, and A260/280 ratio was above 1.82 in all samples apart from one, which had a ratio of 1.79. These values can be

considered acceptable (Fleige and Pfaffl, 2006). RNA integrity gel electrophoresis performed on 10 randomly chosen samples verified the integrity of RNA.

Primers for real-time RT-PCR must be specific for the sequence of interest and must not form primer dimers. Functionality of the primers was tested beforehand by using them in PCR amplification of a cDNA pool from Atlantic cod. PCR products were separated by gel electrophoresis, and presence of only one band in each well on the gel verified functionality and specificity of the primers (Chou et al., 1992). At the end of each real-time PCR assay, a melting curve was generated. DNA of a certain length and GC ratio will denature at a specific temperature (Clark, 2010). If temperature in the wells is increased gradually, DNA denaturation can be detected due to loss of fluorescence from SYBR green, which can only bind to double-stranded DNA (Clark, 2010). All amplification products in each assay had the same melting point, which verifies specificity of the primers. In addition, gel electrophoresis on randomly chosen real-time PCR products showed the presence of a single amplification product per assay, and length of the DNA fragments were correct.

4.1.2 Normalisation of real-time RT-PCR data

Normalisation of real-time PCR data can be done in several ways. For relative quantification of gene expression, comparison of Ct values for the gene in question to those of constitutively expressed housekeeping genes is common (Pfaffl, 2004). For absolute quantification, a standard plot can be applied that relates Ct value to copy number. The objective of the study should determine which method of normalisation to use; however, use of housekeeping gene should be regarded with some scepticism. Whether some genes in the cell's genome are expressed at the same level regardless of conditions is a debated issue. While some genes are tightly regulated to meet the cell's demands, others are needed constitutively and are therefore always expressed, hence they are called housekeeping genes. However, even these genes have been shown to be regulated to a certain extent, and their mRNA levels cannot be considered constant (Arukwe, 2006; Dheda et al., 2005). In cells that do not receive treatment of any kind, many of the housekeeping genes have a constant level of expression, but levels are often altered in toxicology studies where cells are subjected to treatments of different kinds (Dheda et al., 2005). If housekeeping genes are chosen, a thorough evaluation of different genes must be done before choosing one that is not affected by the treatment performed in that specific study (Arukwe, 2006; Dheda et al., 2005; Huggett et al., 2005).

Using known amounts of linear plasmids containing the amplicon of interest gives accurate results, and it is commonly used for absolute quantification of gene expression (Pfaffl, 2004). This is well-validated procedure is commonly used in our laboratory (Arukwe, 2006). A standard plot is made through a serial dilution of plasmids, and these are amplified on the same real-time PCR plate as test samples. The result is a standard plot of Ct values versus logarithm of copy number, and Ct value for samples can be normalized to this standard plot to get exact number of mRNA copies present in the cell (Pfaffl, 2004). In this experiment, real-time PCR data are given as percent of control and a standard plot was created from plasmids containing the *CYP19* (*aromatase*) gene, and this was used to normalize all real-time PCR data.

4.1.3 Evaluation of the statistical analyses

A problem frequently encountered during handling of the data set was a lack of normality. Performing other statistical tests than a parametric one-way ANOVA could easily solve this, but, power of the other tests was lower and it was therefore more difficult to reveal statistically significant differences. Natural variation is expectable in all biological experiments, especially in toxicology since toxicokinetics and toxicodynamics of the tested compound will always vary among individuals. This experiment contained multiple steps: Exposure of fish, dissection process, RNA isolation, synthesis of cDNA, and real-time PCR, and this could have contributed to a further increase in variation (Mannhalter et al., 2000). High variation among the individuals in each exposure group has also had influence on the statistical analyses, since variation decreases the probability of a result being statistically significant. In interpretation of the results in this study non-significant results were also taken into consideration and discussed, although most emphasis were put into results where statistical significance could be proved.

4.2 Biological effects of PFOS and increases in CO_2

4.2.1 Theoretical and observed pH and P_{CO2}

In order to ensure stable water flow and CO_2 input in the exposure tanks, experimental conditions were set up a week before addition of fish, and pH was measured twice daily. Upon addition of fish, pH in the tanks dropped and P_{CO2} increased, caused by CO_2 from cellular respiration of the fish. Although this changed the experimental parameters, change was approximately the same in all CO_2 exposure groups. The purpose of the experiment, exposing cod to different CO_2 levels, was not violated. pH and P_{CO2} gradually returned back to pre-set levels as fish were removed from the tanks

at day 3 and 6 of exposure, and hence fish from day 3 and 6 were exposed to a slightly lower pH and higher P_{CO2} than fish at exposure day 9. This could be an explanation for an overall lack of statistically significant results at day 9, although this is only a mere speculation.

4.2.2 Uptake, accumulation, and tissue distribution of PFOS

Measurements of actual concentration of PFOS in liver were not available, as the analysis of this variable is still in progress. However, previous studies in Atlantic salmon (*Salmo salar*) have shown that PFOS accumulates to high concentrations in liver, and concentration is maintained after ended exposure (Mortensen et al., 2011). This accumulation principle was basis for experimental design in the present study, in which fish were first exposed to PFOS and thereafter to different water CO_2 concentrations, since there was no opportunity for a concurrent exposure. However, lipid contents in livers of Atlantic cod and Atlantic salmon differs: while salmon liver is lean, cod liver is high in lipids (Lie et al., 1988). PFOS has a relatively low tendency of partitioning into lipids (Jones et al., 2003; Long et al., 2013; Mortensen et al., 2011; Zhang et al., 2009). In addition, half-life for PFOS in juvenile Rainbow trout (*Oncorhynchus mykiss*) was determined to 14 days (Martin et al., 2003), and therefore concentration might have been relatively low in cod liver in this experiment.

4.2.3 Effects on growth

All fish were measured and weighed directly after sacrifice. Results from exposure day 9 are presented here, since eventual effects on weight or length can be expected to be greatest at the end of the exposure time (Table 7). There were no significant changes, only a slight and non-significant decrease in weight with increasing PFOS concentration. In studies on the effect of PFOS on zebrafish embryos and larvae (*Danio rerio*), average body length was lower in exposed individuals compared to control group at the end of the exposure period (Shi et al., 2008; Shi et al., 2009). Increased CO_2 level in water has been shown to reduce growth rate in juvenile Atlantic cod of 15-80 g (Moran and Støttrup, 2011). They also compared their results to studies performed on other marine fish species, and they concluded that Atlantic cod are more susceptible to environmental hypercapnia than the other species (Moran and Støttrup, 2011). The short duration of this study and the low PFOS concentration the fish were exposed to compared with the studies Moran and Støttrup (2011) and Shi et al. (2008; 2009) might explain why no significant changes was observed. Also, since fish were not fed and hence growth conditions were not optimal, no significant growth could be expected (Drew et al., 2008).

4.2.4 Effects on lipid homeostasis and oxidative stress response

Effects on PPARs and Acyl-CoA dehydrogenase

A consistent trend showed that increase in water CO_2 had a prominent effect on transcription of *pparβ*. Transcription was increased in all groups subjected to hypercapnic conditions, the only exception being the 0.3 % CO_2 increase group at day 3. *pparβ* is ubiquitously expressed, although level of expression is relatively low in liver (Schoonjans et al., 1996). It activates genes responsible for fatty acid oxidation and energy dissipation in mouse adipose and muscle tissue (Wang et al., 2003). It has been shown that a lowered intracellular pH due to an increase in water CO_2 concentration had the ability of reducing protein synthesis in cultured hepatocytes from the Antarctic cold-water fish species *Pachycara brachycephalum* and *Lepidonotothen kempi* (Langenbuch and Portner, 2003). This was suggested to be due to an overall decrease in non-vital metabolic processes in order to compensate for lower energy availability caused by the stress condition. Since PPARβ has the ability of activating genes involved in fatty acid oxidation, and hence activating energy-generating processes, the increased transcription of *pparβ* might have been a response to increased energy-demand. The reduced transcription caused by 0.3 % CO_2 increase at day 3, might have been a result of an initial decrease of overall protein synthesis. Continuation of hypercapnic exposure returned this decrease in *pparβ* expression back to control level and above, by the previously suggested cause.

PEMT and PISD are involved in phospholipid inter-conversion and they are regulated coordinately (Carson et al., 1984). Expression of *pisd* and *pemt* genes at day 9 showed a clear, but non-significant decrease upon exposure to elevated CO_2 levels, and expression was inversely related to expression of *pparβ*. Expression of *pisd* and *pemt* at day 3 and 6 were ambiguous, although an overall trend suggests a decrease of expression upon exposure to increased CO_2. This might indicate a lowering of metabolic processes considered unnecessary for survival of the organism, as suggested by Lagenbuch and Portner (2003).

Effect of PFOS on *pparβ* expression was minimal, but there was an apparent increase with increasing PFOS concentration at day 3 and 9. Multiple studies show that PFOS is an activator of PPARα, β, and γ in both fish and mammals (Arukwe and Mortensen, 2011; Takacs and Abbott, 2007; Vanden Heuvel et al., 2006; Wågbø et al., 2012). Results from this study are hence in consistence with previous findings, although the response was minimal and non-significant. Changes in level of gene expression are often rapid processes taking place shortly after exposure to

inducing agents. In the study by Arukwe and Mortensen (2011), juvenile Atlantic salmon were exposed to PFOS orally (0.2 mg/kg fish) three times during a period of 6 days, and expression of all three PPAR species in liver was increased 2 days after the first exposure. After day 2, mRNA level returned back to normal for the rest of the exposure period. Hence, it is possible that transcriptional response to PFOS occurred directly after the initial exposure, and that the effect had already transited by the time of the first sampling, which was 8 days after initiated PFOS exposure.

Increased expression of downstream target genes, including *acox* gene and *acod* gene, should be expected in the exposure groups where highest induction of *pparβ* mRNA was observed (Desvergne and Wahli, 1999; Tanaka et al., 2003; Wang et al., 2003). No significant changes in *acod* mRNA levels could be detected; however, an apparent decrease in transcription could be observed at exposure days 3 and 9 upon exposure to 0.3 % CO_2 increase. This was returned back to control level by PFOS, which is in consistence with the small increase in *pparβ by PFOS.* At day 6, expression was reduced only upon exposure to a combination of PFOS and elevated CO_2 concentration. It might seem like a low level of hypercapnia has the ability of decreasing the FA β-oxidation in cod, and that a further increase in hypercapnia combined with PFOS can counteract this effect. In a study investigating effect of PFOS on various fish species, increase in ACOX activity in liver was observed when White sucker (*Catostomus commersoni*), Spottail shiner (*Notropis hudsonius*), and Creek chub (*Semotilus atromaculatus*) were exposed to 3 mg PFOS/L in water for 14 days. The same effect was also observed in Fathead minnow (*Pimephales promelas*) exposed to 0.3 mg PFOS/L in water for 28 days (Oakes et al., 2005). Expression of both *acod* and *acox* are both induced by PPARs, and similar responses can therefore be assumed (Desvergne and Wahli, 1999; Tanaka et al., 2003; Wang et al., 2003). Exposure concentration was higher and exposure duration was longer in the aforementioned studies, which could explain why results were more prominent. Another explanation might be that fish used in the present study was not fed during the acclimation period or during the experiment. Upon starvation, organisms will start utilizing stored reserves of fatty acids for energy and must therefore intensify the lipid catabolism machinery (Li et al., 2006).

mRNA levels for *acod* were measured after a prolonged period of starvation. It is possible that transcription of this gene was already fully up-regulated, and hence, further stimuli for increased transcription by activated PPARs would have had no effect. In a study on rats where transcriptional effects of starvation were investigated using microarray technique, it was shown that transcription of *acox* was increased after one day of fasting and then it decreased at the subsequent days (Li et al.,

2006). The authors suggested that accumulation of urea in the tissues was responsible for inhibiting further transcription of *acox*, however, this is not transferable to teleost fish, where nitrogen is excreted as ammonia which is lost effectively as NH_4^+ across epithelia of the gills (Campbell et al., 2008). The study by Li et al. (2006) is in consistence with other studies, which reported that mRNA levels of *pparα* in mammals increase upon initial starvation (24h) (Kersten et al., 1999) and thereafter decrease upon starvation for 48 and 72 hours (Hashimoto et al., 2000). A possibility is that expression of *acod* was decreased by some unknown mechanism due to or related to starvation. Preventing excessive use of stored lipids could be a strategy for saving energy for more urgent times. Another hypothesis is that the *acod* expression had already been increased at an earlier point of time, by the believed increase in *PPARβ* upon initiation of PFOS exposure.

Effects on antioxidant enzymes

An overall increase in expression of antioxidant enzyme genes was observed. This could be either due to increased formation of ROS by increased β-oxidation, or it could be a direct effect of hypercapnia. It might be argued that the use of gene expression levels for antioxidant enzymes is an uncertain measure of oxidative stress level. The effect depend on many variables such as concentration and duration of exposure, age, gender, species, and environmental conditions, and the use of antioxidant enzymes as biomarkers for oxidative stress are by some considered questionable (Paskerova et al., 2012). However, since this experiment was performed under laboratory conditions, many confounding factors were controlled. There is not necessarily coherency between measured expressional level and activity of the enzyme, since some genes are not regulated at the transcriptional but rather at a post-mRNA level (Clark, 2010). Transcriptional responses can be considered biomarkers of exposure, while changes at enzyme activity level can be considered biomarkers of effect (Giuliani et al., 2013). Albeit *acod* expression was only slightly affected by increased *pparβ* expression, it is possible that ACOD enzyme activity was increased, a hypothesis reinforced by the fact that there was an apparent correlation between expressional levels of *pparβ* and both *CuZn-sod* and *Mn-sod* genes at all exposure durations. The initiating step in β-oxidation of fatty acids by ACOD reduces electron transfer flavoprotein (ETF). ETF is part of the respirational electron transport chain of inner mitochondrial membrane and electrons will be donated here. The electron transport chain is a source of superoxide anion, and hence, an increase in mitochondrial β-oxidation will increase generation of ROS (Kim and Miura, 2004). Since SOD enzymes are scavengers of superoxide anion (Storey, 1996), the increased expression of these genes indicate higher activity of ACOD. At day 9, there was an increase in Cat activity with increasing CO_2, which corresponds to the increase in expression of *pparβ*. The expression of *pparβ* expression at day 3

was reflected in *cat* expression at day 6 and 9, with an apparent decrease by exposure to 0.3 % CO_2 increase. PPARβ-induced increase peroxisomal β-oxidation and subsequent increase in hydrogen peroxide generation can explain the partly corresponding increase in *cat* expression and activity (Jucker et al., 2007; Tanaka et al., 2003; Wang et al., 2003).

A number of studies have reported that PFOS can induce transcription and activity of antioxidant enzymes in fish. ROS concentration in cultured hepatocytes of Freshwater tilapia (*Oreochromis niloticus*), increased significantly as a response to 1 mg PFOS/L (Liu et al., 2007). In another study performed on Zebrafish embryos, PFOS exposure led to significant increase was in SOD and GPx activity at 0.2, 0.4 and 1.0 mg PFOS/L, and in CAT activity at 0.4 and 1.0 mg PFOS/L. Exposure of Atlantic salmon to PFOS resulted in a significant induction in gene expression of *cat* at day 2 and 5, and *sod* at day 2 of exposure. No effects were observed after a 7 days recovery period (Arukwe and Mortensen, 2011). Huang and co-workers (2009) found a concentration-dependent increase in Mn-SOD activity in cultured Atlantic salmon hepatocytes exposed to 15.1 and 35 mg PFOS/L. In the present study, increased expression of *gpx1* at day 3, *gpx3* at day 6, *pparβ* at day 9 and slightly increased GSH levels were observed as a response to PFOS, but none of these could be proven statistically. Expression of *CuZn-sod* was significantly decreased by PFOS at day 3. The few significant responses to PFOS might either indicate that these responses had already occurred, as suggested previously, or that concentration of PFOS in the liver had decreased and hence was too low to give significant changes in expression and activity. The latter will remain a mere speculation until accurate measurements of PFOS concentration are ready.

Expression of *gpx1* at day 3 was increased by all exposure regimes, with highest increase being brought by a combination of 200 μg PFOS/L and a 0.9 % increase in CO_2. Since GPx enzymes are responsible for extra-peroxisomal scavenging of hydrogen peroxide and hence are not directly related to peroxisomal FA β-oxidation, it is likely to assume that induction of this gene was a result of ROS produced otherwise. It is generally accepted that increased CO_2 concentration in plasma and cells can cause oxidative stress. This is either by formation of ROS and RNS from nitrosoperoxycarboxylate (Denicola et al., 1996; Vesela and Wilhelm, 2002), or by increased rate of Fenton reaction, caused by increased dissociation of iron from Tf due to reduced pH in the organism (Garrick and Garrick, 2009; Princiotto and Zapolski, 1975; Sipe and Murphy, 1991). Marine fish are capable of restoring acid-base balance during exposure to hypercapnia in vivo by regulating activities of ion pumps in the gills (Baker et al., 2009; Claiborne et al., 2002). CO_2-mediated effects are therefore more likely to result from an actual increase of CO_2 concentration in the fish than from

a permanent pH change. At day 6 and 9 after initiated CO_2 exposure, it was observed that expression of *gpx1* and *gpx3* decreased with increasing CO_2. This is in agreement with the idea of increased rate of Fenton reaction where hydroxyl radical is formed from hydrogen peroxide. A reduction in the concentration of hydrogen peroxide in cytoplasm would reduce the need for production of more glutathione peroxidase. However, for the Fenton reaction to proceed, iron oxidized in the reaction would have to be reduced back to the ferric form, and this process consumes superoxide anion. A reduction in the concentration of superoxide anion would presumably lead to a decrease in the expression of the different SOD enzymes, which are responsible for scavenging this reactive oxygen species. This was, however, not the case, as expression of *sod* was increased by elevated CO_2 at day 6 and day 9. This further verifies the hypothesis that increased CO_2 level in water induced oxidative stress response through generation of ROS and RNS, and not through a pH-related increase of Fenton reaction.

Malondialdehyde level in cells is measured since it is a metabolite of lipid peroxidation. Lipid peroxidation is often induced by radicals and can therefore be an indicator of increased ROS. However, connection is not absolute, since lipid peroxidation can be induced spontaneously, and since ROS does not necessarily cause lipid peroxidation. Also, the TBARS test, which is most commonly used for measuring MDA, is non-specific and can also react with other oxidation products not generated by lipid peroxidation (Zhou et al., 2006). There were no significant differences in MDA levels in liver of fish from different exposure groups, but there was a small decrease upon exposure to PFOS alone at day 3 and a small increase upon exposure to PFOS alone at day 6. This should be regarded with scepticism as the control group at day 3 and the 200 µg PFOS/L exposure group at day 6 had high standard deviations. Previous studies on the effect of PFOS on lipid peroxidation in fish have presented contradictory results. In vitro exposure of freshwater tilapia (*Oreochromis niloticus*) hepatocytes to PFOS (0, 1, 5, 15 and 30 mg PFOS/L) for 24 hours gave no change in lipid peroxidation level (Liu et al., 2007). In a study where juvenile Atlantic salmon were exposed to PFOS at 0.2 mg/kg fish, there was a significant increase in MDA at day 2 of the exposure, but the increase was not maintained at further exposure days (Arukwe and Mortensen, 2011). A significant increase in MDA content ($p<0.01$) was observed at 1.0 mg PFOS/L (Shi and Zhou, 2010). If PFOS-induced oxidative stress had occurred at an earlier point of time, an eventual induction of lipid peroxidation would have followed directly after this. MDA is readily oxidized to CO_2 (Siu and Draper, 1982), and hence, the MDA generated might already have been metabolized by the cell.

GST activity did not show a clear pattern at day 3, whereas at it was apparently decreased with increasing CO_2 level at day 6 and increased with increasing CO_2 level at day 9. No obvious changes as a response to PFOS were observed. This indicates that GST does not participate in clearance of PFOS. Rather, it indicates involvement in removal of a metabolite produced as a response to CO_2 or PFOS, such as molecules damaged by oxidative attack from ROS (Storey, 1996). GPx activity, GSH levels, *gr* expression and activity, *Mn-sod* expression, and SOD activity displayed an overall increase upon exposure to increased CO_2, previously suggested due to increased ROS and RNS generation. It might be that the defence at day 6 was sufficient, and that the increase in antioxidant capacity of the cell at increased CO_2 levels actually decreased ROS to a level below normal, which is reflected in the lowered activity of GST. At day 9, however, sustained generation of ROS for several days could have overwhelmed defence capacity, and the ROS generated in excess could have attacked biological molecules and therefore increased the need for GST activity. Expression of *gpx1* and *gpx3* was reduced by increased CO_2 at day 9, which might indicate that the cells had decreased the high antioxidant defence system in order to save energy.

Antioxidant enzymes are vital for protection against ROS and RNS. Overwhelming of this system can lead to oxidative damage of important biological macromolecules, resulting in severe consequences for the organism (Livingstone, 2001; Storey, 1996). DNA mutations can reduce fitness of organisms due to malfunctioning gene products, and might potentially contribute to formation of cancerous tumours (Alberts et al., 2008). Oxidative attack on proteins and impairment of their function will generate a need for increased protein synthesis. As this is an energy-demanding process, less energy will be available for other processes such as reproduction (Clark, 2010). Lipid peroxidation can, as mentioned previously, cause leakage of components between compartments (Farber, 1994; Geeraerts et al., 1991). Integrity of membranes is extremely important in oxidative energy-generating processes of mitochondria, which is dependent of an electrochemical gradient of H^+ across the inner mitochondrial membrane. Energy stored in this gradient is responsible for mitochondrial ATP-production by ATPase (Nelson and Cox, 2008). Lipid peroxidation and subsequent leakage of H^+ across inner mitochondrial membrane can therefore impair cell's energy generation (Bindoli, 1988; Farber, 1994).

The results presented here show that both PFOS and increased CO_2 have the ability of causing oxidative stress in Atlantic cod, and in combination the effect might be an overwhelming of cell's antioxidant defence system and potentially a reduction of overall fitness. These consequences implies not only to Atlantic cod, but also to other aquatic animals, both vertebrates and

invertebrates. The on-going climate changes and continuous release of CO_2 to atmosphere will increase CO_2 level in water further (Haugan and Drange, 1996), and adaption of animals to new environmental conditions is important for maintenance of stable ecosystems. PFOS and other POPs are present in organisms in high amounts, giving an additional level of stress which might further decrease ability of adapting.

5 CONCLUSIONS

Altogether, these results indicate that an increase of CO_2 in water has the ability of inducing oxidative stress in Atlantic cod. Also, PFOS might induce oxidative stress in fish, albeit only minor indications of this could be observed. Increase of CO_2 level in water induced increased expression of *pparβ*, and albeit not significant, expression of *acod* showed a small, corresponding increase. Subsequent increase of FA β-oxidation was likely the cause of increased expression levels of *Mn sod* and *CuZn sod* at high CO_2 levels. At day 3, expression of *gpx1* was significantly increased by elevated CO_2, and this effect was potentiated by PFOS. This indicates that hypercapnic conditions with increased CO_2 have the ability of generating ROS independently of increased β-oxidation, which might have been due to generation of ROS and RNS from nitrosoperoxycarboxylate. Few prominent effects of PFOS were observed, likely because these responses happened at an earlier time point, directly after initiated exposure. Increased ROS generation as a response to increased CO_2 will cause oxidative damage to cells with potential consequences being DNA mutations, malfunctioning proteins and lipid peroxidation (Livingstone, 2001; Storey, 1996). Combined with increased energy expenditure due to activation of PPAR, this will reduce viability of cells and fitness of the organism. Since atmospheric and oceanic levels of CO_2 are expected to increase further, this might be relevant information in future risk assessment of CO_2 release. It might also have implications for aquaculture industry. Since aquaculture farms are considered an important source of pollutants of coastal ecosystems, fish farming in closed systems are under investigation (WWF, 2013). In a closed system, cellular respiration of fish will generate hypercapnic conditions in water, which might have the potential of influencing overall fitness and growth rate of fish.

Further research

For future experiments, an additional sampling should be performed a few days after PFOS exposure to investigate initial transcriptional effects. More effort should be put into elucidation of the mechanism behind the hypercapnia-induced oxidative stress observed in this study.

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Appendix A – Exposure regimes

Fish were exposed for one hour daily to PFOS, and thereafter to different CO_2 levels for 3, 6, or 9 days (Table A1).

A – No PFOS, normal CO_2
B – 100 µg PFOS/L, normal CO_2
C – 200 µg PFOS/L, normal CO_2
D – No PFOS, 0.3 % increased CO_2
E – 100 µg PFOS/L, 0.3 % increased CO_2
F – 200 µg PFOS/L, 0.3 % increased CO_2
G – No PFOS, 0.9 % increased CO_2
H – 100 µg PFOS/L, 0.9 % increased CO_2
I – 200 µg PFOS/L, 0.9 % increased CO_2

Table A1. Overview of fish exposure. Fish were exposed to PFOS for one hour daily in five days and for different CO_2 levels for 3, 6, or 9 days. Blue=PFOS exposure, Green=CO_2 exposure. Darker shade of green indicates sampling days. NB. Day number in table does not correlate with day number in Figures 7 and 8.

Day	***A***	***B***	***C***	***D***	***E***	***F***	***G***	***H***	***I***
1	PFOS 1			PFOS 1			PFOS 1		
2	PFOS 2	PFOS 1		PFOS 2	PFOS 1		PFOS 2	PFOS 1	
3	PFOS 3	PFOS 2	PFOS 1	PFOS 3	PFOS 2	PFOS 1	PFOS 3	PFOS 2	PFOS 1
4	PFOS 4	PFOS 3	PFOS 2	PFOS 4	PFOS 3	PFOS 2	PFOS 4	PFOS 3	PFOS 2
5	PFOS 5	PFOS 4	PFOS 3	PFOS 5	PFOS 4	PFOS 3	PFOS 5	PFOS 4	PFOS 3
6	CO_2 1	PFOS 5	PFOS 4	CO_2 1	PFOS 5	PFOS 4	CO_2 1	PFOS 5	PFOS 4
7	CO_2 2	CO_2 1	PFOS 5	CO_2 2	CO_2 1	PFOS 5	CO_2 2	CO_2 1	PFOS 5
8	CO_2 3	CO_2 2	CO_2 1	CO_2 3	CO_2 2	CO_2 1	CO_2 3	CO_2 2	CO_2 1
9	CO_2 4	CO_2 3	CO_2 2	CO_2 4	CO_2 3	CO_2 2	CO_2 4	CO_2 3	CO_2 2
10	CO_2 5	CO_2 4	CO_2 3	CO_2 5	CO_2 4	CO_2 3	CO_2 5	CO_2 4	CO_2 3
11	CO_2 6	CO_2 5	CO_2 4	CO_2 6	CO_2 5	CO_2 4	CO_2 6	CO_2 5	CO_2 4
12	CO_2 7	CO_2 6	CO_2 5	CO_2 7	CO_2 6	CO_2 5	CO_2 7	CO_2 6	CO_2 5
13	CO_2 8	CO_2 7	CO_2 6	CO_2 8	CO_2 7	CO_2 6	CO_2 8	CO_2 7	CO_2 6
14	CO_2 9	CO_2 8	CO_2 7	CO_2 9	CO_2 8	CO_2 7	CO_2 9	CO_2 8	CO_2 7
15		CO_2 9	CO_2 8		CO_2 9	CO_2 8		CO_2 9	CO_2 8
16			CO_2 9			CO_2 9			CO_2 9

Appendix B – Standard curve for real-time PCR

Standard curve for normalization of real-time PCR data was prepared by making a serial dilution of plasmids containing *CYP19* gene (*aromatase*) and amplifying this in a PCR reaction using specific primers. Equation for standard curve was Y=-4.372*log(X)+13.09., R^2=1.000 (Figure A1).

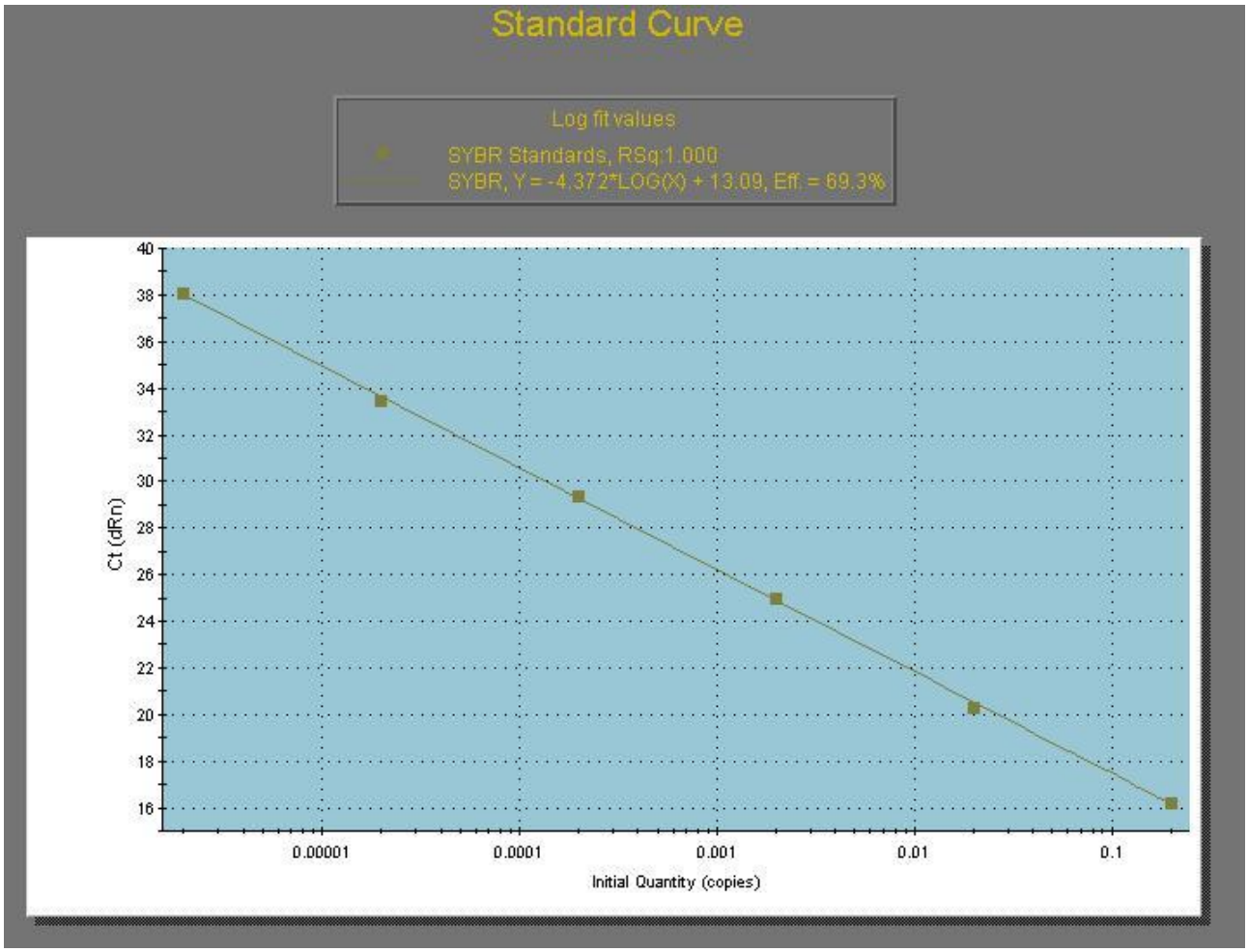

Figure B1. Standard curve for normalization of real-time PCR data.

Appendix C – Reagents used in enzyme assays

Procedure for making reagents used in enzyme assays are listed in Table B1. Required pH is given in protocol in Materials and methods section.

Table C1. Reagents for use in enzyme assays.

Reagent	***Procedure***
Bacitracin 100 mg/mL	Dissolve 100 mg bacitracin in 1 mL dH_2O
CDNB, 50 mM	Dissolve 0.0506 g CDNB in 5 mL methanol
CHP, 200 mM	Add 37 µL CHP 5.2 M to 963 mL methanol
DTNB, 20 mM	Dissolve 0.07962 g DTNB in 10 mL methanol
DTT 100 mM	Dissolve 0.01542 g DTT in 1 mL methanol
EDTA, 100 mM	Dissolve 3.7224 g EDTA in 100 mL dH_2O
GSH, 100 mM	Dissolve 0.0307 g GSH in 1 mL dH_2O
GSSG, 10 mM	Dissolve 0.006566 g GSSG in 1 mL dH_2O
H_2O_2, 1.2 M	Add 100 µL H_2O_2 12 M to 900 µL dH_2O
Homogenizing buffer	100 mL Tris-HCL buffer, 100 µL 100 mM PMSF, 100 µL 100 mg/mL bacitracin, and 1.8 g NaCl
K-phosphate buffer, 100 mM	Dissolve 1.36 g KH_2PO_4 in 100 mL dH_2O. Adjust pH.
NaCl, 1.8 %	Dissolve 1.8 g NaCl in 100 mL buffer
NADPH, 1 mg/mL	Dissolve 1 mg NADPH in 1 mL dH_2O
NADPH, 4 mg/mL	Dissolve 4 mg NADPH in 1 mL dH_2O
NADPH, 20 mg/mL	Dissolve 2 mg NADPH in 100 µL dH_2O
PMSF, 100 mM	Dissolve 0.174 g PMSF in 10 mL methanol
Tris-HCL buffer, 100 mM	Dissolve 1.211 g $C_4H_{11}NO_3$ in 100 mL dH_2O, adjust pH.