Effects of environmental contaminants on Atlantic cod (*Gadus morhua*) from the inner Oslofjord

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II

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Abstract

The present study is part of an ongoing effort to monitor the pollution situation in the inner Oslofjord. The purpose of the study was to investigate effects of pollutants on the resident cod population in the area. The outer Oslofjord was used as a reference site. Several biomarkers were used in order to detect exposure and effects of a broad range of environmental chemicals, including planar organic and inorganic pollutants, and possibly organophosphate and carbamate pesticides. The physiological indices: condition factor, liver somatic index (LSI) and gonadal somatic index (GSI) were used to assess the overall condition, energy reserves, as well as to provide information on reproductive status. Age and size of the fish were used to study possible differences in growth rate of cod from the two areas. There was also a method-optimizing aspect to this study, with regards to the comet assay used to assess DNA damage. Of interest was whether storage duration of samples on lysis buffer would affect the level of DNA damage. As indicated by elevated bile concentrations of polycyclic aromatic hydrocarbons (PAHs), concentration and activity of cytochrome P4501A (CYP1A) and DNA damage as well as decreased activity of δ-aminolevulinic acid dehydratase (ALA-D), cod from the inner Oslofjord appear to be affected by planar organic pollutants, such PAHs and polychlorinated biphenyls (PCBs) as well as inorganic pollutants, such as lead (Pb). Cod from the outer Oslofjord seem to be more affected by chemicals that inhibit acetyl cholinesterase, e.g. organophosphate and carbamate pesticides, compared to cod from the inner Oslofjord. Also, exposure of leukocytes to hydrogenperoxide (H_2O_2) revealed a higher tolerance to oxidative stress, with regards to DNA damage, in cod from the inner Oslofjord compared to cod from the outer Oslofjord. This indicates an adaptive response to chronic exposure to chemicals causing oxidative stress. Physiological indices revealed higher energy reserves at the time of sampling in cod collected from the inner Oslofjord compared to the outer Oslofjord. Growth rate appeared to be slower in the inner Oslofjord, but could not be tested statistically since the size ranges collected only made it possible to compare two-year old individuals. Elevated DNA damage appeared to have resulted from longer storage duration of samples on lysis buffer during the comet assay procedure. This suggests that treatment with lysis buffer may cause elevated levels of DNA damage over the background damage.

Preface

The process of completing this highly exciting and educational project has included a long list of helpful people whom I am forever grateful for. My first thanks go to my main supervisor Ketil Hylland and co-supervisor Tor Fredrik Holth. Thank you Ketil, for always being available with an open door and a quick reply, no matter how much you have got going on. I will never understand your big secret to how you manage to make time. Thank you Tor Fredrik for your tremendous guidance in the lab and field work and for dropping everything to help me when most needed. A big thanks to my co-supervisor Merete Grung at NIVA for performing the HPLC analysis! I would also like to thank the crew of FF Trygve Braarud for assisting with the trawling and the sampling crew which, in addition to Tor Fredrik, comprised of Andrea Lenderink and Kathrin Ellesat. We made a great team! Andrea, you have also been an amazing help and support for me through the whole process with everything from sampling to lab questions and hugs. In regards to my lab work I would also like to thank Vesela Yancheva, Mazyar Yazdani and Marcin Wojewodzic for your assistance, and also Berit Kaasa for letting me borrow your lab when needed.

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Abbreviations

AChE	Acetylcholinesterase
AhR	Aryl hydrocarbon receptor
ALA	δ-aminolevulinic acid
ALA-D	δ-aminolevulinic acid dehydratase
ANOVA	Analysis of variance
AP sites	Apurinic/apyrimidinic sites
ATC	Acetylthiocholine iodide
B[a]P	Benzo(a)pyrene
BSA	Bovine serum albumin
Cd	Cadmium
Cu	Copper
CYP1A	Cytochrome P4501A
DDE	Dichlorodiphenyldichloroethylene
DTNB	Dithiobisnitrobenzoic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EROD	Ethoxyresorufin-O-deethylase
GSI	Gonadal somatic index
H_2O_2	Hydrogenperoxide
Hg	Mercury
HPLC	High-Performance Liquid Chromatography
LMP	Low melting point
LSI	Liver somatic index
NADPH	Nicotinamide adenine dinucleotide phosphate
OH-B[a]P	OH-benzo(a)pyrene

PAHs   Polycyclic aromatic hydrocarbons

Pb   Lead

PBDE   Polybrominated diphenyl ethers

PBG   Porphobilinogen

PBS   Phosphate buffered saline

PCBs   Polychlorinated biphenyls

PCDDs   Polychlorinated dibenzodioxins

PFC   Perfluoroalkyl compounds

Reagent A   Alkaline copper tartrate solution

reagent B   Folin reagent

SOD   Superoxide dismutase

TE buffer   Tris-EDTA buffer

TBT   Tributyltin

TTBS   Tris-tween buffered saline

Zn   Zinc

Table of contents

1 Introduction

1.1 The inner Oslofjord- reasons for concern

The ocean acts as an ultimate recipient to environmental pollutants through atmospheric transport and runoff from land. Coastal areas connected to urbanized or industrial locations are therefore of special concern with regards to levels of pollutants that may affect marine organisms (Green *et al*. 2012). The Oslofjord is located in southern Norway and is physically separated into an inner and outer area by a shallow sill of approximately 20 m at Drøbaksundet. This shallow sill limits the water exchange between the two areas (Berge *et al*. 2011). The inner Oslofjord comprises of two sub-basins, Vestfjorden and Bunnefjorden, which are the two areas of concern regarding environmental pollutants. Vestfjorden undergoes an annual deep-water exchange with water from the outer Oslofjord, while it can take several years between each deep-water exchange in Bunnefjorden (Berge *et al*. 2011). The inner Oslofjord is surrounded by urban areas and with Oslo harbor being Norway's largest with regards to transport of cargo as well as passengers, receiving 50-70 ships through a normal week (Oslo Havn KF 2010). Ship traffic is an important source of pollutants to the fjord, as well as contamination deriving from industry, traffic, landfills and sewage treatment plants connected to the fjord (Berge *et al*. 2011). The sediment is also an important secondary source of legacy persistent pollutants such as polychlorinated biphenyls (PCBs) and metals (Lepland *et al*. 2010, Ruus *et al*. 2012). Due to the lack of water exchange with the outer part of the fjord, chemicals tend to accumulate at this site. The pollution situation in the inner Oslofjord has been monitored for many years and the site is included in the annual monitoring of chemical concentrations in organisms along the Norwegian coast (Berge *et al*. 2011). Pollutants of concern include metals such as lead (Pb), mercury (Hg) and cadmium (Cd), polycyclic aromatic hydrocarbons (PAHs), dioxins, PCBs, tributyltin (TBT), polybrominated diphenyl ethers (PBDEs) and perfluoroalkyl compounds (PFCs), some of which have been detected at relatively high levels in organisms such as cod and mussels (Green *et al*. 2012). Weak effects of endocrine disrupting chemicals, such as increased vitellogenin in male fish, have been observed in cod from the inner Oslofjord (Scott *et al*. 2006). Environmental pollutants may bioaccumulate in organisms and cause toxic effects at a biochemical level as well as on a higher individual, population and ecosystem level (Goksøyr *et al*. 1996).

1.2 Biomarkers

In order to gain knowledge about the health of the organisms inhabiting a polluted site, chemical analyses of pollutants present in the biota and surroundings are insufficient. More information on the actual effect, or no effect, is required. The use of biomarkers is a step further in obtaining this information. A biomarker is defined as a biological response that can be attributed to exposure or effects of environmental chemicals (van der Oost *et al*. 2003). Biomarkers may provide early warning signals and can be considered as intermediates between the sources of pollutants and effects on a higher biological organization (van der Oost *et al*. 2003). Investigating early warning signals provides an opportunity to act before more severe effects, such as cancer, take place. A suite of biochemical biomarkers, as well as a chemical analysis, were used in this investigation to detect exposure and effects of a broad range of environmental pollutants relevant to the Oslofjord (Figure 1.1). Atlantic cod (*Gadus morhua*) was chosen as indicator species. It is widespread in Norway as well as being commercially and recreationally important. Coastal populations are relatively stationary (Godø 1995), which is important in order to relate any observed toxicological effects to point sources. Due to a fatty liver (40-80%) it is a good candidate for environmental monitoring involving accumulation of lipophilic chemicals (Goksøyr *et al*. 1996). Atlantic cod have a diverse diet consisting of both pelagic and benthic organisms such as shrimp, crabs and fish (Hop *et al*. 1992) and may accumulate environmental pollutants from diet or directly from the water through gills (Grung *et al*. 2009). This species has been widely used in environmental monitoring (Goksøyr *et al*. 1994, Beyer *et al*. 1996, Hylland *et al*. 2009), which is advantageous for comparative reasons. Due to health concerns, consumption of cod liver captured in the inner Oslofjord has been advised against by the Norwegian Food Safety Authority due to its high content of PCBs (Økland 2005). The use of Atlantic cod in environmental monitoring is thus important due to its ecological role as well as it's consumption by humans.

Analysis of PAH metabolites in bile has proven to be a good biomarker for environmental PAH exposure (Krahn *et al*. 1984). When PAHs are consumed by fish, they are metabolized by phase I and II enzymes into more hydrophilic compounds and excreted via the bile (Bucheli & Fent 1995). There are two main classes anthropogenic PAH sources; pyrogenic and petrogenic PAHs, originating from incineration processes and fossil fuels respectively (Neff 1979). Since PAHs are metabolized relatively quickly, chemical analysis of parent PAHs in tissues may lead to an underestimation of exposure. Metabolites appears within a

days in the fish bile which means analysis of PAH metabolites in bile can provide an estimate of recent exposure (van der Oost *et al*. 2003). By identifying the types of PAH metabolites in bile, it is possible to identify the sources of origin (Inengite *et al*. 2013). This is useful in environmental monitoring in order to decide what can be done to prevent release. Negative effects of PAHs have been associated with reactive metabolites leading to DNA adducts (Aas *et al*. 2000), neoplasms (Myers *et al*. 2003), immune system modulation (Holladay *et al*. 1998) and developmental impacts (Rhodes *et al*. 2005).

The most sensitive effect biomarkers are considered to be changes in levels or activities of biotransformation enzymes (van der Oost *et al*. 2003). The cytochrome P4501A (CYP1A) enzymes are known to biotransform xenobiotics, e.g. PAHs, planar PCBs, dibenzofurans and dioxins (Goksøyr & Förlin 1992). These chemicals that are metabolized by CYP1A are also responsible for inducing transcription of the enzyme, leading to a higher metabolizing activity. Therefore, investigation of CYP1A induction is a suitable biomarker to combine with chemical analyses, such as PAH metabolites in bile. The induction pathway occurs through binding to the cytosolic aryl hydrocarbon receptor (AhR) which is then transported into the nucleus where it activates transcription of the enzyme (Schlenk *et al*. 2008). The organ that is largely responsible for metabolizing xenobiotics is the liver and it is therefore appropriate to investigate induction of CYP1A in liver tissue. A number of studies have detected elevated CYP1A levels and activity in fish exposed to organic contaminants such as PAHs, PCBs, polychlorinated dibenzodioxins (PCDDs) and PBDEs (Hektoen *et al*. 1994, Aas *et al*. 2001, Chen *et al*. 2001, Olsvik *et al*. 2011). The response in ethoxyresorufin-*O*-deethylase (EROD) activity may vary depending on the mixture of chemicals that the fish are exposed to. In a study of the flounder *Platichthys flesus* the response in EROD activity was lower with exposure to both Cd and benzo(a)pyrene (B[a]P) compared to the response from exposure to B[a]P alone (Hylland *et al*. 1996). In that same study, exposure to the combination of B[a]P and a PCB gave a stronger response in EROD activity than the two of them gave in separate exposures. Measurements of CYP1A on several levels, such as gene expression (mRNA), protein concentration in addition to EROD activity therefore provides a more robust picture of the induction by pollutants. During this investigation, induction of CYP1A was measured on the level of protein concentration as well as the EROD activity.

The metabolism of xenobiotics by CYP1A can generate reactive metabolites or oxidative stress, which are known to cause DNA damage. Detection of single stranded DNA damage as determined by the comet assay (Singh *et al*. 1988) can be used as a biomarker for exposure to

genotoxicants and general oxidative stress. The comet assay is advantageous in that it can measure DNA damage on a single cell level and does not require extraction of DNA. It is also cost efficient. Single-strand breaks may occur through exposure to a large number of chemicals which means this endpoint is not suitable for detecting exposure to specific classes of pollutants. Transient single strand breaks may also occur during repair activities and as a result of apurinic/apyrimidinic sites (AP sites) caused by chemicals (Collins *et al.* 1997). This endpoint can therefore indicate damage as well as repair activities (Collins *et al.* 1997). Strand breaks can thus give information about the sum of exposures to genotoxic chemicals causing strand breaks either directly or indirectly. The function of a cell requires intact DNA. In this study, DNA damage was detected in leukocytes. This cell type was chosen as loss of integrity in the immune system would be relevant for the population's survival.

To detect exposure and effect of organophosphate and carbamate pesticides, the activity of acetylcholinesterase (AChE) has traditionally been used as a biomarker (Payne *et al.* 1996). AChE is an enzyme that breaks down neurotransmitters in the synapses and prevents a continuous signal. Without the breakdown of these transmitters a situation of increased body temperature, heart rate, blood pressure, muscle twitching or tremors may occur (Bradbury *et al.* 2008). Organophosphate binds irreversibly to the enzyme (Bradbury *et al.* 2008). AChE has been shown to be sensitive to organophosphate and carbamate pesticides (Magnotti Jr *et al.* 1994), however speculations have been made to the specificity of this biomarker. Other chemicals, e.g. PAHs (Holth & Tollefsen 2012), urban runoff and effluents from pulp and paper mill (Payne *et al.* 1996) and metals (Olson & Christensen 1980) have also been shown to have inhibitory effect on this enzyme.

A well-known biomarker to investigate exposure to Pb is the activity of δ-aminolevulinic acid dehydratase (ALA-D) (Schmitt *et al.* 2005). This protein is involved in vertebrate heme synthesis, hence generation of hemoglobin. The enzyme uses zinc (Zn) as a cofactor and Pb may inhibit the enzyme activity by replacing Zn (Warren *et al.* 1998, Moraes *et al.* 2003). Strong correlation has been found between blood levels of Pb and ALA-D inhibition (Schmitt *et al.* 2005, Schmitt *et al.* 2007). Although the biomarker has traditionally been used and considered to be specific to Pb, there are studies with findings that have suggested influence of other metals on the enzyme activity as well (Rodriguez *et al.* 1989, Hylland *et al.* 2009).

In addition to biomarkers indicating effects on a biochemical level, physiological biomarkers were also included in this investigation. Liver somatic index (LSI), determined as the ratio of

4

liver weitht to body weight, and condition factor, as determined by the ratio of somatic body weight to length, can be used to assess the energy reserves available to the fish. LSI generally reflects hepatic lipid content while condition factor also reflects protein content in musscle (Lambert & Dutil 1997). Gonadal somatic index (GSI) provides information of the fish' reproductive status. These physiological indices may be affected by environmental chemicals (van der Oost *et al*. 2003, Scholz & Klüver 2009), however, factors such as nutrition status and reproductive status may mask the effects from exposure to chemicals. Therefore these physiological factors are not robust biomarkers for chemical exposures, but can provide useful information when interpreting results from biochemical biomarkers.

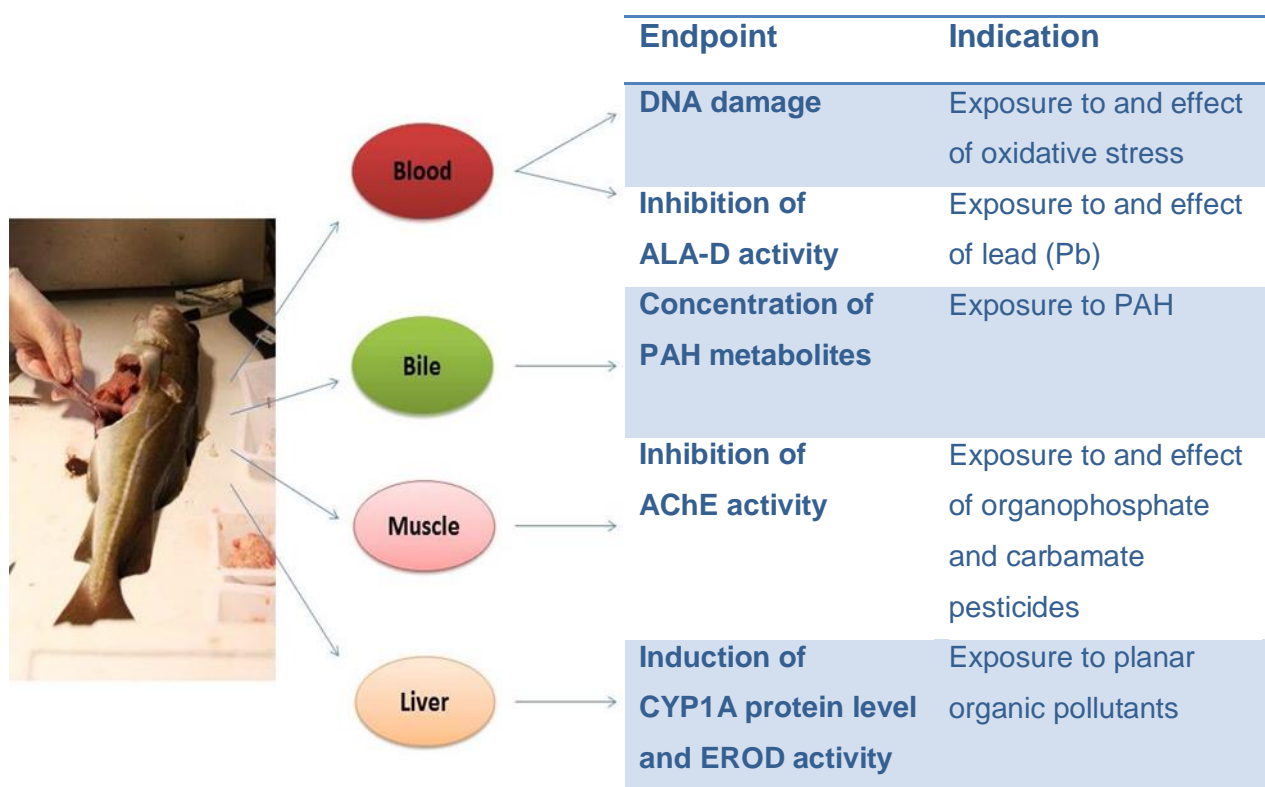| Endpoint	Indication
DNA damage	Exposure to and effect of oxidative stress
Inhibition of ALA-D activity	Exposure to and effect of lead (Pb)
Concentration of PAH metabolites	Exposure to PAH
Inhibition of AChE activity	Exposure to and effect of organophosphate and carbamate pesticides
Induction of CYP1A protein level and EROD activity	Exposure to planar organic pollutants

Figure 1.1 Overview of tissues sampled, biomarkers used and their indications. Sampling photographed by Kathrine Høyrup.

1.3 Aims and hypotheses

The aim of this study was to clarify possible effects of environmental contaminants such as PAHs, PCBs, dioxins, pesticides and heavy metals on cod from the inner Oslofjord. The outer

Oslofjord was used as a reference site. In addition to spatial comparison, earlier studies were considered to provide a temporal overview.

The objectives were:

1. To clarify whether contaminants affect cod that inhabits the inner Oslofjord.
2. To clarify whether cod from the inner Oslofjord is more affected by environmental contaminants than cod from the outer Oslofjord with regards to levels of DNA damage (strand breaks), susceptibility of DNA to oxidative stress, PAH metabolite levels in bile, CYP1A protein levels, EROD activity, ALA-D activity and AChE activity.
3. To clarify whether there is a sex-based difference within each site for any of the biochemical or physiological biomarkers.
4. To clarify whether there is a difference in growth or physiological factors between cod from the inner Oslofjord and the outer Oslofjord.
5. To clarify relationships between any of the biochemical and physiological effects as well as age.
6. To clarify whether storage on lysis buffer in the comet assay would affect the level of DNA damage.

Null hypotheses:

H_0: There was no difference in DNA damage in leukocytes between cod from the inner Oslofjord and the outer Oslofjord or between males and females within each of the two areas.

H_0: There was no difference in DNA susceptibility to oxidative stress between cod from the inner Oslofjord and the outer Oslofjord or between males and females within each of the two areas.

H_0: There was no difference in PAH levels in bile between cod from the inner Oslofjord and the outer Oslofjord or between males and females within each of the two areas.

H_0: There was no difference in hepatic CYP1A protein levels between cod from the inner Oslofjord and the outer Oslofjord or between males and females within each of the two areas.

H_0: There was no difference in hepatic EROD activity between cod from the inner Oslofjord and the outer Oslofjord or between males and females within each of the two areas.

H_0: There was no difference in ALA-D activity of the blood cells between cod from the inner Oslofjord and the outer Oslofjord or between males and females within each of the two areas.

H_0: There was no difference in AChE activity of the muscle tissue between cod from the inner Oslofjord and the outer Oslofjord or between males and females within each of the two areas.

H_0: There was no difference in size between cod, within the same age, from the inner Oslofjord and the outer Oslofjord, and no difference between males and females of the two areas.

H_0: There was no difference in LSI, GSI or condition factor between cod from the inner Oslofjord and the outer Oslofjord or between males and females within each of the two areas.

H_0: There was no correlation between any of the biochemical or physiological effects as well as age

H_0: There was no difference in DNA damage between samples stored on lysis buffer for one week and three weeks.

2 Materials and methods

2.1 Sample collection

Cod were collected by trawling with the research vessel FF Trygve Braarud in the period 21/11/11-24/11/11. The cod were collected from two locations, Midtmeie (Figure 2.1) and Travbanen (Figure 2.2). Midtmeie represented the inner Oslofjord/impacted site and Travbanen represented the outer Oslofjord/reference site. A total of 80 individuals were collected, 40 from each location. Following capture, the fish were kept in a flow-through seawater tank until sampled.

The fish were sacrificed by a blow to the head followed by blood sampling and dissection. The syringes used for blood sampling (1 mL syringe, 0.6 x 30 mm cannula) were rinsed with heparin (10 000 units/mL) to prevent blood clotting. Approximately 1 mL blood was sampled. Upon dissection of each individual, sex (Table 2.1), body length and somatic weight (Table 2.2) as well as liver and gonad weight were noted. The abdomen was opened by cutting from anus up to approximately the location of the pelvic fins. The gall bladder was immediately drained with a syringe (1 mL syringe, 0.5 x 25 mm cannula) to avoid contamination of other tissues. Bile samples were kept in eppendorf tubes on ice and protected from light until storage in a -20ºC freezer. Liver and muscle were sampled in duplicates, approximately 1 g each, using a scalpel and kept in cryotubes in liquid nitrogen until storage in a -80ºC freezer. The tissue was excised from the same area in each individual. Blood samples were kept in eppendorf tubes on ice, from which 200 µl blood was subsampled to new eppendorf tubes and added 1µL heparin (10 000 units/mL), which would be further processed for the comet assay. The remaining blood was centrifuged for 5 minutes at 2000 x g to separate plasma and blood cells. The supernatant comprising plasma was transferred to cryotubes which were kept in liquid nitrogen until storage at -80ºC. The eppendorf tubes holding the pellet containing blood cells were pierced with a cannula to be kept in liquid nitrogen until storage at -80ºC. The sagittal otoliths were collected for determining age and retrieved by cutting down through the top of its head. The otoliths were kept in eppendorf tubes at room temperature.

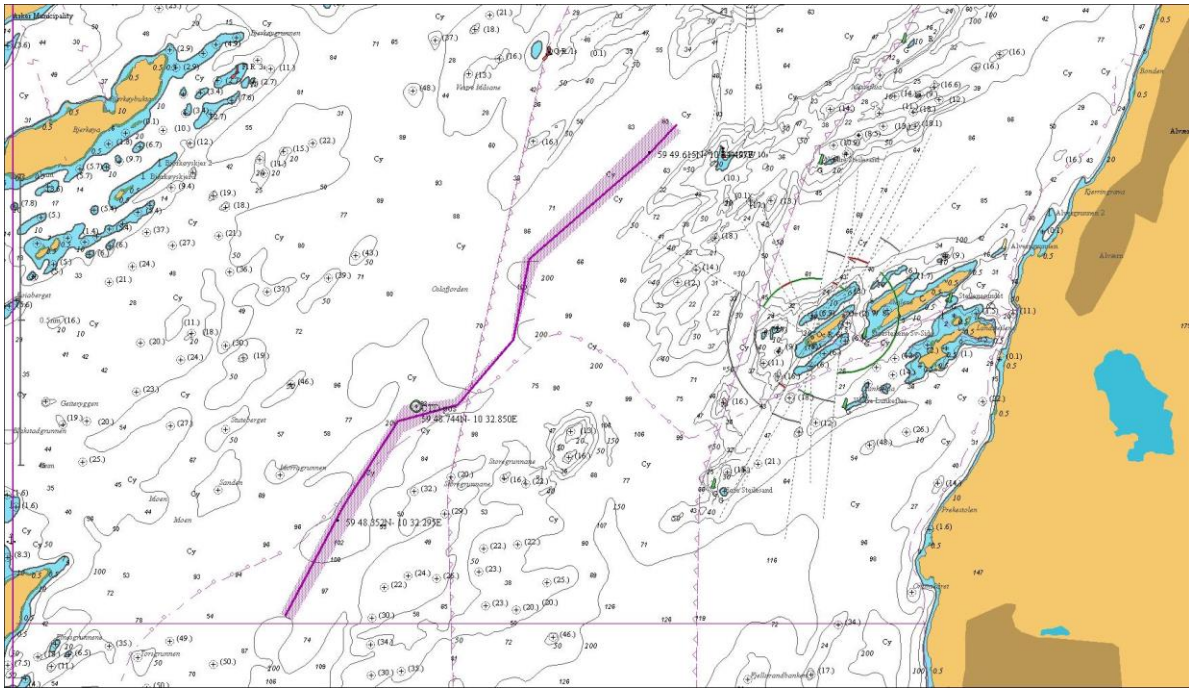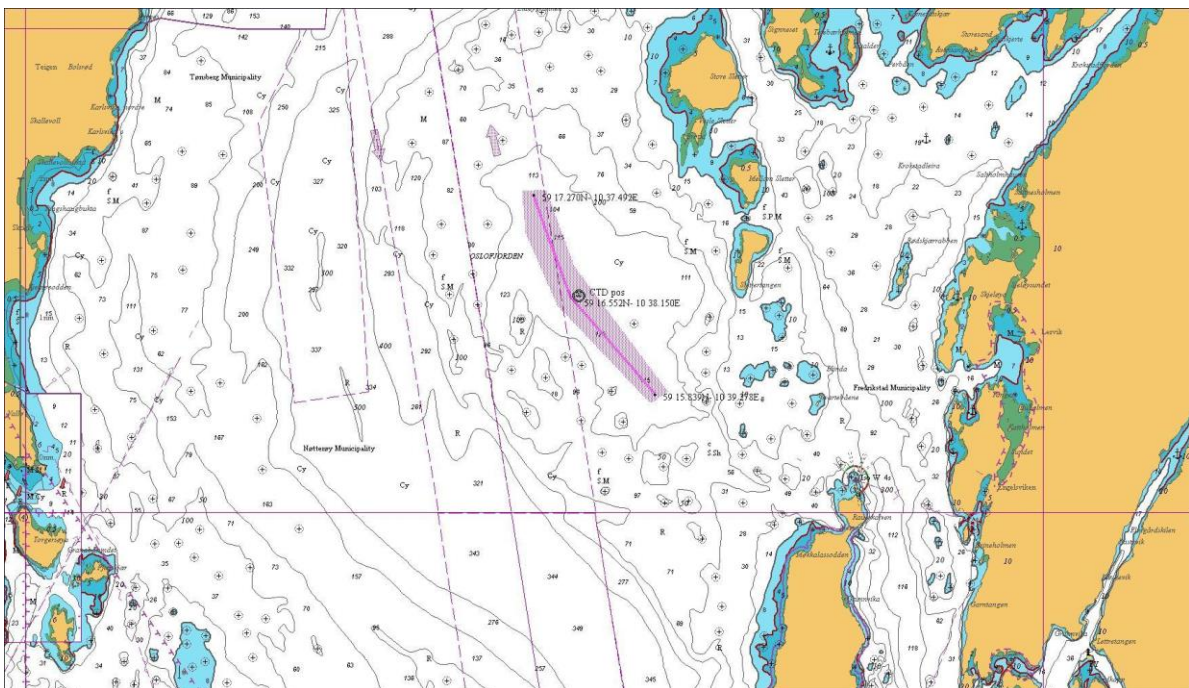**Figure 2.1** The trawling track Midtmeie indicated by the purple (inner Oslofjord).

Figure 2.2 The trawling track Travbanen indicated by the purple (outer Oslofjord).

Table 2.1 The number of male and female fish sampled from each location.

	Female	Male
Inner Oslofjord	19	21
Outer Oslofjord	24	15
Total	43	36

Table 2.2 Somatic weight and length of cod sampled from the inner Oslofjord. Values given are minimum (min), mean, median and maximum (max). Somatic weight is given in grams (g) and total length is given in centimeters (cm).

		Somatic weight			
		Min	**Mean**	**Median**	**Max**
Inner Oslofjord	**Males**	501	888	845	1809
	Females	320	834	893	1233
	Total	320	861	878	1809
Outer Oslofjord	**Males**	365	690	673	1078
	Females	519	1014	899	2329
	Total	365	852	814	2329
		Body length			
Inner Oslofjord	**Males**	39	46	46	60
	Females	36	46	47	54
	Total	36	46	46	60
Outer Oslofjord	**Males**	37	44	44	52
	Females	40	50	49	64
	Total	37	48	47	64

2.2 Age and physiological factors

Age was determined by the method described by Williams & Bedford (1974). As the fish is aging, rings of two different characters, depending on the season, form in the otoliths. These rings can be counted to determine age of the fish. During periods of rapid growth (spring and summer) white, opaque zones form while during periods of slow growth (late autumn and winter) grey, hyaline zones form. The opaque zones are wider than the hyaline zones. The otoliths were prepared by breaking them in half across the nucleus and sanding them to achieve a smooth surface. One half of the otolith was burnt with a gas burner until golden, approximately for five seconds. A drop of glycerol was added to the surface to increase the contrast between hyaline and translucent zones. A dissecting microscope with a fiber optic light source was used to study and count the rings. Physiological factor was calculated as described in Schlenk *et al*. (2008). LSI defines the ratio between liver weight and somatic body weight and was calculated for each individual by the formula: LSI= (liver weight/somatic body weight)* 100. The somatic weight is the weight (g) of the fish excluding the organs. GSI defines the ratio between gonad weight and somatic body weight and was calculated for each individual by the formula: GSI= (gonad weight/somatic body weight)*

100. Fultons condition factor was calculated for each individual by the formula: K= (body weight/body length^3)* 100.

2.3 Tissue preparation

2.3.1 Isolation of leukocytes from blood

The isolation of leukocytes for the comet sssay (assay described in section 2.5) was carried out during the sampling process on the research vessel. Following blood sampling (described in section 2.1) 200 µL of each sample was aliquoted to new eppendorf tubes and 1 µL heparin (10 000 units/mL) was added. The samples were kept on ice until all samples were taken for that day and diluted 5 x in phosphate buffered saline (PBS) ethylenediaminetetraacetic acid (EDTA) (10% PBS, pH 7.4, 10 mM EDTA) containing heparin (1 µL/ mL PBS; 10 000 units/mL heparin).

The procedure to isolate of leucocytes from peripheral blood was based on a method described by Braun-Nesje *et al*. (1981), Ronneseth *et al*. (2007) and Sørensen *et al*. (1997). 500 µL of a Percoll (1.072 g/mL 1.5 M NaCl) was added in a 1.5 mL eppendorf tube. 0.5 mL of a second Percoll (1.055 g/mL 1.5 M NaCl) was carefully added on top as a second layer. Approximately 0.4 mL of the blood sample was carefully placed on top of the two percoll layers with a pasteur pipette before centrifugation at 400 x g for 40 minutes at room temperature. The leukocytes, present as a white band in the interface of the two percolls, were transferred to a new eppendorf tube using a Pasteur pipette. The number of cells was counted in a light microscope at 20 x objective using a Bürker-Türk counting chamber. The leukocytes were then diluted further to a final density of 200 000 cells per mL which in 25 µL agarose would give approximately 500 cells per gel. The samples were further processed for the comet assay (described in section 2.5)

2.3.2 Bile

Preparation of bile samples was done at the Norwegian Institute for Water Research (NIVA) with the purpose of identifying and analyzing relative levels of PAH metabolites present in the bile through High-Performance Liquid Chromatography (HPLC) analysis (described in

section 2.6). The preparation of samples was conducted according to the method described by Krahn *et al*. (1992) and described in further detail by Grung *et al*. (2009).

The bile samples were thawed on ice. 10 µL of an internal standard (triphenylamine) and 20 µL of the bile sample was weighed separately and mixed together in an eppendorf tube followed by the addition of 50 µL distilled water. To hydrolyze the bile samples 20 µL of the enzyme β-glucuronidase/arylsulfatase was added followed by incubation at 37ºC for 60 minutes using a heating cabinet. After incubation 200 µL of methanol was added to the samples which were then centrifuged at 13 000 x g for 10 min at room temperature. Two reference samples, a high and a low concentration, were given the same treatment as the other samples to be included in the HPLC analysis. The supernatant was transferred to HPLC tubes and kept at -20ºC until HPLC analysis. A negative sample control was also included with four replicates.

2.3.3 Liver

The purpose of preparing liver samples was to isolate microsomes for analysis of EROD activity (described in section 2.8) and relative CYP1A concentration (described in section 2.7). The liver samples were prepared according to the method described by Dignam (1990). For each liver sample approximately 1 g was weighed on ice and put in homogenizing tubes where ice cold homogenizing buffer (0.1 M phosphate buffer, pH 7.8, containing 0.15 M KCl, 1 mM dithiotreitol and 5% glycerol) was added immediately to a total volume of 5 mL. The samples were homogenized on ice with a Potter- Elvehjem Teflon homogenizer moving the teflon pestle up and down slowly at 1500 rpm. The homogenate was transferred to centrifugation tubes and centrifuged at 10 000 x g for 30 minutes at 4ºC. The supernatant was transferred to new centrifugation tubes with a Pasteur pipette, avoiding the fat layer on top. The pellet comprising of cell debris was discarded. The supernatant containing cytosol and microsomes was mixed well and further centrifuged at 100 000 x g for 60 minutes at 4ºC to separate microsomes from cytosol. The resulting supernatant was aliquotted into 1.5 mL eppendorf tubes and frozen at -80ºC for possible further analysis. The remaining pellet consisting of microsomes was resuspended in 0.5 mL microsomal buffer (0.1 M phosphate buffer, pH 7.8, containing 20% glycerol) and further homogenized using a Ten Broeck Homogenizer where the glass pestle was slowly moved up and down 10 times. The homogenate was aliquotted into 1.5 mL eppendorf tubes and frozen at -80ºC for future

analysis of EROD activity, CYP1A concentration (Enzyme-linked immunosorbent assay (ELISA)) and total protein concentration (described in section 2.4).

2.3.4 Muscle

The purpose of muscle preparation was to further analyze the tissue for AChE activity (described in section 2.10). The procedure is based on the method first described by Ellman *et al*. (1961) and later modified by Herbert *et al*. (1995). After the samples were thawed on ice, approximately 1 g tissue was weighed and transferred to centrifugation tubes following immediate addition of ice cold potassium phosphate buffer (0.1 M, pH 7.2) to a total volume of 5 mL. The samples were minced and homogenized by a PRO 200 Ultra-Turrax homogenizer using a saw toothed, 10 mm x 115 mm sized generator at a speed of 18 000- 24 000 rpm. The homogenate was then centrifuged at 10 000 x g for 10 minutes at 4ºC. The supernatant was transferred to a 1.5 mL eppendorf tube, mixed well and aliquoted to be frozen at -80ºC for further analysis of protein concentrations (section 2.4) and AChE activity.

2.4 Protein analysis

Liver, muscle and blood tissue were analyzed for protein in order to standardize results from the biological analyses. Total protein could be detected with absorbance measurement due to a blue color development resulting from the reaction between the proteins, an alkaline copper tartrate solution and Folin reagent. The analysis was conducted as described by Lowry *et al*. (1951). Each tissue was tested with five-six samples using several dilutions in order to find the appropriate concentration that would match the standard curve. A dilution factor of 20 x for liver and muscle while 40 x for blood were the best suited concentrations. The samples were thawed on ice, diluted in ice cold Tris buffer (0.1 M, pH 8) and plated out on a flat bottom 96-well microtiter plate (Nunc) in triplicates with 10 µL in each well. The standard was diluted in Tris buffer (0.1 M, pH 8) to five different concentrations (1.5, 1, 0.8, 0.4 and 0.2 mg/mL) from bovine serum albumin (BSA). A stock solution of BSA (15 mg/mL) was made in advance of the analysis and kept at -80˚C. The standard, blank and a reference samples were plated out in triplicates with 10 µL in each well. The following step consisted of the addition of 25 µL alkaline copper tartrate solution (reagent A) and 200 µL diluted Folin reagent (reagent B). Reagent B was added using a multi-channel pipette. The samples were left to incubate in room temperature for 15 minutes before the absorbance was read at 750 nm

using a Synergy MX (BioTek) plate reader with Gen5 software. The standard curve was constructed using linear regression in Excel and applied to calculate the protein concentration in each sample.

2.5 DNA damage in blood leukocytes

Single strand DNA breaks were measured using the alkaline comet assay as described by Singh *et al*. (1988) and according to guidelines adjusted for usage of a plate reader by Tice *et al*. (2000). The preparation of blood samples was as described in section 2.3.1. The remainder of the method can be divided into three main steps: the first was conducted on the research vessel and consisted of suspending the samples in agarose and setting them onto gelbond films followed by exposure to the experimental hydrogenperoxide (H_2O_2) concentrations; the second step was conducted at the Norwegian Institute of Public Health and consisted of horizontal electrophoresis of the gelbond films; the third and final step was to stain the DNA by SybrGold followed by the scoring of the films using fluorescence microscopy. This final step was conducted at the University of Oslo.

All work was performed in dull light.

Low melting point (LMP) agarose (75 mg LMP agarose in 10 mL PBS containing 10 mM EDTA) was heated to the boil in a glass beaker using a microwave until dissolved, aliquotted in eppendorf tubes and cooled to 37ºC using a heating block. 25 µL of each sample was suspended in 225 µL LMP agarose, mixed thoroughly and carefully pipetted onto each of six gelbond films (9.5 x 6.7 cm, cut in lower right corner, labeled by using a diamond pen) as 25 µL droplets. The gelbond films were resting on pre-chilled aluminum cooling plates. After the gels had dried, each sample underwent three different treatments with two replicates; exposure to a high concentration of H_2O_2 (300 µM in PBS EDTA), a low concentration of H_2O_2 (5 µM in PBS EDTA) and a control. The duration of exposure was 15 minutes. The films were then rinsed in distilled water and stored on lysis buffer (44.5 mL lysis stock solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma base, 1% Triton x-100, pH 10), 5 mL distilled water, 0.5 mL Triton x-100), one set of replicates for one week and the second set of replicates for three weeks in plastic boxes at 4ºC. The lysis stock solution was premade and stored at room temperature, while the lysis working solution was made fresh and refrigerated before use.

Prior to the horizontal electrophoresis the films were rinsed briefly in electrophoresis buffer (400 mL stock solution (300 mM NaOH, 1 mM EDTA) in 3600 mL distilled water added 6 mL HCl (37%), pH 13.2). The working solution was made fresh and refrigerated before use. The gelbond films were then transferred to the electrophoresis chamber, located inside a refrigerator, containing 1.4 L of the same buffer. The films were left in the chamber for 20 minutes before horizontal electrophoresis was run for 20 minutes, at 25 V and 0.8 A in a circulating buffer system. The gelbond films were rinsed twice in neutralizing buffer (0.4 M Trizma base, pH 7.5), first for five minutes and then for a further 10 minutes in fresh buffer. Subsequently the gelbond films were rinsed briefly in distilled water before being rinsed for five minutes in 96% ethanol and transferred to fresh 96% ethanol where they were left over night before air-dried. The electrophoresis chamber held four gelbond films per run and the gelbond films were chosen randomly each time.

The DNA was stained for 20 minutes in a plastic box with 50 mL Tris-EDTA (TE) buffer (0.5 M Trizma buffer, 0.5 M EDTA, pH 8) containing 40 µL SybrGold (pre-diluted 10 x in dimethyl sulfoxide (DMSO)) using a rocking table. The diluted SybrGold was frozen as 50 µL aliquots at -20ºC until use. The staining process was conducted in a dark room. After staining the gelbond films were left over night in a moist box at 4ºC. The gelbond films were prepared for scoring by carefully placing them on a plexi glass plate adding droplets of distilled water, both between the plexi glass and the gelbond film and between the gelbond film and the cover slides. Air bubbles were carefully avoided. After staining the DNA appeared as "comets" in the gels consisting of the nucleus as the "head" and the fragmented/damaged DNA as the "tail". The comets were scored using a fluorescence microscope with a 20 x objective, illuminating the cells with an excitation wavelength of 520 nm using an attached mercury lamp. The emission was detected using a 610 nm filter. The microscope was attached to a camera which in turn was connected to a computer running Comet Assay IV software used for scoring the comets. The DNA damage was scored as the fraction of the DNA present in the comet tail expressed as tail intensity. The aim was to score 50 cells per gel and a minimum of 30 cells. Scoring was performed in a random matter and cells close to the edge of the gel were avoided as advised by Tice *et al*. (2000). Abnormal and overlapping cells as well as background noise were also avoided however exceptions were made regarding background noise when the number of cells was otherwise too few. The gelbond films were scored in a random order.

2.6 Polycyclic aromatic hydrocarbons (PAH) metabolites in bile

Bile was analyzed for PAH metabolites using HPLC analysis. This was performed by Merete Grung at the Norwegian Institute for Water Research (NIVA). The HPLC analysis was conducted as described by Krahn *et al.* (1992) and modified by Grung *et al.* (2009). The PAH metabolites OH-Pyrene, OH- phenanthrene and OH-benzo[a]pyrene (OH-B[a]P) were determined by the excitation/emission wavelengths 346/384, 256/380 and 380/430 respectively using a Waters 2695 Separations Module with a 2475 fluorescence detector attached. The column used was a Waters PAH C18 (4.6 x 250 mm) containing 5 µm sized particles. The internal standard (triphenylamine) was determined at excitation/emission wavelengths 300/360. The mobile phase of the separation module consisted of a 0.05 M acetonitrile:ammonium acetate 40:60 gradient (pH 4.1) to 100% acetonitrile which was heated to 35ºC prior to injection of sample. The mobile phase had a flow of 1 mL/min. Two diluted reference samples containing bile as well as blank, five replicates of each, were included in the analysis.

2.7 Hepatic CYP1A levels

The amount of CYP1A protein in liver cells was measured using the ELISA assay as described by Goksøyr (1991). The samples were thawed on ice and diluted to a concentration of 10 µg/mL coating buffer (carbonate-bicarbonate buffer, 1 capsule in 100 mL distilled water). A volume of 100 µL coating buffer (blank), diluted samples and a reference sample were pipetted on to an EIA 96-well microtiter plate (Costar) in quadruplicates. The plate was covered with parafilm and incubated overnight at 4ºC.

Following incubation the plate was washed three times (2 x 10 seconds + 1 min) with Tris-tween buffered saline (TTBS) (20 mM Tris-buffer, pH 8.5, 0.5 M NaCl and 0.05% Tween 20) using a SkanWasher 300. 300 µL blocking solution (TTBS, 1% BSA) was added to each well followed by incubation at room temperature using a vortexer for 30-60 minutes. The plate was then washed three times with TTBS. 100 µL of primary antibody (rabbit anti-fish CYP1A antibody), diluted 1000 x in antibody buffer (TTBS with 0.1% BSA)) was added to each well. The plate was then incubated overnight at 4ºC. The plate was washed three times in TTBS. 100 µL of a secondary antibody (goat anti-rabbit IgG HRP, diluted 3000 x in antibody buffer) was pipetted to each well followed by incubation at 4ºC for six-eight hours. The plate was washed five times (4 x 30 seconds + 1 min) with TTBS. 100 µL color buffer (TMB plus,

ready-to-use-substrate) was pipetted to each well followed by incubation in a dark room at room temperature until color was developed (5-30 minutes). The reaction was stopped by adding 50 µL sulfuric acid (H_2SO_4, 1.5 M) to each well. The timing of this step was carefully noted. Absorbance was measured at 450 nm using a Synergy MX (BioTek) plate reader with Gen5 software. No standard curve was included as only relative measurements were needed.

2.8 Hepatic etoxyresorufin-*O*-deetylase (EROD) activity

EROD activity indicates the activity of CYP1A enzyme and was measured in liver microsomes (preparation of tissue described in section 2.3.3) according to the method described by Burke & Mayer (1974) and modified to using a plate reader by Eggens & Galgani (1992). Due to light sensitivity of 7-ethoxyresorufin and resorufin (standard) all work was performed in absence of direct light. The 7-ethoxyresorufin (0.2 mM in DMSO) and resorufin (0.01 mM in DMSO) were calibrated to 450 nm and 572 nm respectively before stock solutions (0.2 mM 7-ethoxyresorufin and 0.1 mM resorufin) were aliquotted and frozen at -20°C until used. Nicotinamide adenine dinucleotide phosphate (NADPH) (50 mM in 0.1 M potassium phosphate buffer, pH 8) was aliquotted and kept at -80°C until used. 7-ethoxyresorufin, resorufin and NADPH were thawed on ice along with samples which were diluted to 1.2 mg/mL potassium phosphate buffer (0.1 M, pH 8) (protein analysis described in section 2.4). The resorufin (standard) was diluted to 0.64 µM in potassium phosphate buffer in two steps. The first step included a 100 x dilution followed by another 15.5 x dilution. A dilution series was made from 0.64 µM including 0.32, 0.16, 0.08, 0.04, 0.02, and 0.01 µM in potassium phosphate buffer. The reaction solution was made by adding 0.75 mL 7-ethoxyresorufin to 50 mL of potassium phosphate buffer. A vortexer was used to homogenize the solution. The standard was plated out on a flat bottom black 96-well microtiter plate (Nunc) in duplicates with a volume of 275 µL per well. Eight replicates of potassium phosphate buffer (blank) and six replicates of diluted samples and a reference sample were plated out with a volume of 50 µL per well. In order to correct for quenching, half the sample replicates were spiked with 10 µL of 0.64 µM standard. 25 µL diluted NADPH (2.4 mM in potassium phosphate buffer) was added to the blanks and the samples followed by the addition of 200 µL reaction solution. The reaction was read through fluorescence (excitation at 530 nm, emission at 590 nm) over five minutes in eight steps with 39 second intervals, using a Synergy MX (BioTek) plate reader with Gen5 software.

2.9 Aminolevulinic acid dehydratase (ALA-D) activity in blood

The analysis was based on the method described by Hodson (1976) and later modified by Hylland (2004). Dilution buffer (100 mL of 0.1 M phosphate buffer (pH 7) and 1 mL Triton X-100 with distilled water added up to a total of 200 mL) and standard solution (porphobilinogen (PBG), 40 µg/ mL dilution buffer) was made in advance of the analysis. Dilution buffer was kept refrigerated at 4ºC and PBG was aliquotted and kept at -80ºC. δ-aminolevulinic acid (ALA)- reagent (33.5 mg in 50 mL dilution buffer), modified Ehrlichs reagent (175 mg mercury chloride to 3 mL distilled water, 10 mL 70% perchloric acid and acetic acid added up to a total of 55 mL) and precipitation buffer (2 g trichloroacetic acid, 1.35 g mercury chloride with distilled water added up to a total of 50 mL) were made fresh each day. The standard solution was thawed on ice along with the samples, which were added 500 µL dilution buffer and homogenized by a Ten Broeck Homogenizer in which the glass pestle was moved up and down 10 times. The homogenate was centrifuged at 10 000 x g at 4ºC for 15 minutes. The supernatant was transferred to new eppendorf tubes and diluted threefold in dilution buffer. Each sample was aliquotted into six eppendorf tubes of 50 µL each, while 100 µL was frozen at -80ºC for protein analysis (described in section 2.4). All work up until this step was performed on ice. Two hundred µL of dilution buffer was added to three of the replicates, while 200 µL of ALA-reagent was added to the remaining three. Both sets of solutions were held at a temperature of approximately 4ºC. The spike time was noted for each sample followed by immediate incubation at 25ºC for two hours. Each sample had 300 µL of precipitation buffer added exactly two hours later. This step was performed in a fume hood. The samples were left on the bench for at least five minutes followed by a five minute, room temperature centrifugation at 3000 x g. A dilution series was made from the standard solution to the concentrations of 40, 32, 16, 8, 4, 2 and 1 µg/mL dilution buffer. The standard dilution series was plated in duplicates of 150 µL each onto a flat bottom 96-well microtiter plate. 150 µL of each supernatant of the samples was added to a well, resulting in three replicates per sample for each of the two treatments. A reference sample was also included on each plate. For color development, 150 µL of modified Ehrlichs reagent was added to all wells. The plate was left for incubation at room temperature for 15 minutes followed by detection of absorbance at 550 nm using a Synergy MX (BioTek) plate reader with Gen5 software. Results were expressed in ng PBG/min/mg protein.

2.10 Acetylcholinesterase (AChE) activity in muscle

The procedure was conducted according to the method first described by Ellman *et al*. (1961) and later modified by Herbert *et al*. (1995). Potassium phosphate buffer (0.1 M, pH 7.2) was made in advance of the analysis and kept refrigerated at 4ºC, while AChE assay reagent (200 mL potassium phosphate buffer, 1.33 mL of 0.075 M acetylthiocholine iodide (ATC) in distilled water, 6.66 mL of 0.01 M dithiobisnitrobenzoic acid (DTNB) added 1.5 mg/mL sodium bicarbonate in potassium phosphate buffer) was made fresh. The samples were thawed on ice followed by a tenfold dilution with ice cold potassium phosphate buffer. The samples as well as buffer (blank) and a reference sample were plated out on a flat bottom 96-well microtiter plate in quadruplicates of 50 µL in each well. For color development 250 µL AChE reagent was added to each well. The plate was placed in a Synergy MX (BioTek) plate reader set at 25ºC where it was left for temperature adaptation for five minutes. The absorbance was read at 414 nm in five steps over 10 minutes with two minute intervals. The plate was shaken between each reading cycle (medium shake for five seconds) to keep the samples homogenous. The software used was Gen5. The average increase in optical density was calculated and expressed in mmol/min/mg protein.

2.11 Statistical analysis

All data were analyzed by the statistical software JMP 10 and graphically displayed using GraphPad Prism 6 and Excel. The data were analyzed for normal distribution by a Shapiro-Wilk test (Shapiro & Wilk 1965) and homogeneity of variance by Levene's test (Levene 1952). Two-way ANOVA was used on data which met these requirements (length, condition factor, EROD activity). Data which did not immediately meet these requirements were log transformed (Zar 1999). If this resulted in assumptions for parametric tests being met, two-way analysis of variance (ANOVA) was used (weight, LSI, OH- B[a]P, CYP1A protein level, AChE activity) (Zar 1999). For all other data (DNA damage, age, weight and length of 2-yearolds, GSI, OH-phenanthrene, OH-pyrene and ALA-D activity) the non-parametric Wilcoxon test was performed on untransformed data (Wilcoxon 1945). The statistical difference in DNA damage between weeks of storage on lysis buffer were tested by t-tests (Zar 1999) as were samples treated with 5 µM H_2O_2, while the other treatments (0 and 300 µM) were tested by the Wilcoxon test. To investigate possible relationships between measured variables Sperman's rank correlation tests were run (Spearman 1904). In order to

statistically test the difference in susceptibility to oxidative stress-linked DNA damage between the locations, regression lines were drawn based on five random values of the observed tail intensities from each treatment. The slope was determined for each of the five regression lines and the statistical difference between the two locations was tested by the Wilcoxon test. In the cases where a non-parametric test were run, differences by sex were tested initially for the two locations separately. Where statistical differences between sexes were found, male and female cod were compared between the two areas separately, while in cases of no difference by sex, male and female cod were pooled and statistically compared between the two areas. For data that did not show statistical differences between sexes or interactions between sex and location (DNA damage, OH-pyrene in bile, CYP1A concentration, EROD activity and ALA-D activity) male and female cod were pooled for graphical representation.

3 Results

3.1 Optimization of the comet assay

There was a significant difference in DNA damage between samples stored on lysis buffer for one week and three weeks respectively apart from control samples from inner Oslofjord (Wilcoxon tests and t-tests, $p<0.05$). Storage on three weeks resulted in more DNA strand breaks than storage for only one week (Figure 3.1 and 3.2).
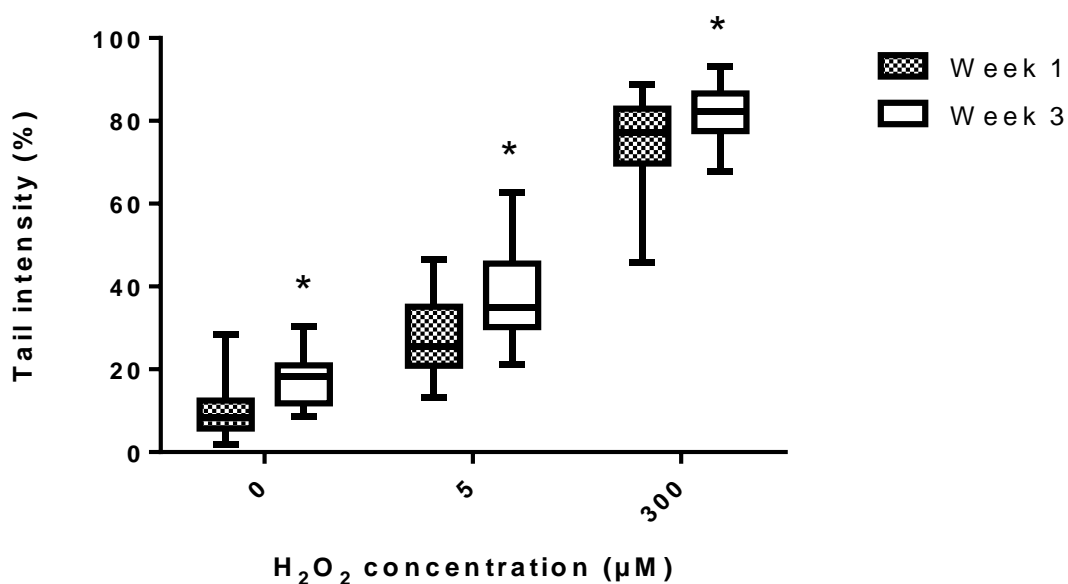
Figure 3.1 DNA damage in cod samples from the outer Oslofjord, stored on lysis buffer for one and three weeks; median, quartiles, minimum and maximum. The symbol * indicates a p-value below the significance level ($p<0.05$).
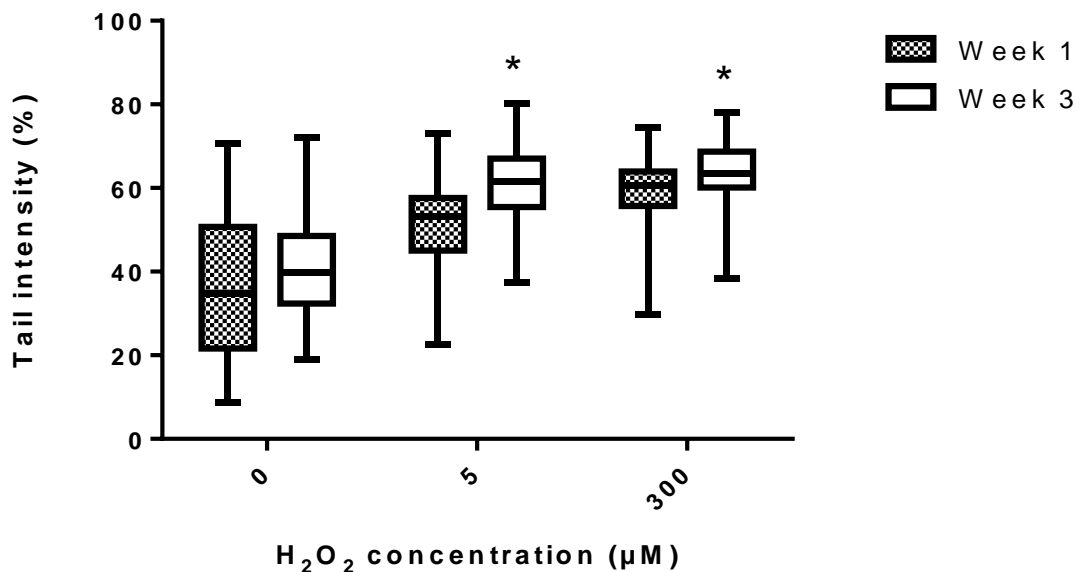
Figure 3.2 DNA damage in cod samples from the inner Oslofjord, stored on lysis buffer for one and three weeks; median, quartiles, minimum and maximum. The symbol * indicates a p-value below the significance level ($p < 0.05$).

3.2 Age, length and weight

There was a significant difference in age of cod between the locations (Wilcoxon test, $p < 0.0001$, Table 3.1). Cod of similar size range collected from the inner Oslofjord were older than cod collected from the outer Oslofjord (Wilcoxon test, $p < 0.0001$). There was no significant difference in age between males and females within the inner Oslofjord (Wilcoxon test, $p = 0.4$) or outer Oslofjord (Wilcoxon test, $p = 0.2$).

For two-year old cod there was no significant difference in somatic body weight between male cod from the two locations (Wilcoxon, $p = 0.9$) or female cod (Wilcoxon, $p = 0.5$). Female cod collected from the outer Oslofjord was significantly heavier than males (Wilcoxon, $p = 0.04$). There was no such difference in somatic body weight between males and females collected in the inner Oslofjord ($p = 1$). The same statistical outcome was reflected for body length. There were too few individuals to statistically compare one-year olds and three-year olds collected from the two locations. There were no four- and five year olds collected in the outer Oslofjord to compare with the inner Oslofjord (Table 3.1).

In a statistical comparison using all age classes there was no significant difference in body length (two-way ANOVA, p=0.6) or somatic body weight (two-way ANOVA on log transformed data, p=0.6) between the locations. Females were significantly longer than males (two-way ANOVA, p=0.02). A significant difference between males and females was not seen for bodyweight (two-way ANOVA on log transformed data, p=0.07). There was also a significant interaction between sex and location. The difference between males and females was larger in outer Oslofjord than inner Oslofjord. This was true for both body length (two-way ANOVA, p=0.03) and weight (two-way ANOVA on log transformed data, p=0.008). The average weight and length within each age class is viewed in Table 3.2 and 3.3.

Table 3.1 Number of individuals of each age class (years), sex and location.

Age	Outer Oslofjord			Inner Oslofjord		
	Male	**Female**	**Total**	**Male**	**Female**	**Total**
1	-	3	3	-	1	1
2	13	19	32	4	4	8
3	1	1	2	8	6	14
4	-	-	-	4	7	11
5	-	-	-	5	1	6

Table 3.2 Length (cm) of male and female cod from all age classes, collected in the inner and outer Oslofjord. Median; quartile.

Age	Outer Oslofjord				Inner Oslofjord			
	Male		Female		Male		Female	
	Length	N	Length	N	Length	N	Length	N
1	-	0	53; 51-56	3	-	0	47	1
2	43; 42-47	13	48; 43-53	19	43; 41-47	4	45; 37-52	4
3	47	1	51	1	44; 42-46	8	44; 42-44	6
4	-	0	-	0	46; 45-53	4	49; 43-50	7
5	-	0	-	0	47; 47-56	5	54	1

Table 3.3 Somatic body weight (g) of male and female cod from all age classes, collected in the inner and outer Oslofjord. Median; quartiles.

Age	Outer Oslofjord				Inner Oslofjord			
	Male		Female		Male		Female	
	Weight	N	Weight	N	Weight	N	Weight	N
1	-	0	1309; 1066-1335	3	-	0	863	1
2	657; 529-853	13	867; 660-1062	19	637; 570-847	4	736; 360-1150	4
3	726	1	1182	1	726; 679-908	8	711; 610-905	6
4	-	0	-	0	881; 823-1287	4	936; 731-1051	7
5	-	0	-	0	1030; 936-1472	5	1106	1

3.3 Physiological biomarkers

There was a significant difference in LSI between the locations (two-way ANOVA on log transformed data, p<0.0001). Cod from the inner Oslofjord had a higher LSI than cod from the outer Oslofjord (Figure 3.3). There was a significant difference between males and females (two-way ANOVA on log transformed data, p=0.005). Females had higher LSI than males (Figure 3.3) No significant interaction was found between sex and location (two-way ANOVA on log transformed data, p=0.7)

Figure 3.3 Log LSI in male and female cod samples in the inner and outer Oslofjord; median, quartiles, minimum and maximum. N= sample size.

GSI was significantly different for male cod between the inner and outer Oslofjord (Wilcoxon test, p<0.0001). Males from inner Oslofjord had higher GSI than males from the outer Oslofjord (Figure 3.4). There was also a significant difference in GSI for female cod between the locations (Wilcoxon test, p=0.0003) with female cod from the inner Oslofjord having a higher GSI than female cod from the outer Oslofjord (Figure 3.4). There was a significant difference between males and females from outer Oslofjord (Wilcoxon test, p<0.0001). Females had a higher GSI than males (Figure 3.4). No such difference was found between males and females from inner Oslofjord (Wilcoxon test, p=0.5).

Figure 3.4 GSI in male and female cod collected in the inner and outer Oslofjord; median, quartiles, minimum and maximum. N= sample size.

The condition factor was significantly different between cod from the two locations (Two-way ANOVA, p=0.001) with cod from the inner Oslofjord having a higher condition factor than cod from the outer Oslofjord (Figure 3.5). There was no significant difference between males and females (Two-way ANOVA, p=0.4), however there was a significant interaction between sex and location (Two-way ANOVA, p=0.006).

Figure 3.5 The condition factor of male and female cod collected in the inner and outer Oslofjord; median, quartiles, minimum and maximum. N= sample size.

3.4 DNA damage in blood leukocytes

There was a significant difference in the natural state DNA damage between cod from the two locations (t-test on log transformed data, p<0.0001). Cod from the inner Oslofjord had a higher DNA damage level than cod from the outer Oslofjord (Figure 3.6). There was a significant difference in DNA damage between cells exposed to H_2O_2 (5 µM and 300 µM) and control cells for both inner and outer Oslofjord (Wilcoxon test, p<0.0001). There was no significant difference between males and females in the control group from the two areas (t-tests, p>0.05). The slope derived from five random selected replicates within each concentration of H_2O_2 was significantly steeper in cod from outer Oslofjord than cod from inner Oslofjord (Wilcoxon test, p=0.009).
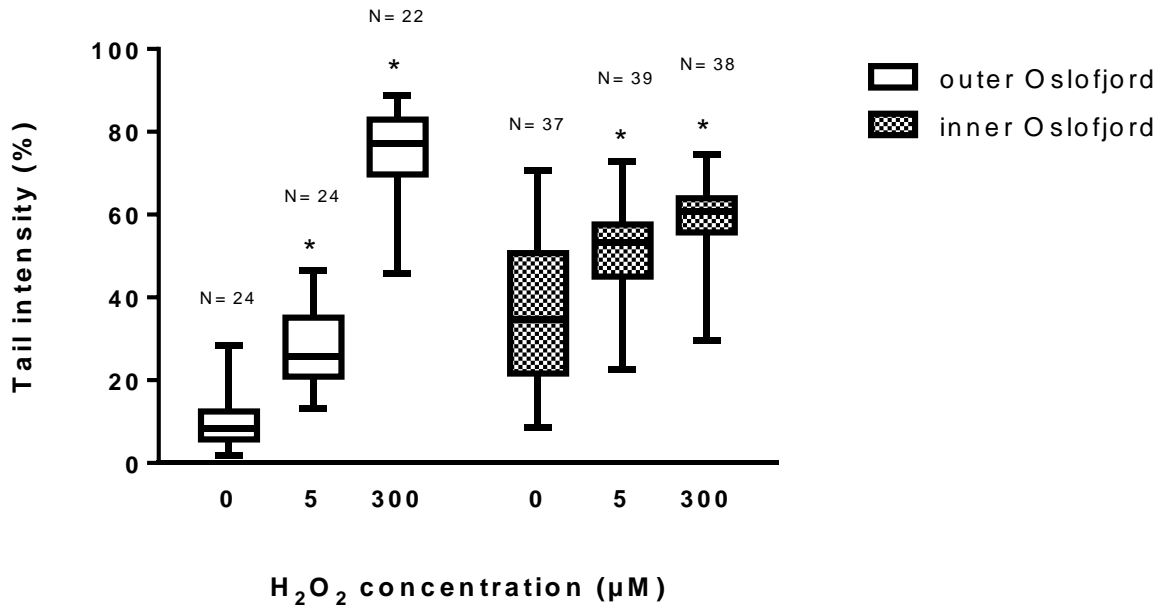
Figure 3.6 DNA damage in leukocytes from cod collected in the inner and outer Oslofjord exposed to 0, 5 and 300 μM H_2O_2; median, quartiles, minimum and maximum. N= sample size. The symbol * indicates a p-value below the significance level (p<0.05).

3.4 PAH metabolites in bile

There was a significant difference in concentration of OH-B[a]P in bile between cod the two locations (two-way ANOVA on log transformed data, p=0.03), in which cod from inner Oslofjord had the higher concentration (Figure 3.7). There was no significant difference between males and females (two-way ANOVA on log transformed data, p=0.4) however there was a significant interaction between sex and location (two-way ANOVA on log transformed data p=0.03) as also indicated by Figure 3.7. Sample number 33 (male from outer Oslofjord) was included in the analysis, even though the log concentration of -0.21 (0.61 ng/g) was slightly below the detection limit at 0.75 ng/g.
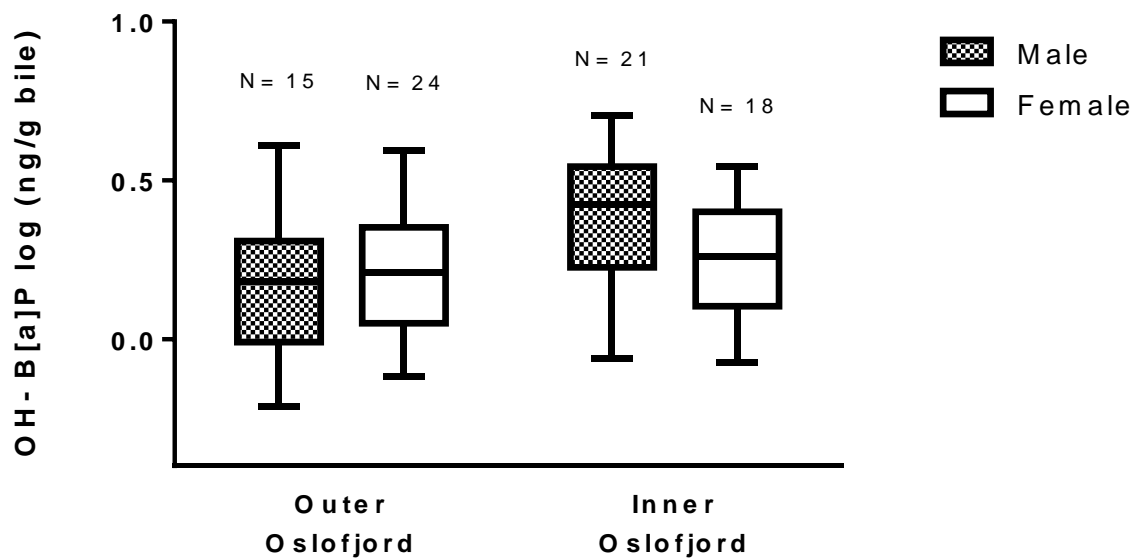
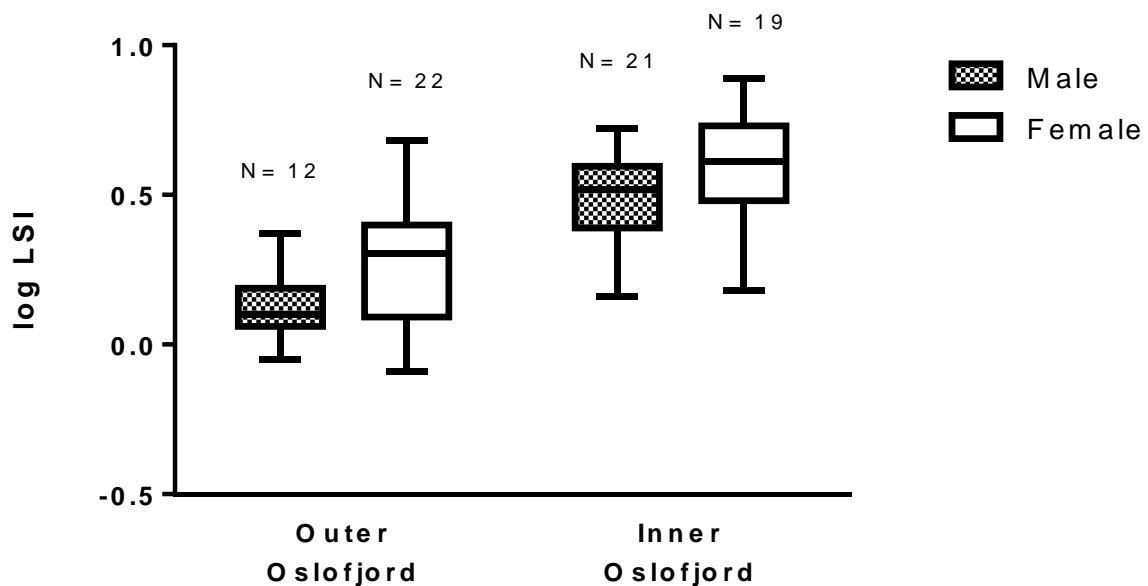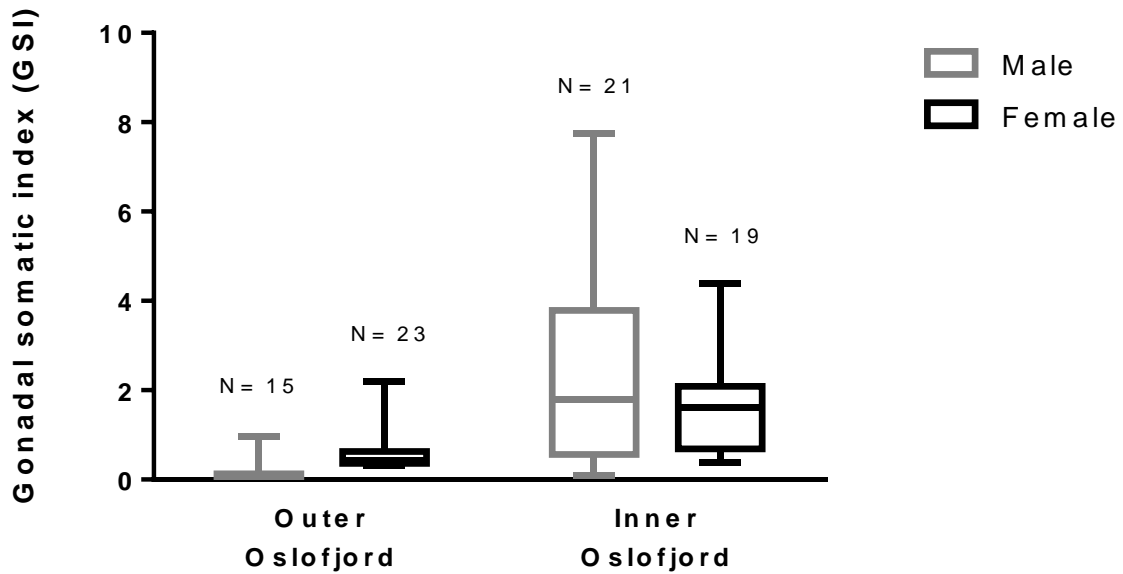**Figure 3.7** Concentration of log OH-B[a]P in bile from male and female cod collected in the inner and outer Oslofjord; median, quartiles, minimum and maximum. N= sample size.

There was a significant difference in concentration of OH-phenanthrene in bile from males between the two locations (Wilcoxon test, $p<0.0001$) as well as between females ($p<0.0001$). Cod collected in the inner Oslofjord had a higher concentration than cod collected in the outer Oslofjord (Figure 3.8). There was a significant difference between males and females from inner Oslofjord (Wilcoxon test, $p= 0.01$) where male cod had the higher concentration (Figure 3.8). No significant difference was found between male and female cod from the outer Oslofjord (Wilcoxon test, $p=0.9$). Sample number 35 (female from outer Oslofjord) was included in the analysis even though it had a concentration of 0.97 ng/g, which was just below the detection limit at 1 ng/g.

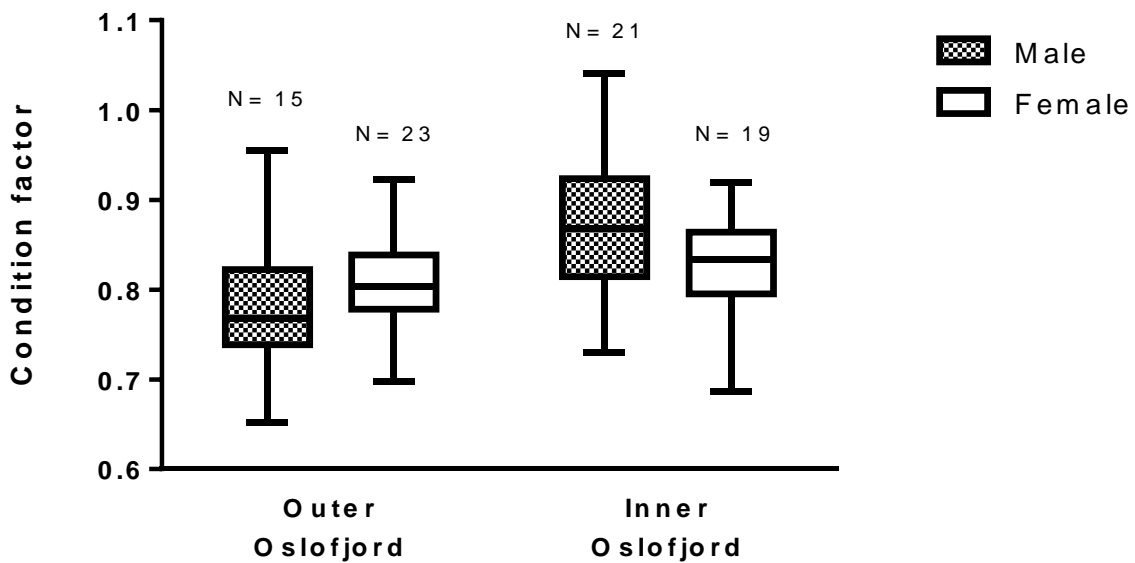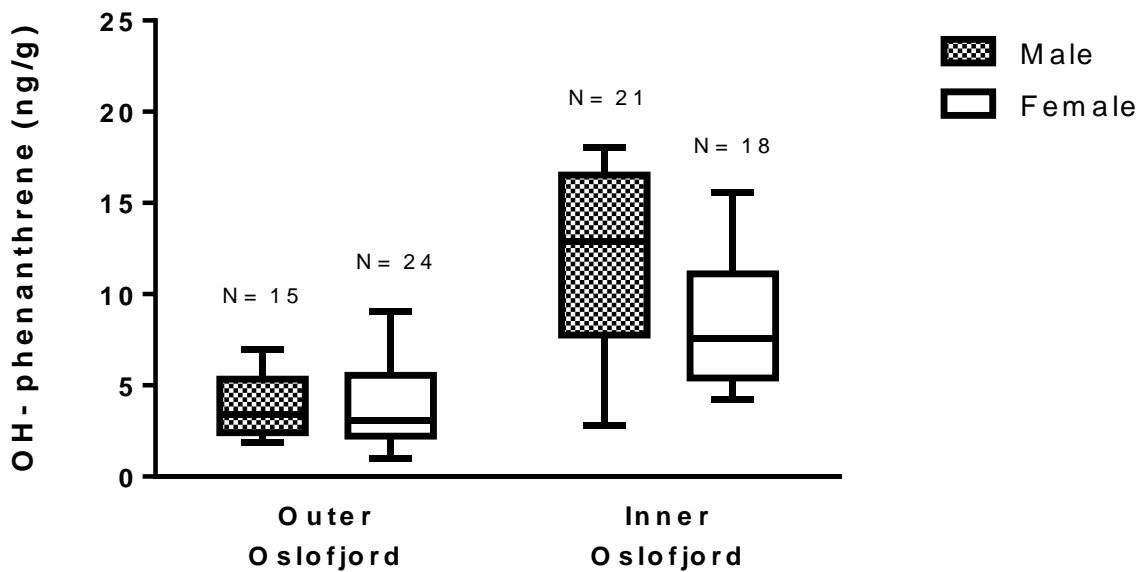**Figure 3.8** Concentration of OH-phenantrene in the bile of male and female cod collected in the inner and outer Oslofjord; median, quartiles, minimum and maximum. N= sample size.

There was a significant difference in the concentration of OH-pyrene in the bile from cod sampled at the two locations (Wilcoxon test, $p<0.0001$). Bile from cod caught in the inner Oslofjord had a higher concentration than bile of cod from outer Oslofjord (Figure 3.9). The concentration of OH-phenanthrene was significantly higher in bile of males than females from inner Oslofjord (Wilcoxon test, $p=0.01$). No such difference was found between males and females in either inner or outer Oslofjord (Wilcoxon test, $p=0.8$).
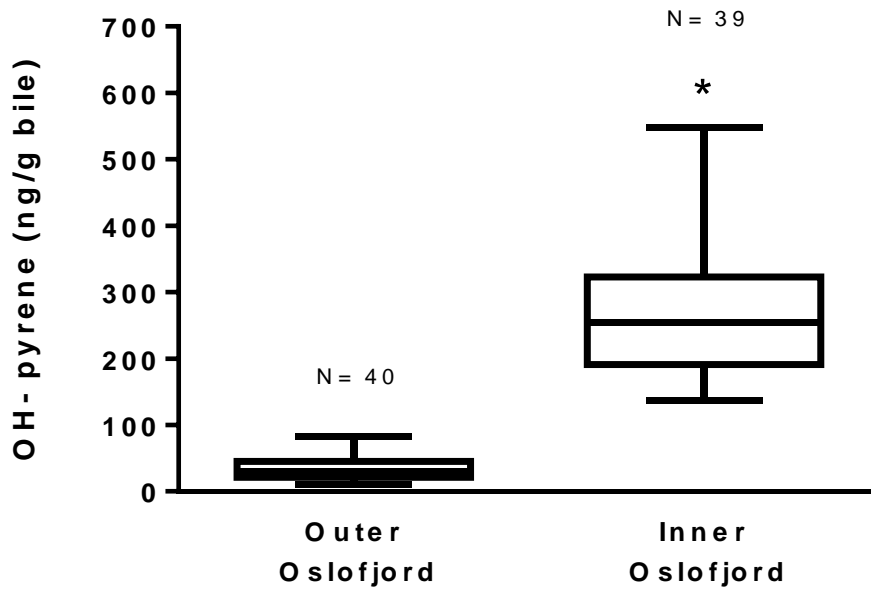
Figure 3.9 Concentration of OH-pyrene in bile from cod collected in the inner and outer Oslofjord; median, quartiles, minimum and maximum. N= sample size. The symbol * indicates a p-value below the significance level (p<0.05).

3.5 Hepatic CYP1A concentration

There was a significant difference in hepatic CYP1A between cod collected at the two locations (two-way ANOVA on log transformed data, p<0.0001). Cod caught in the inner Oslofjord had a higher enzyme concentration than cod caught in the outer Oslofjord (Figure 3.10). There was no significant difference between males and females (two-way ANOVA on log transformed data, p=0.4) and no significant interaction between sex and location (two-way ANOVA on log transformed data, p=0.3).
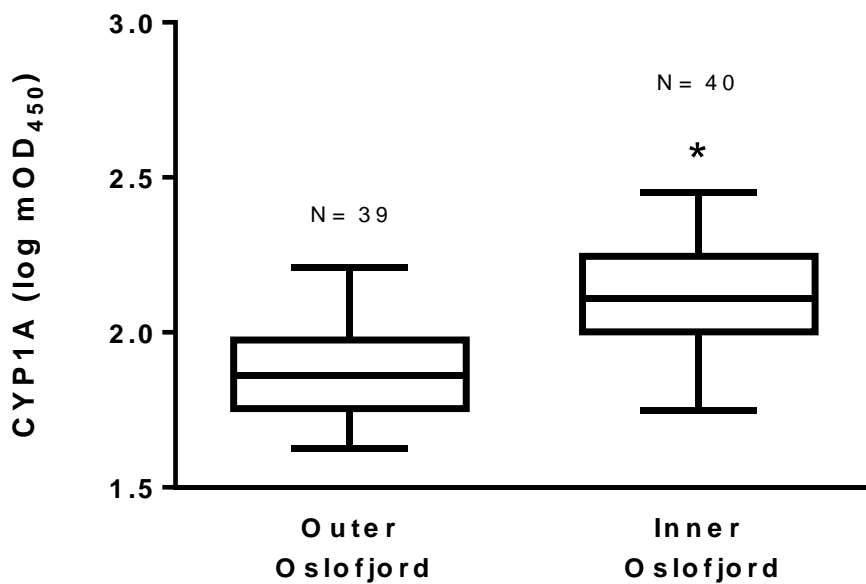
Figure 3.10 Hepatic log CYP1A concentration in liver of cod collected in the inner and outer Oslofjord; median, quartiles, minimum and maximum. N= sample size. The symbol * indicates a p-value below the significance level (p<0.05).

3.6 Hepatic etoxyresorufin-*O*-deethylase (EROD) activity

There was a significant difference in hepatic EROD activity in cod between the two locations (Two-way ANOVA, p=0.0008). Cod from inner Oslofjord had a higher enzyme activity than cod from outer Oslofjord (Figure 3.11). No significant difference was found for either sex or interaction between sex and location (Two-way ANOVA, p=0.4).
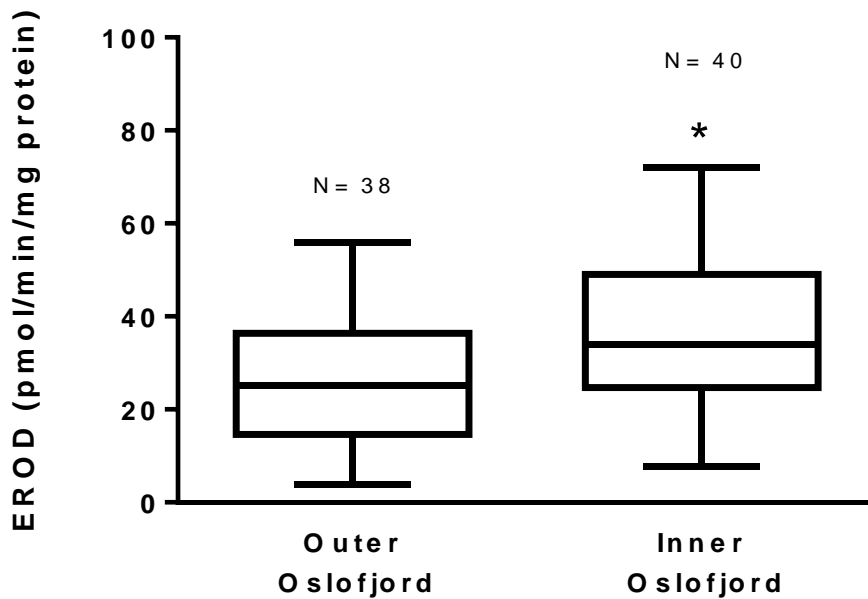
Figure 3.11 Hepatic EROD activity in cod collected from inner and outer Oslofjord; median, quartiles, minimum and maximum. N= sample size. The symbol * indicates a p-value below the significance level (p<0.05).

3.7 Aminolevulinic acid dehydratase (ALA-D) activity in blood

There was a significant difference in ALA-D activity in blood of cod between the two locations (Wilcoxon test, p= 0.01). Cod from the inner Oslofjord had a lower enzyme activity than cod from the outer Oslofjord (Figure 3.12). No such difference was found between males and females from the inner Oslofjord (Wilcoxon test, p= 0.8) or from the outer Oslofjord (Wilcoxon test, p= 0,7). There was larger variability of enzyme activity in cod from the inner Oslofjord compared to cod from the outer Oslofjord (Figure 3.12).
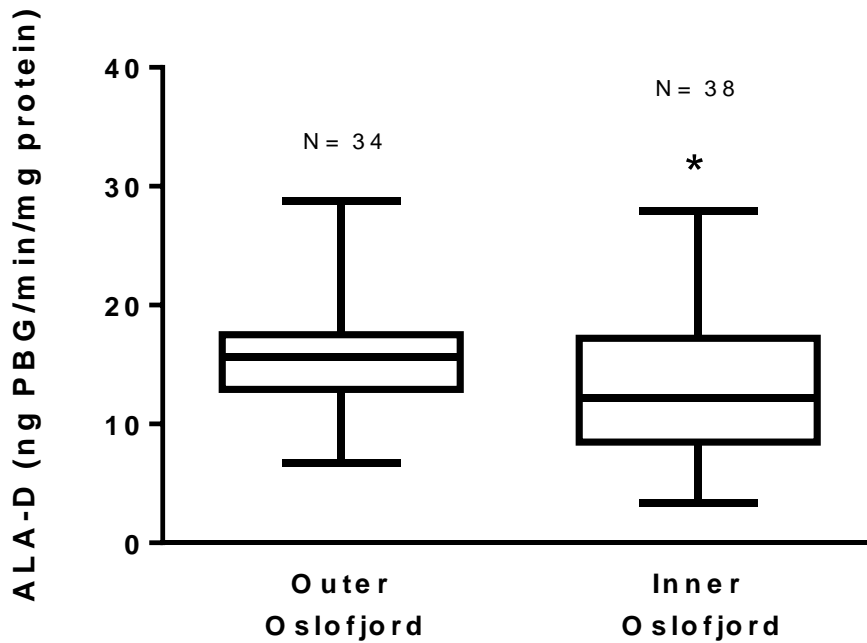
Figure 3.12 ALA-D activity in blood from cod collected in inner and outer Oslofjord; median, quartiles, minimum and maximum. N= sample size. The symbol * indicates a p-value below the significance level (p<0.05).

3.8 Acetylcholinesterase (AChE) activity in muscle

There was a significant difference in AChE activity between cod from the two locations (two-way ANOVA on log transformed data, p=0.02) and also between males and females (two-way ANOVA on log transformed data, p=0.007). Cod from the inner Oslofjord had a lower enzyme activity than cod from the outer Oslofjord and female cod had lower activity than male cod (Figure 3.13). There was no significant interaction between location and sex (two-way ANOVA on log transformed data, p=0.7).
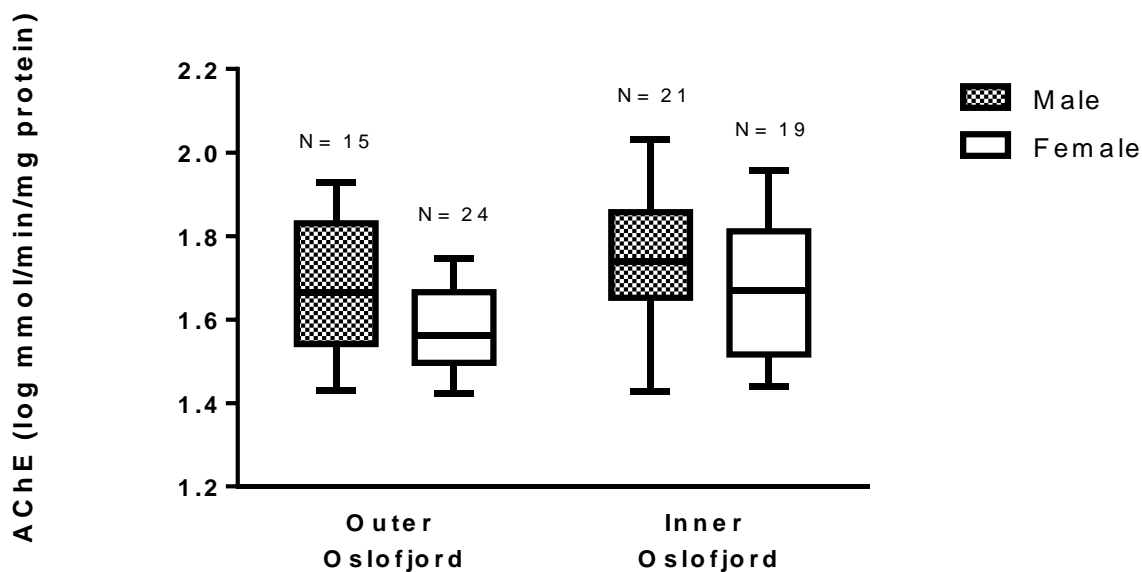
Figure 3.13 Log AChE activity in muscle from male and female cod sampled in the inner and outer Oslofjord; median, quartiles, minimum and maximum. N= sample size.

3.9 Correlations between factors

In order to investigate possible correlation between all biomarkers used in this study, as well as with the age, length and weight of each fish, the non-parametric Spearman's correlation analysis was done on the data. A separation by sex and location was done on comparisons where one or both indices varied between males and females, otherwise a separation was by location only. The variables that correlated in both locations, but only for females were GSI by LSI (positive correlation) (Spearman's correlation analysis, Table 3.4 and 3.5). Variables that correlated in both locations in females and/or males were OH-B[a]P by OH-pyrene, OH-phenanthrene by OH-pyrene, OH-B[a]P by OH-phenanthrene (positive correlations) (Spearman's correlation analysis, Table 3.4 and 3.5). Variables that correlated in female cod, only from the outer Oslofjord, were CYP1A concentration by weight and length, OH-phenantrene by CYP1A concentration and ALA-D activity by LSI (negative correlations) (Spearman's correlation analysis, Table 3.4). Variables that correlated in male cod, only from outer Oslofjord, were OH-pyrene by GSI, DNA damage by LSI, AChE activity by CYP1A concentration (negative correlations) (Spearman's correlation analysis, Table 3.4). Variables that correlated in female cod, only from inner Oslofjord, were GSI by age and condition factor by LSI (positive correlations) (Spearman's correlation analysis, Table 3.5). Variables that

correlated in male cod, only from inner Oslofjord, were AChE activity by GSI (positive correlation), EROD activity by weight and condition factor, AChE activity by weigh and length, ALA-D activity by OH-pyrene as well as by OH-B[a]P (negative correlations) (Spearman's correlation analysis, Table 3.5).

There was a significant positive correlation between EROD activity and CYP1A concentration in cod collected from inner Oslofjord. This positive correlation was also seen in cod collected from outer Oslofjord, although with a relatively high p-value (p=0.05) (Spearman's correlation analysis, Table 3.6). There was a negative correlation ALA-D activity and OH-pyrene in cod collected from the inner Oslofjord (Spearman's correlation analysis, Table 3.6).

Table 3.4 Spearman's correlation analysis for cod collected in the outer Oslofjord. The table shows only correlations that were statistically significant in males and/or females within one location. Spearman's ρ= Spearman's rank correlation coefficient.

Variable	By variable	Male		Female	
		Spearman's ρ	P-value	Spearman's ρ	P-value
CYP1A concentration	length	0.3935	0.2	-0.4792	0.02*
CYP1A concentration	weight	0.4093	0.1	-0.4670	0.02*
GSI	LSI	0.1547	0.6	0.4522	0.03*
OH-phenanthrene	CYP1A concentration	-0.2306	0.4	-0.4565	0.03*
OH-phenanthrene	OH-pyrene	0.3464	0.2	0.8142	<0.0001*
OH-B[a]P	OH-pyrene	0.6571	0.01*	0.6849	0.0002*
OH-B[a]P	OH-phenanthrene	0.3495	0.2	0.8016	<0.0001*
OH-pyrene	GSI	-0.7574	0.001*	-0.1317	0.6
ALA-d activity	LSI	-0.2755	0.5	-0.5108	0.03*
DNA damage	LSI	-0.8095	0.01*	-0.0022	1
AChE activity	CYP1A concentration	-0.7256	0.002*	-0.2520	0.3

* indicates a p-value below the significance level (p<0.05)

Table 3.5 Spearman's correlation analysis for cod collected in the inner Oslofjord. The table shows only correlations that were statistically significant in males and/or females within one location. Spearman's ρ= Spearman's rank correlation coefficient.

Variable	By variable	Male		Female	
		Spearman's ρ	P-value	Spearman's ρ	P-value
AChE activity	Length	-0.5117	0.02*	-0.2112	0.4
AChE activity	Weight	-0.4974	0.02*	-0.2211	0.4
AChE activity	GSI	0.5545	0.009*	-0.0842	0.7
ALA-d activity	OH-pyrene	-0.6105	0.02*	0.0979	0.8
ALA-d activity	OH-B[a]P	-0.5211	0.02*	-0.0440	0.9
EROD activity	Weight	-0.4481	0.04*	0.1316	0.6
EROD activity	Condition factor	-0.4637	0.03*	-0.0951	0.7
GSI	Age	0.1585	0.5	0.4750	0.04*
GSI	LSI	0.2182	0.3	0.5281	0.02*
Condition factor	LSI	0.3347	0.1	0.5082	0.03*
OH-phenanthrene	OH-pyrene	0.8662	<0.0001*	0.6966	0.001*
OH-B[a]P	OH-pyrene	0.9038	<0.0001*	0.7007	0.001*
OH-B[a]P	OH-phenanthrene	0.8226	<0.0001*	0.7090	0.001*

* indicates a ρ-value below the significance level <0.05

Table 3.6 Spearman's correlation analysis for cod collected in the inner and outer Oslofjord. The table shows only correlations that were statistically significant in inner and/or outer Oslofjord. Spearman's ρ= Spearman's rank correlation coefficient.

Variable	By variable	Inner Oslofjord		Outer Oslofjord	
		Spearman's ρ	P-value	Spearman's ρ	P-value
EROD activity	CYP1A concentration	0.4662	0.002*	0.3259	0.05
ALA-D	OH-pyrene	-0.3933	0.03*	-0.1101	0.6

* indicates a ρ-value below the significance level (p<0.05)

4 Discussion

4.1 Optimization of the comet assay

Scoring of the samples stored on lysis buffer for three weeks showed significantly higher DNA damage when compared to samples stored for only one week. This was true for all groups compared apart from control (0 µM H_2O_2) from cod collected in the inner Oslofjord, which indicate that storage duration of samples on lysis buffer may increase the level of DNA damage. These results are highly relevant when working *in situ* where samples may sometimes need to be kept for weeks on lysis buffer until further processed for the comet assay. The response pattern to H_2O_2 and the difference between the two locations were the same after one and three weeks. A natural question would be whether the observed background damage of approximately 10% tail intensity could be caused by storage on lysis buffer for one week. This level of background damage was however also observed elsewhere in other studies with storage for only 90 minutes (Hartl *et al*. 2006). It therefore appears likely that the background level of 10% tail intensity may be a result of natural DNA breakage, a process known to occur thousands of times every day.

4.2 Physiological indices and growth

To minimize possible effects from the fish' body sizes on response to pollutants, the cod collected were homogenous with respect to size. The sizes ranged from approximately 40-60 cm, which is also the size range of cod that were collected in previous studies (Holth 2004, Imrik 2010, Nerland 2011). This allows for more accurate inter-study comparisons. Collecting fish with the same size from both locations may have resulted in the significant difference in age of cod collected for this investigation, with older fish from the inner Oslofjord. The cod collected in the inner Oslofjord had an age span of one to five years while cod collected in the outer Oslofjord only had an age span between one and three years. The similar size, but older cod in the inner Oslofjord suggest slower growth rate for cod from this area compared to the outer Oslofjord. Due to low sample sizes in overlapping ages, only two-year old individuals could be statistically compared for body size between the two areas, for which no significant difference was found. Therefore, a clear conclusion with regard to growth rates cannot be drawn.

Physiological indices, such as LSI, may be affected by physiological status such as nutrition (lipid content) (Lambert & Dutil 1997, Nanton *et al*. 2001). The higher LSI in cod from inner Oslofjord may therefore indicate that the food availability for cod and hence lipid content was higher in the inner Oslofjord than the outer Oslofjord, or that the type of food consumed was different. Diet has been shown to affect LSI in previous studies (Sherwood *et al*. 2007). For example a diet consisting of mostly pelagic organisms has shown to give higher LSI compared to fish with a diet dominated by benthic organisms (Sherwood *et al*. 2007). Increased liver weight has also been observed in fish sampled from contaminated sites and also in fish experimentally exposed to toxicants such as PAHs, PCBs and PCDDs (van der Oost *et al*. 2003). Liver enlargement may be caused by hypertrophy (increased cell size) (Slooff *et al*. 1983) or hyperplasia (increased number of cells) (Poels *et al*. 1980). The higher LSI in inner Oslofjord may therefore be a response to exposure to pollutants, as also indicated by several other biomarkers used in this study. The LSI median values of cod collected from inner Oslofjord was however in the range 3-4%, which is within limits (2-6%) considered normal for wild cod (Jobling 1988). The median LSI values in cod from the outer Oslofjord were below normal at 1-2%.

Previous investigations of cod from these two sites have been variable. A significant difference favouring outer Oslofjord cod was found for LSI in 2010 (Bergland 2012), while investigations from 2002, 2008 and 2009 reported no significant difference between the locations (Holth 2004, Imrik 2010, Nerland 2011). It should be noted here that physiological indices were calculated with body weight that included stomach content in three of these studies (Holth 2004, Imrik 2010, Nerland 2011), which may have contributed to some of the variations in the results. In these previous investigations LSI has been low (1-2%), while the present study reports an increase compared to previous years. Since biomarkers used in previous studies have indicated higher exposure to pollutants in cod from the inner Oslofjord, it seems likely that factors other than exposure to chemicals, as mentioned before, have contributed to the increased LSI in this study. Also, while exposure history could explain the higher LSI in female cod compared to male cod in this study, this is unlikely to be the case here as the biomarkers examined, regarding planar organic pollutants, show either no difference by sex, or higher exposure effects in male cod. A more likely reason for a higher LSI in female cod compared to male cod would be reproductive status. Production of estradiol which increases vitellogenin levels in relation to spawning also increases LSI (Banks *et al*. 1999). The GSI values show that cod from the inner Oslofjord had increased gonadal

development compared to cod from the outer Oslofjord and there was also clearer gonadal development (measured as GSI) in outer Oslofjord females compared to males. As indicated by GSI there may be elevated estradiol levels in the female cod causing increased LSI compared to male cod. Supporting this argument, there was a significant positive correlation between LSI and GSI in females from both locations. There is also a chance that the higher GSI from the inner Oslofjord compared to the outer Oslofjord can partly explain the difference in LSI between females from these two areas.

The higher GSI in cod from inner Oslofjord compared to cod from outer Oslofjord could indicate that cod from the two areas spawn at different times. Two earlier investigations found no difference in GSI between the two areas at the same time of the year (November) (Imrik 2010, Bergland 2012), suggesting that factors other than genetics may affect spawning time. The condition reflects energy reserves and could possibly affect the energy allocated to reproduction. A low general condition in the outer Oslofjord may have affected the population negatively in relation to gonadal development. The higher GSI in cod collected from the inner Oslofjord could possibly be explained by larger energy reserves compared to cod from the inner Oslofjord. There was however no significant correlation between the condition factor and GSI to support this argument. Also, the influence of pollutants on GSI cannot be ruled out. As reviewed by Scholz & Klüver (2009) both increase and reduction of GSI in fish have been associated with contamination. There may be endocrine disrupting chemicals present in the inner Oslofjord (Scott *et al*. 2006), leading to earlier gonadal development than expected. There was a positive correlation between age and GSI in females of the outer Oslofjord. Due to the higher age of cod from the inner Oslofjord this factor may have partly contributed to the difference seen in GSI. This correlation between age and GSI was not seen in males and may indicate that females from the inner Oslofjord become reproductively active at a higher age compared to males.

The condition factor is a measure of the somatic body weight relative to the body length and reflects the fish' overall condition. A high condition factor, as was the case for the cod from inner Oslofjord relative to cod from the outer Oslofjord may reflect higher total energy reserves (Lambert & Dutil 1997). The condition of the fish may be affected by the availability of food (van der Oost *et al*. 2003). When food is limited, energy reserves become depleted, leading to a decrease in condition. With exposure to environmental chemicals for which mode of toxic action changes the cellular energy budget, i.e. decreasing lipid and glycogen energy budget while increasing protein energy budget, condition factor may decline (Smolders *et al*.

2003). Chemical pollution in the inner Oslofjord seems not to have had this effect on cod as the condition factor was higher here than in cod from outer Oslofjord. Condition factor has also shown to increase in fish inhabiting polluted sites (van der Oost *et al*. 2003). For example brown bullhead from a site contaminated with PCBs and dichlorodiphenyldichloroethylene (DDE) had a significant increase in the condition factor compared to brown bullheads from a relatively clean site (Otto & Moon 1996). Whether the increase in condition can be attributed directly to pollution exposure is unclear. The overall condition of cod collected from both areas was above what is considered a risk to survival (0.6), but several individual fish (mostly from the outer Oslofjord) were below what is considered good condition (0.8-1) (Dutil & Lambert 2000). There was a significant positive correlation between the condition factor and LSI in females from the inner Oslofjord. This may indicate that factors increasing or decreasing these two parameters in females collected from the inner Oslofjord may partly be the same, e.g. nutrition. Good correlation between these physiological indices has been found in cod previously and may be explained by the use of muscle proteins as energy source when liver lipids are scarce (Lambert & Dutil 1997).

These physiological factors measured have shown to be different between the locations which may affect the other biomarkers. Such relationships will be discussed in relevant chapters below.

4.3 Exposure to PAH and effects of organic pollutants

4.3.1 PAH metabolites in bile

OH-B[a]P, OH-phenanthrene and OH-pyrene were present at significantly higher levels in the bile in cod sampled from the inner Oslofjord compared to cod samples from the outer Oslofjord. Perhaps not surprisingly, the concentration of the different metabolites correlated significantly. Metabolites measured in bile are an indication of recent exposure to PAHs (van der Oost *et al*. 2003, Beyer *et al*. 2010) where it has been taken up directly from the water through gills or through diet (Grung *et al*. 2009). The significantly higher concentration of metabolites found in cod from the polluted site compared to the reference site therefore indicates a higher recent exposure to PAH in the inner Oslofjord than in the outer Oslofjord. Negative effects caused by PAHs may therefore have a greater impact on the cod population

of the inner Oslofjord. The correlation between the metabolites may indicate that their corresponding PAHs are present in similar ratios, i.e. have similar sources, in the two areas.

The three metabolites, which vary in their ring structure, are generally associated with different sources of origin. These results indicate that there is a clear dominance of exposure to pyrene, followed by phenanthrene and B[a]P. Pyrene is a four-ring PAH of pyrogenic origin (Hylland 2006) and is usually one of the most abundant compounds in these sources (Page *et al*. 1999). Long-term studies of English sole in industrially polluted sites of Puget Sound, Washington, have shown pyrene to be at relatively higher levels than other PAH metabolites (Krahn *et al*. 1987). Important sources to the inner Oslofjord are presumably of both petrogenic origin, i.e. from oil, and pyrogenic origin, such as combustion of fossil fuels and wood fires. Exposure to B[a]P, a five-ring PAH also indicates pyrogenic sources (Inengite *et al*. 2013). Pyrene has also been detected at elevated levels after an oil spill (Jung *et al*. 2011). This shows that pyrene can also originate from petrogenic sources. Phenanthrene is a three-ring PAH generally classified as derived from petrogenic sources (Page *et al*. 1999, Hylland 2006, Inengite *et al*. 2013). The higher level of OH-phenanthrene in cod collected in the inner Oslofjord may therefore indicate that spillage of oil or oil-based products is an important source to PAH exposure.

For OH-B[a]P there was a significant interaction between sex and location, with a larger difference between males and females from the inner Oslofjord than for the two sexes from the outer Oslofjord. Bile of males had higher OH-B[a]P content than females. This indicates that males had higher exposure to B[a]P than females, but only in inner Oslofjord. A similar result was obtained from analysis of OH-phenanthrene where the concentration in bile was significantly higher for males than females in the inner Oslofjord. The difference in exposure between sexes at the polluted site is presumably the result of different feeding behavior. A study by Fordham & Trippel (1999) showed that the feeding habits of males and females vary due to different spawning behaviours. PAH metabolite content of bile has also shown to increase due to starvation (Ariese *et al*. 1993), however contradictive results have also been found with no effect of starvation (Hylland *et al*. 1996).

Previous investigations (Holth 2004, Imrik 2010, Nerland 2011, Bergland 2012) have also shown OH-pyrene to be at relatively high concentrations in the bile of cod from the inner Oslofjord compared to the outer Oslofjord. This suggests that exposure to PAH from pyrogenic sources remains relatively constant. OH-phenanthrene has also been shown to be

significantly higher in cod from inner Oslofjord in 2002 (Holth 2004), which is consistent with this study. Although investigations from the year of 2009 and 2010 (Nerland 2011, Bergland 2012) found no significant difference between the inner and outer Oslofjord, the concentration of phenanthrene in bile seems to have been at similar levels at the polluted site through the years. Comparison with the 2008 investigation is not possible as a large percentage of samples were below the detection limit (Imrik 2010). The consistency of findings regarding exposure to pyrene since the year of 2004 may indicate a continuous input of PAHs into the environment through anthropogenic activities, however PAHs may stay unchanged in sediments for several years (Page *et al.* 1999). This means any reduction in environmental input may not be visible in exposure levels for several years.

Results of analysis of B[a]P have been unclear in previous studies due to a large number of samples reading below the detection limit. However it was observed that more samples read above detection limit in cod from the inner Oslofjord than cod from the outer Oslofjord (Holth 2004, Imrik 2010, Bergland 2012). Nerland (2011) found no significant difference between the locations for OH-B[a]P, although 40-50% of data read below the detection limit and were not included in the analysis. The detection limit in this thesis (0.75 ng/g) was set by adding three times the standard deviation to the average blank sample. The detection limit in previous theses were set to 1.8-2 ng/g (Nerland 2011, Bergland 2012), which would clearly affect the statistical analyses. However, the higher number of samples that have read above detection limit in the inner Oslofjord compared to the outer Oslofjord in addition to the results of this study may indicate that there is a slightly higher long-term exposure to B[a]P in cod from the polluted site than cod from the reference site.

4.3.2 CYP1A level and activity (EROD)

Hepatic EROD activity of cod from the inner Oslofjord was observed to be significantly higher than in cod collected from the outer Oslofjord, an observation which was also supported by a significantly higher CYP1A concentration in cod from the inner Oslofjord. There was no significant difference between males and females and no significant interaction between sex and location. The results indicate that chemicals known to induce CYP1A transcription are present at higher levels in the inner Oslofjord, that male and female cod inhabiting this area are equally exposed to them and that there is no interaction with estrogens at the time of sampling. As EROD activity is expected to decrease in females during periods

of reproductive activity (Whyte *et al*. 2000), it is somewhat interesting that no difference between the sexes was apparent in the present study, given that GSI values indicate gonadal development in the inner Oslofjord. Among other planar organic pollutants, several PAHs are known to be involved in the induction process of CYP1A (McDonald *et al*. 1995, Aas *et al*. 2001, Sturve *et al*. 2006). The analysis of PAH metabolites in bile revealed a higher exposure to PAHs for cod from the inner Oslofjord indicating that these pollutants could be a possible cause of the higher CYP1A level and consequently a higher EROD activity. There was however no significant positive correlation between PAH concentration and CYP1A level or activity. This suggests that the elevated level and activity seen in this study were the result of not only PAH exposure, but also exposure to other planar organic pollutants and that the latter contributed more to inducing CYP1A than PAHs. Other chemicals shown to induce CYP1A transcription and EROD activity are PCBs (Olsvik *et al*. 2011), dioxins (Hektoen *et al*. 1994) and PBDE (Chen *et al*. 2001). Levels of PCB have been persistently higher in cod liver sampled from Steilene in the inner Oslofjord compared to cod from the outer Oslofjord over the last years (Berge *et al*. 2009, Green *et al*. 2010, Green *et al*. 2012b. In 2011, the PCB levels in cod liver from the inner Oslofjord was classified as "markedly polluted" (Green *et al*. 2012). PBDEs have been detected at the highest levels, out of 11 sites tested in Norway, in cod liver from the inner Oslofjord (Green *et al*. 2012). Therefore, combined exposure to a complex mixture of chemicals is a likely cause of the increased CYP1A level and its EROD activity in this study. There was a positive correlation between EROD activity and CYP1A protein level in the inner Oslofjord indicating that the elevated EROD activity can at least partly be explained by induction of CYP1A protein.

When using these two biomarkers in environmental monitoring it is important to know there are also several factors that can reduce EROD activity that may lead to underestimation of exposure. In the natural environment a mixture of chemicals are present in which there are both agonists and antagonists of EROD activity (Forlin & Norrgren 1998). The chemical mixture may result in an effect, different to what the outcome would be when exposed to the chemicals separately (Hylland *et al*. 1996). An *in vitro* study by Willett *et al*. (1998) exposed rat cells to the three-ring PAH fluoranthene together with other PAHs known to induce CYP1A activity. Results from that study showed that the induction of CYP1A by PAHs could be inhibited by fluoranthene. There was a negative correlation between CYP1A level and OH-phenanthrene levels in bile of female cod from the outer Oslofjord, which could possibly indicate that phenanthrene had an inhibitory effect on CYP1A induction in this study. TBT

has also shown to inhibit EROD activity (Fent & Bucheli 1994) and has been classified as "markedly polluted" in mussels of the inner Oslofjord (Green *et al.* 2010). There is a concern that estrogen mimic chemicals can have an inhibitory effect on CYP1A and therefore mask the inducing effect of exposure to planar organic pollutants (Whyte *et al.* 2000). An experimental study using Atlantic salmon (*Salmo salar*) showed a significant reduction of CYP1A protein level and EROD activity with exposure to the estrogenic compound 4-nonylphenol (Arukwe *et al.* 1997). Estrogen-mimicing chemicals can be derived from pharmaceuticals, detergents and personal care products that are released into the water via runoff from urban areas. Elevated levels of vitellogenin have earlier been detected in male cod from the inner Oslofjord (Scott *et al.* 2006), which may indicate exposure to environmental estrogens, presumably deriving from sewage effluents (Pickering & Sumpter 2003). The presence of estrogen-mimicking chemicals in the inner Oslofjord may have decreased the effect of chemical inducers on the CYP1A level and EROD activity seen in this study.

Investigations from previous years have shown, in accordance with this study, a higher EROD activity in cod from the inner Oslofjord compared to the outer Oslofjord, supported by a higher CYP1A concentration (Holth 2004, Imrik 2010, Bergland 2012). An exception to this trend was an investigation in 2009 where no difference between the two locations was observed, which was suggested to have resulted from chemical inhibition (Nerland 2011). Investigations of cod liver from the inner Oslofjord in the time period 1997-2011 showed no significant trend in EROD activity (Green *et al.* 2012). Although studies have revealed a higher enzyme activity in cod from the inner Oslofjord compared to other presumed clean sites, the EROD activity detected has mostly been below background assessment criteria for cod liver with an upper limit of 145 pmol/min/mg protein (ICES 2011). This is also true for the findings in this study and similar investigations in 2002, 2008, 2009 and 2010 (Holth 2004, Imrik 2010, Nerland 2011, Bergland 2012). The insignificant trend in EROD activity may indicate a relatively constant exposure to CYP1A inducing toxicants. It is however worth mentioning here that fish with prolonged exposure to these pollutants have been shown to have reduced CYP1A protein levels and EROD activity responses (Bello *et al.* 2001, Nacci *et al.* 2002), which may also be the case for cod in the inner Oslofjord.

Ratio of fish mass to exposure volume (loading density) may be important in the effect caused by chemicals. Higher EROD activity has been detected in fish with a lower body mass than in fish exposed to the same contaminant level, but of higher body mass (Martel *et al.* 1995). A negative correlation was found between EROD activity and body weight in males from the

inner Oslofjord and between CYP1A protein level and weight as well as length in females from the outer Oslofjord. This suggests that loading density can in part explain the variation seen in CYP1A protein level and EROD activity within each site. The rate at which the fish grow will be of importance in regards to accumulation of chemicals with age. If the fish grow fast the body mass may dilute the concentration while slow growing fish may concentrate the chemicals with greater age. An appearent slower growth of cod collected in the inner Oslofjord could not be concluded in this study. However, previous studies have shown that cod from the outer Oslofjord have been larger for their age than cod from inner Oslofjord (Nerland 2011, Bergland 2012). If this is also true for the cod collected in this study (apart from two-year olds) a possible interference of body mass and age on the observed EROD activity and CYP1A protein level cannot be ruled out.

There was a negative correlation between EROD activity and condition factor. Condition factor is dependent on the fish' energy reserves and is therefore affected by nutrition. Reduced food intake is thought to have a negative effect on EROD activity (Whyte *et al.* 2000). This correlation is therefore the opposite of what would be expected and may be too ambiguous to interpret.

The CYP1A enzyme contributes to biotransformation of chemicals, but may also generate reactive metabolites, which may cause a toxic effect. Metabolites of B[a]P may bind to DNA as adducts possibly causing tumor initiation (Whyte *et al.* 2000). The activity of this enzyme is also known to generate oxygen radicals through redox cycling of some chemicals, such as 1,6-benzo[a]pyrene (Di Giulio & Meyer 2008). Oxygen radicals may cause DNA damage such as DNA strand breaks, which was detected at a higher level in cod from the inner Oslofjord. An increase in EROD activity as seen in cod from this site could potentially lead to these adverse effects comprising the populations' reproduction and survival.

4.4 DNA damage in leukocytes

As detected by the comet assay, the concentration of DNA strand breaks in cod collected from inner and outer Oslofjord were significantly different. There were approximately 3.5 times more strand breaks in the inner Oslofjord cod than in cod from the outer Oslofjord. This may indicate exposure to genotoxicants in the inner Oslofjord. Male and female cod appear to have been equally affected. DNA strand breaks are a type of structural lesion that occurs naturally, but also by interaction between DNA and oxygen radicals or reactive metabolites and by the

action of excision repair enzymes (Lee & Steinert 2003). Oxygen radicals can be generated from environmental pollutants such as PAHs, pesticides, PCBs, dioxins, furans and trace metals through a variety of chemical pathways (Schlenk *et al*. 2008a). For example the common combustion product 1-nitropyrene and a metabolite of B[a]P named 1,6-benzo(a)pyrene quinone can generate oxygen radicals via redox cycling in which CYP1A is the main catalyst (Di Giulio & Meyer 2008). The CYP1A protein concentration and activity (EROD) was higher in cod sampled from the inner Oslofjord than the outer Oslofjord. This could potentially mean that the generation of oxygen radicals by the action of CYP1A has influenced the observed DNA damage. Imrik (2010) observed a significant positive correlation between EROD activity and DNA strand breaks in erythrocytes indicating a possible causal relationship between biotransformation of xenobiotics and DNA damage. A strong correlation between these two biomarkers was also shown in an experimental study exposing rainbow trout to B[a]P and benzo[e]pyrene (B[e]P) (Curtis *et al*. 2011). Interaction between DNA and reactive metabolites affect the activity of repair enzymes. An experiment on human cells showed that activity of excision repair enzymes were involved in most DNA strand breaks caused by exposure to B[a]P (Speit & Hartmann 1995). This shows that it may not be the adduct-forming B[a]P itself that causes strand breaks, but rather the level of repair activity. Exposure of Atlantic cod to low concentrations of crude oil in an experimental study by Aas *et al*. (2000) resulted in elevated formation of DNA adducts. Chemical analysis of PAH metabolites in bile indicated that exposure to B[a]P along with phenanthrene and pyrene was higher in the inner Oslofjord compared to the outer Oslofjord. PAH exposure may have been a contributing factor in the complex mixture of chemicals present in the inner Oslofjord causing the elevated DNA damage.

In accordance with this investigation, previous studies investigating DNA damage have reported larger amounts of DNA strand breaks in different species collected from sites polluted by anthropogenic activity. A study by Hartl *et al*. (2006) showed increased DNA damage in individuals of the clam *Tapes semidecussatus* and the turbot *Scophthalmus maximus* exposed to PAH, organotin and heavy metal contaminated sediment. Pandrangi *et al*. (1995) showed increased DNA damage in Bullheads (*Ameiurus nebulosus*) and carp (*Cyprinus carpio*) from several contaminated sites (based on high concentrations of PAHs and PCBs) compared to individuals of the same species from a clean site. In accordance with that study, higher level of DNA strand breaks in cod from inner Oslofjord was detected by the comet assay in a previous investigation by Imrik (2010), using erythrocytes. Exposure to

heavy metals such as Hg, Cd and copper (Cu) have shown to cause increase DNA strand breaks in mussels (Bolognesi *et al*. 1999). Comparing the results from the present study to previous findings strengthens the argument that the increased DNA strand breaks in leukocytes in cod from the inner Oslofjord indicate a genotoxic effect by environmental pollutants, e.g. through interaction between DNA with oxygen radicals or through repair activities.

H_2O_2 produces DNA strand breaks directly (Singh *et al*. 1991) and was used in this study to introduce oxidative stress linked-DNA damage in order to investigate the relative tolerance to oxidative stress by cod collected from the two sites. All samples were exposed to a low and a high concentration of H_2O_2 and compared with the untreated samples. DNA damage in the treated groups was significantly higher compared to untreated samples indicating that both concentrations of H_2O_2 induced DNA damage in cod samples from both locations. However, the regression line representing the response to the exposure concentrations was significantly steeper for cod from the outer Oslofjord indicating a higher tolerance to oxidative stress by cod from inner Oslofjord. Such increased tolerance may be an adaptive response to chronic exposure to a mixture of chemicals causing oxidative stress in the inner Oslofjord. Possible mechanisms for this tolerance may be higher antioxidant enzyme capacity preventing DNA damage, more efficient repair enzyme system or a combination of the two. A number of studies have showed up-regulation of antioxidant proteins such as superoxide dismutase (SOD) and catalases as a response to higher oxidative damage from exposure to environmental contaminants such as PAHs and PCBs (Di Giulio *et al*. 1993, Livingstone *et al*. 1993). The indicated adaptive response shows that the exposure to genotoxic chemicals in the inner Oslofjord is chronic and it is therefore important to investigate possible negative effects on the population's survival further. Preneoplastic lesions have been linked with level of contamination as well as with adduct formation in subadult English Sole from polluted sites of Puget Sound (Myers *et al*. 1998). Unpublished data by Peden-Adams and Lee in Lee & Steinert (2003) showed a strong negative correlation between T-cell proliferation and DNA strand breaks in white blood cells from dolphins as determined by the comet assay. The ability of T-cells to undergo mitosis is important in the response to infections (Lee & Steinert 2003). It is possible that DNA strand breaks in leukocytes of Atlantic cod also interfere with T-cell proliferation. If that is true, findings from the present study suggest that the cod from inner Oslofjord may have a lower ability to respond to infections compared to cod from outer

Oslofjord. If this is the case it would result in a negative effect on the population in terms of survival and reproduction.

There was a negative correlation between DNA damage and LSI in males collected from the outer Oslofjord. Higher LSI reflects higher lipid content. A possible explanation would be that a liver with higher lipid content may have increased capacity to store lipophilic genotoxic chemicals, leading to decreased bioavailability of these compounds hence decreasing their genotoxic effects.

4.5 Effect of lead (Pb)

ALA-D in red blood cells was significantly lower in cod collected from inner Oslofjord compared to cod collected in the outer Oslofjord. The median enzyme activity of the outer Oslofjord was approximately at 15 ng/min/mg protein, which is within the normal baseline level of activity (10-20 ng/min/mg protein) (OSPAR 2007). Cod from inner Oslofjord on the other hand, had an ALA-D activity within the normal range but also below. This may indicate that Pb is available to cod in the inner Oslofjord and that ALA-D was inhibited in some individuals as a result of the exposure. The ALA-D activity did not differ significantly by sex indicating a similar exposure to both males and females. Sex differences are not generally observed with this biomarker (Schmitt *et al*. 2005, Imrik 2010, Lombardi *et al*. 2010).

ALA-D was also inhibited in the studies performed in 2002 and 2008 (Holth 2004, Imrik 2010), but no difference was found between the two sites in 2009 and 2010 (Nerland 2011, Bergland 2012). Based on these previous investigations Bergland (2012) assumed the presence of a decreasing trend in exposure to lead. The ALA-D inhibition in the present study however does not confirm this trend, although the range of ALA-D activity shows large individual differences with a median value just above 10 ng PBG/min/mg protein in the inner Oslofjord. The individuals with ALA-D below 10 ng PBG/min/mg protein are considered inhibited indicating an increase in Pb exposure compared to the results from the two previous investigations. The large individual variation from the contaminated site could be due to difference in exposure or sensitivity between individuals. Chemical analyses of cod liver from the inner Oslofjord have shown large individual variation of Pb levels (Green *et al*. 2012). There is also a possibility that these individual differences come from differences in the levels of Zn content in blood which may have affected the inhibition caused by Pb. Zn has been shown to reactivate inhibited ALA-D (Lombardi *et al*. 2010) and may alleviate the effect

of Pb (Schmitt *et al.* 2005). This could however also partly explain some of the annual variation in ALA-D activity in inner Oslofjord. In accordance with this study, investigations of cod from inner Oslofjord by NIVA also found a lower ALA-D activity in blood cells compared to other reference sites (e.g. outer Oslofjord), indicating exposure to Pb (Green *et al.* 2012). No significant temporal trend in ALA-D activity was found during the time interval 1997-2011 (Green *et al.* 2012).

Today, important national sources of Pb are products containing Pb such as fishing gear, seeps from landfills, sludge, drains, sediments and storm water. Long distance transport from Europe is also an important source of Pb deposition in Norway (Sørensen 2012). Several actions have been taken to reduce the release of Pb, which has led to approximately 80% decrease in national discharge since 1995 (Sørensen 2012). The results from this study show that the exposure situation is still not quite where it should be. Although ALA-D activity is considered a robust biomarker to Pb exposure, correlations have also been found between ALA-D activity and blood and tissue concentrations of Hg, Cu-Zn and organochlorines in Atlantic cod (Hylland *et al.* 2009). However, due to concentrations below detection limit, Pb could not be included as a predictor in the analysis of that study. The tissue concentration of Pb and Hg correlated significantly at concentrations above the detection limit, which raises the question whether the observed contribution to ALA-D inhibition by other metals was rather due to covariation with Pb. Never the less, the influence of metals and pollutants other than Pb cannot be ruled out.

There was a significant negative correlation between ALA-D activity and LSI for female cod of the outer Oslofjord. This contradicts the previously observed positive correlation between ALA-D and LSI (Hylland *et al.* 2009). The mechanism behind this relationship is unknown. There was also a significant negative interaction between ALA-D activity and the PAH metabolite B[a]P in males from the inner Oslofjord. The negative correlation indicates that with high exposure to PAHs there is also an inhibition or low activity of ALA-D. A possible explanation is that the PAH directly or indirectly leads to inhibition of ALA-D. Another explanation would be that Pb is available for uptake from the same point of exposure to PAHs, e.g. sediment, where pollutants from different sources tend to collect. A similar correlation was observed for ALA-D and OH-pyrene in male and female cod from the inner Oslofjord.

The activity of ALA-D has caused anemia in birds and mammals, but is not thought to be a rate-limiting step in heme synthesis in fish at the concentrations relevant for the environment (Schmitt *et al*. 2007). Negative effects associated with exposure to metals on the population level have been decreased condition factor and fish density (Moraes *et al*. 2003). The inhibition of ALA-D is however an early toxic effect which will occur before further serious effects on the population level. As implied by the condition factors of cod from the inner Oslofjord, the effect of exposure to Pb was not at a point at which energy reserves had declined.

4.6 Effect of organophosphates and carbamates

AChE activity was significantly lower in cod collected from the outer Oslofjord compared to the inner Oslofjord. As AChE is known to be inhibited by some pesticides, these results suggest a higher exposure to organophosphate and carbamate pesticides in the outer Oslofjord compared to the inner Oslofjord. Agriculture is the most common source of pesticide-pollution. The Heiabekken catchment, which drains into the outer Oslofjord, represents a highly cultivated area where 48% of the farmland is used for growing potatoes and vegetables (Stenrød *et al*. 2008). These crops require more frequent use of pesticides than cereals, which dominates the use of farm land in the inner Oslofjord (Hauken *et al*. 2012). In regular testing of pesticide levels by Bioforsk, the Heiabekken catchment has shown more frequent occurrences of unacceptably high pesticide levels than the 12 other monitored catchments (Hauken *et al*. 2012). Even when the released level of each individual chemical is considered safe, the resulting mixture may potentially be unsafe to the environment. Recovery from AChE inhibition has shown to take days or weeks (Straus & Chambers 1995). This could lead to fish becoming more susceptible to cumulative effects. It should be noted here that as sampling took place in late November, enzyme activities might have already recovered, to some extent, from earlier minima. The observed positive correlation between AChE activity and GSI suggest that reproductive state may have contributed to higher enzyme activity in cod collected from the inner Oslofjord compared to the outer Oslofjord. However the same pattern in AChE activity has been found before in cod from those two location, both with equal and different GSI (Imrik 2010, Nerland 2011) indicating that exposure to inhibitory chemicals, e.g. organophosphate and carbamates are a more likely to have affected the enzyme activity than GSI.

The enzyme activity was significantly lower in females compared to males from both sites. It has been suggested by Payne *et al*. (1996) that depression in AChE activity could be related to differences in hormonal control, metabolic rate or physical activity. These factors could be possible explanations to the pattern observed here. AChE activity however, correlated negatively with length and weight in males of the inner Oslofjord. A similar relationship between enzyme activity and size has been documented in *Leuciscus cephalus* (Flammarion *et al*. 2002). In the present study females were significantly longer than males, indicating that body size may partly explain the gender differences in enzyme activity. The difference in body length between the sexes was however larger in the outer Oslofjord as indicated by a significant interaction between sex and location. Therefore the length of the fish is more likely to explain the difference in AChE activity between sexes in cod collected from the outer Oslofjord than for cod from the inner Oslofjord.

There was a significant negative correlation between AChE and CYP1A in males from the outer Oslofjord. This relationship could mean that CYP1A inducing pollutants such as PAHs may also inhibit AChE. An *in vitro* study by Holth & Tollefsen (2012) observed AChE inhibition resulting from exposure to PAHs found in produced water. (Payne *et al*. 1996) also found these two biomarkers to be linked in trout inhabiting water-ways polluted by urban runoff and pulp and paper mills. In that study sources of pesticides were not readily apparent. While PAHs may have some effect in the present study they are not likely a strong influence as the inner Oslofjord would be expected to show highest inhibition in that case. Metals have also shown to have inhibitory effect on AChE activity (Olson & Christensen 1980). The results from ALA-D activity indicate that exposure to Pb or other metals are higher in the inner Oslofjord than the outer Oslofjord. Therefore, a large influence of metals on the inhibited AChE in the cod collected from the outer Oslofjord does not seem likely. The differences seen in AChE activity between the two sites are most likely the result of differences in exposure to organophosphate and carbamate pesticides or possibly effluents from pulp and paper mills.

There was a significant positive correlation between AChE activity and OH-phenanthrene in bile. This is the opposite of what would be expected given what was discussed earlier. The reason for this relationship is not clear. Several parallel Spearman's correlation analyses were run which means there is an increased chance of a Type I error. There was also a negative correlation between AChE activity and body size (length and weight) in males from the outer Oslofjord.

4.7 Conclusions

The biomarkers used in this study have shown that pollutants in the inner Oslofjord affect the resident Atlantic cod population.

DNA damage, PAH metabolites in bile, CYP1A protein levels and EROD activity as well as ALA-D activity, all indicated that cod from the inner Oslofjord was affected by organic and inorganic pollutants. The opposite was true with regards to AChE activity in which the effect from contaminants, e.g. organophosphate and carbamate pesticides, appeared to have affected cod from the outer Oslofjord more than the inner Oslofjord.

Cod from the inner Oslofjord had a higher tolerance to oxidative stress-linked DNA damage compared to cod from the outer Oslofjord, indicating an adaptive response to chronic exposure to chemicals causing oxidative stress.

As indicated by a significant interaction between sex and location, exposure to B[a]P appeared to have affected male cod more than female cod from the inner Oslofjord. The same was true for exposure to phenanthrene with significantly higher concentration of OH-phenanthrene in bile of male cod than female cod from the inner Oslofjord. This may be due to variation feeding between sexes, as associated with spawning behavior. The AChE activity was lower in female cod compared to male cod from the two areas, which may be due to differences in length, hormonal control, metabolic rate or physical activity. LSI was higher in female cod compared to male cod collected in the two areas, which could have been a result of increased estradiol levels.

Cod from the inner Oslofjord appeared to have grown slower than cod from the outer Oslofjord, but had a higher LSI, condition factor and GSI. Cod collected from the inner Oslofjord therefore appeared to have higher energy reserves and an increased gonadal development, at the time of sampling, compared to cod collected from the outer Oslofjord. The difference in GSI may be due to different spawning time, age or possibly exposure to environmental estrogens. Liver enlargement may also have resulted from exposure to pollutants. An apparent difference in growth between the two areas could not be tested statistically since the size ranges collected only made it possible to compare two-year old individuals

All PAH metabolites correlated positively in cod from both locations, indicating that they derive from similar sources within the two areas. The concentration of CYP1A and EROD

activity was related, indicating that at least some of the elevated EROD activity can be explained by higher concentration of CYP1A in the inner Oslofjord compared to the outer Oslofjord. A relationships was found between GSI and LSI which supports the argument that estradiol levels in relation to gonadal development also has contributed to the higher LSI seen in female cod compared to male cod. A negative relationship was found between AChE activity and CYP1A, which raises the question whether chemicals that induced CYP1A also have inhibited AChE. Significant relationships suggest that length and weight have been contributing factors to CYP1A concentration, EROD activity as well as AChE activity. A positive correlation between age and GSI suggests that the higher GSI in cod collected from the inner Oslofjord compared to the outer Oslofjord could partly be explained by a higher age.

The DNA damage level as determined by the comet assay appeared to have been affected by storage time on lysis buffer. A storage duration of three weeks gave significantly more single-strand breaks compared to storage for only one week, suggesting that treatment with lysis buffer may cause elevated levels of DNA damage over the background damage.

4.8 Further research

The use of Zn to reactivate ALA-D after inhibition can be used to calculate reactivation index which represents percentage of enzyme inhibition. This has proven to be a more sensitive biomarker than the measurement of ALA-D activity alone, with stronger correlation to blood levels of Pb (Lombardi *et al*. 2010). In combination with chemical analysis of blood, this biomarker could possibly provide a stronger argument as to whether the ALA-D inhibition in the inner Oslofjord is mainly due to Pb exposure.

The extent of pesticide use on crops varies through the year. As the time of sampling for the present study was conducted in late in the autumn, the presence of organophosphates and carbamates in the environment may have declined from an earlier point in time and the enzyme activity may have been recovering. Therefore, it would be interesting to observe variation in AChE in cod from the Oslofjord through the year, both to see if the activity coincides with the use of pesticides on crops and to detect possible higher extent of inhibition than what was observed in the present study.

As biomarkers can be affected by nutrition status and diet, it would be useful to obtain data on stomach content for future investigations. For example, the level of PAH metabolites in bile

can be affected by nutrition status and may increase with starvation. It was suggested in the present study that nutrition status may have contributed to sex based differences in PAH metabolites in bile and so information on stomach content could clarify as to whether nutrition status has had an effect.

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Appendix

Attachment 1: Sex, age and physiological data of cod collected in the inner Oslofjord.

Lab code	Sex	Age (years)	Length (cm)	Weight (g)	Liver (g)	LSI	Gonads (g)	GSI	Condition
IO 1	M	3	49,5	1023,5	35,2	3,44	13,1	1,28	0,84
IO 2	M	2	48,0	900,5	29,7	3,30	1,0	0,11	0,81
IO 3	F	2	41,0	480,4	11,4	2,37	3,3	0,69	0,70
IO 4	F	1	47,0	863,0	39,7	4,60	29,6	3,43	0,83
IO 5	F	3	42,0	640,0	25,9	4,05	8,4	1,31	0,86
IO 6	F	3	46,5	900,0	48,9	5,43	15,9	1,77	0,90
IO 7	F	3	43,0	690,5	37,0	5,36	12,8	1,85	0,87
IO 8	F	2	53,0	1203,0	45,4	3,77	12,5	1,04	0,81
IO 9	F	4	50,0	994,0	15,0	1,51	8,5	0,86	0,80
IO 10	M	4	54,5	1410,4	20,5	1,45	6,2	0,44	0,87
IO 11	M	5	47,0	967,5	32,0	3,31	2,6	0,27	0,93
IO 12	F	3	40,0	520,0	24,5	4,71	2,0	0,38	0,81
IO 13	M	4	46,0	845,0	17,0	2,01	20,9	2,47	0,87
IO 14	F	4	52,0	1233,0	79,0	6,41	20,0	1,62	0,88
IO 15	F	4	49,0	935,5	33,0	3,53	25,5	2,73	0,80
IO 16	F	2	49,0	992,5	29,1	2,93	4,0	0,40	0,84
IO 17	F	4	42,0	637,5	28,5	4,47	28,0	4,39	0,86
IO 18	M	5	47,0	905,0	25,0	2,76	70,2	7,76	0,87
IO 19	M	5	59,5	1808,8	56,1	3,10	27,2	1,50	0,86
IO 20	M	3	44,0	691,0	26,0	3,76	12,3	1,78	0,81
IO 21	M	3	45,0	948,0	41,7	4,40	43,1	4,55	1,04
IO 22	F	5	54,0	1105,5	60,2	5,45	23,1	2,09	0,70
IO 23	M	3	39,0	501,0	20,0	3,99	9,0	1,80	0,84
IO 24	M	3	44,0	693,5	26,9	3,88	49,0	7,07	0,81
IO 25	M	5	52,0	1135,0	33,2	2,93	36,6	3,22	0,81
IO 26	M	2	41,0	565,0	17,0	3,01	0,5	0,09	0,82
IO 27	F	4	49,0	892,5	41,5	4,65	17,0	1,90	0,76
IO 28	M	3	46,0	788,5	17,2	2,18	24,8	3,15	0,81
IO 29	M	3	41,0	675,5	26,5	3,92	31,2	4,62	0,98
IO 30	F	2	36,0	320,0	5,6	1,75	2,0	0,63	0,69
IO 31	M	2	45,5	688,0	12,6	1,83	1,7	0,25	0,73
IO 32	M	4	46,5	917,0	48,1	5,25	20,8	2,27	0,91
IO 33	M	2	40,0	585,5	10,0	1,71	17,3	2,95	0,91
IO 34	M	5	47,0	1029,5	30,6	2,97	12,5	1,21	0,99
IO 35	F	4	50,0	1051,0	38,3	3,64	18,9	1,80	0,84
IO 36	F	3	44,0	731,0	22,2	3,04	10,4	1,42	0,86
IO 37	M	3	43,0	759,0	37,0	4,87	33,0	4,35	0,95
IO 38	F	4	43,0	731,0	56,6	7,74	22,3	3,05	0,92
IO 39	F	3	48,0	921,5	31,8	3,45	4,4	0,48	0,83
IO 40	M	4	45,0	815,0	34,3	4,21	5,6	0,69	0,89

Attachment 2: Sex, age and physiological data of cod collected in the outer Oslofjord.

Lab code	Sex	Age (years)	Length (cm)	Weight (g)	Liver (g)	LSI	Gonads (g)	GSI	Condition
OO 1	F	2	47,5	841,5	18,4	2,19	3,9	0,46	0,79
OO 2	M	2	43,0	656,5	7,3	1,11	0,5	0,08	0,83
OO 3	M	2	42,5	589,6	91,0	15,43	0,1	0,02	0,77
OO 4	M	3	46,5	725,5	9,1	1,25	6,9	0,95	0,72
OO 5	F	2	47,5	898,5	20,1	2,24	4,5	0,50	0,84
OO 6	F	2	41,5	559,0	11,7	2,09	2,5	0,45	0,78
OO 7	M	2	37,0	416,6	5,3	1,27	0,1	0,02	0,82
OO 8	F	1	51,0	1066,0	14,3	1,34	4,6	0,43	0,80
OO 9	F	2	40,0	519,0	8,1	1,56	1,9	0,37	0,81
OO 10	F	NA	61,0	NA	74,2	NA	24,2	NA	0,00
OO 11	F	2	64,0	2329,0	22,8	0,98	7,4	0,32	0,89
OO 12		3	47,5	819,5	93,4	11,40	4,1	0,50	0,76
OO 13	F	2	49,0	889,0	34,2	3,85	3,3	0,37	0,76
OO 14	M	2	43,0	600,0	5,4	0,90	0,5	0,08	0,75
OO 15	F	2	47,0	789,5	9,2	1,17	2,8	0,35	0,76
OO 16	F	2	52,5	1010,0	NA	NA	15,5	1,53	0,70
OO 17	M	2	44,0	813,5	13,7	1,68	2,0	0,25	0,95
OO 18	F	1	55,5	1309,0	16,3	1,25	5,3	0,40	0,77
OO 19	F	2	43,0	660,0	13,0	1,97	2,4	0,36	0,83
OO 20	F	2	48,0	940,0	10,7	1,14	3,9	0,41	0,85
OO 21	M	2	51,0	1013,5	23,5	2,32	1,3	0,13	0,76
OO 22	M	2	37,0	365,0	5,5	1,51	0,1	0,03	0,72
OO 23	M	2	45,0	673,0	8,2	1,22	0,6	0,09	0,74
OO 24	M	2	44,0	700,5	NA	NA	0,8	0,11	0,82
OO 25	M	2	51,5	1078,0	13,6	1,26	4,0	0,37	0,79
OO 26	F	2	43,0	645,5	15,8	2,45	12,2	1,89	0,81
OO 27	M	2	43,0	518,0	8,0	1,54	0,6	0,12	0,65
OO 28	F	3	51,0	1181,5	56,2	4,76	5,2	0,44	0,89
OO 29	F	2	53,0	1135,0	28,5	2,51	19,0	1,67	0,76
OO 30	F	2	46,0	813,5	22,4	2,75	5,1	0,63	0,84
OO 31	M	2	40,0	539,5	6,0	1,11	0,5	0,09	0,84
OO 32	F	2	41,0	536,2	11,5	2,14	11,8	2,20	0,78
OO 33	M	NA	46,0	772,0	11,7	1,52	0,1	0,01	0,79
OO 34	F	2	45,0	725,0	8,2	1,13	2,9	0,40	0,80
OO 35	F	2	51,0	1061,5	26,4	2,49	4,8	0,45	0,80
OO 36	M	2	49,0	891,5	NA	NA	0,5	0,06	0,76
OO 37	F	2	47,5	866,5	13,7	1,58	3,1	0,36	0,81
OO 38	F	2	53,5	1194,1	19,8	1,66	4,3	0,36	0,78
OO 39	F	1	52,5	1334,5	34,3	2,57	6,5	0,49	0,92
OO 40	F	2	61,0	2021,5	16,4	0,81	12,8	0,63	0,89

Attachment 3: Data for biomarkers from cod collected in the inner Oslofjord.

Lab code	Sex	OH-pyrene	OH-phenanthrene	OH-B[a]P	ELISA	EROD	ALA-D	AChE
IO 1	M	147	2,82	0,88	244	49,51	9,03	26,7
IO 2	M	529	17,38	3,19	174	31,16	5,42	33,0
IO 3	F	291	8,50	2,63	128	23,74	14,10	37,7
IO 4	F	297	15,60	3,50	101	7,75	NA	28,0
IO 5	F	213	5,51	1,31	117	24,48	6,17	32,9
IO 6	F	169	5,10	0,85	163	33,17	9,36	27,5
IO 7	F	292	9,81	1,40	283	46,68	3,89	27,5
IO 8	F	297	12,76	2,57	72	31,14	-1,79	30,0
IO 9	F	237	4,23	2,30	77	38,51	8,82	35,1
IO 10	M	323	14,28	3,56	145	30,93	7,54	29,2
IO 11	M	373	14,20	3,15	100	17,61	7,54	44,5
IO 12	F	138	4,71	1,08	123	16,47	16,68	65,2
IO 13	M	450	15,44	5,07	131	32,92	5,26	70,4
IO 14	F	348	12,97	1,86	102	25,54	26,58	43,8
IO 15	F	183	8,72	2,44	56	19,75	3,61	87,4
IO 16	F	196	7,51	1,15	200	57,75	25,04	59,4
IO 17	F	211	5,93	1,31	103	28,07	2,28	56,2
IO 18	M	165	6,20	2,13	103	20,69	17,43	63,7
IO 19	M	254	8,07	1,83	253	45,66	10,12	42,0
IO 20	M	236	6,60	1,54	101	57,84	10,77	77,6
IO 21	M	249	9,36	2,23	139	35,98	4,54	52,5
IO 22	F	176	6,25	0,91	127	34,66	19,71	46,7
IO 23	M	362	12,01	2,57	177	58,89	5,20	71,6
IO 24	M	329	16,07	3,43	101	49,30	11,42	72,3
IO 25	M	234	10,30	1,12	142	48,40	17,16	80,9
IO 26	M	174	5,18	1,88	172	54,54	NA	45,2
IO 27	F	191	7,65	1,76	169	36,10	2,69	57,8
IO 28	M	514	17,23	4,64	59	30,29	5,54	107,4
IO 29	M	304	15,67	2,83	77	37,57	6,99	66,4
IO 30	F	299	12,04	2,50	258	63,57	18,70	90,8
IO 31	M	387	17,00	3,95	115	72,10	9,04	54,9
IO 32	M	313	18,05	3,05	101	21,45	14,63	47,3
IO 33	M	149	7,45	0,87	173	47,12	11,93	83,7
IO 34	M	284	12,90	2,65	84	33,29	9,39	52,3
IO 35	F	259	10,81	2,58	226	48,39	8,16	53,4
IO 36	F	182	6,29	1,65	184	59,30	7,32	67,8
IO 37	M	549	17,62	4,87	145	23,10	1,57	50,7
IO 38	F	NA	NA	NA	215	23,04	12,44	64,8
IO 39	F	212	4,86	2,16	82	32,88	5,87	35,4
IO 40	M	250	8,14	1,08	189	50,00	7,88	64,0

Attachment 4: Data for biomarkers from cod collected in the outer Oslofjord.

Lab code	Sex	OH-pyrene	OH-phenanthrene	OH-B[a]P	ELISA	EROD	ALA-D	AChE
OO 1	F	17	2,78	1,54	63	20,91	10,65	46,0
OO 2	M	27	3,58	0,89	49	16,29	14,12	51,5
OO 3	M	62	6,98	2,05	92	3,96	13,89	43,9
OO 4	M	15	1,86	1,64	64	14,26	17,13	46,3
OO 5	F	17	1,74	0,97	42	31,66	5,60	41,0
OO 6	F	29	4,33	2,51	74	27,60	15,58	46,3
OO 7	M	26	3,42	1,38	65	14,78	NA	67,7
OO 8	F	39	7,09	2,15	50	25,66	10,25	55,9
OO 9	F	24	2,76	1,47	79	37,06	NA	48,4
OO 10	F	34	4,99	2,25	45	8,85	NA	33,8
OO 11	F	38	3,10	2,20	47	22,00	14,13	37,2
OO 12		53	6,78	2,68	53	10,9	NA	44,2
OO 13	F	45	4,63	1,71	55	10,6	13,44	39,4
OO 14	M	43	5,35	1,52	96	8,9	NA	70,3
OO 15	F	77	5,64	1,22	73	37,3	5,93	46,3
OO 16	F	22	1,57	0,76	83	15,0	7,62	49,8
OO 17	M	13	5,49	1,07	72	6,3	5,86	51,5
OO 18	F	39	6,51	2,25	65	7,8	NA	43,3
OO 19	F	30	4,47	2,01	68	26,1	10,46	53,4
OO 20	F	20	2,82	1,06	64	39,9	19,69	54,3
OO 21	M	36	2,76	1,91	132	22,5	10,49	34,8
OO 22	M	40	5,96	4,08	45	24,8	9,09	84,8
OO 23	M	25	3,79	1,60	81	39,20	3,10	62,0
OO 24	M	11	2,48	1,31	63	25,70	13,30	79,6
OO 25	M	19	2,00	0,79	125	39,55	12,28	27,0
OO 26	F	20	1,73	1,01	131	36,15	9,77	28,5
OO 27	M	23	3,28	0,98	81	35,70	18,47	36,8
OO 28	F	53	6,68	3,65	50	6,85	11,51	31,4
OO 29	F	82	9,08	3,92	71	26,41	13,76	28,3
OO 30	F	15	1,72	1,53	92	27,40	8,63	30,4
OO 31	M	42	2,42	2,03	162	101,13	13,96	30,4
OO 32	F	47	5,88	2,75	86	18,10	5,81	32,1
OO 33	M	46	2,12	0,61	95	55,91	13,74	34,3
OO 34	F	22	2,05	1,11	112	16,75	22,09	35,8
OO 35	F	21	0,97	1,05	NA	NA	13,93	26,4
OO 36	M	45	5,27	2,73	98	51,82	18,58	44,9
OO 37	F	28	2,98	1,15	99	28,48	13,47	31,3
OO 38	F	32	2,71	1,44	104	48,28	14,56	32,6
OO 39	F	59	2,96	2,00	78	22,54	9,69	33,5
OO 40	F	52	5,32	3,56	57	48,64	19,34	28,0

Attachment 5: DNA damage (TI= tail intensity) in leukocytes from cod exposed 0, 5 and 300 µM H_2O_2 respectively and stored on lysis buffer for one and three weeks.

Lab code	Location	Sex	Week	TI at 0 µM H_2O_2	TI at 5µM H_2O_2	TI at 300 µM H_2O_2
IO 1	inner Oslofjord	M	1	45,0	58,9	64,2
IO 2	inner Oslofjord	M	1	54,1	60,6	59,0
IO 3	inner Oslofjord	F	1	57,6	72,9	63,9
IO 4	inner Oslofjord	F	1	70,6	68,4	64,7
IO 5	inner Oslofjord	F	1	30,5	45,1	61,2
IO 6	inner Oslofjord	F	1	50,3	65,0	62,1
IO 7	inner Oslofjord	F	1	49,8	53,4	67,8
IO 8	inner Oslofjord	F	1	43,6	52,2	53,4
IO 9	inner Oslofjord	F	1	NA	49,0	48,4
IO 10	inner Oslofjord	M	1	21,8	43,3	56,7
IO 11	inner Oslofjord	M	1	41,6	64,8	53,7
IO 12	inner Oslofjord	F	1	NA	57,1	61,7
IO 13	inner Oslofjord	M	1	16,6	45,0	54,0
IO 14	inner Oslofjord	F	1	35,8	53,7	57,1
IO 15	inner Oslofjord	F	1	28,2	45,1	56,3
IO 16	inner Oslofjord	F	1	26,7	44,5	60,9
IO 17	inner Oslofjord	F	1	18,7	44,5	57,6
IO 18	inner Oslofjord	M	1	8,6	22,5	29,7
IO 19	inner Oslofjord	M	1	33,8	58,4	66,9
IO 20	inner Oslofjord	M	1	13,8	55,2	NA
IO 21	inner Oslofjord	M	1	13,1	53,7	51,2
IO 22	inner Oslofjord	F	1	10,9	43,8	60,5
IO 23	inner Oslofjord	M	1	19,5	42,3	58,7
IO 24	inner Oslofjord	M	1	19,4	61,1	62,5
IO 25	inner Oslofjord	M	1	31,9	57,3	47,0
IO 26	inner Oslofjord	M	1	NA	NA	NA
IO 27	inner Oslofjord	F	1	22,3	51,6	59,9
IO 28	inner Oslofjord	M	1	56,9	54,6	57,9
IO 29	inner Oslofjord	M	1	55,5	53,3	62,7
IO 30	inner Oslofjord	F	1	28,4	49,0	71,0
IO 31	inner Oslofjord	M	1	21,5	48,6	65,2
IO 32	inner Oslofjord	M	1	35,9	64,6	71,0
IO 33	inner Oslofjord	M	1	25,9	40,0	57,6
IO 34	inner Oslofjord	M	1	56,6	51,0	47,5
IO 35	inner Oslofjord	F	1	34,7	55,6	74,5
IO 36	inner Oslofjord	F	1	51,2	55,7	69,5
IO 37	inner Oslofjord	M	1	35,9	49,3	62,7
IO 38	inner Oslofjord	F	1	53,6	41,9	50,0
IO 39	inner Oslofjord	F	1	45,3	48,0	63,4
IO 40	inner Oslofjord	M	1	54,3	57,6	63,1
OO 17	outer Oslofjord	M	1	9,9	32,2	45,8
OO 18	outer Oslofjord	F	1	7,3	27,2	59,0
OO 19	outer Oslofjord	F	1	9,8	40,6	74,8
OO 20	outer Oslofjord	F	1	12,9	35,8	66,7
OO 21	outer Oslofjord	M	1	6,9	22,5	87,3
OO 22	outer Oslofjord	M	1	10,2	27,6	85,2
OO 23	outer Oslofjord	M	1	13,0	31,0	79,2
OO 24	outer Oslofjord	M	1	7,3	43,6	70,8

OO 25	outer Oslofjord	M	1	24,7	24,1	73,7
OO 26	outer Oslofjord	F	1	4,3	20,8	84,0
OO 27	outer Oslofjord	M	1	6,3	17,7	75,0
OO 28	outer Oslofjord	F	1	28,4	46,6	76,7
OO 29	outer Oslofjord	F	1	1,9	17,3	82,6
OO 30	outer Oslofjord	F	1	16,0	45,8	73,8
OO 31	outer Oslofjord	M	1	13,9	46,0	77,7
OO 32	outer Oslofjord	F	1	9,4	18,9	81,0
OO 33	outer Oslofjord	M	1	7,2	21,6	88,8
OO 34	outer Oslofjord	F	1	9,9	20,9	NA
OO 35	outer Oslofjord	F	1	4,2	23,8	65,9
OO 36	outer Oslofjord	M	1	11,3	33,1	77,9
OO 37	outer Oslofjord	F	1	3,3	17,0	NA
OO 38	outer Oslofjord	F	1	5,5	13,3	81,4
OO 39	outer Oslofjord	F	1	5,2	33,0	48,8
OO 40	outer Oslofjord	F	1	6,9	23,6	86,6
IO 1	inner Oslofjord	M	3	72,2	74,8	71,9
IO 2	inner Oslofjord	M	3	67,1	67,1	66,9
IO 3	inner Oslofjord	F	3	49,8	80,2	74,9
IO 4	inner Oslofjord	F	3	65,8	80,0	67,8
IO 5	inner Oslofjord	F	3	51,1	65,7	52,6
IO 6	inner Oslofjord	F	3	66,7	75,7	68,4
IO 7	inner Oslofjord	F	3	46,4	59,1	63,2
IO 8	inner Oslofjord	F	3	42,2	60,2	62,0
IO 9	inner Oslofjord	F	3	38,9	52,9	63,4
IO 10	inner Oslofjord	M	3	27,2	60,4	51,7
IO 11	inner Oslofjord	M	3	44,0	67,1	62,7
IO 12	inner Oslofjord	F	3	50,0	70,0	69,6
IO 13	inner Oslofjord	M	3	33,3	63,3	53,4
IO 14	inner Oslofjord	F	3	48,5	65,3	65,9
IO 15	inner Oslofjord	F	3	42,6	57,0	65,0
IO 16	inner Oslofjord	F	3	43,5	62,0	66,1
IO 17	inner Oslofjord	F	3	28,3	55,4	61,9
IO 18	inner Oslofjord	M	3	19,0	37,3	38,5
IO 19	inner Oslofjord	M	3	44,8	73,2	77,9
IO 20	inner Oslofjord	M	3	31,1	63,7	NA
IO 21	inner Oslofjord	M	3	36,0	67,0	58,9
IO 22	inner Oslofjord	F	3	23,0	64,4	75,2
IO 23	inner Oslofjord	M	3	34,8	68,5	62,0
IO 24	inner Oslofjord	M	3	32,4	48,6	63,7
IO 25	inner Oslofjord	M	3	40,6	51,4	53,8
IO 26	inner Oslofjord	M	3	NA	NA	NA
IO 27	inner Oslofjord	F	3	27,8	46,7	56,2
IO 28	inner Oslofjord	M	3	54,4	49,9	NA
IO 29	inner Oslofjord	M	3	52,6	57,0	NA
IO 30	inner Oslofjord	F	3	42,5	55,9	73,1
IO 31	inner Oslofjord	M	3	39,2	55,5	68,8
IO 32	inner Oslofjord	M	3	47,1	65,1	64,0
IO 33	inner Oslofjord	M	3	30,6	42,0	64,4
IO 34	inner Oslofjord	M	3	26,9	53,9	60,1
IO 35	inner Oslofjord	F	3	39,4	66,4	77,3
IO 36	inner Oslofjord	F	3	39,5	61,6	55,6
IO 37	inner Oslofjord	M	3	35,6	58,0	63,1

IO 38	inner Oslofjord	F	3	36,0	46,5	60,2
IO 39	inner Oslofjord	F	3	32,0	57,9	69,2
IO 40	inner Oslofjord	M	3	39,8	66,8	60,4
OO 17	outer Oslofjord	M	3	20,4	55,0	75,6
OO 18	outer Oslofjord	F	3	18,3	46,1	79,7
OO 19	outer Oslofjord	F	3	23,6	43,7	81,9
OO 20	outer Oslofjord	F	3	21,2	43,8	77,4
OO 21	outer Oslofjord	M	3	15,5	34,8	89,3
OO 22	outer Oslofjord	M	3	18,5	26,1	87,3
OO 23	outer Oslofjord	M	3	17,5	33,3	76,4
OO 24	outer Oslofjord	M	3	8,7	36,7	81,6
OO 25	outer Oslofjord	M	3	22,0	34,8	85,9
OO 26	outer Oslofjord	F	3	9,5	27,4	87,0
OO 27	outer Oslofjord	M	3	11,7	21,1	78,5
OO 28	outer Oslofjord	F	3	23,4	48,3	77,9
OO 29	outer Oslofjord	F	3	19,5	25,9	86,2
OO 30	outer Oslofjord	F	3	26,4	59,5	82,6
OO 31	outer Oslofjord	M	3	19,4	62,8	88,2
OO 32	outer Oslofjord	F	3	11,7	31,9	81,6
OO 33	outer Oslofjord	M	3	9,9	31,1	84,5
OO 34	outer Oslofjord	F	3	11,8	38,1	86,8
OO 35	outer Oslofjord	F	3	11,3	30,0	69,8
OO 36	outer Oslofjord	M	3	30,4	50,9	82,9
OO 37	outer Oslofjord	F	3	13,0	32,2	93,2
OO 38	outer Oslofjord	F	3	19,7	21,6	83,4
OO 39	outer Oslofjord	F	3	18,4	40,0	67,8
OO 40	outer Oslofjord	F	3	17,3	34,9	76,7

Attachment 7: Chemicals

Chemical	Product No	Producer
Acetic acid	33209	Sigma-Aldrich
acetylthiocholine iodide	A5751	Sigma-Aldrich
Alkaline copper tartrate	500-0113	BioRad
Bovin serum albumin	A3803	Sigma-Aldrich
Carbonate-bicarbonate buffer (capsule)	C3041	Sigma-Aldrich
copper tartrate solution (reagent A)	500-0113	Bio-Rad
Dimethyl sulfoxide	472310	Sigma-Aldrich
disodium ethylenediaminetatraacetate*2H_2O	E7889	Sigma-Aldrich
Dithiobisnitrobenzoic acid	D8130	Sigma-Aldrich
Dithiotreitol	MB1015	Melford lab
Folin reage-nt (reagent B)	500-0114	Bio-Rad
Glycerol	G5516	Sigma-Aldrich
Goat anti-rabbit IgG conjugated with HRP, GAR-HRP	10004301	Cayman Chemical Company
Heparin	H5515	Sigma-Aldrich
hydrogen peroxide (30%)	1.07298.0250	Merck
LMP agarose	A9414	Sigma-Aldrich

Mercury (II) chloride	203777	Sigma-Aldrich
Perchloric acid (70%)	589.293	Prolabo
Percoll	17-0891-01	GE Healthcare
Porphobilinogen	P1135	Sigma-Aldrich
Potassium chloride	1.04936.1000	Merck
potassium phosphate dibasic	221317	Sigma-Aldrich
potassium phosphate monobasic	P5379	Sigma-Aldrich
Rabbit-anti-fish CYP1A antibody	CP226	Cayman Chemical Company
Resorufin ethyl ether	E3763	Sigma-Aldrich
Resorufin sodium salt	R3257	Sigma-Aldrich
Sodium dihydrogenphosphate	71504	Sigma-Aldrich
Sodium hydrogencarbonate	L1703	Biochrom AG
Sodium phosphate dibasic	S5136	Sigma-Aldrich
Sulfuric acid	1.00731.1000	Merck
Tetramethylbenzidine liquid substrate	4390A	KEM EN TEC Diagnostics
Trichloroacetic acid	1.00807.0250	Merck
Tris Hydrochloride	T3253	Sigma-Aldrich
Triton X-100	T8787	Sigma-Aldrich
Trizma base	T-1503	Sigma-Aldrich
Tween-20	P1379	Sigma-Aldrich
β-NADP reduced tetrasodium salt	N1630	Sigma-Aldrich
δ-aminolevulinic acid	A3785	Sigma-Aldrich
ρ-dimethylaminobenzaldehyde	D2004	Sigma-Aldrich

Attachment 8: Solutions

Comet assay		
Phosphate buffered saline (Ca + Mg free) stock solution (PBS)	NaCl	8.5 g
	Na2HPO4	0.85 g
	KH2PO4	0.54 g
	dH2O	up to 100 mL
		pH 7.4
PBS EDTA (Ca + Mg free) working solution	PBS	10 %
	Na2EDTA* 2H2O	10 mM
Lysis buffer stock solution	NaCl	2.5 M
	Na2EDTA* 2H2O	100 mM
	Trizma base	10 mM
	Triton X-100	1 %
		pH 10
Lysis buffer working solution	Lysis stock solution	89 %
	dH2O	10 %
	Triton X-100	1 %
Electrophoresis buffer stock solution	NaOH	3 M
	Na2EDTA* 2H2O	1 mM
Electrophoresis buffer working solution	electrophoresis buffer stock solution	200 mL
	dH2O	1800 mL
	Concentrated HCl	12 mL
		pH 13.2
Neutralising buffer	Trizma base	400 mM
		pH 7.5
LMP Agarose	LMP agarose	0.75 mg
	PBS EDTA working solution	10 mL
	boil in microwave until clear and no crystals, cool at 37˚C	
Tris-EDTA (TE) buffer	Tris Hydrochloride	10 mM
	Na2EDTA* 2H2O	1 mM
		pH 8
Staintining solution (one film)	SYBR Gold prediluted 10 x in DMSO	40 µL
	TE- buffer	50 mL

Protein analysis		
Tris buffer	Tris hydrochloride	0.1 M
	Trisma base	0.1 M
		pH 8

ALA-D activity		
ALA-reagent	δ-aminolevulinic acid (ALA)	33.5 mg
	Dilution buffer	50 mL
Dilution buffer	0.2 M Sodium dihydrogenphosphate	37.5 mL
	0.2 M Disodium hydrogen phosphate	62.5 mL
	Triton X-100	1 mL
	dH$_2$O	up to 200 mL
Modified Ehrlichs reagent	Mercury chloride	350 mg dissolved in 6 mL dH$_2$O
	70% Perchloric acid	20 mL
	Acetic acid	up to 110 mL
	Ehrlich's reagent	2 g
Precipitation buffer	Trichloroacetic acid	4 g
	Mercury chloride	2.7 g
	dH$_2$O	up to 100 mL

Liver preparation		
Buffer stock solution	Sodium dihydrogenphosphate	0.1 M
	Disodium hydrogen phosphate	0.1 M
	Potassium chloride	0.15 M
		pH 7.8
Homogenising buffer	Dithiotreitol	1 mM
	Glycerol	5 %
	Dissolved in buffer stock solution	
Microsomal buffer	Glycerol	20 %
	Dissolved in buffer stock solution	

EROD activity		
Phosphate buffer	potassium phosphate dibasic	0.1 M
	potassium phosphate monobasic	0.1 M
		pH 8
NADPH	NADPH	50 mM
	Dissolved in phosphate buffer	
7-ethoxyresorufin	7-ethoxyresorufin	0.2 mM
	Dissolved in DMSO	
Standard	Resorufin	1 mM
	Dissolved in DMSO	

ELISA		
Anti-body solution	BSA	0.1%
	Dissolved in TTBS	
Blockingsolution	BSA	1 %
	Dissolved in TTBS	
Coatingbuffer	Carbonate-bicarbonate buffer	1 capsule
	Dissolved in 100 mL dH2O	
Stopsolution	Sulfuric acid	1.5 M
	Dissolved in dH2O	
TTBS	Tris-buffer	20 mM
		pH 8.5
	Sodium chloride	0.5 M
	dH2O	up to 10 L
	Tween-20	5 mL

AChE activity		
Potassium phosphate buffer	potassium phosphate dibasic	0.1 M
	potassium phosphate monobasic	0.1 M
		pH 7.2
Dithiobisnitrobenzoic acid (DTNB) solution	DTNB	0.01 M
	Sodium hydrogen carbonate	1.5 mg/mL
	dissolved in potassium phosphate buffer	
AChE assay reagent	Potassium phosphate buffer	30 mL
	75 mM Acetylthiocholine iodide (ATC) dissolved in dH2O	0.2 mL
	DTNB solution	1 mL

Attachment 9: Lab equipment

Instrument/Software	Producer
AccuBlock Digital Dry Bath	Labnet International, Inc.
Analytical scale, AG204	Mettler Toledo
Analytical scale, PB210 S	Sartorius
Centrifuge 5424	eppendorf
Comet Assay IV	Perceptive Instruments
Cooling Incubator, series 6000	Termaks
Electrophoresis system	Norwegian Institure of Public Health
ENDURO MiniMix	Labnet International, Inc.
Gen5	BioTek
LAB pH meter, PHM 92	Radiometer Cobenhagen
Leica DMR	Leica
Micro-Ultracentrifuge, SORVALL MTX 150	Thermo Scientific
Multifuge 3 S-R	Heraceus
Potter-Elvehjem homogenizer	
SkanWasher 300	Skatron Instruments
Stingray	ALLIED Vision Technologies
SynergyMX	BioTek
Ultraturrax, PRO 200	PRO Scientific inc.
Vortex mixer, SA8	Stuart
Waters 26595 Separation Module	Waters
Whirlimixer	Fisons
Wild M3B	Heerbrugg Switzerland