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REPORT

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Abstract

The report presents results from the Water Column Monitoring 2009, performed in collaboration between NIVA and IRIS, with sub-contractors. The objective of the survey was to assess the extent to which produced water discharges from Ekofisk affect organisms living in the water column. The study was designed to monitor bio-accumulation and biomarker responses in organisms held in cages in the vicinity of the water discharge point(s). The study design was identical to that carried out at Ekofisk in 2006 and 2008 with only minor modifications where necessary. The implementation of a produced water treatment system (C-Tour), operational in 2008, aimed to reduce the output of potentially toxic chemicals into the surrounding water column. The monitoring surveys performed pre and post implementation of C-Tour, were designed to assess whether changes in animal health status between the different years have occurred.

The results of the 2009 survey have shown that caged organisms were exposed to low levels of produced water components with highest tissue concentrations in mussels *Mytilus edulis*, located closest to the produced water discharge. Mussels located approximately 1-2 km away showed only background concentrations of the organic compounds measured. Concentrations of polycyclic aromatic hydrocarbon (PAH) and alkyl phenol (AP)-metabolites in bile of caged cod *Gadus morhua*, were slightly elevated suggesting exposure at the Ekofisk stations. There was a signal of exposure with proximity to the discharge for some of the biomarkers measured including CYP1A in fish and micronuclei in mussels. However, all other fish and mussel biomarkers showed no significant exposure effects. The bioaccumulation data and biomarker responses indicated a lower exposure to the produced water effluent than seen previously in 2008 and 2006.

The volume of produced water discharged to the investigated area increased in 2009 from 2008 and 2006. However, the amount of oil discharged with the produced water was less, resulting in an overall reduction of oil to the local area. This reduction in oil discharge was reflected in the bioaccumulation and biomarker data, resulting in a general improvement in the health of caged mussels and fish in 2009 compared to 2008 and 2006. Overall, based on the bioaccumulation and biomarker data in 2009, the current environmental risk of the PW on animals living in the water column in proximity to the platforms is low.

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1. Biomarkører	1. Biomarkers
2. Produsertvann	2. Produced water
3. Offshore	3. Offshore
4. Fisk og blåskjell	4. Fish and mussels

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Preface

The Water Column Monitoring (WCM) programme performs investigations into the potential biological effects of offshore oil and gas activity on the biota living within the water column of the Norwegian sector of the North Sea. The Norwegian Oil Industry Association (OLF), who funded the project, are obliged by the Norwegian authorities to perform biological monitoring offshore. The work has been performed at various fields over the last 20 years, with Ekofisk being the choice for 2009 as well as in 2008 and 2006. The implementation of a produced water treatment system (C-Tour) at Ekofisk in 2008, led to the decision to monitor Ekofisk again in 2008 and 2009, thereby attempting to determine whether environmental benefits of the treatment system could be observed since 2006. The methods used are considered to be the best available technology for the assessment of biological effects monitoring, measuring chemical bioaccumulation and biomarker responses in mussels and fish for an integrated assessment of chemical exposure and organism health status.

The WCM programme has been carried out through collaboration with NIVA and IRIS with subcontractors from the Universities of Stockholm and Vilnius. The work participants from the two leading laboratories are listed below.

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Summary

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The implementation of a produced water treatment system (C-Tour), operational in 2008, aimed to reduce the output of potentially toxic chemicals into the surrounding water column. The monitoring surveys performed pre and post implementation of C-Tour, were designed to assess whether changes in animal health status between the different years have occurred.

The results of the 2009 survey have shown that caged organisms were exposed to low levels of produced water components with highest tissue concentrations in mussels *Mytilus edulis*, located closest to the produced water discharge. Mussels located approximately 1-2 km away had accumulated only background concentrations of the organic compounds measured.

Concentrations of Polycyclic aromatic hydrocarbon (PAH) and Alkyl phenol (AP)-metabolites in bile of caged cod *Gadus morhua*, were slightly elevated suggesting exposure at the Ekofisk stations. There was a signal of exposure with proximity to the discharge for some of the biomarkers measured including CYP1A in fish and micronuclei in mussels. However, all other fish and mussel biomarkers showed no significant exposure effects. The bioaccumulation data and biomarker responses indicated a lower exposure to the produced water effluent than seen previously in 2008 and 2006.

The volume of produced water discharged to the investigated area increased in 2009 from 2008 and 2006. However, the amount of oil discharged with the produced water was less, resulting in an overall reduction of oil to the local area. This reduction in oil discharge was reflected in the bioaccumulation and biomarker data, resulting in a general improvement in the health of caged mussels and fish in 2009 compared to 2008 and 2006. Overall, based on the bioaccumulation and biomarker data in 2009, the current environmental risk of the PW on animals living in the water column in proximity to the platforms is low.

List of symbols

AP	Alkyl phenol
$C_1 - C_9$	referring to the number of carbons in a side chain (e.g. on a PAH or phenol)
COPSAS	ConocoPhillips
CYP1A	Cytochrome P450 1A (CYP1A) proteins
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked ImmunoSorbent Assay
FF	Fixed fluorescence
GC-MS	Gas chromatography – Mass Spectrometry
GST	Glutathione-S-Transferase
IRIS	International Research Institute of Stavanger
NIVA	Norwegian Institute for Water Research
NPD	Naphthalenes, Phenanthrenes and Dibenzothiophenes
OLF	Norwegian Oil Industry Association
PAH	Polycyclic aromatic hydrocarbon
PW	Produced Water
RPM	Revolutions per minute
VTG	Vitellogenin (precursor of egg yolk protein)
WCM	Water Column Monitoring
ZRP	Zona radiata protein (egg shell protein)

1 Introduction

1.1 General purpose of the study

The Water Column Monitoring (WCM) programme is an annual programme that is designed to use the best available biological effects tools to determine the potential impact of the offshore oil and gas activities on the local marine environment. Within the Norwegian sector of the North Sea, the offshore operators are obliged to carry out environmental monitoring within the water column in the vicinity of the offshore installations. This obligation requires that monitoring of the water column should be carried out in at least one offshore oil and gas field each year. Although approval is required by the Norwegian authorities, the operators can choose the study area and the design of the program. The operators then select contractors that perform the study based on the proposed program. It was decided that Ekofisk will be the study site for the WCM programme in 2009. The same location was chosen for the WCM 2009 study as that used in 2006 and 2008. Comparison of the biological effects data between the three years was of principal interest. This was mainly due to the introduction of the produced water (PW) treatment plant (C-Tour) installed at the Ekofisk platform between 2006 and 2008. However, due to operating difficulties, the C-Tour was only working in the latter part of the exposure in the 2008 survey. Consequently, it was decided for the survey to be repeated in 2009. This will enable comparison of the biological effects endpoints between the years and assess the benefits of the treatment system on the local aquatic environment. In addition, the 2009 study will indicate current and future environmental risk on the biota living in the water column at Ekofisk.

1.2 Background

The WCM 2009 was collaboration between the Norwegian Institute for Water Research (NIVA) and International Research Institute of Stavanger (IRIS), with additional sub-contractors. The sub-contractors were the Institute of Applied Environmental Research at Stockholm University, the University of Vilnius and Battelle.

Organisms living in the water column around offshore oil and gas production facilities are predominantly exposed to chemicals through discharge of production water. The amount and composition of PW varies from field to field (Røe, 1998), but is generally a mixture of:

- Formation water contained naturally in the reservoir.
- Injected water used for secondary oil recovery.
- Treatment chemicals added during production.

Typically, produced water contains dissolved inorganic salts, minerals and heavy metals together with dissolved and dispersed oil components and other organic compounds. The specific chemical composition varies between reservoirs and within a reservoir as production proceeds. A target chemical characterisation of four offshore oil production platforms in the North Sea showed that the major organic components were BTEX (benzene, toluene, ethylbenzene and xylene), NPD (naphthalenes, phenanthrenes and dibenzothiophenes), PAHs

(polycyclic aromatic hydrocarbons), organic acids, alkyl phenols (APs) and phenols (Røe and Johnsen, 1996; Utvik, 1999). As a natural consequence of well exploitation, oil content in the reservoirs will decrease and the need to inject water will increase, thus eventually leading to increase in the discharges of PW. Estimates show that the total discharge of PW in the Norwegian sector of the North Sea will increase from approximately 130 million m^3/ year in 2002 to 210 million m^3/ year in 2011 followed by stabilisation and decrease in discharges (Norwegian Petroleum Directorate, 2007).

Some of the organic chemicals found in PW are relatively resistant to biodegradation, have a bioaccumulation potential and may be toxic to organisms in receiving waters (Brendehaug et al., 1992; Tollefsen et al., 1998; Taban and Børseth, 2000; Aas et al., 2000a). This applies in particular to groups of chemicals such as alkylphenols (APs) and polycyclic aromatic hydrocarbons (PAHs) that are known to produce various toxic effects including reproductive disturbances, mutagenicity and carcinogenicity (Landahl et al., 1990; Bechmann, 1999; Lye, 2000; Meier et al., 2002). Studies from the ICES workshop "Biological effects of contaminants in the pelagic ecosystem (BECPELAG)" indicate that toxic compounds can be detected several kilometres away from North Sea oil production platforms using *in vitro* bioassays (Thomas et al., 2006; Tollefsen et al., 2006a) and biomarkers (Regoli et al., 2006). Although there is reason to assume that many of the chemicals that are present in PW effluents may produce biological responses, the ability to assess the potential for adverse effects are limited by the lack of sufficient *in situ* monitoring data using biological effects methods with endpoints reflecting long term (ecological) effects.

Biological indicators or markers (biomarkers) have been developed to measure the biological response related to an exposure to, or the toxic effect of, an environmental chemical (Peakall, 1992). Some biomarkers are specific in terms of their ability to detect and assess the potential for effects through a specific toxic mechanism, whereas others give information about larger groups of chemicals with more diverse mechanisms of action. Common for all of the methods is the capability of performing time-integrating response assessment to complex mixtures over extended periods of time, which is often required in environmental monitoring. Since most of these methods are highly sensitive and responses occur at lower concentrations and/or prior in time to more adverse effects at a higher organisation level, the methods have become convenient early-warning tools for assessing the potential for long term (ecological) effects. The use of biomarkers in sentinel species or specific caging systems with keystone species has consequently facilitated the implementation of such methods in various environmental monitoring programs in freshwater, marine and estuarine areas. Care must be taken to avoid misuse of biomarker data in trials to extrapolate to ecologically relevant effects (Forbes et al, 2006; Lam and Gray, 2003). In recent years, a combination of laboratory and field validation of the different biomarker and effects-based methods has greatly improved the knowledge of the potential and limitations of these methods and made it possible to link responses of biomarker signals to the potential for more adverse effects at the ecological level (Collier et al., 1992; Elliot et al., 2003; Bechmann et al., 2000).

1.3 Objective

The objective of the WCM survey 2009 was to assess the extent to which discharges from an oil production platform (Ekofisk) affect organisms living in the water column. To fulfil this

objective, the survey was designed as described below (chapter 1.4).

In addition, the results of this study were compared to the results of two almost identical studies carried out at Ekofisk in 2006 and 2008. Between 2006 and 2008 a new PW treatment system (C-Tour) was installed at Ekofisk, which was designed to treat the PW prior to discharge (Voldum et al., 2008). Operational problems prior to the survey in 2008 resulted in the decision to conduct a third identical survey in 2009. Comparison of the chemical bioaccumulation and biomarker effect data between the three years provided information on the potential benefits of the C-Tour on improving the water quality around the platform.

1.4 Background on the methods used

This study was designed to monitor bioaccumulation and biomarker responses in cod (*Gadus morhua*) and mussels (*Mytilus edulis*) held in cages at a depth of 15 meters at varying distances from the Ekofisk PW discharge. A number of physicochemical parameters were also determined to provide further information. Background on the methods and the reasons for using them are provided below.

1.4.1 Sea temperature and salinity

In order to collect information about stratification in the sea, conductivity, temperature and depth (CTD) measurements are taken. Such information is considered important since stratification can affect the vertical distribution of the discharged PW. The data may be useful for future modelling studies of plume distribution in the area.

1.4.2 Sea current

Sea current measurements can provide important information on the direction of the discharge plume from the oil platform. This information can then be used to ensure that the cages are situated within the discharge plume during the exposure period. Overall sea current measurements can be useful for future modelling studies of plume distribution in the area.

1.4.3 General biological observations

General biological data as body length, weight and sex is usually recorded in environmental monitoring studies and is used in the interpretation of biomarker data. For the interpretation of biomarkers of reproductive disturbance such as vitellogenin (VTG), the information about sex is crucial for interpretation. A relationship between length and weight can be used as an estimate of the condition of the individual.

1.4.4 Biomarkers in cod

1.4.4.1 PAH-metabolites in bile

The extensive bio-transformation of PAHs by fish greatly prevents the accumulation of these compounds in extra-hepatic tissues (Stein et al., 1987). Consequently, tissue levels of parent PAH do not usually provide an adequate assessment of the PAH exposure level (Varanasi, 1989). PAH metabolites concentrate in the gall bladder of fish following bio-transformation. Analysis of PAH metabolites in the fish bile constitutes a very sensitive method for the assessment of PAH exposure in laboratory and field studies (Beyer et al., 1998; Aas et al., 2001).

Fixed wavelength fluorescence

Generally, the optimal excitation wavelength increases with increasing size of the PAH molecule, i.e. smaller PAHs need more energy (shorter wavelength of the excitation light) than the larger molecules. This variability can be utilised in simple detection methods for PAHs like fixed wavelength fluorescence (FF) detection and synchronous fluorescence spectroscopy (SFS) (Aas et al., 2000b). However, this direct method is not optimal for standardisation and quantification, and should be regarded as a screening method. The metabolites measured with the direct method, are mainly conjugated hydroxyl PAH compounds. Standards of these compounds are difficult and expensive to obtain. With the direct method, different PAH compounds, as well as other natural constituents of the bile, may show interfering fluorescence signals. This may reduce the sensitivity of the method. This is particularly critical when levels are low.

GC-MS

For a more quantitative and qualitative analysis of PAH metabolites, high performance liquid chromatography with fluorescence detection (HPLC/F) or gas chromatography with mass spectrometry in single ion mode detection (GC-MS SIM) can be applied. The GC-MS SIM method is the best suited for the detection of PAH compounds containing 2 to 3 ring structures, namely naphthalenes and phenanthrenes (Jonsson et al., 2003; Jonsson et al., 2004). Both alkyl substituted and non alkyl substituted compounds are detected.

1.4.4.2 AP metabolites in bile

Alkylphenols (APs) are a group of chemicals, which are present in certain discharges from offshore oil activities. Produced water can contain significant levels of APs. As for PAHs the extensive bio-transformation of APs by fish greatly prevents the accumulation of these compounds in extra-hepatic tissues. Exposure studies with radio-labelled APs in fish show that AP metabolites are preferentially excreted through bile (Tollefsen et al., 1998; Sundt et al., 2008a, b). The metabolites concentrate in the gall bladder of fish and specific metabolites of APs from bile can be quantitatively determined by GC/MS. The approach is similar to the detection of PAH metabolites in the bile as a biomarker for exposure to polyaromatic hydrocarbons (Jonsson et al., 2008).

1.4.4.3 Hepatic GST

Glutathione *S*-transferase (GST) is part of the organism's detoxification system converting lipophilic compounds into more hydrophilic compounds that are more easily excreted. Excretion of compounds consists of two major types of reactions: phase I, which involves hydrolysis, oxidation and reduction, and phase II, which involves conjugation. GST is one of the phase II reaction enzymes and catalyses the conjugation of glutathione to compounds with electrophilic centres. The compounds may otherwise be harmful as they may react with macromolecules controlling cell growth, such as DNA, RNA and proteins. Therefore, it is of great importance that the animal is capable of neutralising and excreting these compounds. Changes in the activity of GST may reflect exposure to xenobiotics, and evidence suggests that the level of expression of GST is a crucial factor in determining the sensitivity of cells to a broad spectrum of toxic chemicals. It is also probable that GST is regulated by reactive oxygen species (ROS), and that this would represent an adaptive response to oxidative stress within the cell.

1.4.4.4 CYP1A

The eukaryotic enzyme cytochrome P4501A (CYP1A) is a membrane-bound heme protein belonging to the P450 gene superfamily. It is located in the endoplasmic reticulum (microsomal fraction) of all examined vertebrates and carries out oxidation reactions related to bio-transformation of xenobiotics. CYP1A is induced by certain xenobiotic pollutants, including PCBs and PAHs, and is used as a biomarker of exposure in aquatic organisms. Immunochemical tools, such as Western blots and ELISA, have been used for estimating relative levels of CYP1A in prepared tissues (Goksøyr, 1991).

1.4.4.5 Vitellogenin

The synthesis of the yolk protein vitellogenin (VTG) takes place in the liver of oviparous females under the stimulation of endogenous estradiol (Tata and Smith, 1979). Male and juvenile fish of most species, which only have low levels of circulating estrogens, do not produce appreciable levels of VTG. However, these fish have numerous hepatic oestrogen receptors and are capable of producing high concentrations of VTG when exposed to exogenous estrogens. Induction of this female typical protein in male and juvenile fish has therefore been widely used as a sensitive biomarker for exposure to xenoestrogens (Sumpter and Jobling, 1995). The use of VTG as a biomarker for xenoestrogens in ecologically relevant fish species has been employed for coastal and freshwater environmental monitoring (Hylland et al., 1998; Hylland et al., 1999) and for monitoring of areas that are effected by discharge from oil production activities (Scott et al., 2006). Recent studies with freshwater species such as zebra fish and rainbow trout suggest that induction of VTG occur at concentrations of xenoestrogens that also produce alteration in sexual development when exposed during sensitive early life stages of development (Jobling et al., 1996; Örn et al., 2003)

1.4.4.6 Zona radiata protein

Both the induction of VTG and zona radiata proteins (ZRPs) in male and juvenile oviparous vertebrates have been used as effective and sensitive biomarkers of exposure to xenoestrogens (Arukwe et al., 1997; Arukwe et al., 2000). ZRPs are synthesized in the liver in response to oestrogen stimulation. They are secreted and transported in the blood to the ovary where they form the eggshell that prevents polyspermy, and provides mechanical protection for the developing embryo.

ZRPs have been suggested to be more sensitive than VTG at low concentrations of xenoestrogens (Arukwe et al., 1997). However, it has been identified that when using ZRPs as biomarkers of xenoestrogen exposure, it is important to minimize confounding factors, such as stress (Berg et al., 2004).

1.4.4.7 DNA adducts

The detoxification of genotoxins by the cytochrome P450 mixed function oxygenase systems often results in the production of reactive chemical intermediates that are highly electrophilic and can covalently bind to the bases of DNA to form adducts. Thus, the presence of DNA adducts has been taken as evidence of exposure to specific genotoxicants. A DNA adduct is formed when a non-DNA chemical, e.g. a carcinogenic pollutant chemical, binds covalently to the DNA (normally to the nitrogenous base guanine). Because of the sensitive and consistent responses of hepatic DNA adduct levels to the genotoxic forms of PAH, this parameter is considered to be a reliable biomarker of PAH effect and pro-mutagenic DNA lesions in fish. However, PAHs are not the only chemicals that can form DNA adducts.

The stability of the DNA adduct, i.e. the resistance to DNA repair mechanisms is an important factor. Carcinogenic PAHs form stable DNA adducts after being bio-activated in the cell. And since PAHs are common pollutants in many aquatic environments, this pollutant class has received much attention. In addition to their use as a biomarker for (exposure and) effect of genotoxins, DNA adducts can provide information about the biological effect and potential risk of a chemical. It has been suggested that any chemical that forms stable pro-mutagenic DNA adducts, even at very low levels, should be considered to have mutagenic and carcinogenic potential. In fish DNA adducts are commonly measured in the liver, since this is the key organ for biotransformation of xenobiotics, but other tissues can also be used. In field collected fish, the DNA adduct level provides an indication of long term (typically several months or years) exposure to genotoxins. For further details see Jonsson et al., (2003).

1.4.5 Biomarkers in mussels

1.4.5.1 PAH body burden

The chemical composition of PW is dominated by low molecular PAHs, such as NPDs, decalins and their alkyl homologues (Utvik, 1999). High molecular PAHs such as benzopyrene, pyrene and chrysene are also present in effluents of PW from production platforms in the North Sea, although usually at lower concentrations. Many of the low

molecular weight PAHs have also been detected in caged organisms deployed downstream from known discharge points (Røe, 1998). This applies in particular to alkyl substituted NPDs, which have been found in higher concentrations than their non-alkylated sister compounds in biological tissues and passive sampling devices (Røe, 1998; Ruus et al., 2006). Measurement of contaminant body burdens in caged animals are commonly used to assess the exposure situation in a specified area.

1.4.5.2 Pyrene hydroxylase activity

Pyrene hydroxylase represents an enzymatic activity commonly grouped as mixed function oxidases (MFOs), i.e. cytochrome P450 enzymes. These enzymes metabolise selected PAHs and consequently alter potentially harmful chemicals to non-toxic and readily excretable end products. Pyrene hydroxylase is considered to be a model PAH with a single phase I metabolite i.e. 1-hydroxypyrene. This conjugates to various phase II metabolites that can be enzymatically deconjugated for quantification of total phase I metabolism (Fillman et al., 2004; Jørgensen et al., 2005). Pyrene hydroxylase is thought to be induced by a variety of PAHs and consequently been proposed as a biomarker of exposure to PAH compounds (Fillman et al., 2004).

1.4.5.3 Lysosomal membrane stability

Membrane integrity has been found to be affected by a range of stressors, including metals and organic chemicals. One of the most well-established methods to determine changes in membrane integrity is through measurements on the lysosomal membrane stability (Lowe et al., 1995). The method uses one of a range of available dyes, e.g. neutral red for haemocytes, which will accumulate in the lysosomal compartment of cells. A reduction in membrane integrity will cause the dye to leak back into the cytosol, an effect which can then be quantified. The method is most commonly used with circulating cells, e.g. haemocytes in blue mussels, but methods exist to use a similar method on tissues (Lowe et al., 1995).

1.4.5.4 Micronucleus formation

Chromosomal rearrangements, such as micronuclei, are recognised as a consequence of genome instability (Fenech et al., 1999). The micronuclei test is among the most widely used tools in eco-genotoxicology. Micronuclei are chromatin-containing structures that are surrounded by a membrane and have no detectable link to the cell nucleus. As an index of chromosomal damage, the micronucleus test is based on the enumeration of downstream aberrations after DNA damage and reveals a time-integrated response to complex mixtures of pollutants. The test was developed in several aquatic organisms over the last decade, including mussels (Burgeot et al., 1996; Bolognesi et al., 1996). Cytogenetic damage can result in the formation of micronuclei-containing lagging whole chromosomes or chromosome fragments. Thus, the micronuclei assay provides the evidence of DNA breakage and spindle dysfunction

caused by clastogens and aneuploidogenic poisons (Heddle et al., 1983, 1991; MacGregor, 1991; Seelbach et al., 1993; Kramer, 1998; Zoll-Moreux 1999).

1.4.5.5 Histochemistry

In mussel, histological biomarkers are often analysed in the digestive gland. The digestive gland of molluscs is the main centre for metabolic regulation, participating in the mechanisms of immune defence and homeostatic regulation of the internal medium, as well as in the processes of detoxification and elimination of xenobiotics (Moore and Allen, 2002). In this study, lipofuscin accumulation and neutral lipid content have been chosen as histological biomarkers. Lipofuscin accumulation represents a general response (Viarengo et al., 1990; Regoli, 1992). Elevated lipofuscin accumulation reflects degradation of cellular membrane caused by oxidative damage following the action of different pollutants (Moore, 1988). Neutral lipid accumulation appears to be more strictly linked to organic chemical pollution (Lowe and Clarke, 1989, Cajaraville et al., 1992). Lipophilic xenobiotics may alter the metabolism of neutral lipids leading to abnormal accumulation of that lipid class inside lysosomes (Moore, 1988).

2 Material and methods

2.1 Study design

2.1.1 Source of Cod and Mussels

Farmed cod (*G. morhua*) were obtained from Rygjabø fish farm, Finnøy near Stavanger the same supplier used in the surveys of 2008 and 2006. Farmed cod were used since previous exposure history was known and believed to have been minimal. A veterinary health report confirmed that the farmed cod were in optimal health at the fish farm (Fiskehelse og Miljo AS). Prior to use, fish were held in large external tanks of clean filtered seawater at Rygjabø for at least three weeks prior to deployment in order to reduce pre exposure as much as possible. Fish of similar size and age were used throughout the study (range 1.33 ± 0.56 kg wet weight).

Mussels (*M. edulis*) were obtained from a clean location in Trondheimsfjord, the same population used in the 2008 and 2006 surveys. Mussels were shipped to the IRIS Akvamiljø facility and kept in clean seawater (from 80 m) for 6 days prior to pre-exposure sampling and 3 days prior to field deployment. Mussels of the same size were used throughout the study (length 56.73 ± 4.65 mm).

2.1.2 Cage deployment

A more detailed description of the field work including pre-exposure sampling, deployment cruise and the sampling cruise can be found in the cruise report (Appendix A). A general outline is provided below.

Six stations were positioned along the expected current axis, from close to the PW discharge out to approximately 2000 metres from the installations. Two stations were treated as the reference, with the intention of sampling one (and one as backup, see Figure 1). All stations contained mussels (*M. edulis*) while the two closest to the discharge and the two reference stations also contained cod (*G. morhua*). The fish cages were held at a depth of 10 m at the top of the cage to 16 m at the bottom of the cage. The mussels were attached at the top of the fish cages at a depth between 10 and 12 m. At stations containing mussels only, the mussel cages were held at a depth of 10 to12 m. The fish and mussel cages were deployed for approximately 6 weeks.

Details regarding geographical position of the deployment stations are shown in Table 1, Figure 1. The monitoring approach was based on experiences gained in previous WCM surveys and from the BECPELAG workshop (Hylland et al., 2006).
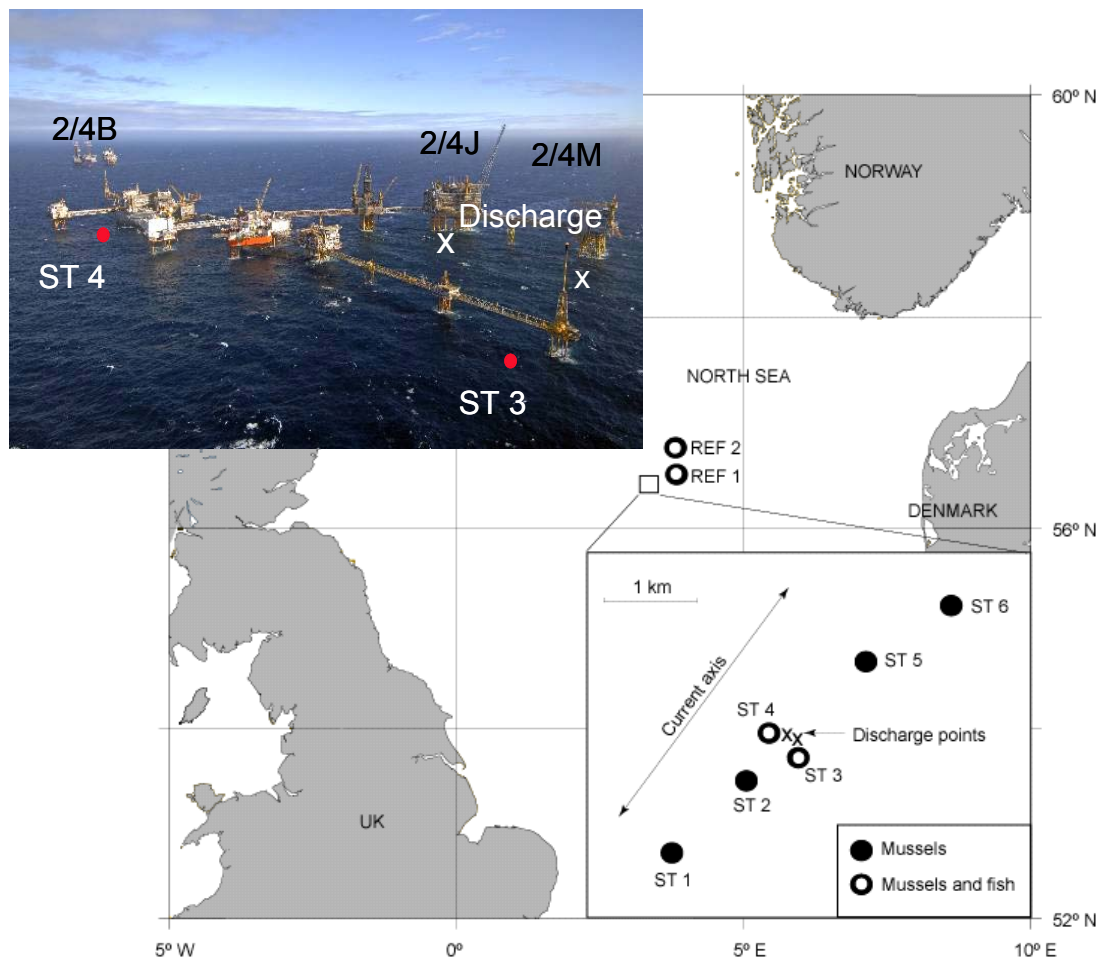
Figure 1. Positions of the caging stations at the Ekofisk field (lower right superimposed panel), and positions of the reference stations in relation to the field. Upper left picture shows the position of station 3 and 4 in relation to the discharge points.

From the same population of mussel and fish used in the caged exposure, pre-exposure samples were taken and the pre-exposure concentrations of contaminants and biomarker responses were determined. The fish and mussel biomarkers selected are listed in Table 2 and 3 respectively, and were carried out on both pre and post exposed animals. In addition, pre-exposure blood samples were taken from 162 fish, which were then pit tagged. The tagged fish were distributed evenly between the cages to enable individual fish to be assessed for VTG and ZRP at the different stations.

Of all the biomarkers analysed only lysosomal membrane stability was required to be performed on live animals immediately after collection. This was carried out on board the vessel with the aid of a field microscope. All other biomarkers were analysed in the laboratory on preserved samples.

Table 1. Locations and designation for stations (WGS 84 co-ordinates).

Sampling station	Description	Location
REF 1	Reference NE of discharge	57° 05.6'N 004° 10.0'E
REF 2	Reference E of discharge	56° 39.9'N 004° 15.0'E
ST 1	1600m SW	56° 32.0'N 003° 11.8'E
ST 2	600m SW Repositioned 2 km S after 5 weeks	56° 32.49'N 003° 12.42'E
ST 3	Off southern flare (100-200 m)	56° 31.56'N 003° 12.09'E
ST 4	Off 2/4J (100 – 200 m)	56° 32.84'N 003° 12.06'E
ST 5	1100m NE	56° 33.30'N 003° 14.20'E
ST 6	2000m NE	56° 33.5'N 003° 14.90'E

Table 2. Overview of Atlantic cod samples for biological and chemical analysis.

Method	Indication of	Matrix	No samples
CYP1A	Exposure to planar organic compounds	liver	100
GST	Exposure to a wide range of xenobiotics	liver	100
VTG	Xenoestrogenic exposure in males	blood plasma	150
ZRP	Xenoestrogenic exposure in males	blood plasma	150
PAH-met., FF	PAH exposure	bile	100
PAH-metabolites, GC/MS	PAH exposure	bile	60
AP met	Alkyl phenol exposure	bile	60
DNA adducts	Genotoxic exposure	liver	60

Table 3. Overview of mussel samples for biological and chemical analysis.

Method	Indication of	Matrix	No samples
Pyrene Hydroxylase	Exposure to PAHs with 4 benzene rings	digestive gland	100
Lysosomal stability	General stress	haemocytes	84
Lipofuscin accumulation	Effects from organic pollutants	digestive gland	100
Neutral lipid accumulation	Peroxidation of lipids	digestive gland	100
PAH concentration	PAH exposure	soft tissue	30
Lipid content	Used for lipid normalization of PAH levels	soft tissue	24
Micronucleus	Genotoxic exposure	haemocytes	100

2.2 Quality Assurance

The following is a description of the quality assurance measures that were taken for each procedure. For all laboratory analysis, standard operating procedures of the analysing laboratory were adhered to. For the chemical analysis, accredited procedures were used.

Procedure	Quality measure
Sampling protocols	All samples were collected by trained scientific personnel. All samples were clearly marked in pre-labelled vials with individual labels and stored in the appropriate conditions prior to analysis. All biological data was recorded in project work books.
PAH body burden	NIVA: Accredited method complying with the requirements of NS-EN ISO/IEC 17025. Battelle: All laboratory and data assessment and reporting activities were conducted under a Quality System defined in the Quality Assurance Manual for the BDO Laboratory. For more detail see the QA section in Appendix D (section 7.4)
Pyrene hydroxylase	The samples were randomised before analysis took place and analysed blind. The samples were analysed within 24 hour of work-up. When determining hydroxy pyrene by HPLC in the samples, two procedural blanks were run for each series of samples. These include 1) a sample without pyrene, and 2) a sample containing pyrene but without microsomes. The median of the blank 2 was subtracted from each sample. A series contained not more than 30 samples. When analysing a series, a standard was run every 10-20 samples, to check that the levels of hydroxy pyrene were correct.
LMS	IRIS SOP NRRT (Lysosomal Membrane Stability) Haemolymph cells . The method is described in Lowe et al., (1995). One batch of neutral red stock solution was used for all individuals. The light level was kept to a minimal tolerable level and maintained throughout the subsequent analysis. Examination time for each slide was kept less than a minute.
Neutral lipid	IRIS 2.2-421 SOP - Neutral lipid accumulation Rev 02 A piece of digestive gland of mussels were immediately put in a cryovial and frozen in liquid nitrogen after dissection. Neutral lipids were detected in cryostat sections (10 μm) by the Oil Red O technique according to Bayliss, (1984). All slides were stained using one batch of the Oil Red O stock solution. Two pictures of each individual were taken using an objective lens of 400 x magnifications and the image analysis program AxioVision (Zeiss). For every picture taken similar settings were used for both microscope and image analysis program. A scale bar was added in each picture. The measurements of neutral lipids were carried out in each picture using an automatic measurement program (AxioVision). Only the secondary tubules in the digestive gland were measured.
Lipofuscin accumulation	IRIS 2.2-423 SOP - Lipofuscin accumulation Rev 02 A piece of digestive gland of mussels were immediately put in a cryovial and frozen in liquid nitrogen after dissection. Demonstration of lipofuscin was performed histochemically in cryostat sections (10 μm) using Schmorl's method (Pearse, 1985). All slides were stained using one batch of the stock solution. Two pictures of each individual were taken using an objective lens of 400 x magnifications and the image analysis program AxioVision (Zeiss). For every picture taken similar settings were used for both microscope and image analysis program. A scale bar was added in each picture. The measurements of lipofuscin were carried out in each picture using an automatic measurement program (AxioVision). Only the secondary tubules in the digestive gland were measured.
Micronuclei formation	All samples were randomised and analysed blind. Only experienced scientists that were trained in the standard operating procedures were allowed to carry out the assessment. Co-ordinates of the micronuclei were recorded to enable assessment checks to be carried out at intervals.

ZRP	All samples were coded, randomised and analysed blind. Four replicates of each sample, a blank and a positive control are added as quality control. The quality is assured by comparing the control samples between plates and previous analysis.
VTG	All samples were coded, randomised and analysed blind. Duplicates of two dilutions of each sample, a blank and a positive control were added as quality control measures. Also two known VTG standard series were transferred to the microplates. The quality was assured by comparing the control samples between plates and previous analysis.
CYP1A	All samples were coded, randomised and analysed blind. Four replicates of the sample, a blank and a positive control were added as quality control measures. The quality was assured by comparing the control samples between plates and previous analysis.
GST	All samples were coded, randomised and analysed blind. Three replicates of each sample, a blank and a positive control were added as quality control measures. The quality was assured by comparing the control samples between plates and previous analysis.
Protein	The total protein concentrations of the samples were determined by a procedure based on the Bradford method (Bradford, 1976). The protein concentrations are controlled with three replicates and reanalyzed until the standard deviation between the replicates does not exceed 5 %. The protein concentrations are determined by extrapolating with a known standard series and compared with previous analysis.
DNA adducts	Several quality control experiments were performed in parallel to the analysis of the various fish tissue samples. Controls used during the analytical work were: a) pure salmon sperm as negative control, b) the standard DNA adduct B[a]PDE-dG-3´p, and c) adducted liver tissue from B[a]P exposed perch (*Perca fluviatilis*). These were processed parallel to the samples and served as quality assurance for all the analytical steps in the 32P-postlabeling method. These quality assurance experiments confirm a faultless assay for the DNA adduct measurements performed in this study.
Data archive	All raw data were stored electronically in the appropriate project folder on the secured NIVA and/or IRIS hard drives. Data will be stored for a minimum of five years.

2.3 Sea temperature and salinity

An STD/CTD – model SD204 was used for measuring, calculating and recording sea water conductivity/salinity, temperature, and depth (pressure) through the water column. The instrument was operated by COPSAS personnel at the Ekofisk platform and lowered on 5 occasions during the 6 week cage deployment. The instrument was set to log data every two seconds.

The specifications for the instrument are as follows:

Conductivity: *Inductive cell*, range: 0-70 mS/cm, resolution: 0.01 mS/cm, accuracy: ± 0.02 mS/cm. Salinity: *Calculated from C, T and D*, range: 0-40 ppt, resolution: 0.01 ppt, accuracy: ± 0.02 ppt. Temperature: range: -2 to +40 °C, resolution: 0.001 °C, accuracy: ± 0.01 °C, response time: <0.5 sec. Pressure range 1000 m, resolution: 0.01 % FS, accuracy: ± 0.02 % FS, Sound velocity: *Calculated from C, T and D*, ranges: 1300-1700 m/s, resolution: 5 cm/s, accuracy: ± 10 cm/s

2.4 Sea current

In order to measure current direction and speed, Aquadopp Current Meter instruments were deployed at ST 2, ST 5 and at REF 1.

The specifications for the instrument are as follows:

Measuring current velocity (range: 0.5 - 500 cm/s, accuracy: 0.5 cm/s or 1 %), current direction (accuracy/ resolution: 2°/ 0.1°), temperature (range: -4 – 40 °C, accuracy/ resolution: 0.1 °C/ 0.01°C) and depth (range 0-200 m, accuracy/resolution: 0.25 %/ better than 0.005 % of full scale per sample). Accuracy of current velocity is dependent on set-up parameters. During the WCM the accuracy was 0.4 cm/s. The instrument measured current velocity as a 60 seconds average and logged data for every 10 minute, diagnostic data were collected every 12 hours. At Ekofisk, the instruments logged data from 1st April to 13th May. At the reference station data were logged from 31st March to 13th May. From the logged data and diagnostics the instrument appeared to work normally through the exposure period and >6000 data points were collected at each station.

2.5 Contamination control during transport

To confirm that organisms were not exposed to PAH contamination during transport, seawater samples from the transportation tanks were collected just before the first fish cage deployment. For each sample, 5 L were collected and used for the ethyl-acetate extraction. The extracts were analysed for PAHs by GCMS (Section 2.7.1.2).

2.6 General biological observations

Total weight and length of each cod was measured in the laboratory onboard the vessel. Fish were sexed by visual examination of their gonad. In order to provide the best possible weight measurements for the liver and gonads, these tissues were wrapped in aluminium foil, frozen at -20ºC and brought to Akvamiljø laboratory for measurements.

The Condition index of each cod was determined by the ratio between total weight and the cube of the fork length of the fish.

$$\text{Condition index} = [\text{Weight (g)/Length (cm)}^3)] \times 100$$

The liver somatic index (LSI, liver index) reflects the animal nourishment status. LSI at 0-sampling (Pre exposure) and at the end of the exposure was calculated as:

$$\text{LSI} = [\text{Liver weight X 100}] / \text{fish weight}$$

The gonadosomatic index (GSI, gonad index) reflects the animals' reproductive status. GSI at the end of the exposure was calculated as:

$$\text{GSI} = [\text{Gonad weight X 100}] / \text{fish weight}$$

2.7 Biomarkers in cod

2.7.1 PAH-metabolites in fish bile

2.7.1.1 Fixed fluorescence

Bile samples were diluted 1:1600 in methanol: water (1:1). Slit widths were set at 2.5 nm for both excitation and emission wavelengths, and samples were analysed in a quartz cuvette. All

bile samples were analysed by FF at the wavelength pairs 290/335, 341/383 and 380/430 nm, optimised for the detection of 2-3 ring, 4-ring and 5-ring PAH metabolites, respectively. The fluorescence signal was transformed into pyrene fluorescence equivalents through a standard curve made by pyrene (Sigma St Louis, USA). Pyrene was measured at the same fluorometer, with the same cuvette, same solvent, and with the same slit settings as the bile samples. It was, however, measured at the optimal wavelength pair of pyrene, 332/374 nm (ex/em). The concentration of PAH metabolites in bile samples was expressed as µg pyrene fluorescence equivalents (PFE)/ mL bile.

2.7.1.2 PAH metabolites in fish bile

Fish bile was prepared for analysis as described by Jonsson et al., (2003; 2004). Briefly, 25–30 µL of bile was weighed accurately into a micro centrifuge vial. Internal standards (2,6-dibromophenol, 3-fluorophenanthrene and 1-fluoropyrene) and β-glucuronidase (3000 units) in sodium acetate buffer (0.4 M, pH = 5) were added and the solution left at 40°C for 2 hours. The OH-PAHs were extracted with ethylacetate (4 times 0.5 mL), the combined extract dried with anhydrous sodium sulphate and concentrated to 0.5 mL. Trimethylsilyl (TMS) ethers of OH-PAHs were prepared by addition of 0.2 mL BSTFA and heating for two hours at 60°C. TPA was added as a GC-MS performance standard before transferring the prepared samples to capped vials.

Trimethylsilyl ethers of OH-PAHs (TMS-OH-PAHs) in fish bile samples were analysed by a GC-MS system consisting of a HP5890 series II Gas chromatograph, Shimudadzu QP2010 GCMS. Helium was used as carrier gas and the applied column was CP-Sil 8 CB-MS, 50 m x 0.25 mm and 0.25 µm film-thickness (Varian). Samples and calibration standards (1 µL) were injected on a split/ splitless injector with splitless mode on for one minute. The temperatures for the injector, transfer-line and ion source were held at 250°C, 300°C and 240°C, respectively, and the GC oven temperature programme was as follows: 80°C to 120°C at 15°C min^{-1}, 120°C to 300°C at 6°C min^{-1} and held at 300°C for 30 min. Mass spectra were obtained at 70 eV in selected ion mode (SIM). Based on the fragmentation pattern of non-alkylated TMS-O-PAHs (Jonsson et al., 2003, Krahn et al., 1992); the molecular ions were selected for determination of both alkylated and non-alkylated TMS-O-PAHs.

2.7.2 AP metabolites in fish bile

Fish bile was prepared for analysis as described by Jonsson et al., (2003; 2004). Briefly, 25–30 µL of bile was weighed accurately into a micro centrifuge vial. Internal standards (2,6-dibromophenol, 3-fluorophenanthrene and 1-fluoropyrene) and β-glucuronidase (3000 units) in sodium acetate buffer (0.4 M, pH = 5) were added and the solution left at 40°C for 2 hours. The OH-PAHs were extracted with Solid Phase Analytical Derivatisation (SPAD).

Trimethylsilyl ethers of OH-APs (TMS-OH-APs) in fish bile samples were analysed by a GC-MS system, Shimudadzu QP2010 GCMS. Helium was used as carrier gas and the applied column was CP-Sil 8 CB-MS, 50 m x 0.25 mm and 0.25 µm film-thickness (Varian). Samples and calibration standards (1 µL) were injected on a split/splitless injector with splitless mode on for one minute. The temperatures for the injector, transfer-line and ion source were held at

280°C, 300°C and 240°C, respectively, and the GC oven temperature programme was as follows: 80°C to 120°C at 15°C min^{-1}, 120°C to 300°C at 6°C min^{-1} and held at 300°C for 30 min. Mass spectra were obtained at 70 eV in SIM. Based on the fragmentation pattern of non-alkylated TMS-O-APs (Jonsson et al., 2003); the molecular ions were selected for determination of both alkylated and non-alkylated TMS-O-APs.

2.7.3 Glutathione-S-transferase (GST) activity

The method used is based on Habig et al., (1974), and optimised for cod tissues. Liver tissue was homogenised with a Potter-Elvehjem glass/teflon homogeniser in four volumes of ice-cold 100 mM KH_2PO_4 buffer, pH 7.8, 0.15 M KCl. The homogenate was centrifuged at 10 000 × g for 30 min aliquoted and stored at −80°C.

Cytosol samples were diluted 5 fold in ice cold phosphate buffer (100 mM KH_2PO_4/K_2HPO_4, pH 7.4), 50 µL of each sample was transferred to 96 microwell plates in triplicates. Each plate contained a negative and a positive control purified porcine GST). The microplates were stored on ice prior to analysis. Reagents (2 mM CDNB, 1 mM GSH) were mixed and 200 µL added to the wells (containing cytosol samples, blanks, or positive controls) using a multi channel pipette. The plate was then transferred to the microplate reader were the absorbance was measured at 340 nm for 2 minute at 22°C. The enzyme activity was normalised against the sample protein concentration.

The activity calculation: (well volume × (Δ Absorbance-blank))/ (sample volume × 9.6 × light-way × [Protein] $_{well}$), where 9.6 is the molar extinction coefficient ($\in$) for the CDNB-GSH conjugate (in mM^{-1}cm^{-1}). GST activities were expressed as nanomoles of substrate converted per minute per mg of protein in the cytosol.

The total protein concentrations of the samples where determined by a procedure based on the Lowry method (Lowry, 1951).

2.7.4 Hepatic Cytochrome P450 1A

From homogenised cod liver tissue in 100 mM KH2PO4 buffer, pH 7.8, the centrifuged cytosolic fraction was centrifuged once more at 50 000 g for a microsomal fraction of hepatocytes used in the CYP1A ELISA assay.

Total protein concentrations of the samples were determined by a procedure based on the Lowry method. Based on the total protein concentrations, the samples were diluted with carbonate-bicarbonate buffer to 10 µg/mL and transferred to a 96 microwell plate, each containing 4 replicates of the sample, a blank and a positive control (cod sample). The plate was sealed with sealing tape and incubated over night in dark at 4 °C.

The second day the plate was washed three times with TTBS. 1% BSA in TTBS was added to the wells to block unspecific binding and the plate was incubated for 1 hour. The plate was washed a second time with TTBS. The primary anti body rabbit-anti-fish CYP1A (CP226) (Biosense) with dilution 1:1000 was added to all wells and the plate was sealed with sealing tape and incubated over night in dark at 4 °C. The third day the plate was washed three times

with TTBS. The secondary anti body goat-anti-mouse HRP conj. (BIORAD) with dilution 1:3000 was added to all wells and incubated at 4 °C for 6 hours. The plate was washed with TTBS. TMB plus (KemEnTec) buffer was added for colour development and the reaction was stopped after 12 min. with 1 M H_2SO_4. The absorbance was read at 450 nm.

2.7.5 Vitellogenin

Blood samples were taken from the caudal vein of each fish with separate syringes containing heparin (10000 IU/mL, Sigma) and the protease inhibitor Aprotinin (5 TIU/mL, Sigma). The blood samples were centrifuged at 2000 g for 5 min in a microcentrifuge before the supernatant (plasma) was removed and transferred to labelled cryovials and snap-frozen in liquid nitrogen. Plasma samples were stored at -80°C until analysis. Vitellogenin was determined in plasma from caged cod using a competitive ELISA with cod vitellogenin standard and competing antigen. The analyses were performed using a kit (V01006401) from Biosense Laboratories AS (Bergen, Norway) with anti-cod antiserum and cod vitellogenin as standard, according to the instructions of the manufacturer.

Plasma samples were diluted 50 and 5000 times in Phosphate buffer saline, pH 7.2. The plasma samples were transferred to 96 well microplates, each containing duplicates of the diluted sample, a blank and a positive control (cod sample). In addition, two VTG standard series were transferred to the microplates. The plates were sealed and incubated for 1 hour at 37 °C. The plates were washed three times in PBS buffer. Detecting antibody with dilution 1:500 was added to the wells and incubated for 1 hour at 37 °C. The plates were washed three times in PBS buffer. Secondary antibody with dilution 1:2000 was added to the wells and incubated for 1 hour at 37 °C. The plates were washed five times in PBS buffer and TMB substrate solution was added to the wells. The plates were incubated in the dark at room temperature for 30 min. The reaction was stopped with 0.3 M H_2SO_4 and the absorbance read at 450 nm. The VTG-concentration in the diluted samples was determined using the equation for the adjusted standard curve from the standard series. The VTG concentration was multiplied with the dilution factor and expressed in ng/mL.

2.7.6 Zona Radiata Protein

Blood samples were taken from cod as described for VTG above. Plasma samples were stored at -80°C until analysis. Zona Radiata Protein (ZRP) was determined in plasma from caged cod using a competitive ELISA with a competing antigen. The plasma samples were diluted 1:2000 in carbonate-bicarbonate buffer and transferred to a 96 micro well plate, each containing 4 replicates of the sample, a blank, and a positive control (cod sample). The plate was sealed with sealing tape and incubated over night in the dark at 4 °C.

The second day the plate was washed three times with 20 mM Tris-buffer, pH 8.5, (TTBS). 1% BSA in TTBS was added to the wells to block unspecific binding and the plate was incubated for 1 hour. The plate was washed a second time with TTBS. The primary anti body rabbit-anti-salmon ZRP (O-146) (Biosense) with dilution 1:400 was added to all wells and the plate was sealed with sealing tape and incubated over night in the dark at 4 °C. The third day the plate was washed three times with TTBS. The secondary anti body goat-anti-rabbit HRP conj. (ZYMED) with dilution 1:3000 was added to all wells and incubated at 4 °C for 6 hours.

The plate was washed with TTBS and TMB plus (KemEnTec) buffer was added for colour development. The reaction was stopped after 12 min with 1 M H_2SO_4 and the absorbance read at 450 nm.

2.7.7 DNA adducts

Deep-frozen liver tissue pieces from cod were semi-thawed. DNA was extracted and purified according to Dunn et al., (1987), Reichert and French, (1994), with minor modifications as described by Ericson et al., (1998) and Ericson and Balk, (2000). DNA adducts were enriched using the Nuclease P1 method, 0.8 µg Nuclease P1/µg DNA, and a 45 min incubation period (Reddy and Randerath, 1986; Beach and Gupta, 1992). Finally the DNA adducts were radiolabelled using 5′-[γ-^{32}P]triphosphate ([γ-^{32}P]ATP) and T_4 polynucleotide kinase (Aas et al., 2000a). Separation and clean up of adducts was performed by multidirectional thin-layer chromatography (TLC) on laboratory produced polyethyleneimine cellulose sheets, described as suitable for adducts formed from large hydrophobic xenobiotics, such as 4- to 6- ring, PAHs (Reichert and French, 1994; Ericson et al., 1999). In addition, several quality control experiments were performed parallel to the analysis of the samples. Detection limit for the method varies among samples due to individual plate background.

2.8 Biomarkers in mussels

2.8.1 PAH body burden

Approximately 15 whole blue mussels were excised from their shell and transferred to high temperature treated (560°C) glass containers. The mussels were frozen and transported to NIVA on dry-ice. The samples were stored at -20°C until analysis. Samples were defrosted, homogenised and divided equally into two with half the homogenised sample refrozen and transported on dry ice to Battelle Laboratories in Massachusetts, US.

2.8.1.1 NIVA analysis

A 5g sub-sample of the homogenate was taken and internal standards added (naphthalene d8, biphenyl d10, acenaphthene d8, phenanthrene d10, anthracene d10, pyrene d10, chrysene d12 and perylene d12) before extraction by saponification. Analytes were then extracted twice with 40 mL cyclohexane and dried over sodium sulphate. The extracts were reduced by a gentle stream of nitrogen and cleaned by size exclusion chromatography. Analysis proceeded by gas chromatography with mass spectrometric detection (GC-MS) with the MS detector operating in selected ion monitoring mode (SIM). The GC was equipped with a 30 m column with a stationary phase of 5% phenyl polysiloxane (0.25 mm i.d. and 0.25 µm film thickness), and the injector operated in 'split less' mode. The initial column temperature was 60°C, which after two minutes was raised stepwise to 310°C. The carrier gas was helium and the column flow rate was 1.2 mL/min. Quantification of individual components was performed by using the internal standard method. The alkylated homologues were quantified by baseline integration of the established chromatographic pattern and the response factors were assumed equal within each group of homologues.

2.8.1.2 Battelle analysis

Previously homogenized blue mussel samples were shipped from NIVA, Oslo on dry ice and were received at the Battelle Duxbury Operations (BDO) Laboratory on 22nd June 2009. Upon receipt of samples, the temperature of the cooler was taken and was found to be within an acceptable range. The samples were stored at -20°C until processing.

Approximately 10 - 20 g of homogenised mussel tissue was used for the analysis. A separate 5 g sub-sample was removed for dry weight determination. The tissue homogenate was placed in a clean 250 mL tall wide mouth glass jar with sodium sulphate for extraction. Surrogate internal standard (SIS) compounds were added and the sample was macerated with a Tissuemizer™ using methylene chloride as the extraction solvent. The Tissuemizer™ extraction was repeated once more, followed by a 1-hour solvent extraction on an orbital shaker table. Between each extraction, the samples were centrifuged to facilitate solvent removal. The extracts were dried over sodium sulphate and concentrated by Kuderna-Danish and N_2 evaporation techniques. The extracts were processed through alumina columns and further cleaned-up through HPLC equipped with a size exclusion column to isolate analytes of interest. The tissue lipid content was determined using a portion of the pre-purified extract. The final extract was spiked with internal standards (IS) and submitted for the analysis of PAH by gas chromatography/mass spectrometry (GC/MS) in the selected ion monitoring (SIM) mode. The PAH analysis was performed based on EPA method 8270 using an Agilent 6890 GC with an Agilent 5973 MSD. The GC was equipped with a 60 m DB-5 column (0.25 mm ID, 0.25 μm film thickness). The concentrations of the individual PAH compounds were calculated by the internal standard method. Target PAH concentrations were quantified using average response factors (RF) generated from the five-point linear calibration. Alkyl homologue PAH series concentrations were determined using the average RF for the corresponding parent compound. Well established alkyl homologue pattern recognition and integration techniques were used to determine alkyl homologues. Final concentrations were determined versus the appropriate surrogate compound. Analytical reporting limits and estimated limits of detections were determined for each sample. The reporting limits were defined as the sample concentration equivalent to the low level standard. The estimated limits of detection were based on a sample concentration equivalent to a signal: noise ratio of 3:1. The data were qualified with a "J" if the measured concentration was below the reporting limit. Non-detections were qualified with a "U" and the null-value was reported.

2.8.2 Pyrene hydroxylase activity

The hydroxylase activity method was adapted from a method described in (Michel et al., 1994). The microsomal fraction (100 μL) was added to sodium phosphate buffer (200 μL, 0.05 M, pH 7.3) containing BSA (2 mg/mL), NADPH (100 μL, 10 mM) and pyrene in acetone (10 μL, 400 μg/mL). The tubes containing the microsomes were incubated on a shaker (room temperature, 30 min.) before the reaction was terminated by adding 500 μL methanol. Internal standard (triphenylamine, 10 μL, 15 μg/mL) was added to the solution and mixed. The tubes were centrifuged to precipitate protein and the supernatant was injected on an HPLC system for the determination of the metabolite formed.

Determination of metabolite by HPLC

HPLC was performed on a Waters 2695 Separations Module equipped with a 2475 fluorescence detector and fitted with a Waters PAH C18 (4.6 ×250 mm, 5 µm) column. The mobile phase consisted of a gradient from 40:60 acetonitrile:water to 100% acetonitrile at a flow of 1 mL/min, at 35°C. The excitation and emission wavelengths used for detection of pyrene and triphenylamine were 346nm, 384nm and 300nm, 360nm respectively. The injection volume used was 25 µL.

2.8.3 Lysosomal membrane stability

The mussels from the pre-exposure group were brought to the IRIS laboratory in Stavanger on ice. The mussels were acclimatised in the laboratory in aquaria with fresh supply of sea-water for two days prior to sampling (to alleviate stress during transport). The field groups were analysed onboard the vessel directly after retrieval of cages.

Haemolymph samples were obtained from 15 individuals at each field station and 15 individuals from the pre-exposure group.

Haemolymph 400 µL was taken from each mussel and mixed with filtered sea water at the ratio 1:1. An aliquot (30 µL) of haemolymph/ seawater-mixture was pipetted out on microscope-slides, and incubated in a light-proof box for 15 min before 30 µL neutral red (concentration 0.1 µg/µL) was added. All analyses were performed blind. For a detailed description of the method see Lowe and Pipe, (1994).

Figure 2. Microscope view (400x magnification) of living and dead mussel haemolymph cells

NR is selectively taken up by haemolymph cells and this adds an extra stress to the membranes. After some time, from 15 to 180 minutes, depending on the health status of the mussels, the integrity of the membrane will weaken and NR will leak out into the cytosol. This causes the cells to become round in shape. The time from when the NR is added to the cells, until the cells become round and perish is observed visually with a microscope. The cells are observed repeatedly at 15, 30, 60, 90, 120, 150 and 180 minutes of incubation with NR. The endpoint of the analysis is when 50% of all cells become round and die. This method is perceived as a general health-parameter, and has been shown to respond to PAH/oil-exposed mussels.

2.8.4 Micronucleus formation

Mussel haemolymph was applied directly on to slides, air-dried and fixed in methanol for 15 min. The slides were cytogenetically analysed by the Institute of Ecology at Vilnius University (Lithuania). Slides were stained with 5% Giemsa solution for 10-20 min. Anonymous scoring of micronuclei was performed on coded slides without knowledge of the exposure status of the samples to eliminate technical variability.

The frequency of micronuclei in haemocytes was determined by scoring at a 1000× magnification using an Olympus BX 51 or Nikon Eclipse 50i bright-field microscope. A minimum of 500 cells per mussel were counted.

Only cells with intact cellular and nuclear membrane were scored. micronuclei were scored when: i) nucleus and micronuclei had a common cytoplasm, ii) colour intensity of micronuclei was the same or lower than the nucleus, iii) the size of the micronuclei was equal or smaller than 1/3 of the nucleus, iv) micronuclei was completely separated from the nucleus, v) cells with multiple micronuclei were not scored.
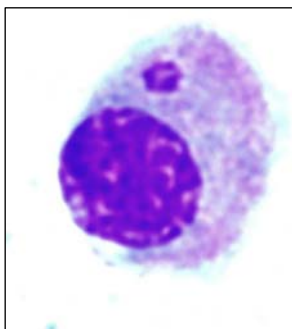
Figure 3. Micronucleus in haemocyte of mussel *M. edulis* (1000x magnification).

2.8.5 Histochemistry in mussels

For histochemical examination, small pieces (5 x 5 x 5 mm) of freshly excised digestive gland tissue were placed in cryovials and snap frozen in liquid nitrogen (-196°C). Prior to sectioning the samples were attached to aluminium chucks.

Cryostat sections (10 μm) were cut in a cryostat with the cabinet temperature below -25°C and the knife cooled to - 20°C. The sections are transferred to "warm" slides (20°C). The slides can be stored in the freezer at -40 °C before use. Cryostat sections were used for analyses of lipofuscin and neutral lipid accumulation.

2.8.5.1 Lipofuscin accumulation

The lipofuscin content of lysosomes was determined using the Schmorl reaction. Cryostat sections were fixed in Baker`s calcium-formol for 15 min, rinsed in distilled water and immersed in the reaction medium containing an aqueous solution of 1% ferric chloride and 1% potassium ferrocyanide in a ratio 1:1 (v:v). Sections were stained for 2 min, rinsed in acetic acid (1%) for 2 min and washed in running water for 10 min and rinsed in distilled

water before mounting using aqueous mounting medium. Slides were subjected to image analysis and results were expressed as pixel density.

2.8.5.2 Neutral lipid accumulation

For the determination of unsaturated neutral lipids, cryostat sections were fixed in Baker`s calcium-formol for 15 min, rinsed in distilled water and transferred into 60% triethylphosphate (v/v with distilled water) for 1 min. Sections were stained in 1% solution of Oil Red O in 60% triethylphosphate for 15 min. Then they were rinsed in 60% triethylphosphate for 30 s, washed in distilled water and mounted using aqueous mounting medium. Neutral lipid accumulation was assessed by computer assisted image analysis. Results were expressed as pixel density.

2.9 Protein determination

Biomarker analyses were normalised with protein concentrations measured using the Lowry method adapted for plate-readers (Lowry et al., 1951) with bovine gammaglobulin as the protein standard. The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. Amino acids reduce the Folin reagent, yielding several reduced species that have a blue colour. The colour has a maximum absorbance at 750 nm and minimum absorbance at 405 nm.

2.10 Statistical methods

Biological responses in individual mussels or fish were subjected to analysis of variance (ANOVA) to clarify whether there were differences between groups (Sokal and Rohlf, 1981). Prior to analyses, homogeneity of variances was checked using the Levene's test. Where necessary variables were log transformed to obtain homogeneity. Where this was not possible, Kruskal-Wallis non-parametric analysis was used. (Sokal and Rohlf, 1981). Where the parametric ANOVA indicated significant differences, groups were compared using Tukey's post-hoc test. The level of significance for rejection of H_0: "no difference between groups" was set to 0.05.

3 Results

In order to provide a visual comparison of the biological effects and chemical bioaccumulation data over the three monitoring periods comparable data from the 2006 and 2008 surveys has been included alongside the 2009 data. This comparison was of particular interest due to the implementation of the C-Tour PW treatment system installed prior to the 2008 monitoring survey.
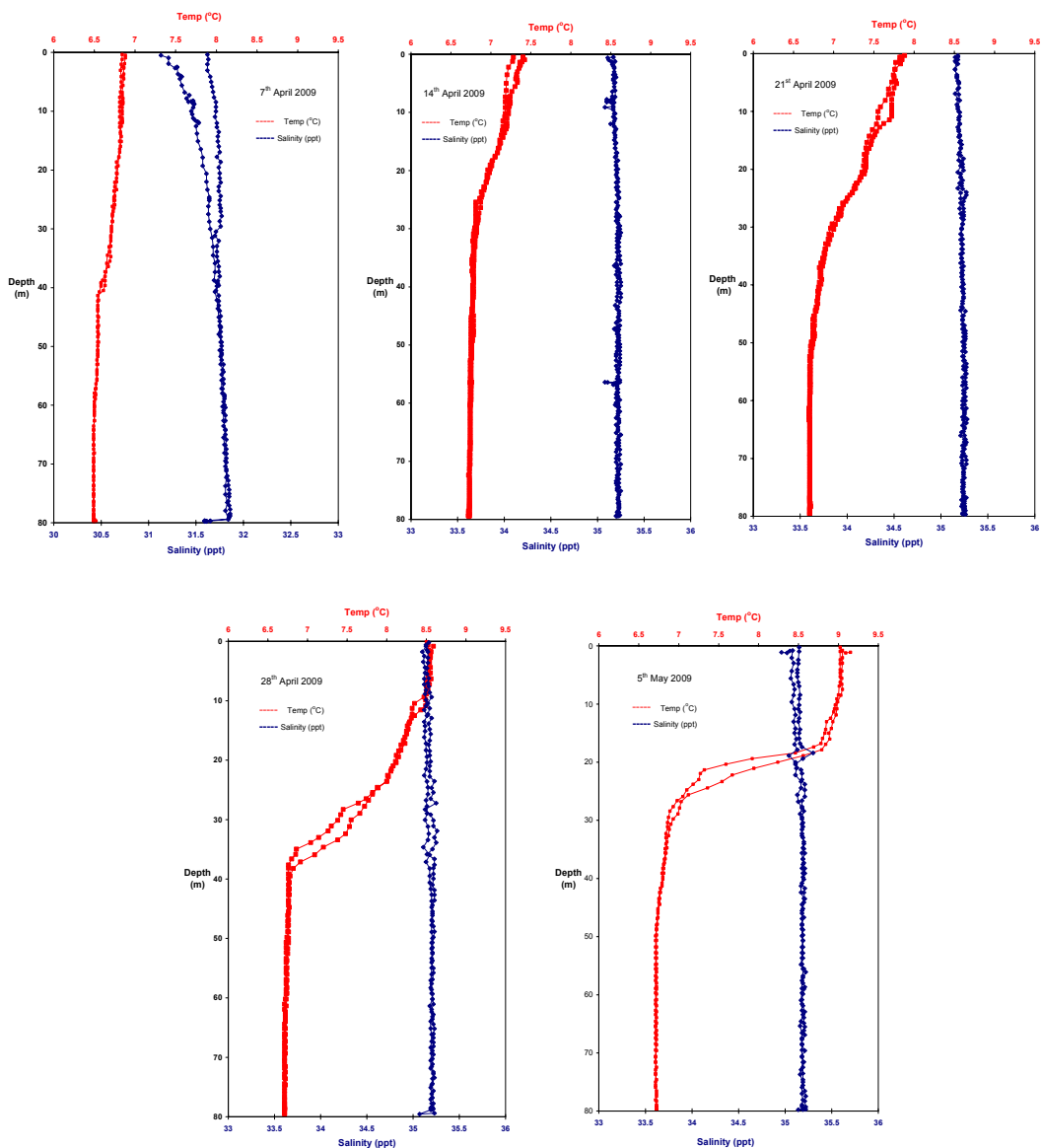
3.1 Sea temperature and salinity measurements

Figure 4. Salinity and temperature profiles taken every week from below the Ekofisk platform 2/4 J during the cage deployment.

The CTD unit was deployed once a week for 5 weeks during the 6 week monitoring program. The data collected is shown in figure 4. With exception to the salinity data retrieved on the first CTD deployment, where the salinity values appeared to be slightly depressed (31-32 ‰), the salinity remained stable over the duration of the study between 35 and 35.5 ‰. Depth had no effect on salinity and a halocline was not present. This corresponds with the salinity data obtained from the 2008 and 2006 surveys (data not shown).

Changes in the temperature depth profile were observed during the survey period with a thermocline developing at a depth of 20 m in the final sampling occasion (5th May 09). Below 40 m the water temperature was stable at approximately 6.5 ºC, with surface water temperatures of 9 ºC. A 20 m thermocline was also observed at the same location and the same time of year in the 2008 and 2006 monitoring surveys (data not shown).

3.2 Current conditions

Ekofisk

The current pattern around Ekofisk was identified by the current meters positioned at station 5. This confirms the pattern found in previous years that the current is predominantly tidal driven, which changes every 6 hours from a SW to NE direction (Figure 5). The tidal influence would be expected to partly prevent the transport of discharged water away from the platform area.

Average current speed at station 5, for the total exposure duration, was 13.7 cm/s, maximum current speed was 37.5 cm/s. For comparison, the average current speed at the same station in 2008 was 9.6 cm/s, maximum current speed was 14.5 cm/s.

The cage at station 2 was unintentionally repositioned on May 6th (week 5 of the 6 week exposure) to a location approximately 2 km south of its original position. Data recorded at the new position showed a different profile with a dominant current in the North-North East direction and was less affected by the tidal currents.

Reference station

Measurements taken at the reference station for 2009 were comparable to previous years with predominantly tidal driven currents in the SW-NE direction (Figure 6).
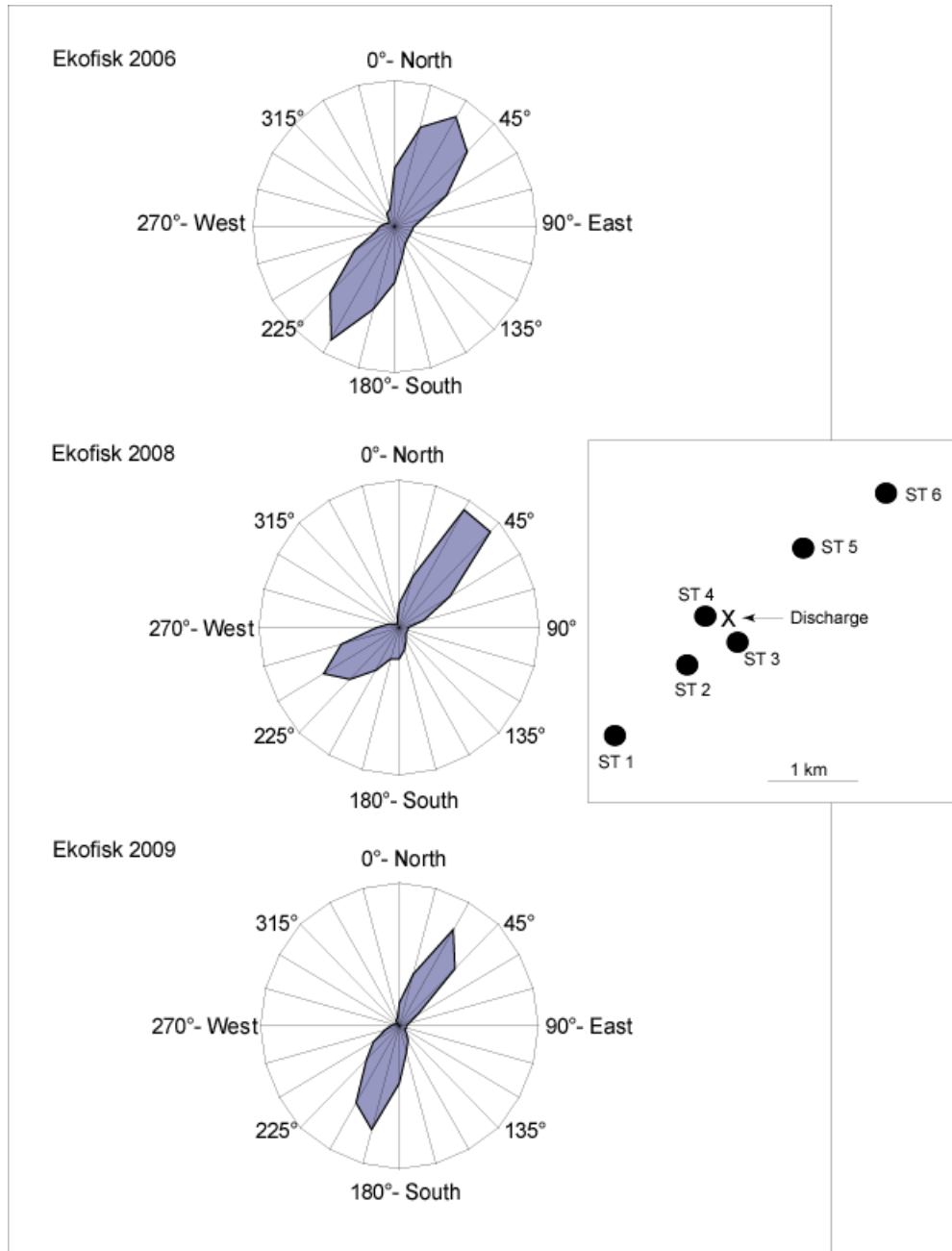
Figure 5. Sum of relative current (in 15º sectors) at Ekofisk in 2006, 2008 and 2009. Positions of cages shown for comparison.
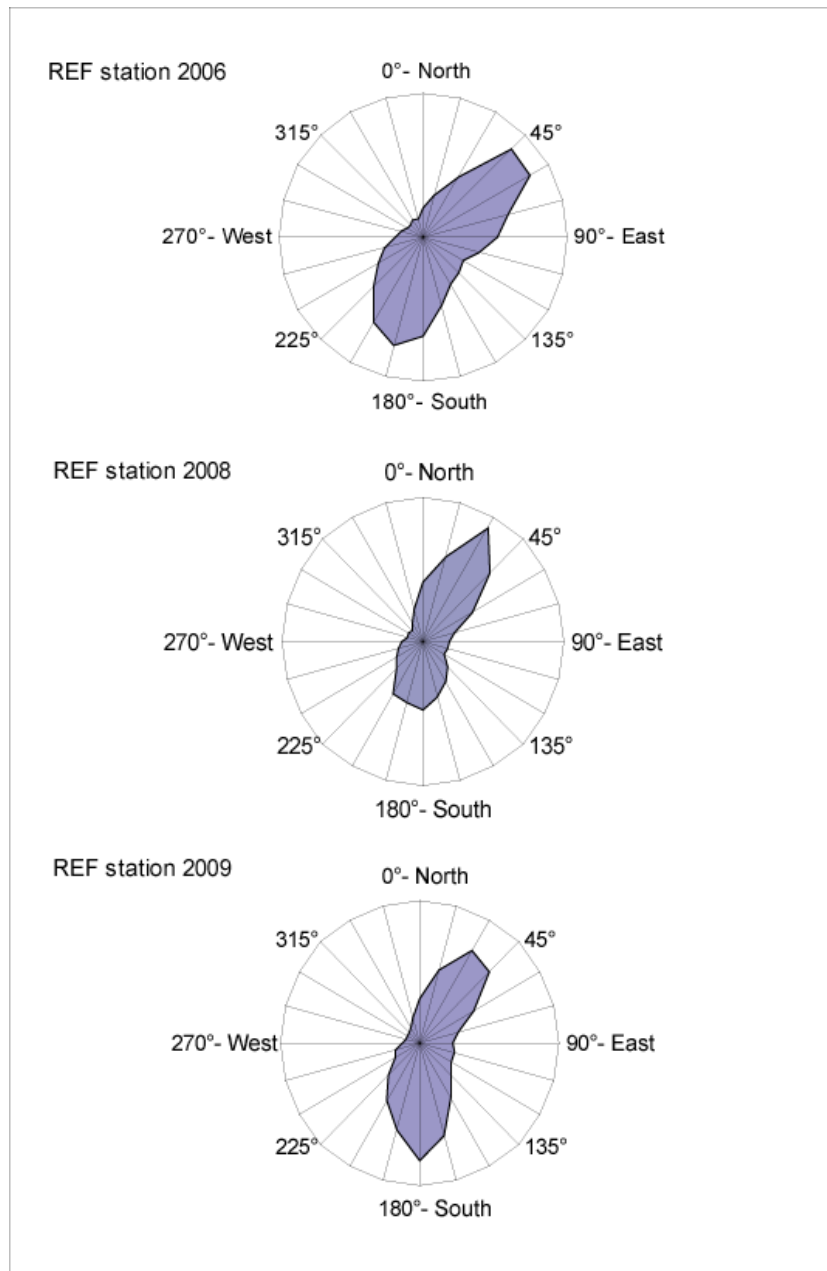
Figure 6. Sum of relative current (in 15º sectors) at the reference station in 2006, 2008 and 2009.

3.3 Contamination control during transport

PAH concentrations were either low or undetected in the seawater samples collected from the vessels storage tanks minutes before the first fish cage deployment (Table 4). This confirms that the fish and mussels used in the study were not exposed to elevated PAH concentrations in the vessels storage tank during transport.

Table 4. PAH concentrations in sea water collected from the vessels transport tanks prior to the first fish cage deployment. (n=3; quantification limit 0.005 µg/L)

Compound	µg/L
Naphthalene	<0.005 (0.003, 0.002, 0.003)
C1-Naphthalene	<0.005 (0.002, 0.002, 0.002)
C2-Naphthalene	<0.005
C3-Naphthalene	<0.005
Acenaphthylene	< 0.005
Acenaphthene	< 0.005
Fluorene	< 0.005
Phenanthrene	< 0.005
Anthracene	< 0.005
C1-Phen/Anthr	< 0.005
C2-Phen/Anthr	< 0.005
Dibenzothiophene	< 0.005
C1-Dibenzothiophene	< 0.005
C2-Dibenzothiophene	< 0.005
Fluoranthene	< 0.005
Pyrene	< 0.005
Benzo(a)anthracene	< 0.005
Chrysene/Triphenylene	< 0.005
C1-Chrysene	< 0.005
C2-Chrysene	< 0.005
Benzo(b,j)fluoranthene	< 0.005
Benzo(k)fluoranthene	< 0.005
Benzo(b,j,k)fluoranthene	< 0.005
Benzo(a)pyrene	< 0.005
Indeno(1,2,3-cd)pyrene	< 0.005
Benzo(g,h,i)perylene	< 0.005
Dibenzo(a,h)anthracene	< 0.005

3.4 General biological observations of experimental fish

The body length and weight distribution in the different groups are shown in Figure 7 and Figure 8. The measurement and tagging of the fish prior to cage deployment enabled pre and post measurements of individual fish to be obtained. No difference in the length of the fish was found between the groups. However, differences in fish weights were found between the groups. The PRE EXP group was found to be overall heavier than all the other groups, although this was only significant for ST4 (ANOVA, Tukey, $p<0.05$).

The reduction in weight of the caged fish was responsible for the significant differences in the calculated condition indices, with the PRE EXP group significantly higher than all three groups of caged fish (Figure 9, Kruskal-Wallis ANOVA, $p<0.05$). This trend was also reflected in the liver-somatic index (Figure 10, ANOVA, Tukey, $p<0.05$).

There was no significant difference in the gonad-somatic indices of the same sex fish between the different stations (Figure 11). The sex ratios were almost identical with a 50:50 split of female to male fish within each group (Figure 12).
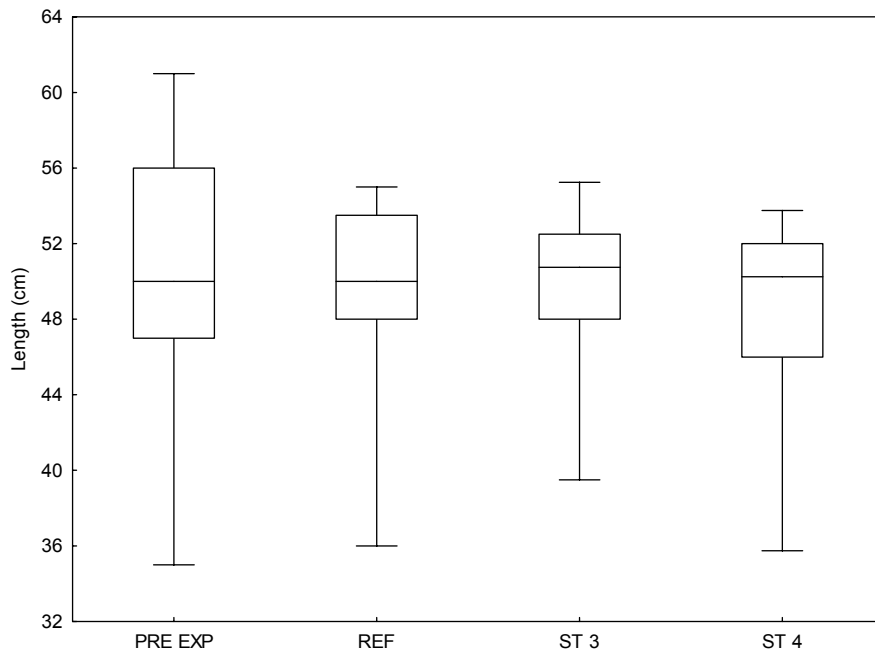
Figure 7. Length (cm) of cod at sampling in the different groups. The figure shows median, quartiles (box) and 10/90-percentiles (whiskers).
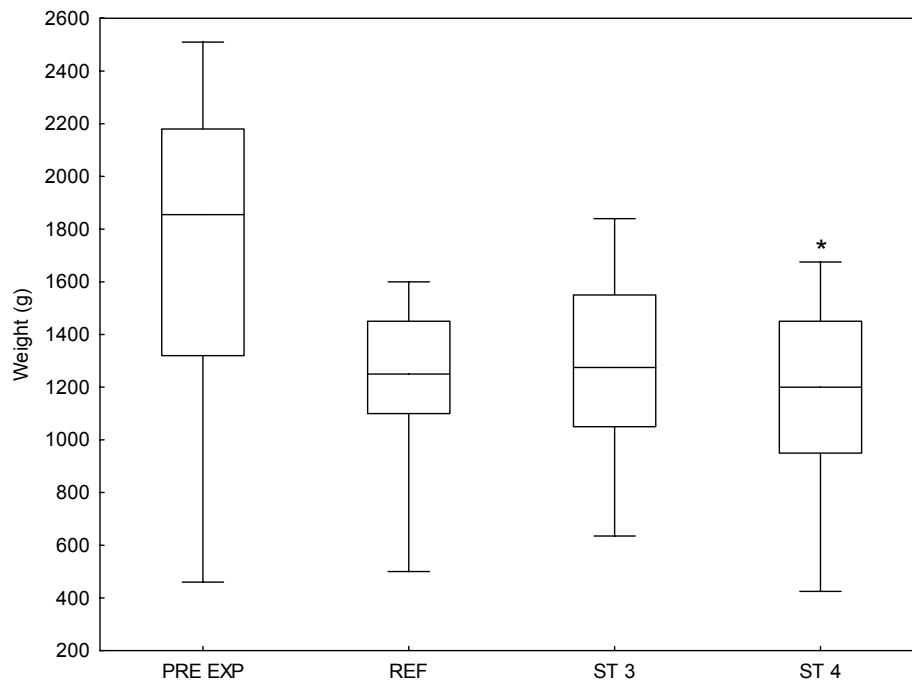

Figure 8. Weight (g) of cod in the different groups. The figure shows median, quartiles (box) and 10/90-percentiles (whiskers). * significant difference from PRE EXP (ANOVA, Tukey, $p < 0.05$).
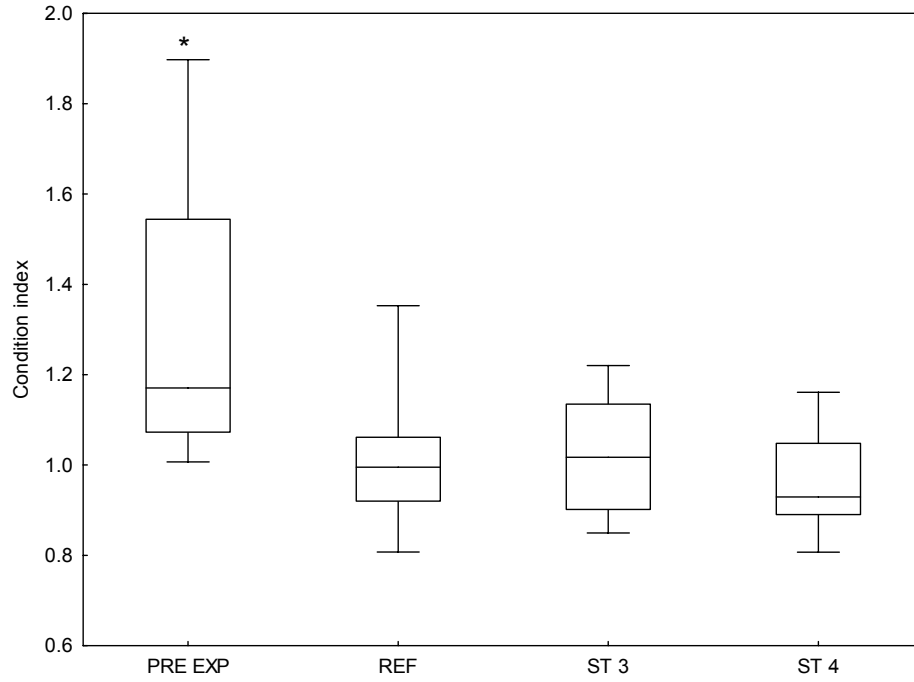
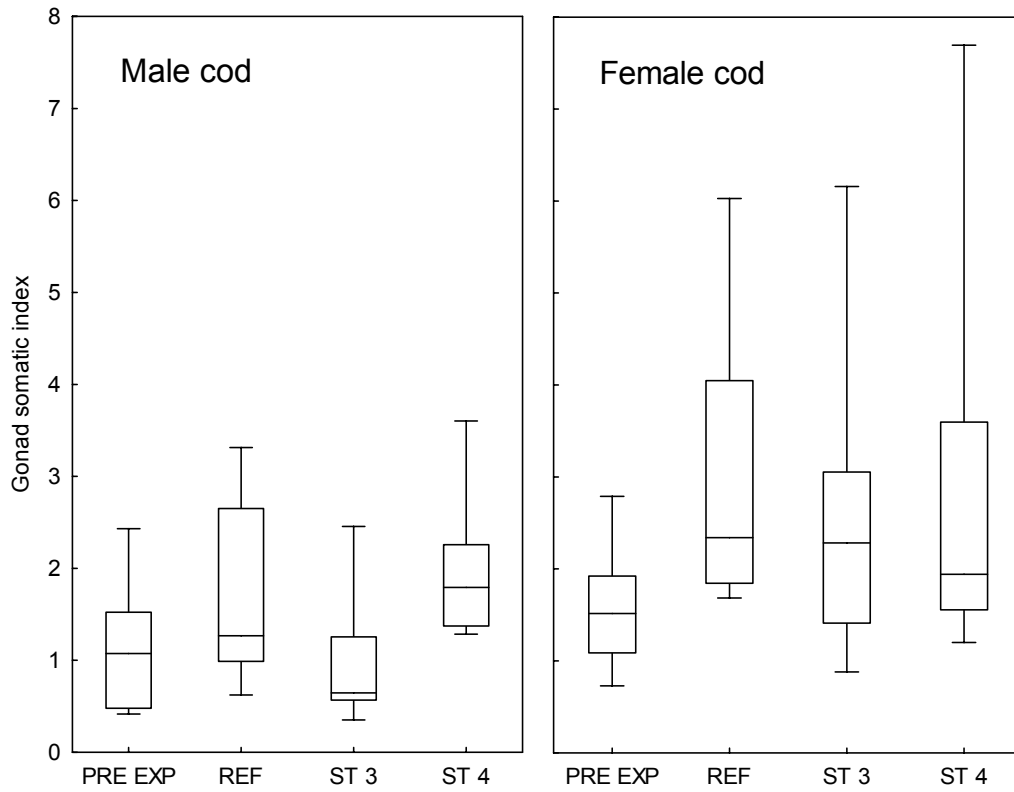Figure 9. Condition of cod in the indicated groups. The figure shows median, quartiles (box) and 10/90-percentiles (whiskers). * significantly different from all other groups (Kruskal-Wallis ANOVA by ranks, p<0.05).
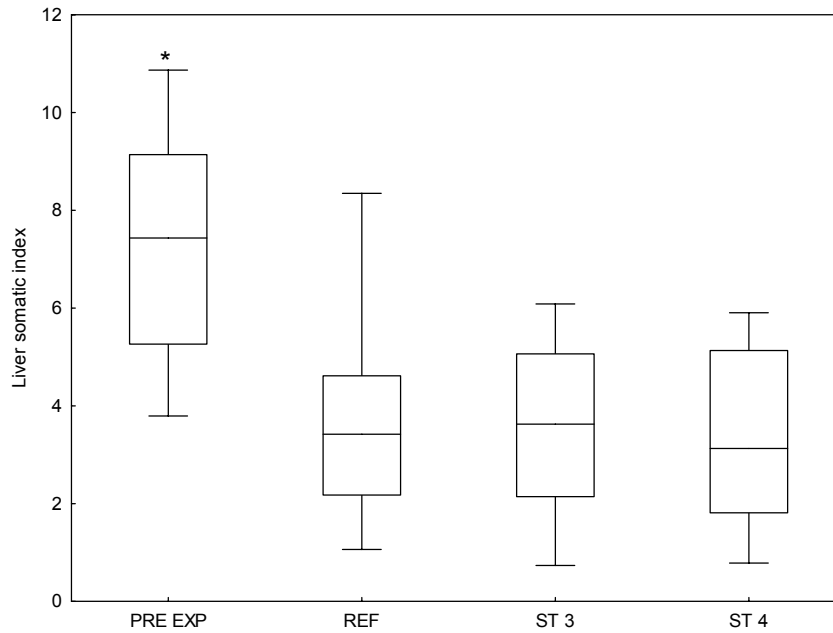
Figure 10. Liver-somatic index of cod from the indicated groups. The figure shows median, quartiles (box) and 10/90-percentiles (whiskers). * significant difference from all other groups (ANOVA, Tukey, p<0.05).

Figure 11. Gonadosomatic index in cod in the indicated groups. Right: females, Left: males. The figure shows median, quartiles (box) and 10/90-percentiles (whiskers).
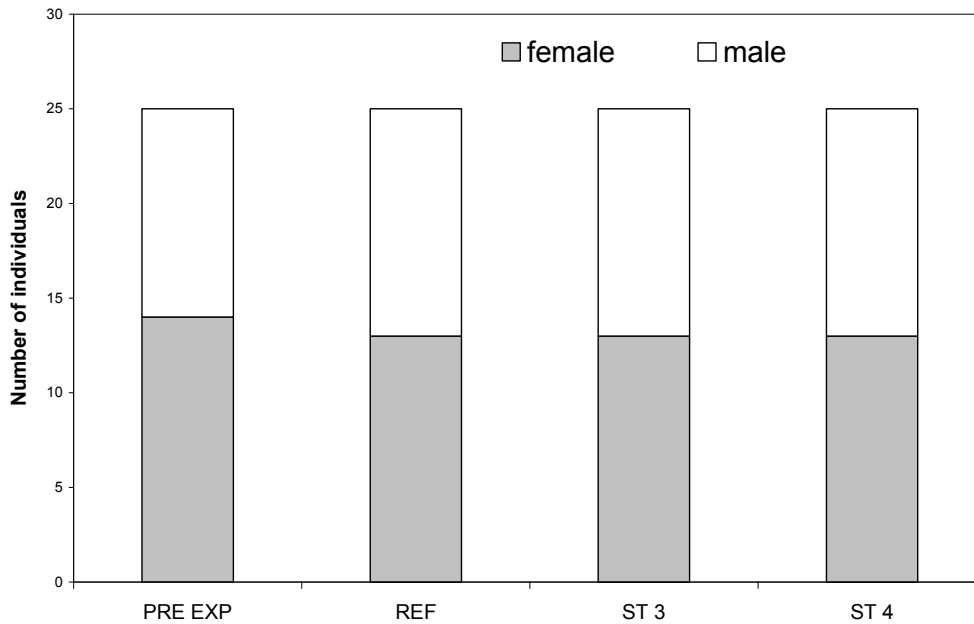
Figure 12. Sex ratios of cod in the groups indicated.

3.5 Biomarkers in cod

3.5.1 Cod - PAH-metabolites in bile

3.5.1.1 Bile fluorescence (PAH-metabolites by FF)

Measured bile fluorescence levels are shown in Figures 13-16. Differences between groups were only found for the wavelength-pair 341/383 (identifying 4 ring structures). The reference station was lower than all other groups (Figure 14). The signal observed for this wavelength-pair in the Pre exposure sampling group is confirmed by a low level of pyrene detected by GC-MS analysis (see section 3.5.2).
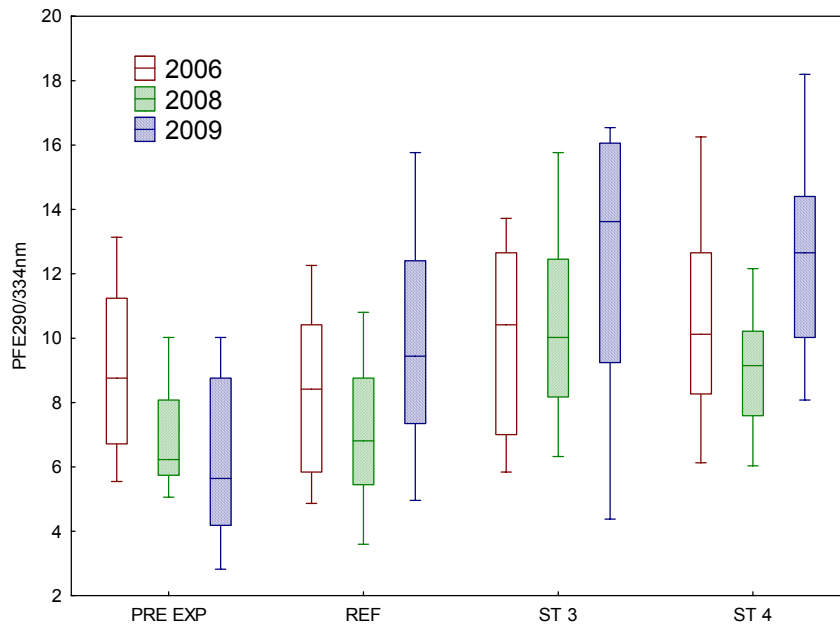
Figure 13. Fixed wavelength (290/334 nm) fluorescence levels in bile from cod in the groups indicated, expressed as pyrene fluorescence equivalents, PFE μg/g. The wavelength pair 290/334 nm identifies 2-3 ring structures. The figure shows median, quartiles and 10/90-percentiles.
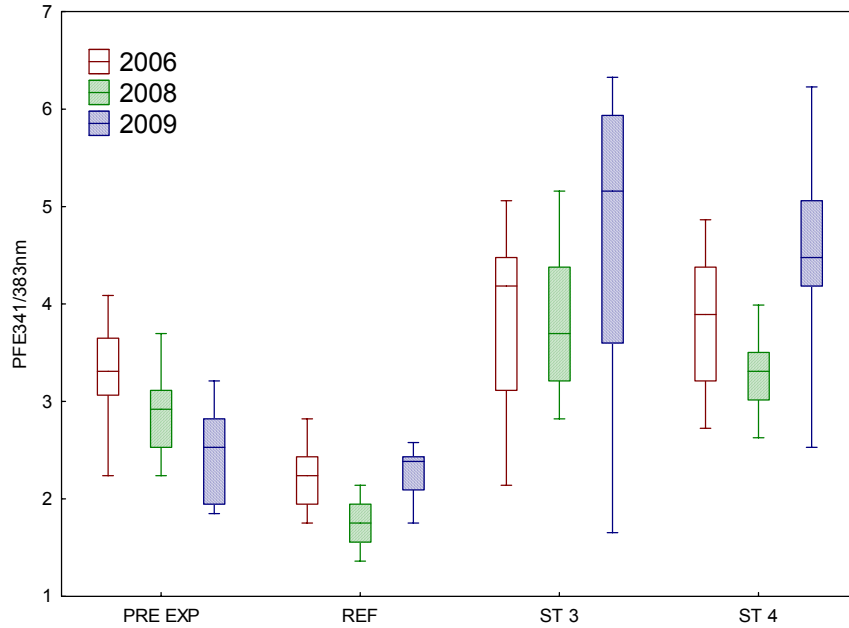
Figure 14. Fixed wavelength (341/383 nm) fluorescence levels in bile from cod in the groups indicated, expressed as pyrene fluorescence equivalents, PFE µg/g. The wavelength pair 341/383 nm identifies 4 ring structures. The figure shows median, quartiles and 10/90-percentiles.
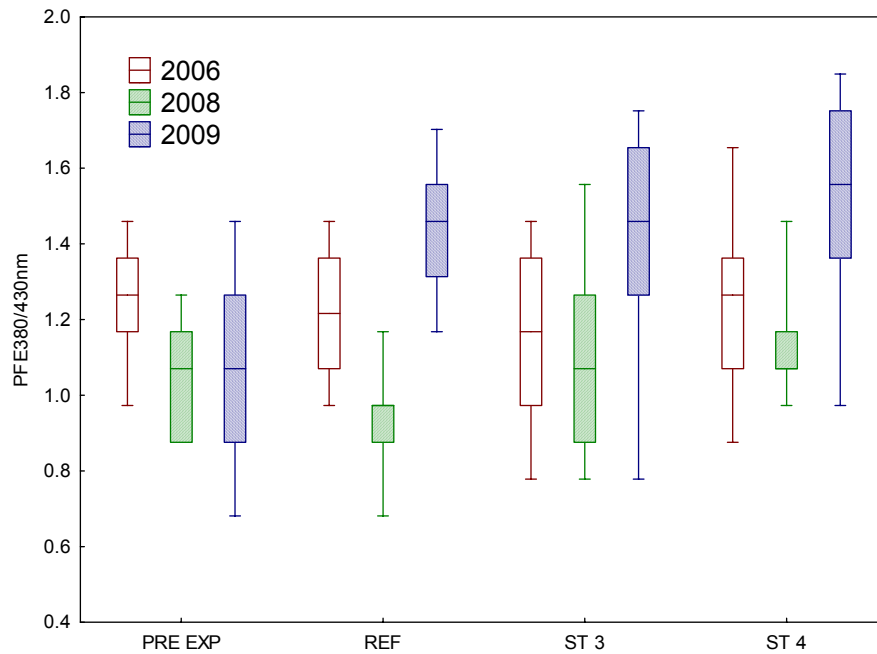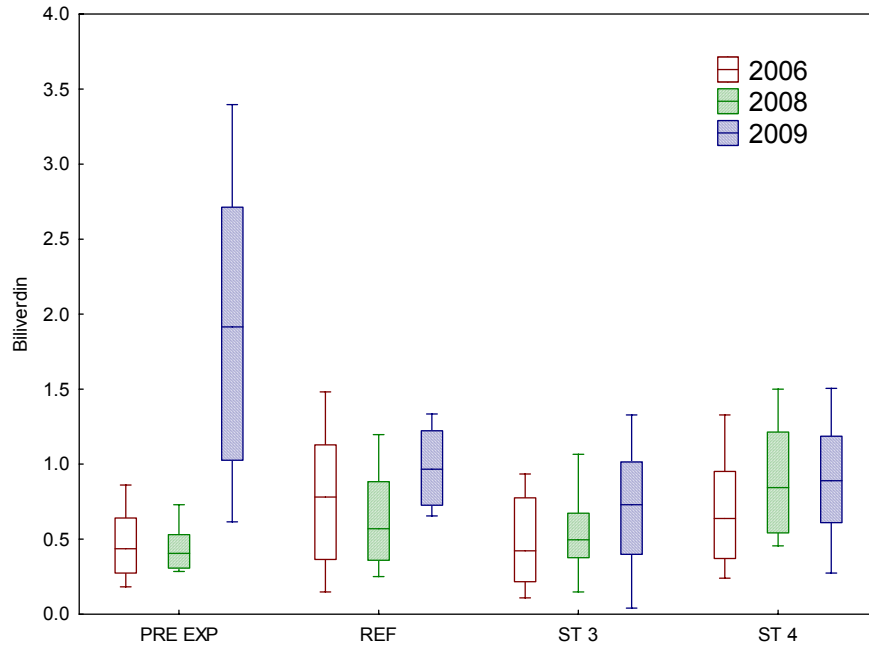

Figure 15. Fixed wavelength (380/430 nm) fluorescence levels in bile from cod in the groups indicated, expressed as pyrene fluorescence equivalents, PFE µg/g. The wavelength pair 380/430 nm identifies 5 ring structures. The figure shows median, quartiles and 10/90-percentiles.

Figure 16. Biliverdin (absorbance 660 nm, mg/l) levels in bile from cod in the groups indicated. The figure shows median, quartiles and 10/90-percentiles.

3.5.1.2 PAH-metabolites by GC-MS

For all metabolite compounds except 1-OH-naphtalene and 1-OH-phenantrene, stations 3 and 4 were significantly different from the reference station ($p < 0.05$, Tukey; Figure17-19). This confirms significant uptake and bio-transformation of PAHs typical for PW to the fish from the two stations close to the discharge. However, the levels measured are only approximately double the quantification limit for analysis methods (e.g. 200 ng/g bile for C1/ C2 naphthalene and 600 ng/g bile for C3 naphthalene) indicating low level of exposure. The levels of PAH metabolites in the 2009 bile material were similar to the levels measured in the 2006 and 2008 material.
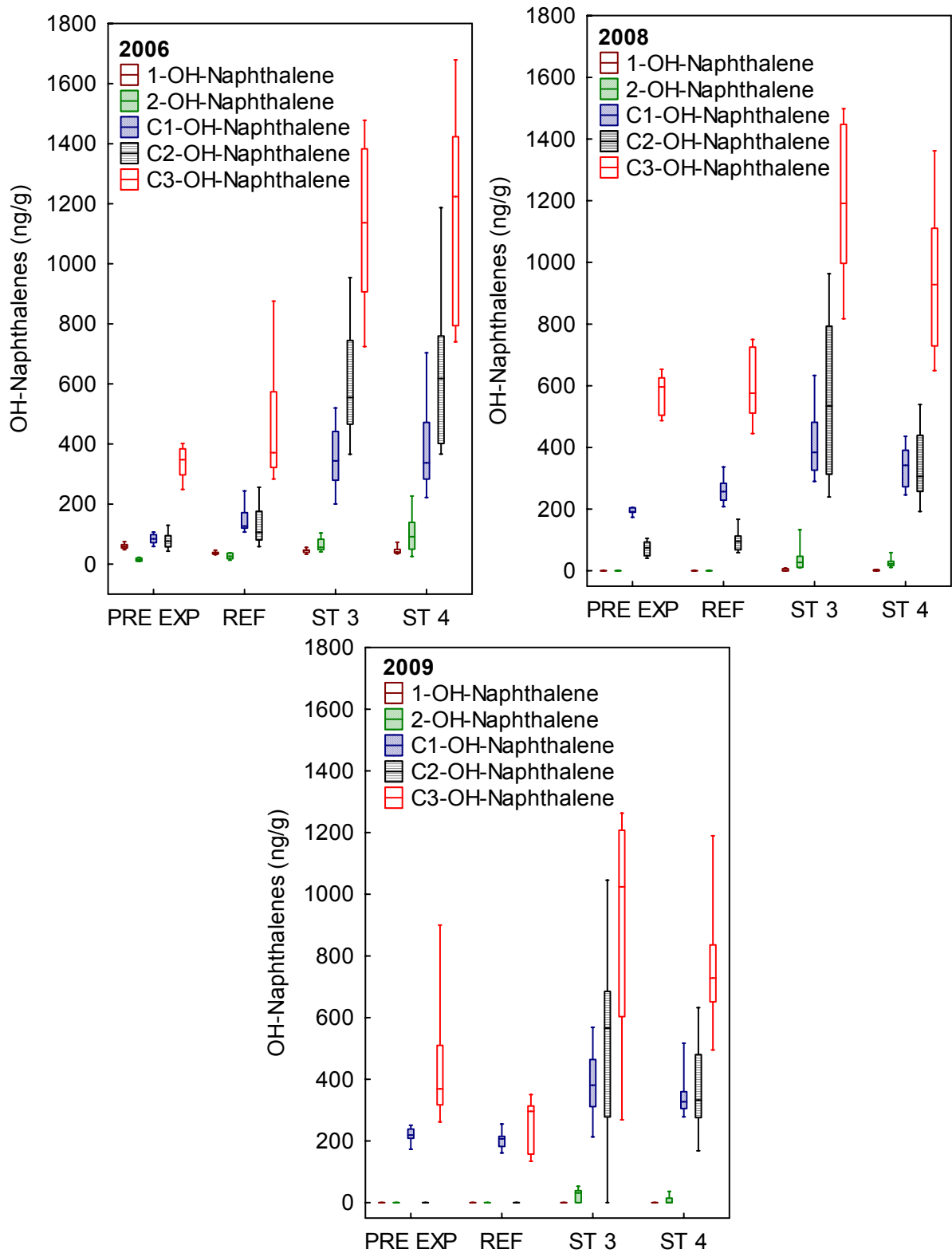
Figure 17. Concentrations (ng/g bile) of OH-naphthalenes in caged cod from the groups indicated. The figure shows median, quartiles and 10/90 percentiles of five individuals from each group.
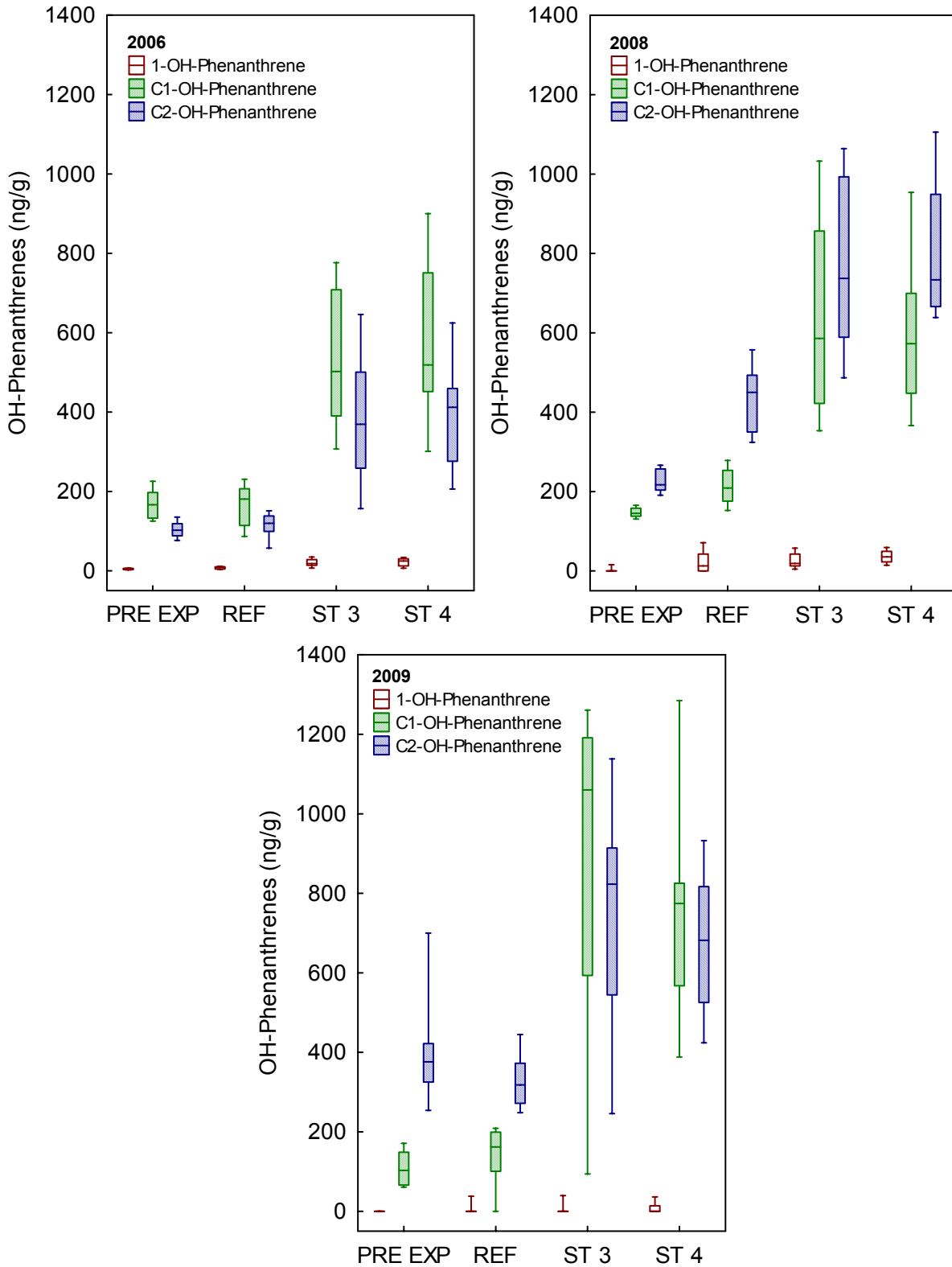
Figure 18. Concentrations (ng/g bile) of OH-phenanthrenes in caged cod from the groups indicated. The figure shows median, quartiles and 10/90 percentiles of five individuals from each group.
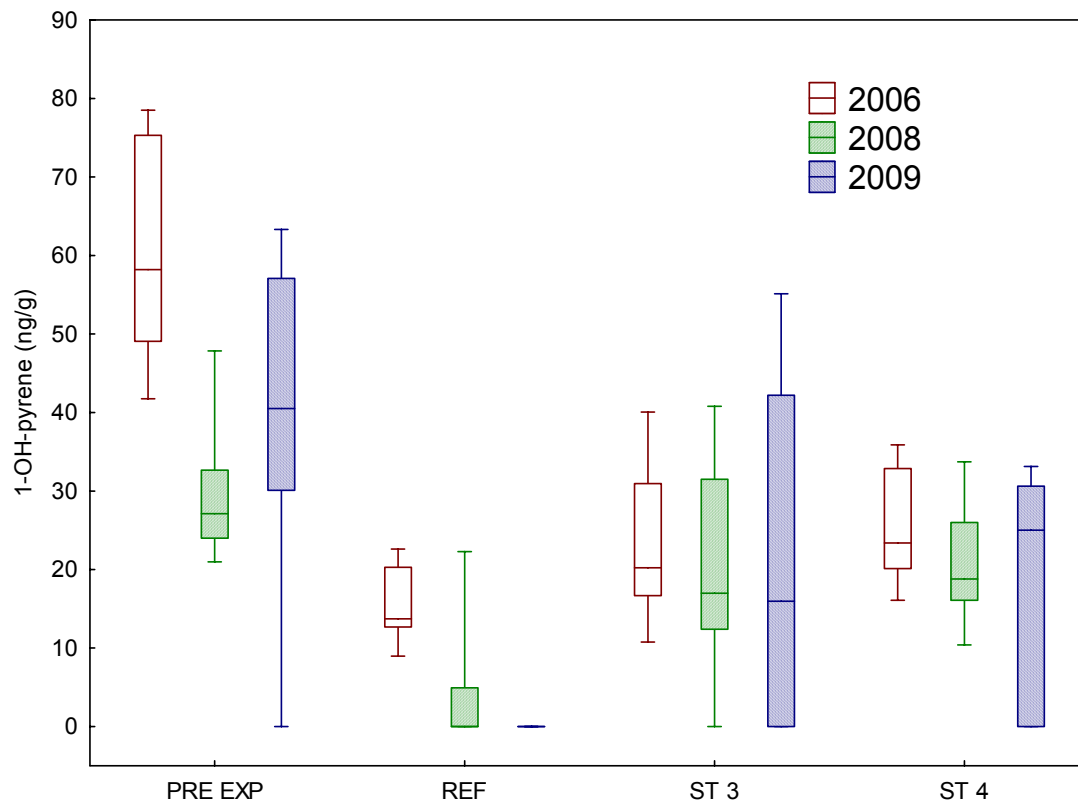
Figure 19. Concentrations (ng/g bile) of 1-OH-pyrene in caged cod from the groups indicated. The figure shows median, quartiles and 10/90 percentiles of five individuals from each group.

3.5.2 AP metabolites in cod bile

Significant differences in levels of 2,4-dimethylphenol and 2,4,6-trimethylphenol metabolites were found between reference station and stations 3 and 4 (Kruskal-Wallis, $p<0.05$, Figure 20). This confirms bio-concentration and bio-transformation of APs typical for PW to the fish caged close to the discharge. However, the levels measured are only approximately double the limit of quantification for analysis methods (30 ng/g bile, for single compounds) indicating low level of exposure.

In 2008, levels of 2-methylphenol, 3- methylphenol and 3,5- dimethylphenol metabolites were elevated in fish caged at station 3 and 4. This finding indicates a reduced exposure level in 2009.
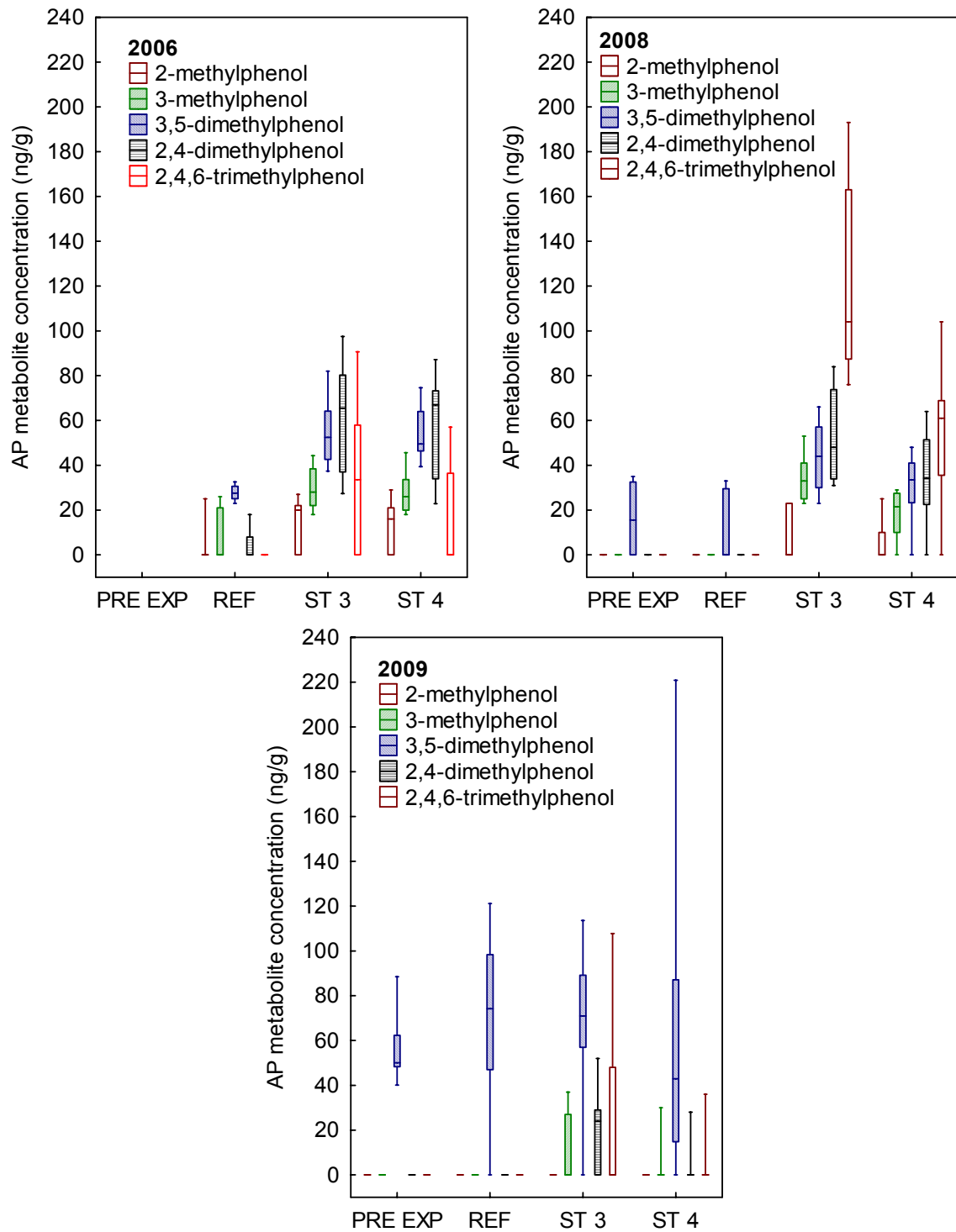
Figure 20. Concentrations (ng/g bile) of AP metabolites in caged cod from the groups indicated. The figure shows median, quartiles and 10/90 percentiles.

3.5.3 Hepatic GST

No differences in hepatic glutathione-*S*-transferase activity (GST) with respect to gender were observed. Therefore, the data has been shown for all fish sampled in 2009 and compared to the same GST data collected in 2006 and 2008 (Figure 21).

GST activity measured in 2009, was significantly lower than that found in 2006 for all stations including the PRE EXP and the REF stations (ANOVA, Tukey, $p<0.05$). For the 2009 data, GST activity in fish from ST4 was significantly lower than all other stations. Overall, there was no evidence of exposure to oil related compounds based on GST activity within 2009, 2008 or 2006
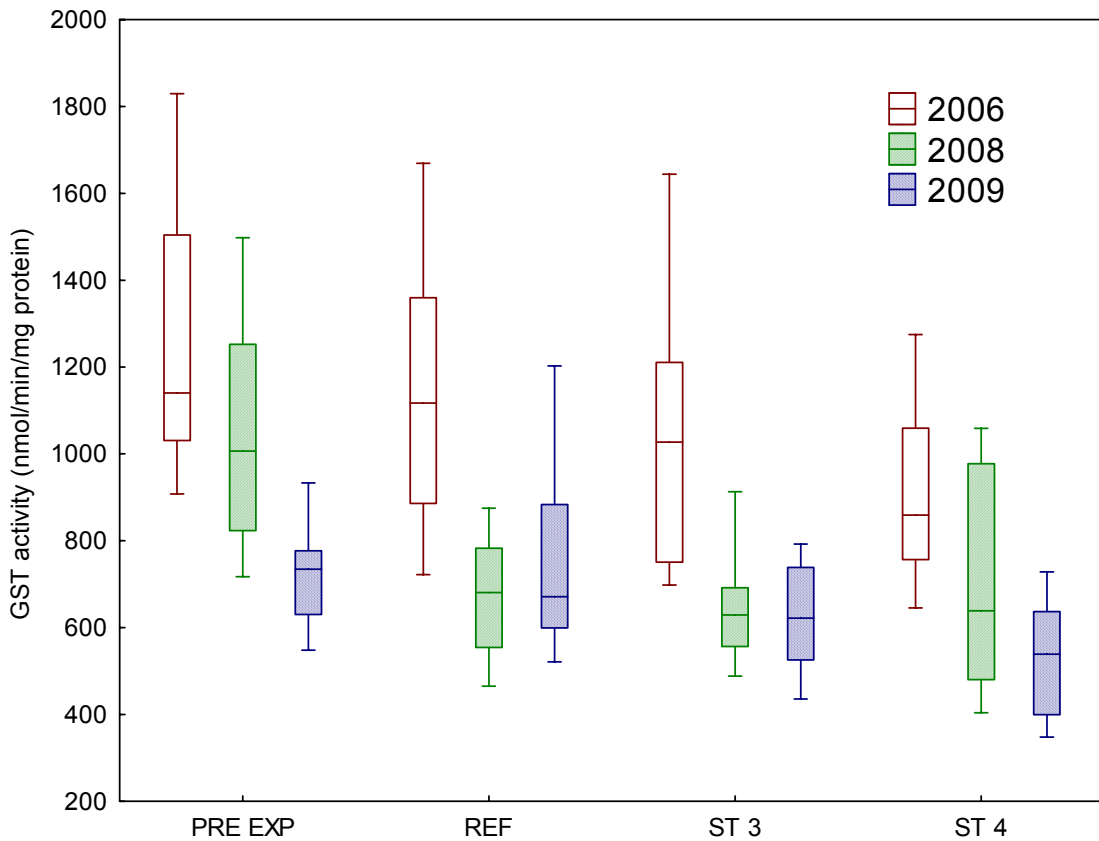

Figure 21. Hepatic Glutathione S-transferase (GST) activity in cod from the indicated groups in 2009, 2008 and 2006 surveys. The figure shows median, quartiles and 10/90-percentiles.

3.5.4 Hepatic Cytochrome P450 1A

Gender differences in hepatic CYP1A activity were not observed and data has been presented for all fish measured for both 2009 and compared to CYP1A data from the WCM 2006 and 2008 surveys (Figure 22).

Problems with the CYP1A analysis (i.e. non-specific binding) were encountered in 2006, which led to higher than expected blank values that were subsequently deducted from the measured concentrations causing them to be artificially lowered. Consequently, the 2006 data cannot be directly compared with the other years.

For the 2009 data, CYP1A activity measured in the fish from the reference site (REF2) was significantly lower then that recorded in fish from the two exposure sites (ST3 and 4; Kruskal-Wallis ANOVA, $p < 0.05$). This suggests some exposure to organic compounds from the PW. A similar pattern can be seen in the 2008 data with ST 3 significantly higher than the reference station. However, the CYP1A activity in 2009 fish were significantly lower than that measured in fish from 2008 (ANOVA, Tukey, $p < 0.05$), and would suggest a reduction in the exposure of the fish to CYP1A inducing compounds such as PAHs in the PW compared to the previous year.
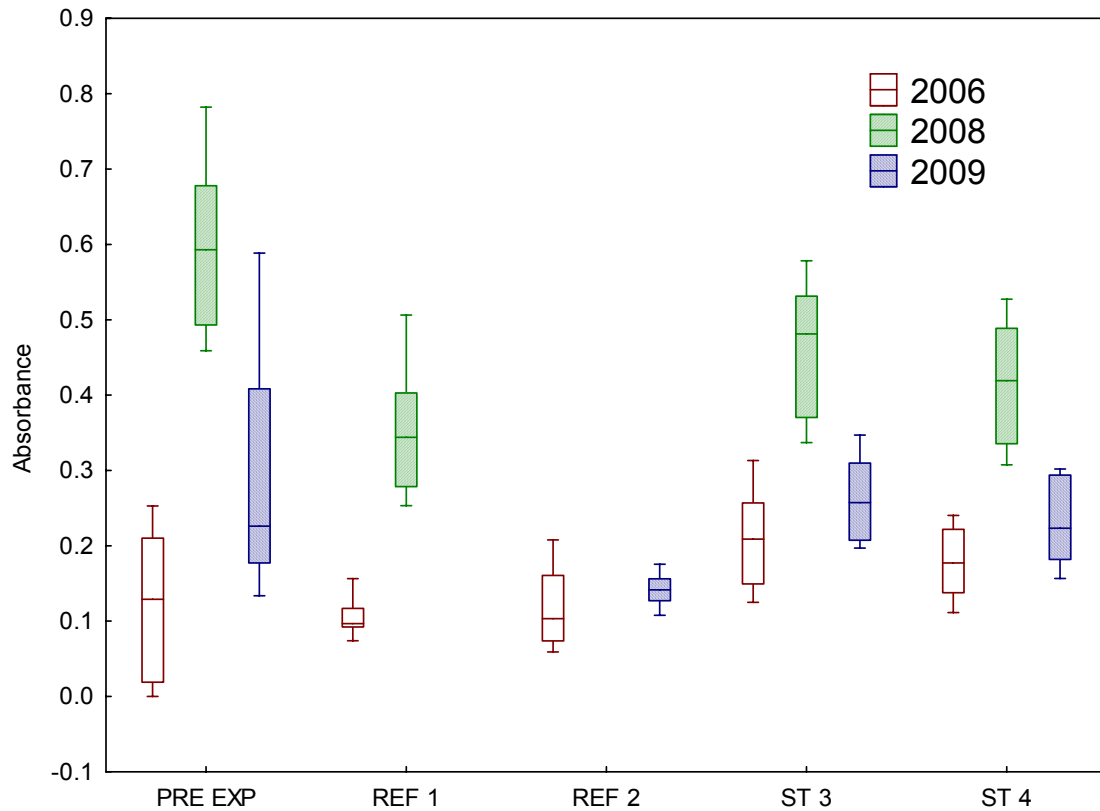

Figure 22. Hepatic cytochrome P450 1A activity in cod from the indicated groups from 2009 – compared with data from the 2006 and 2008 WCM surveys. The figure shows median, quartiles and 10/90-percentiles.

3.5.5 Vitellogenin

Blood samples were taken from all individuals before deployment (pre-exposure). The figure depicts the difference in plasma VTG-concentrations from before and after exposure (ΔVTG = $[VTG]_{after\ exposure} - [VTG]_{before\ exposure}$, Figure 23). In 2009 male cod, there were no significant

differences between the stations. The ΔVTG concentrations in 2009 males were comparable to the male data from 2008 and 2006. The negative vales recorded for some of the data, particularly those measured in 2006 would suggest higher VTG concentrations in the pre-exposed cod.

Higher concentrations of ΔVTG were measured in females, although no significant differences were found either between years of between stations. As observed for the male VTG data, slightly negative ΔVTG values were found in female cod from 2006, which suggests that VTG concentrations were higher in the pre-exposed fish. Large variations in ΔVTG were found in female cod from 2008.
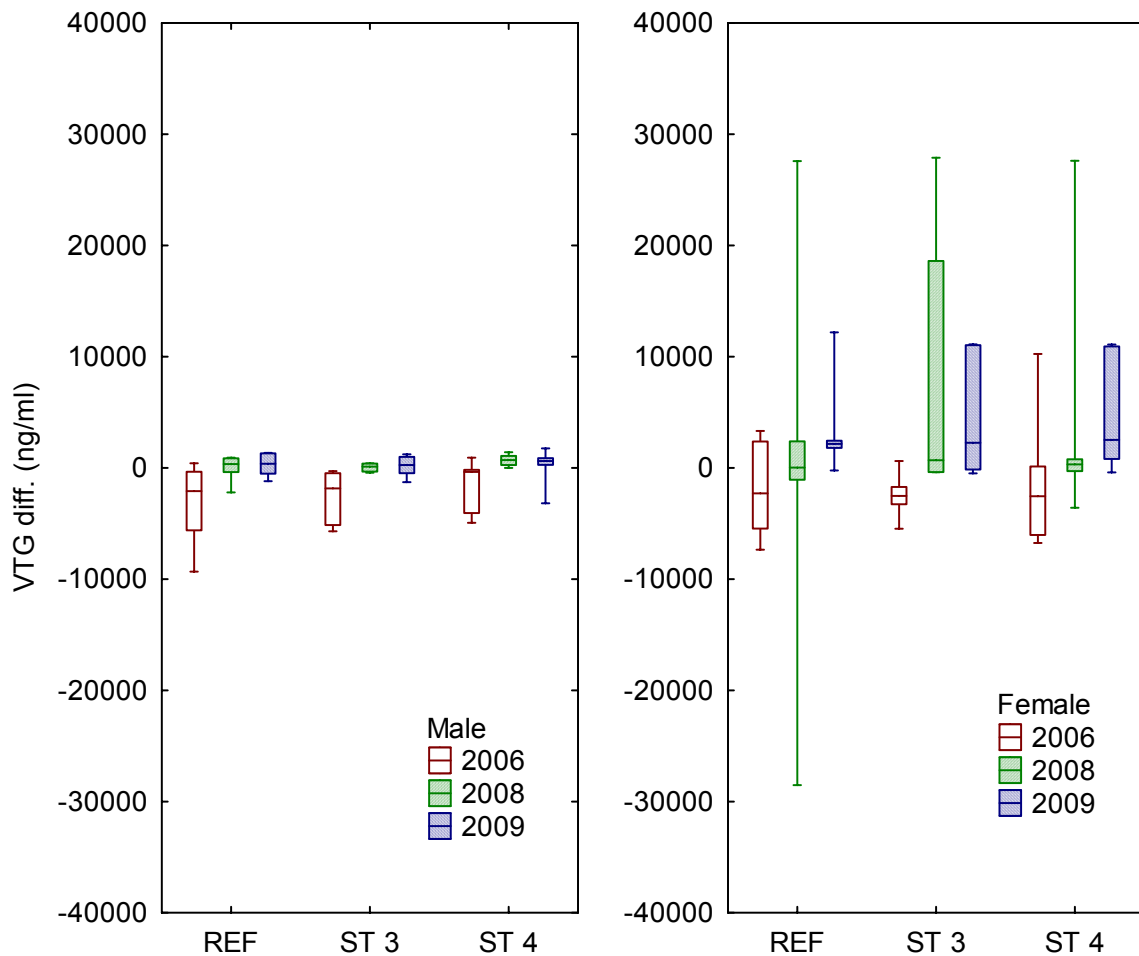

Figure 23. Plasma vitellogenin concentration in caged cod from the groups indicated. The figure shows median, quartiles and 10/90-percentiles and is expressed as the difference in plasma VTG concentrations in individual fish pre and post exposure. Males – left panel; females – right panel.

3.5.6 Zona Radiata Protein

Blood samples were taken from all individuals before deployment (pre-exposure). The figure depicts the difference in plasma ZRP from before to after exposure ($\Delta ZRP = [ZRP]_{after\ exposure} - [ZRP]_{before\ exposure}$; semi-quantitative, Figure 24).

In all fish measured the ΔZRP concentration was small. For male cod measured in 2009, no significant differences in ΔZRP concentration between stations were found. This was also true for ΔZRP concentration measured in male code in 2008 and 2006.

For female cod measured in 2009, no significant differences in ΔZRP concentrations between the stations were found. This was also true for the 2008 and 2006 ZRP data, and firmly concludes that ZRP concentrations in fish from all three sampling cruises were not significantly effected by the cage exposures.
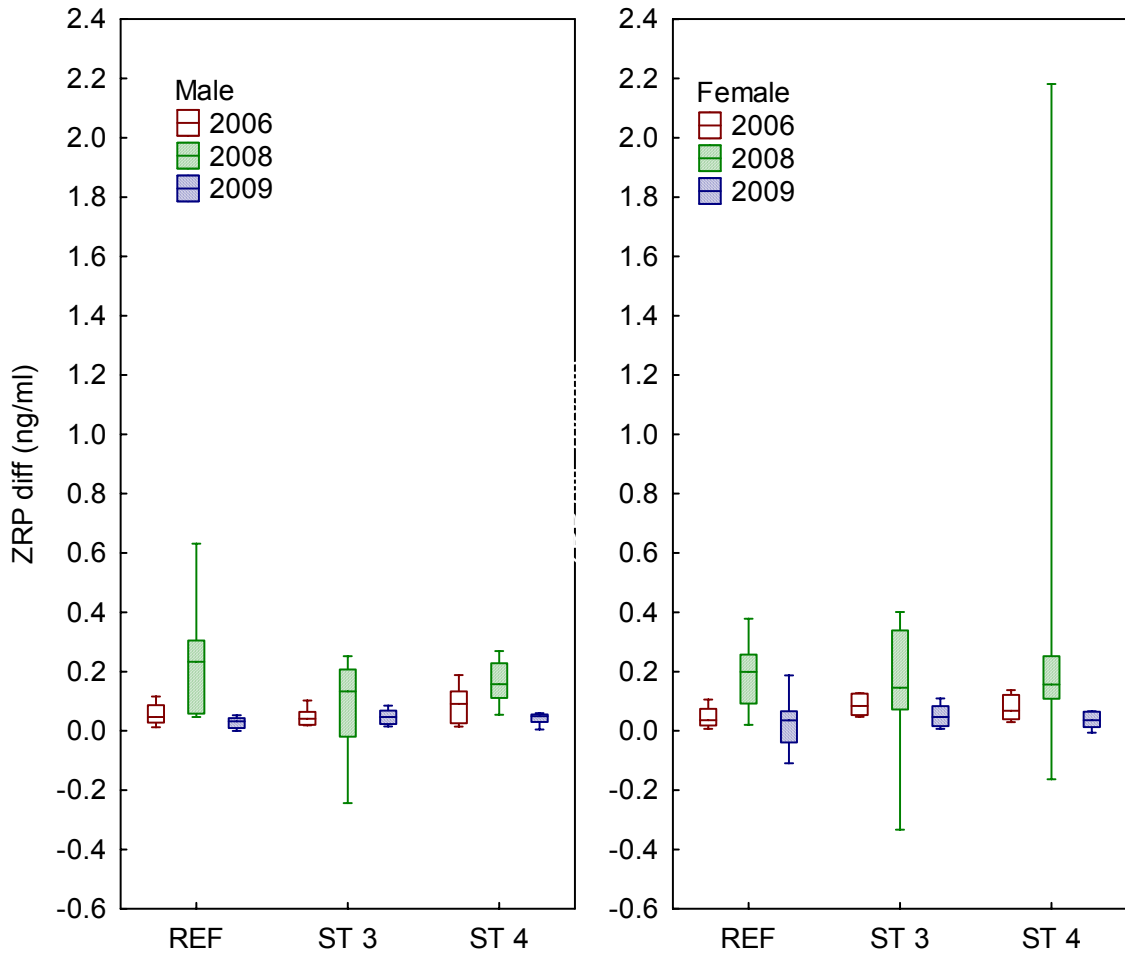

Figure 24. Plasma Zona radiata protein concentration in caged cod from the groups indicated. The figure shows median, quartiles and 10/90-percentiles and is expressed as the difference in plasma ZRP concentrations in individual fish pre and post exposure. Males – left panel; females – right panel.

3.5.7 DNA adducts

The measurement of DNA adducts in exposed cod was carried out by Stockholm University and a full report can be found in Appendix D. The DNA adduct levels in cod from 2009 were low in all groups with no significant difference between the groups (Figure 25). Median concentrations for 2009 cod were below 1 nmol adduct/ mol normal nucleotides in all groups,

and were comparable to the levels of DNA adducts reported in cod from 2006 and 2008. Overall, there was no significant effects of PW exposure on the levels of DNA adducts in cod for all three of the investigative years.
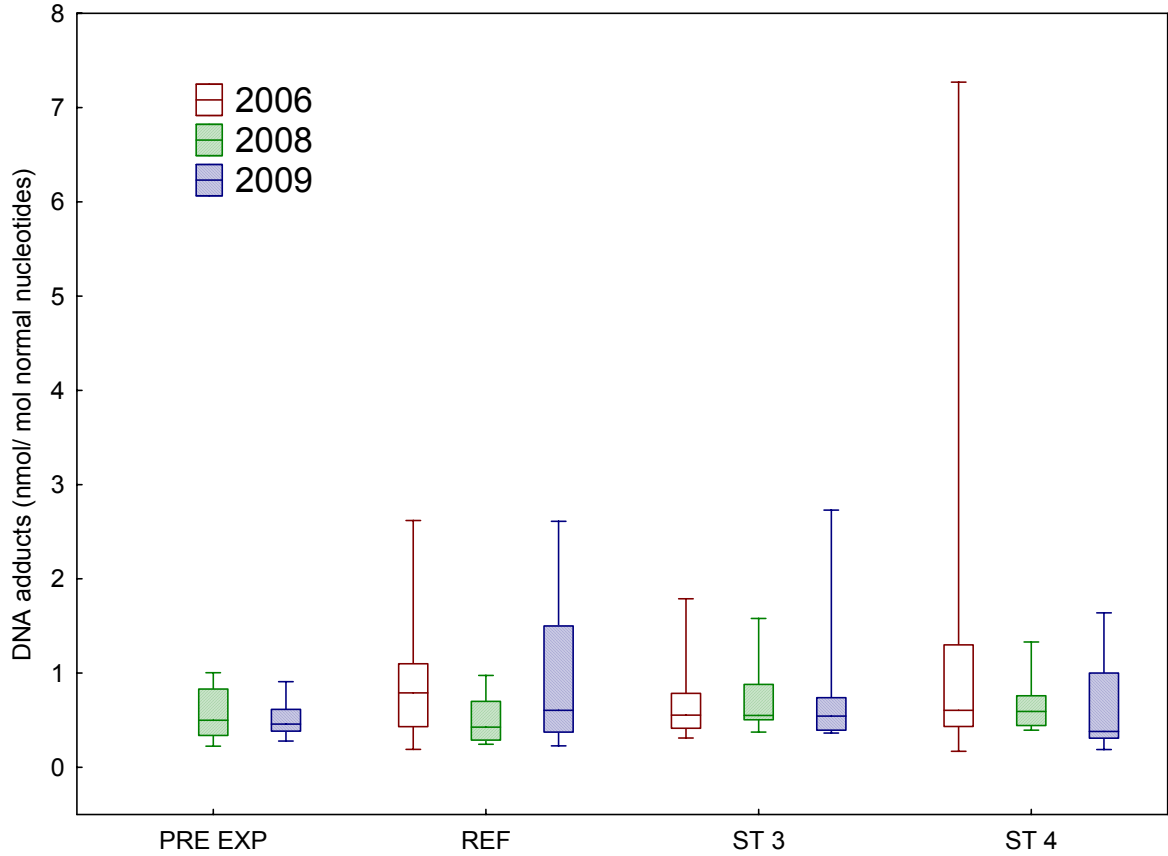

Figure 25. DNA adduct levels in liver (nmol add/mol normal nucleotides) in caged cod from the groups indicated. The figure shows median, quartiles and 10/90-percentiles.

3.6 Biomarkers in mussels

3.6.1 PAH body burden in mussels

Twenty three pooled mussel samples were analysed for PAHs including NPDs and decalins. These included three replicate pooled samples from 6 exposure stations (REF2, ST1, ST3-6) plus the PRE EXP group. ST 2 was relocated approximately 2 km south away from the platform in week 5 of the 6 week exposure. The limited number of mussels available at this station resulted in only a duplicate pooled sample taken for chemical analysis. The chemical analysis was carried out by both NIVA and Battelle and the raw data is provided in Appendix B & D respectively. Both NIVA and Battelle data is shown graphically in the following figures (Figure 26 to 34).

There were no pronounced differences in the mussel lipid content among stations with most lipid content falling within 1 to 2% (Figure Figure 26). Subsequent data for PAHs are presented on a wet-weight basis (µg/kg; Figures 27 to 31). In addition, lipid analysis was not carried out by NIVA on the mussel samples in 2006. Therefore, by presenting the data on a wet weight basis, data for all survey years could be compared.

Due to the small sample size (n=3 in each group), statistical evaluation was not performed. Groups with no overlapping values can be regarded as different. The results are shown as Sum PAH16, total dibenzothiophenes, total phenanthrenes/anthracenes, total naphthalenes and total decalins for both NIVA and Battelle laboratories.
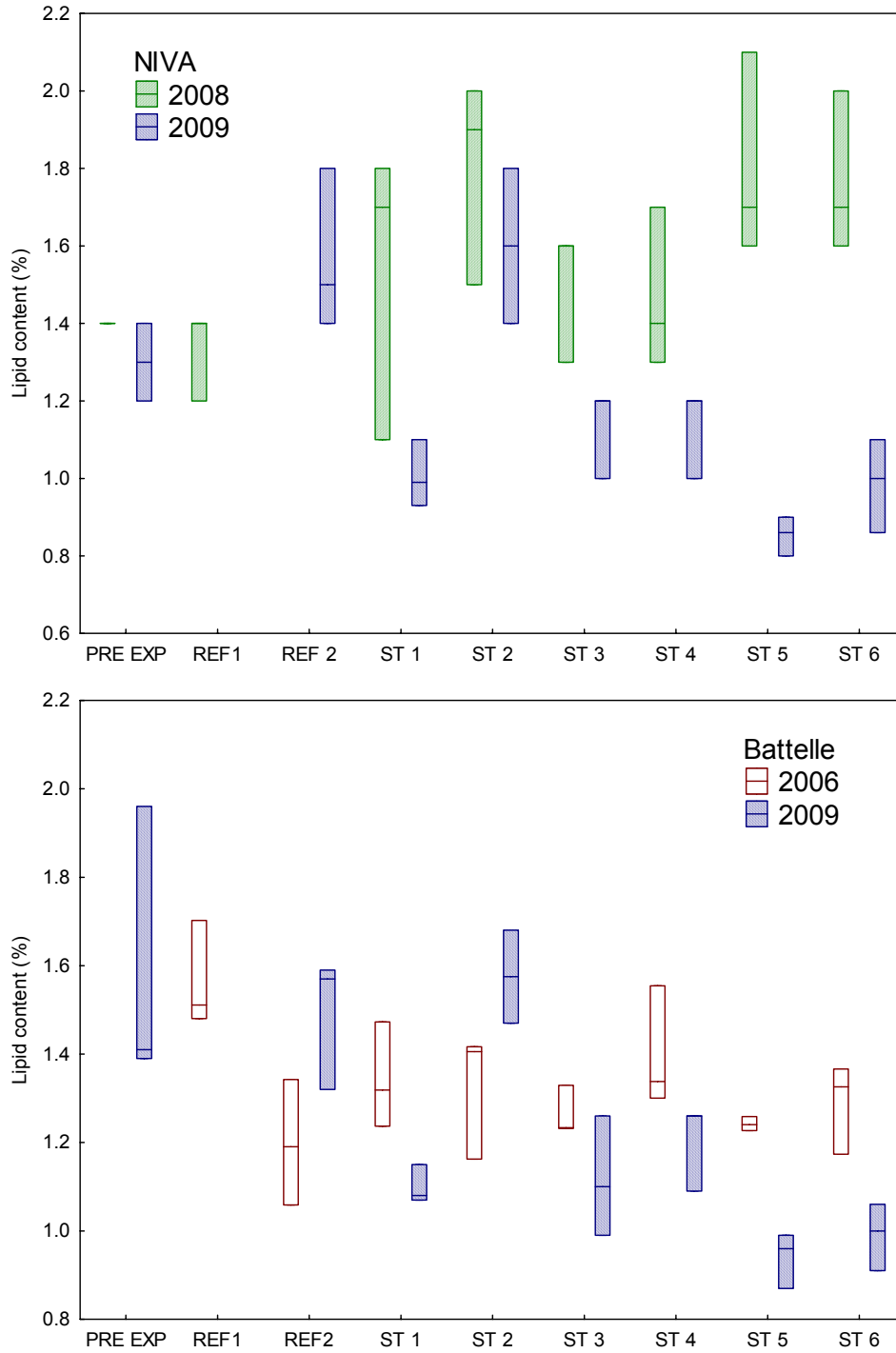
Figure 26. Lipid content of mussels from the groups indicated. The figure shows median and min/max values analysed by NIVA and Battelle laboratories.

Comparison of PAH body burden data between 2006, 2008 and 2009

For the WCM program of 2006 and 2009, mussel PAH body burden analysis was performed by both Battelle and NIVA. In 2008, analysis was only performed by NIVA. Results from both laboratories for all three years are presented. To ensure data are compared correctly separate graphs are shown for the data from each analysing laboratory.

The sum of PAH16 was lowest at the two reference stations, higher in stations 1, 2, 4, 5 and 6, and highest at station 3, which was located closest to the discharge (Figure 27). This pattern associated with distance from the PW discharge was consistent for all other PAHs measured including; dibenzothiophenes, phenanthrenes and anthracenes, naphthalenes and decalins (Figures 28-31).

The sum of PAH16 for 2009 was markedly lower than both 2006 and 2008 data at all sites measured. The same was true for all other PAH concentrations measured. Overall, there was good agreement in PAH body burden data between laboratories with both labs indicating an exposure profile with distance from the PW discharge and a reduction in PAH concentrations in 2009 compared to 2008 and 2006.
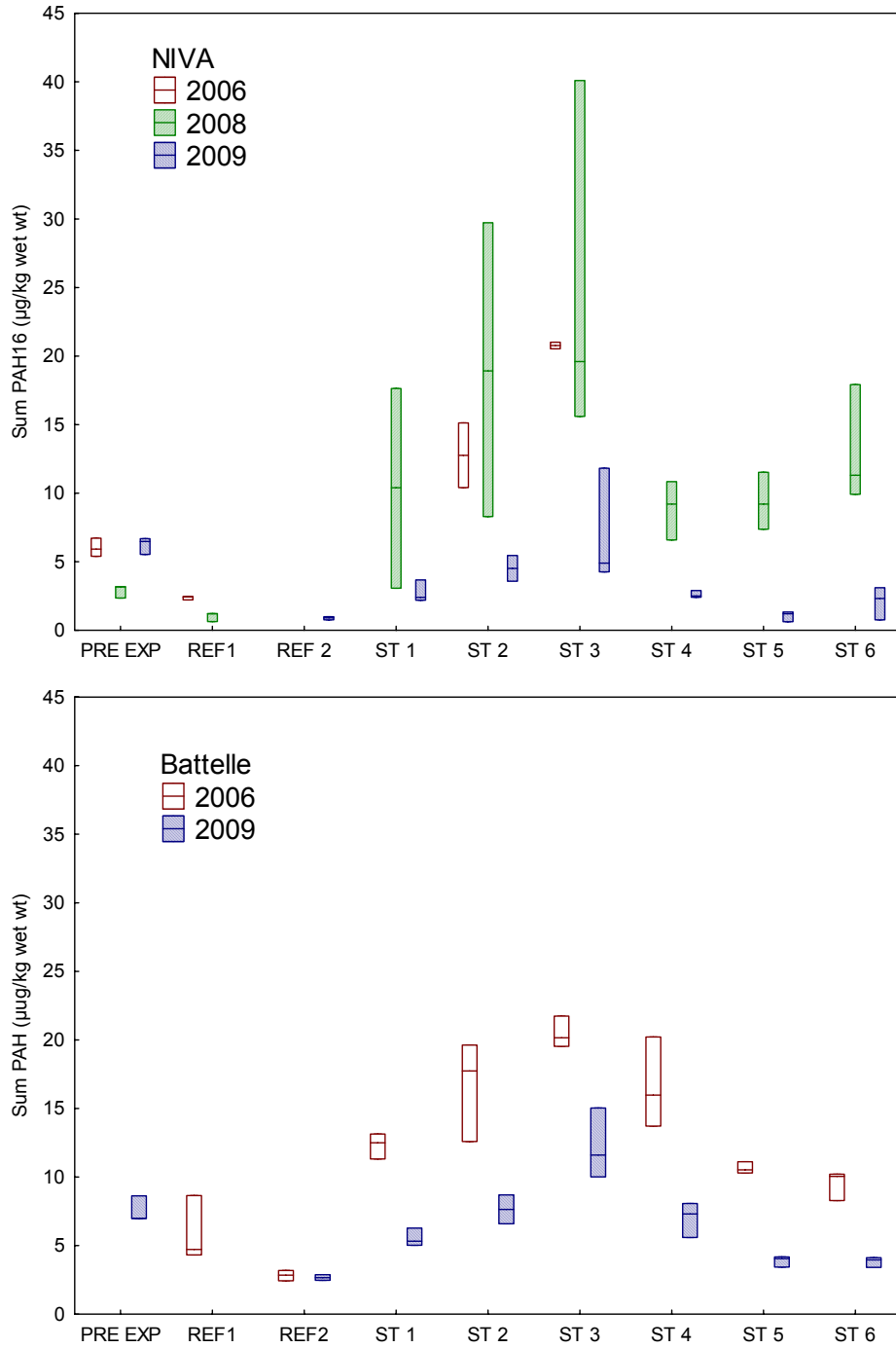
Figure 27. **Sum PAH16** concentrations in mussels from the groups indicated. Boxes depict median and min-max (n=3). Analysis carried out by NIVA and Battelle labs.
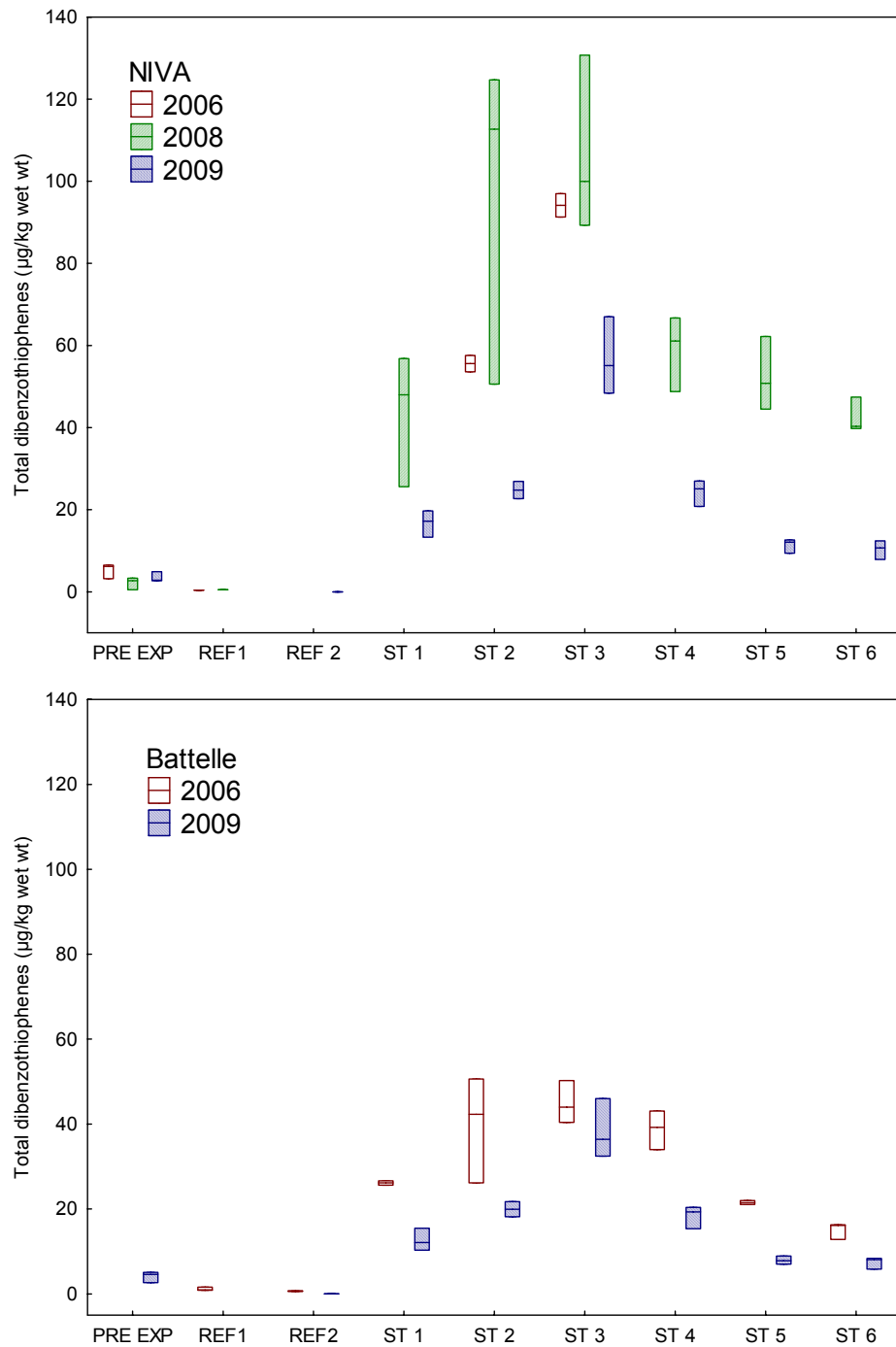
Figure 28**. Total dibenzothiophenes** concentrations shown in mussels from the groups indicated. Boxes depict median and min-max (individual observations, since n=3). Analysis carried out by NIVA and Battelle labs. Data includes the parent compound and alkylated forms. (NIVA – including alkylated C1-C3, Battelle - including alkylated C1-C4).

Figure 29**. Total phenanthrenes and anthracenes** concentrations shown in mussels from the groups indicated. Boxes depict median and min-max (individual observations, since n=3). Analysis carried out by NIVA and Battelle labs. Data includes the parent compound and alkylated forms. (NIVA – including alkylated C1-C3, Battelle - including alkylated C1-C4).
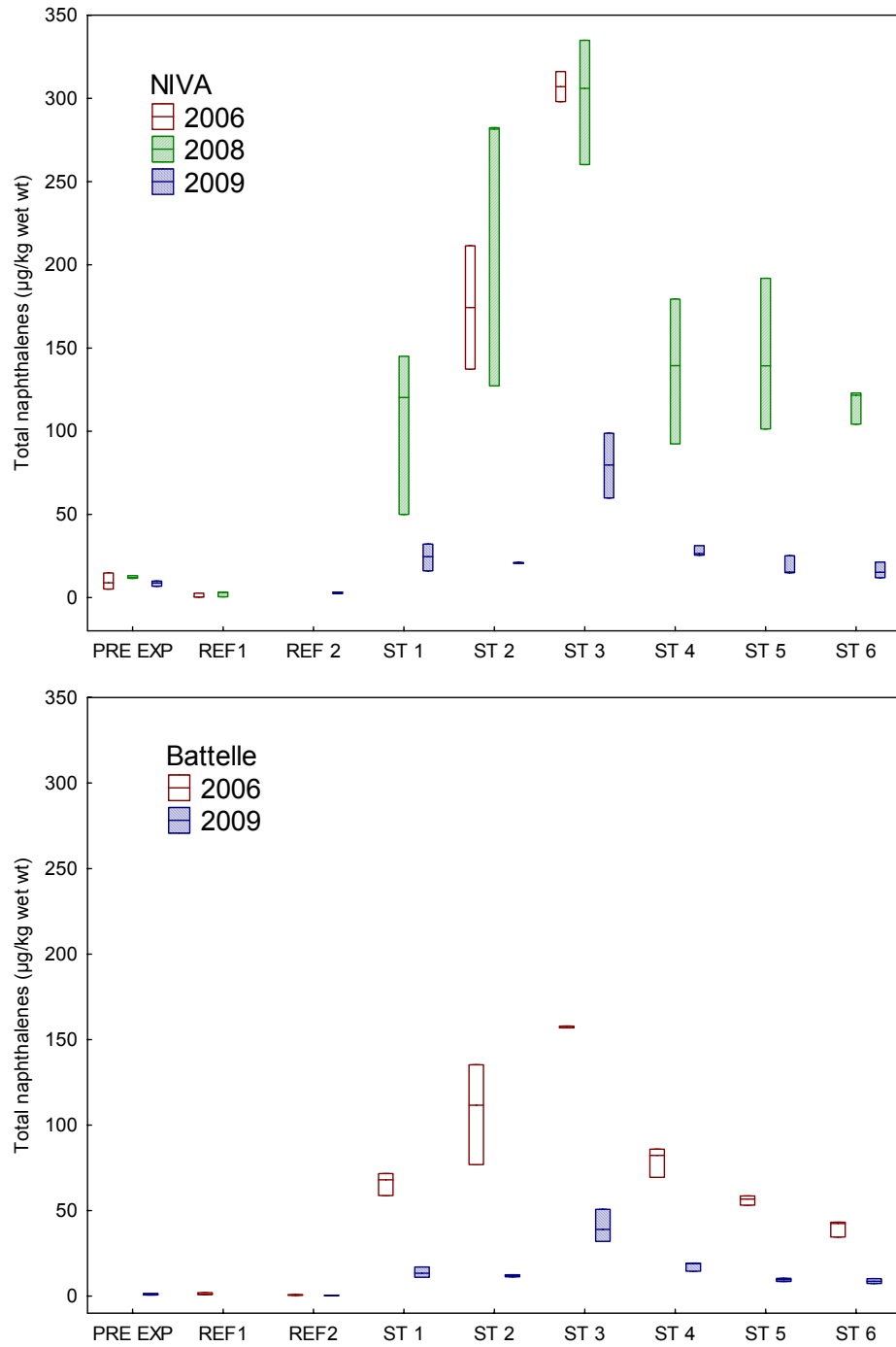
Figure 30**. Total naphthalenes** concentrations shown in mussels from the groups indicated. Boxes depict median and min-max (individual observations, since n=3). Analysis carried out by NIVA and Battelle labs. Data includes the parent compound and alkylated forms. (NIVA – including alkylated C1-C3, Battelle - including alkylated C1-C4).
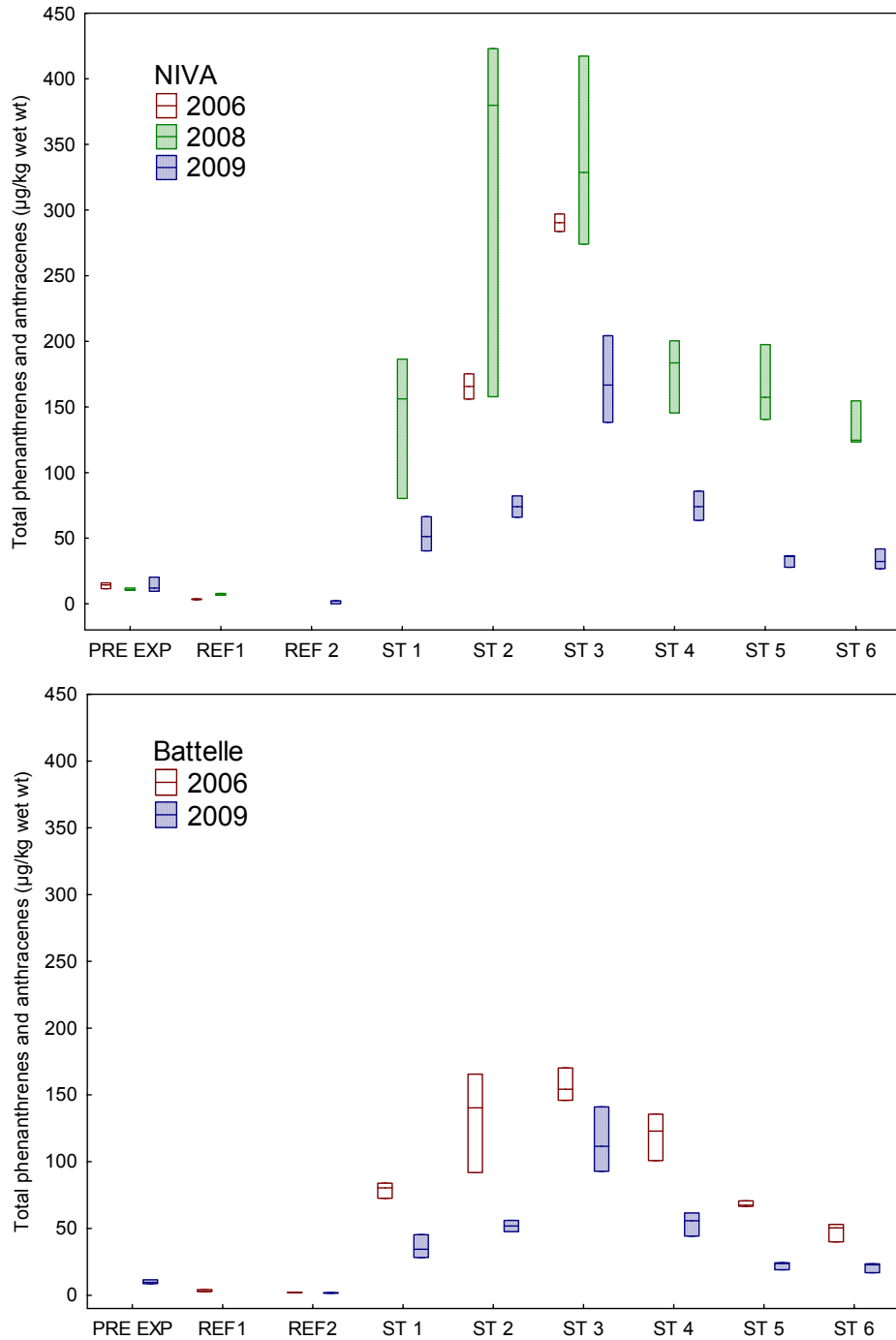
Figure 31. **Decalin** concentrations shown in mussels from the groups indicated. Boxes depict median and min-max (individual observations, since n=3). Analysis carried out by NIVA and Battelle labs. Data includes the parent compound and alkylated forms. (NIVA – including alkylated C1-C3, Battelle - including alkylated C1-C4).
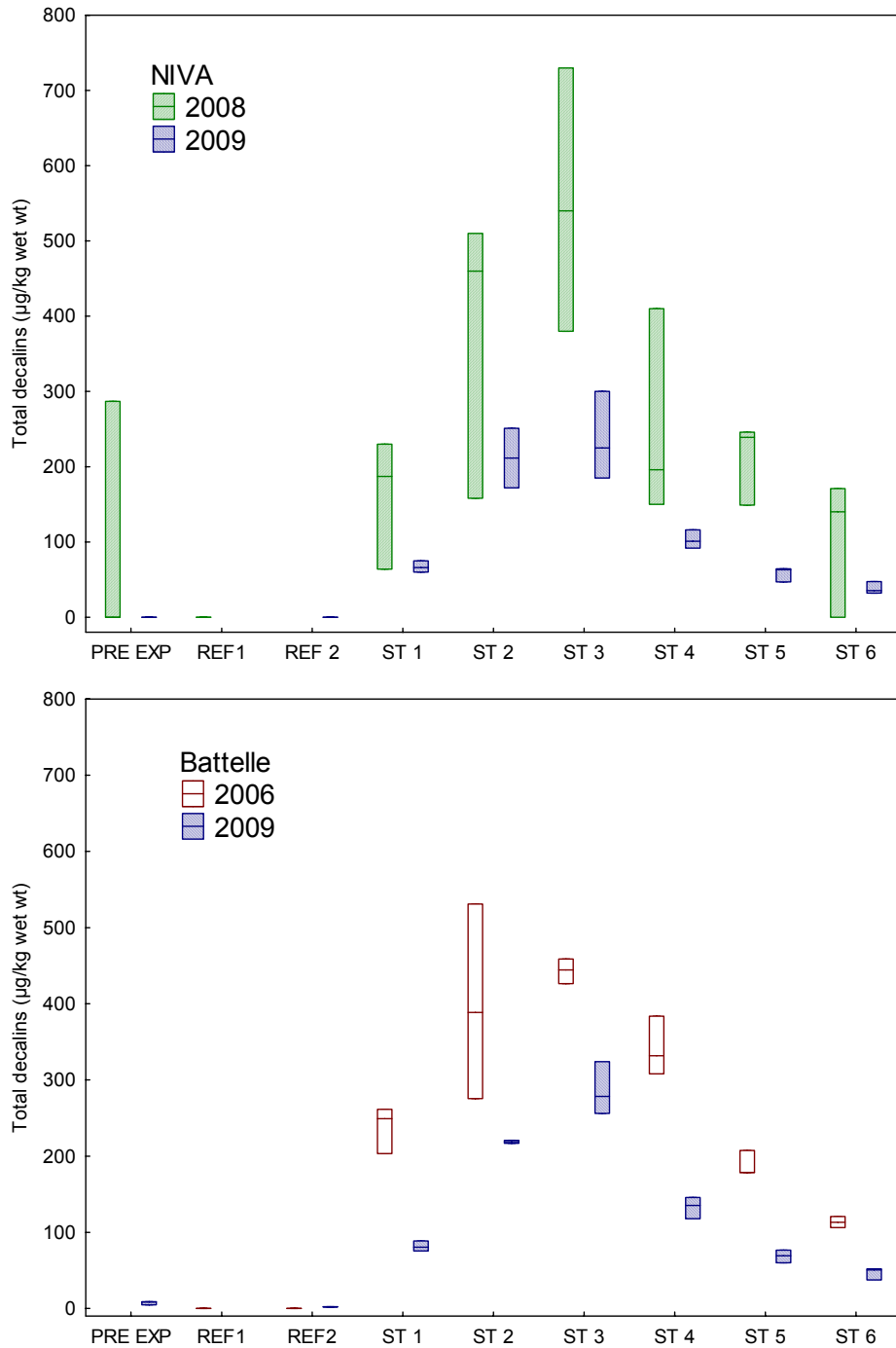
Relative concentrations of alkylated PAHs to parent compounds:

The PAHs found in coal and petroleum often contain one or more methyl (C1), ethyl (C2), propyl (C3), butyl (C4), or (occasionally) higher alkyl substituents on one or more of the aromatic carbons. These alkyl PAHs are generally more abundant than the parent PAHs in petroleum, but are less abundant than the parent PAHs in pyrogenic PAH mixtures. It is earlier shown that mussels caged down-stream of PW discharges from oil platforms accumulate higher concentrations of alkyl-naphthalenes, alkyl-phenanthrenes and alkyl-dibenzothiophenes, than their respective parent compounds (Ruus et al., 2006; Hylland et al., 2005). The ratio of alkyl-compounds: parent-compound for the mussels with respect to the NPD compounds are shown in the following graphs (Figures 32 to 34). As expected, higher ratios were found in the mussels closest to the offshore platform and were much lower at the reference stations. Furthermore, at all Ekofisk stations, the ratio of alkyl-naphthalenes/ naphthalene was lower in 2009 than found in 2008 and 2006. In contrast the ratio of alkyl-phenanthrenes/ phenanthrene was higher at all Ekofisk stations in 2009 compared to 2008 and 2006.

Figure 32. Ratio of alkyl-naphthalenes/ naphthalenes. Boxes depict median and min-max (individual observations, since n=3). Analysis carried out by NIVA and Battelle labs (NIVA – including alkylated C1-C3, Battelle - including alkylated C1-C4).

Figure 33. Ratio of alkyl-phenanthrenes & anthracenes/ phenanthrene and anthracene. Boxes depict median and min-max (individual observations, since n=3). Analysis carried out by NIVA and Battelle labs (NIVA – including alkylated C1-C3, Battelle - including alkylated C1-C4).
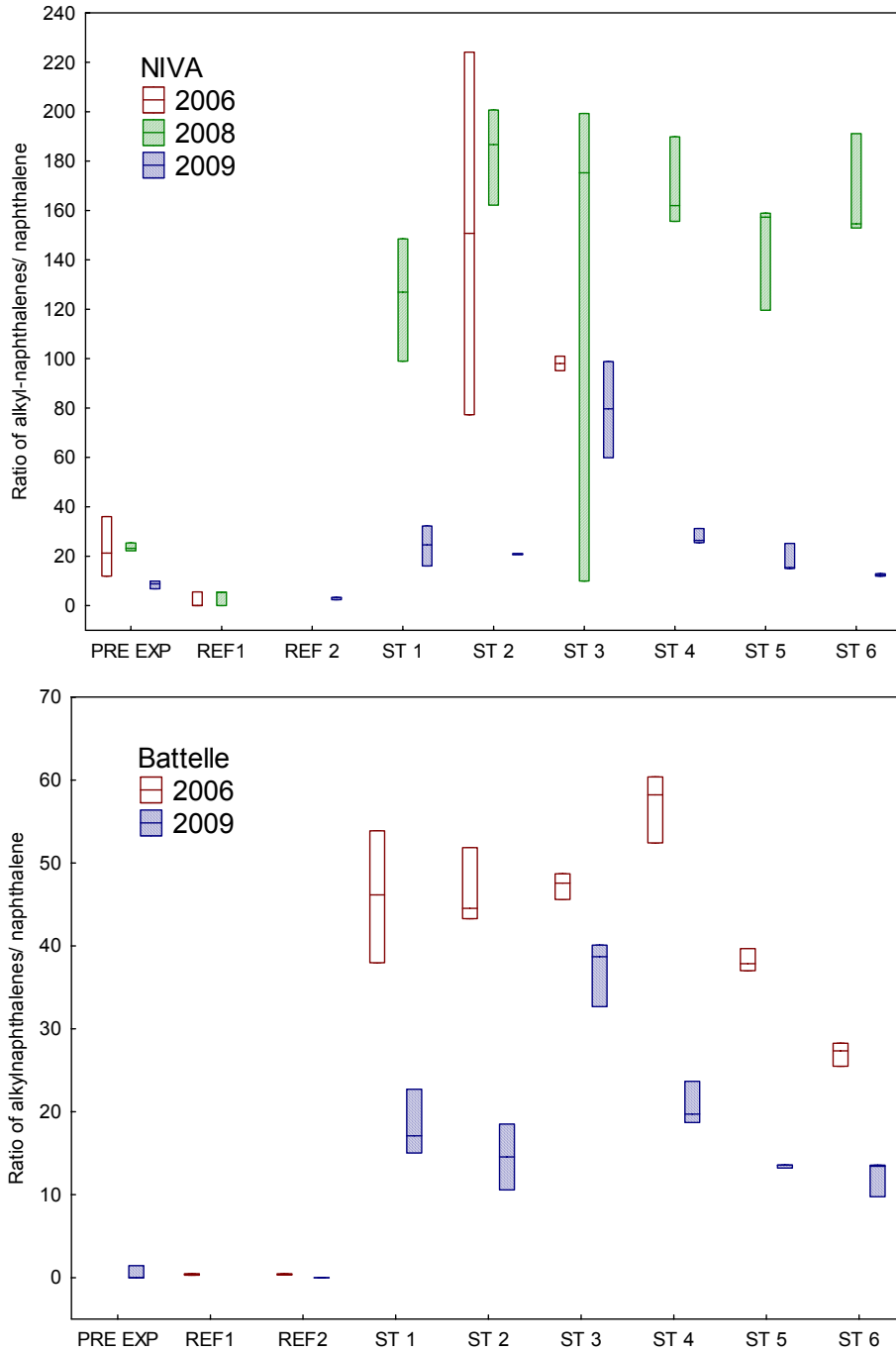
Figure 34. Ratio of alkyl-dibenzothiophenes / dibenzothiophenes. Boxes depict median and min-max (individual observations, since n=3). Analysis carried out by NIVA and Battelle labs (NIVA – including alkylated C1-C3, Battelle - including alkylated C1-C4).
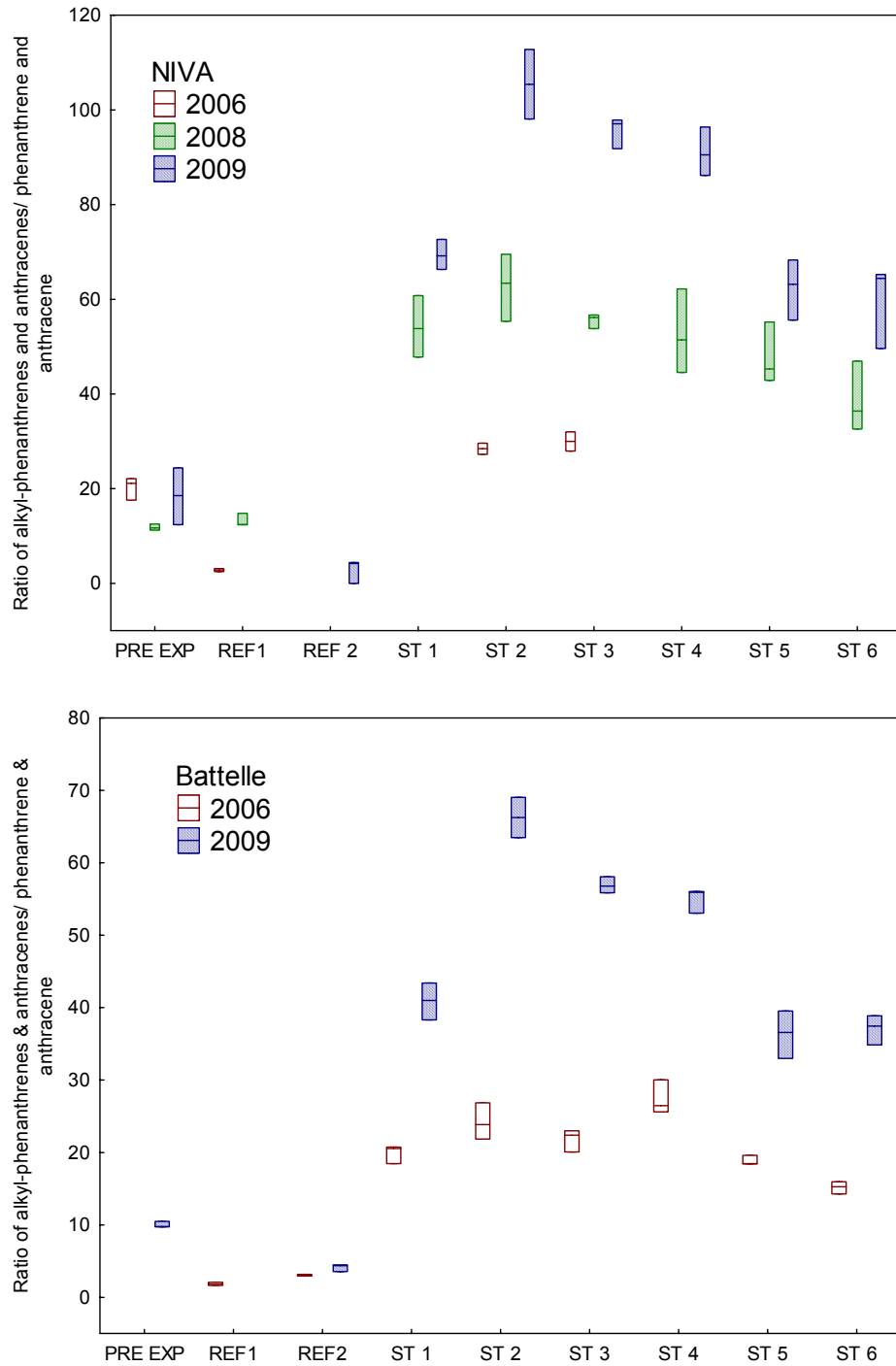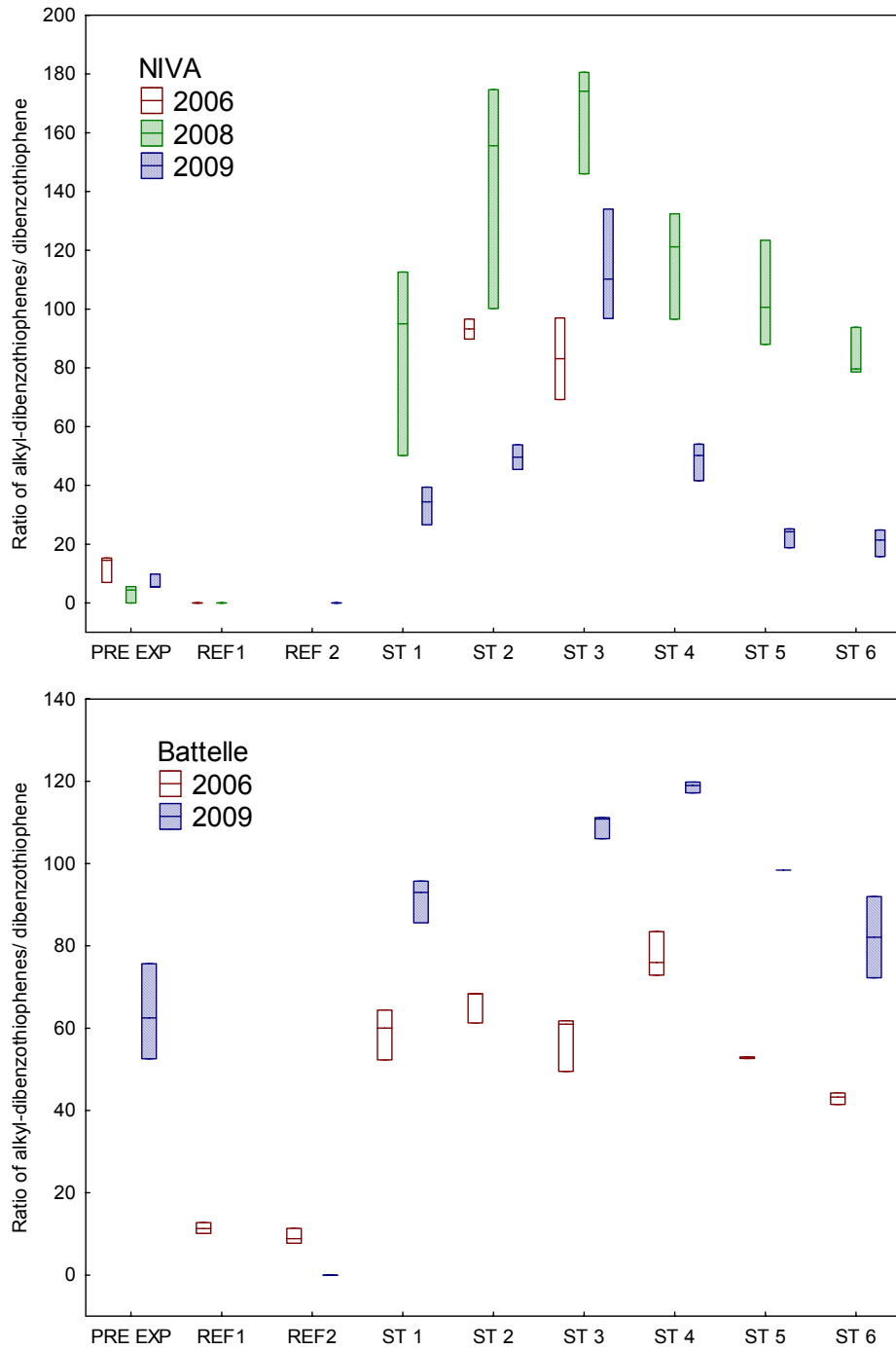
3.6.2 Pyrene hydroxylase activity

For the mussels collected in 2008, significantly higher concentration of pyrene hydroxylase was found at Stations 3, 4 and 5 compared to the REF station. The pyrene hydroxylase activity of the mussels from these exposure stations in 2008, were approximately 2 to 3 fold higher than mussels from the same stations in 2009. Pyrene hydroxylase was not measured in mussels from 2006.

For mussels collected in 2009, pyrene hydroxylase activity at all exposure stations in Ekofisk was not significantly higher than pyrene hydroxylase in mussel from the reference station. All pyrene hydroxylase activity measured at the field stations in 2009 were comparable to the activity levels found in the mussels at the reference station in 2008. The data suggests lower exposure to pyrene compounds in 2009 than that exhibited in 2008.
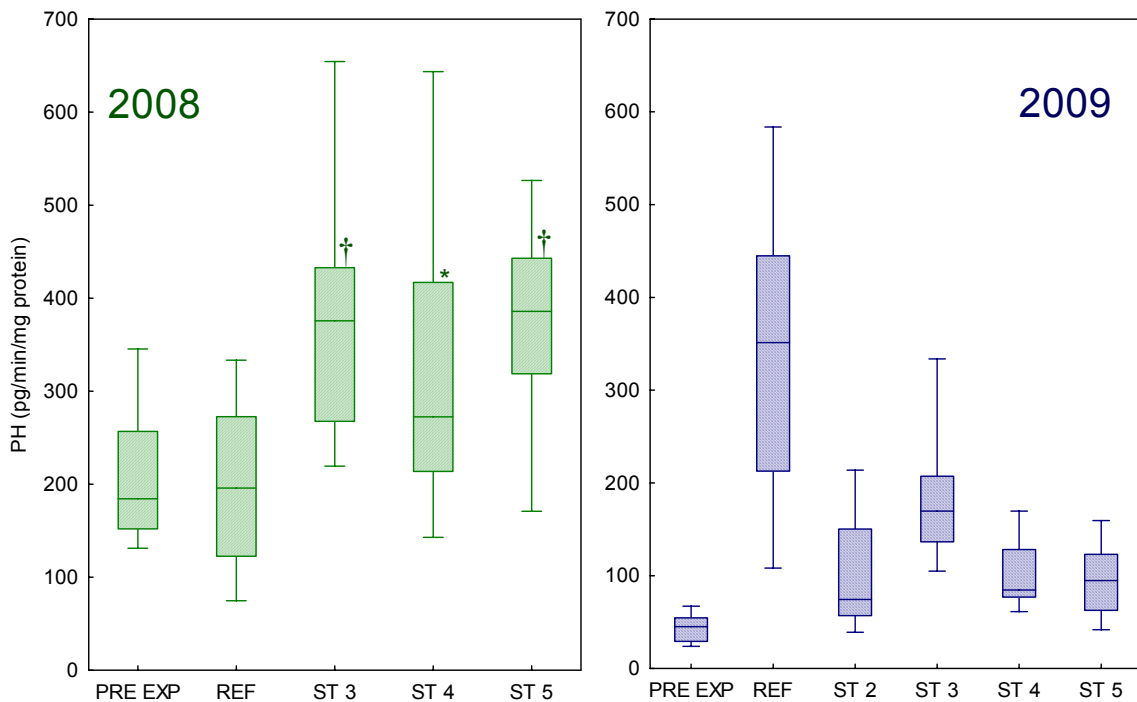
Figure 35. Pyrene hydroxylase activity in mussel digestive gland tissue from the groups indicated. The figure shows median, quartiles and 10/90-percentiles. * significantly different from REF 1, † significantly different from PRE EXP and REF 1.

3.6.3 Lysosomal membrane stability

The observed lysosomal responses in mussel haemocytes from all groups in 2009 were within the normal range of retention times usually observed for blue mussels in unexposed areas. Compared to the results from the 2006 and 2008 monitoring, all Ekofisk stations in 2009 showed lower NRRT levels, indicating a reduced exposure in 2009.
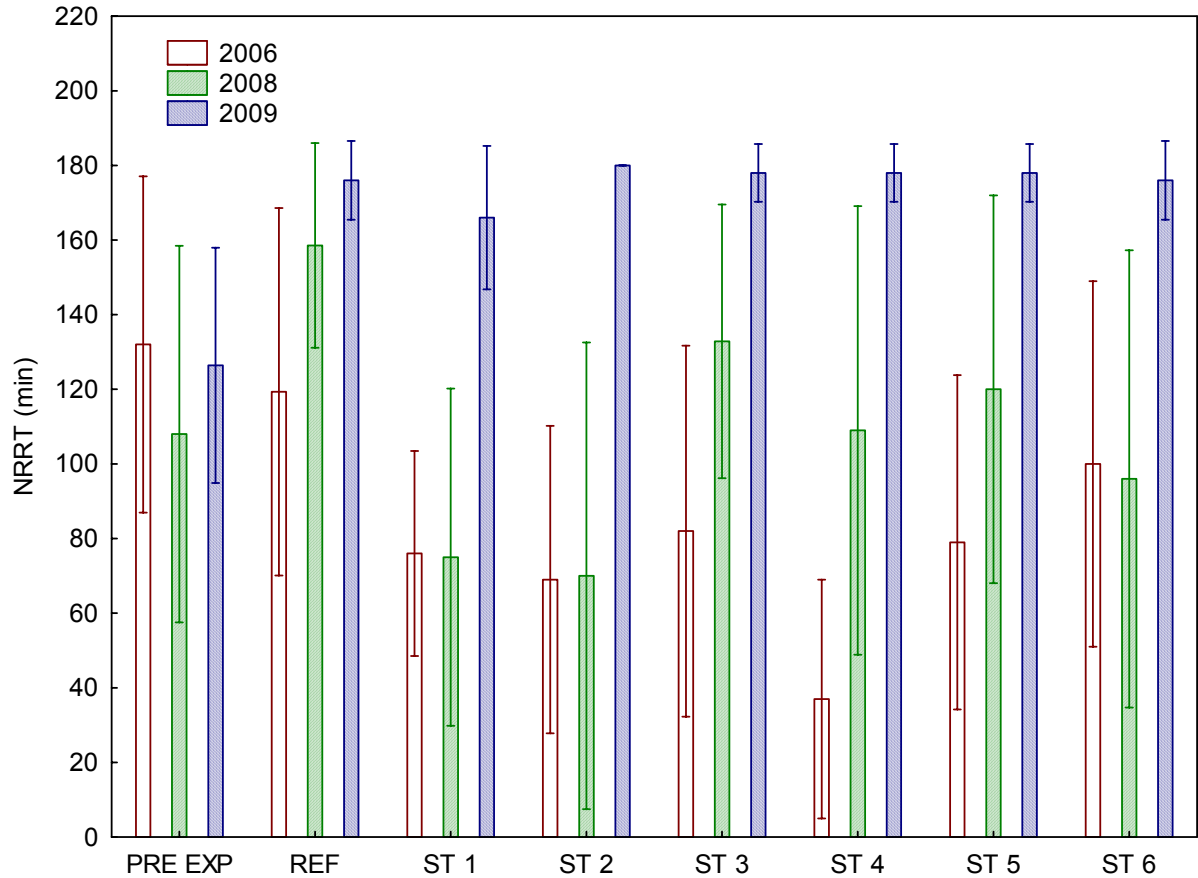
Figure 36. Labilisation period (given as Neutral Red Retention Time (NRRT) defined as the time from the addition of Neutral read to 50% of the cells are dead; min.) of lysosomal membrane in haemolymph cells from mussels from the different experimental groups. The figure shows mean ± standard deviation.

3.6.4 Micronucleus formation

For the mussels exposed in 2009, the frequency of micronuclei varied from 2.78‰ (micronuclei/1000 studied haemocytes) in the mussel reference group to 7.49‰ in mussels caged in station 3. The two stations closest to the PW discharge (ST3 and ST 4) were significantly higher than mussels from the reference station and indicate a clear exposure response. For a more detailed report on the micronuclei data see appendix F.

Compared to previous years, the highest micronuclei frequency at ST3 in 2009 was similar to that found in 2006 at the same station. Unlike many of the other biomarkers analysed, there were no reductions in the frequency of micronuclei at any of the Ekofisk stations compared to levels found in 2008 and 2006.
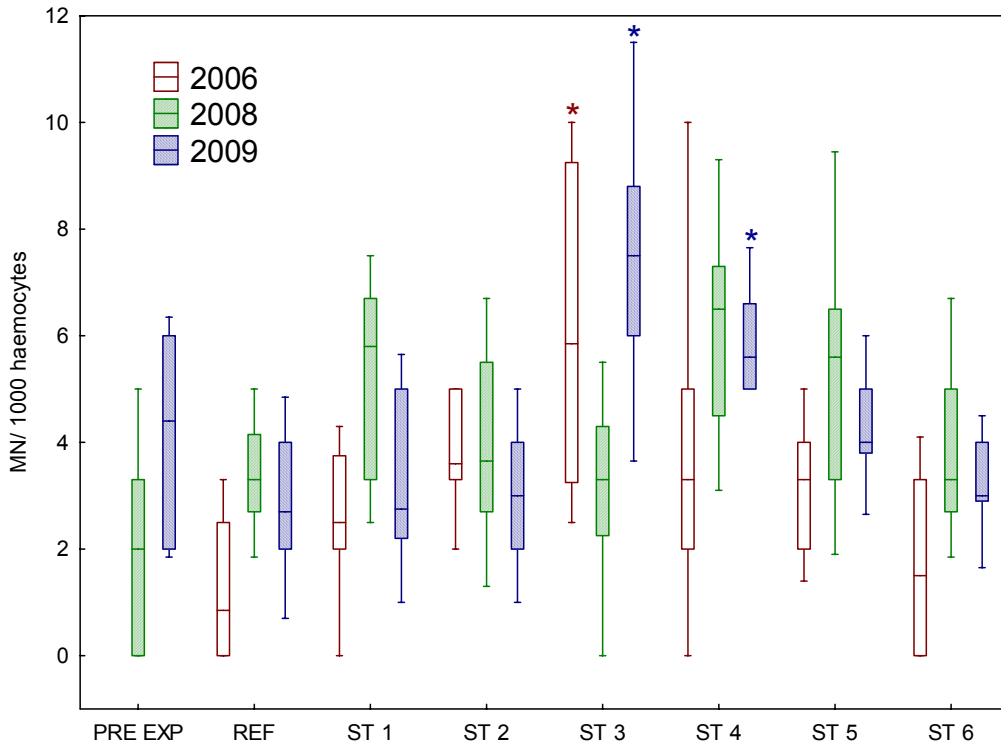
Figure 37. Frequency of micronuclei (micronuclei/1000 haemocytes) in mussels from the groups indicated. The figure shows median, quartiles and 10/90-percentiles.

3.6.5 Histochemistry in mussels

Results from the two mussel histochemical techniques (neutral lipid and lipofuscin) were not suitable for comparison between different sampling years and were therefore presented in different figures.

Neutral lipid accumulation

For Neutral lipid accumulation in 2009, stations 2 and 3 were significantly lower from the reference station. In 2008, the same two stations were significantly different, however the Neutral lipid accumulation were elevated and not lowered as seen in 2009.
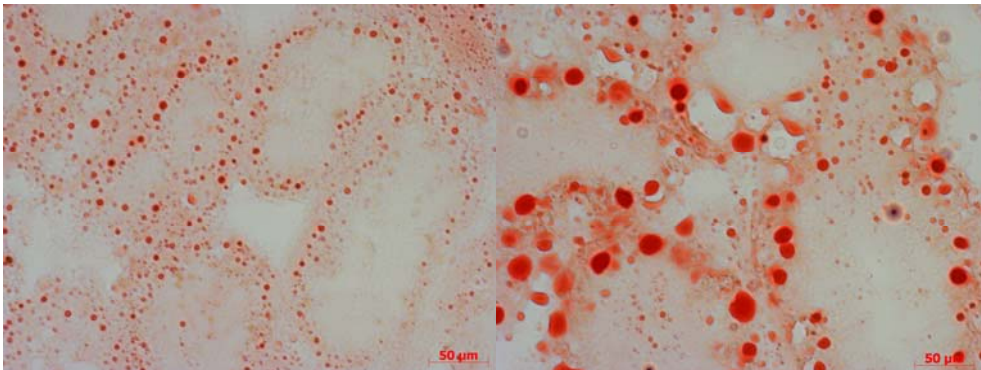
Figure 38. Histological sections (400 X magnification) showing neutral lipid accumulation (arrows) in mussel digestive gland, example of typical (left) and increased (right) accumulation.
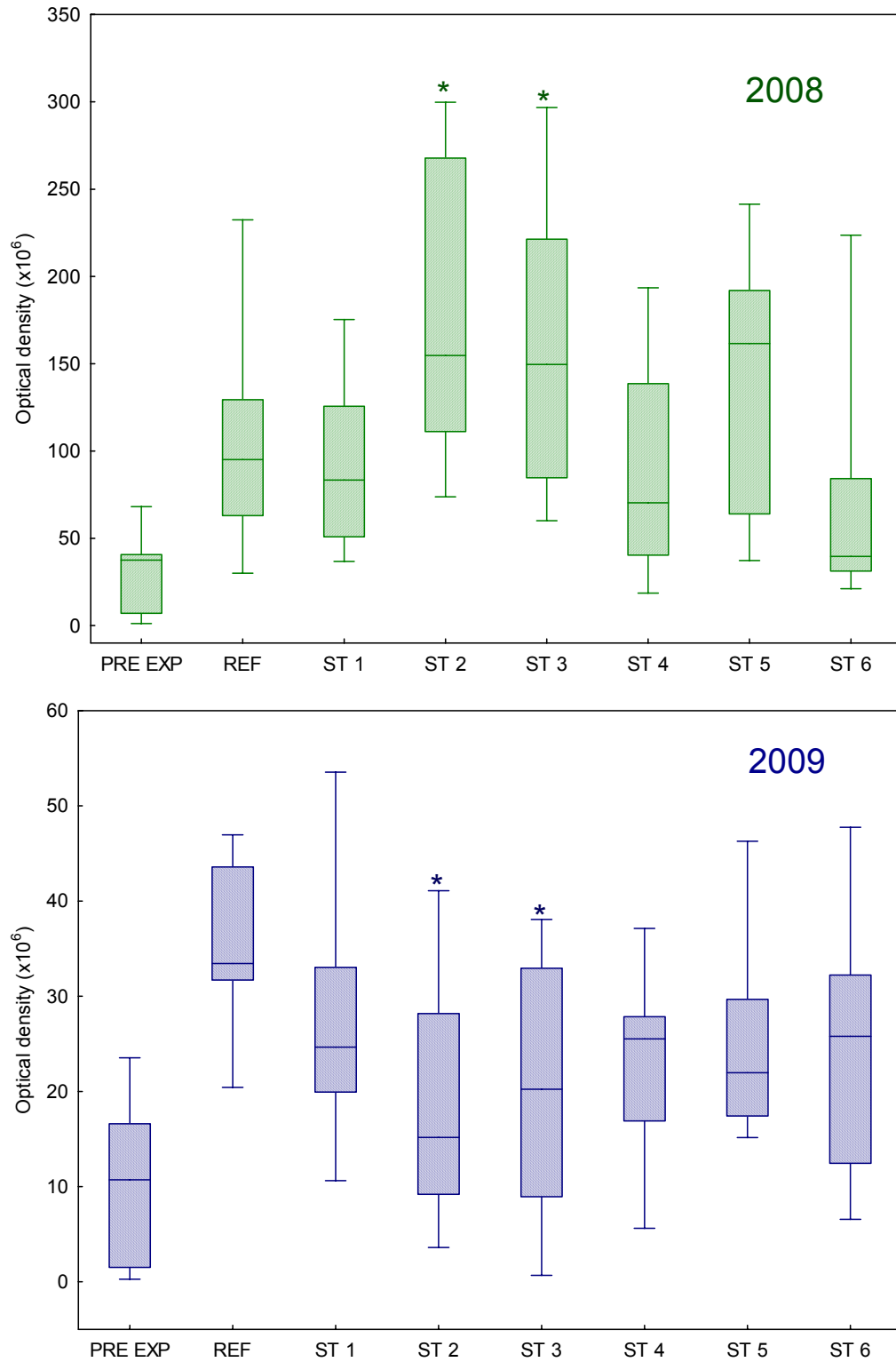
Figure 39. Neutral lipid accumulation given as optical density in mussels from the groups indicated. The figure shows median, quartiles and 10/90-percentiles. * significantly different from the reference station (ANOVA, Tukey p<0.05).

Lipofuscin lysosomal accumulation

Lipofuscin accumulation is the result of peroxidation of autophagocytosed proteins associated with protein aggregates and oxidatively damaged organelles. Lipofuscin accumulation in lysosomes of exposed mussels did not show any significantly differences from the reference.

In the 2008 monitoring survey, significantly different levels were observed at station 2 and 3 indicating a reduction in stress since the last investigation took place.
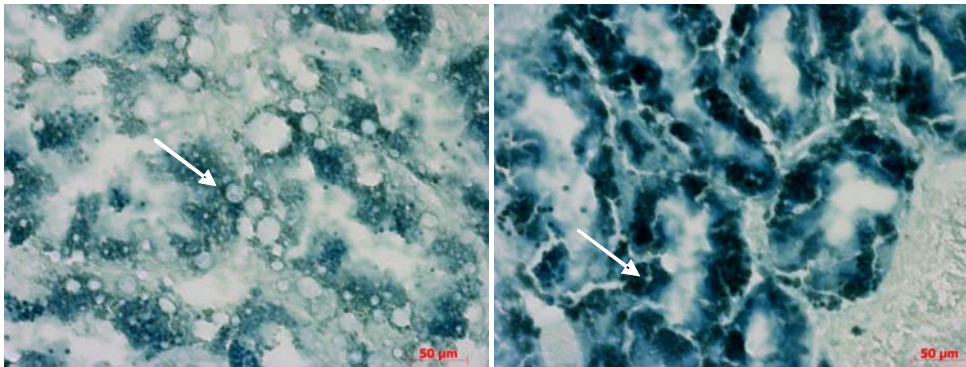
Figure 40. Histological cryostat sections (400 X magnification) showing accumulation of lipofuscin in mussel digestive glands, example of typical (left) and increased accumulation (right). Arrows highlight the presence of autophagy vacuoles.
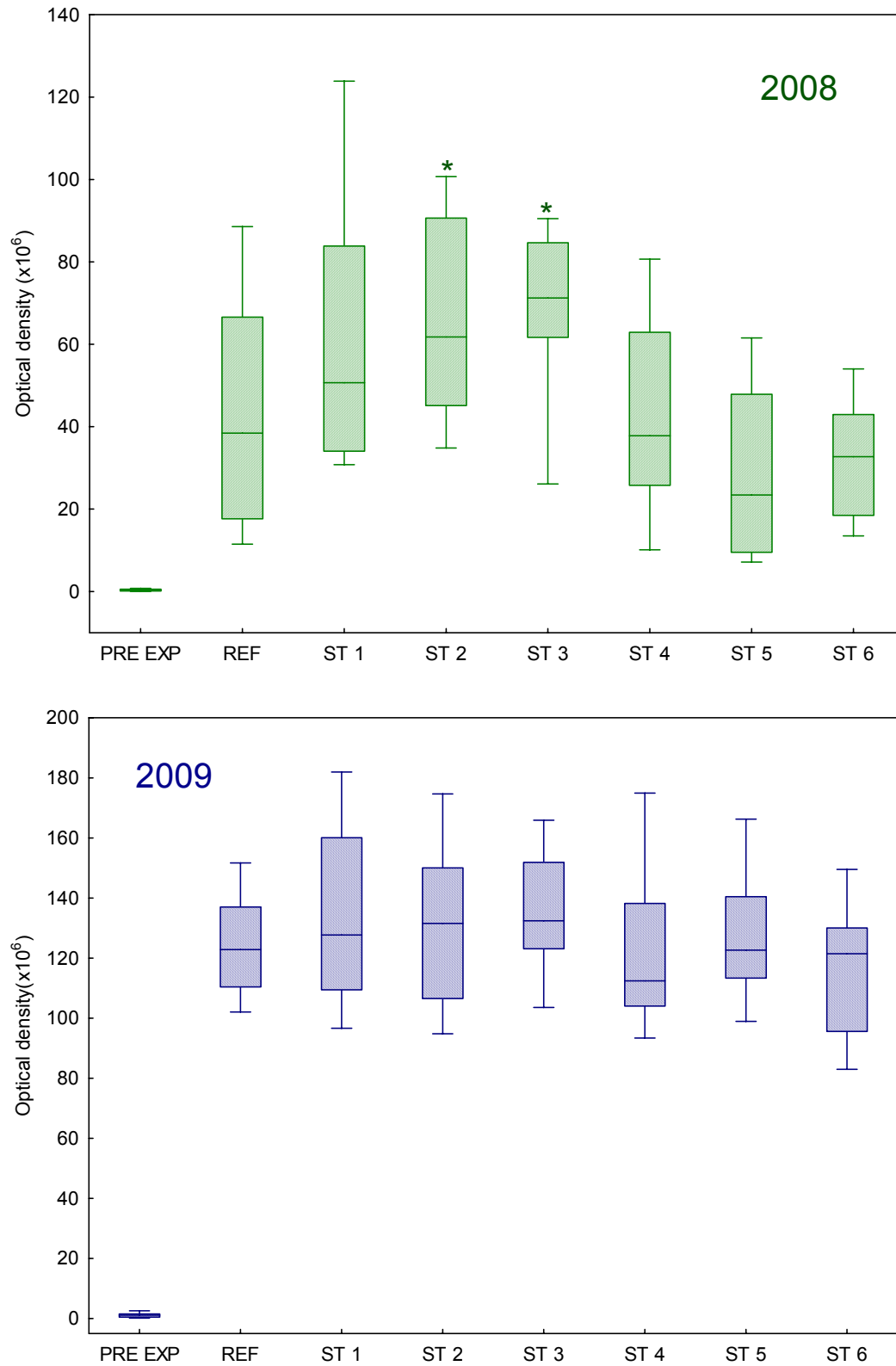
Figure 41. Lipofuscin accumulation in mussel lysosomes given as optical density from the groups indicated. The figure shows median, quartiles and 10/90-percentiles. * significantly different from the reference station (ANOVA, Tukey p<0.05).

4 Discussion

A thermocline was evident at 20 m with the maximum difference in temperature measured at the final CTD deployment. The thermocline would have been expected to strengthen as the temperatures increase with increased sun intensity during the summer months. Such a strong thermocline may have implications with respect to transport of the PW away from the platform. The PW will be prevented from mixing with the colder deeper waters and remain in the top 20 m of the water column. Similar thermoclines were reported in the same area in 2008 and to a lesser extent in 2006, suggesting that it is a regular occurrence in the spring and summer months.

The dominant tidal driven currents within the area would indicate that no strong dispersal mechanisms were taking place that could potentially remove the discharge chemicals. Instead the caged animals were likely to be exposed and re-exposed to the PW as the tide oscillates.

Mussels and fish were caged at a depth of approximately 10-16 m. At this depth the animals have been exposed to water temperatures of approximately 6.3^o C at the start of the deployment rising to around 7.5^{o}C on week 5 (6.5^{o}C to 9°C in 2008). The output of PW appeared to have no effect on the salinity of the seawater under the platform, with a very stable 35 to 35.5 ‰ irrespective of depth or date.

A significantly higher mean condition index in the pre exposure cod, with respect to the cage exposed cod was found that would suggest low food availability within the cages. Blue lights were used in order to entice potential food items into the cages for the fish to eat. Although this may have worked to a certain extent to keep the fish alive during the 6 week deployment with very low mortalities, deterioration in health had occurred. The stress of being within a cage may also have contributed to lower condition indices within caged fish. This was also supported by the significantly lower liver somatic index found in the caged fish compared to the pre-exposure group. Similar observations were reported in the WCM surveys taken place at Ekofisk in 2006 and 2008.

The impact of this on the biomarkers tested was not entirely clear. However, the fish in the reference station also exhibited the same levels of food deprivation as that seen in the exposure stations (ST3 and ST4). Therefore, differences in biomarker response between these stations could be attributed to PW exposure rather than differences related to food availability.

4.1 Tissue levels of PAHs in caged mussels

The results from the 2009 WCM survey show that caged organisms from all locations in the proximity to the Ekofisk PW discharge have been exposed to low levels of PW components. Mussels were exposed to relatively higher concentrations with proximity to the PW discharge as indicated by PAH body burden data for the different stations. However, all mussel body burden data in 2009 was markedly lower than that recorded in previous 2008 and 2006 Ekofisk surveys. This would suggest that the mussels were exposed to lower concentrations of PAHs in the surrounding water column.

For comparison the highest PAH16 values for the WCM 2009 (~ 5 µg/kg wet wt. ST3) were lower than values recorded in mussels over a similar exposure duration in the vicinity of Troll platform (23 µg/kg wet weight, Utvik et al., 1999) and in mussels 500 m from the Statfjord B platform in 2001 (Hylland et al., 2008).

The PAH body burden data from mussels caged at Ekofisk in all monitoring years were in the lower end of the range of the concentrations found in mussels from coastal waters of the Nordic countries, where background concentrations have been found to range from 10 to 111 µg/kg wet weight (Granby & Spliid, 1995). This suggests that the current exposure to PAH compounds from Ekofisk PW represents a limited environmental risk.

4.2 Effect responses in caged mussels

Both the bioaccumulation data and the biological effects measurements have indicated that the Ekofisk caged mussels were exposed to PAH compounds but at lower concentrations than that found in 2008 and 2006. Of the mussel biomarkers measured, micronuclei formation was the only parameter that showed significant effects with highest frequencies of micronuclei found at the closest stations to the PW discharge.

A suggested assessment criteria for micronuclei formation, based on the current literature and results of investigations of environmental genotoxicity in the North and Baltic seas, has been proposed (Baršienė, 2009: Appendix F).

Micronuclei formation ‰(Micronuclei/1000 cells)	Assessment
Up to 2 ‰	background levels
2.1 to 4.0 ‰	minor environmental impact
4.1 and 6.0 ‰	poor environmental conditions,
> 6.0 ‰	bad environmental conditions

Based on this assessment criteria, mussels collected from stations 4 and 5 were considered as 'poor environmental condition' whilst those from Station 3 were identified as 'bad environmental condition'. Whether this is reflective of the environmental situation overall is unclear, particularly since many of the other biomarkers measured had no significant effects with exposure to the PW. However, this could indicate both the sensitivity of the micronuclei technique and exposure to low molecular weight PAHs that may not have been totally removed from the PW by the treatment process, since micronuclei formation (genotoxicity) can occur following exposure to light PAHs.

Pyrene hydroxylase activity, which provides an indication of exposure to pyrene and pyrene like compounds, was low in 2009 at all exposure stations, and showed no significant difference to the reference station. In 2008, pyrene hydroxylase was elevated at the Ekofisk stations compared to the reference station. The pyrene hydroxylase activity in all 2009 stations was similar to the reference values of 2008 and suggests a reduction in exposure.

Slightly elevated levels of pyrene hydroxylase were found in mussels collected from station 3 in 2009, which corresponded to the mussels with the highest PAH body burden. However, the high pyrene hydroxylase activity at the reference station prevents any firm conclusions from being made. For comparison, mussel collected from Aker Brygge, Oslo fjord had pyrene hydroxylase activity levels of 400 pg/ mg protein/ min (Grung, unpublished NIVA data), which represents approximately twice the level as found at Ekofisk in 2009.

The observed lysosomal responses in mussel haemocytes from the pre exposure sampling and the reference station were within the normal range of retention times usually observed for

mussels in unexposed areas. In addition, based on the health assessment criteria from OSPAR, (2007) all neutral red retention times of mussels caged within Ekofisk were indicative of a 'healthy' population, with no significant effects following exposure to the PW discharge. Based on the lysosomal membrane stability measurement, mussel health was markedly improved in 2009 compared to that found in 2008 and 2006.

4.3 PAH- and AP metabolites in cod bile

Low but quantifiable concentrations of pyrene metabolites in the bile of pre exposure fish indicate that the cod had been exposed to low levels of PAHs prior to deployment. Pyrene contamination measured as OH-pyrene in bile of fish from coastal waters has previously been recorded but the observed levels were not believed to significantly affect the quality of the material for the purpose of monitoring.

For most PAH and two of the AP metabolite compounds measured, both stations at Ekofisk were significantly different from the reference station. This confirms uptake and bio-transformation of PAHs and APs typical for PW exposure in fish from the two stations close to the discharge. However the levels observed are low. AP metabolite levels in fish bile was at least an order of magnitude lower than the levels needed to cause xenoestrogenic effects (measured as increased VTG levels) in Ekofisk PW exposed fish in the laboratory (Sundt et al., 2008b). This was also confirmed by the lack of VTG response in the Ekofisk exposed cod as discussed below.

4.4 Exposure and effect responses in caged cod

The results for PAH-metabolites in the bile of cod suggest that exposure levels have been evident but low, at least during the last week prior to sampling (due to continuous removal by excretion). For cod caged at station 3, the exposure was sufficient to induce elevated amounts of hepatic cytochrome P450 (CYP-1A) enzymes in the fish. Several studies have indicated that P450 induction may be the first step in a series of toxic symptoms, such as immunosuppression, vitamin and hormonal imbalance, and reproductive failure (Reviewed by Safe, 1994).

There was no increase in plasma VTG concentrations in males at any stations. The same was found for ZRP. Arukwe et al., (1997) have shown that ZRP proteins are sensitive markers for low dosages of xenoestrogens. Results from both the present and the 2008 and 2006 investigations, supports previous conclusions about the levels needed to cause estrogenic effects in cod (Sundt et al., 2008b).

The levels of DNA adducts found were in overall low, with no significant differences between the groups. The same was found in 2008 and 2006. A suggest assessment criteria of 2 times the reference value was not exceeded by the exposure groups. This indicates that fish were exposed to genotoxic pollutants beyond their short term DNA repair capacity.

For comparison, DNA adduct levels in cod from the Barents Sea have been found to be 0.75 ± 0.58 ($\pm$ SD) nmol add/mol normal nucleotides (Aas et al., 2003). This is comparable to the levels of DNA adducts found at Ekofisk over the three survey years.

4.5 Produced water discharge Ekofisk – 2006, 2008 and 2009

Since 2006, the discharged volume of produced water from Ekofisk has increased by 14% in 2008 and by 32% of the 2006 level in 2009 (Figure 42). For all three years there have been two PW discharge points, external platforms 2/4J and 2/4M (see Figure 43). However, in 2006 approximately 90% of all discharge was from the 2/4J platform. In 2008 and 2009, the first half of the exposure period was almost equal proportions in discharge volume between the 2/4J and 2/4M platforms, although in the last half of the cage exposures there was almost no discharge from the 2/4M.
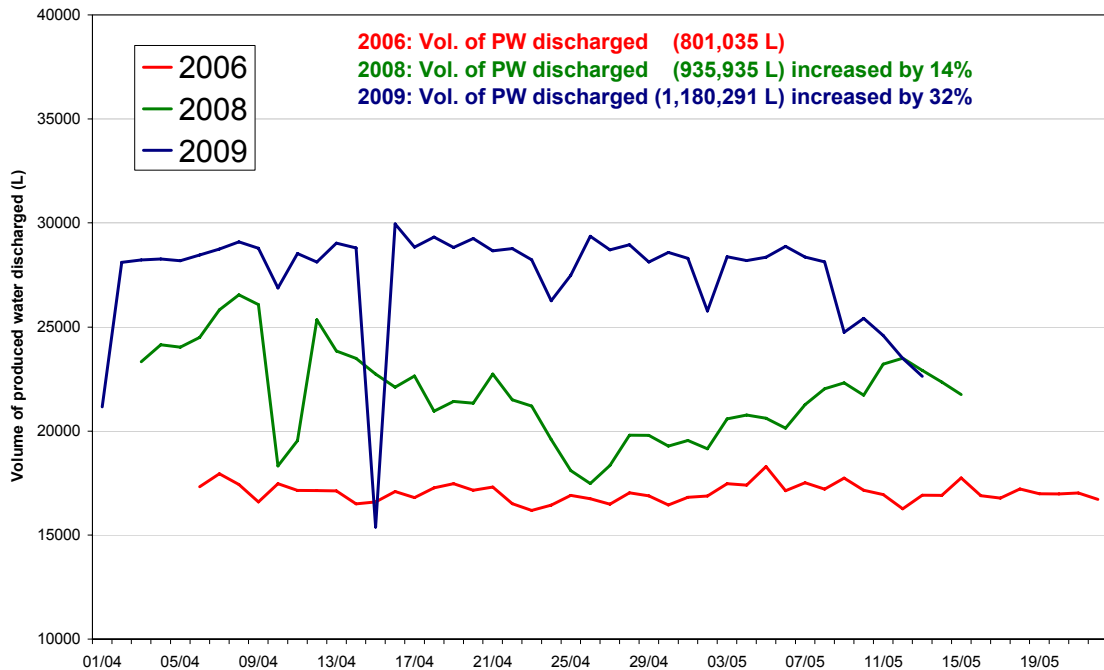

Figure 42. Volume of produced water discharged during the 6 week cage exposure. Data provided by ConocoPhillips. (values in litres).

Since the 2006 WCM survey, new treatment technology (C-Tour) was implemented (Voldum et al., 2008). However, during the 2008 WCM survey some operational problems occurred, which are reflected by the peaks in the oil in water (OiW) values that occurred on certain days (Figure 44). Despite these peaks, the average percentage of oil in the PW for the period of cage exposure was reduced by approximately 30% of that recorded in 2006. During the 2009 WCM survey the performance of the C-tour was reported to have been stabile during the cage exposure. In this case, the concentration of oil in the PW during the caged exposure was found to reduce by 58% of the concentration reported in 2006.

Despite the increase in the volume of PW discharge from the platform in 2008 and 2009 compared to 2006, the total discharge of oil was found to reduce by 19% and 38% in 2008 and 2009 respectively (Figure 45). This was due to the reduction in the mean concentration of oil in water recorded in 2008 and particularly 2009 as a result of the improvement in removing the oil from the PW. The reduction in the total discharge of oil due to improved treatment systems are likely to have environmental benefits, some of which may have been demonstrated in this project with lower bioaccumulation concentrations of PAH compounds and the reduction in biological effect coinciding with the reduced oil discharge.
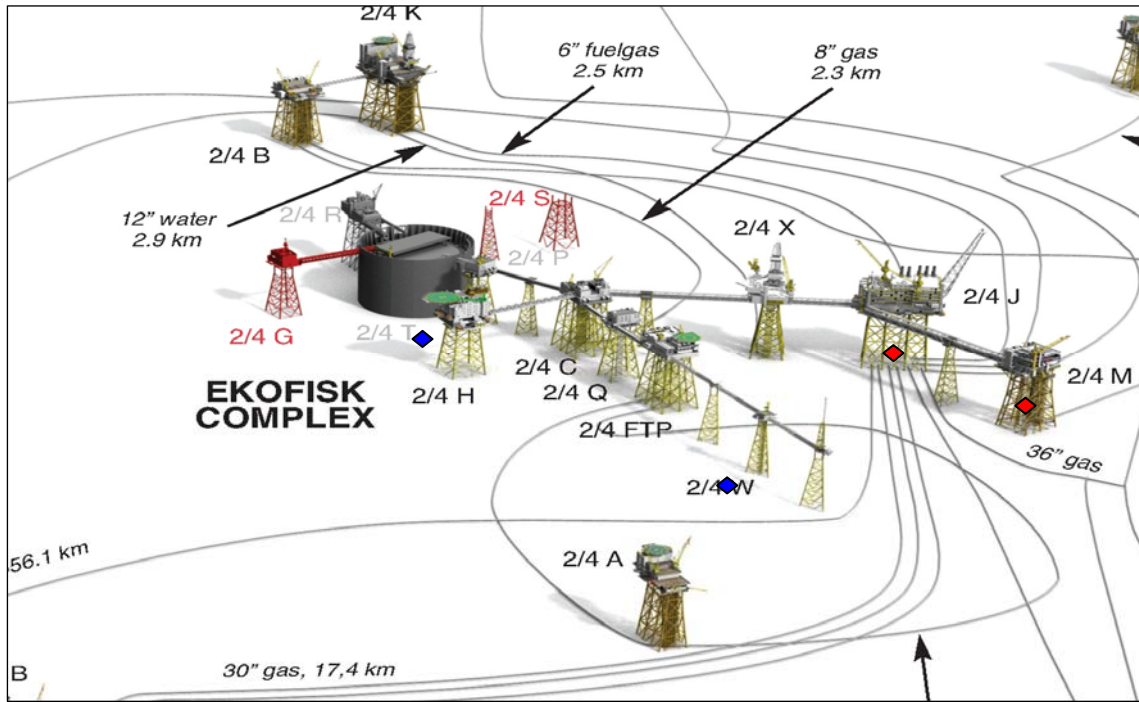
Figure 43. Map of the Ekofisk complex with the discharge points (red diamonds at 2/4J and 2/4M) and approximate positions of the two closest stations containing fish and mussels (blue diamonds). (Source: ConocoPhillips).
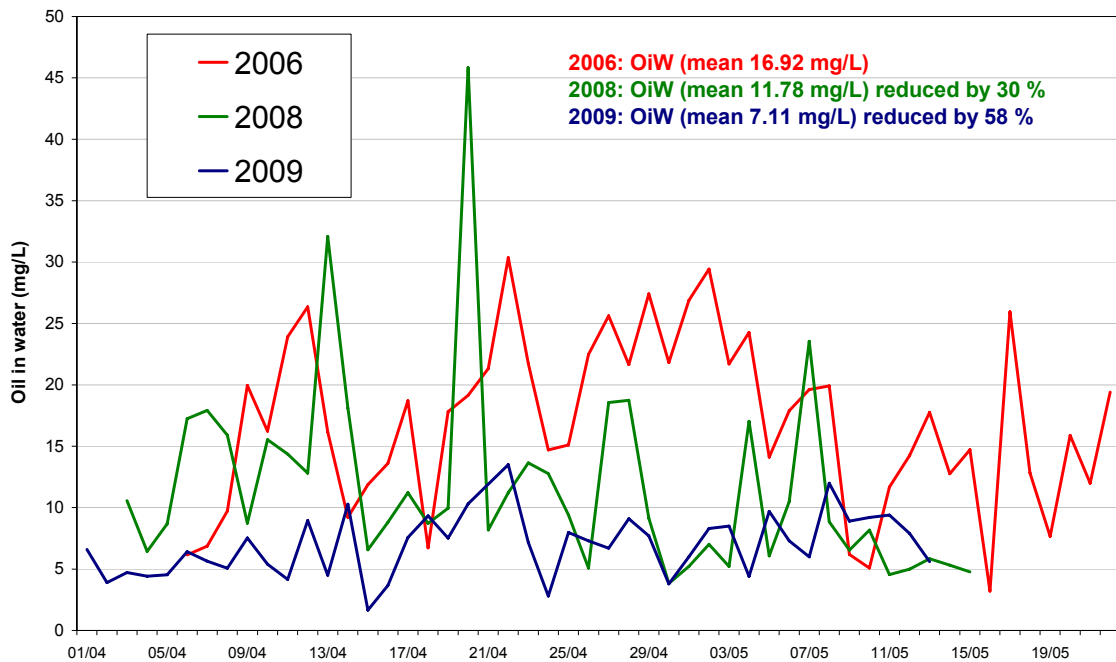

Figure 44. Oil in water measurements at the Ekofisk field for the duration of the cage exposures. Data provided by ConocoPhillips. (Daily average values in mg/L).
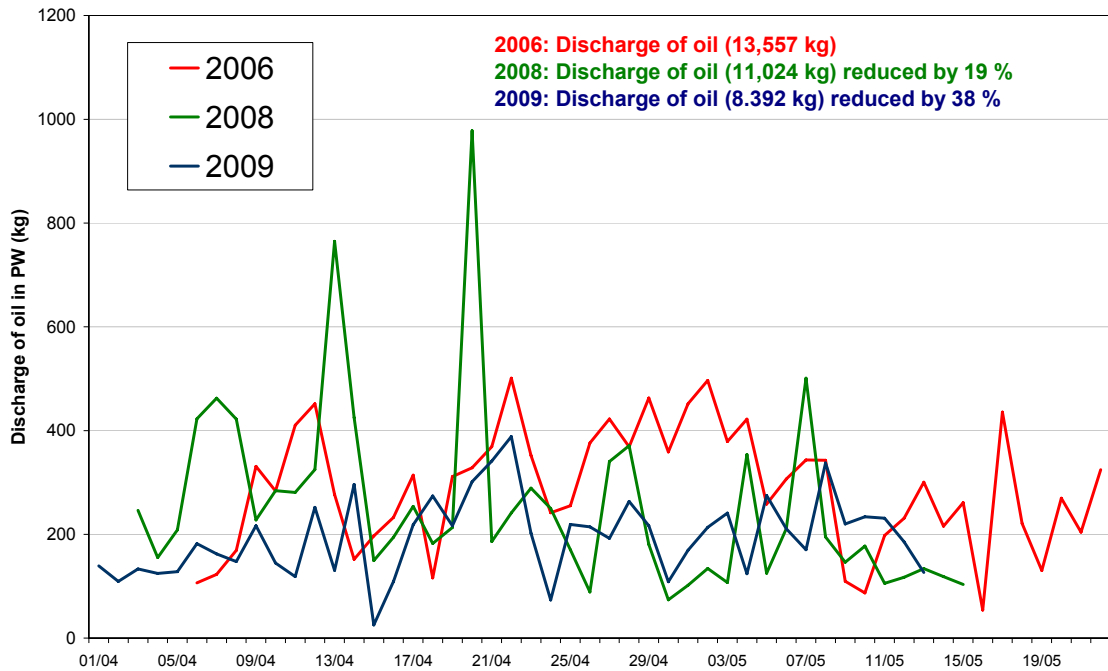
Figure 45. Discharge of oil in produced water at the Ekofisk field for the duration of the cage exposures. Data provided by ConocoPhillips. (values in kg).

4.6 Confounding factors - biomarkers

From previous WCM surveys, certain biomarkers have sometimes failed to show a clear distance-response relationship from the PW discharge. For example, VTG and GST markers have been found to be equal or lower in caged fish closest to the discharge source than farther away. The lack of biological effect may simply be due to the exposure concentration of the compounds being below the threshold level for an effect to occur. However, an important factor to consider is that PW components such as PAHs, alkylphenols, and organic acids have been found to have multiple mechanisms of effect, which can lead to induction and inhibition of an enzyme system simultaneously (Tollefsen et al., 2008). For example, certain alkyl phenol isomers at sub-lethal concentrations have been found to have estrogenic and anti-estrogenic properties (Meier et al., 2007). Therefore, exposure of fish to mixtures of alkylphenols as typically found in PW discharge may interfere with oestrogen exposure biomarkers such as VTG and ZRP. This was thought to be one possible reason for the reduction in female VTG at the closest cage to the Statfjord oil platform during the WCM 2004 survey.

Furthermore, chemicals can induce effects in one biological endpoint and inhibit other endpoints that are normally affected by totally different compounds. For example, weakly estrogenic alkylphenols and low molecular PAHs have been proposed to inhibit the CYP1A-mediated induction of EROD activity (Navas and Segner, 2000; Hasselberg et al., 2005), whereas EROD inducers such as high molecular weight PAHs are able to inhibit the estrogenic activity of endogenous estrogens (Navas and Segner, 2000). Such interactions can have profound effects in terms of monitoring, with the lack of biological response potentially

underestimating the environmental risk. However, it is thought that such interactions are only likely to take place at relatively high chemical concentrations. Since most of the PW chemicals have low persistence and are rapidly diluted, the inhibition of biological effect should only occur, if at all, in fish closest to the PW discharge.

A recent report attempted to answer the question as to whether compounds common to PW can mask the biological effects of other compounds and lead to the absence of the effect in biomarker response (Tollefsen et al., 2008). This study confirmed several interactions: 1) that EROD and CYP1A induction was due to 4 and 5+ ring PAHs and vitellogenin induction by alkylphenols; 2) inhibition of EROD and CYP1A was predominantly due to 3-, 4- and 5-ring PAHs as well as alkylphenols such as 4-t-butylphenol and; 3) inhibition of VTG production was associated with the presence of 4 and 5+ ring PAHs. However, due to limited data, it was inconclusive as to whether these effects are caused at environmentally realistic concentrations. Nonetheless, it is important to consider such interactions when investigating the biological effects of mixture toxicity.

4.7 Preliminary ecological risk assessment

For the WCM 1999 an evaluation of environmental risk was carried out based on PAH body burden in mussels (Neff, 2000). The evaluation performed by Battelle concluded that the discharge of PW at Ekofisk did not represent any environmental risk for marine life 0.5 km or further away from the discharge point. Uptake of organic compounds in mussels was compared with body burden threshold levels for acute lethality and chronic effects by dividing the threshold level for acute lethality with an application factor of 100.

The WCM 2006 provided a better evaluation of environmental risk since the biomarkers gave a more direct measure of the organism's health status. These biomarkers cover more types of chemical stress than critical levels based on chemical load are capable of. Acute lethality/ chronic effects are related to a stress caused by a particular type(s) of chemical compound(s). For example, in cases where the stress on the organism is caused by compounds other than those measured PAHs, this situation would be intercepted with the biomarker approach but not with the chemical based approach.

Biomarker based risk assessment is limited by the lack of connections between biomarker level and effect data, information that will be provided in the future. However, for lysosomal stability by NRRT (OSPAR, 2007), DNA adduct (NMMP, 2001) and micronuclei formation (Baršienė, 2009: Appendix F) assessment levels of effect have been established.

Based on NRRT data from WCM 2006 we concluded the same as in Battelle's report (Neff 2000) and obtained a more balanced evaluation due to more reliable data (Figure 46). In 2006, stress was indicated in mussels from all stations situated in the proximity of Ekofisk, however only Station 4 (situated close to the discharge Figure 1) showed NRRT levels indicating severe stress. The 2008 survey showed that the effect situation had improved and none of the groups could be defined as severely stressed. In the 2009 survey NRRT data indicated healthy mussels at all caging stations, reflecting the improved environmental health status of these mussels compared to previous years. This corresponds with the lower PAH bioaccumulation data found in mussels at Ekofisk in 2009.

For the DNA adduct data all groups of exposed fish from the monitoring in 2006, 2008 and 2009 had DNA adduct levels below the threshold level (Figure 47).
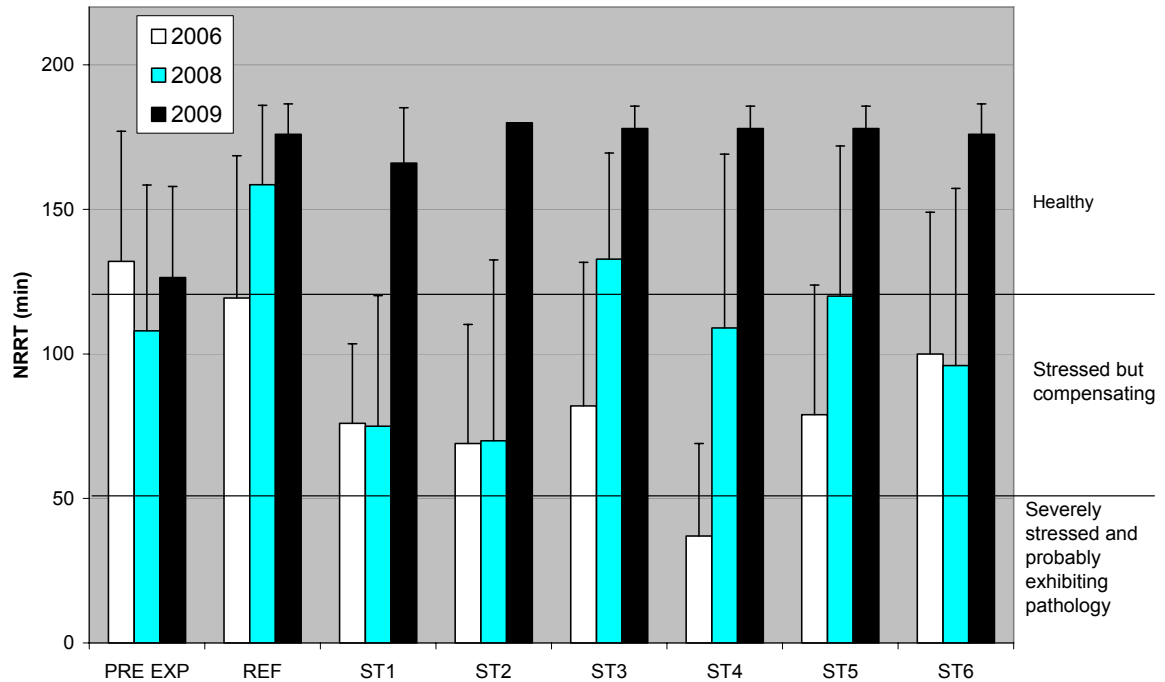
Figure 46. Lysosomal membrane stability in mussels from WCM 2006, 2008 and 2009 (given as average Neutral Red Retention Time, NRRT) with the stress definitions (assessment criteria) defined in OSPAR (2007) (mean ± SD).

In contrast to DNA adducts in cod liver and LMS in mussels, the frequency of micronuclei formation was significantly increased at the two closest stations (ST3 and ST4) to the PW discharge in 2009 compared to the reference station. From an assessment criteria based on over 10 years of data collection in the North Sea, ST3 and ST4 were considered to reflect 'bad' environmental condition. Most of the outer stations in 2009 were considered as 'minor impact' including the reference station. This was different to that found in 2008 when only ST4 was considered as 'bad' environmental condition, with 'poor' environmental condition observed in two outer stations (ST1 and ST5). The profile in 2006 was similar to that described for 2009, but with slightly lower micronuclei formation in almost all stations. Therefore, micronuclei formation did not show any improvement in environmental condition with time.
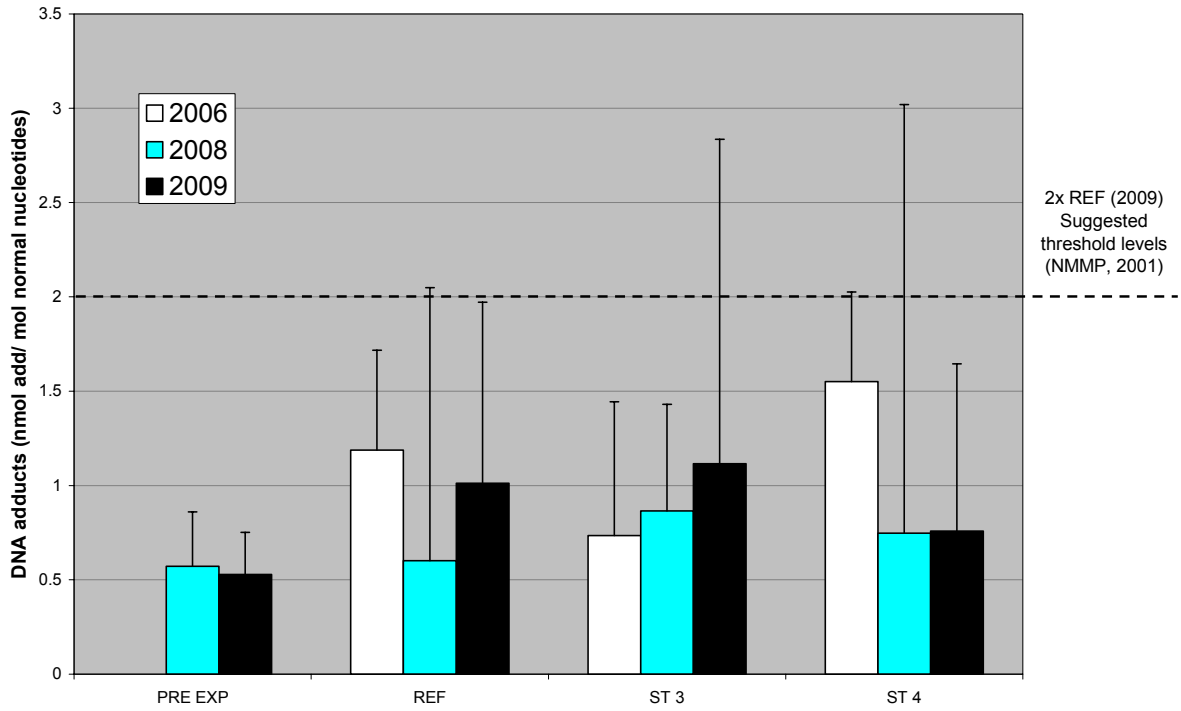
Figure 47. DNA adduct levels in cod liver (nmol add/mol normal nucleotides) in caged cod from the groups indicated from WCM 2006, 2008 and 2009. Suggested threshold value, 2x appropriate reference value (NMMP, 2001) (mean ± SD).

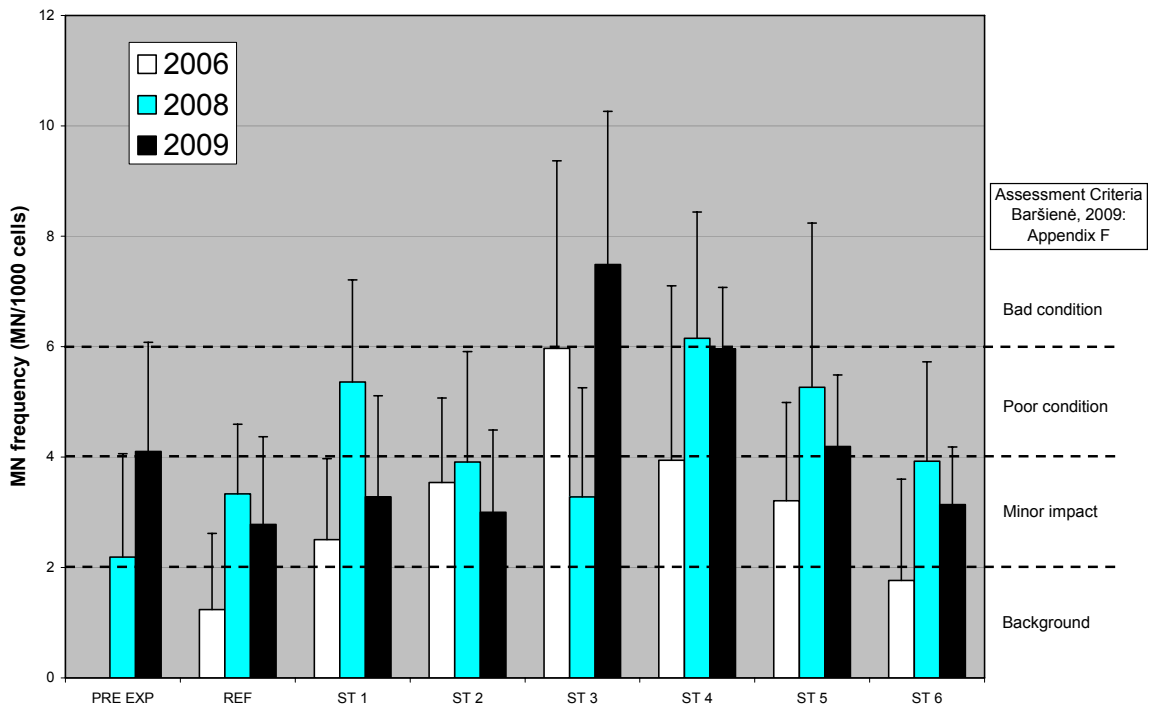Figure 48. Micronuclei formation in haemocytes (micronuclei/ 1000 cells) of caged mussels from the groups indicated from WCM 2006, 2008 and 2009. Suggested assessment criteria (Baršienė, 2009: Appendix F) (mean ± SD).

5 Conclusions and recommendations for future WCM

Results show that deployed organisms from the whole investigated area contained low levels of hydrocarbons expected to originate from the PW discharge. Based on the mussel bioaccumulation data and the overall biomarker responses it appears that the concentrations and effects in 2009 were lower than in 2008 and 2006. The profile for the bioaccumulation data, with highest concentrations of PAHs in mussels located at the closest station to the discharge was in agreement to the biomarkers that showed significant responses i.e. micronuclei in mussels, CYP1A in cod.

The mussel bioaccumulation data clearly showed a lower bioaccumulation of PAHs including NPDs and decalins than previously found in 2006 and 2008. The bioaccumulation data in mussels from all stations in 2009 was in good agreement between the two separate analysing laboratories (NIVA and Battelle). Based on the ratio of alkylated PAH to parent compound, it was demonstrated that the PAHs that were bioaccumulated in mussels originated from a petroleum source and were very likely to be from the PW discharge.

Only two of ten biomarkers measured showed a significant response at the exposure stations compared to the reference stations. These significant responses were found at the Ekofisk station (station 3) closest to the discharge and corresponded to the PAH bioaccumulation data in mussels and PAH metabolite data in cod. However, it was unlikely that only the PAHs measured were responsible for the biological effects, but many different compounds known to have toxicity effects, are also present in PW (e.g. organic acids), which may have contributed. For example, the micronuclei formation in 2009 was significantly higher than in previous years despite the reduction in bioaccumulated PAHs. This increase in micronuclei could therefore be attributed to other compounds within the PW that were not measured.

Histological alterations including neutral lipid and lipofuscin accumulation in 2009 were not found to be related to the distance from the PW discharge and/or the bioaccumulation of PAHs. This was in contrast to the 2008 data were a relationship between the two histological endpoints and PAH bioaccumulation were recorded.

For most PAH and two AP metabolite compounds scored in cod bile, both stations at Ekofisk were significantly different from the reference station. This confirms uptake and bio-transformation of PAHs and APs typical for PW to the fish from the two stations close to the discharge (ST 3 and 4, 100 - 200 m from PW discharge). The fish metabolite data was comparable to the concentrations measured in the bile of fish from 2008 and 2006, and showed no reduction in exposure that was present in the mussels.

PAH exposure data from both cod and mussels indicated a higher exposure at station 3, which may reflect the change in the discharge pattern discussed in chapter 4.5 bringing a part of the plume closer to station 3. The same pattern was seen for some other parameters (e.g. micronuclei formation & CYP1A).

Both VTG and ZRP data were not elevated at the exposure stations compared to the reference stations in any of the three years. This would suggest that the fish were not exposed to xenoestrogens at concentrations that would stimulate a significant response. Xenoestrogens that have been identified in PW include alkyl phenols and certain organic acids (Tollefsen et al., 2006). Therefore, it would be expected that these estrogenic compounds, if present in the PW, were adequately diluted below biological threshold levels by the receiving water.

Results on hepatic DNA adducts indicate no differences between the exposure stations and the reference stations in either of the three survey years.

The reduction in the mussel bioaccumulation data and the biomarker responses in general indicate that the exposure signal in 2009 was markedly lower than that measured in 2008 and 2006. This reduction coincides with a reduction in the overall discharge of oil in the PW over the exposure duration. The reduction in the overall oil discharge was mostly due to the C-Tour that was operational for the entire exposure duration in 2009, but absent in 2006 and only in operation for some of the exposure in 2008. Overall, based on the bioaccumulation and biomarker data in 2009, the current environmental risk of the Ekofisk PW on the animals living in the water column in the vicinity of the platforms is low.

Based on experience from WCM 2009 we propose the following recommendations for future WCMs:

The use of a larger number of mussel cages is a good model for deployment. Cages of mussels are less resource demanding than fish, which allows greater coverage and ensures that some locations are exposed to the plume. The mussel bioaccumulation of PAHs has proven to be an extremely effective tool for monitoring organism exposure, with a clear distinction with distance from the PW discharge. To complement this it is recommended for passive samplers to be used and in particular POCIS, since they are known to be more effective in measuring alkyl phenols in the water column.

The use of VTG and ZRP biomarkers for the assessment of estrogenic exposure in cod appears to be a duplication of information. Since the VTG method is more developed and robust, VTG alone is recommended for use in future monitoring programs. Since compounds with estrogenic potential such as APs, and organic acids (e.g. naphthenic acids) have been identified in PW, it is important that estrogenicity is measured. The most sensitive biological marker for estrogenic exposure remains to be VTG synthesis in fish. Therefore, it is recommended that fish are used for future monitoring programs for this purpose.

There is uncertainty of the sensitivity of the GST method. The method measures all GST activity, since GST has many isomers performing a wide range of biological functions they are thought to be naturally up-regulated within the body. Consequently, a large exposure to a GST stimulating compound is required to elevate GST above background levels. For this reason it appears inappropriate to measure GST to detected exposure to environmental concentrations of PAH related compounds.

There is generally a lack of information about the importance of the effect of particle density on the bioavailability of lipophilic organic pollutants. PW related compounds bind to biological surfaces like microalgae, which may affect results generated by offshore monitoring programs. Knowledge about the importance of these issues could be obtained by introduction of laboratory grown microalgae into diluted PW in controlled experiments. This type of information would be helpful when interpreting monitoring results from different periods.

Since PW is composed of a large range of organic compounds that can illicit a wide range of biological effects in different target tissues a rapid screening tool that can include many thousands of biological endpoints in a single sample would prove extremely useful. The microarray available for both the cod and the blue mussel could be used as a screening tool for future monitoring programs. The microarray is an extremely sensitive tool that could provide a plethora of information on the potential biological impact of the PW.

Considerable amounts of results from monitoring at Ekofisk are now available. We suggest that the findings are quality assessed and made available for the scientific community. Dissemination of the data should include both the presentation at international conference(s) and publication in an international peer review journal.

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7   Appendix list

7.1 Appendix A: Cruise report NIVA

7.2 Appendix B: Data report NIVA

7.3 Appendix C: Data report IRIS

7.4 Appendix D: Data report Battelle

7.5 Appendix E: Data report, University of Stockholm

7.6 Appendix F: Data report – University of Vilnius

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