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Biological effects monitoring of the discharge water from the Ormen Lange gas facility, Norway

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Norwegian Institute for Water Research
– an institute in the Environmental Research Alliance of Norway

REPORT

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Abstract

The mussel, *Mytilus edulis* has been used to assess the biological effects of produced water (PW) discharged from the Ormen Lange gas processing plant on the West coast of Norway. An integrated monitoring approach was applied, using a combination of sensitive health index parameters in the mussel including lysosomal membrane stability (LMS) and micronuclei (MN) formation in haemocytes of live mussels, cell type composition, lysosomal membrane stability and peroxisome proliferators in mussel digestive gland, and contaminant body burden concentrations in whole tissue homogenates. In addition, semipermeable membrane devices (SPMDs) were used to support the biological effects data. The biological and chemical endpoints were measured in mussels from three different exposure scenarios: 1) native mussels found on the shore in the vicinity of the produced water discharge point; 2) mussels placed in cages at known distances from the produced water discharge point; and 3) mussels exposed to known concentrations of produced water in a laboratory controlled flow-through dosing system. The results of the biological effects data and chemical analysis are presented. For the native shore mussels, good agreement between biological effects measurements was observed. Mussels located at sites closest to Ormen Lange showed clear stress responses indicative of poor health status, compared to the reference mussels. For the caged mussels, contradictory biological effects measurements were found, which were thought partly due to the overall poor health of the mussels used. Consequently no firm conclusions were drawn about the cage exposure. In the controlled laboratory exposure to PW, biological effects were found in mussels exposed to 0.01% PW and above compared to a control group. Overall, although some were anomalous, the results indicate that Ormen Lange PW may have detrimental effects on mussel health.

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2. Produsert vann	2. Produced water
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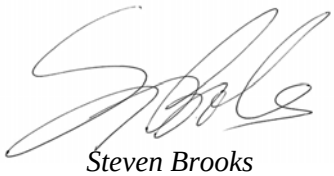

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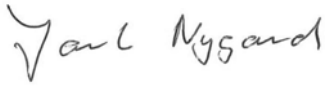

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Biological effects monitoring of the discharge water from the Ormen Lange Gas facility, Norway.

Source: Hanne Fosnes

Preface

This work is an investigation into the effects of a produced water discharge from Ormen Lange gas processing plant on the local aquatic environment. The work is one aspect of a larger study investigating the potential environmental impacts of Ormen Lange processing plant. NIVA have been subcontracted by Den Norske Veritas (DNV) to carry out the study, with Shell the principal customer.

Oslo, February 2009

Steven Brooks

Contents

Summary

The mussel, *Mytilus edulis* has been used to assess the biological effects of produced water (PW) discharged from the Ormen Lange gas processing plant on the West coast of Norway. An integrated monitoring approach was applied, using a combination of sensitive health index parameters in the mussel including lysosomal membrane stability (LMS) and micronuclei (MN) formation in haemocytes of live mussels, cell type composition, lysosomal membrane stability and peroxisome proliferators in mussel digestive gland, and contaminant body burden concentrations in whole tissue homogenates. In addition, semipermeable membrane devices (SPMDs) were used to support the biological effects data. The biological and chemical endpoints were measured in mussels from three different exposure scenarios: 1) native mussels found on the shore in the vicinity of the produced water discharge point; 2) mussels placed in cages at known distances from the produced water discharge point; and 3) mussels exposed to known concentrations of produced water in a laboratory controlled flow-through dosing system. The results of the biological effects data and chemical analysis are presented. For the native shore mussels, good agreement between biological effects measurements was observed. Mussels located at sites closest to Ormen Lange showed clear stress responses indicative of poor health status, compared to the reference mussels. For the caged mussels, contradictory biological effects measurements were found, which were thought partly due to the overall poor health of the mussels used. Consequently no firm conclusions were drawn about the cage exposure. In the controlled laboratory exposure to PW, biological effects were found in mussels exposed to 0.01% PW and above compared to a control group. Overall, the Ormen Lange PW was found to have detrimental effects on mussel health.

1. Introduction

The Ormen Lange gas processing plant is situated on the island of Gossa situated on the West coast of Norway where it processes gas condensate received by pipeline from the Ormen Lange gas fields 100 km offshore in the North Sea. Ormen Lange is the largest natural gas field in development on the Norwegian continental shelf with potential gas reserves in the region of 397 billion cubic meters.

Produced water (PW) from the onshore processing plant is diluted with cooling water within the Ormen Lange system before it is discharged into the surrounding coastal water environment by a single outfall pipe. The potential biological impact of this PW has been evaluated using an integrated approach of biological effects measurements and tissue body burdens using the blue mussel, *Mytilus edulis*. The integration of the biological effects is essential for a more comprehensive assessment of organism health (Brooks et al., 2009). In addition, semipermeable membrane devices (SPMDs) have also been used to support the biological effects measurements.

The biological effects measurements used in this study provide a sensitivity evaluation of mussel health. The health status of the mussel can be directly related to the amount of environmental stress imposed on the individual mussel through a variety of factors including contaminant exposure. Therefore, the health status of a mussel can provide important information on its surrounding environment including water quality. The biological effects tools that were used in the mussel include lysosomal membrane stability (LMS(h)) and micronuclei formation (MN) in haemocytes, relative volume density of basophilic cells (VvBAS), lysosomal membrane stability in digestive cells – LMS(dc)- and Acyl-CoA oxidase (AOX) activity in digestive gland tissue. In addition, concentrations of metals and polycyclic aromatic hydrocarbons (PAH) and alkylated naphthalenes, phenanthrenes and dibenzothiophenes (NPD), were determined from whole mussel homogenates.

Lysosomal membrane stability in haemocytes (LMS (h))

The membrane integrity of lysosomes has been found to be affected by a range of environmental stressors, including metals and organic chemicals (Lowe et al., 1995). The method measures the ability of the lysosomes of haemocytes to retain a neutral red dye. A reduction in membrane integrity will reduce the retention time of the dye and cause it to leak back into the cytosol, an effect which can be quantified. This is a well established method, which is recommended by ICES as a biological effects tool for environmental monitoring.

Micronuclei formation

The MN test is among the most widely used tools in eco-genotoxicology, and provides a measure of DNA damage in target organisms. 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 quantification 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).

Digestive gland histopathology

The molluscan digestive gland is a target organ widely used in environmental toxicology. It accumulates pollutants and actively participates in their detoxification and elimination (Marigómez et al., 2002; Moore and Allen, 2002). Mussel digestive gland is organised into clusters of alveolotubular units connected by secondary ducts to primary ducts that communicate with the stomach. The digestive gland epithelium is greatly dynamic and morphological changes in digestive alveoli may occur normally during digestion (Morton, 1983). Stress sources including exposure to pollutants (Lowe et al., 1981; Vega et al., 1989; Cajaraville et al., 1992) provoke changes in alveoli beyond their normal range of variability. In particular, atrophy of the digestive gland is characterised by the extreme thinning of the digestive tubule walls (Couch, 1984; Ellis et al., 1998). In addition, parasitic burden and pathological status may also occur after sublethal exposure to pollutants (Kim et al., 1998). Overall, histopathological examination of bivalve digestive gland provides sensitive, useful and potential indications for the diagnosis of the ecosystem health status (Kim et al., 2006; Au, 2004).

Cell type replacement (digestive cell loss)

The epithelium of the digestive alveolus is comprised by two cell types: digestive and basophilic cells (Morton, 1983). Digestive cells are involved in the intracellular digestion of food materials and posses a well-developed endo-lysosomal system, whereas basophilic cells are less abundant secretory cells believed to contribute to extracellular digestion and metabolic regulation (Marigómez et al., 2002; Robledo et al., 2006; Izagirre et al., in press). Under normal physiological conditions the digestive cells outnumber basophilic cells, but under different stress situations, including exposure to pollutants, the relative occurrence of basophilic cells is apparently augmented (Zaldibar et al., 2007). Since the first reports (Rasmussen et al., 1983) these changes in the cell type composition have been attributed to basophilic cell proliferation, but it has been recently concluded that it mainly results from digestive cell loss (Zaldibar et al., 2007). Thus, digestive cell loss resulting from environmental stress, measured

in terms of VvBAS, constitutes a sensitive indication of sublethal damage due to contaminant exposure.

Lysosomal responses

Lysosomes are cell organelles specialised in digestion of both endogenous and exogenous materials. Impairment of lysosomes and, hence, of food assimilation, can result in severe alterations of cells and whole organisms. Lysosomes of the digestive cell of mussels, apart from their main functions in intracellular digestion of ingested material (Robledo et al., 2006, Izagirre et al., 2008) and autophagic processes (Moore et al., 2007), play an important role in responses to toxic compounds through the sequestration and accumulation of toxic metals and organic xenobiotics. Lysosomal responses to pollutants are widely accepted cellular biomarkers of biological effect, especially in mussels and other bivalve molluscs, whose digestive cells posses a very well developed endo-lysosomal system (UNEP/RAMOGE 1999, ICES 2004). It has been thoroughly reported that environmental stressors cause reduction in lysosomal membrane stability, usually measured in terms of reduced labilisation period (LP) (UNEP/RAMOGE 1999, ICES 2004).

Peroxisome proliferation

Peroxisomes are membrane-bound cytoplasmic organelles involved in lipid metabolism, oxyradical homeostasis and several other important cell functions (Cancio and Cajaraville, 2000). One of the unique features of peroxisomes is their ability to proliferate and to enhance their metabolic activity under exposure to certain organic chemical compounds, a phenomenon termed "peroxisome proliferation" (Fahimi and Cajaraville 1995). Laboratory and field studies have shown that phthalate ester plasticizers, PAHs, oil derivatives, Polychlorinated biphenyls (PCBs), certain pesticides, bleached kraft pulp and paper mill effluents, alkylphenols and estrogens all provoke peroxisome proliferation in different fish or bivalve mollusc species (Cajaraville et al., 2000). Peroxisome proliferation consists of an increase in peroxisome volume and numerical densities, which is accompanied usually by the induction of some peroxisomal enzyme activities, particularly those of the fatty acid ß-oxidation system, such as acyl-CoA oxidase or AOX (Fahimi and Cajaraville 1995). In molluscs, peroxisome proliferation, measured in terms of AOX activity induction, has been proposed as an exposure biomarker for organic pollutants (Cajaraville et al., 2000).

1.1 Objectives

The overall objective of the study was to apply a suite of mussel biological effects techniques combined with contaminant body burdens to provide an assessment of mussel health status in three different exposure scenarios.

1) To determine the longer term impact of the Ormen Lange processing plant using biological and chemical markers in native shore mussels.
2) To determine the biological effects and contaminant body burden concentrations in field caged mussels exposed to the produced water discharged from Ormen Lange.
3) To determine the biological effects and contaminant body burden concentrations in mussels exposed to known concentrations of the produced water from Ormen Lange, under controlled laboratory conditions.

This will help in evaluating the potential impact of the Ormen Lange PW discharge on the local marine environment.

2. Methods

2.1 Collection of the field mussels

With the aid of a small boat, blue mussels were located and collected from three sites in and around Ormen Lange on the morning of 3rd Sept. 2008. The location of the three sites can be seen in figure 1. The three sites include a reference site and two exposure sites. Mussels were collected from below the water line at each site and stored in a cooler box containing seawater dampened paper towels. This was designed to maintain the mussels in optimum condition prior to assessment. All mussels were brought back to the field laboratory and processed within 6 hours of collection from the shore.

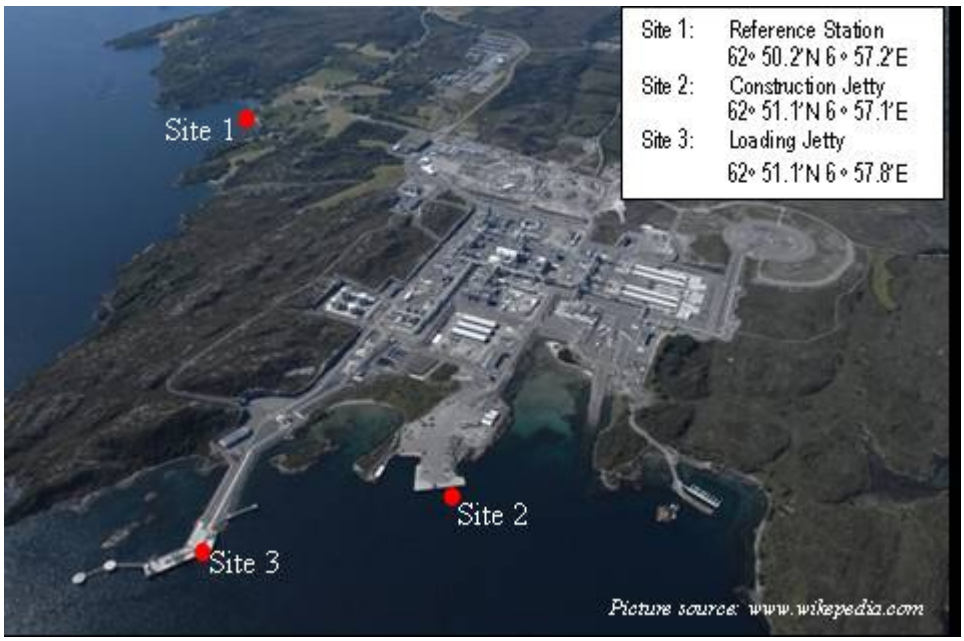

Figure 1. Location of the native mussels collected in and around Ormen Lange.

2.2 Deployment and retrieval of caged mussels

Mussel cages consisting of nylon mesh bags were attached together with Semipermeable Membrane Devices (SPMDs) to secured buoys and positioned at known distances from the Ormen Lange produced water discharge point. The position and co-ordinates of all 6 mussel cages and SPMDs, in relation to the discharge point, can be seen in table 1 and figure 2. The M/S Emilie and crew were used to deploy the buoys safely and to ensure placement at the desired location. Due to boating traffic at Ormen Lange, it was recommended to deploy the buoys at a depth of approximately 20 m from the surface. The mussel cages and SPMDs were placed at approximately 5 m below the buoys. The buoys were secured to the seabed using rope and anchor. The precise depths of the mussels at 3 of the 6 sites were confirmed by an underwater Remotely Operated Vehicle (ROV). The confirmed depths are displayed in table 1. The cages were deployed on the 3rd September 2008 and retrieved on the 15th and 16th October 2008, resulting in an exposure period of six weeks.

The mussels used in the cages were considered clean mussels obtained from a shellfish supplier in Rissa, Norway (www.snadderogsnaskum.no). The mussels were collected from the supplier on ice and transported in person to Ormen Lange. Mussels were placed in nylon mesh cages in groups of 10-20, with approximately 100 mussels at each site. Three SPMDs were used at each site. All sites were deployed within 4.5 h of each other.

Table 1. Information on the mussel caged sites including location and position of cages within the water column (* depths confirmed by ROV). Cages deployed 3rd Sept 08 and retrieved 15/16th Oct 08.

Site	Latitude/ Longitude	Time of deployment (hh:mm)	Total Depth (m)	Buoy depth (m)	Cage depth (m)
1	62° 51.181'N 6 ° 57.229'E	13:00	44	21*	27.5*
2	62° 51.202'N 6 ° 57.287'E	14:00	74	15.5*	21.5*
3	62° 51.225'N 6 ° 57.193'E	14:30	69	19	24
4	62° 51.251'N 6 ° 57.243'E	15:30	90	20*	27.5*
5	62° 51.954'N 6 ° 54.099'E	17:00	65	0	25
6	62° 52.118'N 6 ° 53.833'E	17:30	41	0	20

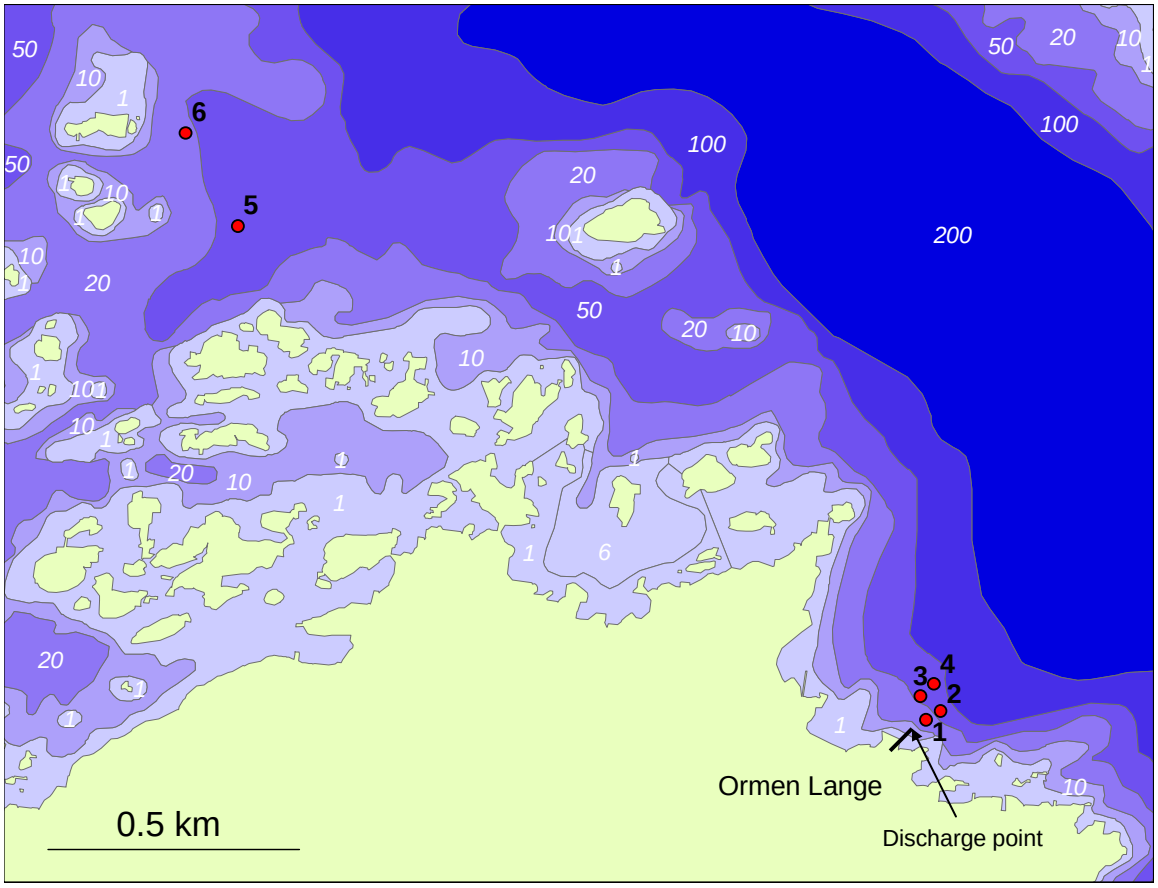

Figure 2. The approximate positions of the mussel cages with respect to the discharge outlet at Ormen Lange. Cage 1 – 50 m from discharge, Cages 2 and 3 – 100 m from discharge, Cage 4 – 200 m from discharge, Cages 5 & 6 - Reference stations.

Mussels and SPMDs were retrieved after 6 weeks with the aid of divers from the M/S Emilie. Divers were used to collect the buoys from 20 m below the surface. Six sites were collected over two days with three sites collected each day. This was to ensure that the mussels were processed on the same day as sample collection.

On collection, mussels were sorted and rinsed briefly in local seawater onboard M/S Emilie. They were then placed in chilled cooler boxes and wrapped in seawater soaked paper towels for transportation back to the field laboratory. All mussels were processed within 7 hours of sample collection.

Figure 3. Retrieval of the mussels and SPMDs after 6 weeks exposure.

2.3 Laboratory flow-through dosing experiment

A laboratory flow-through dosing system was set up at the NIVA marine field station at Solbergstrand near Drøbak. This system was designed to expose mussels and SPMDs to known and stable concentrations of the produced water collected from the Ormen Lange gas facility. The mussels used were from the same source as the caged mussels (i.e. www.snadderogsnaskum.no). Mussels were transported on ice by overnight courier and placed in the exposure tanks on the morning of arrival at Solbergstrand.

A large volume of produced water (circa 3000L) was collected in 3 x 1000 L plastic containers by Shell employees at the Ormen Lange plant and transported by road to Solbergstrand. At the time of collection the Ormen Lange facility was operating at 50% production (personal communication). The produced water was diluted with clean filtered seawater from the Solbergstrand research station to produce a concentration series of 1%, 0.5%, 0.1% and 0.01% of the original produced water concentration. The concentration of the original PW was measured for main contaminant concentrations at the start of the experiment. The clean seawater and the produced water were mixed within a 'mixer tank' prior to exposure to the mussel and SPMDs (Figure 4). The residency time of the water in each mixer tank was approximately one hour.

The seawater flow rate was calculated at 2.3 L/min, which was based on a mussel clearance rate of 0.033 L/min and 70 mussels in each exposure tank. This was to ensure that each mussel was exposed to fresh exposure medium. Physicochemical readings including pH, temperature, salinity and dissolved oxygen, as well as flow rates, were checked on a daily basis. Feeding and general health checks of the mussels and the dosing system were made every two days during the 5 week exposure (Start: 23/09/08 – end 28/10/08).

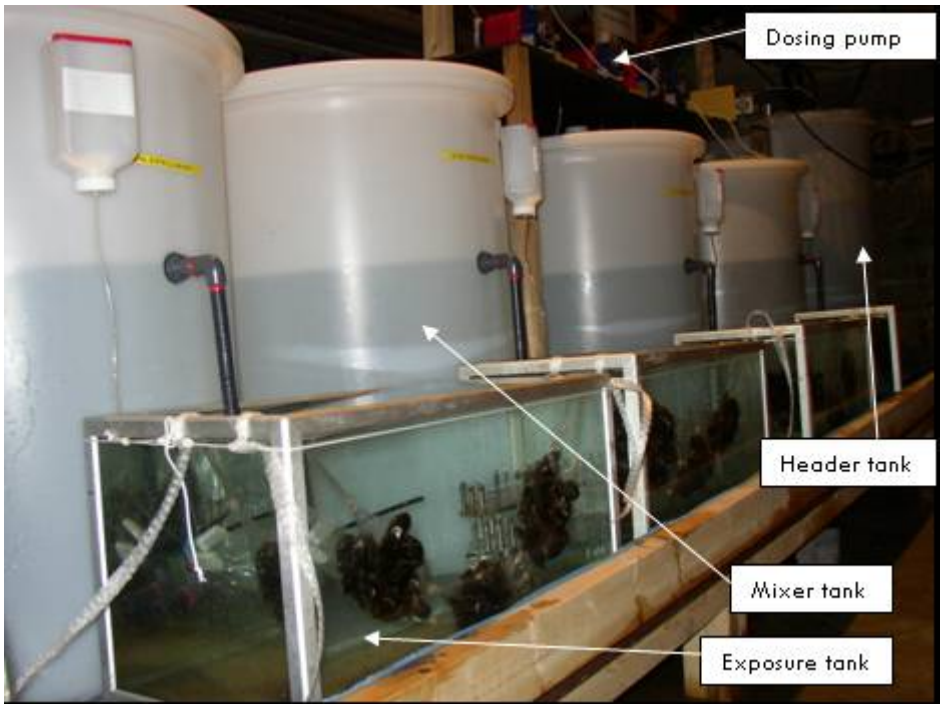

Figure 4. Laboratory flow-through experiment at the NIVA research station, Solbergstrand

2.4 Measuring biological responses and tissue chemistry in mussels

2.4.1 Lysosomal membrane stability by neutral red retention in mussel haemocytes

Lysosomal stability was measured in mussel haemocytes using the Neutral Red Retention (NRR) procedure adapted from Lowe and Pipe (1994). Approximately 0.1 ml of haemolymph was removed from the adductor muscle of the mussel with a syringe containing approximately 0.1 ml of physiological saline. The haemolymph/ saline solution was placed in a microcentrifuge tube, from which a 40 µl sample was removed and pipetted onto the centre of a microscope slide. The slide was left in a dark humid chamber for 15 min to allow the cells to adhere to the slide. Excess liquid was removed from the slide after this time and 40 µl of neutral red solution added (Sigma). The neutral red solution was taken up inside the haemocytes and stored within the lysosome. The ability of the lysosome to retain the neutral red solution was checked every 15 min by light microscopy (x40). The

test was terminated and the time recorded when greater than 50% of the haemocytes leaked the neutral red dye out of the lysosome into the cytosol.

2.4.2 Micronuclei formation in mussel haemocytes

Approximately 0.1 ml of haemolymph was removed from the posterior adductor muscle of each mussel with a hypodermic syringe containing 0.1 ml of PBS buffer (100 mM PBS, 10 mM EDTA). The haemolymph and PBS buffer were mixed briefly in the syringe and placed on a microscope slide. The slide was then placed in a humid chamber for 15 min to enable the haemocytes to adhere to the slides. Excess fluid was drained and the adhered haemocytes were fixed in 1% glutaraldehyde for 5 min. Following fixation, the slides were gently rinsed in PBS buffer and left to air-dry overnight. The dried slides were brought back to the laboratory for further processing.

Slides were stained with 1 µg/ml bisbenzimide 33258 (Hoechst) solution for 5 min, rinsed with distilled water and mounted in glycerol McIlvaine buffer (1:1). The frequency of micronuclei formation was measured on coded slides without knowledge of the exposure status of the samples to eliminate bias. The frequency of micronuclei in haemocytes was determined microscopically at 1000x magnification. A total of 2000 cells were examined for each experimental group of mussels.
Only cells with intact cellular and nuclear membrane were scored. MN were scored when: i) nucleus and MN have a common cytoplasm, ii) colour intensity and texture of MN is similar to the nucleus, iii) the size of the MN is equal or smaller than 1/3 of the nucleus, iv) MN are apparent as spherical structures with a sharp contour.

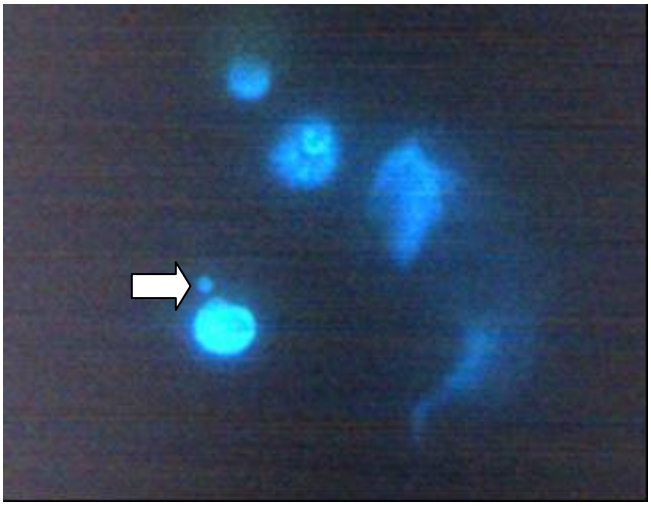

Figure 5. Micronuclei formation in a mussel haemocyte. (x1000 magnification)

2.4.3 Mussel histochemistry

Mussel histochemistry was carried out by colleagues at the University of the Basque Country, Spain. Digestive gland and gonad tissue were removed from individual mussels and preserved by either snap freezing in liquid nitrogen or submersion in formalin. The preserved tissues were transported to Spain by 24 h courier.

Digestive gland histopathology

Fixed tissues were dehydrated in alcohols and embedded in paraffin. Histological sections (7 µm) were cut with the aid of a rotary microtome, stained with haematoxylin/eosin (H/E) and mounted. Prevalence of parasites, haemocyte infiltration and general condition of the digestive epithelium, the interstitial connective tissue and the gonad tissue were systematically recorded.

Digestive cell loss (Vv_{BAS})

As an indication of whether cell-type replacement occurred or not, the volume density of basophilic cells (Vv_{BAS}) in the digestive gland of mussels was determined by means of stereology. A Weibel graticule (M-168; Weibel, 1979) was superimposed on 7 µm paraffin sections stained with H/E with the aid of a drawing tube attachment. Randomly selected fields were counted (20x objective). The volume density of basophilic cells was calculated as Vv_{BAS} = VBAS/VDT where V = volume; BAS = basophilic cell and DT = digestive gland epithelium.

Lysosomal membrane stability

The determination of lysosomal membrane stability was based on the time of acid labilization treatment required to produce the maximum staining intensity according to UNEP/RAMOGE (1999), after demonstration of hexosaminidase (Hex) activity in digestive cell lysosomes.

Slides were put at 4 ºC for 30 min and then 10 min at RT prior to staining. Serial cryotome sections (10 µm) were subjected to acid labilization in intervals of 0, 3, 5, 10, 15, 20, 30 and 40 min in 0.1 M citrate buffer (pH 4.5 containing 2.5 % NaCl) in a shaking water bath at 37 ºC, in order to find out the range of pre-treatment time needed to completely labilise the lysosomal membrane. Following this treatment, sections were transferred to the substrate incubation medium for the demonstration of Hex activity. The incubation medium consisted of 20 mg naphthol AS-BI-N-acetyl-β-D glucosaminide (Sigma, N 4006) dissolved in 2.5 ml of 2-methoxyethanol (Merck, 859), and made up to 50 ml with 0.1 M citrate buffer (pH 4.5) containing 2.5 % NaCl and 3.5 g of low viscosity polypeptide (Sigma, P5115) to act as a section stabiliser. Sections were incubated in this medium for 20 min at 37 ºC, rinsed in a saline solution (3.0 % NaCl) at 37 ºC for 2 min and then transferred to 0.1 M phosphate

buffer (pH 7.4) containing 1mg/ml of diazonium dye Fast Violet B salt (Sigma, F1631), at RT for 10 min. Slides were then rapidly rinsed in running tap water for 5 min, fixed for 10 min in Baker's formol calcium containing 2.5 % NaCl at 4 ºC and rinsed in distilled water. Finally, slides were mounted in Kaiser's glycerine gelatine and sealed with nail varnish.

The time of acid labilization treatment required to produce the maximum staining intensity was assessed under the light microscope as the maximal accumulation of reaction product associated with lysosomes (UNEP/RAMOGE 1999). Four determinations were made for each animal by dividing each section in the acid labilization sequence into 4 approximately equal segments and assessing the labilization period in each of the corresponding set of segments. The mean value was then derived for each section, corresponding to an individual digestive gland.

Palmitoyl-CoA Oxidase Activity

Digestive glands were individually homogenised in a Braun-Potter homogeniser using TVBE buffer (1 mM sodium bicarbonate, 1 mM EDTA, 0.1% ethanol and 0.01% Triton X-100; pH=7.6). After homogenisation, samples were centrifuged at 500 g for 15 minutes. Supernatants were removed and diluted appropriately to perform the assays. Peroxisomal palmitoyl-CoA oxidase activity was measured as described by Small et al. (1985). Total protein of all samples was measured according to the Lowry method using a commercial protein as standard (BioRad, California).

2.4.4 Tissue chemistry

For each mussel group/ treatment, triplicate samples were taken for analysis of selected metals, PAHs and NPDs. Five whole mussels per sample were removed from their shells and placed in high temperature treated (560 ^{o}C) glass containers. The mussels were frozen and transported to NIVA on dry ice. All samples were stored at -20 ^{o}C until analyses.

Samples were defrosted, homogenised and a sub sample taken of approximately 5 g. 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 40 ml cyclohexane and dried over sodium sulphate. The extracts were reduced by a gentle stream of nitrogen and cleaned by size exclusion chromatography. Analysis proceeded by gas chromatography with mass spectrometric detection (GC-MS) with the MS detector operating in selected ion monitoring mode (SIM). The GC was equipped with a 30 m column with a stationary phase of 5% phenyl polysiloxane (0.25 mm i.d. and 0.25 µm film thickness), and the injector operated in splitless mode. The initial column temperature was 60 ^{o}C, which after two minutes was raised

stepwise to 310 ^{o}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.

2.5 Semipermeable membrane devices (SPMDs)

The semipermeable membrane device (SPMD) developed by Huckins et al. (1990) is the best described passive sampling device for hydrophobic contaminants in the aquatic environment. It consists of a flat tube of low-density polyethylene filled with triolein. Hydrophobic chemicals (log K_{ow} > 3.0), including many classical environmental contaminants such as PAHs, diffuse into and become concentrated within the SPMD. The rate of this uptake is controlled by the physicochemical properties of the analyte and by external environmental factors such as water flow and temperature. The effects of the environmental factors can be accounted for by adding known concentrations of performance reference compounds (PRCs) to the samplers before deployment. The release rate of these PRCs can be used to calculate the uptake of target compounds and provide a time integrated measure. This has clear advantages over spot sampling, since variations in compound concentrations over time are accounted for. Other advantages include the ability to detect low environmental concentrations of compounds and the ability to measure the freely dissolved and bioavailable fraction.

Deployment

SPMDs were wound around stainless steel deployment spiders (Figure 6), which were either placed directly in exposure tanks for laboratory studies or in commercially available stainless steel containers for field stations (Environmental Sampling Technologies, St Joseph, USA). In all cases, three replicates per tank/ station were used. At field stations, cages were fastened directly to the ropes of deployment rigs at the same depths as the mussels. SPMDs were spiked with a mixture of deuterated PAH as PRCs and were obtained from ExposMeter (Tavelsjo, Sweden).

Sampler extraction and chemical analysis

The exterior of the SPMDs were briefly cleaned before extraction by dialysis with hexane and clean up by gel permeation chromatography (GPC) to remove interferences. Resulting extracts were reduced in volume and analysed by gas chromatography-mass spectrometry (GC-MS) for PAH, largely as described for mussel samples. Quantification of individual components was performed by using the relative response of internal standards. Detection limits were typically low ng/SPMD levels.

In order to correct for any possible contamination during study procedures, control or 'blank' SPMDs were used at each stage of the project. These included field controls (FCs) that are exposed to the air during deployment and retrieval (SPMDs are also efficient air samplers) and laboratory controls (LCs) that follow exposure to solvents, glassware etc. during work up. At least one of each type of control was used per 10 exposed samplers. Initial (time zero) concentrations of PRCs were also established from LCs.

Figure 6. SPMD ready for use, mounted on a stainless steel spider in a deployment cage. *(Photo source: Christopher Harman).*

Calculation of sampling rates and water concentrations

An empirical model, described in detail by Huckins et al. (2006), was used in the calculation of water concentrations from SPMD accumulations. In this model compound specific or intrinsic effects are adjusted based on the log K_{ow} of the analyte and site-specific or extrinsic factors arising from differences in environmental variables are adjusted by using the PRC data. In this way the uptake for each individual compound at each sampling station was established (expressed as a sampling rate, L/d). The use of the sampling rate term allows a more direct comparison to traditional batch extraction techniques (sampling rate × exposure time = volume of water sampled). Where individual analytes were not detected in SPMDs then the analytical detection limit was used in calculations to provide a maximum theoretical concentration in the water.

3. Results

3.1 Field mussels collected from the shore

3.1.1 Lysosomal membrane stability (LMS) in haemocytes (h)

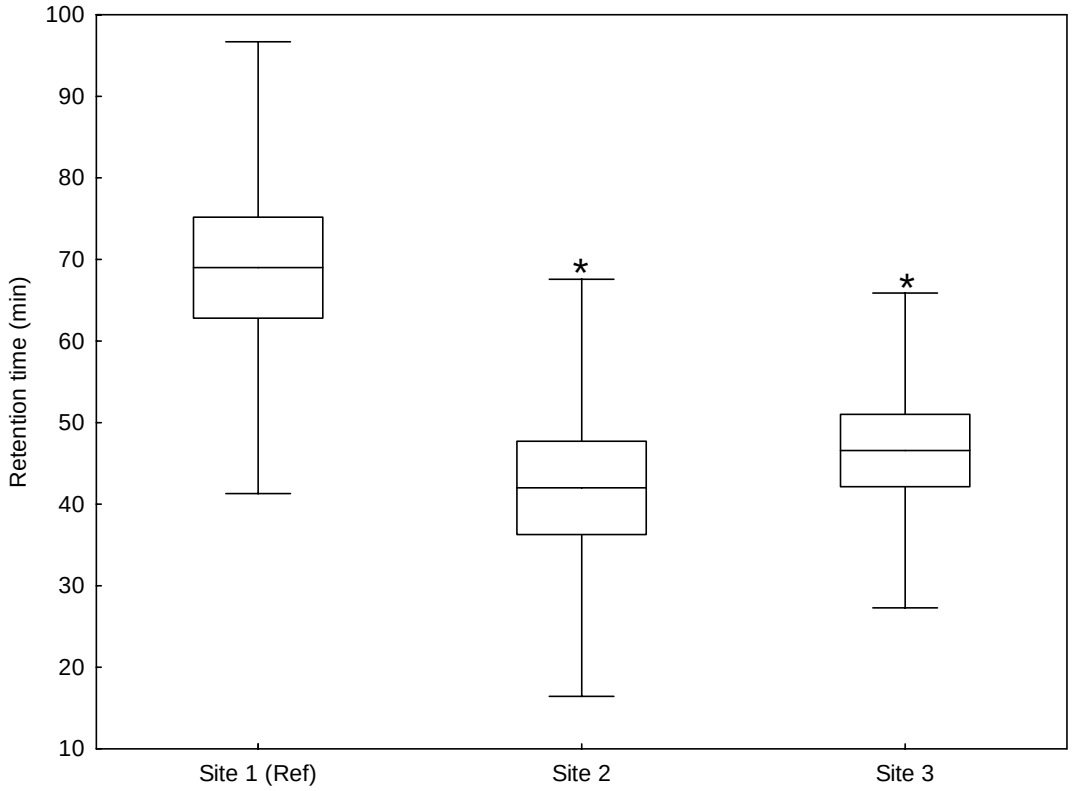

Figure 7. Comparison of NR retention time between the three mussel groups from native populations. Data expressed as mean, standard error (box) and standard deviation (outer line). * significant difference from reference site (ANOVA, Tukey P<0.05).

From the lysosomal stability test with mussel haemocytes, significantly shorter retention times were observed in mussels collected from the exposure sites (2 & 3) compared to the reference site 1 (ANOVA, Tukey, p<0.05, Figure 7). This suggests that the mussels from sites 2 and 3 were exposed to some kind of environment stress that resulted in an apparent reduction in health status compared to the reference mussels.

3.1.2 Micronuclei (MN) formation

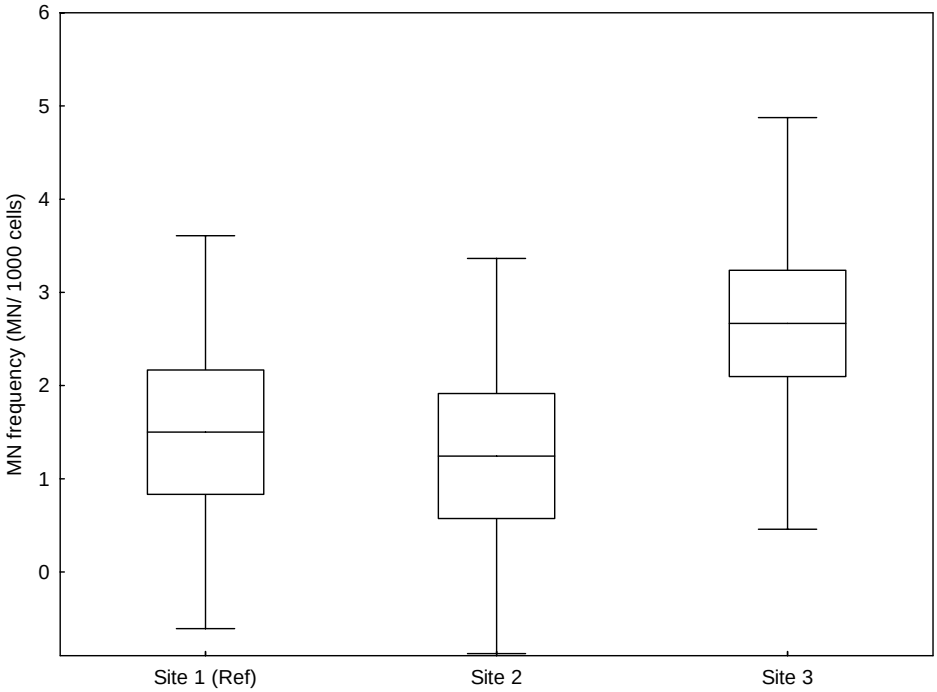

Figure 8. The frequency of micronuclei formation in native shore mussels collected from the three sites. Data expressed as mean, standard error (box) and standard deviation (outer line).

The formation of micronuclei has been used as a sensitive indicator of organism health and provides an assessment of exposure to genotoxic compounds. A slight increase in micronuclei formation was found in mussels collected from Site 3 compared to the other two sites, although this apparent increase was not found to be statistically significant ($P>0.05$).

3.1.3 Mussel histochemistry

Digestive gland histopathology

The histological analysis performed in the digestive gland tissue of the mussels indicated marked differences between the studied stations and experimental conditions. Prevalence of parasites, haemocyte infiltration and general condition of the digestive epithelium were examined. Overall, no significant parasitic infestation or pathological lesion was found in any case.

For the shore native mussels, the digestive gland tissue presented a normal histological integrity in the 3 studied sites (Fig. 9A-C), with a well organized interstitial connective tissue (ICT), apparently unaltered epithelia in stomach and digestive gland and food material being processed in the mid-gut lumen. However, in mussels from Site 3, and to a lesser extent in those from Site 2, thinning and vacuolisation of the digestive gland epithelium as well as an apparent reduction in the size of the

digestive alveoli were recorded (Figs 9B-C), which seems to suggest that, mainly in Site 3, mussels were subjected to some kind of environmental stress.

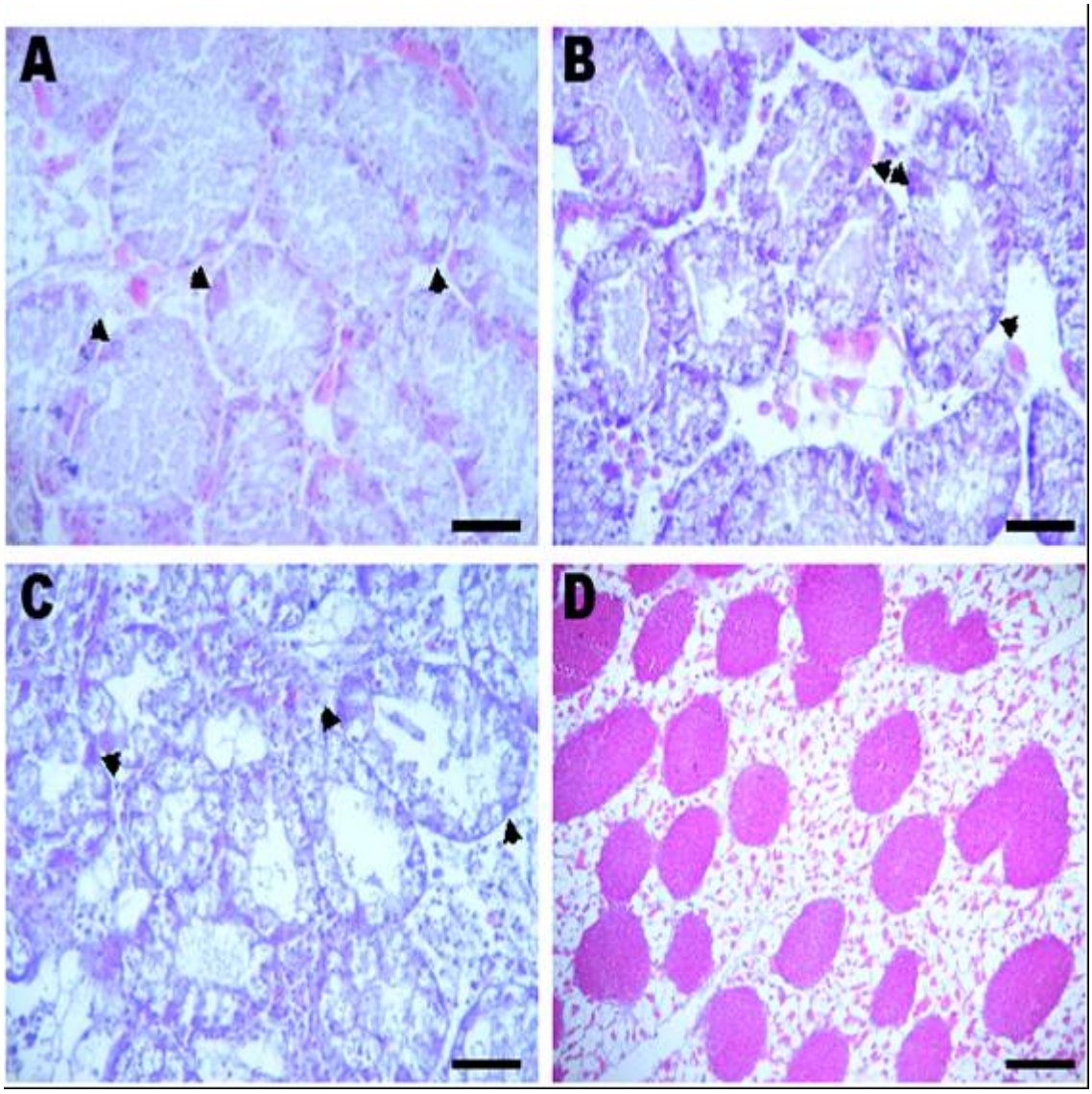

Figure 9. Micrography of the digestive gland and gonad tissue of mussels collected from the shore. A: digestive alveoli of mussels from the Site 1; B: digestive alveoli of mussels from Site 2; C: digestive alveoli of mussels from the Site 3; D: male gonad of mussels from the Site 1. Arrows indicate the presence of basophilic cells in the digestive epithelium. Scale bar: A, B and C 50 µm; D 250 µm.

Digestive cell loss (Vv_{BAS})

Vv_{BAS} provides a sensitive indication of general stress (Zaldibar et al., 2007). Vv_{BAS} values below 0.10 $\mu m^3/\mu m^3$ indicate a healthy condition; whereas Vv_{BAS} values higher than 0.12 $\mu m^3/\mu m^3$ indicate a stress situation (Marigómez et al, 2006). In native shore mussels (Fig. 8A), Vv_{BAS} values recorded in Site 3 were significantly higher than those recorded in Site 1 (reference site). The Vv_{BAS} values in Site 1 were below 0.10 $\mu m^3/\mu m^3$ which, according to the critical values (Marigómez et al, 2006), would indicate good health. Vv_{BAS} values in Site 2 were higher but very variable and thus not significantly

different from those recorded in Site 1. It must be mentioned that reference values in Biscay Bay vary with season with baseline Vv_{BAS}.below 0.10 $\mu m^3/\mu m^3$ in summer but below 0.05 in spring and autumn (Marigómez et al, 2006), and thus it can not be discarded that baseline Vv_{BAS} values in North Sea in late summer-autumn might be lower than 0.10 $\mu m^3/\mu m^3$ and therefore also mussels from Site 1 might be subjected to some source of environmental stress, although to a much lesser extent than Sites 2 and 3.

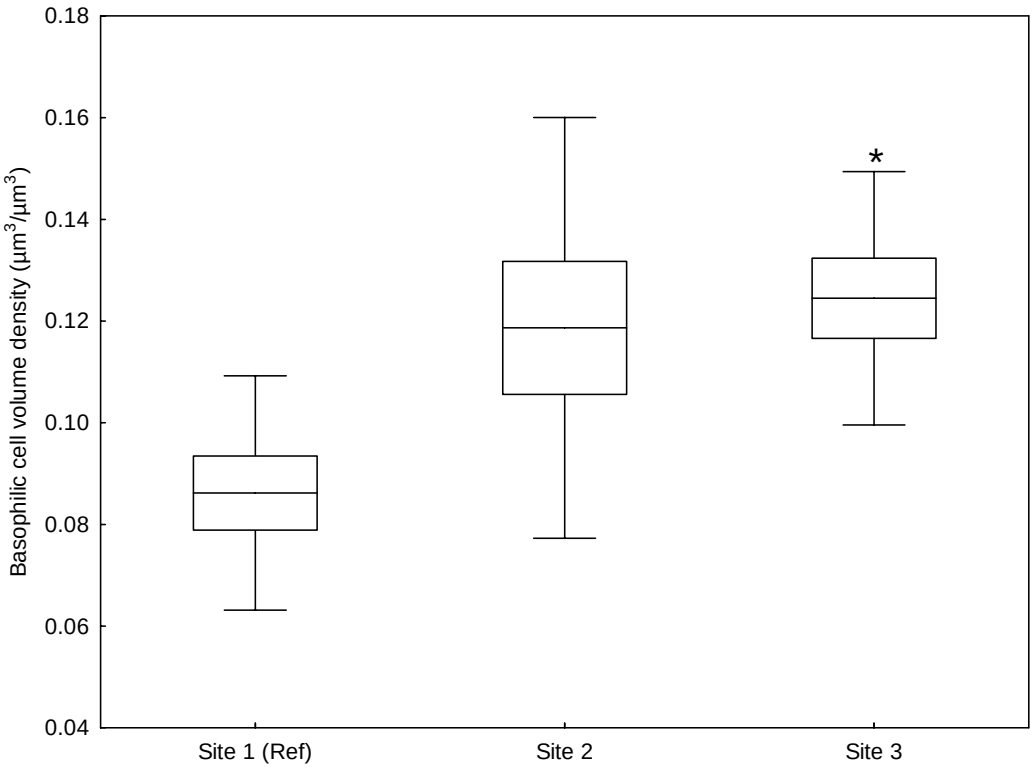

Figure 10. Volume density of basophilic cells in the digestive gland of native shore mussels. Data expressed as mean, standard error (box) and standard deviation (outer line).* indicates significant differences ($P < 0.05$) from reference site (Site 1).

Lysosomal membrane stability (LMS) test in digestive gland (dg) cells

The LMS test (UNEP/RAMOGE, 1999; Marigómez et al., 2005) provides a very sensitive indication of general stress. Generally, LP values over 20 min indicate a healthy condition; whereas LP values lower than 10 min indicate a severe stress situation (Viarengo et al., 2000). In native shore mussels (Figure 11), LP values recorded in Sites 2 and 3 were significantly lower than those recorded in Site 1 (reference site). The LP values in Site 1 were around 15 min which, according to consensus critical values (Viarengo et al., 2000), may be considered as an indication of a certain degree of stress, but the LP values in Sites 2 and 3 approached 5 min .which reveals a marked stress condition.

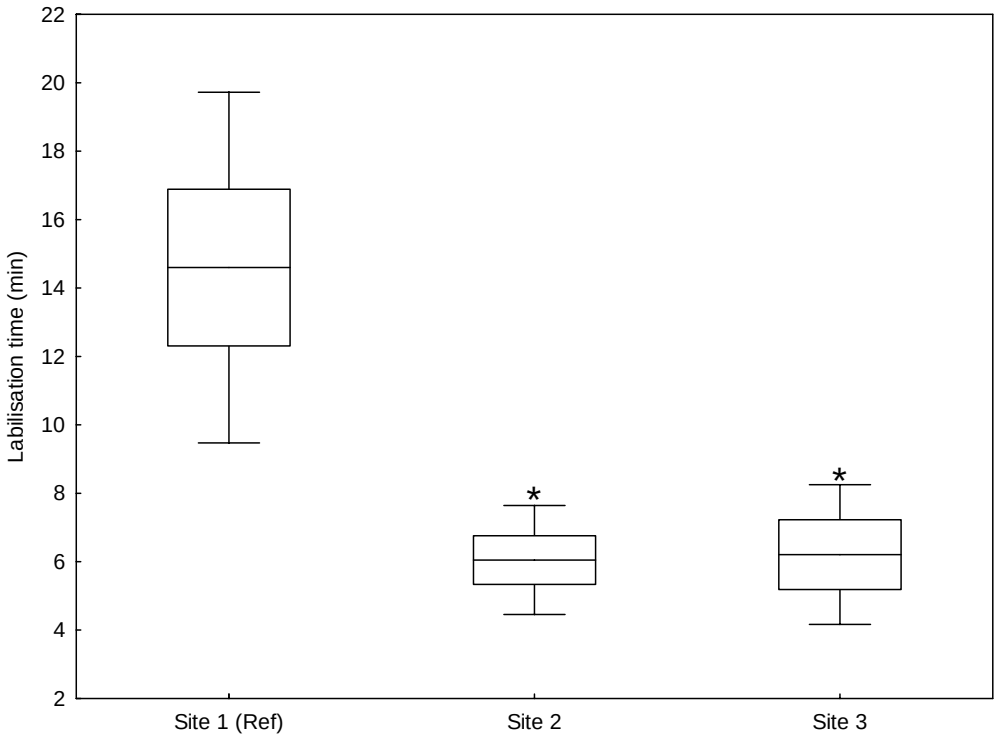

Figure 11. Labilization period for lysosomes in the digestive gland of native shore mussels. Data expressed as mean, standard error (box) and standard deviation (outer line). * indicates significant differences ($P < 0.05$) from reference site (Site 1).

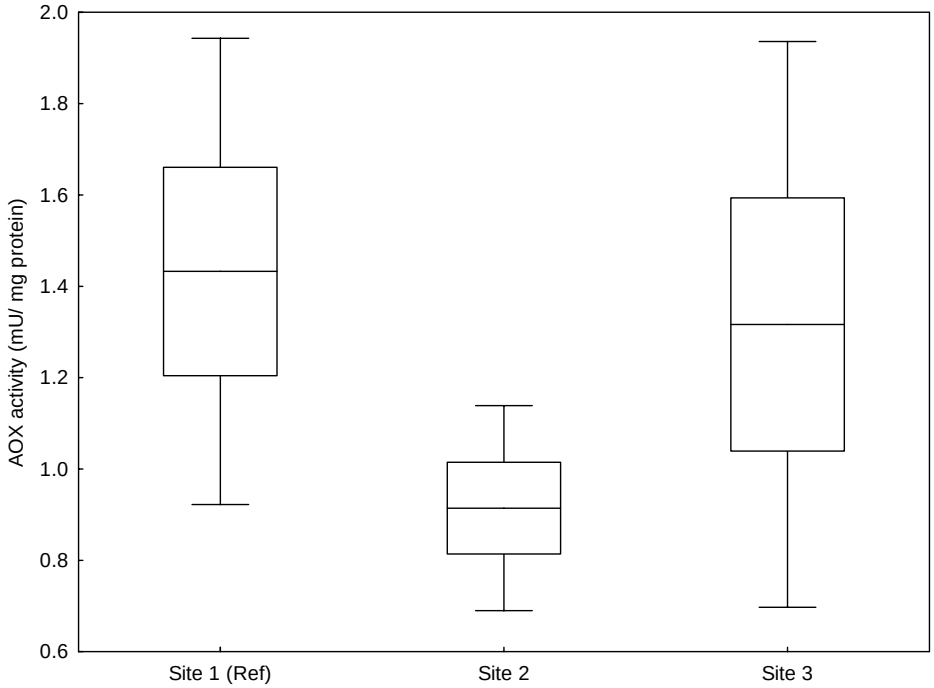

Figure 12. AOX activity in the digestive gland of native shore mussels. Data expressed as mean, standard error (box) and standard deviation (outer line).

Palmitoyl-CoA Oxidase Activity

In native shore mussels, AOX is apparently reduced in Site 2 in comparison with Site 1 but differences were not significant due to the great intravariability in samples (Figure 12). No sustained conclusion can be obtained regarding exposure to peroxisome proliferators.

3.1.4 Mussel chemistry

Low or undetected concentrations of PAHs and metals were measured in the whole mussel homogenates collected from the three sampling sites. These denote background concentrations of the measured compounds with no significance differences between sites.

Table 2. PAH body burden in native shore mussels collected from the three sampling sites (mg/ kg).

Compound	Site 1			Site 2			Site 3		
	1	2	3	1	2	3	1	2	3
Naphthalene	<0.0045	<0.0045	<0.0045	<0.0045	<0.0045	<0.0045	<0.0045	<0.0045	<0.0045
Acenaphthylene	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010
Acenaphthene	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010
Fluorene	**0.0012**	**0.0015**	**0.001**	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010
Phenanthrene	**0.0032**	**0.0041**	**0.0035**	**0.0015**	**0.0014**	**0.0011**	<0.0010	**0.0016**	**0.0011**
Anthracene	**0.0018**	**0.0018**	**0.0014**	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010
Fluoranthene	**0.0037**	**0.0047**	**0.0047**	**0.001**	**0.0013**	<0.0010	<0.0010	**0.0011**	<0.0010
Pyrene	**0.0018**	**0.0023**	**0.0021**	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010
Benzo(a)anthracene	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010
Chrysene	<0.0010	**0.0011**	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010
Benzo(b)fluoranthene	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010
Benzo(k)fluoranthene	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010
Benzo(a)pyrene	<0.0015	<0.0010	<0.0015	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010
Dibenz(a,h)anthracene	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010
Benzo(g,h,i)perylene	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010
Indeno(1,2,3-cd)pyrene	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010	<0.0010
Sum PAH16	0.0117	0.0155	0.0127	0.0025	0.0027	0.0011	n.d	0.0027	0.0011
Cadmium	0.13	0.115	0.099	0.11	0.122	0.14	0.13	0.154	0.15
Copper	0.88	1.01	0.9	0.9	0.8	0.97	1.16	1.13	0.99
Mercury	0.01	0.01	0.008	0.01	0.009	0.01	0.01	0.009	0.008
Lead	0.05	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.07
Zinc	16.4	20.5	17.4	13.1	15.4	14	17.1	19.1	20.2
Lipid %	1.4	1.7	1.7	1.4	1.3	1.6	1.6	1.8	1.5

3.2 Caged mussels

3.2.1 Lysosomal membrane stability (LMS) in haemocytes (h)

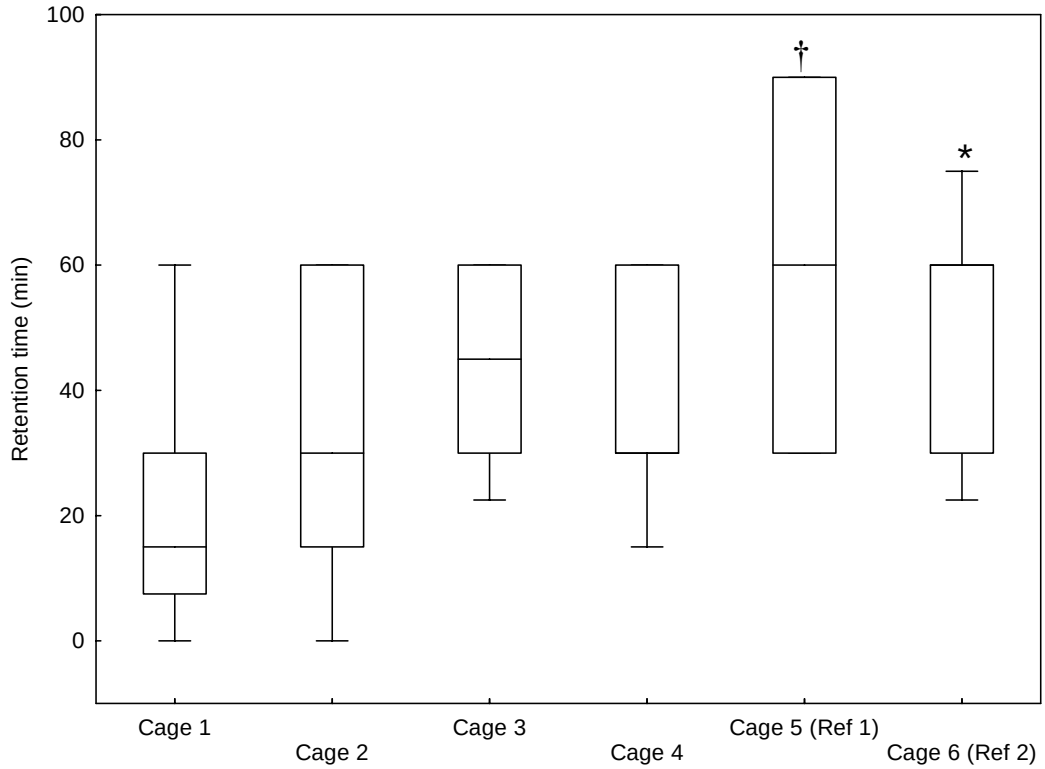

Figure 13. Comparison of NR retention time between the caged mussels at varying distances from the produced water discharge. The figure shows median, quartiles and 10/90-percentiles.* significant difference from Cage 1; † significantly different from Cage 1 and 2 (P<0.05, ANOVA, Tukey)

Highest retention times were found in mussels from the two reference sites (Cage 5 &6), which were significantly higher than those found in mussels closest to the PW discharge (Cage 1 and 2). However, it should be noted that the retention times from all mussels were low, which suggest a compromised health at all stations including the reference stations.

3.2.2 Micronuclei (MN) formation

Higher frequency of MN were found in haemocytes of mussels from cage 1 compared to all other cages, although this was only found to be statistically significantly different from cage 3 ($p<0.05$, ANOVA).

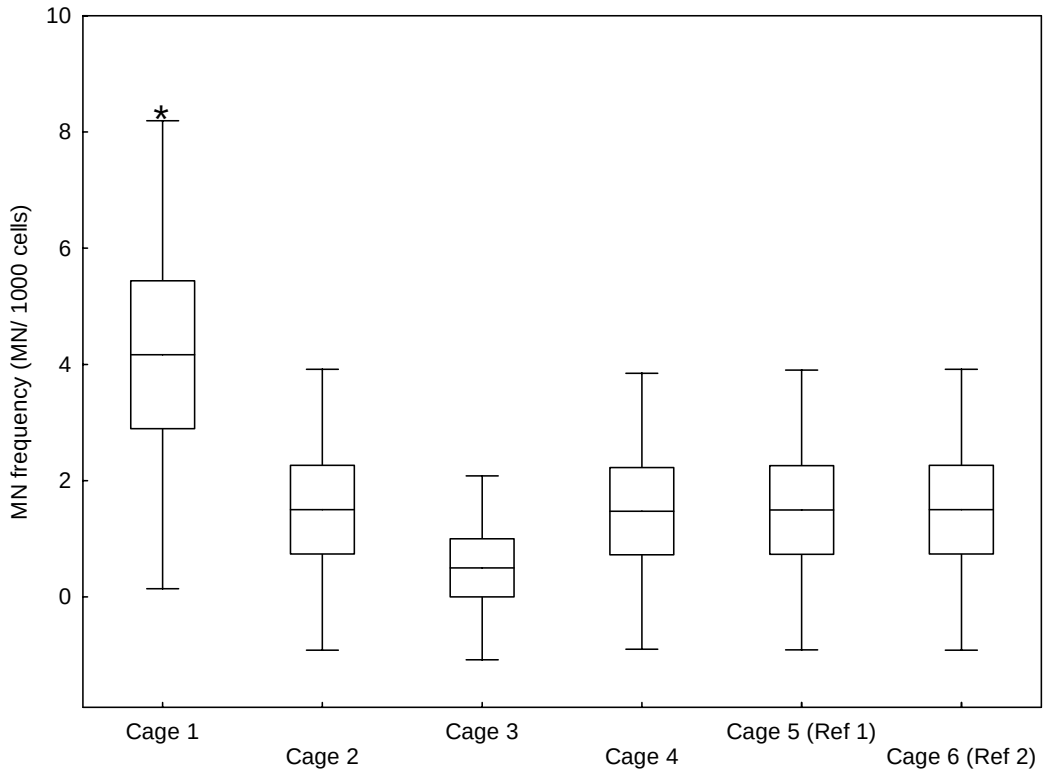

Figure 14. The frequency of micronuclei formation in caged mussels from the six sites. Data expressed as mean, standard error (box) and standard deviation (outer line). * denotes significant difference from Cage 3 (ANOVA, Tukey, P<0.05).

3.2.3 Mussel histochemistry

Digestive gland histopathology

In general, the digestive gland tissue of mussels located at cages 1 and 2 (Figs. 15A, 15B & 15E) exhibited a histological integrity similar to that observed in the native shore mussels from Site 1 (Figure 9A-B). In contrast, the digestive tissue of mussels from cages 3-6 (Figure 15C-D) showed some alterations that included a certain reduction in the extent of the diverticular mass, disorganization of the ICT and thinning of the epithelium in the digestive alveoli.

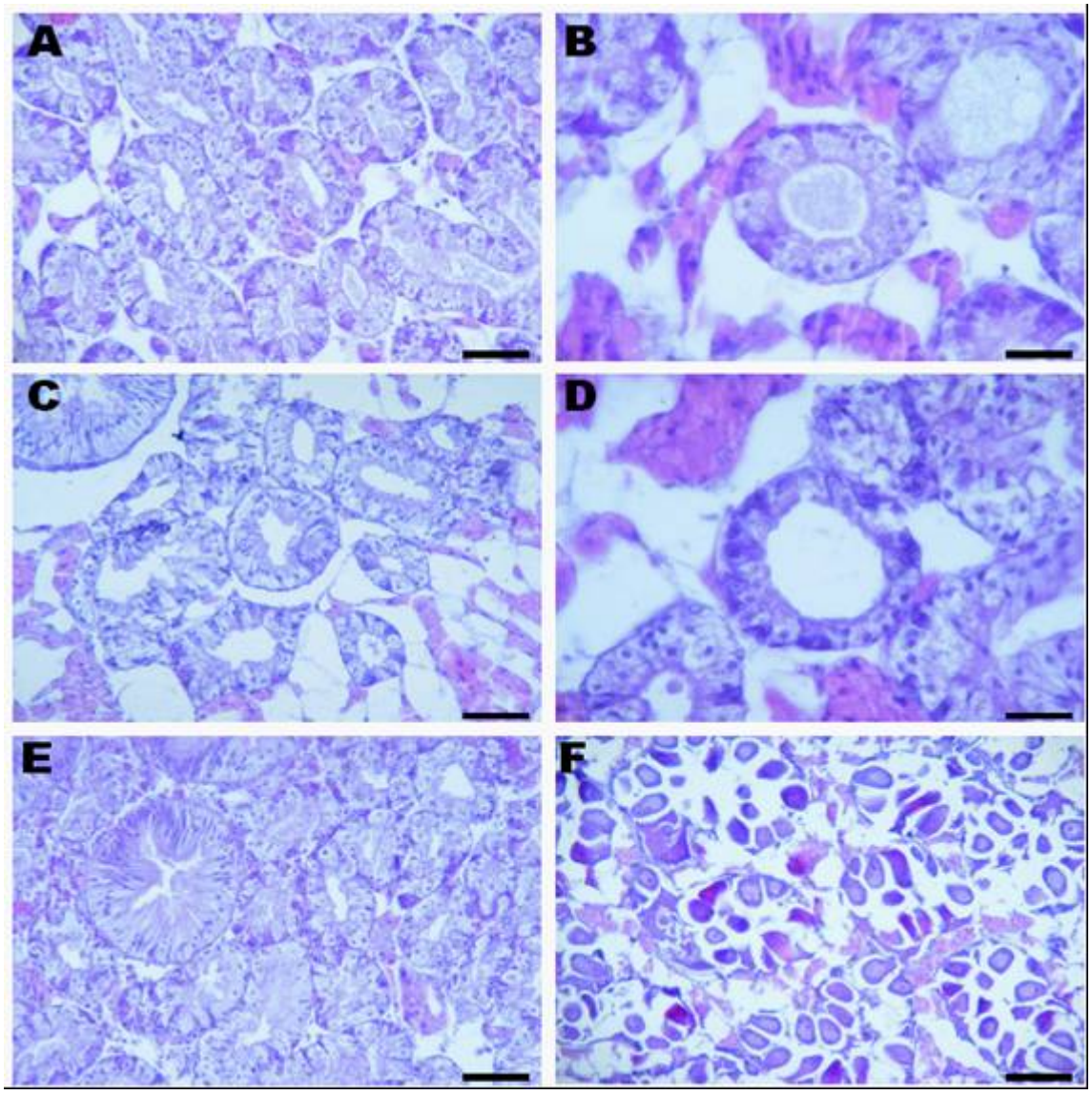

Figure 15. Micrography of the digestive gland and gonad tissue of mussels caged at different sites to the discharge outlet. A and B: digestive alveoli of mussels from cage 1; C and D: digestive alveoli of mussels from cage 4. E: digestive alveoli of mussels from cage 2; F: female gonad of mussels from cage 1. Scale bar: A, C and E 50 µm; B and D 25 µm; F 100 µm.

Digestive cell loss (Vv_{BAS})

In caged mussels (Figure 16), Vv_{BAS} values were significantly lower in Cage 1 than Cage 5, although overall Vv_{BAS} values were above 0.10 $\mu m^3/\mu m^3$ in Cages 3 to 6 (certain degree of environmental stress) and below this value in Cage 1 and Cage 2 (healthier; under the assumption made due to limited knowledge of annual and geographical variability in baseline values).

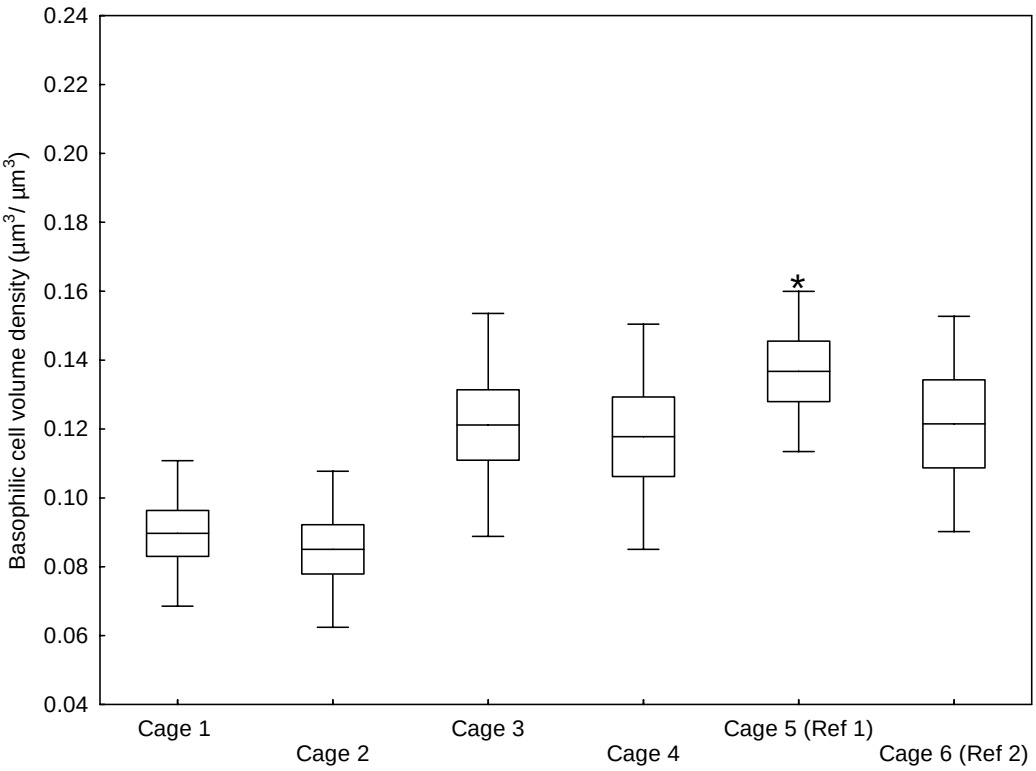

Figure 16. Volume density of basophilic cells in the digestive gland of caged mussels. * indicate significant differences ($P < 0.05$) from cage 1. Data expressed as mean, standard error (box) and standard deviation (outer line).

Lysosomal membrane stability (LMS) test in digestive gland (dg)

After examination at the light microscope of cryotome sections of digestive gland where lysosomes had been visualized by N-acetyl hexosaminidase cytochemistry differences in the appearance of lysosomes between native shore, and caged, and laboratory mussels, were readily evident (Figure 17). Native shore mussels presented deep purple stained small lysosomes, whereas caged and laboratory mussels presented violet stained large lysosomes that, at first, were less stable than the small ones. This may indicate reduced health of the caged and laboratory mussels compared to the native shore mussels.

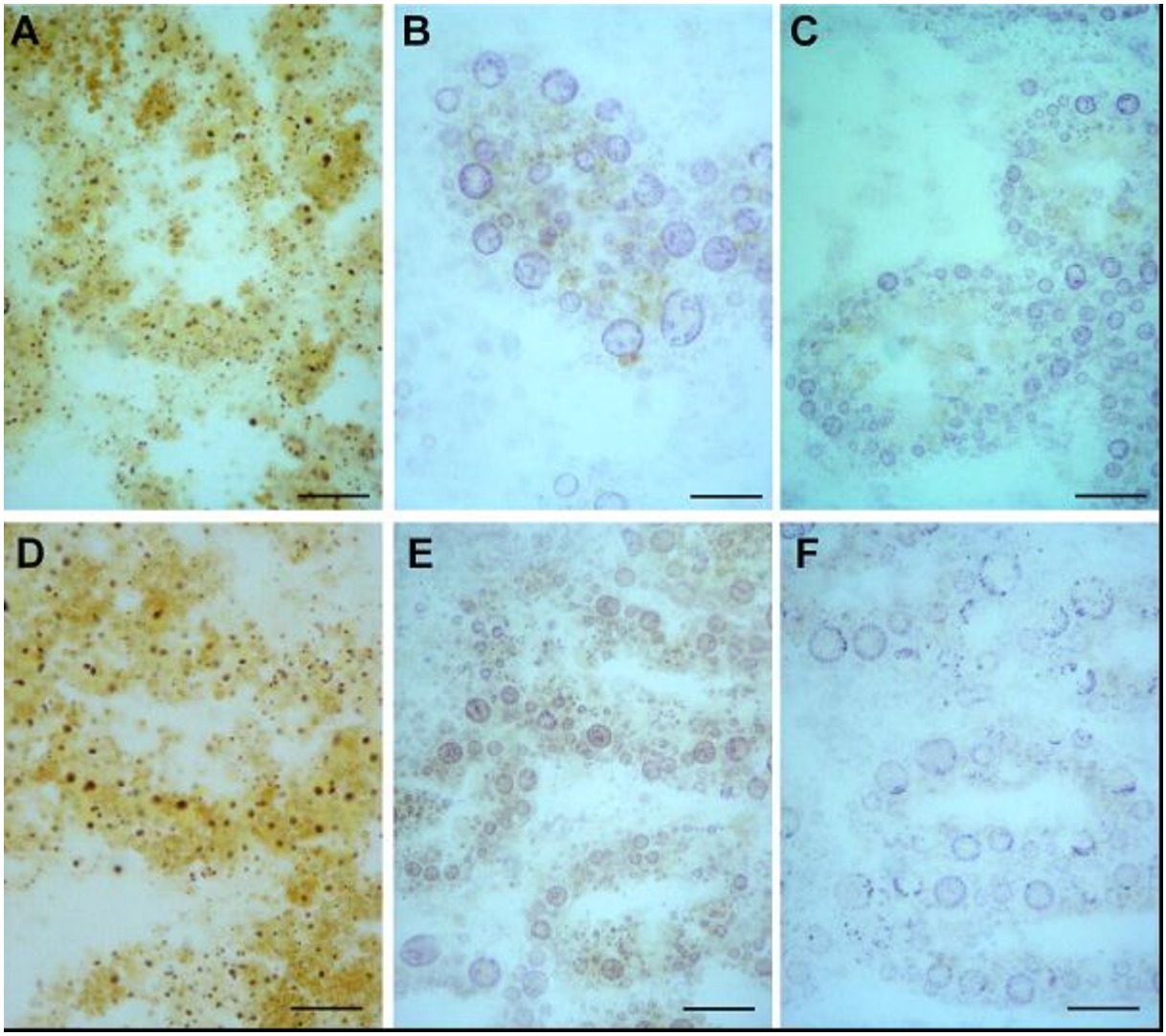

Figure 17. Micrography of lysosomes stained by the histochemical demonstration of hexosaminidase in cryotome sections. Scale bars: 30µm. A: Shore site 1; B: Cage 1; C: Sol 1; D: Shore site 2; E: Cage 6; F: Sol 5.

In caged mussels (Figure 18), LP values were significantly higher in the high exposure cage 1 than in all the other groups. LP values were below 15 min in cage 1 indicating a certain degree of environmental stress in this group of caged mussels, more marked stress in cage 2 (LP around 10 min) and a severe affection in mussels from cages 3 to 6, where LP values remained below 10 min. The data suggests the reference mussels were stressed more than the mussels located closest to the discharge, possible reasons for this will be discussed later.

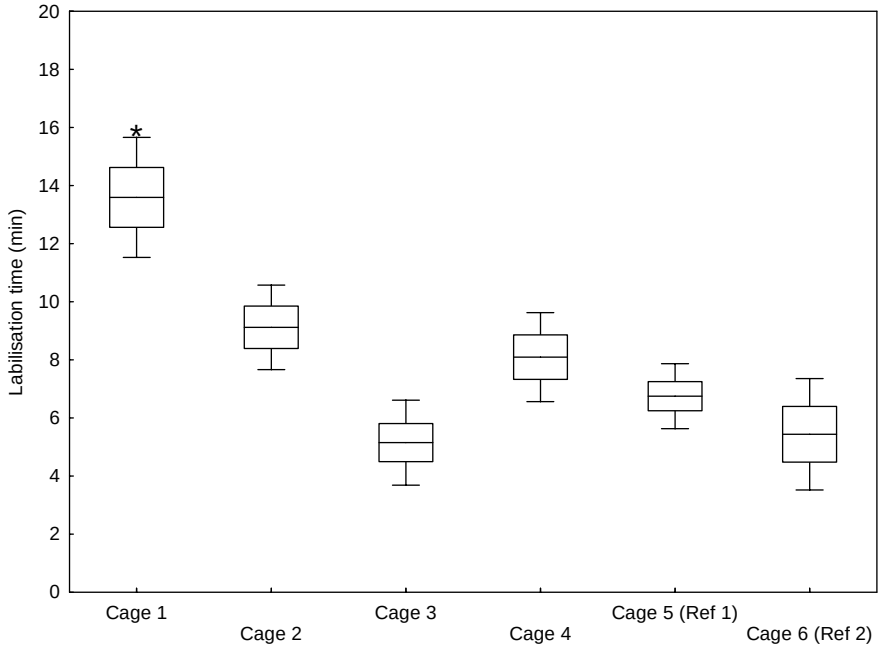

Figure 18. Labilization period for lysosomes in the digestive gland of caged mussels. Data expressed as mean, standard error (box) and standard deviation (outer line). * indicates significant differences (ANOVA, Tukey, $P < 0.05$) from all other cages.

Palmitoyl-CoA Oxidase Activity

AOX activities obtained after caging were very low (<0.5, Figure 19). Significantly higher AOX activity was found at the reference site (cage 5) (ANOVA, Tukey, $P<0.05$).

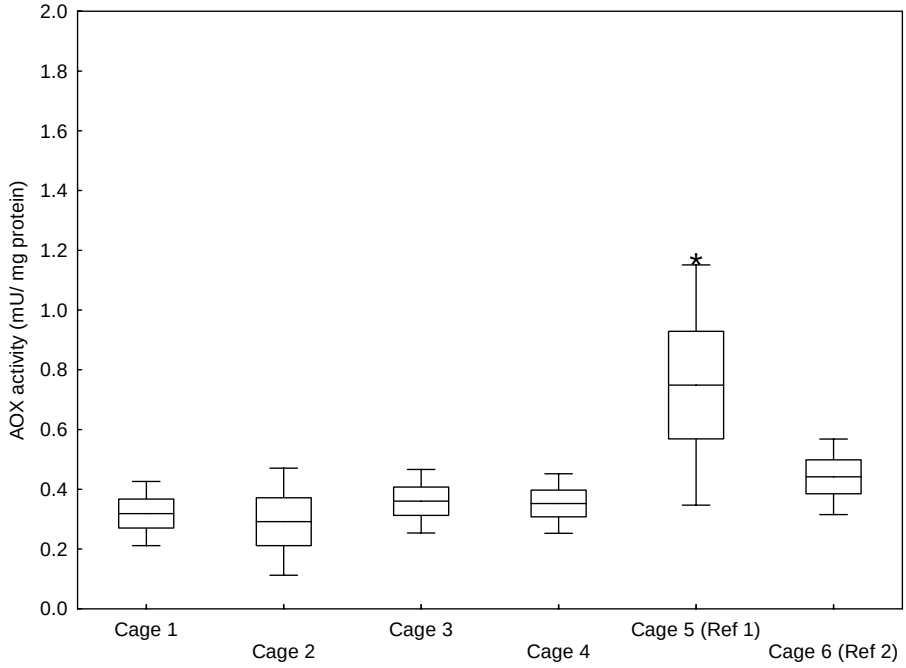

Figure 19. AOX activity in the digestive gland of caged mussels. * indicate significant differences (ANOVA, Tukey, $P < 0.05$) from cage 1, 2 and 4. Data expressed as mean, standard error (box) and standard deviation (outer line)

3.2.4 Mussel chemistry

Table 3. PAH concentration of whole mussel homogenates from the 6 cage sites.

	Cage 1			Cage 2			Cage 3			Cage 4			Cage 5			Cage 6		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Naphthalene	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.75	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8
C1-Naphthalenes	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	2.6	<2
C2-Naphthalenes	<2	<2	<2	2.2	<2	<2	2.8	<2	2.2	<2	<2	<2	<2	2.4	<2	<2	2.1	4
C3-Naphthalenes	<2	<2	<2	2.1	3.3	<2	2.8	<2	3.6	<2	2.2	2.7	3.4	3.2	2.2	2	3.9	3.1
Phenanthrene	<0.5	0.72	0.89	0.92	0.88	0.89	0.83	<0.5	0.94	0.8	0.84	0.88	0.94	1.1	0.88	0.96	0.83	0.88
C1-Phenanthrenes	<2	<2	<2	2.5	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
C2-Phenanthrenes	<2	2.4	3.6	3.5	3.3	2.5	3.6	<2	3	2.9	2.7	3.4	3.1	3.9	3.6	3.3	2.6	2.8
C3-Phenanthrenes	<2	<2	3.5	5	3.3	2.6	3	<2	<2	<2	<2	<2	3.1	<2	<2	<2	<2	<2
Dibenzothiophene	<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
C1-Dibenzothiophenes	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
C2-Dibenzothiophenes	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
C3-Dibenzothiophenes	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	3.3	<2	<2	<2	2.1	<2
Sum NPD	<19.8	<20.42	<23.29	<25.52	<24.08	<21.29	<24.28	<19.8	<23.04	<21	<21.04	<22.28	<25.14	<23.9	<21.98	<21.56	<23.43	<24.08
Acenaphthylene	<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
Acenaphthene	<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
Fluorene	<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
Anthracene	<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
Fluoranthene	<0.5	0.63	0.91	0.79	0.59	0.55	0.88	<0.5	0.67	0.85	0.79	0.97	0.86	1.1	0.93	0.68	0.74	0.68
Pyrene	<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
Benzo(a)anthracenes	<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
Chrysene	<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
Benzo(b)fluoranthene	<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	0.57	0.5	<0.5	<0.5
Benzo(k)fluoranthene	<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
Benzo(e)pyrene	<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
Benzo(a)pyrene	<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
Perylene	<1.0	0.65	<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
Indeno(1,2,3-cd)pyrene	<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
Dibenz(a,h)anthracene	<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
Benzo(g,h,i)perylene	<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	<28.3	<28.7	<31.7	<33.81	<32.17	<29.34	<32.66	<27.8	<31.21	<29.35	<29.33	<30.75	<33.5	<32.5	<30.48	<29.74	<31.67	<32.26
Sum PAH16	<8.3	<8.65	<9.1	<9.01	<8.77	<8.74	<8.96	<8.3	<8.91	<8.95	<8.93	<9.15	<9.1	<9.5	<9.18	<8.94	<8.87	<8.86

Table 4. Metal concentration of mussels collected from the six cage sites.

Cage No.	Rep	Distance from discharge (m)	Ag	Al	As	Cd	Cr	Cu	Fe	Hg	Ni	Pb	Zn
1	1	50	0.024	2.5	2.56	0.169	0.1	1.28	10	0.007	0.12	0.04	13.4
	2		0.013	4.3	2.67	0.141	0.2	1.48	12	0.007	0.15	0.03	11.9
	3		0.021	4.2	2.87	0.168	0.2	1.47	12	0.007	0.16	0.04	14.5
2	1	100	0.013	4.6	2.76	0.152	0.1	1.53	13	0.007	0.13	0.05	17.1
	2		0.008	3.1	2.75	0.149	0.2	1.26	10	0.006	0.13	0.04	12.9
	3		0.012	3.6	2.63	0.138	0.3	1.09	12	0.007	0.15	0.03	11.7
3	1	100	0.013	4.6	2.91	0.147	0.1	1.47	12	0.008	0.11	0.04	15.2
	2		0.015	4.5	2.99	0.159	0.1	1.53	12	0.007	0.12	0.04	19.2
	3		0.014	2.8	2.82	0.153	0.2	1.26	11	0.007	0.14	0.04	11.3
4	1	200	0.017	3.2	2.61	0.135	0.2	1.07	11	0.007	0.14	0.04	13.1
	2		0.02	3.4	2.76	0.150	0.1	1.66	11	0.006	0.11	0.04	13.4
	3		0.016	3.2	2.88	0.143	0.2	1.05	12	0.007	0.10	0.03	14.7
5	1	2000	0.011	2.9	2.84	0.161	0.1	1.33	10	0.008	0.12	0.03	14.3
	2		0.019	4.2	2.95	0.166	0.1	2.12	12	0.008	0.12	0.04	13.0
	3		0.015	3.7	2.93	0.147	0.1	1.91	11	0.007	0.11	0.04	12.5
6	1	2000	0.012	3.0	2.81	0.134	0.1	1.56	11	0.007	0.12	0.04	12.6
	2		0.011	2.9	2.85	0.140	0.2	1.67	11	0.007	0.12	0.03	15.8
	3		0.011	3.5	2.97	0.166	0.2	1.18	12	0.007	0.15	0.04	14.8

Background concentrations of PAHs and metals were found in mussels from all six cages. There were no apparent differences in mussel contaminant concentrations between the different cage sites.

3.2.5 Semipermeable membrane devices (SPMDs)

PAH concentrations were either low or undetected at all six mussel cage sites. Those PAHs that were detected in the water column include fluorene, fluoranthene, dibenzothiophene and phenanthrene. The performance reference compound (PRC) data (results not presented) confirmed that the SPMDs worked correctly in accordance with the uptake model used. Each SPMD sampled between 100-500L of water (depending on the compound) during the six week deployment. However, many target compounds were still not detected, which strongly suggests that only background concentrations of PAHs were present. No difference in PAH concentration between the cage sites was found from the SPMD data.

Table 5. PAH concentrations calculated from SPMDs. SPMDs were placed in the water column at 6 locations for 6 weeks in the vicinity of a produced water discharge. Data converted to ng/L. a = high blank values prevent reporting of this compound.

	Cage 1			Cage 2			Cage 3			Cage 4			Cage 5			Cage 6		
ng/L	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Naphthalene	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Acenaphthylene	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07	<0.08	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07	<0.07
Acenaphthene	<0.19	<0.19	<0.2	<0.19	<0.18	<0.19	<0.19	<0.2	<0.18	<0.18	<0.18	<0.18	<0.19	<0.19	<0.17	<0.18	<0.18	<0.19
Fluorene	0.25	0.26	0.27	0.29	0.19	0.2	0.29	0.3	0.16	0.11	0.1	0.14	0.15	0.15	0.15	0.09	0.13	0.15
Dibenzothiophene	0.08	0.08	0.08	0.09	0.06	0.06	0.08	0.08	0.05	0.05	0.05	0.06	0.06	0.06	0.06	0.05	0.07	0.06
Phenanthrene	0.92	0.91	0.99	1.18	0.8	0.68	1.03	1.01	0.58	0.52	0.52	0.8	0.83	0.92	0.73	0.63	0.8	0.83
Anthracene	<0.03	<0.03	<0.04	<0.03	<0.03	<0.03	0.04	<0.04	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03
Fluoranthene	0.16	0.15	0.15	0.15	0.15	0.12	0.15	0.14	0.12	0.13	0.13	0.15	0.29	0.28	0.26	0.23	0.24	0.28
Pyrene	0.05	0.05	0.05	0.05	0.05	0.05	0.06	0.05	0.05	0.04	0.04	0.05	0.1	0.1	0.08	0.07	0.08	0.09
Benz[a]anthracene	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.01	<0.02	<0.02	<0.02
Chrysene	<0.02	<0.02	<0.02	<0.02	0.03	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	0.02	0.04	0.03	0.03	<0.01	0.02	0.03
Benzo[b.j]fluoranthene	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	0.03	0.03	0.02	0.02	0.02	0.03
Benzo[k]fluoranthene	<0.02	<0.02	<0.03	<0.02	<0.02	<0.02	<0.02	<0.03	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.01	<0.02	<0.02	<0.02
Benzo[e]pyrene	<0.02	<0.02	<0.03	<0.02	<0.02	<0.02	<0.03	<0.03	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Benzo[a]pyrene	<0.02	<0.02	<0.03	<0.02	<0.02	<0.02	<0.02	<0.03	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Perylene	0.04	<0.02	<0.03	<0.02	0.07	<0.02	<0.02	<0.03	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	0.04
Indeno[1.2.3-cd]pyrene	0.07	<0.03	<0.03	<0.03	<0.02	<0.03	<0.03	<0.03	<0.02	<0.02	<0.02	<0.02	<0.03	<0.03	0.04	0.03	<0.02	<0.03
Dibenzo[ac/ah]anthracene	0.03	<0.02	<0.03	<0.03	<0.02	<0.02	<0.03	<0.03	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Benzo[g.h.l]perylene	0.05	<0.03	<0.04	<0.03	<0.03	<0.03	<0.03	<0.04	<0.03	<0.02	<0.03	<0.03	0.03	0<.03	0.03	<0.02	<0.03	<0.03
SUM PAH	<2.07	<1.98	<2.13	<2.31	<1.82	<1.62	<2.16	<2.18	<1.43	<1.31	<1.31	<1.69	<1.97	<2.05	<1.75	<1.53	<1.81	<1.97
PAH EPA16	<1.93	<1.86	<1.99	<2.17	<1.66	<1.51	<2.03	<2.04	<1.35	<1.23	<1.23	<1.59	<1.87	<1.94	<1.66	<1.44	<1.71	<1.85

3.3 Laboratory exposure

3.3.1 Lysosomal membrane stability (LMS) in haemocytes (h)

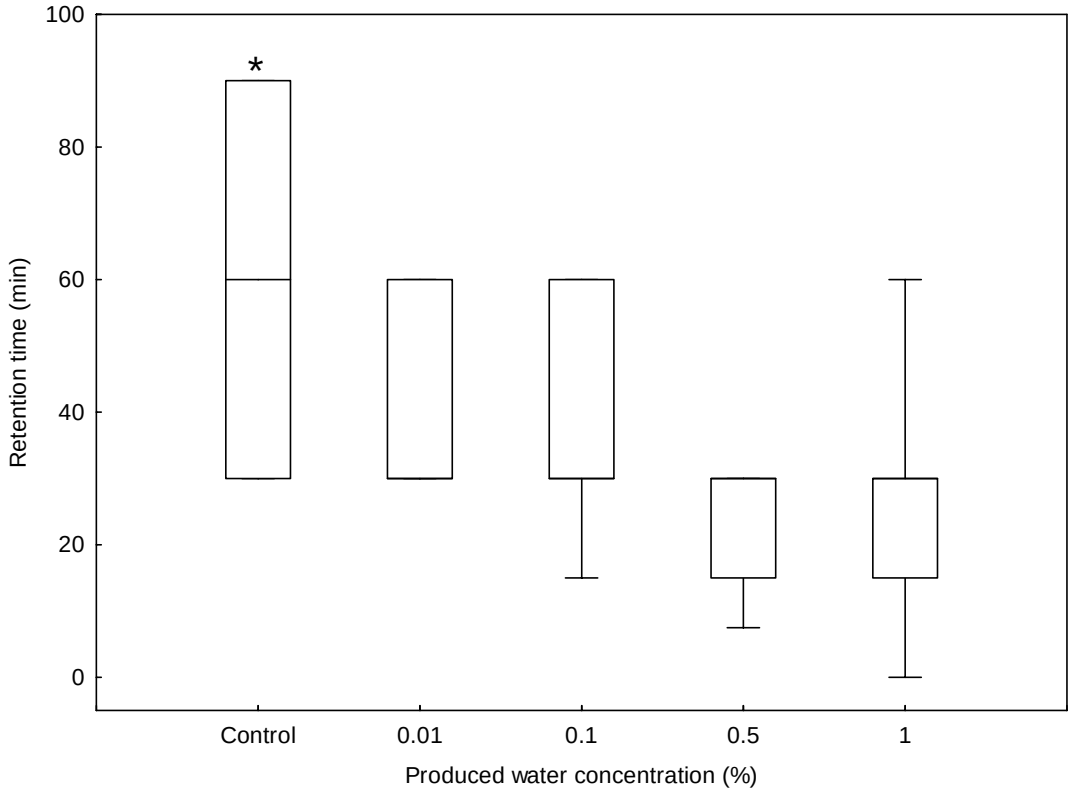

Figure 20. Comparison of NR retention time between the mussels exposed to known concentrations of produced water. The figure shows median, quartiles and 10/90-percentiles. * denotes significant different from all other groups (P<0.05, ANOVA, Tukey).

Highest retention times were found in mussels from the control group, which was found to be significantly higher than all other groups. However, it should be noted that the retention times from all mussels were low, which suggest a compromised health at all treatments including the control group.

3.3.2 Micronuclei (MN) formation

There were no differences in the prevalence of MN formation in mussel haemocytes between the exposure groups, with a low prevalence of MN in all groups.

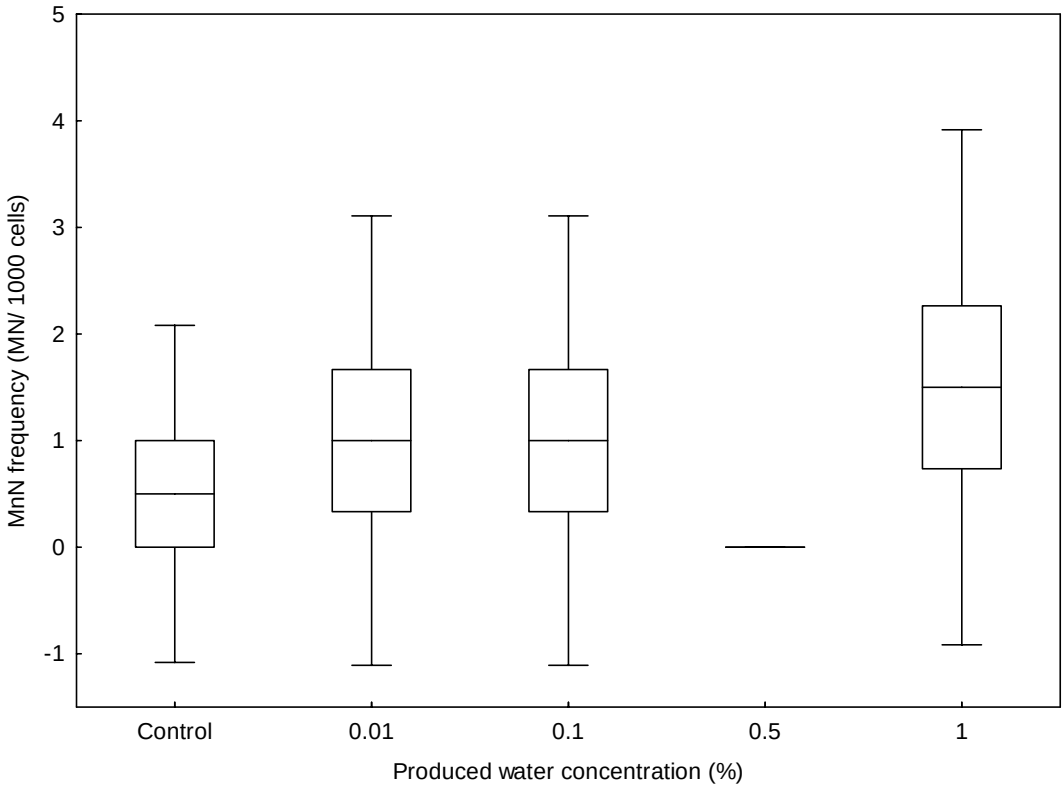

Figure 21. The frequency of micronuclei formation in mussels exposed to known concentrations of produced water effluent. Data expressed as mean, standard error (box) and standard deviation (outer line).

3.3.3 Mussel Histochemistry

Digestive gland histopathology

Laboratory PW exposure provoked clear histopathological alterations (Figure 22), although some degree of loss of histological integrity in the digestive gland tissue and certain epithelial thinning in digestive alveoli were also found in experimental control groups (Figure 22A). Overall, PW exposed mussels showed a severe reduction in the numbers of digestive diverticula, which appeared sparse throughout a highly disorganized and eventually fibrous ICT. Moreover, extreme thinning of the digestive gland epithelium and high prevalence of digestive alveoli with reconstituting appearance were characteristics in mussels exposed to high PW concentrations.

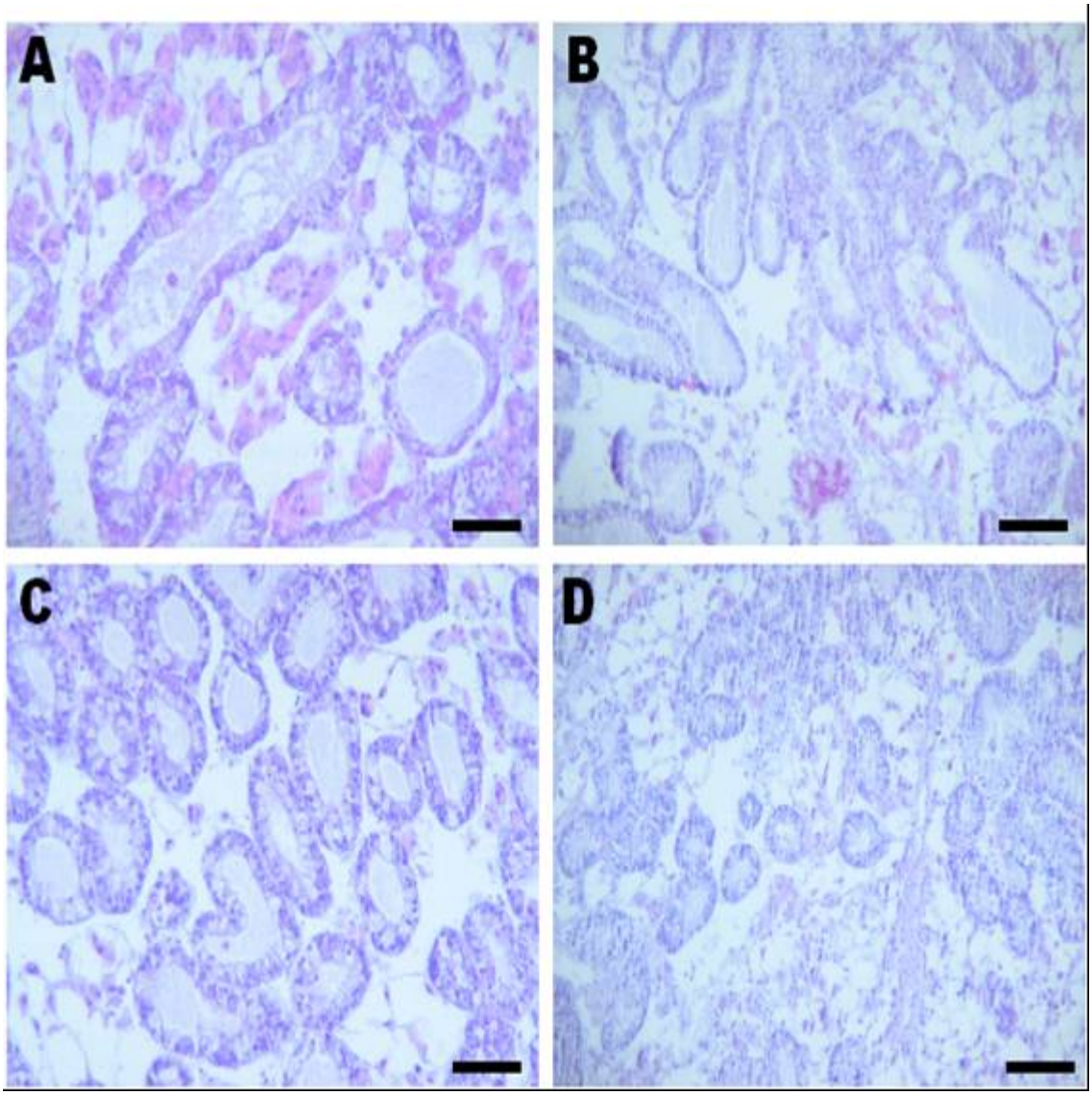

Figure 22. Micrography of the digestive gland tissue of PW exposed mussels in the laboratory. A: digestive alveoli of reference mussels; B: digestive alveoli of the low intermediate dose; C: digestive alveoli of the highest dose; D: digestive alveoli of the low dose. Note the difference in size of the digestive alveoli and the amount of connective tissue between the alveoli. Scale bar: A and C 50 µm; B and D 100 µm.

Digestive cell loss (Vv_{BAS})

In laboratory exposed mussels (Figure 23), Vv_{BAS} values were not significantly different between treatment groups. However, it is worth noting that (a) all the Vv_{BAS} values recorded (also in controls) were always above 0.12 $\mu m^3/\mu m^3$, which appears to suggest that all the mussels used for laboratory exposure experiments were not in good condition; (b) there existed a very high variability between mussels from the same experimental group; and (c) Vv_{BAS} values were highest at exposure to 0.1% PW. Overall, it seems that PW exposure provokes digestive cell loss but the results are not definitively evident due to the condition of the mussels used for experimental laboratory exposures.

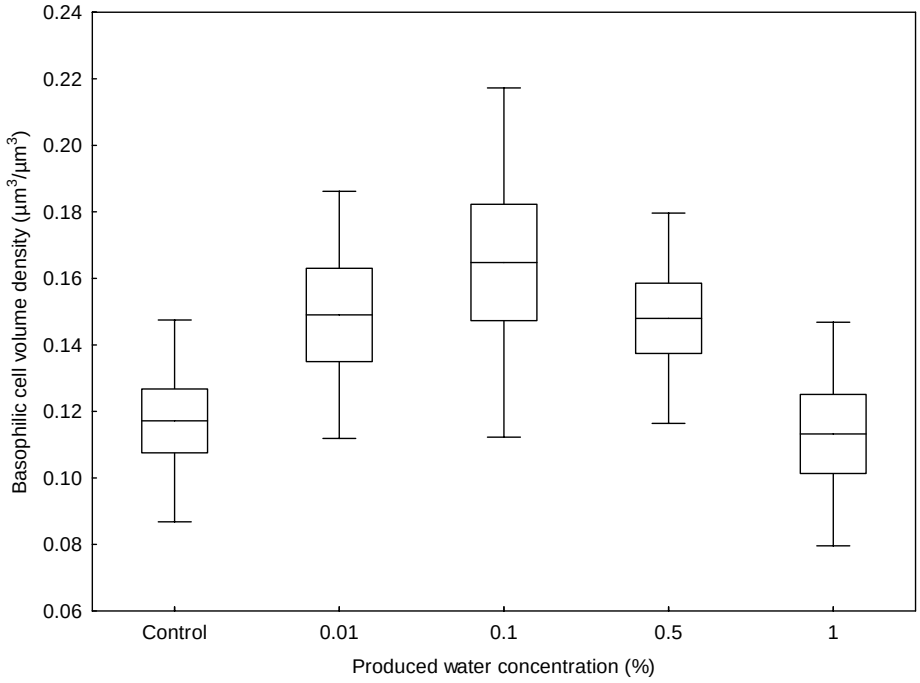

Figure 23. Volume density of basophilic cells in the digestive gland of mussels exposed to known concentrations of produced water. Data expressed as mean, standard error (box) and standard deviation (outer line).

Lysosomal membrane stability (LMS) test in digestive gland (dg)

In laboratory exposed mussels (Figure 24), LP values were significantly lower at exposures of 0.01, 0.1 and 0.5% PW than in the control ($P<0.05$). However, LP values of mussels exposed to the highest PW concentration (1%PW) were similar to control values at approx 10-15 min. This also indicates a stress condition, although a more severe affection is clearly envisaged in mussels exposed to 0.01 to 0.5%PW (~ 5 min).

Palmitoyl-CoA Oxidase Activity

Although no significant differences were found in AOX activity between the different exposure groups, there was a tendency for peroxisomal proliferation to increase with exposure to PW. Highest AOX activity was found in mussels exposed to 0.5%PW.

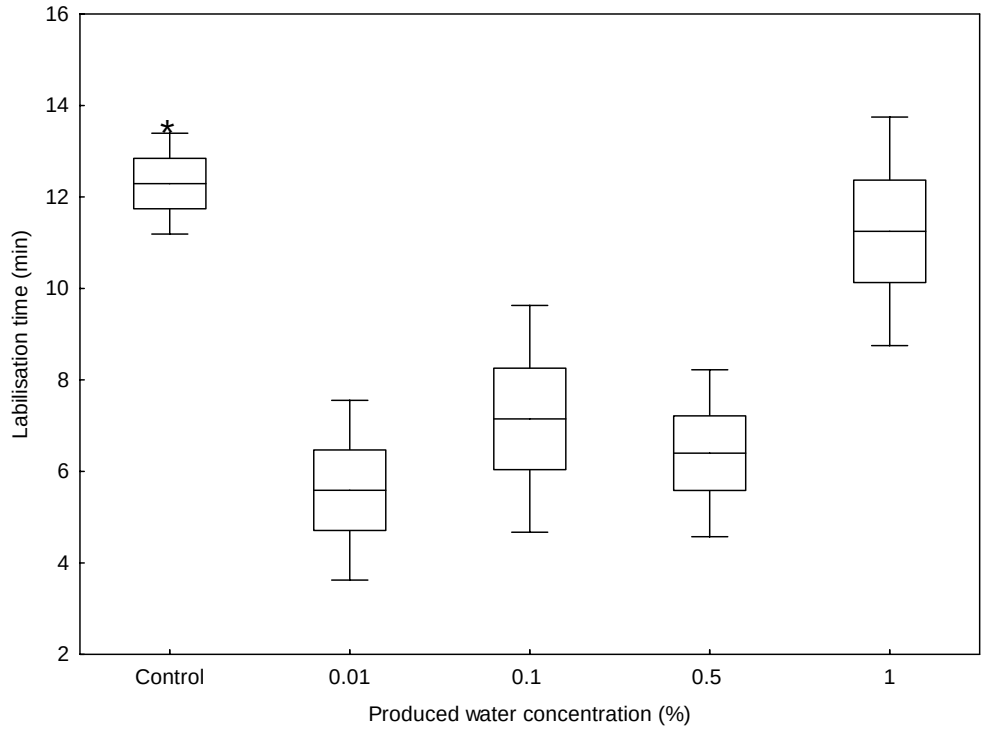

Figure 24. Labilization period for lysosomes in the digestive gland of mussels exposed to known concentrations of produced water. * indicates significant differences (P < 0.05) from 0.01, 0.1 and 0.5%PW. Data expressed as mean, standard error (box) and standard deviation (outer line)

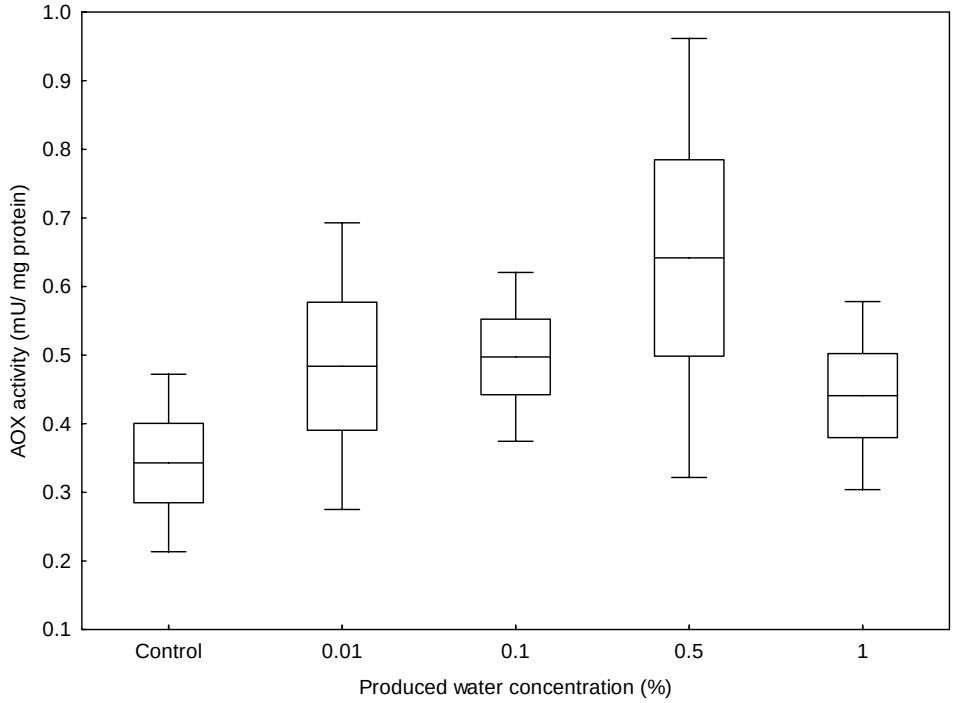

Figure 25. AOX activity in the digestive gland of mussels exposed to known concentrations of produced water. Data expressed as mean, standard error (box) and standard deviation (outer line).

3.3.4 Mussel chemistry

Table 6. The PAH concentration of whole mussel homogenates from mussels exposed for 5 weeks to different concentrations of produced water.

	Tank 1 (control)			Tank 2 (1% PW)			Tank 3 (0.5% PW)			Tank 4 (0.1%PW)			Tank 5 (0.01%PW)		
µg/Kg ww	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Naphthalene	<0.8	2.0	3.2	<0.8	2.9	1.3	0.92	0.90	2.9	4.1	2.8	4.3	2.0	2.0	1.4
C1-Naphthalenes	<2	<2	<2	<2	<2	<2	<2	<2	2.6	2.1	<2	<2	<2	<2	<2
C2-Naphthalenes	4.8	8.9	6.0	12	8.4	6.4	3.8	4.7	11	9.5	5.8	3.0	5.6	7.5	5.6
C3-Naphthalenes	7.4	12	10	7.9	13	12	7.7	9.6	23	18	12	7.9	14	14	12
Phenanthrene	1.2	1.1	0.80	1.1	2.1	1.0	0.93	0.75	1.8	1.9	1.4	1.9	1.2	1.6	1.8
C1-Phenanthrenes	5.1	<2	2.3	2.4	4.1	3.4	2.2	<2	4.8	4.5	3.8	2.5	4.5	5.6	6.8
C2-Phenanthrenes	3.0	2.3	<2	2.7	3.1	2.5	<2	<2	3.6	5.7	4.0	4.5	3.8	4.0	3.8
C3-Phenanthrenes	5.0	2.5	<2	4.6	3.8	4.4	2.2	<2	4.4	3.4	3.1	2.2	5.2	5.0	12
Dibenzothiophene	<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
C1-Dibenzothiophenes	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
C2-Dibenzothiophenes	<2	<2	<2	<2	<2	<2	<2	<2	<2	2.1	<2	2.5	<2	<2	<2
C3-Dibenzothiophenes	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Sum NPD	<35.8	<39.3	<34.8	<40	<45.9	<39.5	<28.25	<30.45	<60.6	<55.8	<41.4	<35.3	<44.8	<48.2	<51.9
Acenaphthylene	<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
Acenaphthene	<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
Fluorene	<0.5	<0.5	<0.5	0.61	0.61	<0.5	<0.5	<0.5	0.72	0.91	0.72	0.56	0.65	0.64	0.61
Anthracene	<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
Fluoranthene	0.97	0.76	<0.5	0.75	1.3	0.88	0.72	0.73	0.88	1.2	1.3	0.97	1.1	1.4	0.77
Pyrene	1.2	0.85	0.84	1.0	1.3	1.0	0.66	0.89	1.5	1.4	1.6	1.3	1.4	1.7	0.94
Benzo(a)anthracenes	<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
Chrysene	<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
Benzo(b)fluoranthene	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0.53	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Benzo(k)fluoranthene	<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
Benzo(e)pyrene	0.71	0.52	<0.5	0.55	0.64	<0.5	<0.5	<0.5	0.96	0.58	0.73	0.70	0.60	0.82	0.52
Benzo(a)pyrene	<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
Perylene	<0.5	0.70	0.57	<0.5	<0.5	<0.5	<0.5	<0.5	0.55	<0.5	<0.5	<0.5	<0.5	<0.5	0.57
Indeno(1,2,3-cd)pyrene	<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
Dibenz(a,h)anthracene	<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
Benzo(g,h,i)perylene	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0.61	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Sum PAH	<45.18	<48.13	<43.21	<48.91	<55.75	<48.38	<36.63	<39.07	<70.85	<65.89	<51.75	<44.83	<54.55	<58.76	<60.81
Sum PAH16	<10.17	<10.71	<11.34	<9.76	<13.71	<10.18	<9.23	<9.27	<13.44	<15.01	<13.32	<14.53	<11.85	<12.84	<11.02
Lipid-%	1.8	2.0	1.9	1.5	1.8	1.8	1.9	1.8	1.6	1.7	1.7	1.3	1.8	0.3	1.6

Low or undetected concentrations of PAHs and metals were found in all exposure tanks with no noticeable differences between the exposure concentrations. The PAH concentrations that were detected include naphthalenes, phenanthrenes, fluorine, fluoranthene and pyrene.

Table 7. Metal concentrations of whole mussel homogenates from mussels exposed for 5 weeks to different concentrations of produced water.

Tank	Rep	Ag	Al	As	Cd	Cr	Cu	Fe	Hg	Ni	Pb	Zn
Tank 1 (control)	1	<0.005	4.3	2.06	0.098	1.2	1.19	18	0.009	0.48	0.07	11.3
	2	<0.005	4.5	1.84	0.099	1.0	1.13	18	0.008	0.38	0.04	10.0
	3	<0.005	4.1	1.75	0.094	0.71	1.12	14	0.007	0.33	0.04	11.9
Tank 2 (1% PW)	1	0.005	3.8	1.81	0.092	0.51	1.03	13	0.007	0.26	0.05	12.4
	2	0.009	4.8	1.95	0.106	0.49	1.05	14	0.008	0.26	0.05	10.0
	3	0.008	4.1	2.09	0.088	0.42	1.26	12	0.008	0.24	0.05	13.7
Tank 3 (0.5% PW)	1	0.009	3.1	1.92	0.092	0.55	1.50	14	0.009	0.38	0.05	13
	2	0.007	3.1	2.22	0.079	0.81	1.00	16	0.008	0.54	0.06	10.4
	3	0.008	6.2	1.94	0.109	1.0	0.94	20	0.008	0.71	0.05	13.8
Tank 4 (0.1% PW)	1	0.007	4.0	1.86	0.100	1.2	1.07	14	0.009	0.40	0.05	10.9
	2	0.005	5.4	1.73	0.084	1.2	1.16	16	0.008	0.41	0.05	10.8
	3	0.006	2.7	1.88	0.105	0.65	1.10	14	0.008	0.42	0.06	10.3
Tank 5 (0.01% PW)	1	0.005	6.1	1.75	0.106	1.1	1.10	17	0.008	0.30	0.05	11.4
	2	0.007	4.9	1.64	0.093	1.7	1.01	16	0.008	0.35	0.04	10.0
	3	0.007	3.1	1.96	0.107	0.97	0.74	14	0.008	0.29	0.06	11.6

3.3.5 Semipermeable membrane devices (SPMDs)

PAH concentrations were either low or undetected in all experimental tanks. The PAHs that were detected were similar to that shown in the field study and include fluorene, phenanthrene, fluoranthene and pyrene. These compounds were detected at background concentrations. The PRC results (data not presented) showed that the total volume of water extracted during the five week laboratory exposure was 28-161 L. This was less than the field study, which would be expected due to higher water flows around the field deployed SPMDs and the shorter exposure period. There were no noticeable differences in PAH concentration between the exposure concentrations.

Table 8. The PAH concentration calculated from SPMDs exposed for 5 weeks to different concentrations of produced water. a = high blank values prevent reporting of this compound.

	Control			0.01% PW			0.1% PW			0.5% PW			1% PW		
ng/L	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Naphthalene	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Acenaphthylene	<0.11	<0.10	<0.10	<0.10	<0.09	<0.09	<0.10	<0.11	<0.11	<0.15	<0.15	<0.13	<0.12	<0.10	<0.11
Acenaphthene	0.10	<0.09	0.09	0.11	0.08	<0.07	0.09	0.13	0.11	0.14	0.16	0.12	<0.10	0.09	0.11
Fluorene	0.34	0.32	0.27	0.25	0.17	0.19	0.27	0.39	0.33	0.57	0.51	0.45	0.35	0.29	0.40
Dibenzothiophene	<0.08	<0.07	<0.07	<0.07	<0.06	<0.06	<0.07	0.10	<0.08	0.11	<0.11	<0.10	<0.08	<0.07	<0.08
Phenanthrene	0.71	0.67	0.56	0.43	0.26	0.30	0.51	0.86	0.65	1.27	0.94	0.91	0.75	0.63	0.81
Anthracene	<0.07	<0.06	<0.06	<0.06	<0.05	<0.05	<0.06	0.07	<0.07	<0.1	<0.1	<0.09	<0.07	<0.06	<0.07
Fluoranthene	0.14	0.14	0.13	0.10	0.08	0.09	0.11	0.14	0.12	0.19	0.16	0.14	0.14	0.11	0.14
Pyrene	0.11	0.10	0.09	0.08	0.06	0.07	0.09	0.11	0.11	0.16	0.13	0.12	0.13	0.11	0.13
Benz[a]anthracene	<0.06	<0.05	<0.05	<0.05	<0.04	<0.04	<0.05	<0.06	<0.05	<0.09	<0.09	<0.08	<0.06	<0.05	<0.06
Chrysene	<0.05	<0.05	<0.05	<0.04	<0.03	<0.04	<0.05	<0.05	<0.05	<0.08	<0.08	<0.07	<0.06	<0.05	<0.06
Benzo[b.j]fluoranthene	<0.05	<0.05	<0.05	<0.04	<0.04	<0.04	<0.05	<0.05	<0.05	<0.08	<0.08	<0.07	<0.06	<0.05	<0.06
Benzo[k]fluoranthene	<0.06	<0.06	<0.05	<0.05	<0.04	<0.04	<0.05	<0.06	<0.06	<0.10	<0.10	<0.08	<0.07	<0.05	<0.07
Benzo[e]pyrene	<0.07	<0.06	<0.06	<0.05	<0.04	<0.05	<0.06	<0.07	<0.07	<0.11	<0.11	<0.09	<0.08	<0.06	<0.07
Benzo[a]pyrene	<0.07	<0.06	<0.06	<0.05	<0.04	<0.04	<0.06	<0.07	<0.06	<0.10	<0.10	<0.09	<0.07	<0.06	<0.07
Perylene	<0.07	<0.06	<0.06	<0.05	<0.04	<0.04	<0.06	<0.07	<0.06	<0.10	<0.10	<0.09	<0.07	<0.06	<0.07
Indeno[1.2.3-cd]pyrene	<0.08	<0.08	<0.07	<0.07	<0.05	<0.06	<0.07	<0.08	<0.08	<0.13	<0.13	<0.11	<0.09	<0.07	<0.09
Dibenzo[ac/ah]anthracene	<0.07	<0.07	<0.06	<0.06	<0.05	<0.05	<0.06	<0.07	<0.07	<0.11	<0.11	<0.10	<0.08	<0.06	<0.08
Benzo[g.h.I]perylene	0.09	<0.08	<0.08	<0.07	<0.06	<0.06	<0.08	<0.09	<0.09	<0.14	<0.14	<0.12	<0.10	<0.08	<0.09
SUM PAH	<2.32	<2.17	<1.94	<1.74	<1.29	<1.38	<1.88	<2.58	<2.22	<3.73	<3.32	<2.97	<2.49	<2.04	<2.57
PAH EPA16	<2.11	<1.97	<1.76	<1.56	<1.15	<1.23	<1.69	<2.35	<2.01	<3.41	<2.99	<2.69	<2.26	<1.86	<2.35

4. Discussion

4.1 Shore mussels

A summary of the main biological and chemical results for the native shore mussels is shown in Table 9. The biological effects endpoints show clear differences in the overall health status of the mussel groups, with the mussels from sites 2 and 3 showing evidence of stress responses indicating impaired health compared to the reference mussels. Lysosomal stability in haemocytes and digestive gland tissue both identified a reduction in health status of mussels from sites 2 and 3 compared to the reference group. This was supported by the pathological assessment of the digestive gland tissue, where increased thinning and vacuolisation of the digestive gland epithelium as well as reduction in the size of the digestive alveoli were found to occur in mussels from sites 2 and 3. In addition, VvBAS measurements in mussels from sites 2 and 3 were indicative of poor health compared to that found in the reference group. This was further supported by the increased frequency of MN in mussels from Site 3 compared to the reference site.

Table 9. Summary table of the biological and chemical endpoints in the shore collected mussels.

Shore site	Description	LMS (h)	MN	DG pathology	Digestive cell loss (VvBAS)	LMS (dg)	AOX	Mussel Chemistry
Site 1	Reference	Slightly stressed			<1	slightly stressed		
Site 2	Construction Jetty	Sig. lower retention times than control mussels (high stress)		Thinning & vacuolisation of the digestive gland epithelium and apparent reduction in the size of the digestive alveoli – more severe in Site 3	>1 poor health - stress response	sig diff. showing severe stress response	No sig. diff. between the three mussel groups.	Background concentrations for PAHs, NPDs and metals at all sites,
Site 3	Loading Jetty		Higher frequency of MN					

Despite the clear differences in many of the biological endpoints measured the mussel body burden data did not show any differences between the sites and were either low or undetected in all cases. Therefore, it was highly unlikely that the measured changes in biological endpoints were caused by either elevated concentrations of PAHs or metals, but rather other contaminants that were not measured in this study.

The biological effects exhibited by the shore mussels can not be directly related to the PW discharge but rather the general impact of the Ormen Lange processing plant. The mussels exhibiting the greatest effects were those collected from site 2 and particularly those from site 3, which were the construction jetty and the loading jetty respectively. The movement of the water outside the Ormen Lange plant has been previously found to follow an eddying motion (DNV, pers. comm.), which would likely result in a mixing of the water body near to the produced water discharge. Consequently, mussels collected from the construction and loading jetties, were likely to have been exposed to the PW discharge to a certain extent, although dilution of this discharge would likely to reduce the PW concentration to negligible concentrations. Other factors that may influence mussel health include, inputs from man-made structures and boats such as anti-foaling paints and corrosion inhibitors, as well as the discharge of ballast water in the area from large ocean going vessels (pers. comm.), and increased boating activity in the area. These factors may have contributed to the reduced health status of the mussels within the Ormen Lange bay area.

Although clear differences in health status were found between the reference mussels and those collected from the two sites within the Ormen Lange bay area. The mussels from the reference site were found to exhibit a low level stress response, identified from the lysosomal stability test in both haemocytes and digestive gland samples. The reason for this is not known, although it may suggest an additional source of exposure, such as from a nearby river, which has not yet been identified, or from other industrial and/or man-made activities upstream.

4.2 Caged mussels

The biological effects measured in the caged mussels produced contradictory results (see Table 10). The LMS in mussel haemocytes responded as expected with reduced retention times in mussels closest to the produced water discharge. This was also supported by the MN test, which measured a significantly higher frequency of MN in the haemocytes of mussels closest to the discharge. However, the mussel histochemistry did not agree with the findings of the LMS (h) and MN formation tests. In contrast, VvBAS, LMS (dg) and general digestive gland pathology all found the mussel from the two sites closest to the discharge to be in relatively better health compared to the mussels from the other cages, including the reference cages. However, it is important to consider that all mussels measured in the study were found to display a certain level of stress response irrespective of proximity to the PW discharge, indicating that the mussels used were in poor health. In addition, higher than expected mortalities (~ 40%) were observed in mussels cages from five of the six sites, with exception of cage 5 (~ 15% mortality), suggesting that the mussels were unduly stressed during the six week exposure

from additional factors. These factors include: 1) the depth at which the mussels were held; 2) handling and transport pressures prior to deployment; and 3) an additional point or diffuse source of contamination influencing mussel health during the exposure period.

Table 10. Summary table of the biological and chemical endpoints in the cage mussels

Shore site	Approx. Distance from discharge	LMS (h)	MN	DG pathology	Digestive cell loss (VvBAS)	LMS (dg)	AOX	Mussel Chem	SPMDs
Site 1	50 m	Significantly lower retention times than reference groups (poor health)	Sig. higher frequency of MN formation	similar histological integrity	vVBAS value <1 suggest healthier mussels	Highest value but denotes some level of environmental stress		Background concentrations detected at all sites	Background concentrations detected at all sites
Site 2	100 m					Lower LMS values – mussels in poor health and high environmental stress			
Site 3	100 m			similar histological integrity – initial signs of stress.	Higher VvBAS values suggest mussels in poorer health				
Site 4	200 m								
Site 5	2000 m (REF 1)	Lower retention times than expected in reference mussels.					Highest AOX value – high stress		
Site 6	2000 m (REF 2)								

Due to boating traffic in the area, it was required for the mussels to be held within the water column at a minimum depth of 20 m. Whether the increased depth could have unduly stressed the mussels due to either, pressure, temperature and/ or food availability was uncertain. However, a previous study measured histopathological endpoints in mussels located at a depth of 450 – 650 m (Powell et al 1999). The authors reported no detrimental effects of depth on the histopathological endpoints measured. Therefore, holding mussels at 20 to 27.5 m in the present study was unlikely to have caused the high mortalities and the anomalies in the biomarker results. The availability of food to the mussels may have been critical if mussels were held below the thermocline, thereby reducing food availability.

However, the mussel cages were found to be fouled with algae upon collection, suggesting that food availability was not a limiting factor.

The caged mussels were obtained from a mussel hatchery on the West coast of Norway (Snadder & Snaskum). Although the mussels were considered clean in terms of contaminant body burden, undue stress on the animals can occur during transport and transplantation into a new environment. To reduce possible impact of transport on the mussels, the mussels were collected in person from the hatchery and transported on ice to the field. The quality and size range of the mussels was checked by hand prior to transplanting into the water column. However, despite this, mussel mortalities did occur. It was not possible to know whether this was the cause of the undue stress and high mortalities, however, for future studies it is recommended that mussels be held in clean flowing seawater for at least two weeks prior to field deployment, to ensure optimum mussel health.

The changes in biological responses observed were not supported by the chemical data since only background concentrations of metals, PAHs, and NPDs typical of coastal waters were found in mussel tissue and SPMDs. This was despite up to 500 L of seawater sampled with the SPMDs.

4.3 Controlled laboratory exposure

There was reasonable agreement found between the biomarker endpoints for the controlled laboratory exposures. Mussels exposed to PW at 0.01% and above demonstrated biomarker responses including a reduction in lysosomal stability in both haemolymph and digestive gland samples, increased digestive cell loss (vVBAS), increased expression of AOX, and adverse digestive gland histopathology. Changes in MN formation were not observed, which may be related to the shorter exposure time (5 months) for significant effects in MN formation to be established.

It should be noted once again that mussels in the control group were found to be exhibiting stress responses for some of the biomarkers investigated (i.e. LMS (h), LMS (dg) and VvBAS). Confounding factors including food availability and water quality can be ruled out since animals were fed daily and physicochemical measurements (i.e. temp, pH, dissolved oxygen) were taken almost daily and were found to remain stable during the exposure duration (See Appendix, Figure 1). The mussels used for the laboratory exposure were from the same supplier as that used for the cage study, which were found to have high mortalities. However, mussel mortality was minimal (< 1%) in the laboratory exposure suggesting that these mussels were in good health prior to the test exposure.

The chemistry data for the mussel and SPMDs were not found to differentiate between the exposure groups and could not account for the biological effects observed. In this case the biological responses were most likely caused by other contaminants not measured. Although PAHs and NPDs are a crucial component of PW, there are also many other chemicals that have not been measured. Some examples include; alkylphenols, organic acids (such as naphthanic acids), and decalins, which may have contributed towards the biological effects observed. It is however, time consuming and expensive to measure the thousands of chemicals that could be present and highlights the benefits of sensitive biological effects measurements for the assessment of environmental risk.

Comparison of the PAH, NPD data between the cage exposure and the laboratory exposure reveal slightly higher concentrations in the laboratory, even in the control tank. This may suggest elevated baseline concentrations of PAH in the dilution water from the research facility at Solbergstrand, which obtains its seawater from the Oslo fjord at a depth of approximately 60 m. However, whether these slightly elevated baseline concentrations are contributing to the apparent biological responses observed in the control mussels is uncertain.

Table 11. Summary table of the biological and chemical endpoints in mussels from controlled laboratory exposures of produced water (PW). PW obtained from Ormen Lange prior to missing with cooling water.

Tank No.	PW conc. (%)	NRR	MN	DG pathology	Digestive cell loss (VvBAS)	LMS (dg)	AOX	Mussel Chem	SPMDs
Tank 1	Control	Partly stressed	No significant differences between the exposure groups	Reduction in the number of digestive diverticula, thinning of the DG epithelium and high prevalence of digestive alveoli.	VvBAS >1 in all groups including the control (environmental stress), highest values at 0.1% PW.	Partly stressed	Elevated concentrations of AOX	Background concentrations of PAH, NPDs and metals in all groups	Background concentrations of PAH in all groups
Tank 5	0.01	Significantly lower retention times than mussels from the control group.				Severely stressed			
Tank 4	0.1								
Tank 3	0.5								
Tank 2	1					Partly stressed			

4.4 Conclusions

Shore mussels

Good agreement between the biological effects measurements indicating that mussel health was impacted at the two exposure sites located in the inner harbour of Ormen Lange, compared to the reference group. There was no link between biological effect and mussel body burden, which suggests that the biological responses observed were caused by other contaminants not measured. Due to the position of the shore mussels, it was not possible to directly relate the biological effects with PW exposure but rather the overall impact of the Ormen Lange processing plant. The biological effects observed in the reference mussels suggest a second source of exposure other than Ormen Lange.

Cage mussels

Contradictory results were found for the biological effects measurements. This was thought to be partly due to the overall poor health of the caged mussels used, identified by biological effects in the reference groups and high mortalities observed in five of the six cages. Consequently, no clear conclusions could be drawn from the cage mussel study.

Laboratory mussels

Good agreement between biological effects measurements showing that exposure to PW concentrations at 0.1% and above had a marked effect on mussel health. However, no relationship was found between the biological effects and the contaminant concentrations measured, which suggests that other PW contaminants not measured was responsible for the biological effects observed.

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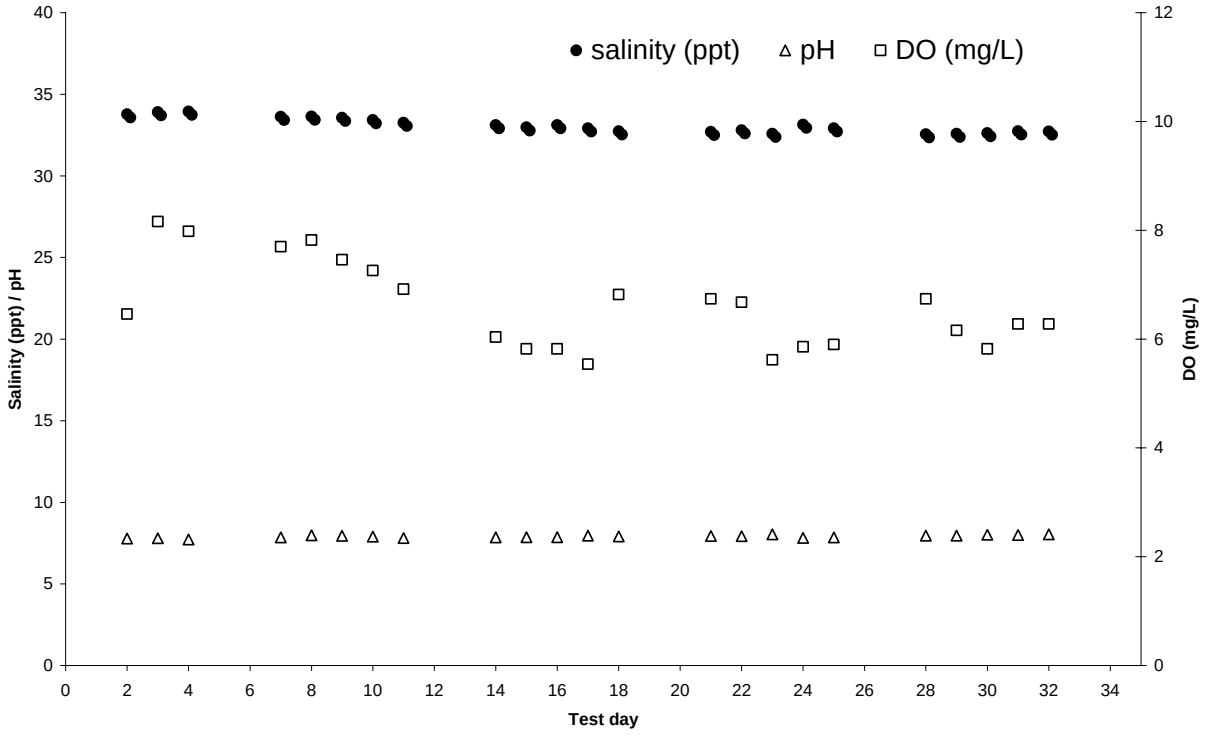
Appendix A.

Table 1. Metal and PAH including NPD concentration of the 100% Ormen Lange produced water used for the laboratory flow through system.

Metal	mg/L	PAH	ng/L
Cadmium	<0.001	Acenaphthylene	<2
Copper	0.007	Acenaphthene	<2
Iron	0.0068	Fluorene	<2
Mercury	3.5	Anthracene	<2
Zinc	0.0067	Fluoranthene	<2
PAH - NPDs	ng/L	Pyrene	<2
Naphthalene	9.6	Benzo(a)anthracenes	<2
C1-Naphthalenes	20	Chrysene	<2
C2-Naphthalenes	220	Benzo(b)fluoranthene	<2
C3-Naphthalenes	420	Benzo(k)fluoranthene	<2
Phenanthrene	<2	Benzo(e)pyrene	<2
C1-Phenanthrenes	<10	Benzo(a)pyrene	<2
C1-Dibenzothiophenes	<10	Perylene	<2
C2-Phenanthrenes	<10	Indeno(1,2,3-cd)pyrene	<2
C3-Phenanthrenes	<10	Dibenz(a,h)anthracene	<2
Dibenzothiophene	2.3	Benzo(g,h,i)perylene	<2
C2-Dibenzothiophenes	<10	Sum PAH	765.9
C3-Dibenzothiophenes	10	Sum PAH16	39.6
		Sum NPD	733.9

Figure 1. Physicochemical values of the dosing system maintained over the duration of the laboratory exposure.



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