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New biomarkers

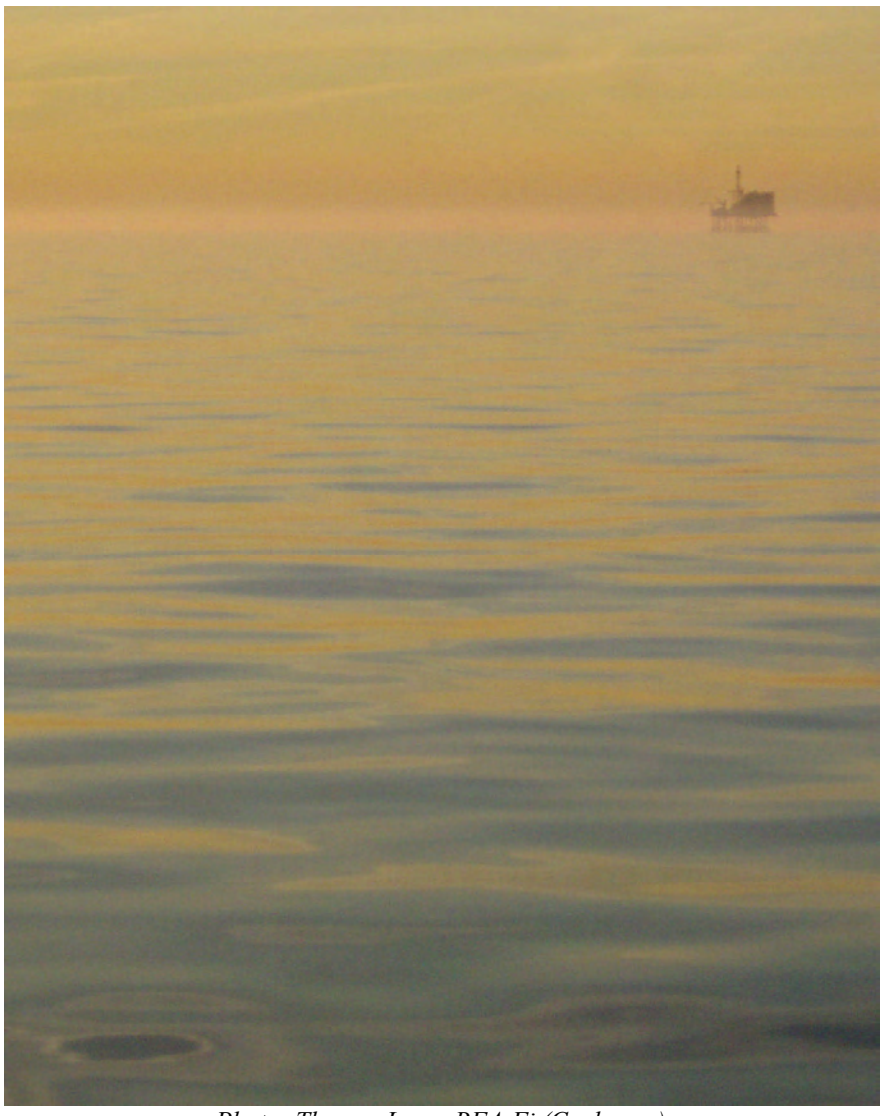

Photo: Thomas Lang, BFA-Fi (Cuxhaven)

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REPORT

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Abstract

Large volumes of produced water are released into the North Sea and there is still some uncertainty as to the environmental impacts. Following the BECPELAG workshop in 2001-2002, water column monitoring near selected platforms has been performed using a combination of chemical and biological effects methods. The aim of the present study was to investigate some new candidate biomarker methods. At the two chosen exposure levels, 4300- and 137000-fold diluted produced water, PAH concentrations in exposed blue mussels were similar to those found following deployment offshore. PAH bile metabolites in cod were not significantly increased. There was a clear response in micronucleus formation in mussel haemocytes. Other methods that could become useful for monitoring in the future included mixed xenobiotic defence (MXR) components in cod red blood cells, peroxisomal effects in mussel and cellular energy allocation in mussel. No exposure-related effects were seen for glutathione S-transferase in either species or MXR in blue mussel haemocytes.

4 keywords, Norwegian	4 keywords, English
1. biomarkør	1. biomarker
2. offshore	2. offshore
3. blåskjell	3. blue mussel
4. torsk	4. Atlantic cod

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New Biomarkers

Preface

This project was initiated to test out alternative methods to monitor environmental effects from effluents from offshore activities. OLF's contact person has been Ingunn Nilssen, Statoil. Work within the project has been performed by NIVA personnel, micronucleus analyses in collaboration with Goran Klobučar, Zagreb University (Croatia).

Oslo, 24.04.2006

Ketil Hylland

Contents

Summary

Produced water is discharged in substantial amounts into the North Sea, and in the Norwegian sector reached 143 million m^3 in 2004. Produced water discharges are immediately diluted in surrounding seawaters and reach close to background levels within a few kilometres downstream the platform. There have been few methods available to detect or monitor effects of contaminants on organisms in the water column, a contrast to the situation for benthic systems. This situation was one of the driving factors for the international workshop held 2001-2002 on effects of contaminants in pelagic ecosystems (BECPELAG). Following this workshop, selected methods were indicated as appropriate to monitor environmental effects in the water column from offshore activities. Most of those methods have been used during the last two years of water column effects monitoring. Results from monitoring studies have however indicated that many of the methods in use may not be sufficiently sensitive to detect effects at the exposure levels found offshore, even in the vicinity of oil platforms. The aim of this project was to test some relevant methods under conditions that would simulate produced water exposure near North Sea offshore platforms.

Exposure levels were chosen to reflect environmentally relevant concentrations, nominally 4300- and 137 000-fold dilutions of produced water from Statfjord B. Three replicate tanks were used for each treatment and the duration of the experiment was 5 weeks, in accordance with cage deployment for water column monitoring programmes.

The methods selected for study were cellular energy allocation (CEA) in blue mussel, P-glycoprotein induction (MDR/MXR) in both blue mussel and cod, a biochemical assay for peroxisomal proliferation in blue mussel, micronucleus formation (tested in 2004) in both species and, finally, isoenzyme-specific analyses of glutathione *S*-transferase activity (GST) in both blue mussel and cod.

Blue mussels accumulated PAHs to concentrations similar to those seen following deployment offshore in 2003 and 2004, confirming the environmental relevance of the exposure levels and period. As was the case for both WCM 2003 and 2004, there were no significant increases in the concentrations of PAH metabolites in the bile of cod held in the same tanks.

There were clear dose-dependent effects of the exposures on micronucleus formation in haemocytes from blue mussels. No such effects were seen with red blood cells from cod, which does not appear to be a good matrix for such studies. Micronucleus formation in haemocytes has shown similar results during earlier tests offshore and appears appropriate for inclusion in offshore monitoring programmes.

The results from this study also indicated that some methods should be further investigated for possible use in offshore monitoring. They include mixed xenobiotic defence (MXR) components in cod red blood cells, peroxisomal effects in mussel and cellular energy allocation in mussel.

No effects from the exposures were seen for glutathione *S*-transferase (GST) activities in either species, measured using CDNB and DCNB as substrates. Although seen as promising from the results in other studies, GST needs further research prior to routine application in monitoring programmes. Further work is also needed for MXR in blue mussel haemocytes before the method can be recommended for monitoring applications.

1. Introduction

Produced water is discharged in substantial amounts into the North-sea, and in 2004 reached 143 million m^3. The discharges are diluted in surrounding seawaters and reach near background levels within some kilometres downstream the platform. In the BECPELAG workshop the PAH concentrations for the Statfjord B were estimated from PAH concentrations in SPMDs and blue mussels in comparison with DREAM modelling (Utvik & Gärtner, in press). The dilution 500 meters from the platform was estimated to be about 10 000 for naphthalenes by the DREAM model, more dilution was estimated for her 2-3 ring PAHs, and less dilution for the 4-5 ring PAHs (Utvik & Gärtner, in press).

There have been few methods available to detect or monitor effects of contaminants on organisms in the water column, a contrast to the situation for benthic systems. This situation was one of the driving factors for the international workshop held 2001-2002 on effects of contaminants in pelagic ecosystems (BECPELAG; Hylland et al., 2002a). Following this workshop, a range of methods was indicated as appropriate to monitor environmental effects in the water column from offshore activities (Hylland et al., 2002b). Some of those methods have been used during the last two years of water column effects monitoring (Børseth et al., 2003; Hylland et al., 2004). Results from this monitoring has however indicated that some of the methods in use may not be sufficiently sensitive to detect effects at the exposure levels found offshore, even in the vicinity of oil platforms. The aim of this project was therefore to test some relevant methods under conditions that would simulate exposure in the North Sea, including the level of produced water components.

Exposure levels were chosen to reflect environmentally relevant concentrations, nominally 2000- and 100 000-fold dilutions of produced water. A range of relevant biomarkers were then evaluated and a limited set selected for testing with blue mussel and Atlantic cod. Conditions were set to mimic the exposure scenarios during WCM campaigns offshore, so the duration of the experiment was set to 5 weeks.

Methods not included in the 2004 programme, but may be appropriate are; cellular energy allocation (CEA), P-glycoprotein induction (MDR/MXR), assays for peroxisomal proliferation, micronucleus formation (tested in 2004) and isoenzyme-specific analyses of glutathione *S*-transferase activity (GST). Further description of the methods and why they may be relevant to detect effects of offshore effluents can be found below.

Cellular energy allocation (CEA) is a biochemical version of scope for growth, i.e. determination of energy reserves and current energy consumption, making it possible to estimate whether an organism is "overspending" for some reason or whether food intake is sufficient to generate more biomass (somatic growth or reproduction; de Coen & Janssen, 1997). The method thus requires chronic exposure to contaminants, i.e. an accumulation of physiological stress from the handling of contaminants (metabolism and repair processes).

The method has been used both with invertebrates and fish, but mainly the former. The method was used for cod, blue mussels, zooplankton and fish larvae during the BECPELAG workshop, but has otherwise mainly been used with freshwater organisms (de Coen & Janssen, 2003; Smolders et al., 2002, 2004). The results from those freshwater studies indicate that it is a robust technique that provides a trustworthy general background for the physiological status of organisms. Some comparative data indicates that it may be more sensitive than the more widely used scope for growth assay, at least in the crustacean *Neomysis* (Verslycke et al., 2004). No comparison has been performed between scope for growth and CEA in blue mussel.

Damage to DNA can result in adduct formation (covalent binding of reactive substances to DNA) and/or breaks in DNA. The latter can lead to breaks in the chromosome and thus "free-floating" bits of chromosomal DNA in the nucleus. Following the next cycle of cell division such DNA may be broken down or split off a separate unit within the cell, i.e. a micronucleus. The prevalence of micronuclei varies between species and tissues, but has been demonstrated to be contaminant-dependent in both blue mussels and fish (Brunetto et al., 1988; Dailianis et al., 2003; de Lemos et al., 2001; Rodriguez-Cea et al., 2003). Dose-dependent responses in micronucleus formation were seen for blue mussel during the WCM 2004 campaign. Nuclear DNA in cells exposed to clastogenic agents (that cause breaks in chromosomes) may be split, resulting in a small nucleus outside the true nucleus with some DNA. Micronucleus formation can theoretically be used with any dividing cells, but has most commonly been used with circulating cells such as haemocytes in mussels or blood cells from fish.

Peroxisomal proliferation (PP) is a cellular process that involves increased cellular volume of peroxisomes (as the name indicates) and induction of enzymes involved in lipid catabolism. There is also a link to oxidative stress through induction of e.g. catalase. Peroxisomal proliferation may lead to cancer and can be strongly induced following exposure to some agents/contaminants, including oil. As for oxidative stress, the process involves many components and it is not really sufficient to measure single responses. The methods used generally involve histochemical analyses of tissues (volume of peroxisomes) in addition to biochemical assays (palmitoyl CoA, catalase activity).

Several studies have shown peroxisomal proliferation in mussels following exposure to naphthalene (Cajaraville et al., 1990) or the water accommodated fraction (WAF) of oil (Cajaraville et al., 1992, 1997), although some studies found no clear response following exposure to WAF (Cancio et al., 1998). Peroxisomal proliferation was one of the endpoints investigated during the BECPELAG workshop, in which a clear response was seen for the enzyme palmitoyl CoA in caged mussels at all three locations in the plume compared to mussels at the reference location. An assessment of peroxisomal proliferation was included in WCM 2003 and partly in 2004 as part of the histopathology programme. Results were less clear in 2003 and 2004 compared to BECPELAG, possibly due to lower exposure levels.

Peroxisomal proliferation is a mechanism more or less specific to some groups of organisms; some rodents are good models for PP, but it is not well expressed in most other mammals. A similar situation has been observed for fish – PP is not expressed in all species (Pretti et al., 1998). From previous studies it is not clear whether peroxisomal proliferation is a relevant mechanism for e.g. gadoids Results from BECPELAG show that the receptor required for PP is expressed in cod, but there was no evidence of PP in caged fish. The main reason for this was probably methodological problems associated with the exceedingly fatty livers of gadiids (Cajaraville, personal communication). Further research is needed to clarify the usefulness of this method for fish. Peroxisomal proliferation is expressed in blue mussels and appears to respond to oil-related components.

Glutathione *S*-transferases (GSTs) are a family of isoenzymes that catalyse the binding of a water-soluble ligand to hydrophobic substances (endogenous or exogenous) destined for excretion. The standard methodology for GST analyses was one of the methods that showed a dose-dependent response during the BECPELAG workshop (using a general method that will cover most isoforms of the enzyme family), but there were no responses during the subsequent monitoring surveys. The constitutive overall GST activity, measured using a substrate that will reflect the activity of most isoenzymes (CDNB; 1-chloro-2,4-dinitrobenzene), is very high in tissues and any contaminant-induced changes must therefore be substantial before changes can be detected (George, 1994). This may be the reason why in a majority of studies no effects have been detected following exposure to contaminants in the field or in the lab (van der Oost et al., 2003). There are recent indications that GST may be particularly relevant in an offshore context (Danischewski et al., in press).

Some studies have however shown more contaminant-specific responses in fish, e.g. Machala et al. (1998) and Egaas et al. (1999). The presence of multiple isoforms in fish has recently been confirmed through characterisation of genes (Donham et al., 2005a, b).

GST is a dimeric protein and the major subunits in mammals belong to five different families: alpha, mu, pi, sigma and theta (Hayes & Pulsford, 1995). It is known that there is a non-inducible membrane bound form and a form in mitochondria, designated kappa (Pemble et al., 1996). Early studies indicated that the major isoform in fish could be pi or theta like isoforms (Dominey et al., 1991, Leaver et al., 1993, respectively), but recent results suggest that it differs from the enzymes found in mammals and may be given a separate name, i.e. rho (Konishi et al., 2005). The latter form was exclusively induced by CDNB, but other genes were also identified with different substrate specificities. Work by Donham et al. (2005b) support the presence of at least four isoform subunits in salmonid fish (alpha, theta, pi and mu). Studies using catalytic activity support the presence of different isoforms in fish as well as in mammals, i.e. different activities in different species towards substrates thought to be specific to one or more isoforms.

Multidrug resistance (MDR) was initially recognised as a mechanism by which cancerous cells could excrete chemotherapeutic chemicals, but has later also been investigated as an inducible system in both fish and invertebrate tissues as a response to contaminant exposure, termed MXR (multixenobiotic resistance; Kurelec, 1992; Smital & Kurelec, 1998). Recent studies have shown the complexity of the system, it includes a variety of P-glycoproteins that pump organic contaminants out of cells. The use of P-gps or MXR in aquatic ecotoxicology has recently been reviewed (Bard, 2000). MXR has been used in ecotoxicological studies with both mussels (Smital et al., 2000) and fish (Bard et al., 2005a, b). A range of substances have been shown to affect this system and components present in produced water would be expected to interact with MXR (Bard et al., 2002b; Smital et al., 2004). P-glycoproteins remove putative toxins from cells and environmental contaminants may affect the system through two main mechanisms: induction of MXR protein synthesis (thereby increasing excretion and decreasing toxicity of contaminants), inhibition of the MXR pumps (thereby decreasing excretion of contaminants and increasing the toxicity of other substances). The relative contribution of the two can be clarified through pharmacokinetic analyses using appropriate substrates and inhibitors. Both mechanisms may be relevant for produced water components.

2. Materials and methods

2.1 Exposure system

Produced water was collected at Statfjord B platform one week prior to start of the exposure. The produced water was transported in inert metal tanks each containing approximately 40 litres each. The tanks were air-tight and were kept at 10°C during storing and use. During exposure studies, metal tanks with produced water were left unopened until the previous tank was empty. The exposure system was set up at NIVAs research station Solbergstrand (MFS) and consisted of three replicate 1 m^3 tanks for each of three treatments with a continuous flow of 5 L/min seawater (from 20 m depth in the outer Oslofjord). The produced water was added in appropriate amounts to the influent seawater by means of peristaltic pumps.

The treatments (in triplicate) were: control (seawater only), low (nominally 100 000-fold diluted) and high (nominally 2 000-fold diluted). The exposure was started at 6th September 2005 and terminated on 23rd October 2005. Temperature varied between 11 and 16.5 °C and salinity between 19.5 and 29.5 during the exposure period (daily means). The salinity was lower and the variation in salinity larger than found in oceanic waters (offshore). Although there is evidence that the response of some biomarkers is affected by salinity (Hylland et al., 1998; Ringwood et al., 1998; Schlenk & El Alfy, 1998), it is not likely that this variation had a substantial influence on results from this study as all treatments had identical conditions.

2.2 Organisms – origin and maintenance

Blue mussels (5-6 cm) were purchased from Snadder & Snaskum, Trøndelag. They were maintained in continuous flow systems at NIVAs marine research station Solbergstrand (MFS) for two weeks prior to the start of experiments. Atlantic cod (200-400 g) were collected in the vicinity of Jomfruland (wild fish). The cod were maintained at MFS for at least two weeks prior to start of exposures. Cod were transferred to the tanks one week prior to start of the experiment. Blue mussels were held in stockings, 30 individuals in each tank. Twenty-six cod were held in each tank (with mussels). Oxygen was monitored on a regular basis, salinity and temperature monitored continuously.

2.3 Sampling

An overview of samples taken for analyses can be found below (Table 1).

Table 1. Overview of samples taken

species	tissue	analysis	Number of samples
Atlantic cod	blood	micronucleus	9 x 8
Atlantic cod	blood	MXR	9 x 8
Atlantic cod	liver	GST	9 x 8
Atlantic cod	bile	PAH metabolites	9 x 4
blue mussel	hemolymph	micronucleus	9 x 8
blue mussel	hemolymph	MXR	9 x 8
blue mussel	hepatopancreas	GST	9 x 8
blue mussel	hepatopancreas	AOX (PP)	9 x 8
blue mussel	soft parts	CEA	9 x 15
blue mussel	soft parts	PAH analyses	9 x 2 (pooled samples)

2.3.1 Blue mussels

Mussels were weighed and measured by their maximal diameter after exposure. The mussels for chemical analysis were then opened, the shell was removed and two pooled samples made from 5 animals each, from each aquarium. The mussels for CEA analysis were opened, the shell was removed and their soft tissue weight determined. The soft tissue put in cryo tubes with 1 mL Hendrickson's buffer (50% glycerol, 20 mM phosphate buffer (pH 7.4), 5 mM mercaptoethanol, 0.5 mM EDTA, and 0.02% bovine serum albumin) and snap frozen in liquid nitrogen. For the micronucleus assay, animals were carefully opened and hemolymph withdrawn from the anterior adductor mussel into an equal amount of PBS with 10 mM EDTA in a 1 mLsyringe and a 23G needle. A smear was prepared on a clean glass slide from a small drop of hemolymph. The smear was left for 15 minutes in a humid chamber at room temperature, allowing the haemocytes to adhere to the glass. Excess haemolymph was gently removed by placing the edge of the slide towards a filter paper. The haemocytes were fixed with 1% glutaraldehyde in PBS for 5 minutes, dried and the slide placed in the dark at 4°C prior until further processing for analyses. The remaining animals were carefully opened, and hemolymph collected from the anterior adductor mussel with a 1 mL syringe and a 23G needle, both pretreated with PBS. The hemolymph was mixed with 3 mL ice cold PBS and kept on ice until the MXR assay. Subsequent to this, the mussels were fully opened, and two small pieces (approximately 0.5 cm^3 each) cut from hepatopancreas. The pieces were put on separate cryo tubes, and snap frozen in liquid nitrogen.

2.3.2 Atlantic cod

Cod were weighed and measured after exposure. Blood was drawn from the caudal vein, using a hypodermic 5 ml syringe and a 23G needle, both pretreated with heparin. A drop of blood was applied to a clean glass slide and air-dried for micronucleus assay. 500 µl of blood was mixed with an equal volume of RPMI 1640 medium supplemented with 25% FBS and 20% DMSO. The sample was snap-frozen in liquid nitrogen. 1 ml of the remaining blood in each sample was then diluted 1:9 in ice-cold PBS in separate glass tubes, and kept on ice until the MXR-assay. The cod was then killed with a blow to the head and dissected. Bile was drawn from the gall bladder, completely emptying this, using a 1mL syringe and a 19G needle. The bile was transferred to eppendorf-tubes, and kept on ice and in the dark until analysis could be performed. Liver and gonads from each animal were removed and weighed. Two pieces of liver, approximately 1 cm^3 each, was cut from the anterior end, close to the main veins carrying blood to the liver. The two pieces were put on separate cryo tubes, and snap-frozen in liquid nitrogen.

2.4 Chemical analyses

2.4.1 PAH in blue mussels

The biological matter was homogenised, internal standards added and saponified. The PAHs were extracted with n-pentane and the extract dried over sodium sulphate. The extraction volume was reduced, the solvent exchanged to DCM, and the extracts cleaned by GPC and solvent then exchanged to cyclohexane. The samples were further cleaned by DMF partitioning and silica column elution. The extracts were analysed by GC/MS. The MS detector was operated in selected ion monitoring mode (SIM), and the analyte concentrations in the standard solutions were in the range 5-1000 ng/µL. The GC was equipped with a DB5, 30 m, column with (0.25 mm i.d. and 0.25 µm film thickness), and an inlet operated in the splitless mode. The initial column temperature was 60°C, which was raised isothermally to 310°C. The injector temperature was 300°C, the transfer line temperature was 280°C, the MS source temperature was 230°C and the column flow rate was 1.2 mL/min. Quantification of individual components was performed by using internal standards.

2.4.2 PAH-metabolites in bile from Atlantic cod

Approximately 20 mg of bile was diluted with water and treated with β-glucuronidase/arylsulfatase and incubated at 37 °C for 1 h. Triphenylamine was added to the samples as an internal standard, and methanol was added to give a final concentration of approximately 80%. After centrifugation, 25 µL of the supernatant was injected into a heated (30°C) HPLC column (Waters PAH C18 column with 5µm; 4.6×250mm). The mobile phase was a linear increase of acetonitrile in water, starting at 40 % acetonitrile. The flow was 1 mL/min. The analytes were detected by fluorescence at optimised wavelengths during the elution. The excitation/emission wavelengths were 256/380 (1-hydroxyphenanthrene), 346/384 (1-hydroxypyrene), 300/360 (triphenylamine) and 380/430 (3-hydroxy B[a]P).

2.5 Biomarker analyses

2.5.1 Cellular energy allocation (CEA)

Samples were thawed and transferred to tubes containing 500 µL homogenisation buffer and homogenised with a rod-type homogeniser with rotating knives. The total volume was adjusted to 5 mL with homogenization buffer. 200 µl of homogenate was taken out for lipid analysis, and 300 µL each for protein and carbohydrate analysis. These samples were stored at -80°C until they were analysed. The remaining volume was diluted with homogenization buffer to an appropriate concentration (25 times dilution for blue mussel) and immediately used for the ETS analysis.

2.5.2 Activity of the Electron Transfer System

All work was performed on ice for this analysis. 100 µL Buffered Substrate Solution (BSS) was pipetted into microwells, 50 µl diluted homogenate was added followed by 50 µl NAD(P)H solution. To initiate the reaction, 100 µL of freshly prepared INT was added. A change in absorbance was immediately measured at 490 nm at 20°C during 10 min (one reading each 15th second).

2.5.3 Measurement of lipid content

This procedure was as follows: 200 µL homogenate was thawed, 500 µL chloroform added and the solution thoroughly mixed by vortexing. 500 µL methanol and 250 µl distilled water were then added and the solution again mixed by vortexing. The preparation was then centrifuged at 14000 rpm for 5 minutes producing two phases – 100 µL of the chloroform (bottom) phase was transferred to a glass tube and 500 µL concentrated H_2SO_4 was carefully added and mixed. A standard dilution series was prepared from tripalmitine in chloroform; 500 µL of H_2SO_4 was added and the standard solutions mixed. All preparations were then charred at 200°C for 15 min. The solutions were then cooled to room temperature and water added, following which 200 µL of each sample or standard was transferred to the wells of a microtiter plate and total lipid content was quantified photometrically at 340 nm.

2.5.4 Measurement of total protein- and carbohydrate content

Three hundred µL of sample was thawed, 100 µL 15% Trichloroacetic acid (TCA) added and the preparation mixed by vortexing and centrifuged at 14000 rpm for 4 min. The supernatant was transferred to new eppendorf tubes and stored on ice for carbohydrate analysis. 100 µL 5% TCA was added and the preparation mixed by vortexing and centrifuged at 14000 rpm for 4 min. Supernatants were combined in eppendorf tubes and kept on ice until analyses.

Protein measurement

The remaining pellet from the centrifugation above was resuspended in 500 µL 1N NaOH and the preparation mixed by vortexing. The preparation was incubated at 60°C for 30 min. 300 µL 1.67 N

HCl was then added and the preparation mixed by vortexing. A standard curve was prepared from 1.5 mg/mL bovine immunoglobulin. Protein was determined as described by Lowry et al. (1951) using a method adapted for plate reader. 10 µL of appropriately diluted sample or standard was pipetted into microwells in 96-well plates, 25 µL of reagent A was then added to each well, followed by 200 µL of reagent B. The microtiter plates were then agitated gently for 5 seconds and then incubated on the bench for 15 minutes, following which absorbance was measured at 750 nm.

Carbohydrates

Standards were prepared from dilutions of 1.5 mg/mL glycogen; 50 µL of sample or standard was pipetted into microwells; 200 µL H_2SO_4 was carefully added to each; 50 µL 5% phenol was added to each and the plates incubated for 30 min at room temperature. The absorbance was then measured at 490 nm. The samples had to be diluted before measurement, to avoid too high absorbance (and hence samples outside the range of the standard curve). All carbohydrate samples were diluted 20 times.

2.5.5 Micronucleus formation

Haemocytes were fixed in 1% glutaraldehyde in PBS for 5 minutes. This step was skipped for blood smears. The slides were then rinsed with PBS, stained with 1 µg/mL *bis*-benzimide 33258 (Hoechst) for 5 minutes, and rinsed with distilled water. They were then mounted in glycerol-McIlvaine buffer (1:1) and covered with a cover slide. The slides were kept in the dark at 4°C until analysis.

The cells were counted using a fluorescence microscope (1000x, excitation filter 355 nm, barrier filter 465 nm). For haemocytes, a minimum of 200 cells per slide were counted. Due to low cell densities, fewer cells (min 90) were counted on some of the slides. For erythrocytes, a minimum of 2000 cells per slide were counted. Only intact cells with distinct nuclear and cellular membranes were scored. Identification of micronuclei were performed according to the following criteria: sharp contour, diameter less than one third of cell nucleus, colour and texture similar to nucleus, no contact with nucleus. To verify some of the micronuclei found, pictures were taken and digitally manipulated (picture colour layers, contrast and brightness) to check whether the criteria were fulfilled.

2.5.6 Peroxisomal CoA oxidase (AOX)

The digestive glands were individually homogenised in a Potter-Elvehjem homogeniser using TVBE buffer (1 mM sodium bicarbonate, 1 mM EDTA, 0.1% ethanol, and 0.01% Triton X-100), pH 7.6, in a proportion of 4 mL buffer per gram of tissue. The samples were then centrifuged at 500 x *g* at 4°C for 15 minutes to remove debris. The supernatants were split into aliquots and stored at -80°C until the measurements were performed.

Palmitoyl-CoA solution and samples were thawed on ice. Reaction media was thawed in a water bath, 25°C, 10-15 mins before the assay was started. The samples were diluted 10 times in ice cold TVBE buffer. 100 µl diluted sample was added to one aliquot of reaction medium (1.9 mL) and incubated in a water bath, 25°C, for 5 minutes. Two aliquots (1 mL) of the reaction medium were then placed in spectrophotometer cuvettes. Substrate was added to one of the cuvettes (10 µL 3mM palmitoyl-CoA) and the solution mixed thoroughly. The cuvettes were placed in a spectrophotometer and absorbance of the sample was read against the blank for 5 minutes following an incubation period of 4 minutes. Protein was determined according to Lowry et al. (1951) with bovine gamma globulin as standard.

2.5.7 Glutathione *S*-transferase (GST)

At the time of sample preparation, the tissue was thawed on ice, weighed and homogenized with ten strokes in a Potter-Elvehjem glass/Teflon homogenizer in 4:1 (w/v) of ice cold 20 mM sodium phosphate buffer, pH 7.4, containing 0.15 mM KCl and 0.1 mM phenylmethyl-sulfonylfluoride. The homogenate was centrifuged at 10 000 *g* for 20 mins and the supernatant stored at -80°C.

Glutathione-S-Transferase was measured essentially as described by Habig et al. (1974) with microtiter plate modifications described by Stephensen et al. (2002).The analysis measures the change in substrate concentration (CDNB or DCNB) over time. This is then used to calculate enzyme activity, generally proportional to the concentration. For CDNB, 50 µL of 50-fold diluted sample was added in triplicates to microtiter plates and then added 200 µL of 0.1M sodium phosphate buffer containing 2 mM CDNB and 1 mM GSH. For the DCNB substrate, 4 mM DCNB and 5 mM GSH was added to 25 times diluted samples in a similar manner. Extinction coefficients of CDNB (9600 M^{-1} cm^{-1}) and DCNB (8500 M^{-1} cm^{-1}) were used to calculate GST activity.

2.5.8 Multixenobiotic response (MXR)

All cells have membrane proteins that pump out substances from the interior, commonly referred to as p-glycoproteins (pg-p), MDR (mammalian cells) or MXR (other organisms). The concentration and activity of such protein pumps can be induced by various substances (e.g. some environmental contaminants). The pumps can also be inhibited by other chemicals, so-called chemosensitisers, which also include environmental contaminants (Smital et al., 2003). MXR activity is most commonly measured using fluorescent dyes that are substrates for MXR (i.e. will be pumped out) in combination with agents that are known to inhibit the pump, i.e. cyclosporin A (used with blue mussel here) or verapamil (used with Atlantic cod). Fluorescence is then measured for a specific number of cells with or without inhibitor. Fluorescence without inhibitor will give information about chemosensitising effects, fluorescence with inhibitor about specific modulation of the pumps and the ratio between the two an integrated value for effects on MXR.

The cell density in each of the blood (cod) and hemolymph (blue mussel) was determined using a coulter counter. The samples were then diluted in phosphate buffered saline (0.1 M PBS, pH 7.8; 8.5 mL NaH_2PO_4 (stock 0.2 M dissolved in distilled water); 91.5 ml Na_2HPO_4 (stock 0.2 M dissolved in distilled) in 100 ml water and adjusted for the salinity in the sampling area with 2.4% w/v NaCl) to obtain a final cell density of 200000 cells/mL. The diluted cell suspension was applied to the wells of a 96-well microplate (Falcon) with 200 µL per well (corresponding to 40000 cells per well). Samples from each individual were applied in 8 replicates. The microplates were then left for 24 hours at 15ºC to allow for the cells to settle and adhere to the bottom of the wells.

A sample of cell suspension (100 µL) was carefully removed from the top layer of each well. Fifty µL of the fluorescent MXR-substrate rhodamine B (Sigma Aldrich) was applied to each well to obtain a final concentration of 0.1µM rhodamine B in wells with mussel haemocytes and 1µM rhodamine B in wells with cod blood cells. To 4 of the 8 replicates for each individual sample, 50 µL of cyclosporin A (MXR-inhibitor) was applied, to the other 4 wells, 50 µL PBS was added. For mussel haemocytes the inhibitor was cyclosporin A, in a final concentration of 5 µM. For cod blood cells, the inhibitor was verapamil, in a final concentration of 1 µM. The plates were incubated at room temperature and in the dark on an orbital shaker set at 300 rpm for 30 mins. The cells were washed by removal of 100 µl of fluid from the top layer of each well, and adding of 100 µL PBS. Both steps conducted carefully so as not to disturb the cell layer at the bottom of the well. This washing step was repeated once. All fluid was then carefully removed from each well, and 100 µL triton x-100 (0.1% in PBS) added to lyse the cells. Fluorometric readings were performed on the plate reader CytofluorTM 2300. Excitation and emission wavelengths were 530 nm and 590 nm, respectively.

MXR activity was expressed as fluorescence in treated cells relative to fluorescence in control cells.

2.6 Statistical analyses

Differences between treatment groups were assessed using a nested ANOVA model with aquaria (3 replicates for each treatment) nested within treatment. All variables were tested for homogenous

variances using Levene's test. If it was not possible to achieve homogenous variances by transformation, variables were compared using Kruskal-Wallis' test (without nesting).

Wherever no effects of aquaria were found, results are presented as box plots of raw data (median, quartiles and 10/90 percentiles). If aquaria contributed to explaining a significant component of variance in the ANOVA model, results are presented as least squares means from the nested model (means and 95% confidence intervals).

3. Results and discussion

3.1 Exposure

The dosing system was set to deliver a 2000-fold dilution of produced water to three aquaria and a 100 000-fold dilution of produced water in three aquaria. An additional three aquaria received no produced water and served as reference. The dilutions were chosen to simulate 5 times the concentrations at 500 m distance from Statfjord B and 1/10 of that same concentration. DREAM modelling estimated the PAH concentration to be approximately 10 000 times diluted from the PW discharges to 500 meters from the platform (Utvik and Gärtner, *in press*), consequently a dilution of nominally 2000-fold and 100 000-fold of produced water were chosen as exposure concentrations. By back-calculation from the amount of produced water pumped in the tanks, the true dilutions were 1/4300 and 1/137000.

An aliquot of the produced water used in the exposure studies was analysed for PAHs, and the results are given in appendix A. The estimated PAH concentrations based on a dilution factor of 4300 and 137 000 for the exposure concentrations are given in the same appendix. Weekly drawn SPE-extracts from water of one of the highest dosed tanks were also analysed for PAHs, although most of the compounds were expected to be below the limit of detection for the analytical method. The concentrations measured in the SPE-extracts match fairly well with expected concentrations for the 1/4300 dilutions regarding the lighter PAH compounds that were present above the detection limit. The concentrations of most of the heavier PAH components were below the detection limit (see appendix A for details).

The PAH concentrations in the seawater for the highest exposure were also estimated from the mussel tissue PAH concentrations; assuming a steady state situation as described in detail in the work by Neff and Burns (1996). The estimated PAH concentrations are given in appendix A. For the majority of the compounds, the estimated PAH concentrations in the water corresponded well with the predicted water concentration based on calculation by the dilution factor of 4300 of the produced water.

3.1.1 PAH in blue mussel

Blue mussels held in tanks receiving 2000-fold or 100 000-fold dilutions of produced water (nominal concentrations) accumulated PAHs to higher concentrations than in mussels held in reference tanks (Table 2, Figure 1). A full list of PAHs determined can be found in Table 2. Differences between treatments were significant for some PAH components, e.g. C3 napthalenes (Kruskal-Wallis, $p = 0.004$), C2 phenanthrenes (Kruskal-Wallis, $p = 0.003$) and EPA sum PAHs (Kruskal-Wallis, $p = 0.01$).

Table 2. Results from analyses of PAH in blue mussels; pooled samples of 5 individual mussels were used for each analysis; NAP: naphthalene, ACNLE: , ACNE:

	1/4300-fold dilution						1/137 000-fold dilution						reference					
	1	1	2	2	3	3	4	4	5	5	6	6	7	7	8	8	9	9
NAP	<1	<1	<1	<1	1.3	1.3	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
NAPC1	4.9	5.2	<2	4.2	5.9	6	4.2	3.5	3.4	3.3	2.5	3.1	4.4	3.1	2.1	<2	9.1	10
NAPC2	9.6	9.1	7.9	6.3	8	7.9	8.4	6	7.4	7.4	6.2	5.7	5.3	4.5	4.9	3.9	16	14
NAPC3	19	16	17	15	17	15	9.8	11	13	10	13	9.1	11	9.1	8.8	10	15	14
ACNLE	<0.5	0.51	0.56	<0.5	<0.5	<0.5	<0.5	<0.5	0.51	<0.5	<0.5	<0.5	0.59	<0.5	<0.5	<0.5	0.72	0.55
ACNE	<0.5	<0.5	<0.5	<0.5	0.79	0.6	0.51	0.67	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
FLE	1.5	1.5	1.3	1.3	1.6	1.4	1.4	1.3	1.5	1.4	1.2	1.6	1.3	1.5	0.84	1.2	1.5	1.3
ANT	0.79	0.78	0.62	0.6	0.88	0.8	0.65	0.64	0.61	0.63	0.61	0.61	0.61	0.53	<0.5	<0.5	0.57	0.56
PA	4.3	4.2	3.2	3.2	5.1	5.1	3.7	3.6	3.2	3.2	3.5	3.5	2.7	2.7	2.7	2.6	2.9	2.9
PAC1	5.1	6.1	4.2	4.3	5.4	4.9	2.9	2.8	2.9	2.9	3.1	3.3	3.3	2.7	2.6	2.6	3	3
PAC2	9.6	9.5	7.6	6.6	7.7	8.4	5.4	4.7	5.5	6	6.5	8.8	4.8	4.8	4.6	5	5.8	4.7
PAC3	5.1	4.6	4.5	3.7	4.9	4.8	2.4	3.4	3.3	2.8	2.5	4.9	3.5	<2	2.7	2.3	2.9	3
DBTHI	0.6	0.58	<0.5	<0.5	0.67	0.63	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
DBTC1	2.2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
DBTC2	5.8	4.1	4.1	4.4	4.7	4.2	2.9	4	3.6	3.2	3.6	3.7	3.5	2.5	2.7	2.5	4.6	4.2
DBTC3	7.1	7.5	6.4	6	5.9	5.8	3.8	5.4	4.4	4.2	4.2	6.5	3.4	3.7	3.4	2.7	5	3.9
FLU	4.2	4.1	2.7	2.6	5.3	5.2	2.9	2.9	2.8	2.7	3.3	3.3	2.5	2.5	2.2	2.3	3.1	3.1
PYR	2.9	2.8	2.3	2.1	4.1	4	2.6	2.5	2.5	2.4	2.8	2.8	2	1.9	1.9	1.9	2.5	2.4
BAA	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
CHRTR	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
BBF	<0.5	<0.5	0.54	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
BJKF	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
BEP	1.2	1.1	0.84	0.77	0.87	0.84	0.62	0.73	0.94	0.99	0.78	0.75	0.71	0.71	0.54	0.57	0.75	0.72
BAP	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
PER	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
ICDP	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
DBA3A	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
BGHIP	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
sum PAH	83.9	77.9	63.8	61.1	80.1	76.9	52.2	53.1	55.6	51.1	53.8	57.7	49.6	40.2	40.0	37.6	73.4	68.3
16 EPA	13.7	13.9	11.2	9.8	19.1	18.4	11.8	11.6	11.1	10.3	11.4	11.8	9.7	9.13	7.64	8.0	11.2	10.8
3-ring	6.59	6.99	5.68	5.1	8.37	7.9	6.26	6.21	5.82	5.23	5.31	5.71	5.2	4.73	3.54	3.8	5.69	5.31
4-ring	7.1	6.9	5	4.7	9.4	9.2	5.5	5.4	5.3	5.1	6.1	6.1	4.5	4.4	4.1	4.2	5.6	5.5

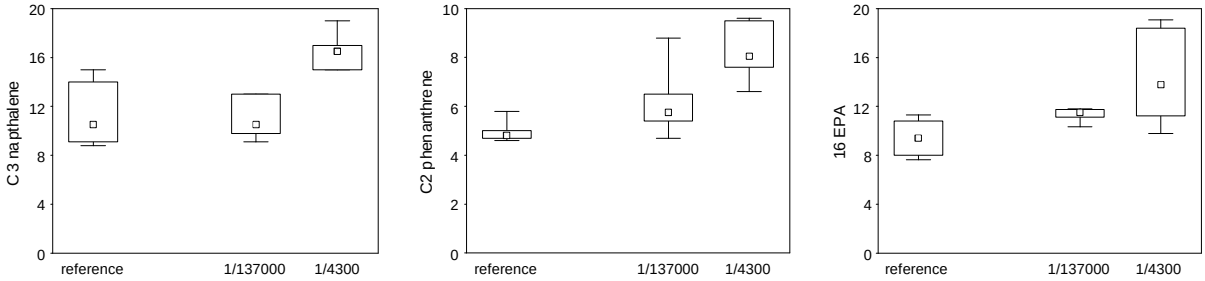

Figure 1. Concentrations of C3 napthalenes, C2 phenanthrenes and 16 EPA PAHs in blue mussels held in tanks receiving the indicated treatments; median (square), quartiles (boxes) and 10/90 percentiles (whiskers) are shown.

Background concentrations in the mussels used in this study were between those found during BECPELAG and WCM 2003/2004, within the range assumed to be background concentrations along the Norwegian coast. Mussels came from the same source in this study as for WCM2003/2004 and there is no obvious explanation for the slightly higher concentrations seen here. The mussels held in the tanks receiving 4300-fold diluted produced water accumulated napthalenes to similar concentrations as those found in mussels following cage deployment under WCM 2004, but the concentration of EPA 16 PAH was higher in mussels exposed during this study compared to WCM 2003, similar to WCM 2004 and lower than BECPELAG.

3.1.2 PAH-metabolites in cod

There were no significant differences between concentrations of the three metabolites 1-OH-phenanthrene, 1-OH-pyrene and 3-OH-BaP between the three groups (Figure 2). There was however a trend towards increased concentrations in produced water exposed groups for 1-OH-phenanthrene and 1-OH-pyrene. There was no significant accumulation of PAH metabolites in the bile of cod exposed to diluted produced water during the exposure period although there was a tendency towards increased three- and four-ring metabolites in the two exposed groups compared to the reference group. The fish were starved for two days prior to sampling to ensure sufficient bile would be available.

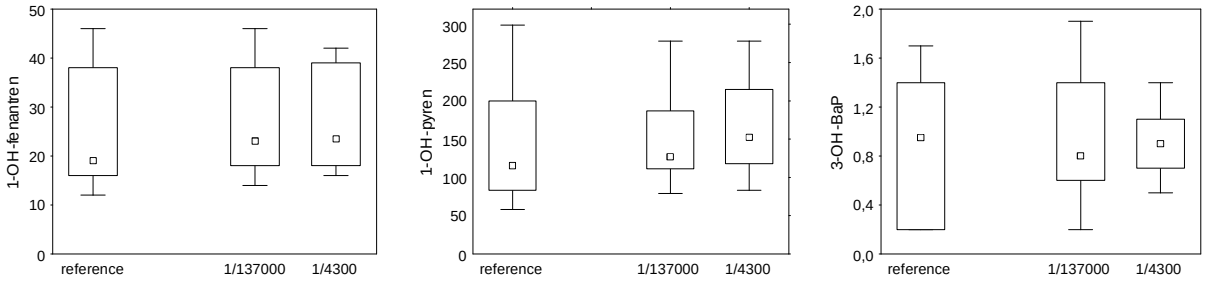

Figure 2. Concentrations of 1-OH-phenanthrene, 1-OH-pyrene and 3-OH-BaP (µg/g) in bile of cod held in tanks receiving the indicated treatments; median (square), quartiles (boxes) and 10/90 percentiles (whiskers) are shown. Note that values below detection limit for 3-OH-BaP have been set to 0.2 (half the detection limit) for this figure.

The results indicate that the exposure of cod in the tanks to PAHs was low. Median concentrations were slightly higher for 1-OH-pyrene in the two treated groups compared to the reference group, but the concentrations were not significantly different. The concentration of PAH-metabolites in the bile of reference group cod was within the background range observed for cod at uncontaminated locations elsewhere along the Norwegian coast (Green et al., 2004; Ruus et al., 2003).

3.2 Cellular energy allocation

Cellular energy allocation is an endpoint that includes energy resources of an organism (carbohydrates, lipids and protein) as well as a measure for cellular respiration. Whole blue mussel were analysed in the present study. There were obvious differences between treatments (Figure 3), but also between replicate tanks (results not shown). Results were analysed using a nested ANOVA, which made it possible to separate the two sources of variability in the statistical analysis. There is no obvious reason why there should be differences between replicate tanks within each treatment. Neither produced water exposure nor environmental parameters (temperature, salinity) were found to differ. The results indicate that the method is very sensitive, but also that further investigation into possible sources of variability is needed.

The figures show the effect of treatments from a nested ANOVA model with tank nested in treatment (variability due to differences between tanks has been taken account of). Mussels kept in tanks with 1/4300 dilution of produced water had highest concentrations of carbohydrates ($p = 0.002$), whereas the reference group had more lipid than both the produced water exposed groups ($p < 0.0001$). There were no significant differences in protein content between the groups.

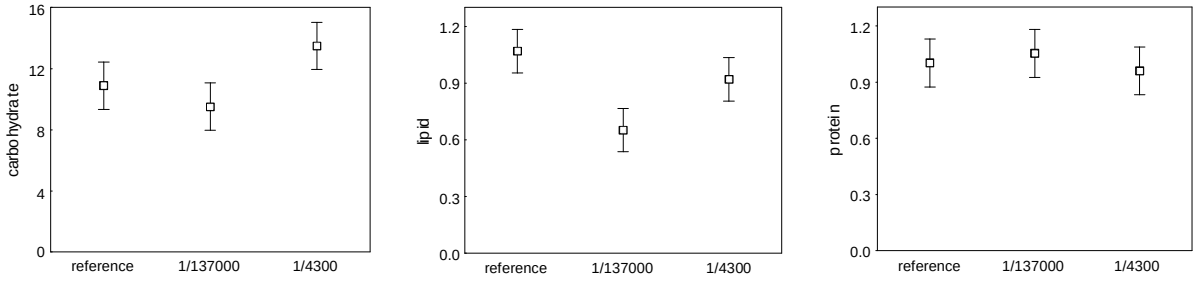

Figure 3. Least squared means from nested ANOVA models (tank, treatment) of carbohydrate (left), lipid (middle), protein (right) for blue mussels held in tanks receiving the indicated treatments; mean (squares) and 95% confidence intervals (whiskers) are shown.

Although the results for the composition of blue mussels indicate some effects of the treatment, it is not clear why there should be impacts at the lowest exposure level and not the highest. The clearest result for the three components was seen for lipid, which was lower in both exposed groups compared to the reference group. This result suggests an effect from the treatment and an effect on lipid storage is in accordance with results seen by Smolders et al. (2002) for zebra mussel. Smolders et al. (in press) assessed CEA in blue mussels during the BECPELAG workshop. Results from that study also suggested decreased lipid levels at all stations in the plume (500 m, 2 000 m, 10 000 m) compared to mussels from the reference location, but differences were not statistically significant.

Energy equivalents from the three components listed above can be summed to total available energy resources. In this case carbohydrates contributed a major component to this value and the groups were significantly different (Figure 4- left; $p = 0.002$ in a nested model). There were however no differences between groups for cellular respiration (Figure 4 – right).

Cellular energy allocation is generally measured as a change from start until the end of the exposure, but can also be assessed by comparing energy equivalents and respiration of exposed groups to the same in a reference group (as done here).

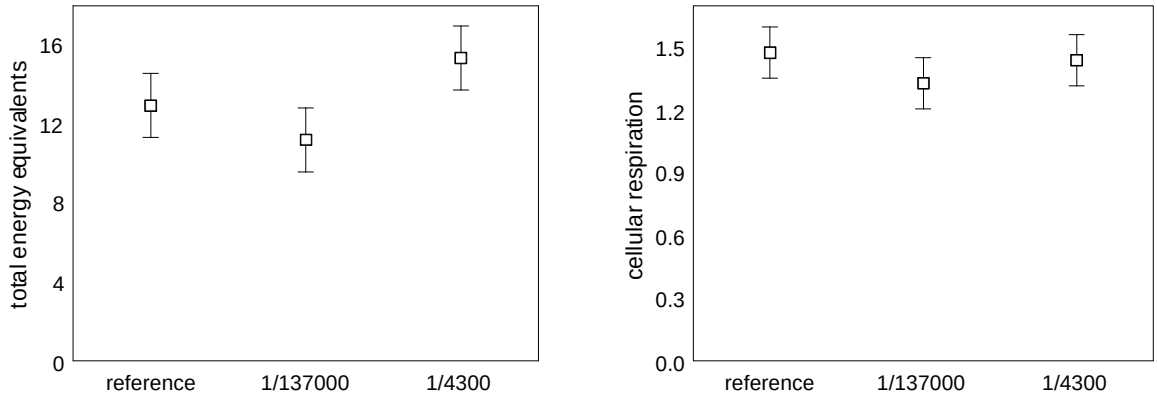

Figure 4. Least squared means from nested ANOVA model (tank, treatment) of summed total energy equivalents (left) and cellular respiration (right) for blue mussels held in tanks receiving the indicated treatments; mean (squares) and 95% confidence intervals (whiskers) are shown.

Results for components of cellular energy allocation (CEA) were confusing. Although there were differences between groups, trends were not consistent. There did however appear to be an influence of the exposure on lipid concentration in the mussels. This is only the second study with blue mussel and further work is needed to understand the kinetics of CEA, as compared to scope for growth, in this species. The method appears to be sensitive to effects of produced water components and has the advantage of being amenable to direct translation to ecologically relevant impacts (growth and reproduction).

3.3 Micronucleus formation

3.3.1 Blue mussels

There were obvious differences between the experimental groups in the level of micronucleus formed (Figure 5). There were significant differences between the groups in a non-parametric ANOVA (KW, $p = 0.001$), but only the group that had received 1/4300 diluted produced water had micronucleus values that differed from those of the reference group (M-W, $p < 0.05$).

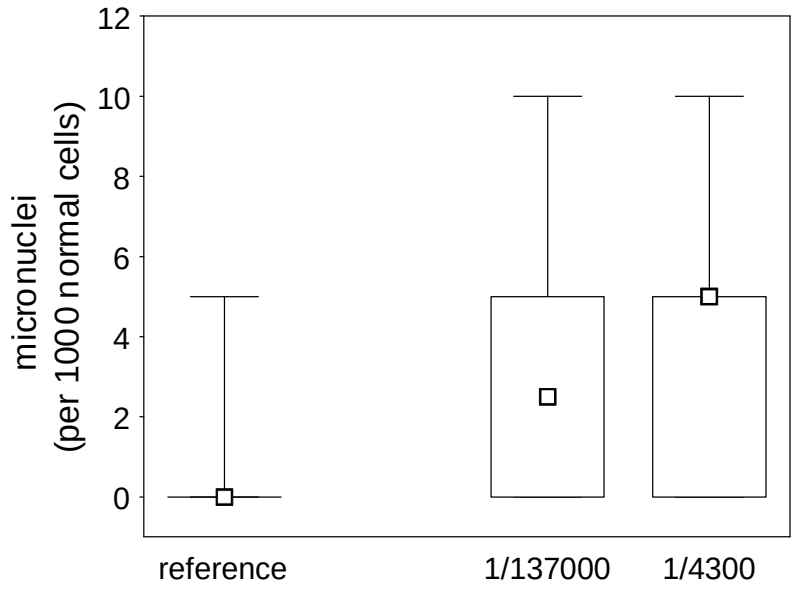

Figure 5. Micronucleus formation in haemocytes from blue mussels held in tanks receiving the indicated treatments; median (square), quartiles (boxes) and 10/90 percentiles (whiskers) are shown.

3.3.2 Atlantic cod

There were no significant differences between the groups in formation of micronucleus in red blood cells of cod (Figure 6). In fact, the reference group had more fish with expressed micronuclei than the two exposed groups. This result was due to two individuals with a high incidence of micronuclei. Since earlier results with kidney cells indicated possible produced-water related effects during WCM 2004 (Hylland et al., 2005), this result suggest that red blood cells in cod is not an optimal tissue for the determination of produced-water related generation of micronuclei. The fish were kept in the same tanks as the mussels for which results were reported above.

There was a clear dose-dependent effect of produced water exposure on micronucleus formation in haemocytes from blue mussels. This is in accordance with results found during the 2004 water column monitoring campaign (Hylland et al., 2005). There was no indication of the effect on micronucleus formation in cod blood cells, in contrast to results from WCM 2004 where a trend was seen (not statistically significant in that study either). The two studies can not be compared directly since analyses during WCM 2004 were done on kidney samples.

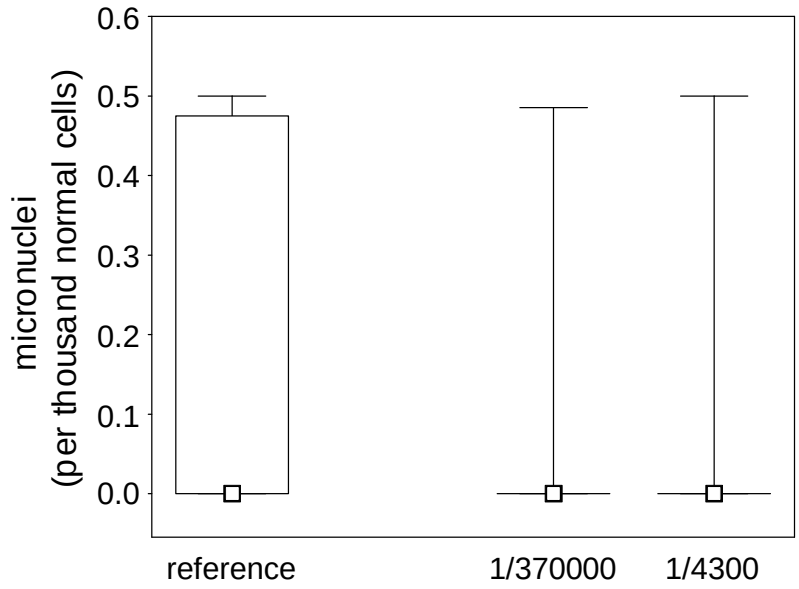

Figure 6. Micronucleus formation in red blood cells from cod held in tanks receiving the indicated treatments; median (line), quartiles (boxes) and 10/90 percentiles (whiskers) are shown.

3.4 Peroxisomal acyl-CoA oxidase (AOX)

There were no significant differences in the activity of peroxisomal acyl-CoA oxidase in hepatopancreas from mussels that received the three treatments in this study (Figure 7).

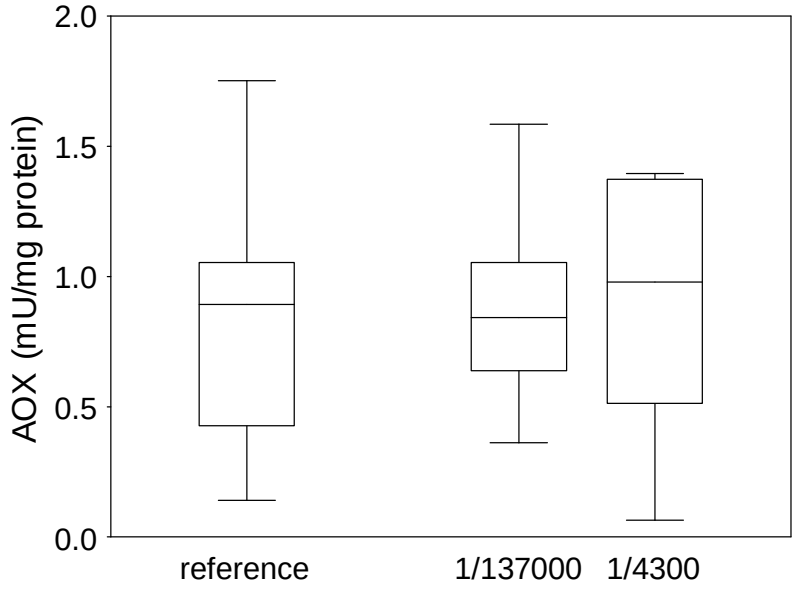

Figure 7. Peroxisomal acyl-CoA oxidase (AOX) in hepatopancreas from blue mussels held in tanks receiving the indicated treatments; median (line), quartiles (boxes) and 10/90 percentiles (whiskers) are shown.

The median AOX activity was higher in the treatment group receiving 4300-fold diluted produced water, but the large variability within each group, especially the group receiving the highest

concentrations of produced water, precluded statistically significant differences. Although no clear differences were seen between groups, the results for the highest exposure group suggest that there may be effects and that with further refinement of the method and a larger sample size AOX may provide a useful tool for oil-related effects. This has indeed been indicated in earlier studies by Cajaraville and her research group (Cajaraville et al., 1992, 1997; Cancio et al., 1998). An important difference between past studies and the present study is the lower concentration of contaminants used in the latter. The current study reflects an environmentally relevant exposure in a produced water plume whereas other studies are more relevant to concentrations that will be found during acute spills.

3.5 Glutathione *S*-transferase (GST)

3.5.1 Blue mussels

There were no significant differences in GST activity in hepatopancreas from mussels exposed to diluted produced water measured using either CDNB or DCNB as substrates (Figure 8). There was a tendency for decreased CDNB activity in mussels exposed to produced water and really no differences between groups for DCNB.

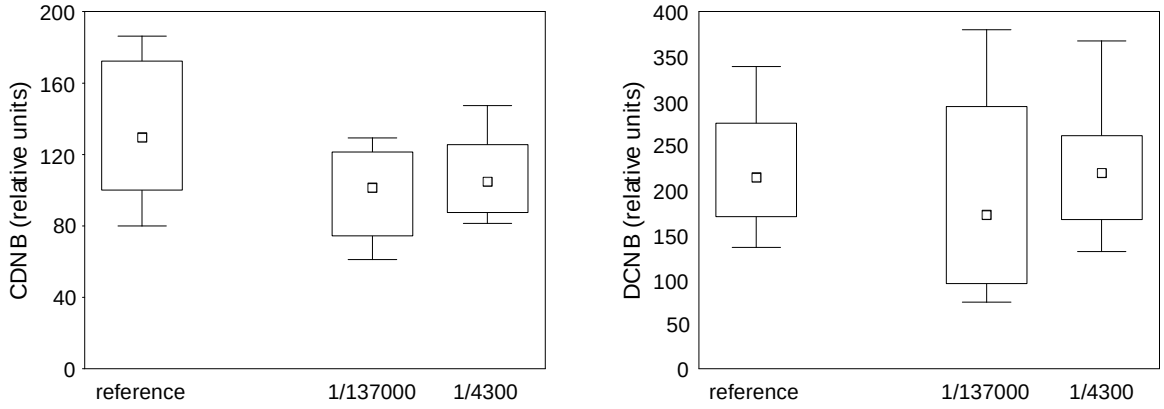

Figure 8. Glutathione *S*-transferase activity in blue mussel hepatopancreas measured as CDNB activity (left) or DCNB activity (right); the mussels were held in tanks receiving the indicated treatments; median (squares), quartiles (boxes) and 10/90 percentiles (whiskers) are shown.

3.5.2 Atlantic cod

There were no significant differences in hepatic GST activity between cod exposed to diluted produced water and the reference group (Figure 9). Many individuals in all groups had values close to the detection limit for the method. The largest number of individuals with high values of DCNB was found in the group exposed to 2000-fold diluted produced water.

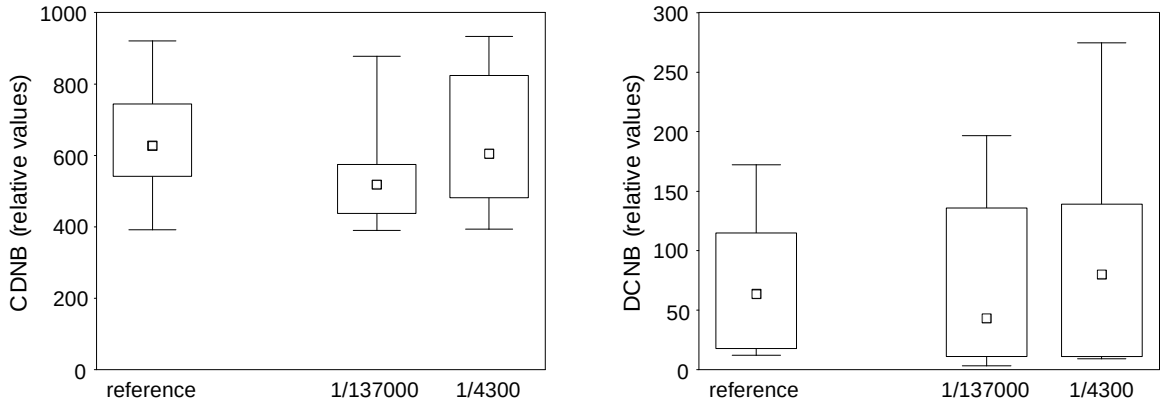

Figure 9. Hepatic glutathione *S*-transferase activity in cod measured as CDNB activity (left) or DCNB activity (right); the cod were held in tanks receiving the indicated treatments; median (squares), quartiles (boxes) and 10/90 percentiles (whiskers) are shown.

Earlier observations of GST induction caused by produced water effluents were not reproduced in this study. If anything, there was a decrease in GST, as determined using CDNB, in mussels exposed to diluted produced water. Although not different in ANOVA, there were still differences between the groups in the number of individuals that appeared to respond to 4300-fold diluted produced water compared to the reference group (a larger number of individuals responded). There appears to be a proportion of low-responding individuals in all three groups with regard to this GST endpoint.

3.6 Multixenobiotic response (MXR)

3.6.1 Blue mussels

There were no significant differences in Rhodamine B retention (P-glycoprotein activity) without or with inhibitor in haemocytes from mussels exposed to diluted produced water (Figure 10).

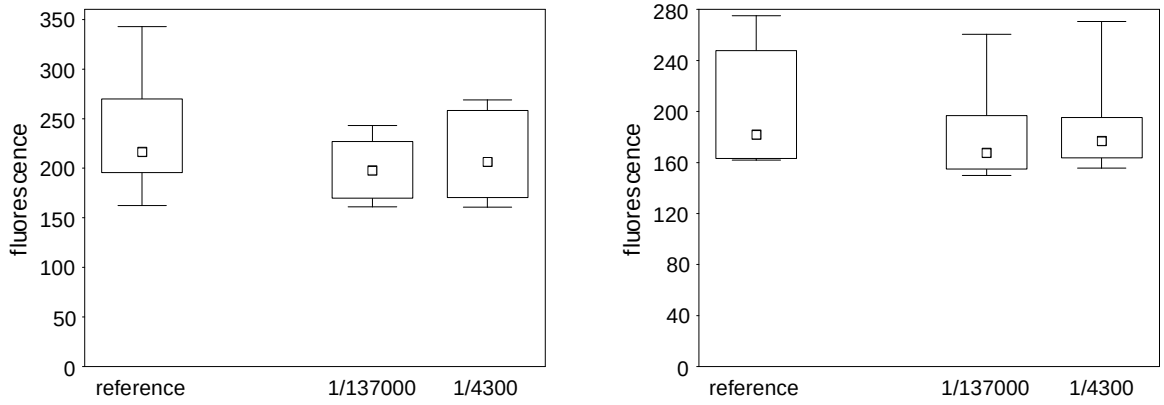

Figure 10. Rhodamine B retention in haemocytes (measured as fluorescence) in the absence (left) or presence (right) of the MXR-inhibitor cyclosporin A. Blue mussels were held in tanks receiving the indicated treatments; median (squares), quartiles (boxes) and 10/90 percentiles (whiskers) are shown.

Although not significantly different, there was a tendency for increased overall MXR-activity in the two groups exposed to diluted produced water compared to the reference group (Figure 11).

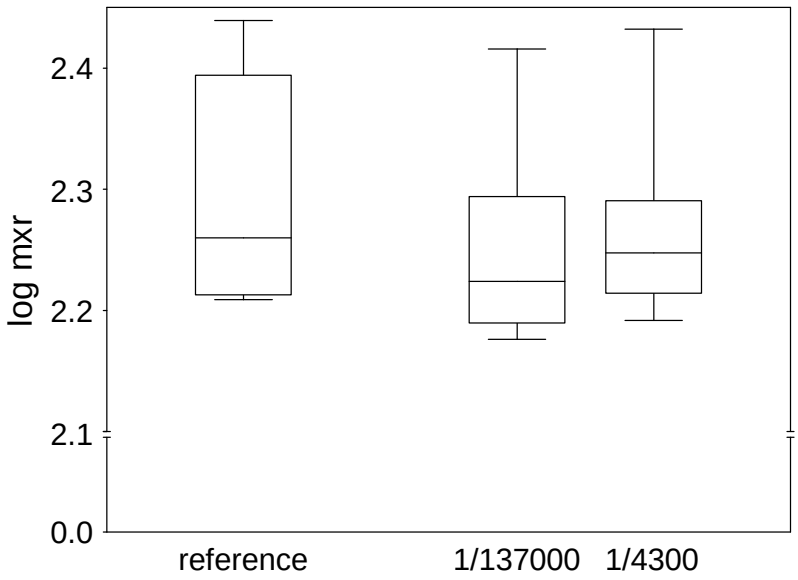

Figure 11. MXR response in haemocytes (ratio) from blue mussels held in tanks receiving the indicated treatments; median (line), quartiles (boxes) and 10/90 percentiles (whiskers) are shown. Note log-axis.

There were no significant treatment-dependent responses in MXR in haemocytes from blue mussels, possibly due to large variability (medians were higher in exposed groups). Although MXR has been determined in blue mussels before, there are no previous studies with produced water. As mentioned above, produced water components could be expected to affect the system. There was no obvious effect on MXR in blue mussel hemocytes using Rhodamine B as substrate and cyclosporin A as inhibitor (established during a MSc project; Røysland 2005). Further research is needed into the fundamental mechanisms of P-glycoprotein pumps in this species before the method can be applied in a monitoring context.

3.6.2 Atlantic cod

There was apparently more efficient removal of the fluorescent probe rhodamine B from red blood cells in the reference group compared to the two groups that were exposed to diluted produced water (Figure 12). This was the case for samples both with and without inhibitor. Nested ANOVA models were significant (p = 0.05 and 0.008, respectively) in both cases (with and without inhibitor), but only the 1/137 000 group treated with inhibitor differed significantly from the reference (p = 0.06, 0.04, respectively, for the two concentrations, blood incubated without inhibitor).

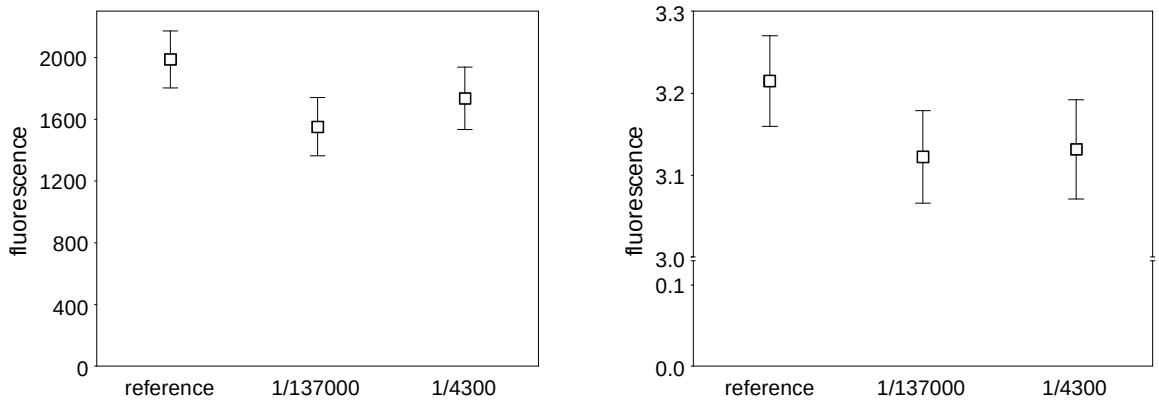

Figure 12. Least squared means from nested ANOVA model (tank, treatment) of Rhodamine B retention in red

blood cells (measured as fluorescence) in the absence (left) or presence (right; note log axis) of the MXR-inhibitor verapamil. Cod were held in tanks receiving the indicated treatments; mean (squares) and 95% confidence intervals (whiskers) are shown.

There were no significant differences in MXR activity of red blood cells from cod exposed to diluted produced water or seawater (Figure 13).

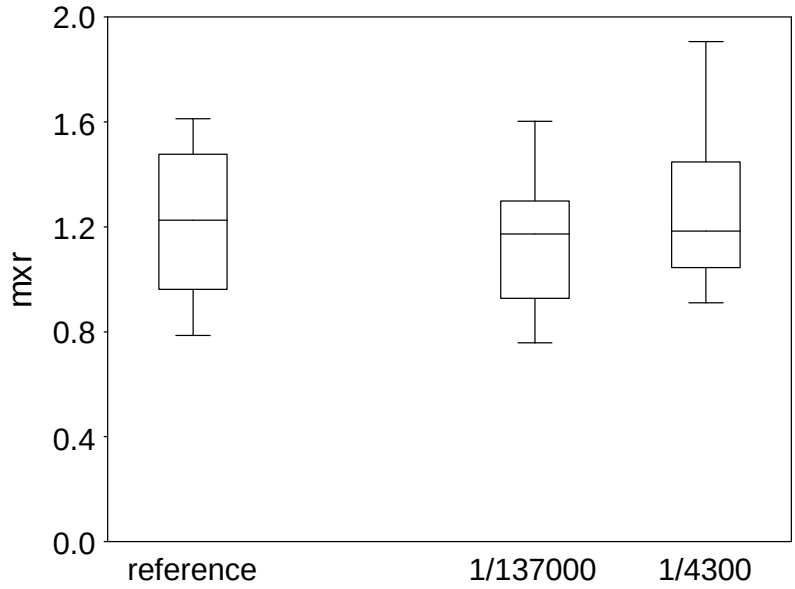

Figure 13. MXR response in blood cells (ratio) from cod held in tanks receiving the indicated treatments; median (line), quartiles (boxes) and 10/90 percentiles (whiskers) are shown.

Red blood cells from produced water-exposed cod had higher capacity to pump foreign substances out of the cells than red blood cells from reference cod. The results indicate that the treatment increased MXR capacity in red blood cells from cod and that the inhibitor used (verapamil) was largely ineffective (the affected MXR component was not inhibited by verapamil). MXR in cod red blood cells appear promising as a method, but other inhibitors should be tested alongside verapamil.

4. Conclusions and recommendations

4.1 Exposure

PAHs accumulated in blue mussels exposed to diluted produced water. The PAH concentrations found in blue mussels following the exposure levels used in this study show that the experiment reflected environmentally relevant concentrations of produced water. As was the case for both WCM 2003 and 2004, there were no significant increases in concentrations of PAH metabolites in cod held in tanks receiving 4300- and 137000-fold diluted Statfjord B produced water.

Produced water for the exposures were kept cool in closed metal containers until use. Analyses of produced water in an unopened container at the end of the exposure indicated that there was limited breakdown or modification of produced water components during the exposure period.

It will be clear from the above that the concentrations used were lower than in most other studies, but presumably close to concentrations actually found in the vicinity of production platforms.

4.2 Effects

The results for cellular energy allocation (CEA) measured in blue mussels did not show clear effects, contrary to the results seen by Smolders et al. (in press). There were apparent effects on lipid concentration in the mussels. Further work is needed with this method before it should be applied in monitoring.

There were clear dose-dependent effects of the exposures on micronucleus formation in hemocytes from blue mussels. No such effects were seen with red blood cells from cod, which does not appear to be a good matrix for such studies. Micronucleus formation in hemocytes has shown similar results during earlier tests offshore and appears appropriate for inclusion in offshore monitoring programmes.

There were indications of possible effects from 4300-fold diluted produced water on peroxisomal proliferation, determined as AOX activity in blue mussel hepatopancreas. Apparent differences were not statistically significant. Further work is needed with this method, which may be useful to monitor acute spills.

No effects from the exposures were seen on glutathione *S*-transferase (GST) activities in either species, measured using CDNB or DCNB as substrates. Although seen as promising from the results in other studies, GST needs further research prior to routine application in monitoring programmes.

Produced water exposure did not have large effects on blue mussel hemocyte MXR, although significant differences would have been found if variability had been lower. Other substrates and inhibitors should be tested specifically for produced water effects. On the contrary, the exposures did appear to affect MXR in red blood cells from cod. The inhibitor used (verapamil) appeared to be largely ineffective. More baseline studies are needed before this method is applied in monitoring.

4.3 Recommendations

The results from this study shows that micronucleus formation in mussel hemocytes should be included in future WCM programmes.

The results also indicate that some methods should be further investigated for possible use in offshore monitoring. They include mixed xenobiotic defence (MXR) components in cod red blood cells, peroxisomal effects in mussel and cellular energy allocation in mussel.

Further work is needed with MXR in blue mussel hemocytes and glutathione S-transferase isoenzymes, possibly using molecular tools (qPCR), before the methods can be recommended for use in monitoring.

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Appendix A. PAH exposure estimates

PAH analysis of produced water from Statfjord B, calculated PAH concentrations for the highest exposure concentration, predicted water concentrations based on mussel tissue, and median values of the weekly drawn SPE extracts from the highest exposure concentration. All values are given in ng/l.

	Analysis PW Statfjord B	Calculated waterconc. 1/4300	Predicted water conc. Mussel tissue 1/4300	Median value of weekly SPE extracts 1/4300
Napthtalene	360000	83,7	18,3	38,7
C1 Napthtalenes	150000	34,9	24,1	31,7
C2 Napthtalenes	110000	25,6	12,1	16,4
C3 Napthtalenes	>300000	>70	6,2	37,7
Acenaphthen	1100	0,26	2,9	2,1
Acenaphthylene	<200	<0,05	1,6	nd
Fluorene	8800	2,05	3,4	1,9
Anthracene	220	0,05	0,8	nd
Phenanthrene	13000	3,02	4,2	3,9
C1 Phenanthrenes	12000	2,79	1,4	5,6
C2 Phenanthrenes	3100	0,72	1,0	nd
C3 Phenanthrenes	13000	3,02	0,2	nd
Dibenzothiophene	5100	1,19	0,7	nd
C1-Dibenzothiophenes	7400	1,72	1,1	nd
C2-Dibenzothiophenes	6700	1,56	0,5	nd
C3-Dibenzothiophenes	3500	0,81	0,5	nd
Fluoranthene	<200	<0,05	0,96	nd
Pyrene	<200	<0,05	0,72	nd
Benzo(a)antracene	<200	<0,05	0,00	nd
Chrysene	<200	<0,05	0,00	nd
Benzo(b)fluoranthene	<200	<0,05	0,03	nd
Benzo(j,k)fluoranthene	<200	<0,05	0,00	nd
Benzo(e)pyrene	<200	<0,05		nd
Benzo(a)pyrene	<200	<0,05	0,00	nd
Perylene	<200	<0,05		nd
Indeno(1,2,3-cd)anthracene	<200	<0,05	0,00	nd
Dibenz(a,h)anthracene	<200	<0,05	0,00	nd
Benzo(ghi)perylene	<200	<0,05	0,00	nd

Appendix B. Biomarkers – blue mussel

AOX

tank	protein	mU/mg protein
1	12.09	0.43
1	8.36	0.55
1	10.99	0.29
1	12.75	0.96
1	11.84	1.20
1	10.48	1.42
1	12.61	0.53
2	9.70	0.84
2	15.30	0.96
2	10.44	1.21
2	5.88	0.35
2	9.99	1.01
2	10.51	1.40
3	13.16	1.39
3	12.49	0.85
3	11.37	2.02
3	10.01	0.73
3	10.95	1.05
4	7.89	2.23
4	12.09	0.70
4	11.39	0.54
4	11.72	0.90
4	12.20	0.67
4	11.32	0.82
4	12.41	0.60
5	13.91	0.88
5	11.03	2.63
5	14.57	0.82
5	11.73	0.92
6	6.57	0.00
6	11.97	1.00
6	11.54	1.16
6	12.48	1.04
6	11.85	1.05
6	11.63	1.62
6	10.39	0.77
7	10.28	0.91
7	5.09	2.25
7	12.59	0.41
7	13.27	0.32
7	16.43	0.57
7	12.89	0.83
8	10.06	0.00
8	13.77	1.10
8	11.87	0.31

8	9.58	1.11
8	10.25	1.78
8	13.81	2.14
8	10.12	1.31
8	10.64	0.88
9	12.31	1.19
9	10.96	1.71
9	11.87	1.02
9	8.41	0.93

glutathione *S*-transferase

tank	protein	DCNB/prot	CDNB/prot
1	12	173	105
1	21	167	95
1	16	260	122
1	13	261	212
1	17	150	87
1	15	85	147
1	13	377	110
1	12	130	67
2	9	177	91
2	13	367	108
2	20	201	94
2	10	131	228
2	10	326	128
2	17	144	89
2	12	205	113
2	12	223	122
3	16	261	83
3	19	219	86
3	8	227	126
3	15	179	96
3	10	498	127
3	16	256	81
3	15	311	77
4	9	339	166
4	11	380	121
4	17	297	85
4	15	208	51
4	16	135	105
4	15	68	112
4	19	165	129
4	16	327	111
5	8	493	123
5	22	96	75
5	13	77	79
5	17	206	74
5	19	71	61
5	16	248	67
5	19	159	125

5	16	87	122
6	19	74	96
6	17	106	152
6	13	94	111
6	23	179	57
6	24	120	62
6	3	776	88
6	21	261	98
6	18	291	105
7	15	104	183
7	13	378	58
7	8	304	153
7	10	211	100
7	11	149	137
7	17	275	95
7	19	236	130
8	14	312	80
8	10	238	172
8	18	253	104
8	13	339	186
8	17	198	141
8	17	170	94
9	13	187	181
9	19	212	120
9	16	217	154
9	17	190	105
9	12	166	123
9	11	136	249
9	7	641	264
9	21	53	69
9	18	227	129

micronuclei

tank	slide no	cell count	total cells MN	per thousand
1	1	200		0
1	2	200	1	5
1	3	200	1	5
1	4	200	2	10
1	5	500	1	2
1	6	230	1	4.347826087
1	7	200	1	5
2	8	200	1	5
2	9	200	2	10
2	10	200		0
2	11	200	1	5
2	12	200		0
2	13	200	1	5
2	14	200	1	5
2	15	200	1	5

3	16	200		0
3	17	210	2	9.523809524
3	18	200		0
3	19	203	1	4.926108374
3	20	200		0
3	21	200		0
3	22	200	2	10
3	23	200	2	10
3	24	200	1	5
4	25	200		0
4	26	200	1	5
4	27	200	1	5
4	28	200		0
4	29	200	1	5
4	30	200		0
4	31	200		0
4	32	200		0
5	33	200	1	5
5	34	200	2	10
5	35	200		0
5	36	200	1	5
5	37	200	1	5
5	38	200	1	5
5	39	200	2	10
5	40	200	2	10
6	41	200	2	10
6	42	200		0
6	43	200		0
6	44	200		0
6	45	200	1	5
6	46	90		0
6	47	200		0
6	48	200		0
7	49	200	1	5
7	50	200		0
7	51	124		0
7	52	200		0
8	53	200		0
8	54	200		0
8	55	200		0
8	56	200		0
8	57	105		0
8	58	200		0
8	59	200		0
9	60	200	1	5
9	61	200		0
9	62	200		0
9	63	200		0
9	64	200		0
9	65	200		0
9	66	200		0

MXR

tank	no inhibitor	with inhibitor	ratio
1	187	159	1.17
1	193	193	1.00
1	163	150	1.09
1	162	142	1.14
1	204	171	1.19
1	220	185	1.19
1	207	177	1.17
2	258	164	1.57
2	171	156	1.10
2	273	195	1.40
2	152	179	0.85
2	227	164	1.39
2	269	271	0.99
2	288	1116	0.26
2	243	243	1.00
3	260	177	1.47
3	216	250	0.86
3	267	174	1.53
3	206	172	1.20
3	161	158	1.02
3	213	321	0.66
3	157	170	0.92
3	180	187	0.96
4	198	179	1.11
4	206	162	1.27
4	230	171	1.35
4	170	151	1.12
4	161	167	0.97
4	236	246	0.96
4	258	261	0.99
5	193	155	1.24
5	224	166	1.35
5	198	150	1.32
5	186	168	1.11
5	169	148	1.14
5	227	269	0.84
5	170	153	1.11
6	179	160	1.12
6	212	181	1.17
6	243	258	0.94
6	171	168	1.02
6	152	197	0.77
7	217	178	1.21
7	171	143	1.20
7	196	229	0.85
7	211	170	1.24
7	208	569	0.37
8	204	174	1.17
8	221	248	0.89
8	239	163	1.46

8	272	275	0.99
9	378	224	1.68
9	343	245	1.40
9	270	272	0.99
9	162	162	1.00
9	233	182	1.28
9	163	162	1.00

cellular energy allocation

aquarium	wwt	protein	lipid	glycogen	total energy	dA/dt
1	3.3	0.64	0.87	16.80	18.31	1.53
1	4.2	0.99	0.70	11.58	13.27	2.00
1	4.6	0.65	0.59	10.87	12.10	1.37
1	2.6	1.10	1.62	23.51	26.22	1.75
1	3.2	1.09	1.46	9.38	11.92	1.47
1	2.9	1.11	1.72	20.23	23.05	0.99
1	4.3	1.00	1.33	17.77	20.10	1.55
1	2.9	1.27	1.16	21.87	24.29	1.49
1	3.4	0.84	1.74	18.99	21.57	1.32
1	4.3	1.42	1.56	16.45	19.43	1.30
2	5.4	0.76	0.58	11.75	13.09	2.07
2	2.6	1.11	1.34	15.05	17.50	0.88
2	4.5	0.41	0.89	17.38	18.68	1.33
2	5	0.49	1.13	12.24	13.86	1.93
2	3.4	0.79	0.94	10.64	12.37	1.36
2	4.2	1.13	0.88	12.73	14.74	1.95
2	4.4	0.83	0.54	4.87	6.25	1.14
2	3.6	1.34	1.69	8.32	11.36	1.66
2	4.4	0.79	0.92	13.55	15.26	2.22
2	3.7	0.92	1.64	21.43	24.00	1.62
3	4.3	0.75	0.59	9.34	10.68	1.52
3	2.8	1.32	0.64	8.56	10.52	1.06
3	3.1	0.45	0.83	4.51	5.79	1.25
3	4.5	0.43	0.09	11.07	11.59	0.69
3	4.3	1.14	0.60	12.69	14.43	1.07
3	3.8	1.31	0.43	20.24	21.97	1.73
3	4.9	1.17	0.24	11.89	13.31	1.49
3	2.7	1.17	0.37	8.94	10.48	0.71
3	4	0.85	0.31	8.46	9.62	1.20
3	3.2	1.52	0.23	13.52	15.27	1.60
4	4	0.61	0.73	8.49	9.83	1.44
4	4.2	1.02	0.53	5.76	7.32	1.49
4	4	1.51	0.50	4.32	6.33	1.08
4	4	1.01	0.32	6.12	7.44	0.62
4	3.4	1.22	0.41	13.54	15.17	1.12
4	3.5	2.21	0.48	17.05	19.74	1.53
4	3.2	1.38	0.37	11.24	12.99	1.11
4	3.2	1.35	0.73	18.27	20.35	1.19
4	3.6	1.04	1.02	18.55	20.62	1.51
4	3.6	0.69	0.70	12.10	13.48	1.20

5	4.4	1.08	0.41	12.26	13.75	1.48
5	4.1	0.84	0.41	6.74	8.00	1.31
5	3.6	0.91	0.64	5.68	7.22	1.35
5	4.3	1.56	0.89	7.33	9.79	1.16
5	3.2	0.23	0.77	1.74	2.74	1.68
5	3.6	0.69	0.39	5.12	6.20	1.21
5	2.8	1.10	0.59	5.76	7.45	0.91
5	3.5	0.74	0.50	6.97	8.20	1.69
5	2.2	1.56	0.68	3.91	6.16	0.62
5	3	0.53	0.65	9.23	10.41	0.99
6	5.2	0.97	0.79	14.86	16.62	1.57
6	2.6	1.50	1.39	18.95	21.84	1.35
6	4.2	0.77	0.85	10.56	12.18	1.58
6	3.8	0.51	1.14	9.09	10.74	1.24
6	4.2	1.21	0.48	9.33	11.02	1.57
6	4.2	1.02	0.68	6.03	7.73	1.72
6	3.8	0.73	0.64	5.89	7.25	1.91
6	3.8	1.46	0.61	12.10	14.17	1.31
6	4.1	1.33	0.67	9.37	11.37	1.59
6	3.8	0.82	0.58	8.97	10.37	1.39
7	3.5	0.76	1.03	4.14	5.93	1.08
7	4.1	1.22	0.60	4.84	6.66	1.04
7	3.8	1.37	0.91	4.32	6.60	1.11
7	4.8	0.67	0.44	4.25	5.36	1.56
7	4.9	0.83	0.42	4.45	5.70	1.42
7	3.8	1.28	0.34	2.64	4.26	1.29
7	1.9	1.43	0.38	3.16	4.97	0.81
7	5.2	1.09	0.44	2.70	4.23	2.25
7	2.7	1.09	0.48	6.55	8.11	1.12
7	4.2	0.85	0.66	12.10	13.61	1.19
8	3.4	0.62	1.63	17.28	19.54	1.99
8	3.6	0.99	1.47	6.70	9.16	1.49
8	4.2	0.85	0.87	18.15	19.87	1.99
8	3.6	1.16	1.15	20.51	22.83	1.98
8	3	0.95	0.67	18.51	20.14	1.21
8	2.8	1.22	1.13	14.05	16.40	1.25
8	3.7	1.45	1.44	17.06	19.95	1.30
8	2.8	1.14	1.17	16.34	18.65	1.32
8	3.6	0.59	1.80	20.23	22.62	1.40
8	2.7	1.01	1.51	23.17	25.68	1.15
9	4.2	1.22	1.75	17.58	20.55	2.12
9	2.9	1.85	1.03	16.14	19.02	1.47
9	5.9	0.48	0.74	4.36	5.58	1.63
9	5.4	1.29	1.23	7.18	9.69	1.89
9	5.9	0.99	1.13	7.37	9.48	1.09
9	5.4	0.93	1.12	11.06	13.11	1.94
9	3.1	0.71	2.13	17.02	19.85	1.10
9	7.8	0.34	1.17	7.22	8.73	1.87
9	3.7	0.83	1.83	8.41	11.07	1.52
9	4.7	0.86	1.42	9.19	11.47	1.74

Appendix C. Biomarkers – cod

PAH metabolites
nd: not detectable

tank	wt bile	abs 380 nm	1-OH-phenanthrene	1-OH-pyrene	3-OH-BaP
1	2024	0.16	99	516	nd
1	2008	0.53	58	357	2.63
1	1916	0.37	48	301	2.42
1	1959	0.12	74	347	nd
1	1900	0.32	68	365	2.17
1	1925	0.60	70	414	2.85
1	2002	0.81	82	517	2.73
1	1958	0.17	75	431	4.02
2	1943	0.28	83	457	2.54
2	1981	0.25	163	853	4.37
2	1960	0.49	84	468	2.45
2	1943	0.27	124	772	4.12
2	1914	0.38	111	737	2.39
2	1871	0.34	53	361	3.85
2	1935	0.17	126	661	5.17
3	1908	0.42	73	470	1.65
3	1894	0.15	120	820	6.00
3	1972	0.19	116	787	4.26
3	1981	0.41	51	379	2.42
3	1976	0.16	149	863	3.73
3	2011	0.30	95	645	1.64
3	1954	0.36	109	801	2.24
4	1948	0.25	92	506	2.79
4	1945	0.36	48	315	2.25
4	1940	0.70	98	415	1.87
4	1969	0.28	189	1135	6.91
4	1960	0.60	65	311	2.66
4	1939	0.36	122	403	1.66
4	2164	0.13	103	460	nd
5	1931	0.28	51	285	nd
5	2054	0.16	89	646	4.43
5	1970	0.24	96	483	2.92
5	1904	0.15	136	660	nd
5	2134	0.42	88	383	2.86
5	1946	0.03	256	941	11.76
5	1921	0.83	35	232	3.52
6	1965	0.27	125	506	4.91
6	1905	0.19	106	707	7.45
6	1950	0.45	84	450	2.66
6	1928	0.18	98	617	3.28
6	1837	0.44	105	632	4.55
6	2457	0.66	32	169	1.22
6	2082	0.39	79	311	3.57
7	2011	0.27	63	291	5.22

7	1919	0.11	114	838	nd
7	1915	0.22	55	382	4.61
7	2102	0.61	57	607	2.77
7	1954	0.22	115	539	3.69
7	1868	0.16	115	669	7.64
7	1975	0.30	101	591	3.38
8	1944	0.17	103	630	nd
8	1985	0.28	2	2	nd
8	2061	0.25	173	654	5.12
8	1941	0.40	87	499	4.99
8	1920	0.25	80	480	nd
8	1963	0.72	135	436	2.08
9	1952	0.62	96	485	0.97
9	1968	0.19	63	401	nd
9	1862	0.15	110	649	7.79
9	1944	0.11	148	537	nd
9	2092	0.31	2	9	nd
9	2142	0.20	79	559	4.46
9	1931	0.38	120	572	6.27

glutathione *S*-transferase

tank	protein	DCNB/prot	CDNB/prot
1	28	94	633
1	21	66	903
1	16	116	394
1	26	122	473
1	21	125	400
1	16	218	603
1	21	9	530
1	14	17	904
2	14	275	820
2	8	280	1250
2	14	10	829
2	25	129	574
2	28	94	331
2	11	25	933
2	28	9	362
2	22	11	641
3	15	149	490
3	16	201	1067
3	22	47	774
3	22	37	607
3	9	536	720
3	24	8	579
3	20	8	540
3	25	11	416
4	15	0	972
4	23	72	891
4	20	13	878
4	23	98	448

4	18	231	482
4	24	17	504
4	22	3	497
4	24	8	279
5	19	47	362
5	22	185	532
5	23	39	630
5	26	120	580
5	21	185	451
5	21	3	560
5	18	18	390
5	16	9	569
6	27	116	566
6	24	71	537
6	7	463	642
6	19	197	506
6	25	152	402
6	24	17	408
6	30	6	427
6	24	15	562
7	12	22	785
7	26	36	627
7	16	192	829
7	20	80	556
7	22	154	327
7	25	5	627
7	25	15	392
7	26	18	337
8	14	172	1079
8	21	50	586
8	15	68	962
8	22	85	626
8	25	86	587
8	14	213	514
8	17	10	664
8	27	12	527
9	23	33	509
9	22	73	921
9	5	59	570
9	22	160	692
9	20	129	704
9	23	101	702
9	16	18	897
9	20	14	641

micronuclei

tank	cell count	total cells MN	per thousand
1	2000	1	0.50
1	2000		0.00
1	2060		0.00

1	2040		0.00
1	2020		0.00
1	2000		0.00
1	2000		0.00
1	2000		0.00
2	2000	1	0.50
2	2080	1	0.48
2	2010	1	0.50
2	2000		0.00
2	2000		0.00
2	2000		0.00
2	2000		0.00
2	2000		0.00
3	2060		0.00
3	2000	1	0.50
3	2050		0.00
3	2040		0.00
3	2020		0.00
3	2000		0.00
3	2000		0.00
3	2000		0.00
4	2000		0.00
4	2030		0.00
4	2030		0.00
4	2030		0.00
4	2020		0.00
4	2000		0.00
4	2000		0.00
4	2000		0.00
5	2030		0.00
5	2010		0.00
5	2030		0.00
5	2020		0.00
5	2060	1	0.49
5	2000	36	18.00
5	2000		0.00
5	2000		0.00
6	2000		0.00
6	2070		0.00
6	2150		0.00
6	2000		0.00
6	2010		0.00
6	2000		0.00
6	2000		0.00
6	2000	12	6.00
7	2030	1	0.49
7	2010		0.00
7	2010		0.00
7	2010		0.00
7	2000		0.00
7	2000		0.00
7	2000		0.00
7	2000		0.00
8	2070		0.00

8	2020		0.00
8	2106	1	0.47
8	2010		0.00
8	2000		0.00
8	2000		0.00
8	2000		0.00
9	2100	3	1.43
9	2004		0.00
9	2040	1	0.49
9	2030	2	0.99
9	2030		0.00
9	2000		0.00
9	2000		0.00
9	2000	1	0.50

MXR

tank	no inhibitor	with inhibitor	ratio
1	966	845	1.14
1	2064	2231	0.92
1	1732	1315	1.32
1	2484	1020	2.43
1	1964	1684	1.17
1	2409	2006	1.20
1	1266	1946	0.65
2	1858	1849	1.00
2	2348	1304	1.80
2	2140	1737	1.23
2	2001	1880	1.06
2	1536	1712	0.90
2	1949	1893	1.03
2	1581	1424	1.11
3	1272	806	1.58
3	984	929	1.06
3	2035	1289	1.58
3	1568	780	2.01
3	1110	914	1.21
3	1746	1404	1.24
4	1047	1003	1.04
4	1214	945	1.29
4	1123	1701	0.66
4	1750	1506	1.16
4	1700	1061	1.60
4	1960	1493	1.31
4	1355	1078	1.26
5	1305	1005	1.30
5	2341	1350	1.73
5	2096	1628	1.29
5	1517	2540	0.60
5	1235	918	1.35
5	1424	1158	1.23
5	2279	2088	1.09

5	1173	1000	1.17
6	1680	2217	0.76
6	1400	1509	0.93
6	1037	903	1.15
6	1991	912	2.18
6	1310	1015	1.29
6	1692	1555	1.09
6	2057	2569	0.80
6	1125	1226	0.92
7	2146	2731	0.79
7	1681	1108	1.52
7	2216	1837	1.21
7	2460	1167	2.11
7	1322	1044	1.27
7	2561	1617	1.58
7	1405	871	1.61
7	1297	1043	1.24
8	1968	1894	1.04
8	1740	1854	0.94
8	1704	1730	0.98
8	2498	1699	1.47
8	2440	1717	1.42
8	2591	1790	1.45
8	1473	1396	1.06
8	2478	1323	1.87
9	1679	1573	1.07
9	1687	2777	0.61
9	2375	1628	1.46
9	2801	4108	0.68
9	1582	1601	0.99
9	1510	1653	0.91
9	1530	1831	0.84
9	2589	1746	1.48