Effects of long-term exposure to produced water components on Atlantic cod (*Gadus morhua* L.) measured by lysosomal alterations and peroxisome proliferation

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Förord

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Abstract

Discharge from offshore activities to the sea may seriously affect the surrounding environment and cause disturbances in communities and ecosystems close to oil production installations. A major part of the waste released during oil production comes from produced water, including organic chemicals, metals and chemicals added in the separation and production process. The aim of this study was to investigate and quantify the effects of environmental relevant concentrations of produced water on fish in a long-term exposure. Gender-differences and changes over time were also investigated. Atlantic cod (*Gadus morhua* L.) were exposed to different concentrations of polycyclic aromatic hydrocarbons (PAHs) and alkylphenols (APs) for a total period of 44 weeks. The exposure consisted of three different treatments; "low" (2000 x diluted produced water), "high" (200 x diluted produced water) and "pulsed" (alternating control and high treatment on a weekly basis). The "control" treatment was made of water and acetone. Samples from the head kidney of cod taken before the start of the exposure and after 2, 16 and 33 weeks were analyzed using lysosomal membrane stability (LMS) and peroxisomal acyl-CoA oxidase (AOX) activity as biomarkers.

Cod exposed to "high" and "pulsed" treatment had significantly lower lysosomal membrane stability than cod exposed to "control" treatment. No differences in lysosomal membrane stability between weeks of exposure or between male and female cod were found. Levels of peroxisomal AOX activity in the head kidney were significantly lower in cod sampled at 33 weeks of exposure compared to the activity found in cod sampled before start of the exposure. Significant differences in peroxisomal AOX activitiy were also found between male and female cod. There were no significant differences in cod head kidney AOX activity with regard to treatment groups. A correlation was observed between levels of PAH metabolites (1-OH-pyrene) measured in bile and lysosomal membrane stability in the head kidney of cod.

The results from the present study clearly demonstrate that environmentally relevant concentrations of produced water components may have subtle effects on the health of fish in receiving waters. The results also revealed low and variable AOX activity in head kidney peroxisomes, indicating that the tissue may not be suitable for AOX activity measurements.

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1. Introduction

1.1 Oil production and environmental pollution

The oceans are increasingly affected by anthropogenic activities. The North Sea receives input from agriculture and offshore oil and gas production, as well as direct and indirect impacts from fisheries (OSPAR, 2000). Anthropogenic inputs such as these are polluting the marine environment and may cause disturbances in populations, communities and ecosystems of the marine life.

Norway has been producing oil since the beginning of the 70's. With 57 fields in production on the Norwegian continental shelf, Norway is ranked as the eleventh largest oil producer in the world, producing 2.6 million barrels of oil per day (Oljedirektoratet, 2008). During the operation and production phase, large volumes of waste are discharged from the platforms to the sea, affecting the marine environment. Pollution will arise from drilling and well operations and production of oil and gas, including discharge of drilling waste, produced water and acute oil spills (OLF, 2008). Discharge to the sea from offshore installations is regulated according to the OSPAR Convention[1] and the environment surrounding the platforms is monitored continuously to document conditions and changes due to the petroleum activities (OLF, 2008; Oljedirektoratet, 2008).

There are serious concerns about how the marine life surrounding the platforms is affected by the discharge. The concern is of both ecologically and as well as economically motives. The wellbeing of Atlantic cod (*Gadus morhua* L.) populations are of special interest, since it is one of the most economically important fish species in Norway.

Acute effects in marine organisms due to offshore oil production are rarely discovered, but large volumes of discharge released to the sea comes from produced water which may give rise to potential long-term effects.

[1] Convention of the Protection of the Marine Environment of the North-East Atlantic

1.2 Produced water

Produced water is water that has been trapped by geological formations for several million years (OLF, 2008). The water is pumped up to the surface from reservoirs during oil and gas production, separated from the oil and reinjected into the reservoir or cleaned and discharged to the sea. Produced water consists of several components harmful to the environment, including dispersed oil, dissolved hydrocarbons, organic acids, phenols, metals and chemicals added in the separation and production process. The chemical content of the water depends on the oilfield where it is pumped from (Utvik, 1999). The amount of water produced and discharged increases with the age of the field (Somerville et al., 1987).

The discharge of produced water to the sea increases every year (OLF, 2008). The amount of water produced on the Norwegian continental shelf in 2007 was 183 million m^3 and from this amount, about 162 million m^3 was discharged to the sea (OLF, 2008; SFT, 2008). Organic acids are the dominant compounds in the water followed by BTX (benzene, toluene and xylene), alkylphenols (APs), phenols and polycyclic aromatic hydrocarbons (PAHs). PAHs and APs are of special interest, having shown to cause unwanted negative effects in marine organisms. The total amount of PAHs released with produced water was about 74 tonnes in 2007 and the total amount of alkylphenols (ranged from C1 to C9) released was over 350 tonnes, with alkylphenols from C1-C3 making up 96 % of the total discharge of AP. Maximum concentration of oil allowed in water discharged from the platforms is set to 30 mg/L and average oil concentration in produced water discharged to the sea was found to be 9.5 mg/L in 2007. It is predicted that the increase in produced water to the sea will continue until 2012-2014, with an annual discharge of 200 million m^3 (OLF, 2008).

Even though produced water components are rapidly diluted when entering the sea and taken up by marine organisms in low concentrations (Strømgren et al., 1995), they may have the ability to cause chronic effects (Beyer et al., 2001). APs have been found to cause endocrine disruption in cod and may also contribute to oxidative stress in fish (Hasselberg et al., 2004; Meier et al., 2007), while PAHs have been found to have genotoxic effects in fish (Reichert et al., 1998).

This study has focused on effects of long-term exposure to produced water components in Atlantic cod (*Gadus morhua* L.) using environmentally relevant concentrations of PAHs and APs in the exposure water (table 2-1). The concentrations used in the study were based upon

produced water from the Statfjord area. 200 and 2000 times dilutions of produced water were used, which are representing dilutions found in situ. Samples of the fish were taken at different time periods until terminating the exposure at 44 weeks, making it possible to investigate changes over time.

1.3 Biomarkers

Exposure of xenobiotics and effects of this exposure can be measured in organisms by the use of biomarkers (Shugart et al., 1992). A biomarker is defined in various ways (Peakall, 1994; van Gestel et al., 1996). After Shugart et al. (1992) a biomarker is defined as a "biochemical or cellular response that can be measured and that indicates that the organism has been exposed to a chemical or anthropogenic stress". Biomarkers are used as sensitive indicators in environmental monitoring by measuring toxic effects at cellular levels in organisms sampled from contaminated areas. Cellular biomarkers are referred to as short-term indicators of long-term biological effects since they can predict changes within populations, communities or ecosystems (Cajaraville et al., 2000). These markers make it possible to control and manage pollution before severe environmental damage has taken place (McCarthy and Shugart, 1990; van der Oost et al., 2003).

Fish are often used as indicator species in marine pollution studies, and Atlantic cod have been used in several exposure studies where different biomarkers have been investigated (Goksøyr et al., 1994; Beyer et al., 1996; Aas et al., 2000, 2001; Sturve et al., 2006). Atlantic cod is one of the most important commercial fish species in Norway, and it is found along the Norwegian coastline and around the Barents Sea area. Most Atlantic cod populations will migrate for some period during their life span (living in one location and spawning in another) while others are more stationary (Moen et al., 2004). Cod is found from the littoral zone to 600 metres depths and is known as a predatory species with a diverse diet (Moen and Svensen, 2004). Biomarkers are often measured in cod liver. The liver is a suitable organ for measurements of lipophilic organic contaminants since it is large and lipid rich (Goksøyr et al., 1994), but other tissues including the bile, kidney, gonad, and muscle are also widely used (Beyer et al., 1996; Sturve et al., 2006; Meier et al., 2007). The fish kidney is involved in the regulation of osmotic homeostasis and is the site of excretion of xenobiotic metabolites (Au, 2004). The kidney of teleostean fish is divided into the head kidney and the

excretory kidney. The head kidney consists of haematopoietic and lymphoid tissue, whereas the excretory kidney is mostly composed of nephrons, with interstitial haematopoietic and lymphoid tissue as support (Takashima and Hibiya, 1995; Roberts, 2001). In this study, Atlantic cod head kidney was analysed by using two different biomarkers; lysosomal membrane stability and peroxisomal acyl-CoA oxidase (AOX) acitivity.

1.3.1 Lysosomal alterations

Lysosomes are cell organelles containing acid hydrolases, such as acid phosphatase, for degradation of materials brought to the organelle. This includes materials that are foreign to the cell, components that are damaged and molecules that are no longer needed (Becker et al., 1996). Many environmental contaminants have the ability to affect the cellular functions of marine organisms, and the lysosomal system has been found to be a specific target for toxic effects of xenobiotics. Pathological alterations have been discovered in lysosomes exposed to different kind of discharges, such as oil spill and industrial waste (Moore, 1990; Fernley et al., 2000; Galloway, et al., 2004; Moore et al, 2006). The lysosomal system of digestive gland cells of mussels and hepatocytes of fish have proven to be particularly susceptible to pollution (Moore, 1985; Köhler, 1991) as these are the central organs for xenobiotic accumulation, detoxification and biotransformation (Köhler, 1991; Köhler et al., 1992; Krishnakumar et al., 1994).

Changes in contents, fusion events and permeability of the lysosome membranes are common reactions of exposure to xenobiotics (Hawkins, 1980). Lysosomal membrane stability is frequently used as a biomarker of stress in marine organisms (Cajaraville et al., 2000; Köhler et al., 2002; Zorita et al., 2008) and has been used to detect effects in molluscs and fish exposed to organic components and metals (Moore et al., 1987; Regoli, 1992; Roméo et al., 2000; Viarengo et al., 2000; Cajaraville et al., 2006; Aarab et al., 2008; Hylland et al., 2008). Reduced LMS may also be provoked by stressors such as hypoxia, hyperthermia, reduced diet and osmotic shock (ICES, 2004). There may also be seasonal differences in LMS. Variations in LMS have been observed in liver of flounder sampled at different seasons, with reduced lysosomal membrane stability during winter (Köhler, 1991).

In this study Atlantic cod head kidney samples were analysed by using changes in lysosomal membrane stability as a biomarker. LMS is most commonly measured using *N*-acetyl-β-

hexosaminidase activity as a marker enzyme (Marigómez et al., 2005), while fewer studies using acid phosphatase activity have been made. In this study LMS was measured in the head kidney of Atlantic cod, using acid phosphatase, since it was successfully applied in an earlier study on Atlantic cod kidney (Marigómez et al., 2004).

1.3.2 Peroxisome proliferation

Peroxisomes are enzyme-containing organelles found in eukaryotic cells, and are especially prominent in liver cells. Several different functions have been discovered such as detoxification of hydrogen peroxide by catalase, detoxification of harmful components and oxidation of fatty acids (β-oxidation) (Becker et al., 1996). Peroxisomes can under certain circumstances undergo changes in size, shape and number, a phenomenon referred to as peroxisome proliferation. An induction of some of the peroxisomal enzymes (primarily β-oxidation enzymes) is also usually seen during proliferation (Reddy and Mannaerts, 1994). Peroxisome proliferation was first reported in liver cells of male rats exposed to a hypolipidemic drug (Hess et al., 1965). Since then, a number of environmental pollutants have shown proliferating properties in different organisms. The response of proliferation depends greatly on the species, gender and tissue. Rodents such as rats and mice are very sensitive to peroxisomal proliferators, while humans, monkeys and guinea pigs have demonstrated to be less responsive (Keller et al., 2000). Aquatic organisms are continuously exposed to environmental pollution and previous studies have revealed that contaminations such as bleached kraft mill effluents, PCBs, some pesticides, PAHs and alkylphenols can cause peroxisome proliferation in various species of molluscs and fish. Peroxisome proliferation as a biomarker is assessed either as increased volume density or increased peroxisomal acyl-CoA oxidase (Porte et al., 2001; Marigómez et al., 2006; Gorbi et al., 2008).

The β-oxidation pathway in peroxisomes consists of three different enzymes: acyl-CoA oxidase (AOX), peroxisomal hydratase-dehydrogenase-isomerase (multifunctional enzyme) and thiolase. All of these enzymes are usually induced during peroxisome proliferation. Peroxisomal acyl-CoA oxidase activity can be used as a biomarker of exposure to organic contaminants in the environment since the AOX activity is often induced in marine organisms when exposed to contaminants such as PAHs and APs (reviewed in Cancio and Cajaraville, 2000; Cajaraville et al., 2003; Cajaraville and Ortiz-Zarragoitia, 2006).

Induction of AOX is made by receptor-mediated mechanisms and involves activation of genes coding for the peroxisomal enzyme by peroxisome proliferator-activated receptors (PPARs) (Reddy and Mannaerts, 1994). These receptors play an important roll in cell proliferation, differentiation and apoptosis (programmed cell death) (Keller et al., 2000), and activation of PPARs by peroxisome proliferators may therefore cause severe effects in exposed organisms.

Peroxisome proliferation was used as a biomarker in this study by measuring the increased activity of peroxisomal acyl-CoA oxidase in Atlantic cod head kidney.

1.4 Aim of the study

The overall aim of the study was to investigate and quantify the effects of produced water (containing PAHs and alkylphenols) on Atlantic cod (*Gadus morhua* L.) in a long-term exposure. This study is part of a larger research project, and some results from other measurements have therefore been included wherever necessary.

The following questions were investigated:

- Does exposure to produced water components affect the stability of lysosomal membranes of the cod head kidney and are there differences between males and females?

- Does exposure to produced water components induce acyl-CoA oxidase activity in cod head kidney peroxisomes and are there differences between males and females?

- Are changes over time observed in the stability of lysosomal membranes and enzyme activity of peroxisomes during the exposure?

2. Materials and Methods

Chemicals and concentrations of the exposure are listed in table 2-1. LMS and AOX measurements and other data of the fish are listed in appendix A. Equipment used in the analysis is listed in appendix B and chemicals in appendix C.

2.1 Exposure

Atlantic cod (*Gadus morhua* L.) aged one year were transported by Marine Invest A/S (Norway) to Solbergstrand Marine Research Station (NIVA) and placed in aquaria connected to an exposure setup (figure 2-1). 75 fish were placed in each aquarium of which ten fish were used in another experiment but exposed to the same water. The weight and length of the fish were measured and blood samples were taken. Each fish was marked for identification by injecting a PIT-Tag (Biomark Inc.) in the abdominal cavity. The fish were held in the aquaria for two to four weeks due to acclimatisation before starting the exposure.

The exposure consisted of produced water components containing PAHs and alkylphenols (table 2-1) and was divided into three different treatments; “low” (2000 times diluted produced water), “high” (200 times diluted produced water) and “pulsed” (alternating “control” and “high” treatment at two weeks intervals). “Pulsed” treatment was set up to investigate effects in fish that move between areas with different exposure. In addition to the three treatments a fourth “control” treatment was set up which consisted of water and acetone. 13 aquaria were used; four for the “control” treatment and three for each of the “low”, “high” and “pulsed” treatment. The exposure setup consisted of a primary header tank, four secondary header tanks and four stock solutions connected to the aquaria (figure 2-1). The primary header tank received seawater from a depth of 60 meter and the temperature and salinity of the water was monitored continuously. The water was distributed from the primary header tank to four secondary header tanks. The stock solution of the chemicals used for the exposure was diluted with a 1:1 mixture containing distilled water and acetone in 30 liter glass tanks and pumped to the secondary header tanks. The secondary header tanks were connected to the aquaria and the fish were exposed to produced water for a total period of 44 weeks.

Table 2-1 Chemicals and concentrations of the exposure.

Chemical name	Conc. in PW (µg/l)	2000x dilution (µg/l)	200x dilution (µg/l)
Naphthalene	310.00	0.155	1.55
Acenaphthylene	0.0050	2.50E-06	2.50E-05
Acenaphtene	1.70	8.50E-04	8.50E-03
Fluorene	12.37	6.19E-03	6.19E-02
Anthracene	0.7239	3.62E-04	3.62E-03
Phenanthrene	16.00	8.00E-03	8.00E-02
Pyrene	1.2740	6.37E-04	6.37E-03
Dibenzothiophene	3.80	1.90E-03	1.90E-02
2-methylnaphthalene	340.00	0.17	1.70
2,6-dimethylnaphthalene	189.00	9.45E-02	0.945
2-ethyl-6-methylnaphtalene	113.00	5.65E-02	0.565
2-methylanthracene	20.00	1.00E-02	0.1
9,10-dimethylanthracene	20.00	1.00E-02	0.1
4-methyldibenzothiophene	4.60	2.30E-03	2.30E-02
4-ethyldibenzothiophene	6.60	3.30E-03	3.30E-02
9-methylphenanthrene	21.61	1.08E-02	0.108
9-ethylphenantrene	23.70	1.19E-02	0.119
9-n-propylphenanthrene	3.51	1.76E-03	1.76E-02
Phenol	1400	0.70	7.00
p-cresol	1500	0.75	7.50
4-ethylphenol	630	0.315	3.15
4-n-propylphenol	123	6.15E-02	0.615
4-tert-butylphenol	35	1.75E-02	0.175

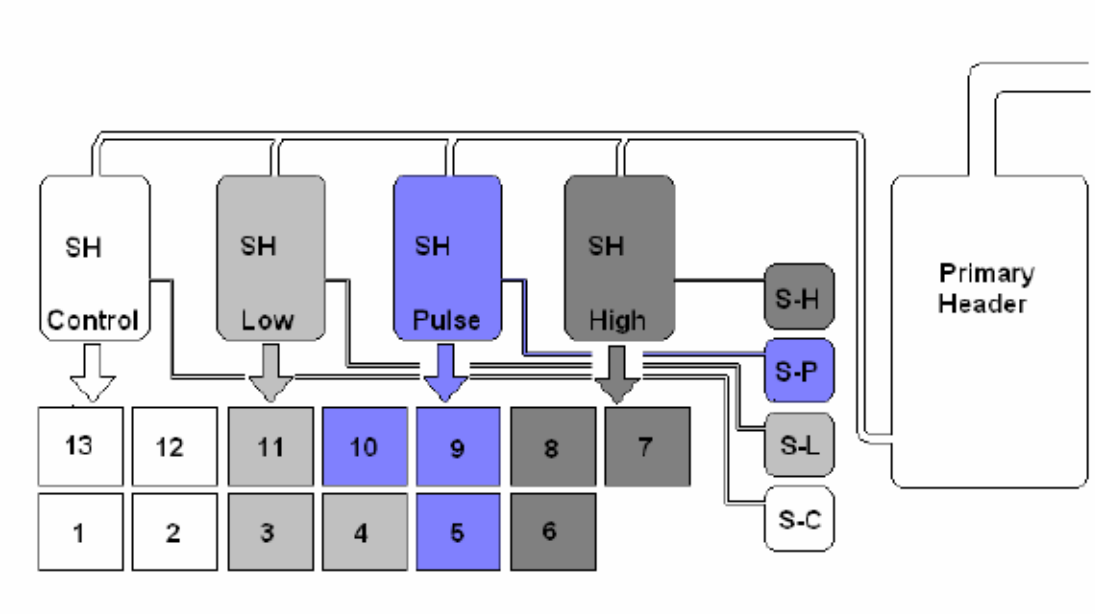

Figure 2-1 The aquarium setup. The setup included four aquaria for "control" treatment and three aquaria for each of "low", "pulse" and "high" treatments. Each of the secondary header tanks (SH) connected to the aquaria received water from the primary header tank. Chemicals used in the exposure were transported to the secondary header tanks from the four stock solutions; S-H, S-P, S-L and S-C.

The fish were fed two times a week. The food consisted of frozen fish during the first 18 weeks and then changed to specially produced pellets for gonad maturation.

Water samples were taken both from the aquaria and the primary header tank to check the exposure, and the oxygen concentration was regularly measured for each aquarium. Sunlight was used as a light source in this experiment, keeping the day rhythm of the fish as natural as possible.

2.2 Sampling

The experiment was terminated after 44 weeks of exposure. During this time period samples were taken after 2, 4, 8, 16, 24, 33 and 44 weeks. Samples of the cod had also been taken before starting the exposure. For each sampling the fish were taken out of the aquarium with a net and identified by their PIT-Tag using a FS2001F-ISO Reader (Biomark Inc.). The fish were terminated and the length and weight were measured. Samples were taken from the head kidney, liver, bile and blood, placed in tubes and frozen in liquid nitrogen and stored at -80°C for later processing.

Samples containing the head kidney were placed in liquid nitrogen and transported to and analysed at the Laboratory of Cell Biology and Histology at the University of the Basque Country in Bilbao, Spain.

2.3 Biomarker assays

2.3.1 Samples

Samples of the head kidney taken before the exposure, and after 2, 16 and 33 weeks of exposure were used when analysing lysosomal membrane stability (LMS) and peroxisomal acyl-CoA oxidase (AOX) activity.

A total of 155 individuals were analysed for LMS including six individuals of each sex from each treatment in the selected sampling periods (only five females from “low” treatment were analysed from samples taken at 16 weeks). For AOX activity, the same samples were analysed as for LMS.

Table 2-2 The number of fish used in the study, and weight, liver-somatic index (LSI), condition factor (CF) and PAH-metabolites (1-OH-pyrene) measured for each treatment and sampling period, expressed by the median and max/min values.

Weeks of exp.	Treatment	No.	Weight (g)	LSI	CF	1-OH-pyrene (ng/g bile)[2]
0	Control	♂ 6	600 (528-732)	6.39 (4.73-10.44)	1.23 (1.13-1.49)	109.5 (70-145)
		♀ 6	503 (344-662)	9.36 (6.89-11.21)	1.17 (0.96-1.31)	117 (1-174)
2	Control	♂ 6	423 (268-450)	6.99 (3.75-10.27)	0.84 (0.60-0.93)	84 (50-126)
		♀ 6	437 (308-718)	9.29 (6.46-10.14)	0.95 (0.77-1.03)	70 (26-141)
2	Low	♂ 6	321 (294-442)	7.19 (5.44-8.03)	0.81 (0.75-0.88)	345
		♀ 6	507 (364-646)	8.54 (6.94-41.63)	0.97 (0.81-1.29)	102 (77-248)
2	Pulse	♂ 6	409 (310-520)	8.32 (7.65-11.77)	0.89 (0.85-1.07)	1134.5 (747-2258)
		♀ 6	449 (312-576)	8.61 (0.75-9.56)	0.84 (0.79-0.96)	1701 (891-3151)
2	High	♂ 6	463 (346-626)	7.84 (7.11-8.87)	0.90 (0.78-2.67)	519 (420-986)
		♀ 6	414 (276-504)	7.87 (5.50-11.18)	0.92 (0.73-1.23)	1466 (407-1524)
16	Control	♂ 6	468 (236-624)	6.38 (3.31-7.70)	0.91 (0.76-1.09)	29 (19-51)
		♀ 6	578 (422-662)	5.29 (4.55-6.53)	0.88 (0.80-1.00)	49 (16-123)
16	Low	♂ 6	430 (324-568)	5.63 (4.28-7.81)	0.91 (0.70-0.96)	108 (32-193)
		♀ 5	438 (312-724)	5.07 (1.06-9.23)	0.90 (0.70-0.98)	115.5 (29-350)
16	Pulse	♂ 6	481 (408-764)	6.68 (2.38-8.26)	0.87 (0.77-1.07)	305.5 (141-1069)
		♀ 6	541 (406-820)	7.17 (4.06-9.50)	0.98 (0.90-1.11)	530 (483-767)
16	High	♂ 6	615 (412-762)	7.14 (5.15-9.86)	0.94 (0.79-1.03)	466 (224-1255)
		♀ 6	580 (262-940)	6.47 (4.24-7.45)	0.93 (0.76-1.00)	415.5 (191-9177)
33	Control	♂ 6	721 (560-1086)	7.25 (4.16-8.31)	0.93 (0.90-1.05)	36 (16-47)
		♀ 6	720 (544-820)	5.62 (3.18-8.98)	0.96 (0.85-1.08)	37.5 (11-70)

[2] Analysed by Merete Grung (NIVA).

33	Low	♂ 6	572 (450-684)	5.81 (1.84-8.54)	0.90 (0.82-1.95)	222.5 (157-436)
		♀ 6	644 (410-742)	4.66 (3.09-5.97)	0.85 (0.79-0.95)	165 (54-267)
33	Pulse	♂ 6	794 (480-1042)	6.75 (3.21-10.07)	0.95 (0.79-1.18)	761.5 (374-1392)
		♀ 6	678 (348-1020)	4.20 (2.96-6.80)	0.83 (0.69-0.94)	856 (125-1207)
33	High	♂ 6	717 (516-1208)	7.57 (2.94-10.52)	0.96 (0.87-1.04)	1162.5 (396-1831)
		♀ 6	823 (418-1346)	7.35 (1.41-10.30)	0.98 (0.73-1.09)	1182 (145-2195)

2.3.2 Lysosomal membrane stability (LMS)

The procedure for lysosomal membrane stability was performed according to Broeg et al. (1999) and the stability was determined as described in Moore (1988).

Cryostat sections

Pieces of frozen cod head kidney from six different individuals of the same sex, treatment and time period were cut in a Leica CM3000 cryostat at a cabinet temperature below -25°C using a 15° knife angle. Each sample was glued to a cryostat chuck using a tissue freezing medium. Tissue sections of 10 μm thickness were cut and transferred to warm slides (at room temperature) for flash-drying. For each group of the six individuals ten slides were made. The slides were stored in the cryostat during the cutting process, transferred to a freezer and stored at -40°C until staining. Only half part of the sample volume was used for preparing cryostat sections, leaving the rest of the tissue for measuring peroxisomal acyl-CoA oxidase activity.

Buffers, solutions and media

To carry out the analysis different buffers, solutions and media were prepared. To make 0.1 M citrate buffer, 2.94 g Na-citrate was mixed with 2.5 g NaCl in 100 mL dH_2O and the pH fixed at 4.5. In making 0.1 M phosphate buffer, 2.892 g $Na_2HPO_4*12H_2O$ and 0.265 g $NaH_2PO_4*H_2O$ were dissolved in 100 mL dH_2O. 2.5 g NaCl was added to the mixture and the pH was fixed at 7.4. 3% NaCl was made by dissolving 3 g NaCl in 100 mL dH_2O. To make the fixative solution (Baker's formula), 1 g $CaCl_2$ was mixed with 90 mL dH_2O, 10 mL 40 % neutralised formaldehyde solution and 2.5 g NaCl. The solutions were then kept at 4 °C until used.

Incubation medium was made by dissolving 3.5 g POLYPEP (a low viscosity polypeptide that acts as a section stabiliser) in 50 mL 0.1 M citrate buffer (pH 4.5) on a heating plate and 10 mg naphthol AS-BI phosphate was dissolved in 1 mL DMSO. The two solutions were then mixed together. The incubation medium was prepared just 5 minutes before use. To make staining medium, 50 mg Fast Violet B salt and 50 mL 0.1 M phosphate buffer (pH 7.4) were mixed and dissolved for 30 minutes in the dark using aluminium foil.

Staining

The method was carried out as described in Broeg et al. (1999). Every step of the following staining procedure was performed in Hellendahl jars. The cryostat slides prepared earlier for staining were taken out of the -40°C freezer, air-dried in the refrigerator and defrosted at room temperature five minutes before starting the staining. The tissues of the slides were destabilized in 0.1 M citrate buffer at 37°C in a shaking water-bath for different time periods (0, 3, 5, 10, 15, 20, 30, 40, 50 and 60 minutes). After destabilization, the slides were transferred to the incubation media, and incubated for 15 minutes at 37°C in the shaking water-bath. The slides were washed in 3 % NaCl at 37°C in the shaking water-bath for five minutes and stained in staining media at room temperature for nine minutes. During the staining period, the slides were protected from light using aluminium foil. The slides were gently washed in running tap water at room temperature for ten minutes and fixed in fixative solution at 4°C for 15 minutes. They were rinsed in distilled water, mounted in Kaiser's

glycerol-gelatine and cover slips were placed on the slides. After the slides were dry, the staining reaction was visualized under a light microscope.

The slides were treated for different time periods in order to determine the lysosomal membrane destabilization time, which is the period of acid labilization needed to destabilize the membrane (Köhler, 1991). When the lysosomal membrane is destabilized in citrate buffer, the substrate (naphthol) in the incubation medium penetrates the destabilized membrane and reacts with the hydrolases (acid phosphatase) inside the lysosomes. Acid phosphatase catalyzes a hydrolytic release of the naphthol AS-BI phosphate group, and a reaction with the dye Fast Violet B in the staining medium follows, producing a bright-violet product (Sturve et al., 2005). When the lysosomal membrane was totally destabilized a maximum staining intensity was observed. The destabilization time was measured manually using a light microscope (20 x objective).

Measuring lysosomal membrane stability

The LMS measurements were made in the interstitial tissue of the kidney. When starting to measure the destabilization time, each slice of tissue on the cryostat slides was divided into four areas to compare the staining intensity. The staining intensity was compared both within the same slice and between the ten slides containing the same sample. The measurements were performed by starting with the slide that had the shortest treatment time (3 minutes) and within the four areas on the first sample estimate if there was a visible decrease in the staining intensity compared with the next slide that had been treated for a longer time period (5 minutes). The same procedure was done for all of the slides until an increase in staining intensity could be observed and a peak was reached, followed by a decrease in the staining intensity. The peak indicated the destabilization time. A value was derived from each of the four areas and a mean value for the whole sample was calculated as described by Moore (1988). All the measurements were done without knowledge of the origin by covering the sample number with a label and all samples were measured by the same person.

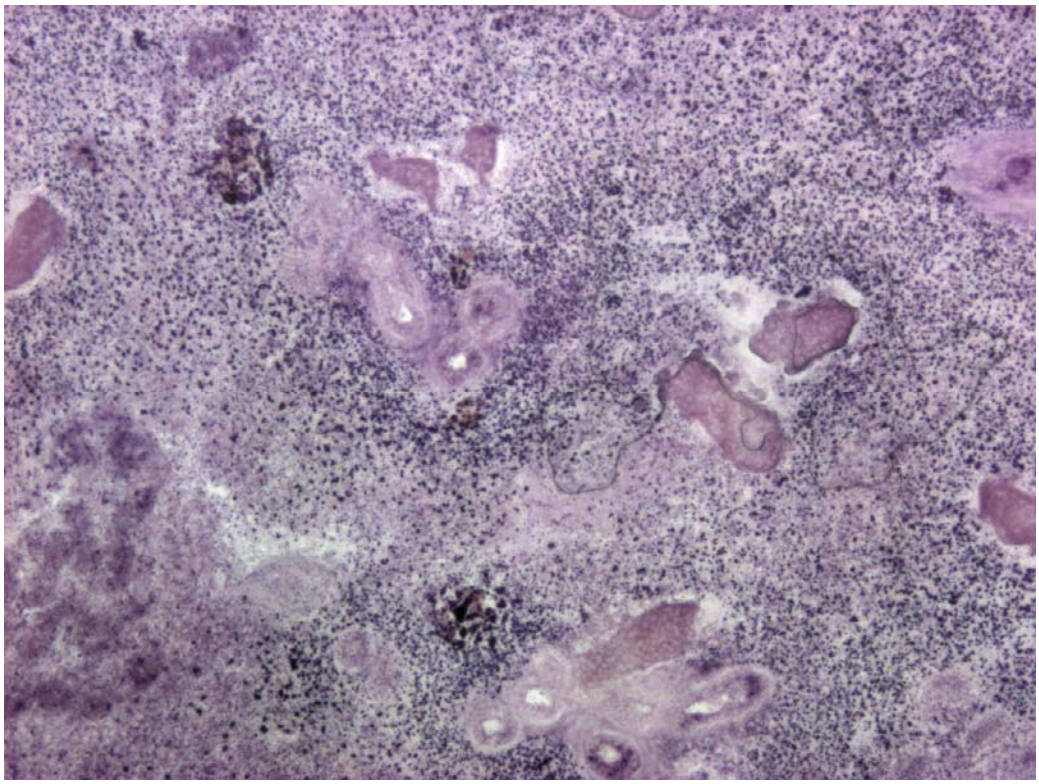

Figure 2-2 Cryostat section (10 µm) of Atlantic cod head kidney exposed for "high" treatment viewed using a light microscope (10 x objective). The section shows lysosomes in haematopoietic tissue after staining.

2.3.3 Peroxisomal acyl-CoA oxidase (AOX) activity

The same head kidney samples of Atlantic cod used for LMS were also used for measuring peroxisomal acyl-CoA oxidase (AOX) activity and a total of 152 individuals were analysed (five males from "control" treatment and five females from "pulsed" treatment at 16 weeks, and five males from "pulsed" treatment at 33 weeks were measured instead of six).

The analysis was performed by using a spectrophotometric assay described by Small et al. (1985). AOX activity was measured by determining the amount of peroxisomal H_2O_2 that is produced during oxidation of the substrate palmitoyl-CoA, when using an exogenous horseradish peroxidase (figure 2-3) (Small et al., 1985; Zorita et al., 2008). For all samples, the total protein concentration was determined according to the Lowry method (Lowry et al., 1951) using the DC protein assay and bovine γ-globulin as standard. AOX activity is given as mUnits AOX/mg protein equivalent to nmol H_2O_2/min/mg protein.

$$R-CH_2-CH_2-CO-SCoA+O_2 \xrightarrow{AOX} R-CH-CH-CO-SCoA+H_2O_2$$

$$Leuco-DCF+H_2O_2 \xrightarrow{HRP} DCF+2H_2O$$

Figure 2-3 Reaction principle of the spectrophotometric assay of peroxisomal acyl-CoA oxidase activity.

Buffer, medium and solutions

For analysis of peroxisomal acyl-CoA oxidase activity in the head kidney, the standard operating procedure (SOP) developed by Orbea and Cajaraville within the framework of the BEEP project was applied. Before starting the measurements all samples were weighed and the buffer, medium and solutions were prepared. Homogenization buffer (TVBE buffer) was made by mixing 84 mg $NaHCO_3$ with 10 mL 0.1 M EDTA, 1 mL ethanol and 1 mL triton X-100 (10 %). Distilled water was added up to 1000 mL and pH was fixed at 7.6. To make 0.5 M potassium phosphate buffer, 680 mg KH_2PO_4 was dissolved in 10 mL dH_2O and 1140 mg

$K_2HPO_4*3H_2O$ was dissolved in 10 mL dH_2O. The first solution was then used to fix the pH in the second solution to 7.4. All solutions were kept at 4°C until used.

Leuco-DCF solution was prepared by dissolving 3.25 mg Leuco-DCF in 250 µL DFA and 2.25 mL 0.01 M NaOH.

The reaction medium was prepared a day prior to measurements of AOX activity. Reaction medium was made by mixing 2 mL 0.5 M potassium phosphate buffer (pH 7.4), 2 mL Leuco-DCF solution, 1 mL Horseradish peroxidase (1200 U/mL), 1 mL 4 M sodium azide and 200 µL triton X-100 (10 %). Distilled water was added up to 100 mL and the solution was mixed well. During the preparation it was important that the reaction medium was protected against light using aluminium foil. 1.9 mL of reaction medium was transferred to 2 mL aliquots and stored in the dark at -20°C until use, but not longer than one week.

Preparing the samples

Samples were taken out of the -80°C freezer and weighed. Each sample was homogenized in TVBE buffer kept on ice and 4 mL buffer per gram tissue was added. Ethanol in the TVBE buffer functions as a substrate for catalase and preserves its activity, making TVBE a good buffer to measure catalase activity and activities of peroxisomal oxidases (Cancio et al., 1998). A glass-Teflon homogeniser held in an ice-water cooled bath was used. The samples were transferred to 2 mL aliquots and centrifuged at 500 g for 15 minutes at 4°C using an Allegra 25R centrifuge. The supernatants were removed from the centrifuged tubes and transferred to new tubes and then kept on ice for dilution.

1:10 dilutions were made by mixing 450 µL TVBE buffer (pH 7.6) with 50 µL of the sample in eppendorf tubes and then kept on ice during the whole measurement. In the same time, tubes were prepared for protein measurements by transferring 50 µL of the sample to 200 µL eppendorf tubes and stored at -80°C until protein measurement.

Measuring AOX activity

10 minutes before starting measuring the AOX activity aliquots containing the reaction medium were taken out of the -20°C freezer and put in a water bath at 25°C, covered against light. The reaction medium was not allowed to defrost for longer than 30 minutes. 100 µL of diluted sample (1:10) was transferred to an aliquot containing 1.9 mL of reaction medium, mixed well and incubated in the water bath at 25°C for 5 minutes. After incubation, 1 mL of the reaction medium containing the sample was placed in a spectrophotometer cuvette and 10 µL of 3 mM palmitoyl-CoA were added. The rest of the reaction medium left in the aliquot were placed in another cuvette and used as a blank. Both cuvettes were placed in a recording Shimadzu UV-1603 spectrophotometer and the absorbance of the sample was read against the blank at 502 nm during 4 minutes. When measuring the absorbance, the increase had to be linear. If the measurement was not linear, a second measurement had to be performed. Each sample was measured twice and if the difference between the measurements was more than 10 %, a third measurement was done. When not measured, the rest of the samples were kept on ice.

The protein concentration was measured in 96 well microplates using a Bio-Rad DC protein assay based on the method described by Lowry et al. (1951). A standard solution was prepared by mixing 1.5 mg/mL bovine γ-globulin with TVBE buffer (pH 7.6). The standard solutions used were 0.00, 0.15, 0.30, 0.60 and 1.00 mg/mL. All preparations were made on ice.

1:25 dilutions were made by adding 240 µL TVBE buffer to 10 µL sample (prepared for protein measurements earlier) in eppendorf tubes and mixed well. 5 µL of each sample mixture was transferred to the microplate starting with the standard. 25 µL of Reagent A and 200 µL of Reagent B from the Bio-Rad DC protein assay kid were added to each well. The protein concentrations were measured at 750 nm in a THERMO LABSYSTEM Multiskan Spectrum spectrophotometer. The measurement was done twice for each microplate, directly and after 15 minutes.

Calculating AOX activity

The absorbance and protein concentration (mg protein/mL) of the samples were used to calculate the activity in mUnits AOX/mg protein by following equations:

$$\frac{\text{mUnits}}{\text{ml}} = \frac{\Delta\text{OD}}{\text{min}} \times \frac{\text{Reaction volume}\,(1000\,\text{mL})}{\text{Sample volume}\,(50\,\text{mL})} \times \frac{\text{Dilution}\,(10)}{0.091}$$

$$\frac{\text{mUnits}}{\text{mg prot}} = \frac{\text{mUnits}}{\text{ml}} \div \frac{\text{mg prot}}{\text{ml}}$$

2.4 Statistical analysis

The statistical analysis was done using the data analysis software system Statistica version 7.0 (Statsoft). The data obtained from lysosomal membrane stability and peroxisomal acyl-CoA oxidase activity measurements were analysed by a general linear model using a nested ANOVA (Zar, 1999). By having sex, time and treatment as fixed factors, and aquarium nested in treatment as random factor, the data were tested for homogeneity of variance by Levene's test (Levene, 1960). If Levene's test showed significant differences in the data, a log10-transformation was made and the data were tested for homogeneity of variance again. If Levene's test did not show statistically significance, the hypothesis of homogenous variances was accepted and Dunnett's test (Dunnett, 1955), a post-hoc multiple comparison test, was performed (based on the statistically significant outcome of the ANOVA). Nonparametric Spearman rank R was carried out to investigate correlations between variables (Zar, 1999). The level of significance was set to $p<0.05$ in all tests used.

Graphs showing box plots were used to present the data. The box plots consist of a median (point/line), 25-75 % percentiles and 10/90 percentiles (whiskers). Nested ANOVA was presented as table when significant values were present.

3. Results

3.1 Lysosomal membrane stability (LMS)

Cryostat sections of cod head kidney displayed large abundant lysosomes filling all the tissue after staining for acid phosphatase. Both sections with and without tubules containing small lysosomes were observed when investigated under the microscope.

A downward trend in the membrane stability of cod head kidney lysosomes was observed for all treatments (low, pulsed and high) during the exposure period, when compared to cod exposed to "control" treatment. No differences in LMS between sampling times along the exposure period were observed in the cod (figure 3-1).

Significant differences in the membrane stability of cod head kidney lysosomes were found between treatment groups in a nested ANOVA with treatment, time and sex as fixed factors and aquarium nested in treatment as random factor ($p=0.005$; table 3-1). When further tested significant differences in head kidney LMS were found between "control" and "pulsed" treatment (Dunnett's test, $p=0.0005$) as well as between "control" and "high" treatment (Dunnett's test, $p=0.000007$). There were no significant differences in LMS between males and females (nested ANOVA, $p=0.11$) and no significant differences were found in LMS for weeks of exposure (nested ANOVA, $p=0.10$). No significant differences were found between aquaria within the same treatment with regard to lysosomal membrane stability in the head kidney of cod (nested ANOVA, $p=0.09$).

Table 3-1 Analysis of variance (ANOVA) of lysosomal membrane stability measured in Atlantic cod head kidney with treatment as factor; aquaria were nested in treatments.

	Df	F	P
Sex	1	2.57	0.11
Weeks of exposure	3	2.11	0.10
Treatment	3	7.98	0.005
Aquarium(Treatment)	9	1.72	0.09
Error	138		

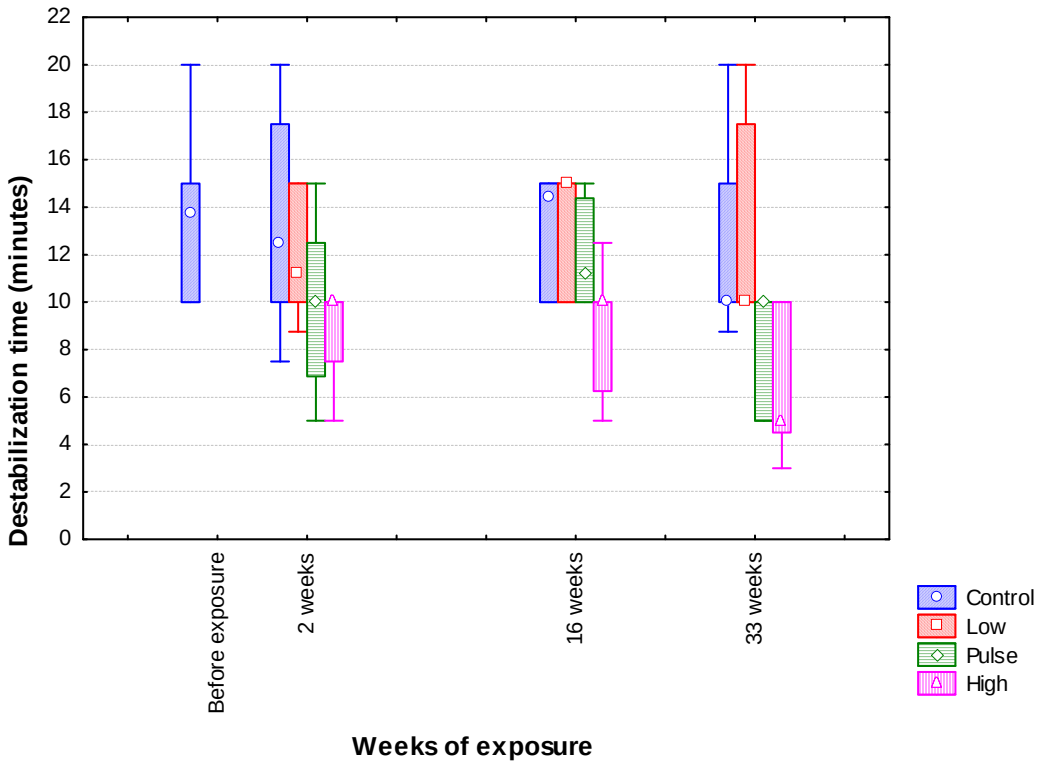

Figure 3-1 Lysosomal membrane stability in cod head kidney samples exposed to three different treatments and "control" treatment for 2, 16 and 33 weeks; median (point), 25/75 percentiles and 10/90 percentiles (whiskers).

3.2 Peroxisomal acyl-CoA oxidase (AOX) activity

Peroxisomal AOX activity was low and variable in cod head kidney. Male cod in "control" treatments had reduced peroxisomal kidney AOX activity after 2, 16 and 33 weeks of exposure compared to before exposure. An increase in AOX activity within treatment groups (low, pulsed and high) was seen at 2 weeks. Male cod held in aquaria with "low" and "pulsed" treatment had increased AOX activity at 16 weeks compared to 2 weeks, followed by a decrease in the activity at 33 weeks. A downward trend in AOX activity was observed over time in male cod treated with "high" exposure (figure 3-3).

Female cod had lower peroxisomal head kidney AOX activity than male cod. The AOX activity in female cod held in aquaria with "control" treatment was at same level during the whole exposure period. An increase in AOX activity within treatment groups (low, pulsed and high) was seen in females at 2 and 16 weeks, though the activity was decreased at 33 weeks. AOX activity in female cod exposed to "high" treatment at 2 weeks was lower than the activity found in females held in aquaria with "control" treatment at the same time period. Head kidney of female cod exposed to "high" treatment had an increased AOX activity after 16 weeks of exposure, while a decrease in the activity was seen at 33 weeks (figure 3-4).

In a nested ANOVA with treatment, time and sex as fixed factors and aquarium nested in treatment as random factor, significant differences in peroxisomal AOX activities were found between male and female cod ($p<0.000001$; table 3-2) and between weeks of exposure ($p<0.000001$; table 3-2). Males were significantly different from females (Dunnett's test, $p=0.000007$). For weeks of exposure significant differences in AOX activity were found between cod sampled at 33 weeks of exposure compared to samples taken before the start of the exposure (Dunnett's test, $p=0.0006$). There were no significant differences in cod head kidney AOX activity with regard to treatment groups (nested ANOVA, $p=0.97$; table 3-2). No significant differences in AOX activity were found in cod held in aquaria with the same treatment (nested ANOVA, $p=0.77$; table 3-2). All values for ANOVA and Dunnett's test were log10 transformed.

Table 3-2 Analysis of variance (ANOVA) of peroxisomal AOX activity measured in Atlantic cod head kidney with treatment as factor; aquaria were nested in treatments.

	Df	F	P
Sex	1	32.06	<0.000001
Weeks of exposure	3	13.46	<0.000001
Treatment	3	0.08	0.97
Aquarium(Treatment)	9	0.63	0.77
Error	135		

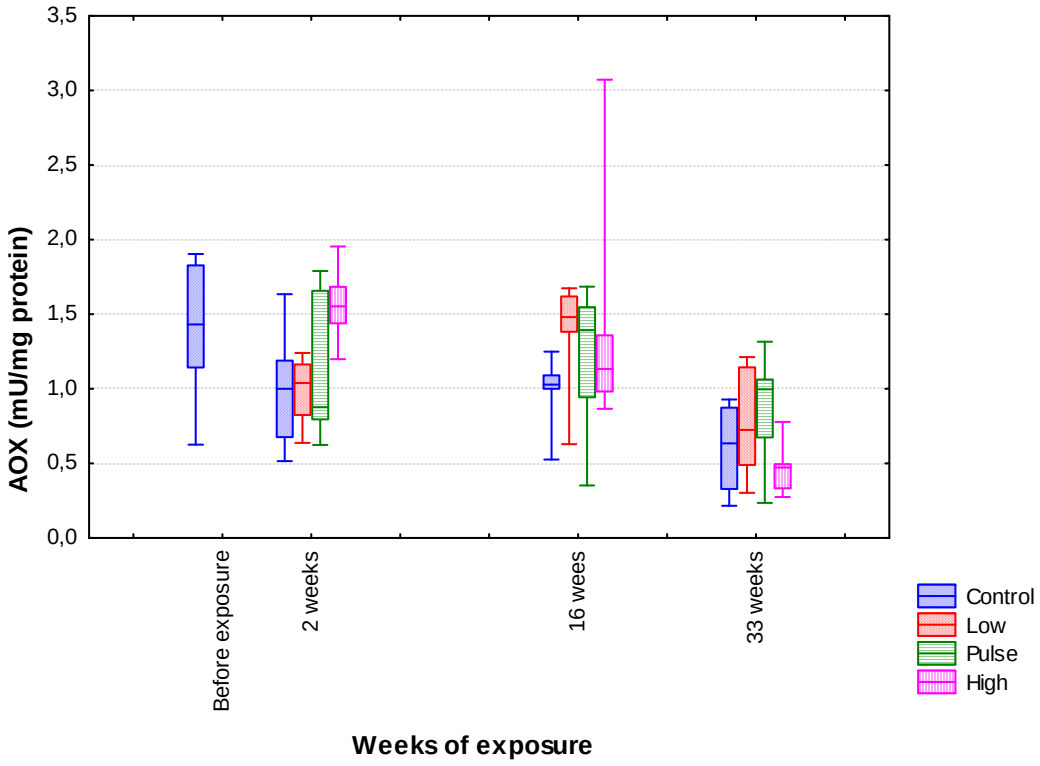

Figure 3-3 Peroxisomal acyl-CoA oxidase (AOX) activity in male Atlantic cod head kidney exposed to three different treatments and "control" treatment for 2, 16 and 33 weeks; median (line), 25/75 percentiles and 10/90 percentiles (whiskers).

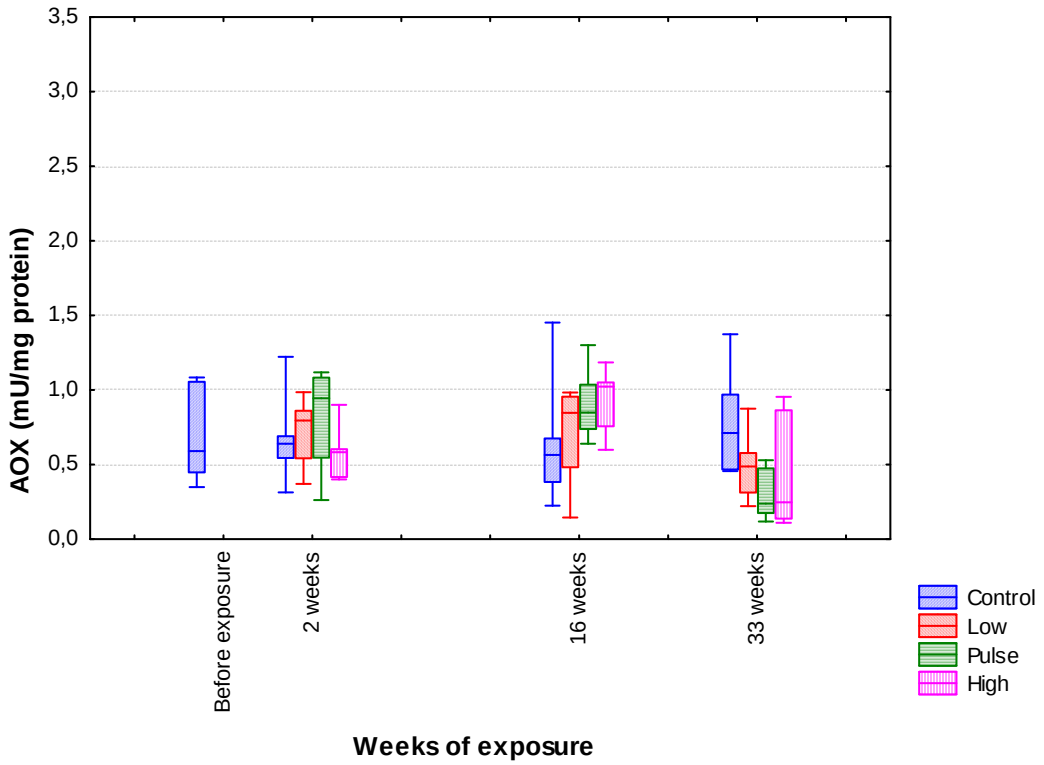

Figure 3-4 Peroxisomal acyl-CoA oxidase (AOX) activity in female Atlantic cod head kidney exposed to three different treatments and "control" treatment for 2, 16 and 33 weeks; median (line), 25/75 percentiles and 10/90 percentiles (whiskers).

3.3 Correlations

A correlation was found between levels of 1-OH-pyrene in bile and lysosomal membrane stability in head kidney of the cod (Spearman, r=-0.399; p=0.000002; figure 3-5). No correlation was found between levels of 1-OH-pyrene in bile and activity of AOX in head kidney peroxisomes of the cod (Spearman, r=-0.016; p=0.854; figure 3-6). No correlation was found between condition factor and lysosomal membrane stability (Spearman, r=0.084; p=0.302; figure 3-7) or between condition factor and AOX activity (Spearman, r=0.057, p=0.484; figure 3-8).

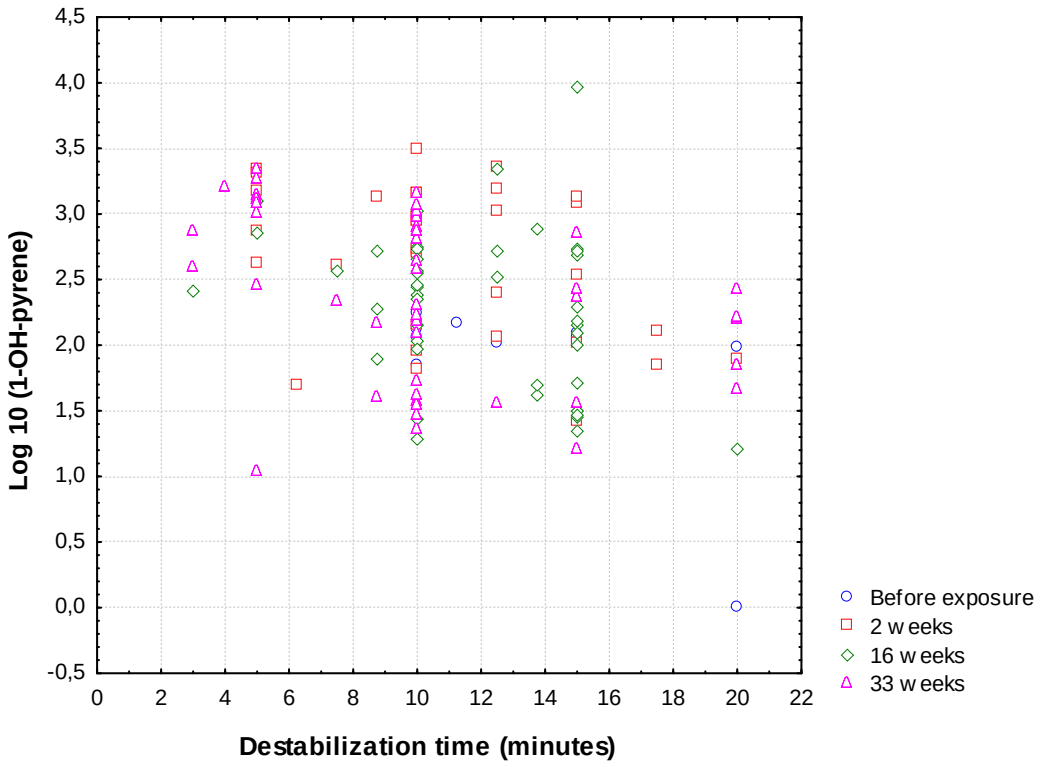

Figure 3-5 Relationship between levels of log10-transformed 1-OH-pyrene (ng/g bile) and lysosomal membrane stability in cod exposed to "control", "low", "pulsed" and "high" treatment for 2, 16 and 33 weeks.

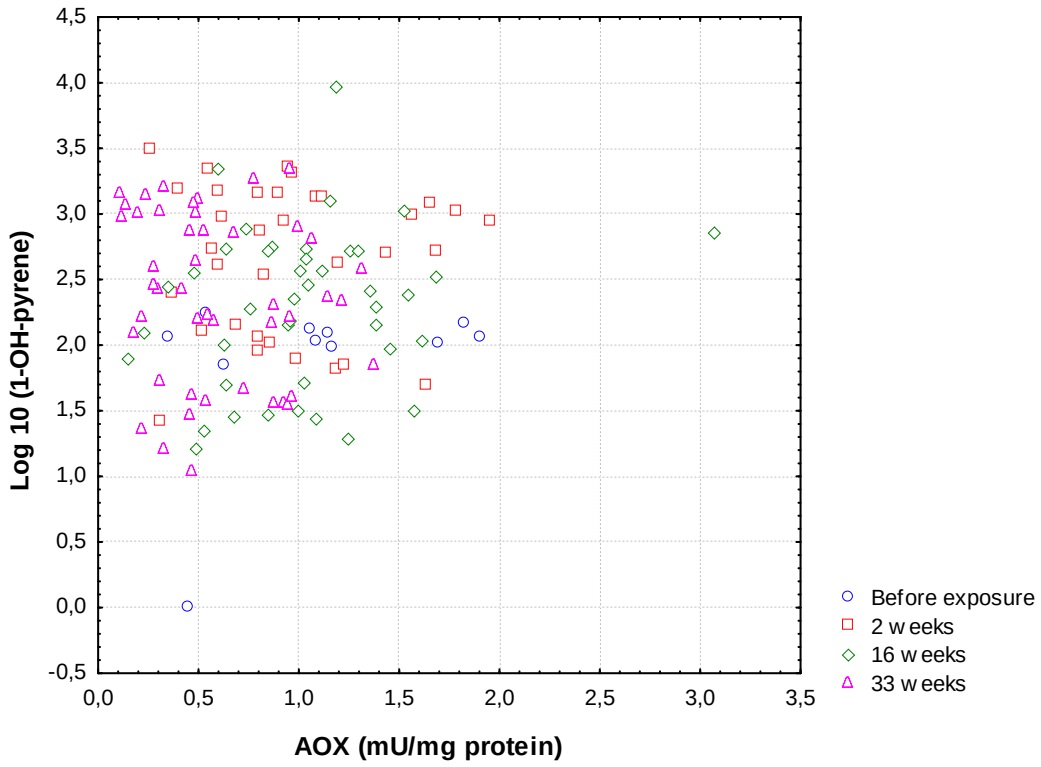

Figure 3-6 Relationship between levels of log10-transformed 1-OH-pyrene (ng/g bile) and peroxisomal acyl-CoA oxidase (AOX) activity in cod exposed to "control", "low", "pulsed" and "high" treatment for 2, 16 and 33 weeks.

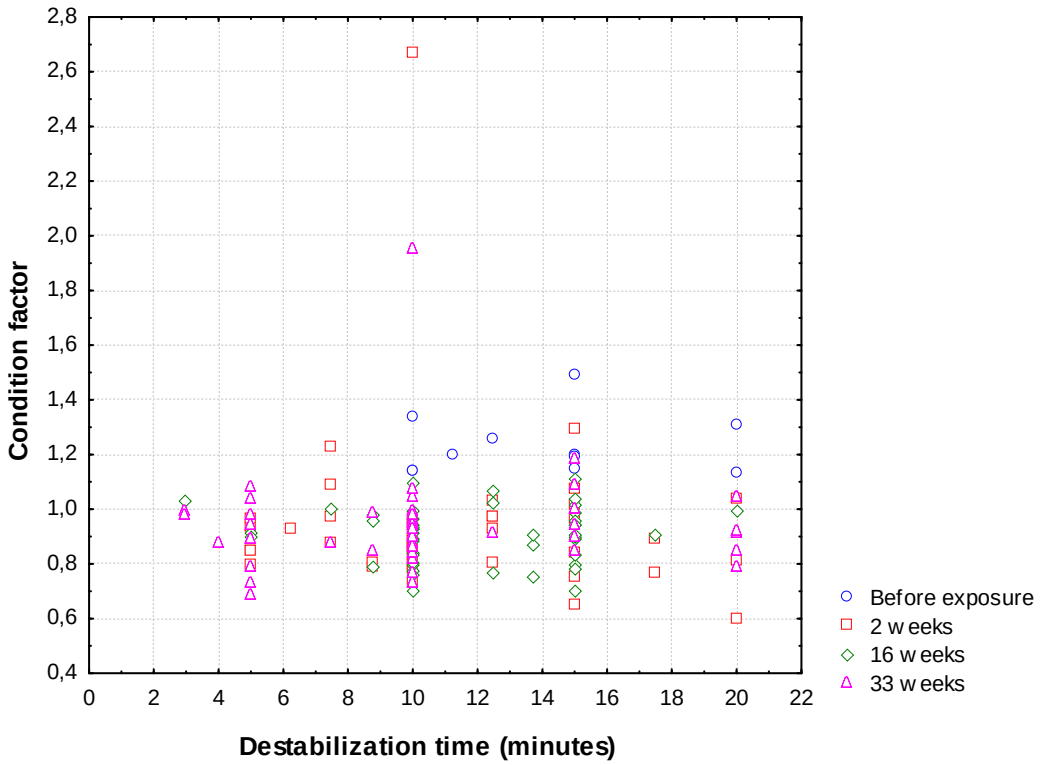

Figure 3-7 Relationship between condition factor and lysosomal membrane stability in cod exposed to "control", "low", "pulse" and "high" treatment for 2, 16 and 33 weeks.

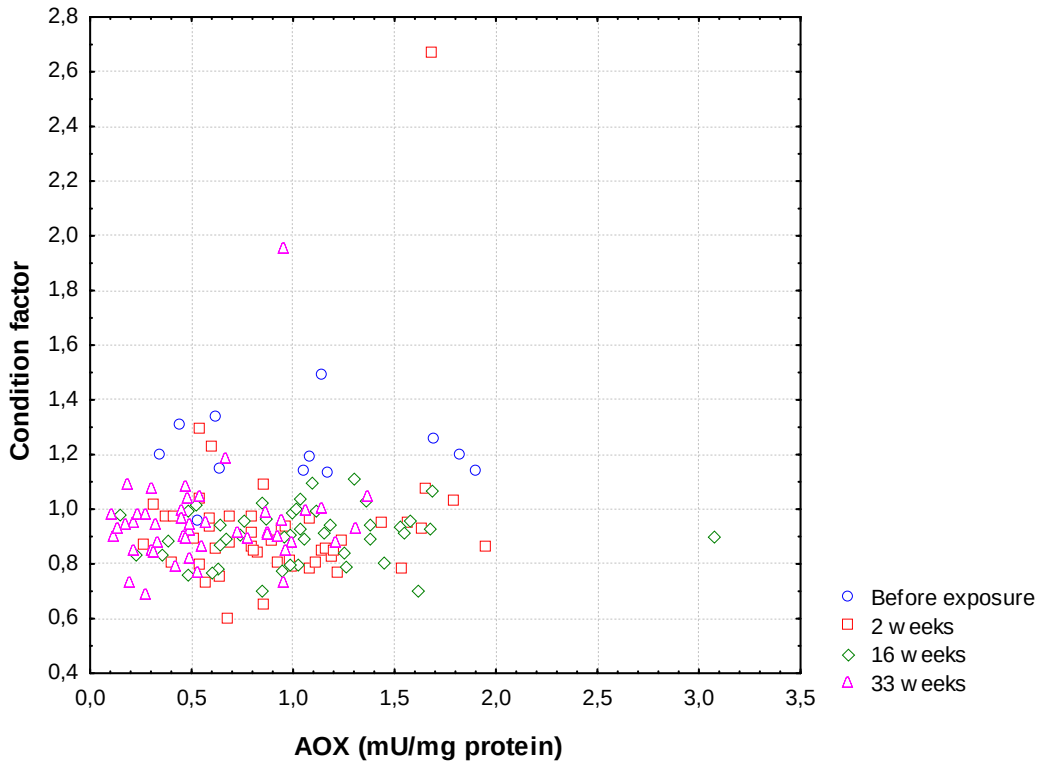

Figure 3-8 Relationship between condition factor and peroxisomal acyl-CoA oxidase (AOX) activity in cod exposed to "control", "low", "pulse" and "high" treatment for 2, 16 and 33 weeks.

4 Discussion

4.1 Lysosomal membrane stability (LMS)

The results from the present study clearly indicate that lysosomal membrane stability may be affected by produced water. Cod that had been exposed to "high" and "pulsed" treatment had significantly lower lysosomal head kidney membrane stability than cod held in aquaria with "control" treatment. This is indicating a higher degree of stress in cod treated with 200 times dilution of produced water found near oil platforms. No significant differences in LMS values of head kidney were found between male and female cod. The effect on lysosomal membrane stability was evident already after two weeks, but then remained stable for 33 weeks. There was no sign of increasing response over time or adaption to the treatment.

Lysosomal membrane stability is often measured in the digestive glands of molluscs and liver of fish (Moore, 1976; Regoli, 1992; Broeg et al., 1999; Koukouzika and Dimitriadis, 2005; Sturve et al., 2005; Baršienė et al., 2006; Zorita et al., 2008), and not many studies have been made on fish kidney. Lysosomal membrane stability has been studied in the head kidney of sea bass (*Dicentrarchus labrax* L.) by Roméo et al. (2000) and in the kidney of Atlantic cod by Marigómez et al. (2004). This is most likely the first study where lysosomal membrane stability has been measured in Atlantic cod head kidney.

In this study, the lysosomal membrane stability of cod ranged from 3 to 20 minutes, with a median LMS value of 10 minutes in "high" and "pulsed" treatment and 12-14 minutes for "control" and "low" treatment. In mussels, it have been found that LMS levels between 20 and 40 minutes indicates good general health and levels below ten minutes indicates severe stress (Viarengo et al., 2000). The low LMS value in higher treatments in the present study indicates a higher degree of stress, presumably due to exposure of PAHs and APs in the treatment water. Reduced lysosomal membrane stability is used as an indication of general stress to environmental pollution, but LMS has also been recommended as a biomarker for induced liver injury and carcinogenesis in fish due to xenobiotic exposure (Köhler et al., 2002). When the lysosomes are damaged, accumulated chemicals and acid hydrolases are released causing irreversible pathological alterations in the cell such as cell death (Broeg et

al., 2005). Destabilization of the lysosomal membrane may cause an increase in lysosomal fusion. A result of this could be increased autolytic activity followed by cell degeneration and dysfunction in the target tissue (Moore, 1985). Correlations between lysosomal membrane stability and degree of histopathological injuries in liver of flounder (*Platichthys flesus* L.) have been reported in previous studies (Köhler, 1991). In flounder from the Wadden Sea it was observed that LMS values below 10 minutes correspond to severe cell damage in the liver (Köhler and Pluta, 1995).

Levels of PAH metabolite 1-OH-pyrene measured in bile were higher in treatments with higher concentration of PAHs, indicating that the cod were exposed. A correlation was observed between levels of 1-OH-pyrene and lysosomal membrane stability, which suggests that the lysosomal alterations are related to increased concentration of PAHs. Laboratory experiments have in fact shown that increased concentrations of anthracene and phenanthrene may result in reduced lysosomal membrane stability in the digestive glands of mussels (Nott and Moore, 1987). Similarly, in a field study where flounder was sampled from an oil spill contaminated area in the Baltic Sea, reduced lysosomal membrane stability in the liver and increased levels of PAH metabolites bile was observed (Baršienė et al., 2006).

The low LMS values in higher treatments found in the present work are in agreement with observations made in field studies. In a study by Marigómez et al. (2004) lower LMS was found in kidney of Atlantic cod exposed in cages for six weeks in the vicinity of an oilfield in the North Sea (Statfjord), when compared to reference areas. Similarly, saithe (*Pollachius virens* L.) sampled close to the Statfjord field had significantly reduced liver LMS when compared to saithe sampled from a reference area (Bilbao et al., 2006b). In another study Bilbao et al. (2006a) reported reduced lysosomal membrane stability in the digestive glands of mussel (*Mytilus edulis* L.) caged at Statfjord for five to six weeks, when compared to mussels caged at reference locations.

No gender-differences in lysosomal membrane stability were found in the present study, which corresponds with observations made in earlier studies. Broeg et al. (1999) reported no differences in LMS between male and female flounder sampled from polluted locations in the German Bight. Gender-differences have been discovered in cellular responses in

flounder hepatocytes exposed to sublethal doses of pro-oxidants (benzo[a]pyrene, nitrofurantoin and hydrogen peroxide), though no gender-differences in lysosomal membrane stability (determined by NR retention) was observed (Winzer et al., 2002).

Measurements of the membrane stability of lysosomes have in previous studies proven to be a good indicator of cellular and general health in marine organisms exposed to environmental pollution (Moore, 1985; Köhler, 1991; Regoli, 1992; Köhler and Pluta, 1995; Broeg et al., 1999; Viarengo et al., 2000; Köhler et al., 2002; Zorita et al., 2008) and the biomarker was successfully applied in the present study. The study has also shown that cod kidney is a suitable tissue for LMS measurement. It was possible to perform measurements of lysosomal membrane stability in cod head kidney tissues even though samples were composed of not only haematopoietic tissue, but also of tubules. However, it is easier to do the measurements in a more homogenous tissue, such as the liver, but unfortunately the fat content in cod liver was too high to measure lysosomal responses (Bilbao et al., 2006a).

4.2 Peroxisomal acyl-CoA oxidase (AOX) activity

No differences between treatment groups were found in levels of peroxisomal AOX activity in cod head kidney. However, significant differences in peroxisomal head kidney AOX activity were detected between males and females and between weeks of exposure. Head kidney tissue of cod exposed for 33 weeks had lower AOX activity than tissue from cod sampled before start of the exposure.

As for lysosomal membrane stability, peroxisomal acyl-CoA oxidase activity is often measured in the digestive glands of mussel and the liver of fish (Marigómez et al., 2004, 2006; Bilbao et al., 2006a, 2006b; Orbea and Cajaraville, 2006; Gorbi et al., 2008; Zorita et al., 2008). No literature on AOX activity measured in fish kidney was available, thus this study is the first where AOX activity has been measured in Atlantic cod head kidney.

Levels of AOX activity in cod head kidney found in the study were generally low, with median values below 2 mUnits AOX/mg protein in males and values below 1.5 mUnits AOX/mg protein in females. However, the activity seemed to be at same level as those

recorded in other studies using Atlantic cod as sentinel organism (Marigómez et al., 2004; Bilbao et al., 2006a).

Induced levels of peroxisomal AOX activity in marine organisms is an indication of pollution to organic contaminants, including PAHs and APs (Cajaraville et al., 2003; Cajaraville and Ortiz-Zarragoitia, 2006; Orbea and Cajaraville, 2006), as have been demonstated in various studies. In a study by Orbea and Cajaraville (2006), the highest levels of AOX activity in mussels were discovered in samples from the most PAH polluted area. The indifference between treatment groups found in the present study is however corresponding to earlier observations in Atlantic cod. In previous studies it has been discussed whether cod is suitable for AOX activity measurements since the activity has been found to be low (Marigómez et al., 2004; Bilbao et al., 2006a). Marigómez et al. (2004) reported low AOX activity in liver peroxisomes of Atlantic cod caged for six weeks along pollution gradients in the North Sea. The activity did not differ from cod caged in reference areas. In the same study, differences in AOX activity in digestive glands of mussels caged in the same area and during the same length of time as cod were observed. This suggests that the AOX activity may not be induced in cod. Similarly, no differences in AOX activity between sampling areas were found in liver of cod caged for five to six weeks along a contamination gradient in the Statfjord field (Bilbao et al., 2006a). Induced AOX activity has though been reported in other fish species. Bilbao et al. (2006b) reported induced peroxisomal AOX activity in liver of saithe (*Pollachius virens* L.) from the North Sea.

The AOX activity in the head kidney of male cod was reduced during the exposure period, while the activity of female head kidney peroxisomes seemed more stable. A similar reduction in AOX activity over time has been reported in mussels exposed to benzo[a]pyrene, phthalates and cadmium in a laboratory setup (Orbea et al., 2002). The reduction in activity over time in the present study may be a result of the exposure treatments, though it may also have been caused by other factors. Several biotic and abiotic factors such as age, gender, diet, reproductive cycle, temperature, salinity and season may have an effect on peroxisomes (reviewed in Cajaraville et al., 2003). Peroxisomal AOX activity in mussels has proven to be affected by seasonal changes, with highest activity in spring and lowest in winter (Cancio et al., 1999). There is limited knowledge on whether seasonal changes are also affecting peroxisome proliferation in fish or not (reviewed in Cajaraville et al., 2003). In this study, the AOX activity of male cod head kidney

peroxisomes was higher than what was found in female cod peroxisomes. Studies on gender differences in AOX activity of fish are scarce. Ibabe et al. (2005) reported no differences in the AOX activity of zebrafish. No literature on gender differences linked to AOX activity in cod was found, making it difficult to compare the results.

Although head kidney is mostly composed of haematopoietic and lymphoid tissue (Takashima and Hibiya, 1995; Roberts, 2001), tubules were observed in several samples when measuring lysosomal membrane stability. As head kidney of teleostean fish is supposed to be free of tubules, it is possible that there may have been some parts of the excretory kidney that were taken together with the head kidney during sampling. The mixture of head and excretory kidney tissue may have contributed to the variability in AOX activity. Another hypothesis for the low and variable peroxisomal AOX activity found in this study is the choice of tissue. Peroxisomes are found in various tissues, though highest number and volume is found in the liver (Yang et al., 1990). AOX activity is often measured in fish hepatocytes since acyl-CoA oxidase have been identified in liver peroxisomes of several fish species (reviewed in Cancio and Cajaraville, 2000). Haematopoietic tissue of kidney may on the other hand not be a tissue with prominent peroxisomes and it is possible that the enzyme activity is lower in kidney peroxisomes than in liver peroxisomes. Due to the lack of available literature on AOX activity measured in kidney of fish, it is difficult to support this theory. However, tissue differences have been discovered in studies on rodents (Nemali et al., 1988). Finally, since low activity has been reported in liver peroxisomes of Atlantic cod in earlier studies (Marigómez et al., 2004; Bilbao et al., 2006a), it is possible that the cod is a fish species not suitable for AOX measurements.

5 Conclusions

This study has demonstrated that Atlantic cod exposed to environmental relevant concentrations of produced water components PAHs and APs were affected on a cellular level. Membrane stability of head kidney lysosomes was significantly reduced in treatments where 200 times dilutions of produced water were used, indicating that cod were affected by the exposure. No changes in lysosomal membrane stability were found between sampling times along the exposure period. No differences were observed with regard to sex. A correlation was found between levels of PAH metabolites in bile and lysosomal membrane stability of cod head kidney, which suggests that cod were exposed to the chemicals in the treatment. This study has shown that the head kidney of cod is a good tissue for LMS measurement.

Peroxisomal AOX activity was low in cod head kidney and no significant differences between treatments were found. This is in agreement with observations made in other studies using cod as sentinel organisms. Atlantic cod may therefore not be suitable for measurements of AOX activity, though the reason for the variable results in this study may be due to low purity of head kidney samples. The low AOX activity measured in cod may also be due to naturally low constitutive enzyme activity in the kidney tissue and cod kidney may therefore not be a suitable tissue for AOX activity measurements.

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Appendix A: Data from measurements

Sex	Exp[3].	Weeks of exposure	Aq.	Weight (g)	Length (cm)	Liver (g)	LSI	CF	LP (min)	AOX (mU/mg prot.)	1-OH-pyrene (ng/g bile)
M	C	0	1	612	34,5	38,1	6,23	1,49	15,0	1,14	125
M	C	0	1	588	36,0	27,8	4,73	1,26	12,5	1,69	105
M	C	0	2	536	35,5	35,1	6,55	1,20	11,5	1,83	145
M	C	0	2	732	40,0	44,5	6,08	1,14	10,0	1,90	114
M	C	0	12	650	36,5	48,1	7,40	1,34	10,0	0,63	70
M	C	0	12	528	36,0	55,1	10,44	1,13	20,0	1,17	98
M	C	2	1	430	37,0	30,2	7,02	0,85	10,0	1,14	0
M	C	2	1	268	35,5	12,4	4,63	0,60	20,0	0,68	0
M	C	2	2	402	36,5	28,0	6,97	0,83	10,0	1,19	65
M	C	2	2	450	36,5	46,2	10,27	0,93	6,3	1,63	50
M	C	2	12	416	40,0	15,6	3,75	0,65	15,0	0,86	103
M	C	2	12	434	36,5	37,4	8,62	0,89	17,5	0,52	126
M	L	2	3	294	32,5	16,0	5,44	0,86	10,0	1,16	0
M	L	2	3	296	33,5	20,6	6,96	0,79	8,8	0,99	0
M	L	2	10	428	38,0	23,8	5,56	0,78	10,0	1,09	0
M	L	2	10	442	37,5	34,3	7,76	0,84	15,0	0,82	345
M	L	2	11	346	34,0	25,7	7,43	0,88	10,0	1,24	0
M	L	2	11	294	34,0	23,6	8,03	0,75	15,0	0,64	0
M	P	2	4	414	36,5	33,5	8,09	0,85	10,0	0,62	964
M	P	2	4	310	33,0	26,5	8,55	0,86	10,0	0,80	1419
M	P	2	5	404	33,5	30,9	7,65	1,07	15,0	1,66	1224
M	P	2	5	520	37,0	42,1	8,10	1,03	12,5	1,79	1045

[3] C=control, L=low, P=pulsed, H=high

M	P	2	9	332	33,0	32,6	9,82	0,92	12,5	0,95	2258
M	p	2	9	502	39,0	59,1	11,77	0,85	5,0	0,81	747
M	H	2	6	364	36,0	31,2	8,57	0,78	10,0	1,54	0
M	H	2	6	626	42,0	44,5	7,11	0,84	5,0	1,20	420
M	H	2	7	346	23,5	30,7	8,87	2,67	10,0	1,68	519
M	H	2	7	510	39,0	39,0	7,65	0,86	10,0	1,95	871
M	H	2	8	500	37,5	38,0	7,60	0,95	10,0	1,44	498
M	H	2	8	426	35,5	34,2	8,03	0,95	10,0	1,56	986
M	C	16	1	518	39,5	29,6	5,71	0,84	10,0	1,25	19
M	C	16	12	456	38,5	15,1	3,31	0,80	15,0	1,03	51
M	C	16	12	480	36,5	36,4	7,58	0,99	15,0	1,00	31
M	C	16	12	624	38,5	44,0	7,05	1,09	10,0	1,09	27
M	C	16	13	434	35,0	33,4	7,70	1,01	15,0	0,53	22
M	C	16	13	236	31,5	10,3	4,36	0,76	13,8	0,00	41
M	L	16	3	416	36,0	23,2	5,58	0,89	15,0	1,38	193
M	L	16	3	510	38,0	24,9	4,88	0,93	10,0	1,67	0
M	L	16	10	430	39,5	18,4	4,28	0,70	10,0	1,62	108
M	L	16	10	568	39,0	44,0	7,75	0,96	15,0	1,58	32
M	L	16	11	430	38,0	24,4	5,67	0,78	15,0	0,63	99
M	L	16	11	324	32,5	25,3	7,81	0,94	15,0	1,38	143
M	P	16	4	764	41,5	63,1	8,26	1,07	12,5	1,68	336
M	P	16	4	408	37,5	9,7	2,38	0,77	10,0	0,94	141
M	P	16	5	450	38,5	22,5	5,00	0,79	8,8	1,26	524
M	P	16	5	440	37,5	32,6	7,41	0,83	10,0	0,35	275
M	P	16	9	512	38,0	41,3	8,07	0,93	10,0	1,53	1069
M	P	16	9	604	40,5	36,0	5,96	0,91	10,0	1,55	240
M	H	16	6	762	42,0	75,1	9,86	1,03	3,0	1,36	254
M	H	16	7	528	40,5	27,2	5,15	0,79	10,0	0,98	224
M	H	16	7	636	40,0	61,7	9,70	0,99	10,0	1,11	371
M	H	16	7	412	35,0	29,9	7,26	0,96	10,0	0,87	561
M	H	16	8	699	42,5	48,8	6,98	0,91	5,0	1,15	1255
M	H	16	8	594	40,5	41,7	7,02	0,89	5,0	3,07	703

M	C	33	1	560	39,0	23,3	4,16	0,94	15,0	0,33	16
M	C	33	1	1086	47,0	78,5	7,23	1,05	10,0	0,54	37
M	C	33	2	742	43,5	54,0	7,28	0,90	15,0	0,93	36
M	C	33	2	652	41,0	54,2	8,31	0,95	10,0	0,22	23
M	C	33	12	700	42,5	45,6	6,51	0,91	20,0	0,73	47
M	C	33	12	752	43,5	58,5	7,78	0,91	12,5	0,87	36
M	L	33	10	450	38,0	14,1	3,13	0,82	10,0	0,49	436
M	L	33	10	604	41,0	43,8	7,25	0,88	7,5	1,21	214
M	L	33	10	452	28,5	8,3	1,84	1,95	10,0	0,95	165
M	L	33	11	638	40,0	54,5	8,54	1,00	15,0	1,14	231
M	L	33	11	684	42,0	57,3	8,38	0,92	20,0	0,50	157
M	L	33	11	540	40,0	23,6	4,37	0,84	15,0	0,30	270
M	P	33	4	1042	44,5	79,5	7,63	1,18	15,0	0,67	720
M	P	33	4	848	44,0	84,7	9,99	1,00	10,0	1,06	636
M	P	33	5	844	45,0	49,6	5,88	0,93	10,0	1,32	374
M	P	33	5	560	38,5	20,8	3,71	0,98	5,0	0,24	1392
M	P	33	9	744	45,5	74,9	10,07	0,79	5,0	0,00	1282
M	P	33	9	480	38,0	15,4	3,21	0,87	10,0	1,00	803
M	H	33	6	1208	49,5	97,3	8,05	1,00	3,0	0,46	742
M	H	33	6	674	41,0	48,1	7,14	0,98	3,0	0,27	396
M	H	33	7	516	38,0	36,0	6,98	0,94	5,0	0,50	1306
M	H	33	7	760	44,0	60,9	8,01	0,89	5,0	0,78	1831
M	H	33	8	1078	47,0	113,4	10,52	1,04	5,0	0,49	1019
M	H	33	8	670	42,5	19,7	2,94	0,87	4,0	0,33	1609
F	C	0	1	516	35,5	44,5	8,62	1,15	15,0	0,65	0
F	C	0	2	662	37,0	69,1	10,44	1,31	20,0	0,45	1
F	C	0	12	536	35,5	47,5	8,86	1,20	15,0	0,35	117
F	C	0	13	446	33,5	50,0	11,21	1,19	15,0	1,08	109
F	C	0	13	490	35,0	48,3	9,86	1,14	10,0	1,06	133
F	C	0	13	344	33,0	23,7	6,89	0,96	10,0	0,54	174
F	C	2	1	368	34,0	34,9	9,48	0,94	10,0	0,59	0
F	C	2	1	308	31,0	19,9	6,46	1,03	20,0	0,54	0

F	C	2	2	456	39,0	45,8	10,04	0,77	17,5	1,22	70
F	C	2	2	718	42,0	56,8	7,91	0,97	10,0	0,69	141
F	C	2	12	500	38,5	45,5	9,10	0,88	7,5	0,69	0
F	C	2	12	418	34,5	42,4	10,14	1,02	15,0	0,31	26
F	L	2	3	646	39,0	51,3	7,94	1,09	7,5	0,86	0
F	L	2	3	578	35,5	240,6	41,63	1,29	15,0	0,54	0
F	L	2	10	364	33,5	32,0	8,79	0,97	12,5	0,80	115
F	L	2	10	502	38,0	41,6	8,29	0,91	10,0	0,79	89
F	L	2	11	512	37,5	60,8	11,88	0,97	12,5	0,37	248
F	L	2	11	428	37,5	29,7	6,94	0,81	20,0	0,99	77
F	P	2	4	440	37,0	41,8	9,50	0,87	10,0	0,26	3151
F	P	2	4	312	34,0	21,2	6,79	0,79	5,0	0,55	2188
F	P	2	5	468	36,5	3,5	0,75	0,96	15,0	1,08	1343
F	P	2	5	576	39,5	51,6	8,96	0,93	5,0	0,97	2039
F	P	2	9	458	38,5	43,8	9,56	0,80	8,8	1,12	1363
F	P	2	9	390	36,5	32,2	8,26	0,80	10,0	0,92	891
F	H	2	6	504	34,5	27,7	5,50	1,23	7,5	0,60	407
F	H	2	6	472	36,5	48,6	10,30	0,97	7,5	0,42	0
F	H	2	7	288	34,0	19,3	6,70	0,73	10,0	0,57	531
F	H	2	7	276	31,5	24,1	8,73	0,88	10,0	0,90	1466
F	H	2	8	468	36,5	52,3	11,18	0,96	5,0	0,60	1488
F	H	2	8	360	35,5	25,2	7,00	0,80	12,5	0,40	1524
F	C	16	1	554	40,0	29,3	5,29	0,87	13,8	0,64	49
F	C	16	1	566	40,0	29,9	5,28	0,88	10,0	0,38	0
F	C	16	12	596	41,5	31,5	5,29	0,83	15,0	0,23	123
F	C	16	12	422	37,5	19,2	4,55	0,80	10,0	1,45	94
F	C	16	13	590	40,5	38,5	6,53	0,89	15,0	0,68	28
F	C	16	13	662	40,5	38,1	5,76	1,00	20,0	0,49	16
F	L	16	3	416	39,0	16,5	3,97	0,70	15,0	0,85	29
F	L	16	3	312	34,5	3,3	1,06	0,76	10,0	0,48	350
F	L	16	10	724	42,0	66,8	9,23	0,98	8,8	0,15	77
F	L	16	10	518	38,5	31,4	6,06	0,91	17,5	0,98	0

F	L	16	11	438	36,5	22,2	5,07	0,90	15,0	0,96	154
F	P	16	4	536	39,0	34,2	6,38	0,90	15,0	0,00	483
F	P	16	4	546	37,5	43,5	7,97	1,04	15,0	1,04	543
F	P	16	5	536	38,5	30,4	5,67	0,94	10,0	0,64	541
F	P	16	5	606	39,0	57,6	9,50	1,02	12,5	0,85	519
F	P	16	9	820	42,0	69,5	8,48	1,11	15,0	1,30	518
F	P	16	9	406	35,5	16,5	4,06	0,91	13,8	0,74	767
F	H	16	6	616	41,0	38,3	6,22	0,89	10,0	1,05	287
F	H	16	6	416	35,5	30,2	7,26	0,93	10,0	1,03	458
F	H	16	6	262	32,5	11,1	4,24	0,76	12,5	0,60	2158
F	H	16	6	544	38,5	36,6	6,73	0,95	8,8	0,76	191
F	H	16	7	940	45,5	48,0	5,11	1,00	7,5	1,01	373
F	H	16	8	671	41,5	50,0	7,45	0,94	15,0	1,19	9177
F	C	33	1	610	40,0	49,0	8,03	0,95	10,0	0,95	35
F	C	33	1	802	42,0	72,0	8,98	1,08	5,0	0,47	11
F	C	33	2	820	45,0	30,8	3,76	0,90	10,0	0,47	42
F	C	33	2	544	40,0	17,3	3,18	0,85	8,8	0,97	40
F	C	33	12	644	39,5	22,7	3,52	1,04	20,0	1,37	70
F	C	33	12	796	43,5	59,5	7,47	0,97	10,0	0,46	29
F	L	33	10	720	43,0	43,0	5,97	0,91	10,0	0,88	200
F	L	33	10	742	44,5	22,9	3,09	0,84	10,0	0,31	54
F	L	33	10	410	36,5	18,0	4,39	0,84	20,0	0,22	161
F	L	33	11	504	40,0	16,2	3,21	0,79	20,0	0,42	267
F	L	33	11	652	41,0	32,2	4,94	0,95	10,0	0,58	151
F	L	33	11	636	42,0	35,0	5,50	0,86	10,0	0,55	169
F	P	33	4	754	47,0	51,3	6,80	0,73	10,0	0,20	1028
F	P	33	4	602	40,0	31,4	5,22	0,94	10,0	0,18	125
F	P	33	5	898	46,5	32,0	3,56	0,89	5,0	0,48	1207
F	P	33	5	348	37,0	10,3	2,96	0,69	5,0	0,28	283
F	P	33	5	404	37,5	13,7	3,39	0,77	10,0	0,53	752
F	P	33	9	1020	48,5	49,3	4,83	0,89	10,0	0,12	960
F	H	33	6	958	46,0	67,8	7,08	0,98	8,8	0,86	145

F	H	33	6	418	38,5	5,9	1,41	0,73	5,0	0,95	2195
F	H	33	7	992	45,0	102,2	10,30	1,09	15,0	0,18	0
F	H	33	7	626	40,0	37,3	5,96	0,98	10,0	0,11	1466
F	H	33	8	1346	50,0	102,7	7,63	1,08	10,0	0,31	1069
F	H	33	8	688	42,0	52,5	7,63	0,93	10,0	0,14	1182

Appendix B: Equipment

Equipment	Supplier
Aliquots	
Centrifuge (-4°C)	BECKMAN COULTER (Allegra 25R Centrifuge)
Coverslips	VWR (Ref: ECN 631-1575)
Cryostat chucks	
Cryotome (LEICA CM3000)	Leica Instruments GmbH, Nussloch, Germany
Cuvettes	SARSTEDT (Ref: 67.742)
Eppendorf tubes	EPPENDORF (Ref: 0030 120.086)
Freezer (-40°C)	SANYO
Freezer (-80°C)	THERMOFORMA
FS2001F-ISO Reader	Biomark Inc.
Glass slides	MENZEL-GLASER (ca. 76 x 26 mm)
Glass-Teflon homogeniser	
Hellendahl jars	LABOLAN (Ref: 29675250)
Light microscope	OLYMPUS BX51
Microplates	SARSTEDT (Ref: 82.1581)
pH meter	
PIT-Tag	Biomark Inc.
Shaking water bath	SELECTA (Unitronic OR)
Software for Lowry-Amaia	THERMO LABSYSTEM Multiskan Spectrum
Spectrophotometer	Shimadzu UV-1603, Munich, Germany

Spectrophotometer (protein concentration)	THERMO LABSYSTEM Multiskan Spectrum
Statistica	Statsoft
Tissue freezing medium	JUNG (Ref: 0201 08926)

Appendix C: Chemicals

Name	**Supplier**
Bovine γ-globulin	Bio-Rad
$CaCl_{2\ (2H2O)}$	Sigma-Aldrich
Dimethylformamide (DFA)	Merck
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Ethanol	Panreac
Fast Violet B	Sigma
Formaldehyde, 40%	Panreac
Horseradish peroxidase (HRP)	Sigma
$K_2HPO_4*3H_2O$	Sigma
Kaiser's glycerol-gelatine	Merck Biosciences
KH_2PO_4	Sigma
Leuco-DCF	Molecular Probes
$Na_2HPO_4*12H_2O$ (disodium phosphate)	Fluka
Na-Citrate (2H20)	Fluka
NaCl	Sigma
$NaH_2PO_4*H_2O$ (monosodium phosphate)	Probus
$NaHCO_3$ (sodium bicarbonate)	Riedel-de Haën
NaOH.	Fluka
Naphtol AS-BI phosphate	Sigma-Aldrich
Palmitoyl-CoA	Sigma

POLYPEP	Sigma
Sodium azide	Sigma
Triton X-100 (10 %)	Sigma
Bio-Rad DC protein assay	Bio-Rad, USA
Reagent A	Bio-Rad, USA
Reagent B	Bio-Rad, USA