Water Column Monitoring 2008

Rolf C Sundt, Steven J Brooks, Anders Ruus, Merete Grung, Nadia Aarab,
Brit F. Godal, Janina Baršienė, Halldóra Skarphéðinsdóttir

Report IRIS 2008 / 354
Water Column Monitoring 2008

Report IRIS - 2008/354

Project number: 7151832
Project title: Water Column Monitoring 2008
Client(s): ConocoPhillips on behalf of the OLF WCM coordination group
Distribution restriction: Open

Rolf C Sundt
Project Leader

Jan Fredrik Børseth
Project Quality Assurance(IRIS)

Päivi A. Teivanen-Lædre
Vice President
IRIS Biomiljø a/s
Contents

1 INTRODUCTION .................................................................................................................. 8

1.1 General purpose of the study ......................................................................................... 8

1.2 Background ..................................................................................................................... 8

1.3 Objective ......................................................................................................................... 11

1.4 Description of methods ................................................................................................. 11

1.4.1 Sea temperature and salinity .................................................................................... 11

1.4.2 Sea current ................................................................................................................ 11

1.4.3 General biological observations .............................................................................. 11

1.4.4 Biomarkers in cod .................................................................................................... 12

1.4.4.1 PAH-metabolites in bile ..................................................................................... 12

1.4.4.2 AP metabolites in bile ....................................................................................... 13

1.4.4.3 Hepatic GST ....................................................................................................... 13

1.4.4.4 CYP1A ............................................................................................................... 13

1.4.4.5 Vitellogenin ........................................................................................................ 14

1.4.4.6 Zona radiata protein ......................................................................................... 14

1.4.4.7 DNA adducts .................................................................................................... 14

1.4.5 Biomarkers in mussels ............................................................................................. 15

1.4.5.1 PAH body burden ............................................................................................ 15

1.4.5.2 Pyrene hydroxylase activity .............................................................................. 15

1.4.5.3 Lysosomal membrane stability ......................................................................... 16

1.4.5.4 Micronucleus formation ................................................................................... 16

1.4.5.5 Histology ........................................................................................................... 16

2 MATERIAL AND METHODS ............................................................................................. 18

2.1 Source of Cod and Mussels ......................................................................................... 18

2.2 Cage deployment ......................................................................................................... 18

2.3 Quality assurance ....................................................................................................... 21

2.4 Sea temperature and salinity ....................................................................................... 22

2.5 Sea current .................................................................................................................. 22

2.6 Contamination control during transport ..................................................................... 23

2.7 General biological observations ................................................................................. 23

2.8 Biomarkers in cod ....................................................................................................... 24

2.8.1 PAH-metabolites in fish bile .................................................................................. 24

2.8.2 Fixed fluorescence ................................................................................................. 24
2.8.3 GC/MS .......................................................... 24
2.8.4 AP metabolites in fish bile .................................. 25
2.8.5 Glutathion-S-transferase (GST) activity ................. 25
2.8.6 Hepatic Cytochrome P450 1A................................. 26
2.8.7 Vitellogenin ..................................................... 26
2.8.8 Zona Radiata Protein ......................................... 27
2.8.9 DNA adducts ................................................... 27
2.9 Biomarkers in mussels ........................................... 28
  2.9.1 PAH body burden .......................................... 28
  2.9.2 Pyrene hydroxylase activity ............................... 28
  2.9.3 Lysosomal membrane stability ......................... 29
  2.9.4 Micronucleus formation .................................... 30
  2.9.5 Histology in mussels ....................................... 31
2.10 Protein determination ........................................... 31
2.11 Statistical methods ............................................. 32
3 RESULTS .................................................................. 32
  3.1 Sea temperature and salinity measurements at Ekofisk 32
  3.2 Current conditions .............................................. 34
  3.3 Contamination control during transport .................... 36
  3.4 General biological observations ............................. 37
  3.5 Cod - PAH-metabolites in bile ............................... 40
    3.5.1 Bile fluorescence (PAH-metabolites by FF) .......... 40
    3.5.2 PAH-metabolites by GC/MS ............................ 43
  3.6 AP metabolites in cod bile .................................... 45
  3.7 Hepatic GST ...................................................... 46
  3.8 Hepatic Cytochrome P450 1A ................................ 47
  3.9 Vitellogenin ...................................................... 48
  3.10 Zona Radiata Protein .......................................... 48
  3.11 DNA adducts .................................................... 49
  3.12 PAH body burden in mussels ............................... 50
  3.13 Pyrene hydroxylase activity ................................ 56
  3.14 Lysosomal membrane stability ............................ 57
  3.15 Micronucleus formation ..................................... 58
  3.16 Histology in mussels .......................................... 59
4 DISCUSSION ................................................................. 62
  4.1 Tissue levels of PAHs in caged mussels .................... 62
  4.2 Effect responses in caged mussels ......................... 62
4.3 PAH- and AP metabolites in cod bile ................................................................. 63
4.4 Exposure and effect responses in caged cod ...................................................... 63
4.5 Produced water discharge Ekofisk - 2006 and 2008 ........................................ 64
4.6 Confounding factors - biomarkers ..................................................................... 64
4.7 Preliminary ecological risk assessment based on lysosomal stability in mussels. ................................................................. 66

5 CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WCMS ............ 67

6 REFERENCES ........................................................................................................ 70

7 APPENDIX LIST ..................................................................................................... 78
  7.1 Appendix A: Cruise report IRIS ......................................................................... 78
  7.2 Appendix B: Data report NIVA ......................................................................... 78
  7.3 Appendix C: Data report IRIS ........................................................................... 78
  7.4 Appendix D: Data report ITM, University of Stockholm .................................. 78
  7.5 Appendix E: Data report – University of Vilnius ............................................. 78
  7.6 Appendix F: Survey report BiotaGuard ......................................................... 78
  7.7 Appendix G: Gill histology .............................................................................. 78
Summary

The report presents results from the Water Column Monitoring 2008, carried out in collaboration between IRIS and NIVA, with sub-contractors. The objective of the survey was to assess the extent to which 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 points. The study design was identical to that carried out at Ekofisk in 2006 with only minor modifications. The results from the survey show that caged organisms have been exposed to low levels of produced water components. Mussels accumulated PAHs, with levels following the expected gradient with distance from the discharge. Concentrations of PAH- and AP-metabolites in bile of caged cod were moderately elevated suggesting low exposure levels. There was clear signal from the biological responses for several of the methods employed. As expected in animals that were kept close to the discharge, moderate biological responses were observed. The ultimate health effects on the organisms are, however, unknown at this stage. For both bioaccumulation of contaminants and biological effects a gradient with distance from the discharge was observed. The volume of PW discharged to the investigated area have increased but the exposure situation in the investigated area was similar to the situation in 2006 confirming the environmental benefit of the new treatment technology implemented at the field since the 2006 survey.

Work participants

IRIS: Rolf C Sundt, Nadia Aarab, Brit F. Godal, Jan Fredrik Børseth, Solveig Apeland, Kjell Birger Øysæd, Atle Nævdal, Stig Westerlund.

NIVA: Steven Brooks, Merete Grung, Anders Ruus, Eivind Farmen Finne, Christopher Harman, Kevin Thomas, Sigurd Øxnevad, Oscar Fogelberg, Anja Julie Nilsen, Tor Fredrik Holth.
# List of symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP</td>
<td>Alkylated Phenol</td>
</tr>
<tr>
<td>PH</td>
<td>Pyrene Hydroxylase</td>
</tr>
<tr>
<td>C&lt;sub&gt;1&lt;/sub&gt; – C&lt;sub&gt;9&lt;/sub&gt;</td>
<td>referring to the number of carbons in a side chain (e.g. on a PAH or phenol)</td>
</tr>
<tr>
<td>COPSAS</td>
<td>ConocoPhillips</td>
</tr>
<tr>
<td>CYP1A</td>
<td>Cytochrome P450 1A (CYP1A) proteins</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked ImmunoSorbent Assay</td>
</tr>
<tr>
<td>FF</td>
<td>Fixed fluorescence</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography – Mass Spectrometry</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathion-S-Transferase</td>
</tr>
<tr>
<td>IRIS</td>
<td>International Research Institute of Stavanger</td>
</tr>
<tr>
<td>MN</td>
<td>Micronuclei</td>
</tr>
<tr>
<td>NIVA</td>
<td>Norwegian Institute for Water Research</td>
</tr>
<tr>
<td>OLF</td>
<td>Norwegian Oil Industry Association</td>
</tr>
<tr>
<td>PW</td>
<td>Produced Water</td>
</tr>
<tr>
<td>PH</td>
<td>Pyrene hydroxylase</td>
</tr>
<tr>
<td>rpm</td>
<td>Rounds per minute</td>
</tr>
<tr>
<td>VTG</td>
<td>Vitellogenin (precursor of egg yolk protein)</td>
</tr>
<tr>
<td>WCM</td>
<td>Water Column Monitoring</td>
</tr>
<tr>
<td>ZRP</td>
<td>Zona radiata protein (egg shell protein)</td>
</tr>
</tbody>
</table>
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 effect 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 obligated 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. In 2008, it was decided that Ekofisk will be the study site for the WCM programme. The same location was chosen for the WCM study in 2006 and comparison of the biological effects data between the two years was of principal interest. This interest was mainly due to the introduction of the produced water cleaning plant (C Tour) installed at the Ekofisk platform between 2006 and 2008. Therefore, comparison of the biological effects endpoints between the years would enable an assessment of the benefits of the cleaning plant installed on the local aquatic environment.

1.2 Background

The WCM 2008 was carried out in collaboration between the Norwegian Institute for Water Research (NIVA) and International Research Institute of Stavanger (IRIS), with sub-contractors. The sub-contractors were the Institute of Applied Environmental Research at Stockholm University and the University of Vilnius.

Organisms living in the water column around offshore oil and gas production facilities are predominantly exposed to chemicals through discharge of production water (OLF 2000). The amount and composition of produced water (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 (naphtalenes, phenanthrenes and
dibenzothiophenes), PAHs (polyaromatic hydrocarbons), organic acids, alkylphenols (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 shows that the total discharges 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 are detectable several kilometres away from a North Sea oil production platform using in vitro bioassays (Thomas et al. 2006; Tollefsen et al. 2006) and biomarkers (Balk et al. in press; Regoli et al. in press). 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).
The present study is a repetition of an almost identical study that was carried out at Ekofisk in 2006. Since the 2006 study, a PW treatment system (CTour) has been installed at the Ekofisk platform, which is designed to clean the PW prior to discharge. The study has been repeated in 2008 in an attempt to assess the benefits of CTour on improving the water quality around the platform by comparing the biological effect endpoints and contaminant concentrations between the two years.
1.3 Objective

The objective of the WCM survey 2008 was to assess the extent to which discharges from an oil production platform 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 will be compared to the results of an almost identical study carried out at Ekofisk in 2006, in order to evaluate changes in biological effect endpoints and tissue chemistry following the introduction of cleaning technology at Ekofisk.

1.4 Description of methods

This study was designed to monitor bioaccumulation and biomarker responses in fish and mussels held in cages at 15 meters depth in the vicinity of the Ekofisk field. A number of physicochemical parameters were also determined to provide further information, a description of the methods is provided.

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 produced water. 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 potential adverse effects of PAHs have resulted in many years of concentration monitoring in water, sediment and biota. However, 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 usually not provide an adequate assessment of the PAH exposure level (Varanasi 1989). The 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 assessment of PAH exposure in laboratory and field studies (Beyer et al. 1998; Aas et al. 2001).

Fixed wavelength fluorescence

A characteristic feature of PAH compounds is their fluorescing properties. All PAH molecules absorb ultraviolet light followed by emission of light of a longer wavelength. This UV-fluorescence phenomenon occurs because PAH molecules contain delocalised electrons. The fluorescence properties (i.e. optimal excitation and emission wavelengths and signal intensity), vary between PAH compound and is dependent on size, structure and eventual substituents on the molecule. 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 spectrography (SFS) (Aas et al. 2000). However, this direct method is not optimal for standardisation and quantification, and should be regarded a screening method. The metabolites measured with the direct method, are mainly conjugated hydroxy PAH compounds. Standards of these compounds are impossible or 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.

GCMS

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

The alkylphenols (APs) are a group of chemicals which are relevant to discharges from the offshore oil industry. Produced water, which is released in large volumes from many platforms, includes 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 alkylphenols in fish show that AP metabolites are preferentially excreted through the bile pathway (Tollefsen et al. 1998; Sundt et al. 2008). 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 biliary PAH metabolites as a biomarker for exposure to polyaromatic hydrocarbons (Jonsson et al. 2008).

1.4.4.3 Hepatic GST

Glutathione S-transferase (GST) is a 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 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 eucaryotic 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 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 affected by discharge from oil production activities (Scott et al., 2006). Recent studies with freshwater species such as zebrafish 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 (Zr-proteins) in male and juvenile oviparous vertebrates has been used as an effective and sensitive biomarker of exposure to xenoestrogens (Arukwe et al., 1997; Arukwe et al., 2000). Zr-proteins 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. Zr-proteins have been suggested to be more sensitive than VTG at low concentrations of xenoestrogens (Arukwe et al., 1997). In applying the Zr-proteins as xenoestrogen biomarkers, it is important to minimize confounding factors, such as stress (Berg et al., 2004).

1.4.4.7 DNA adducts

The detoxification of genotoxicants by the inducible 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 forming adducts. Thus, the presence of DNA adducts has been taken as evidence of exposure to specific genotoxicants. 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 may 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 produced water is dominated by low molecular PAHs (naphthalenes, phenanthrenes, dibenzothiophenes, commonly denoted NPDs), decalins and their alkylated homologues (Utvik, 1999). High molecular PAHs such as benzopyrene, pyrene and chrysene are also present in effluents of produced water from production platforms in the North Sea, although at lower concentrations. Many of the low molecular PAHs have also been detected in caged organisms deployed downstream from known discharge points (Røe, 1998). This applies in particular to alkylated 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 (PH) 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. PH is considered to be a model PAH compound with a single phase I metabolite i.e. 1-hydroxypyrene (1-HP). This conjugates to various phase II metabolites that can be enzymatically deconjugated for quantification of total phase I metabolism (Filman et al., 2004, Jørgensen et al., 2005). PH is thought to be induced by a variety of PAHs and consequently been proposed as a biomarker of exposure to PAH compounds (Filman 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. 1994). 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. 1994).

1.4.5.4 Micronucleus formation

Chromosomal rearrangements, such as micronuclei (MN), are recognised as a consequence of genome instability (Fenech et al., 1999). The MN 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 MN-containing lagging whole chromosomes or chromosome fragments. Thus, the MN 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 Histology

Histopathological alterations in selected organs and tissues are conceived as histopathological or tissue-level biomarkers. By looking at the structure/morphology of digestive glands, it is possible to follow the metabolic activity. Digestive gland alterations are a reflection of disturbances at the molecular level and identification of these disturbances can aid in the understanding of whole animal impact due to pollutants and other stress factors. Histopathological characteristics of specific organs express condition and represent time-integrated endogenous and exogenous impacts on the organism stemming from alterations at lower levels of biological organisation (Stebbing 1985).

Histological biomarkers provide powerful tools to detect and characterise the biological endpoints of toxicant and carcinogen exposure (Hinton et al., 1992; Moore & Simpson, 1992). As such, the utility of histological lesions as sensitive and reliable indicators of the health of wild fish populations has been demonstrated in several European and
North American studies (Kranz & Dethlefsen, 1990; Myers et al., 1998; Köhler, 1991, 1992; Lang et al., 1999). Several laboratory and mesocosm studies have also demonstrated causal links between exposure to xenobiotics and the development of toxicopathic hepatic lesions (Malins et al., 1985a; Malins et al., 1985b; Moore & Myers, 1994).

In mussel, histopathological 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). The biomarkers selected for this study are lipofuscin and neutral lipid accumulation in mussel digestive gland. The digestive gland of bivalves is made by a complex endo-lysosomal system that is primarily in the uptake and digestion of food as well as in process of pollutant accumulation and detoxification (Cajaraville et al., 1992). The lysosomal lipid content may change due to environmental stress. In this study, lipofuscin accumulation and neutral lipid content had been chosen as histological biomarkers. Lipofuscin accumulation represents a general response (Viarengo et al., 1990; Regoli et al. 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, Cjaraville, 1991). Lipophilic xenobiotics in fact 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 Source of Cod and Mussels

Farmed cod (*G. morhua*) were obtained from Rygjabø fish farm, Finnøy near Stavanger, the same supplier used in the 2006 survey. 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 0.76 ± 0.19 kg wet weight).

Mussels (*M. edulis*) were obtained from a clean location in Trondheimsfjord, the same population used in the 2006 survey. 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).

2.2 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, approximately 50 fish were placed within each cage. Approximately 100 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 to 12 m. The fish and mussel cages were deployed for approximately 6 weeks.

Details regarding the geographical position of the deployment stations with respect to the PW discharge point are shown in Figure 1 & Table 3. The monitoring approach was based on experiences gained in previous water column monitoring surveys and from the BECPELAG workshop (Hylland et al. 2006).

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 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. Overview of samples for biological and chemical analyses of Atlantic cod (*Gadus morhua*).

<table>
<thead>
<tr>
<th>Method</th>
<th>Indication of</th>
<th>Matrix</th>
<th>No samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A</td>
<td>Exposure of planar organic compounds</td>
<td>liver</td>
<td>100</td>
</tr>
<tr>
<td>GST</td>
<td>Exposure of a wide range of xenobiotics</td>
<td>liver</td>
<td>100</td>
</tr>
<tr>
<td>VTG</td>
<td>Xenoestrogenic exposure in males</td>
<td>blood plasma</td>
<td>75</td>
</tr>
<tr>
<td>ZRP</td>
<td>Xenoestrogenic exposure in males</td>
<td>blood plasma</td>
<td>75</td>
</tr>
<tr>
<td>PAH-met., FF</td>
<td>PAH exposure</td>
<td>bile</td>
<td>100</td>
</tr>
<tr>
<td>PAH-metabolites, GC/MS</td>
<td>PAH exposure</td>
<td>bile</td>
<td>60</td>
</tr>
<tr>
<td>AP met</td>
<td>Alkylphenol exposure</td>
<td>bile</td>
<td>60</td>
</tr>
<tr>
<td>DNA adducts</td>
<td>Genotoxic exposure</td>
<td>liver</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 2. Overview of samples for biological and chemical analyses of mussels (*Mytilus edulis*).

<table>
<thead>
<tr>
<th>Method</th>
<th>Indication of</th>
<th>Matrix</th>
<th>No samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrene Hydroxylase</td>
<td>Exposure of PAH with 4 benzenic rings</td>
<td>digestive gland</td>
<td>100</td>
</tr>
<tr>
<td>Lysosomal stability</td>
<td>General stress</td>
<td>haemocytes</td>
<td>84</td>
</tr>
<tr>
<td>Lipofuscin accumulation</td>
<td>Effects from organic pollutants</td>
<td>digestive gland</td>
<td>100</td>
</tr>
<tr>
<td>Neutral lipid accumulation</td>
<td>Peroxidation of lipids</td>
<td>digestive gland</td>
<td>100</td>
</tr>
<tr>
<td>PAH concentration</td>
<td>PAH exposure</td>
<td>soft tissue</td>
<td>30</td>
</tr>
<tr>
<td>Lipid content</td>
<td>Used for lipid normalization of PAH levels</td>
<td>soft tissue</td>
<td>24</td>
</tr>
<tr>
<td>Micronucleus</td>
<td>Genotoxic exposure</td>
<td>haemocytes</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 3. Locations and designation for stations.

<table>
<thead>
<tr>
<th>St designations</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>REF 1</td>
<td>Reference NE of discharge</td>
</tr>
<tr>
<td>REF 2</td>
<td>Reference E of discharge</td>
</tr>
<tr>
<td>ST 1</td>
<td>1600m SW</td>
</tr>
<tr>
<td>ST 2</td>
<td>600m SW</td>
</tr>
<tr>
<td>ST 3</td>
<td>Off southern flare</td>
</tr>
<tr>
<td>ST 4</td>
<td>Off 2/4J</td>
</tr>
<tr>
<td>ST 5</td>
<td>1100m NE</td>
</tr>
<tr>
<td>ST 6</td>
<td>2000m NE</td>
</tr>
</tbody>
</table>

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 placing of station 3 and 4 in relation to the discharge points.
### 2.3 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.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Quality measure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sampling protocols</td>
<td>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.</td>
</tr>
<tr>
<td>PAH body burden</td>
<td>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)</td>
</tr>
<tr>
<td>Pyrene hydroxylase</td>
<td>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.</td>
</tr>
<tr>
<td>LMS</td>
<td>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.</td>
</tr>
<tr>
<td>Neutral lipid</td>
<td>IRIS 2.2-421 SOP - Neutral lipid accumulation Rev 02</td>
</tr>
<tr>
<td></td>
<td>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.</td>
</tr>
<tr>
<td>Lipofuscin accumulation</td>
<td>IRIS 2.2-423 SOP - Lipofuscin accumulation Rev 02</td>
</tr>
<tr>
<td></td>
<td>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.</td>
</tr>
<tr>
<td>Micronuclei formation</td>
<td>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. Coordinates of the micronuclei were recorded to enable assessment checks to be carried out at intervals.</td>
</tr>
<tr>
<td>ZRP</td>
<td>All samples were coded, randomised and analysed blind. Four replicates of each sample, a</td>
</tr>
</tbody>
</table>

- 21 -
<table>
<thead>
<tr>
<th><strong>IRIS &amp; NIVA</strong> Water Column Monitoring 2008</th>
</tr>
</thead>
</table>

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-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.

### 2.4 Sea temperature and salinity

An STD/CTD – model SD204 was used for measuring, calculating and recording sea water conductivity/salinity, temperature, depth (pressure) and sound velocity/water density through the water column. The instrument was operated by COPSAS personnel at the Ekofisk platform and lowered on 2 occasions during the 6 week cage deployment (April 20th and April 27th). The instrument was set to log data every two seconds.

The specifications for the instruments 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**: ranges: 500, 1000, 2000, 6000 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

### 2.5 Sea current

Current instruments were deployed at ST 4 and at REF 1.

At Ekofisk (ST 4) the instrument type Recording Current Meter RCM9 was used, measuring current velocity (range: 0 - 300 cm/s, accuracy: 2 cm/s or 2 %), current direction (accuracy/resolution: 0.35°; accuracy ±5° for 0-15° tilt), temperature (range: -
2.46 – 36.04 °C, accuracy/resolution: 0.05 °C) and depth (range 0-2000 m, accuracy/resolution: 0.1 %/0.2 %). The instrument logged data every 10th minute. The instruments were deployed in the water from 19:07 hours the 3rd of April and recorded until 05:49 the 23rd of April, 895 data points were collected.

For the reference station the used instrument were of the type Aquadopp Current Meter 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) 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 WC monitoring 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 hour.

The instrument was deployed in the water from 21:54 hours the 3rd of April 2008 until 15:54 the 11th of May 2008. From the logged results data and diagnostics the instrument seems to work normal through the test period and a total of 5400 data points were collected. For pre-programmed set-up see appendix.

2.6 Contamination control during transport

To confirm that organisms were not exposed to PAH contamination during the transport, seawater samples from the transportation tanks were collected. For each sample, 5 litres were used for the ethyl-acetate extraction. The extracts were analysed for PAHs by GCMS (2.5.2.).

2.7 General biological observations

Fish were sexed by visual examination of gonads and liver weight was recorded. Total weight of cod was measured in the lab onboard the vessel. In order to provide best possible measurements of liver and gonads, these tissues were wrapped in aluminium foil, frozen at -20°C and brought to Akvamjø lab for measurements.

Condition was determined as the ratio between total weight and the cube of the fork length of the fish.

\[
\text{Condition index} = \left[ \frac{\text{Weight (g)}}{\text{Length (cm)}^3} \right] \times 100
\]

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

\[
\text{LSI} = \left[ \frac{\text{Liver weight} \times 100}{\text{fish weight}} \right]
\]

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

\[
\text{GSI} = \left[ \frac{\text{Gonad weight} \times 100}{\text{fish weight}} \right]
\]
2.8 Biomarkers in cod

2.8.1 PAH-metabolites in fish bile

2.8.2 Fixed fluorescence

Fixed Fluorescence (FF) is a semi-quantitative and semi-qualitative screening method for direct fluorescence detection of groups of PAH metabolites (Aas et al. 2000b). 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.

2.8.3 GC/MS

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 (Instrument Teknikk A.S., Oslo, Norway). 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.
2.8.4 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 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-APs 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-APs (TMS-OH-APs) in fish bile samples were analysed by a GC-MS system consisting of a HP5890 series II Gas chromatograph, Shimadzu 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 (Instrument Teknikk A.S., Oslo, Norway). 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-APs (Jonsson et al. 2003); the molecular ions were selected for determination of both alkylated and non-alkylated TMS-O-APs.

2.8.5 Glutathion-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 KH₂PO₄ 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 (100mM KH₂PO₄/K₂HPO₄, 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 × (∆ Absorbance−blank))/ (sample volume × 9.6 × light-way × [Protein]ₘₜₜ), where 9.6 is the molar extinction coefficient (ε) for the CDNB-GSH conjugate (in mM⁻¹cm⁻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 were determined by a procedure based on the Lowry method (Lowry, 1951).

2.8.6 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 to 10 µg/ml 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 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 antibody 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 antibody 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 H2SO4. The absorbance was read at 450 nm.

2.8.7 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$_2$SO$_4$ 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.

### 2.8.8 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$_2$SO$_4$ and the absorbance read at 450 nm.

### 2.8.9 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’-[$\gamma$-$^{32}$P]triphosphate ([$\gamma$-$^{32}$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.
2.9 Biomarkers in mussels

2.9.1 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 a sub sample taken of approximately 5g. Internal standards were 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 40mL 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.

2.9.2 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, 0.05M, 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
The HPLC used was a Waters 2695 Separations Module with a 2475 fluorescence detector attached. The column was a Waters PAH C18 (4.6 x250 mm) with 5 µm particles. The mobile phase consisted of a gradient from 40:60 acetonitrile:water to 100% acetonitrile at a flow of 1 mL/min, and the column was heated to 35°C. The excitation and emission wavelengths used for detection of pyrene and triphenylamine were 346, 384 and 300, 360 respectively. A 25 µL extraction volume was injected for each analysis.
2.9.3 Lysosomal membrane stability

The mussels from the pre-exposure group were brought to the lab in Stavanger on ice. The mussels were acclimatised in the lab 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.

0.4 ml haemolymph was sampled from each mussel and mixed with filtered sea water at the ratio 2:1. 40 µl haemolymph/seawater-mixture was pipetted out on microscope-slides, and incubated in a light-proof box for 20 min before 35 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).

NR is selectively taken up by haemolymph cells and this adds an extra stress to the membranes. After some time, from 15 to 200 minutes, depending of the health status of the mussels, the membrane will start to burst and NR will leak out in the cytosol. This causes the form of the cells to change from irregular to round shaped. The time from NR is added the cells and until they 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.

Figure 2. Microscope view (400× magnification) of living and dead mussel haemolymph cells
2.9.4 Micronucleus formation

Haemolymph of mussels were applied directly on slides, air-dried and fixed in methanol for 15 min. The slides were then shipped and cytogenetic analysis was done in 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 Olympus BX 51 or Nikon Eclipse 50i bright-field microscope. A total of 20000-30000 cells were examined in each caged experimental group of mussel. In some mussel slides, the deficiency of appropriate cells for the micronuclei analysis was noted. Nevertheless, 500 haemocytes was a minimum amount of cells suitable for the analysis. Therefore, in mussels micronuclei were counted in 500-2000 haemocytes from each specimen.

Only cells with intact cellular and nuclear membrane were scored. MN are scored when: i) nucleus and MN have a common cytoplasm, ii) colour intensity of MN is the same or lower than the one of the nucleus, iii) the size of the MN is equal or smaller than 1/3 of the nucleus, iv) MN must be completed separated from the nucleus, v) cells with multiple MNs are not scored.

Figure 3. Micronucleus in haemocyte of mussel *Mytilus edulis* (1000× magnification).
2.9.5 Histology in mussels

For cytochemical examination small pieces (5x5x5mm) of freshly excised digestive gland tissues from animals are placed on metal cryostat chucks. Each chuck is then placed for 1 min in a small bath of n-Hexane that has been pre-cooled to -196°C (using a surrounding bath of liquid nitrogen). The metal chuck plus the quenched (super-cooled) solidified tissues are then sealed by double-wrapping in parafilm and stored at -40°C until required for sectioning.

Cryostat sections (8µm) are cut in a cryostat with the cabinet temperature below -25°C and the knife cooled -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.

Lipofuscin accumulation

The lipofuscin content of lysosomes was determined using the Schmorl reaction. Cryostat sections were fixed in 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). Section were stained for 5 min, rinsed in acetic acid (1%) for 2 min and washed in running water for 10 min and rinsed in distilled water before mounting. Slides were subjected to image analysis and results were expressed as pixel density.

Neutral lipid accumulation

For the determination of unsaturated neutral lipids, cryostatic section were fixed in calcium-formol for 15 min, rinsed in distilled water and transferred into 60% triethylphosphate (v/v with distilled water) for 1 min. Section 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.

2.10 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 maximum absorbance at 750 nm and minimum absorbance at 405 nm.

2.11 Statistical methods

Biological responses in individual mussel or fish were subjected to analysis of variance (ANOVA) to clarify whether there were differences between groups (Sokal & Rohlf, 1981). Prior to analyses, homogeneity of variances was checked using the Levene’s test. Variables were transformed as appropriate to attain homodascicity. Where this was not possible, the non-parametric Kruskal-Wallis analysis was used (Sokal & 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 H₀: “no difference between groups” was set to 0.05.

3 Results

Where comparable data from both 2006 and 2008 exist, the data are presented together. This renders possible a comparison of the exposure situation in the two periods of monitoring. Such a comparison was of particular interest since new PW treatment technology had been implemented prior to the 2008 monitoring.

3.1 Sea temperature and salinity measurements at Ekofisk

The salinity remained relatively stable at ~35‰ throughout the water column on both occasions (April 20th and 27th). A thermocline was evident in the water column on both sampling occasions, although this was more pronounced on the 27th April. On this day a thermocline was seen at a depth of approximately 20 m with a change in temperature from around 7.8°C in the surface waters to 6.8°C at a depth > 25 m. (Figure 4).
Figure 4. Salinity and temperature profiles taken from below the Ekofisk platform 2/4 J on two occasions during the cage deployment (April 20th and 27th).
3.2 Current conditions

*Ekofisk*

Measurements indicate that the current in the area is affected by tide with an axis stretching SW-NE (Figure 5). The tidal influence reduces transport of discharged water away from the platform area. The temperature increased from 6.3 to 8.0°C during the deployment period for both measuring points, showing a natural spring situation. For comparison the temperature increased from 5.5 to 9.5 in 2006. The measuring depth was approximately 19 meters in both investigations. Average current speed was 9.6 cm/s, maximum current speed was 14.5 cm/s and overall current direction was 45.8°.

![Diagram showing current conditions at Ekofisk in 2006 and 2008](image)

**Figure 5.** Sum of relative current (in 15° sectors) at Ekofisk in 2006 and 2008. Positions of cages shown for comparison.
Reference station
As for the Ekofisk area, measurements at the reference station show tidal driven current with an axis stretching SW-NE (Figure 6). The temperature increased from 6.3 to 7.8°C during the deployment period (5 to 9°C in 2006). The measuring depth was 22 metres (12 and 14 metres in 2006). Average current speed was 14.3 cm/s (15.1 in 2006), maximum current speed was 33.8 cm/s and overall current direction was 32°.

Figure 6. Sum of relative current (in 15° sectors) at reference station in 2006 and 2008.
3.3 Contamination control during transport

Results from GCMS analysis of PAHs in sea water from the vessels storage tanks showed only background levels. This confirms that the organisms used in the study were not contaminated by PAHs from water during the transport.

Table 4. PAH (µg/L) and standard deviations (stdev) in sea water collected from the vessels transport tanks. (n=3; quantification limit 0.005 µg/L)

<table>
<thead>
<tr>
<th>Compound</th>
<th>µg/L</th>
<th>stdev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>0.008</td>
<td>0.001</td>
</tr>
<tr>
<td>C1-Naphthalene</td>
<td>0.013</td>
<td>0.004</td>
</tr>
<tr>
<td>C2-Naphthalene</td>
<td>0.019</td>
<td>0.006</td>
</tr>
<tr>
<td>C3-Naphthalene</td>
<td>0.010</td>
<td>0.002</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>&lt; 0.005</td>
<td>-</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>&lt; 0.005</td>
<td>-</td>
</tr>
<tr>
<td>Fluorene</td>
<td>&lt; 0.005</td>
<td>-</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>&lt; 0.005</td>
<td>-</td>
</tr>
<tr>
<td>Anthracene</td>
<td>&lt; 0.005</td>
<td>-</td>
</tr>
<tr>
<td>C1-Phen/Anthr</td>
<td>&lt; 0.005</td>
<td>-</td>
</tr>
<tr>
<td>C2-Phen/Anthr</td>
<td>&lt; 0.005</td>
<td>-</td>
</tr>
<tr>
<td>Dibenzothiophene</td>
<td>&lt; 0.005</td>
<td>-</td>
</tr>
<tr>
<td>C1-Dibenzothiophene</td>
<td>&lt; 0.005</td>
<td>-</td>
</tr>
<tr>
<td>C2-Dibenzothiophene</td>
<td>&lt; 0.005</td>
<td>-</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>&lt; 0.005</td>
<td>-</td>
</tr>
<tr>
<td>Pyrene</td>
<td>&lt; 0.005</td>
<td>-</td>
</tr>
<tr>
<td>Benzo(a)anthracene</td>
<td>&lt; 0.005</td>
<td>-</td>
</tr>
<tr>
<td>Chrysene/Triphenylene</td>
<td>&lt; 0.005</td>
<td>-</td>
</tr>
<tr>
<td>C1-Chrysene</td>
<td>&lt; 0.005</td>
<td>-</td>
</tr>
<tr>
<td>C2-Chrysene</td>
<td>&lt; 0.005</td>
<td>-</td>
</tr>
<tr>
<td>Benzo(b,j)fluoranthene</td>
<td>&lt; 0.005</td>
<td>-</td>
</tr>
<tr>
<td>Benzo(k)fluoranthene</td>
<td>&lt; 0.005</td>
<td>-</td>
</tr>
<tr>
<td>Benzo(b,j,k)fluoranthene</td>
<td>&lt; 0.005</td>
<td>-</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>&lt; 0.005</td>
<td>-</td>
</tr>
<tr>
<td>Indeno(1,2,3-cd)pyrene</td>
<td>&lt; 0.005</td>
<td>-</td>
</tr>
<tr>
<td>Benzo(g,h,i)perylene</td>
<td>&lt; 0.005</td>
<td>-</td>
</tr>
<tr>
<td>Dibenzo(a,h)anthracene</td>
<td>&lt; 0.005</td>
<td>-</td>
</tr>
</tbody>
</table>
3.4 General biological observations

The body length and weight distribution in the different groups are shown in Figure 7 and Figure 8. No difference in the length of the fish was found between the groups. However, differences in fish weights were found between the group, with the caged cod from REF 1 and ST 3 showing a significant reduction in weight compared to the pre-exposure group (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-exposure group significantly higher than all three groups of caged fish (Figure 10, ANOVA, Tukey, p<0.05). This trend was also reflected in the liver-somatic index (Figure 11). The sex ratios were similar between the groups with a slight bias of female to male fish (Figure 12). There was no significant difference in the gonad-somatic indices of fish between the different stations (Figure 13).

![Figure 7](image_url)

**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 (ANOVA, Tukey, p<0.05).
Figure 10. Liver-somatic index in cod in the indicated groups. Right: females, Left: males. The figure shows median, quartiles (box) and 10/90-percentiles (whiskers).

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

3.5 Cod - PAH-metabolites in bile

3.5.1 Bile fluorescence (PAH-metabolites by FF)

Measured bile fluorescence levels are shown in Figure 13-15. Differences between groups were only found for the wavelength-pair 341/383 (identifies 4 ring structures). The reference station was lower than all other groups (Figure 14). The signal observed for this wavelength-pair in the 0-sampling group is confirmed by a low level of pyrene detected by GCMS analysis (see 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) levels in bile from cod in the groups indicated. The figure shows median, quartiles and 10/90-percentiles.
3.5.2 PAH-metabolites by GC/MS

For all metabolite compounds (except 1-OH-phenantrrene, p=0.29 and p=0.06 station 3 and 4 respectively), stations 3 and 4 were significantly different from the reference station (p<0.0035, Wilcoxon; Figure 17 and 18). This confirms significant uptake and bio-transformation of PAHs typical for produced water 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 2008 bile material were similar to the levels measured in the 2006 material.

![OH-naphthalene concentrations](image)

**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.
3.6 AP metabolites in cod bile

Significant differences in AP metabolite levels (all measured compounds) were found between reference and station 3 and 4 (Kruskal-Wallis, P<0.05). This confirms bio-concentration and bio-transformation of APs typical for produced water to the fish caged close to the discharge. However, the levels measured are only approximately double the limit of quantification for the analysis method (30ng/g bile, for single compounds) indicating low level of exposure.

![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.](image-url)
3.7 Hepatic GST

Since there were no differences in hepatic glutathione-S-transferase activity (GST) with respect to gender, data is shown for all fish sampled in 2006 and 2008 (Figure 21). Comparable concentrations of GST were measured in 2006 and 2008 fish with highest median concentrations found in the pre-exposure group. However, for both 2006 and 2008, there were no significant differences in GST levels between the groups.

![Figure 21. Hepatic Glutathione S-transferase, GST, (nmol/min/mg protein) activity in cod from the indicated groups from the 2006 and the 2008 surveys. The figure shows median, quartiles and 10/90-percentiles.](image-url)
3.8 Hepatic Cytochrome P450 1A

Lower levels of CYP1A were reported in 2006 samples due to substantial non-specific binding in these samples. This caused higher than usual concentrations in the blank controls, which were subsequently deducted from the concentrations in the groups, artificially lowering the CYP1A activity. Therefore the CYP1A data from 2006 could not be directly compared to the CYP1A data from 2008 and has not been included in the graph (Figure 22). For the 2008 data, gender differences in hepatic CYP1A activity were not observed and data has been presented for all fish measured for the 2008 survey. CYP1A activity in the pre-exposed fish was significantly higher than all field exposed fish (ANOVA, Tukey, p<0.05). Highest concentrations of CYP1A in field exposed fish were measured in fish from station 3, which was significantly higher than the concentration measured in fish from the reference station (ANOVA, Tukey, p<0.05). This may suggest exposure of the station 3 fish to higher concentrations of CYP1A inducer compounds such as PAHs from the produced water discharge.

CYP1A levels are known to be affected by the temperature the fish experience prior to sampling, with lower temperature typically resulting in higher CYP1A activity (Sleiderink et al., 1995). This is a possible explanation for the relatively higher level observed in the pre-exposure group.

![Figure 22](image-url). Hepatic cytochrome P450 1A activity in cod from the indicated groups from the 2008 survey. The figure shows median, quartiles and 10/90 percentiles. * significantly different from all other groups. † significantly different from REF 1.
3.9 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 \( (\Delta VTG = [VTG]_{after\ exposure} - [VTG]_{before\ exposure}, \text{Figure 23}) \). The VTG concentrations in male cod were consistently low in all groups and were comparable for both 2006 and 2008 surveys. Higher concentrations of VTG were measured in females, although no significant differences were found either between years or between stations.

Figure 23. Plasma vitellogenin concentration in caged cod from the groups indicated. The figure shows median, quartiles and 10/90-percentiles. Males – left panel; females – right panel.

3.10 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 \( (\Delta ZRP=ZRP_{after\ exposure} - ZRP_{before\ exposure}; \text{semi-quantitative, Figure 24}) \). For male cod, there was no significant difference in \( \Delta ZRP \) concentration between stations for either 2006 or 2008. Higher median concentrations of \( \Delta ZRP \) were found in male cod from
2008, although due to the large within group variability no significant differences were found.

For the female cod measured in 2006, significantly higher ΔZRP concentrations were found in fish from station 3 compared to the reference site (ANOVA, Tukey p<0.05). This significant increase in ΔZRP concentration from female cod in 2006 has been masked by the high variability of ΔZRP concentration in female cod of 2008. Reasons for the large variability are unclear, but no significant differences in ΔZRP concentration between the 2008 stations or between the two years were found.

![Figure 24](image-url)  
Figure 24. Plasma Zona radiata protein concentration in caged cod from the groups indicated. The figure shows median, quartiles and 10/90-percentiles. Males – left panel; females – right panel.

### 3.11 DNA adducts

No significant differences in DNA adducts were found between the groups (ANOVA on log-transformed data). Average levels in cod from the pre-exposure group were 0.57 ± 0.24, the reference station was 0.60 ± 0.31 nmol add/mol normal nucleotides (all given as average ± 95% confidence level), levels from station 3 were 0.86 ± 0.41, and levels from station 4, 0.75 ± 0.27.
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.

3.12 PAH body burden in mussels

Overall, there were 24 pooled samples of mussels available for PAH analyses; 3 replicates from pre exposure sampling, and 3 replicates from 7 different stations. The results of these are provided in Appendix B. Due to the large within station variation in 2008, there was no significant differences between the mussel lipid content from the different stations (Kruskal-Wallis, p>0.05, Figure 26). Therefore, PAH data are presented on a wet-weight basis (µg/kg; Figures 27 to 34). Due to the small sample sizes (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, respectively.
Comparison of data between 2006 and 2008

For the WCM program of 2006, mussel PAH body burden was performed by both Battelle and NIVA. In some cases, good comparisons where observed between the laboratories (i.e. sum of PAH16). However in other cases, such as for the NPDs, the data was not comparable. Consequently, the 2006 data has been shown only for those values that are comparable to the 2008 data. This includes the Battelle data for sum PAH16 and the NIVA 2006 analysis, measured at 4 stations only, for all other PAHs. Decalin analysis was not carried out by NIVA in 2006. Therefore, the comparability of the NIVA and Battelle decalin data were unknown. For this reason no between year comparisons for the decalin data were made.

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

Moderate differences in mussel lipid levels were observed between the 2006 and 2008 material. However and the differences are not expected to have significant consequence for the wet weight based PAH body burden. The lipid level in mussels is generally low (below 2%) compared to other biological matrixes typically used for PAH analyses.
Figure 27. Concentrations (µg/kg wet wt) shown as **Sum-PAH16** in caged mussels from the groups indicated. Boxes depict median and min-max (individual observations, since n=3).

Figure 28. Concentrations (µg/kg wet wt) shown as **total dibenzothiophenes** in caged mussels from the groups indicated. Boxes depict median and min-max (individual observations, since n=3). Data includes the parent compound and alkylated forms.
Figure 29. Concentrations (µg/kg wet wt) shown as total phenanthrenes and anthracenes in caged mussels from the groups indicated. Boxes depict median and min-max (individual observations, since n=3). Data includes the parent compound and alkylated forms.

Figure 30. Concentrations (µg/kg wet wt) shown as total naphthalenes in caged mussels from the groups indicated. Boxes depict median and min-max (individual observations, since n=3). Data includes the parent compound and alkylated forms.
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 produced water discharges from oil platforms accumulate higher concentrations of alkynaphthalenes, alkylphenanthrenes and alkylbenzothiophenes, than their respective parent compounds (Ruu et al. 2006; Hylland et al. 2005). Figures 32-34 show this pattern, with higher alkyl-compounds:parent-compound ratios in mussels in the vicinity of the offshore platform.
Figure 32. Ratio of alkyl-naphthalenes/naphtalenes. Boxes depict median and min-max (individual observations, since n=3).

Figure 33. Ratio of alkyl-phenanthrenes & anthracenes/phenanthrene & anthracene. Boxes depict median and min-max (individual observations, since n=3).
3.13 Pyrene hydroxylase activity

In 2006, benzo(a)pyrene hydroxylase activity was not detected in mussel digestive samples. The reasons for this were unclear, although it was thought not to be due to the exposure conditions the mussels experienced during deployment. The development of a similarly related enzyme system, pyrene hydroxylase (PH), has taken place at NIVA, and has led to its inclusion in the 2008 survey. Consequently, comparison between 2006 and 2008 could not be made for PH. For the 2008 data, significantly higher concentrations of pyrene hydroxylase were found at Stations 3, 4 and 5 compared to the PRE EXP and REF 1 stations (ANOVA, Tukey, p<0.05). There was no significant difference between the reference station and pre-exposure.
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.

### 3.14 Lysosomal membrane stability

The observed lysosomal responses in mussel haemocytes from the pre exposure sampling, the reference station and stations 3 and 5 from 2008 are within the normal range of retention times usually observed for blue mussels in unexposed areas. Only stations 1 and 2 were significantly lower than the reference station in 2008 (Kruskal-Wallis, p<0.05) The high degree of stress indicated in mussels from station 4 in 2006 was not supported by the 2008 data, with mussels from the two closest stations (3 and 4) having NRRT indicative of good health.

NRRT results are further used in a preliminary ecological risk assessment in chapter 4.7.
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.

### 3.15 Micronucleus formation

The lowest frequency of micronuclei (2.2 MN/1000 cells) was found in pre exposure group. The highest MN induction level (5.87 MN/1000 cells), was found in mussels caged at station 4. This is 2.7-times higher than in pre-exposed mussels and 1.8-times higher than in mussels deployed to the reference site. The frequency of micronuclei in mussels deployed at the reference station and station 3 was equal to 3.3 MN/1000 cells. In mussels from stations 2 and 6, the MN frequency of 3.9 MN/1000 cells was registered. Stations 1, 4 and 5 differed significantly from the reference station (p<0.015, Wilcoxin).

Compared to the investigation in 2006, relatively high responses were detected in mussels deployed some distance away from the discharge point, at stations 5 (5.27 MN/1000 cells) and at station 1 (5.36 MN/1000 cells; Fig. 2). The MN frequency pattern in the near field (ST 3 and 4) did not reflect the PAH body burden as well as in 2006.
3.16 Histology in mussels

Both mussel histology techniques (netral lipid and lipofucin) have been improved since the 2006 investigation. The change in how the techniques are performed has led to limitations for direct comparison with previous data. Implementation of improved techniques for comparison with future results was considered more important than the possibility of comparability with WCM 2006 results.

Neutral lipid accumulation

Neutral lipid accumulation was only significantly higher in stations, 2 and 3 compared to the reference site (Dunnets, p= 0.005 and 0.049 respectively).
Figure 38. Neutral lipid accumulation given as optical density in mussels from the groups indicated. The figure shows median, quartiles and 10/90-percentiles.

Figure 39. Histological sections (400 X magnification) showing neutral lipid accumulation (arrow) in mussel digestive gland from the groups indicated.

**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 mussel digestive gland was significantly higher in station 2 and 3 compared to the reference site (Dunnets, p= 0.011 and 0.005 respectively).
Figure 40. Lipofuscin accumulation in mussel lysosomes given as optical density from the groups indicated. The figure shows median, quartiles and 10/90-percentiles.

Figure 41. Histological sections (400 X magnification) showing accumulation of lipofuscin in mussels digestive gland from the groups indicated. Arrows highlight the presence of autophagy vacuoles in stations around the discharge, indicating a high stress condition.
4 Discussion

At caging depth, temperature increased from 6.3 to 7.8 °C during the deployment period (5.5 to 9.5 °C in 2006). The salinity was generally stable during the deployment period at ~35‰.

Due to low availability of food in the cages the experimental cod lost weight during deployment, as expressed by higher mean condition index in the pre exposure group, than in the other groups. In general, the material was homogeneous and suitable for comparison among groups.

4.1 Tissue levels of PAHs in caged mussels

The results from the present Water Column Monitoring survey show that caged organisms from all locations in the proximity of Ekofisk have been exposed to low levels of produced water components. Mussels were exposed to higher concentrations with proximity to the produced water discharge as indicated by PAH body burden data for the different stations.

For comparison, the concentrations of $\Sigma$PAH16 accumulated in mussels closest to the produced water discharge (Station 3) were at the same level as for WCM 2006. Concentrations were an order of magnitude higher than in mussels 500 m from the Statfjord B platform in the 2004-monitoring (Hylland et al. 2005). The concentrations were comparable with those found earlier in the vicinity of Troll (23 µg/ kg wet weight; Utvik et al. 1999) and 500 m from Statfjord B in 2001 (BECPELAG; 34.5 µg/ kg wet weight).

4.2 Effect responses in caged mussels

Both the chemistry data and the biological effects measurements indicated that the caged mussels were exposed to PAHs during the approximate 6 week deployment.

Elevated levels of pyrene hydroxylase (PH) were found in mussels collected from the stations closest to the produced water discharge, which corresponded with the mussel PAH body burden data. Field data on PH activity in mussels is limited in the scientific literature. Since dose response information is not available for this parameter interpretation must be done with caution. However, PH activity in mussels collected from the inner harbour of Aker Brygge, Oslo fjord were found at similar levels (i.e. 400 pg/ mg protein/ min) than those found for mussels close to the Ekofisk discharge (M. Grung NIVA, personal observation).

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. Some stations from Ekofisk showed shorter retention time indicating stress, however the stress levels were generally lower than in 2006. In the near field, particularly data from station 3 indicate less stress than what was seen in 2006.
A similar situation was observed for micronuclei formation in mussel haemocytes. Micronuclei formation indicates that the PW contains genotoxic compounds in sufficient amounts to cause effects in the water close to the discharge.

In addition, the mussel histopathological investigations indicated that the caged mussels located close to the discharge were stressed. The results showed higher levels of neutral lipids in two stations (2 and 3). Intracellular accumulation of neutral lipids in lysosomes digestive cells has been used as a complementary indication of exposure to organic pollutant (Lowe and Clarke, 1989, Cajaraville, 1991; Regoli, 1992).

Lipofuscin accumulation was elevated in mussels from stations 2 and 3, suggesting increased stress compared to the mussels from the reference station. Previous studies have shown increased lipofuscin accumulation with exposure to metal and organic contaminant concentrations (Moore, 1988; Regoli, 1992).

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 AP metabolite compounds measured, both stations at Ekofisk were significantly different from the reference station. This confirms significant uptake and bio-transformation of PAHs and APs typical for produced water exposure in fish from the two stations close to the discharge. However the levels observed are low. AP metabolite levels in fish bile is 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 laboratory (Sundt et al. 2008b).

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. Median increase in VTG-concentrations in females were highest on station 4, however, this was not significant. A similar trend could be read from the zona radiata protein (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 2006 investigation and supports previous conclusions about levels needed to cause xenoestrogenic effects in cod (Sundt et al. 2008b).

The levels of DNA adducts found were in general low, however some individual fish from all stations (including the field reference station) showed elevated levels of DNA
adducts. This indicates that fish were exposed to genotoxic pollutants beyond their short term DNA repair capacity.

Variability in DNA adducts among individuals is also recorded for cod, saithe and haddock from the Ling/Egersund bank area. Few studies on DNA adduct levels in wild cod from the open sea have been published. For comparison, Aas et al. (2003) studied DNA adduct levels in ten individual cod from the Barents Sea. Six of the ten had detectable adduct levels, with an average of 0.75 ± 0.58 (± SD) mmol add/mol normal nucleotides (average of individuals with adducts only), to be compared with 2.28 (n=1), 1.49 (n=5) and 1.41 (n=3) for reference station, station 3 and 4 respectively in the present study (2.19, 1.55 and 3.32 in 2006).

Levels of DNA adducts in wild bottom dwelling fish from the Tampen area showed 10-fold higher levels (Beyer et al. 2004). This may indicate the presence of heavier adduct forming PAH compounds in the bottom sediments compared to that found in the PW. Cod collected near to discharge water from an aluminium smelter showed elevated levels (up to 50-fold higher) of DNA adducts compared to what was found in the present WCM study (Aas et al. 2001). The low levels of DNA adducts found in the present study and the 2006 study, suggests low levels of adduct forming compounds in the PW discharge.

4.5 Produced water discharge Ekofisk - 2006 and 2008

Since the 2006 WCM survey, new treatment technology was implemented (Voldum et al. 2008). However, during the initial phase, the treatment facility faced technical problems causing the treatment process to run below optimal performance. The average concentrations of oil in the PW have been reduced by on average by approximately 36% during the exposure period. Since 2006, the discharged volume has increased, partially from additional supply of PW from an external platform (2/4 J). At the same time, some of the PW has been discharged from the platform (2/4 M).

The total discharge of oil in PW has been reduced on average by 14% during the exposure period. When comparing the monitoring data from 2006 and 2008 the difference in amounts of discharged hydrocarbons between the two years are reflected relatively well.

Even though the cleaning process has been working with reduced efficiency, comparison with 2006 data confirms the environmental benefit of the new treatment technology implemented at the field since the last survey.

4.6 Confounding factors - biomarkers

From previous WCM surveys, certain biomarkers have sometimes failed to show a clear distance-response relationship from the produced water 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 response 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 produced water 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., 2007). For example, certain alkylphenol
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 produced water 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 produced water chemicals have low persistence and are rapidly diluted, the inhibition of biological effect should only occur, if at all, in fish closest to the produced water discharge.

A recent report attempted to answer the question as to whether compounds common to produced water 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+ 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 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.

The potential endocrine disrupting effects of the PW from platforms could be examined in future monitoring programmes, using a range of in vitro techniques including: Yeast oestrogen screen (YES); anti-yeast oestrogen screen (anti-YAS); yeast androgen screen (YAS); and anti-yeast androgen screen (anti-YAS). These would provide information on the potential masking effects of the PW mixture and help to evaluate the potential potency of the PW to organisms living within the water column close to offshore platforms.
4.7 Preliminary ecological risk assessment based on lysosomal stability in mussels.

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 of chemical compound. In cases where stress other than by PAHs influences the situation this would also 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 (NRRT) levels of effect have recently been established (OSPAR, 2007). For this method, animals are considered to be healthy if NRRT is >120 minutes; stressed but compensating if <120 but >50 minutes and severely stressed and probably exhibiting pathology if <50 minutes. Based on NRRT data from WCM 2006 we concluded the same as in Battelles report (Neff 2000) and obtained a more balanced evaluation due to more reliable data (Figure 42).

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. In the 2008 survey, the NRRT data showed a reduction in mussel stress, which may reflect a reduction in exposure to PW related compounds. Whether this reduction in exposure of mussels to PW related compounds was brought about by an improved treatment system or other confounding factors is not entirely clear. Further biomonitoring investigations are required before assumptions concerning the treatment system can be made. Particularly since in 2008, the treatment system was only operational for part of the 6 week exposure.
Figure 42. Lysosomal membrane stability in mussels from WCM 2006 and 2008 (given as average Neutral Red Retention Time, NRRT) with the stress definitions defined in OSPAR (2007).

5 Conclusions and recommendations for future WCMs

Results show that deployed organisms from the whole investigated area contained moderate levels of hydrocarbons expected to originate from produced water. The levels were within the range found in previous years. This would likely also apply to other oil related compounds than PAH. Caged mussels did accumulate PAHs during the, field exposure especially 2- and 3-ring components. Bioaccumulation levels followed the expected gradient with distance to the discharge along the current axis in the area. However a minor change in the exposure pattern in the near field was observed, probably due to a new discharge point for some of the PW. The lowest levels were found in mussels from the pre-exposure group and in mussels caged at the reference location.

Apart from a reduced stress level observed at station 3, micronucleus formation in haemocytes of mussels caged close to the discharge generally followed the gradient along the current axis as was seen in 2006. It is known that micronucleus formation is commonly observed as a response to PAH exposure.

There were histological changes in mussels from two stations off Ekofisk. Mussels from stations 2 and 3 were significantly different measured by both neutral lipid accumulation and lipofuscin lysosomal accumulation, reflecting the PAH exposure levels.

For most PAH and 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 produced water to the fish from the two stations close to the discharge (ST 3 and 4).

PAH exposure data from both cod and mussels indicate a higher exposure in station 3, this might 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 is seen for some other parameters.
(e.g. gill lesions, Lipofuscin, Neutral lipid accumulation, pyrene hydroxylase, Cyp 1a) but not for micronucleus formation and lysosomal membrane stability).

The exposure was sufficient to induce elevated amounts of hepatic cytochrome P450 (CYP) 1A enzymes in the fish from station 3. There was no increase in plasma VTG-concentrations in males at any stations.

Results on hepatic DNA adducts indicate no differences between sampling locations.

PAH body burden, lysosomal membrane stability and micronucleus formation indicate a gradient in the signals with distance from the discharge however not as clear as in 2006. Some of the changes in “near field” response pattern between the two investigations are believed to be cause by the change in points of some of the PW to be discharged in 2008.

Observed differences in exposure signal from 2006 to 2008 are reflecting the actual discharges from the Ekofisk field in a good manner.

Despite technical problems during the start up of the new treatment technology, exposure data from the 2008 survey show that the overall discharge situation has improved, reflecting the environmental benefit of the new technology.

*Based on experience from WCM 2006 we propose the following recommendations for future WCMs:*

SOPs for CTD measurements should be closely followed by platform crew.

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. However, in case of a repeated investigation at the Ekofisk field, additional fish stations further away from the discharge could answer questions related to the geographical extent of conditions causing gill lesions and other effects.

Due to operational problems with the new treatment technology, the expected full environmental benefit potential of the CTour cleaning has not been biologically documented. A repetition of the survey once the CTour treatment is working optimally is therefore suggested.

Another argument for a repeated monitoring at the Ekofisk field is the tidal driven current system in that area. Since the dispersal of the discharge out of the near field is restricted, this area may be considered as a “North Sea worst case PW exposure scenario”. Lack of effects in the Ekofisk area could therefore render probable the potential for environmental impact at other fields in the region and hence reduce the need for similar monitoring at other fields (given that the chemical and volume properties of the other discharges are known).
There is generally a lack of information about the importance of the effect of particle density on the bioavailability of lipophilic organic pollutants. Since PW related compounds binds to biological surfaces like micro algae this may affect results generated by offshore monitoring programs. Knowledge about the importance of these issues could be obtained by introduction of laboratory grown micro algae into diluted PW in controlled experiments. This type of information would be helpful when interpreting monitoring results from different periods.

The development of the pyrene hydroxylase assay in mussel tissue proved to be a sensitive tool that was capable of discriminating between stations of high and low PAH exposure, as described by the PAH mussel body burden data. It would be useful in future monitoring programs to expand this technique to caged cod, since preliminary observations have shown the technique to be more sensitive in this species compared to the mussel.
6 References


Ericson, G. and Balk, L. (2000) DNA adduct formation in northern pike (Esox lucius) exposed to a mixture of benzo(a)pyrene, benzo(k)fluoranthene and 7H-dibenzo(c,g)carbazole: time-course and dose-response studies. Mutation Research 454, 11-20.


Ericson, G., Noaksson, E. and Balk, L. (1999) DNA adduct formation and persistence in liver and extrahepatic tissues of northern pike (Esox lucius) following oral exposure to benzo(a)pyrene, benzo(k)fluoranthene and 7H-dibenzo(c,g)carbazole. Mutation Research 47, 135-145.


Landahl, JT, BB McCain et al. (1990) Consistent associations between hepatic lesions in English sole (Parophrys vetulus) and polycyclic aromatic hydrocarbons in bottom sediment. Environ Health Perspect. 89: 195-203.


contaminants on CYP1A levels in North Sea dab (Limanda limanda). Aquatic Toxicology, 32: 189-209.


Voldum K, Andersen NO, Brun Henriksen I 2008. The CTour Process, an option to comply with "zero discharge legislation” in Norwegian waters. SPE-118012-PP


Aas E, Liewenborg B, Grosvik BE, et al. 2003 DNA adduct levels in fish from pristine areas are not detectable or low when analysed using the nuclease P1 version of the P-32-postlabelling technique Biomarkers. 8, 6: 445-460
7 Appendix list

7.1 Appendix A: Cruise report IRIS
7.2 Appendix B: Data report NIVA
7.3 Appendix C: Data report IRIS
7.4 Appendix D: Data report ITM, University of Stockholm
7.5 Appendix E: Data report – University of Vilnius
7.6 Appendix F: Survey report BiotaGuard
7.7 Appendix G: Gill histology