Environmental harm assessment of a wastewater discharge from Hammerfest LNG: A study with biomarkers in mussels (*Mytilus sp.*) and Atlantic cod (*Gadus morhua*)

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Abstract

Biologically treated wastewater (WW) from the Hammerfest LNG (liquefied natural gas) plant is discharged to the sea. A study using biomarkers in mussels and Atlantic cod was performed to examine whether this discharge meets a zero harmful emission requirement. Caging of mussels close to the outfall and exposure of mussels and fish to WW in the laboratory were conducted, and a suite of contaminant responsive markers was assessed in exposed animals. In mussels the markers included chemical contaminant levels, haemocyte lysosomal instability and nucleus integrity, cellular energy allocation, digestive gland and gonad histopathology and shell-opening behaviour. In fish, biliary PAH metabolites and gill histopathology biomarkers were measured. A consistent cause-effect relationship between WW treatments and markers measured in test animals was not found. The results therefore indicate that the WW emission is unlikely to represent a significant stress factor for the local marine environment under the conditions studied.

Introduction

The Hammerfest LNG processing plant at the island of Melkøya, Norway, receives a multiphase gas/condensate flow through a 140 kilometre pipeline from the Snøhvit offshore natural gas field. The liquid component of the pipe flow consist mainly of a combination of hydrocarbon condensate and water mixed with monoethylene glycol (MEG). MEG is added to maintain flow fluidity. At the LNG plant the MEG is regenerated and the aqueous component of the pipeflow is extracted and processed in a wastewater (WW) treatment facility equipped with a biological treatment system for bacterial downgrading of contaminants (Lyngmo, 2007). After the cleaning process, the WW is mixed with a much larger volume (normally >5000x) of hot seawater used in the plants cooling system. The final mix is subsequently discharged to the adjacent sea at a depth of approx. 40 m. According to demands in the LNG plants licence to operate, the marine WW discharge should meet a zero harmful emission requirement.

For pollution risk assessment studies the application of toxicant responsive biomarkers has gained much attention (van der Oost et al., 2003). A broad definition of the term "biomarker" in this connection is any change which can be detected in an individual living organism as a consequence of exposure to a harmful chemical (or chemicals). Study organisms in marine biomarker studies are often filter feeder bivalves such as common mussels (Brooks et al., 2011; Cajaraville et al., 2000; Garmendia et al., 2010; Hagger et al., 2009; Martin-Diaz et al., 2008) or suitable species of fish (Balk et al., 2011; Jung et al., 2011; Sundt et al., 2012; van der Oost et al., 2003). A wide array of biomarkers has been evaluated in relation to their possible use in pollution assessment studies or in environmental risk assessment (ERA), e.g. (Beyer et al., 2011; Jemec et al., 2010; Sole et al., 2010; van der Oost et al., 2003). Depending on the type of environmental pollution problem being studied; such as toxic metals, genotoxic aromatic compounds, endocrine disruptors, etc., certain selections of biomarkers can be

chosen. Some markers, such as lysosomal membrane instability in blood cells of mussels (Lowe et al., 1995a), are responsive to a broad range of toxic stresses, whereas other markers are selective towards specific stressors, for example the presence of metabolites of benzo[a]pyrene or other toxic poly-aromatic hydrocarbons (PAH) fish bile (Beyer et al., 2010). In the case of histopathology biomarkers in aquatic organisms, all sorts of stressor specificity (from broad to narrow) can be found, e.g. (Teh et al., 1997; Wedderburn et al., 2000). Physiological status indicators can give valuable information about the general health condition in a study organism in situations of long term toxic stress. In this connection, the assessment of energy reserves (in terms of protein, carbohydrate and lipid contents) as a marker to evaluate the organisms' energetic budget and general healthiness has attracted attention (Arzate-Cardenas and Martinez-Jeronimo, 2012; De Coen and Janssen, 2003; Mouneyrac et al., 2010). Also specific animal behaviours can be applied as markers for detection of chemical stresses in a study organism, examples of such behavioural biomarkers are traits related to mussel filter-feeding activity and fish swimming performance (Amiard-Triquet, 2009; Beitinger, 1990; Glegg and Rowland, 1996; Vieira et al., 2009). Normally a biomarker based study will involve a set of markers. The actual selection of a biomarker set will depend on the nature of the addressed ecotoxicology study and the amount of information which is available. Using biomarkers which have broad contaminant responsivity is often the choice in cases when there is uncertainty about which contaminants that possibly could be present, as in the case of the present study.

Environmental risk predictions had concluded that the discharge of treated wastewater from the Hammerfest LNG plant should not constitute any significant environmental stress factor for the marine recipient (Dagestad et al., 2005). A monitoring program was therefore designed to provide documentation about the environmental condition of the recipient (Velvin et al., 2009). The study presented in this paper was one of the studies performed. The work includes

two parts; a field study with caging exposure of mussels close to the WW outfall and a laboratory part in which mussels and fish were exposed to selected test concentrations of WW representing both moderate and high exposure levels. In mussels, a suite of chemical markers and biomarkers were measured. In fish, biliary PAH metabolites and gill histopathology biomarkers were studied. The obtained results are presented herein and the rationale for using contaminant responsive biomarkers in marine organisms as tools for assessing environmental implications of mixed industrial effluent discharges is discussed.

Method description

Mussel caging study

A group of roped mussels, average length and weight 56 mm and 17 g, was obtained from a mussel farm at the island Seiland 19 km from the LNG plant at Melkøya (Figure 1). Two stainless steel cages (well perforated) were deployed at 10 m depth roughly 100 m from the WW outfall (Figure 2) and two similar cages were placed at a reference location at the island Seiland. The cages were deployed for three months during the summer season of 2008. A field trial with the sea-current measurement and caging setup was performed in 2006, before the start-up of the LNG plant. Water temperatures and salinity conditions were measured at deployment and retrieval of cages. The sea-current off Melkøya was measured at three water depths (4, 16 and 25 m) by means of an Aqua-Dopp profiling current sensor (NorTek AS, Bergen, Norway). A diagram showing the prevailing current direction at 4 m depth at the Melkøya caging site is included in Figure 1. After caging, the steel cages were retrieved and transported to a nearby lab facility for sample collection. The time from cages retrieval to sample collection was less than 4 hours. 10 mussels were obtained from each test group and weight and shell length were measured. Haemolymph (containing haemocytes for lysosomal stability and nucleus integrity assessments) was obtained from the posterior adductor muscle

with use of a syringe. Shells were opened by severing the adductor muscles. A narrow cross section along the middle standard plane for histological examination of gonadal maturity and a piece of digestive gland for histological biomarker assessments were removed (further described below). Finally, a pooled batch sample of 20 mussels per group was frozen (in their shells) for chemical analyses.

Laboratory exposure of mussels and fish

A flow-through system for exposure of mussel and fish to different concentrations (dilutions) of WW was developed in the laboratory. The design was based on a produced water exposure system which previously has been reported (Sundt et al., 2009). WW was collected into multiple five-litre polyethylene cans during week 39 (2009). The WW tapping point was located ultimately after the water treatment plant (i.e. before the dilution with cooling water). The collected WW was frozen at -20 °C, transported to the laboratory in Stavanger and kept frozen until used. A group of roped mussels, average length and weight 56 mm and 22 g, was supplied from the same mussel farm as for the caging study. Transport of mussel took less than 12 hours. At arrival, the mussels (in net bags containing 50 individuals each) were transferred to the experimental glass fibre tanks (volume 250 L) and acclimated for 1 day to the control condition (clean seawater, 34‰, 12°C). Atlantic cod (21-57 g) were obtained from a local aquaculture facility close to Stavanger. The cod were allowed to acclimatise in 34‰, 12°C seawater for 1 week. The test animals were fed moderately with commercial mussel feed (Shellfish diet 1800, Instant Algae®) and cod feed (3 mm cod pellets, Skretting A/S). The test animals were exposed in two replicates to three test concentrations of WW, namely WW diluted 1:4,000, 1:8,000 and 1:20,000 times in seawater. The exposure lasted for four weeks. In addition, one single group of mussels were exposed to a higher concentration (200 times dilution) for a shorter period (1 week) to provide samples for method validation. All controls were kept in clean seawater. The laboratory facility is equipped with seawater piped

from 80m depth and filtered through sand filters. Seawater temperature and oxygen saturation levels were monitored during the exposure period and the values varied within 12-13 ^{o}C and 70-80%, respectively (data not shown). In each replicate treatment group there were 30 mussels in total and 20 cod. During sampling 16 mussels (8+8) and 20 cod (10+10) were taken from each exposure concentration.

Sample preparation and analyses

Samples were coded for anonymity before analysis. Caged mussels were analysed for a set of PAH contaminants (Table 1), a selection of metals and methyl mercury (Table 2) and organic tin compounds (data very low, not shown). Five samples of frozen WW were analysed for aromatic hydrocarbons, focussing in particular on the naphthalene content (Table 3).

Mussel haemolymph was applied on a glass slide for haemocyte lysosome membrane stability analysis using the neutral red retention (NRR) method as described by Lowe et al. (1994; 1995b). Haemolymph was also applied on glass slides for microscopy scoring of nuclear anomalies (increased tendency of nuclear buds and micronuclei) in haemocytes as biomarkers of chromosomal damage and genotoxic stress. The presence of abnormal nuclei in haemocytes from the different groups of exposed mussels was assessed by scoring a minimum of 2000 cells per individual specimen for micronuclei as well as for nuclear buds. A tissue sample including gonad and digestive gland tissues was fixed in formalin for histological examination. For histochemistry of mussels in the laboratory exposure study pieces of freshly excised mussel digestive glands (3±4 mm^3) were placed on an aluminium cryostat chuck and cooled for 40 s in N-hexane that had been precooled to -70ºC with liquid nitrogen as described by Aarab et al. (2011; 2008). Cryostats sections were prepared and used for analyses of the “aging pigment” lipofuscin (LF) using the method described by Pearse (1985).

In homogenised whole mussels, cellular energy allocation was evaluated by means of protein, carbohydrate and lipid analyses and by the assessment of energy consumption measured by an electron transfer system (ETS) assay, largely as described by King and Packard (1975) (ETS) and by De Coen and Janssen (1997) using a microplate (Perkin Elmer, spectraplate, 96 wells) assays and a Perkin Elmer 1420 Multilabel counter. Protein content was measured according to Bradford (1976) using bovine serum albumin as standard. Carbohydrate content was determined against a standard curve of glycogen from bovine liver. Lipid contents were determined against a standard curve of tripalmitin. ETS measurements were done directly after homogenisation to minimise loss of activity. The three fractions of energy available were transferred into energetic equivalents using their respective energy of combustion (24000 mJ mg-1 protein, 17500 mJ mg-1 glycogen and 39500 mJ mg-1 lipid) (Gnaiger, 1983).

A biosensor based approach using mussel valve gape detection as a biomarker was tested. The valve gape sensor system was developed by IRIS together with Biota Guard AS (Stavanger, Norway). In three days eight acclimatized mussels were kept under control conditions (clean sea water + algae-feeding), the next three days they were held in the highest exposure concentration (1:200 WW dilution + algae-feeding), and the subsequent three days they were again kept under control conditions (clean sea water + algae-feeding). Valve gape measurements were measured continuously throughout the nine days of the test, and the mussel valve gape activity during the three different exposure conditions was compared.

Bile and gill samples were obtained from the cod for detection of PAH metabolites and gill histopathology, respectively. Only a selection of fish bile samples (two samples from the 1:4000 group and two samples from the reference group), were measured for a set of PAH metabolites with a special attention to PAHs with less than 4 aromatic rings and alkylated forms of these (Table 4) by using gas-chromatography based analysis as described by Jonsson et al. (2003). Produced water from offshore formations is likely to contain a certain level of

parent and alkylated PAHs with less than 4 aromatic rings. Such metabolites in fish bile are sensitive exposure markers for determining low concentration situations (Beyer et al., 2010). For the gill histology, 18-20 cod from each WW exposure concentration were scored. The first left gill was fixed in neutral-buffered formalin (4% formaldehyde), paraffin-embedded, subsequently sliced in approx. 5 μm sections, dewaxed in xylene, rehydrated in graded ethanols, and stained in haematoxylin and eosin using standard histology protocols. A selection of traits indicative of histological stress (aneurysms, epithelial lifting, epithelial thickening, lamellar fusion, lamellar clubbing, excess mucus, proliferation of epithelium and necrosis) was assessed in each gill sample and the presence of these traits was scored in the following categories: 0: normal (histopathology not seen), 1: mild, 2: mild to moderate, 3: moderate, or 4: severe (common).

Statistical treatment of data

Statistical analysis of data was performed using JMP (version 5.1, SAS Institute, Cary, NC, USA). Parametric data were tested for normality and homogeneity. Normal distributed data were tested for difference between experimental and control groups by the use of Dunnett's test. The level of significance to reject the H_0 hypothesis (no difference between groups) was set as $p < 0.05$.

Results

Field study

The sea current at the caging location at Melkøya was found to move preferably north/northeast along the shoreline of Melkøya, but a shift to the opposite direction, most likely due to the tidal cycle, was also observed (Figure 1). The CTD measurements revealed no stratification in the water column at the Melkøya location, whereas a thermocline and

halocline was observed at 10-15 m depth at the reference location (CTD data not shown). No clear difference in mortality of mussels between the two study locations was noted during the retrival of mussel cages. It was observed that algae had attached at the outer surface of the cages. The algae diversity was different on the Melkøya cages compared with the reference cages, the latter contained green algae species typical for sheltered waters whereas brown algae laminaria species grew at the Melkøya cages.

The lysosomal stability analyses of the caged mussels did not reveal any significant differences between the two caging locations or the replicate groups (Figure 3A). The mussels, which were obtained from the Seiland mussel farm start group, sampled before caging, also fell within the range observed for the caged groups. No significant difference between the Seiland and Melkøya cage groups or the start group was observed for the two nucleus integrity markers (Figure 3B and 3C). There was no clear difference between groups for the gonad histological status and adipogranular (ADG) cell development or for the scorings of cellular atrophy in digestive gland tissue (data not shown). The measurement of chemical contaminants in the caged mussels showed generally very low levels of the chemicals detected but a weakly increased concentration of alkylated isomers of the more volatile organic compounds (naphthalene, phenanthrene, dibenzothiophene group) was observed in Melkøya mussels when compared to the reference mussels (Table 1). For metals and methyl mercury the concentration was similar across the two locations (Table 2). Analyses of various organic tin contaminants in the caged mussels did not reveal any measurement signals over the limit of chemical detection for any of the mussel test groups (data not shown).

Laboratory exposure study

No mortality was observed for the mussels during the four weeks of exposure, while a single cod was found dead (in control tank) during the experiment. A small variation in temperature

of intake seawater was seen during the four-week experiment, with temperatures ranging from 10.5 °C - 13.5 °C, with an average value of 12.3 °C (± 0.86 STD, n = 21). The oxygen level ranged from 6.7 to 8.4 mg/L with a mean value of 7.57 mg/L (± 0.38 STD, n = 19). Salinity of the intake water remained stable at 34.5 per thousand.

The chemical analyses indicated that low levels of naphthalene and alkylated naphthalenes were present in four of five WW samples analysed (Table 3). According to the Norwegian guidelines for the classification of environmental quality in fjords and coastal waters (TA-2229/2007) the measured levels of naphthalene in the water samples was at the condition class II (good) classification level. During water extraction, a tendency of emulsification in the test sample was observed; caused by components of unknown identity. This observation could indicate a presence of surface active substances in the sample; in that case this may to an unknown degree have influenced the analytical precision of the GC analysis. Furthermore, the chemical analyses of the laboratory exposed mussel samples gave little evidence of PAH exposure. The only PAH component which could be detected with a sufficient analytical security was phenanthrene, but the moderately high level detected was more or less the same for all treatment groups (measured range 2.9 - 4.7 ng/g w.w.), and did not reflect the different WW exposure concentrations. This observation indicates that the analysis was not successful and the data should therefore be treated with caution.

In mussel haemocytes, the lysosomal membrane stability was not significantly different in exposed groups in comparison to the controls (Figure 4A). There was a statistically significant increase in irregular nucleus shapes in both the replicates treatment groups for the lowest exposure concentration, i.e. the 1:20,000 times wastewater dilution treatment (Figure 4B). Also in the 1:8,000 treatment replicates there was seemingly an increase tendency of irregular nuclei but this increase was only statistically valid when the two replicate groups (A & B) were merged before statistical testing (not shown). No obvious effect on this marker was

observed in the two 1:4,000 dilution replicate groups or in the more short term extreme dose treatment (1:200 dilution). It is unclear whether the noted effect was caused by the wastewater exposure or came as a result of methodological uncertainty. Also the micronuclei results seemed to indicate an increasing tendency in the lowest treatment concentrations, but this tendency was not statistically significant (Figure 4C). The measurement of lipofuscin aging pigment and cellular atrophy tendency in digestive gland and the histological assessment of adipogranular (ADG) cells, atresia (tissue degeneration), spawning status and presence of inflammatory cells in gonad tissue did not reveal any effects that could be logically explained by differences in WW exposure (data not shown).

The analyses in mussels of the three major forms of nutritional molecules (protein, carbohydrates and lipids) used as markers of available energy and the summarised parameter of all three energy rich molecules together, showed some weak signs of variability between groups (Figure 5), but no differences between groups was statistically significant.

The valve gape test-trial in mussels proceeded successfully in the sense that the biosensor was able to continuously record the valve gape activity in the test group of eight individual mussels. However, the data did not reveal any significant acute stress responses between day 3 and 4, when the test group was transferred from clean seawater to the highest exposure concentration (1:200 dilution of WW). Instead there was apparently a decrease in valve gape activity at day 8, i.e. in the recovery phase (Figure 6).

In fish, the exposure markers measured in bile indicated very low contamination levels caused by WW exposure. One bile sample from the 1:4000 dilution treatment contained low detectable levels of C3-OH-Naphtalene and C2-OH-Phenantherene (Table 4). The level, however, was so low that the detection result was determined uncertain. The other PAH metabolites were generally below the detection limit. The examination of fish gills revealed

that several histopathological traits could be detected within the sample material as a whole, but there was no consistent dose-wise trend that could be explained with the exposure to WW (Figure 7 A-D). A similar lack of dose related effects was true for the other examined gill histopathologies, i.e. aneurisms, lamellar clubbing, excess mucus secretion and necrosis (data not shown).

Discussion

The data in the present study did not show significant differences between controls and groups exposed to the treated WW from Hammerfest LNG. The results thus give no support for suggesting that the discharge represents a hazard to the sea environment at Melkøya. On the contrary, since the WW exposure level in the laboratory part was higher than what realistically can be expected to occur in the field, the observed lack of stress-related effects might be taken as a conservative signal of the satisfactory environmental safety level for this particular WW discharge. The Melkøya location is characterised by an extreme hydrography with strong currents and regularly heavy waves and apart from a narrow shelf around the island the bottom topography slopes quickly towards deeper waters. In the caging study, it was therefore a challenge to find a position at Melkøya where the mussel cages could be deployed safely but at the same time with an optimal likelihood of being exposed to the plume. The CTD measurements showed that the water column at the caging location at Melkøya was practically non-stratified between 0 and 30 m. Thus, the effluent, which initially is significantly warmer and less dense than the surrounding seawater, will under such conditions be likely to reach the surface and then disperse further in the upper part of the water column. The recorded sea-current data confirmed the strong and tidally shifting current conditions at this site and with a prevailing direction along the Melkøya shoreline. However, the data does not clarify whether the mussel actually were exposed to the plume. The

exposure and biomarker data from the caging study indicated that the Melkøya mussels were not more exposed or stressed than the mussels caged at the reference site. However, if the deployed cages were left outside the discharge plume, then the WW exposure would naturally also be zero. Because of this uncertainty it was decided to perform the exposure study in the laboratory, in which the study organisms could be exposed to the WW at more controlled dilutions.

The results from the laboratory exposure study corroborated the data from the caging study in the sense that consistent and significant effects were not observed in the WW exposed organisms. Some odd and weak tendencies of differences were, however, observed within and between treatment groups, e.g. for some histopathology markers and the irregular nucleus shape marker in mussel haemocytes. But these finds were not logically consistent and could have been a result of biological variability or methodological uncertainty. It is not so likely that the lack of clear responses was caused by a too mild exposure regime. Actually, the WW exposure concentrations which were used in the present laboratory exposure study was “worst case” or above. At the LNG plant, the treated wastewater is always mixed with a significantly larger volume of process-cooling seawater before it is released to sea. This pre-release dilution will vary by a factor between 5,000 - 30,000 times, depending on technical variation in the LNG production process, according to information provided by the client. Thus, the “high exposure” treatment used in the laboratory thus represented an environmental concentration greater than at the point of effluent release. Even the lowest exposure concentration, the 20,000x dilution, can be considered as “high-dose”, when taking the plume dispersal into consideration. Interestingly, also the short-term "extreme dose" exposure at 200x dilution, which was run for one week and included mainly for method validation purpose, also failed to produce a consistent effect, although not all markers were analysed for these samples. The chemical analyses of the WW indicated that the contaminant levels in the

mixture were generally low. However, it can be questioned whether the freezing of the WW samples was influencing, i.e. decreasing, the toxicity of the WW mixture. Compounds including volatiles could have been lost from the collected samples despite freezing, resulting in exposure of the test organisms to a different mixture to that experienced in the field situation. It may also be questioned whether the four weeks of rather modest exposure in the laboratory was sufficient to produce clear and statistically significant effects which the employed biomarkers were able to detect. And with regard to the field study there is obviously much uncertainty related to the exposure of the caged mussels to the dispersing effluent plume. Hence, it is not possible with 100% confidence to state that the discharge from the Hammerfest LNG plant have no effect, since the results observed in this study may simply indicate no exposure.

Still, the conducted study with caging, a reasonable controlled exposure and biomarker assessment may represent valuable tools for ecotoxicity assessment of this type of industrial effluents, although the data presented in this study indicate a "no effect" situation. A deviance from biomarker control levels clarifies that some component(s) in the test medium make the test organism respond, although the response may not necessarily represent a toxic action *per se*. Investigators would normally value markers that positively indicate a condition of harm (e.g. genotoxicity). However, also markers which signal exposure or general physiological condition have value for the assessment. When a study shows a "no effect situation", as in the present study, the specific exposure situation is indicated to be benign. In particular, this type of biomarker data is relevant in connection with assessment of a complex effluent discharge. Mussels and fish are often used in biomarker based studies. Mussels are in particular useful since they are immobile and relatively long-lived filter feeders that bioaccumulate a wide range of chemical contaminants and they are very easy to use both in laboratory as well as in field caging studies (Pereira et al., 2011; Tsangaris et al., 2011). Fish, on the other hand, have

a more advanced physiology and offer a bigger selection of toxicity markers that can be used to asses chemical stress (van der Oost et al., 2003). Caging of fish are also much used as a study approach for biomarker based ecotoxicity studies, e.g. (Beyer et al., 1997; Beyer et al., 1996).

The central task in this study was to challenge the client's predictions that the discharge of processed WW from Hammerfest LNG does not cause any measurable environmental effect in the marine recipient. In sum, the data presented in the present study tend to indicate that this assumption is true. Field and laboratory based WW exposure studies have been conducted and no significant stress relevant signals above typical background were found. This investigation is useful as an example of how the environmental quality performance of an industrial WW discharge to sea can be assessed. The good experience with the laboratory exposure in the present study suggests that this approach is favourable in comparison to the caging approach in the case of a repeated environmental assessment of the treated WW discharge from Hammerfest LNG.

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References:

Aarab, N., Godal, B.F., Bechmann, R.K., 2011. Seasonal variation of histopathological and histochemical markers of PAH exposure in blue mussel (*Mytilus edulis* L.). Mar. Environ. Res. 71, 213-217.

Aarab, N., Pampanin, D.M., Naevdal, A., Oysaed, K.B., Gastaldi, L., Bechmann, R.K., 2008. Histopathology alterations and histochemistry measurements in mussel, *Mytilus edulis* collected offshore from an aluminium smelter industry (Norway). Marine Pollution Bulletin 57, 569-574.

Amiard-Triquet, C., 2009. Behavioral Disturbances: The Missing Link between Sub-Organismal and Supra-Organismal Responses to Stress? Prospects Based on Aquatic Research. Hum. Ecol. Risk Assess. 15, 87-110.

Arzate-Cardenas, M.A., Martinez-Jeronimo, F., 2012. Energy reserve modification in different age groups of Daphnia schoedleri (Anomopoda: Daphniidae) exposed to hexavalent chromium. Environ. Toxicol. Pharmacol. 34, 106-116.

Balk, L., Hylland, K., Hansson, T., Berntssen, M.H.G., Beyer, J., Jonsson, G., Melbye, A., Grung, M., Torstensen, B.E., Børseth, J.F., Skarphedinsdottir, H., Klungsøyr, J., 2011. Biomarkers in natural fish populations indicate adverse biological effects of offshore oil production. PloS One 6, e19735, 19710 pp.

Beitinger, T.L., 1990. Behavioral reactions for the assessment of stress in fishes. Journal of Great Lakes Research 16, 495-528.

Beyer, J., Aas, E., Borgenvik, H.K., Ravn, P., 1997. PAH-exposure in fish adjacent to Hydro Aluminium Karmøy: Bioavailability of PAH in gas scrubbers outfall water evaluated by biliary fluorescence measurements in caged Atlantic cod *(Gadus* morhua L.). Hydro Aluminium Karmøy, N-4265 Håvik, Karmøy, Norway, p. 14 pp.

Beyer, J., Jonsson, G., Porte, C., Krahn, M.M., Ariese, F., 2010. Analytical methods for determining metabolites of polycyclic aromatic hydrocarbon (PAH) pollutants in fish bile: A review. Environ. Toxicol. Pharmacol. 30, 224-244.

Beyer, J., Sandvik, M., Hylland, K., Fjeld, E., Egaas, E., Aas, E., Skåre, J.U., Goksøyr, A., 1996. Contaminant accumulation and biomarker responses in flounder (*Platichthys flesus* L) and Atlantic cod (*Gadus morhua* L) exposed by caging to polluted sediments in Sørfjorden, Norway. Aquat. Toxicol. 36, 75-98.

Beyer, J., Sundt, R., Sanni, S., Sydnes, M., Jonsson, G., 2011. Alkylphenol metabolites in fish bile as biomarkers of exposure to offshore oil industry produced water in feral fish. Journal of Toxicology and Environmental Health - Part A - Current Issues 74, 569-581.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248-254.

Brooks, S., Harman, C., Zaldibar, B., Izagirre, U., Glette, T., Marigomez, I., 2011. Integrated biomarker assessment of the effects exerted by treated produced water from an onshore natural gas processing plant in the North Sea on the mussel Mytilus edulis. Marine Pollution Bulletin 62, 327-339.

Cajaraville, M.P., Bebianno, M.J., Blasco, J., Porte, C., Sarasquete, C., Viarengo, A., 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. Sci. Total Environ. 247, 295-311.

Dagestad, K., Lyngmo, V., Myhrvold, A., Bryne, K.H., Åsnes, K., 2005. Miljøovervåkingsprogram for Snøhvit. Statoil, p. 193.

De Coen, W.M., Janssen, C.R., 1997. The use of biomarkers in *Daphnia magna* toxicity testing. IV. Cellular energy alloction: a new methodology to assess the energy budget of toxicant-stressed *Daphnia* populations. Journal of Aquatic Ecosystem Stress and Recovery 6, 43-55.

De Coen, W.M., Janssen, C.R., 2003. The missing biomarker link: Relationships between effects on the cellular energy allocation biomarker of toxicant-stressed Daphnia magna and corresponding population characteristics. Environmental Toxicology and Chemistry 22, 1632-1641.

Garmendia, L., Soto, M., Cajaraville, M.P., Marigomez, I., 2010. Seasonality in cell and tissue-level biomarkers in Mytilus galloprovincialis: relevance for long-term pollution monitoring. Aquatic Biology 9, 203-219.

Glegg, G.A., Rowland, S.J., 1996. The Braer oil spill - Hydrocarbon concentrations in intertidal organisms. Marine Pollution Bulletin 32, 486-492.

Gnaiger, E., 1983. Calculation of energetic and biochemical equivalents of respiratory oxygen consumpition, in: Gnaiger, E., Forstner, H. (Eds.), Polarographic Oxygen Sensors. Aquatic and Physiologaca Applications. Springer Verlag, Berlin, pp. 337-345.

Hagger, J.A., Galloway, T.S., Langston, W.J., Jones, M.B., 2009. Application of biomarkers to assess the condition of European Marine Sites. Environ. Pollut. 157, 2003-2010.

Jemec, A., Drobne, D., Tisler, T., Sepcic, K., 2010. Biochemical biomarkers in environmental studies-lessons learnt from enzymes catalase, glutathione S-transferase and

cholinesterase in two crustacean species. Environmental Science and Pollution Research 17, 571-581.

Jonsson, G., Beyer, J., Wells, D., Ariese, F., 2003. The application of HPLC-F and GC-MS to the analysis of selected hydroxy polycyclic hydrocarbons in two certified fish bile reference materials. J. Environ. Monit. 5, 513-520.

Jung, J.H., Kim, M., Yim, U.H., Ha, S.Y., An, J.G., Won, J.H., Han, G.M., Kim, N.S., Addison, R.F., Shim, W.J., 2011. Biomarker responses in pelagic and benthic fish over 1 year following the Hebei Spirit oil spill (Taean, Korea). Marine Pollution Bulletin 62, 1859-1866.

King, F.D., Packard, T.T., 1975. Respiration and the activity of the respiratory electron transport system in marine zooplankton. Limnology and Oceanography 20, 849–854.

Lowe, D.M., Fossato, V.U., Depledge, M.H., 1995a. Contaminant-induced lysosomal membrane damage in blood cells of mussels *Mytilus galloprovincialis* from the Venice Lagoon: An in vitro study. Marine Ecology Progress Series 129, 189-196.

Lowe, D.M., Pipe, R.K., 1994. Contaminant-induced lysosomal membrane damage in marine mussel digestive cells: an in vitro study. Aquat. Toxicol. 30, 357-365.

Lowe, D.M., Soverchia, C., Moore, M.N., 1995b. Lysosomal membrane responses in the blood and digestive cells of mussels experimentally exposed to fluoranthene. Aquat. Toxicol. 33, 105-112.

Lyngmo, V., 2007. Waste Water - Cleaning System on Melkøya. Statoil, Stavanger.

Martin-Diaz, M.L., DelValls, T., Riba, I., Blasco, J., 2008. Integrative sediment quality assessment using a biomarker approach: review of 3 years of field research. Cell Biology and Toxicology 24, 513-526.

Mouneyrac, C., Perrein-Ettajani, H., Amiard-Triquet, C., 2010. Influence of anthropogenic stress on fitness and behaviour of a key-species of estuarine ecosystems, the ragworm *Nereis diversicolor*. Environ. Pollut. 158, 121-128.

Pearse, A.G.E., 1985. Pigments and pigment precursors, in: Pearse, A.G.E. (Ed.), Histochemistry, 4th edn. Churchill Livingstone, London, UK, pp. 874–928.

Pereira, C.D.S., Martin-Diaz, M.L., Zanette, J., Cesar, A., Choueri, R.B., Abessa, D.M.D., Catharino, M.G.M., Vasconcellos, M.B.A., Bainy, A.C.D., de Sousa, E., Del Valls, T.A., 2011. Integrated biomarker responses as environmental status descriptors of a coastal zone (Sao Paulo, Brazil). Ecotox. Environ. Safe. 74, 1257-1264.

Sole, M., Shaw, J.P., Frickers, P.E., Readman, J.W., Hutchinson, T.H., 2010. Effects on feeding rate and biomarker responses of marine mussels experimentally exposed to propranolol and acetaminophen. Anal. Bioanal. Chem. 396, 649-656.

Sundt, R., Ruus, A., Jonsson, H., Skarphéðinsdóttir, H., Meier, S., Grung, M., Beyer, J., Pampanin, D., 2012. Biomarker responses in Atlantic cod (*Gadus morhua*) exposed to Produced Water from a North Sea oil field: laboratory and field assessments. Marine Pollution Bulletin 64, 144-152.

Sundt, R.C., Meier, S., Jonsson, G., Sanni, S., Beyer, J., 2009. Development of a laboratory exposure system using marine fish to carry out realistic effect studies with produced water discharged from offshore oil production. Marine Pollution Bulletin 58, 1382 - 1388.

Teh, S.J., Adams, S.M., Hinton, D.E., 1997. Histopathologic biomarkers in feral freshwater fish populations exposed to different types of contaminant stress. Aquat. Toxicol. 37, 51-70.

Tsangaris, C., Hatzianestis, I., Catsiki, V.A., Kormas, K.A., Strogyloudi, E., Neofitou, C., Andral, B., Galgani, F., 2011. Active biomonitoring in Greek coastal waters: Application of the integrated biomarker response index in relation to contaminant levels in caged mussels. Sci. Total Environ. 412, 359-365.

van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57-149.

Velvin, R., Beyer, J., Walday, M., Vögele, B., Evenset, A., 2009. StatoilHydros miljøovervåkingsprogram for Snøhvit: Marin overvåkingsundersøkelse av kystmiljø i forbindelse med drift på LNG anlegg på Melkøya 2008. Akvaplan-niva, Tromsø, Norway, p. 164.

Vieira, L.R., Gravato, C., Soares, A., Morgado, F., Guilhermino, L., 2009. Acute effects of copper and mercury on the estuarine fish Pomatoschistus microps: Linking biomarkers to behaviour. Chemosphere 76, 1416-1427.

Wedderburn, J., McFadzen, I., Sanger, R.C., Beesley, A., Heath, C., Hornsby, M., Lowe, D., 2000. The field application of cellular and physiological biomarkers, in the mussel *Mytilus edulis*, in conjunction with early life stage bioassays and adult histopathology. Marine Pollution Bulletin 40, 257-267.

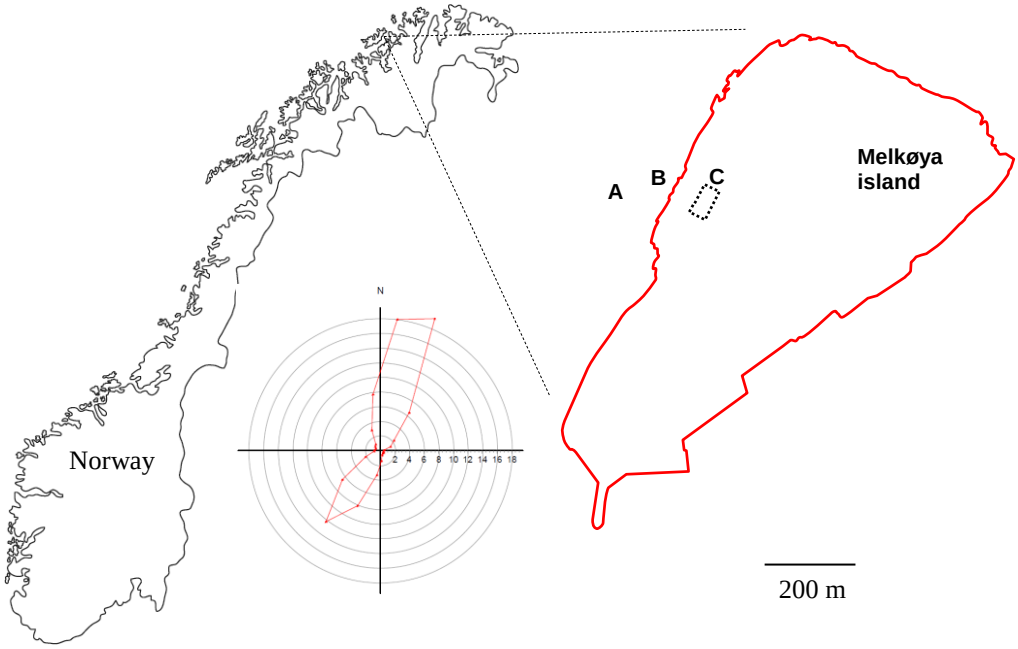


Figure 1: Localization and outline of the island Melkøya just outside the city of Hammerfest in Northern Norway. The island houses the Hammerfest LNG plant. (A) Approximate position of the discharge point for the effluent at 40 m water depth. (B) Position of mussel cages at 10 m depth. (C) Position of the canteen building. The included data chart shows the prevailing direction of sea currents (% of total) at 4 m depth at position B.

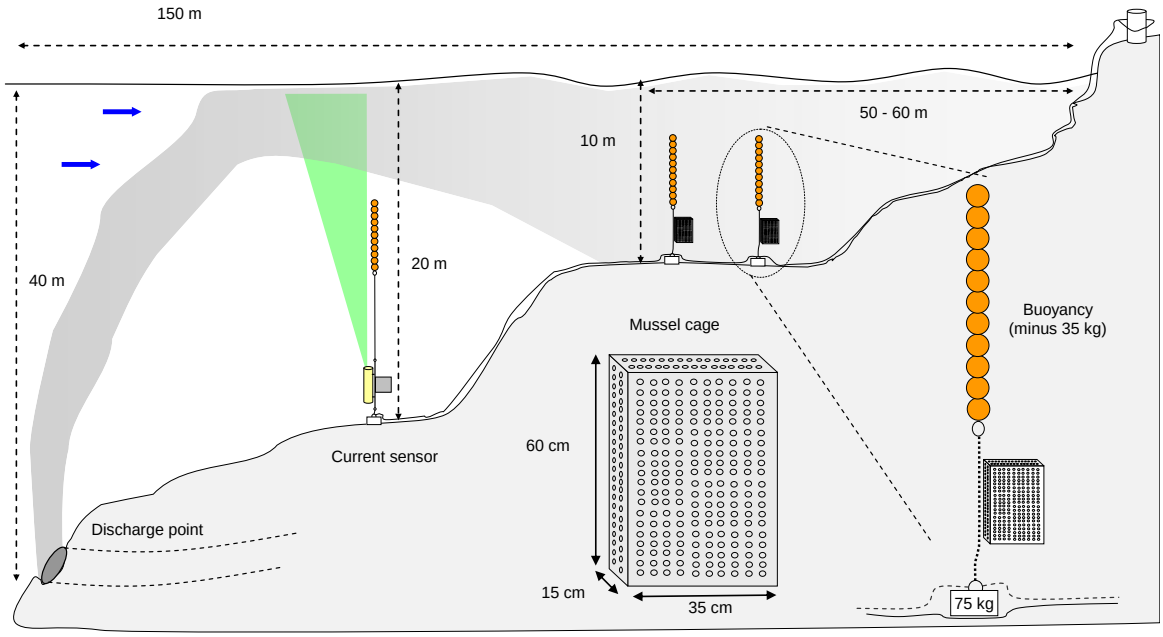


Figure 2: Sketch of the cage rig and how the cages and current sensor were placed relative to the effluent outfall. The effluent is warmer than the sea and will rise towards the surface in the first part of the dispersal, but when the temperature difference is equalized the plume will mix downwards in the continued dilution process.

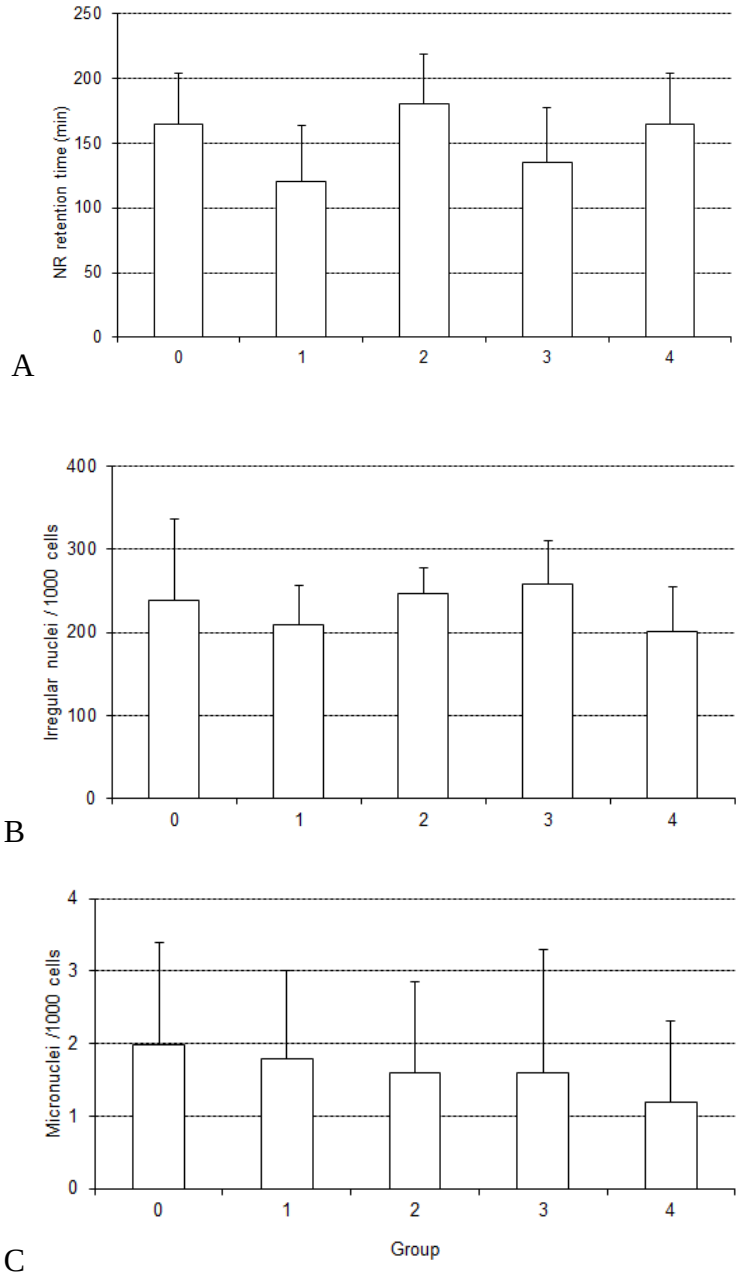


Figure 3: Biomarker data in mussels in Melkøya caging study. Groups: 0: mussels obtained from mussel farm (not caged). 1 & 2: mussels caged at reference site (Seiland); 3 & 4: mussels caged at Melkøya, close to wastewater discharge point. Data: A: Haemocyte lysosomal stability measured as the time (in minutes) to loss of lysosomal retention of neutral red in 50% of scored cells. B: Frequency of irregular nuclei in haemocytes (number per thousand cells). C: Frequency of micronuclei in haemocytes (number per thousand cells). Data are shown by the group mean + one standard deviation and *n*=10 in all groups.

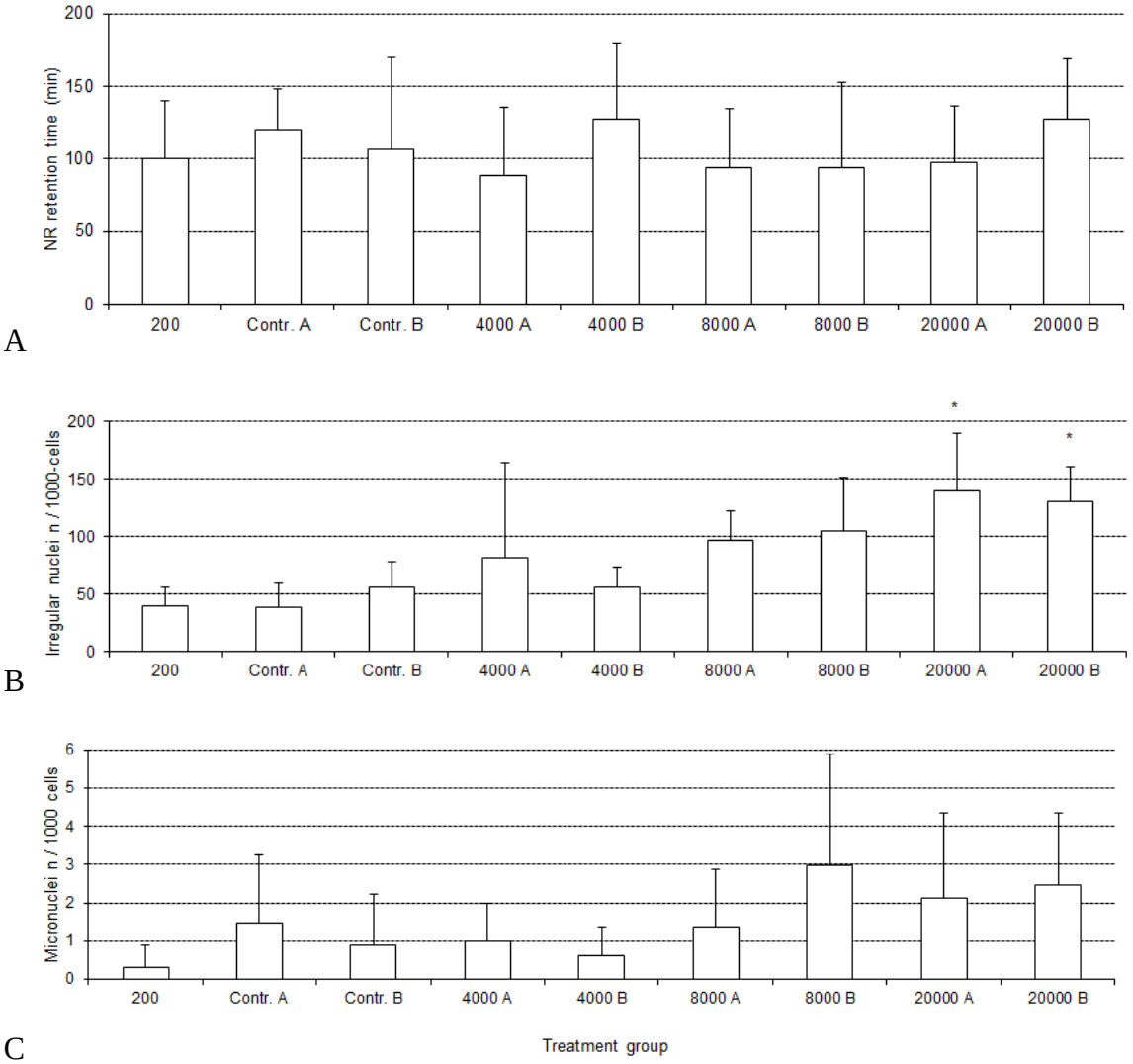


Figure 4: Biomarker data in mussels exposed to wastewater in the laboratory. A: Lysosomal stability (neutral red retention time) in haemocyte cells. B: Relative frequency of irregular nuclei in haemocytes. C: Relative frequency of micronuclei in mussel haemocytes. Treatment concentrations: 1:200 (one replicate), control groups (2 replicates A & B), 1:4000 (A & B), 1:8000 (A & B) and 1:20000 (A & B). Data are shown as group mean (+stdev) (*n*=8) in each replicate group. *Significantly different from control group (Dunnett's test of means).

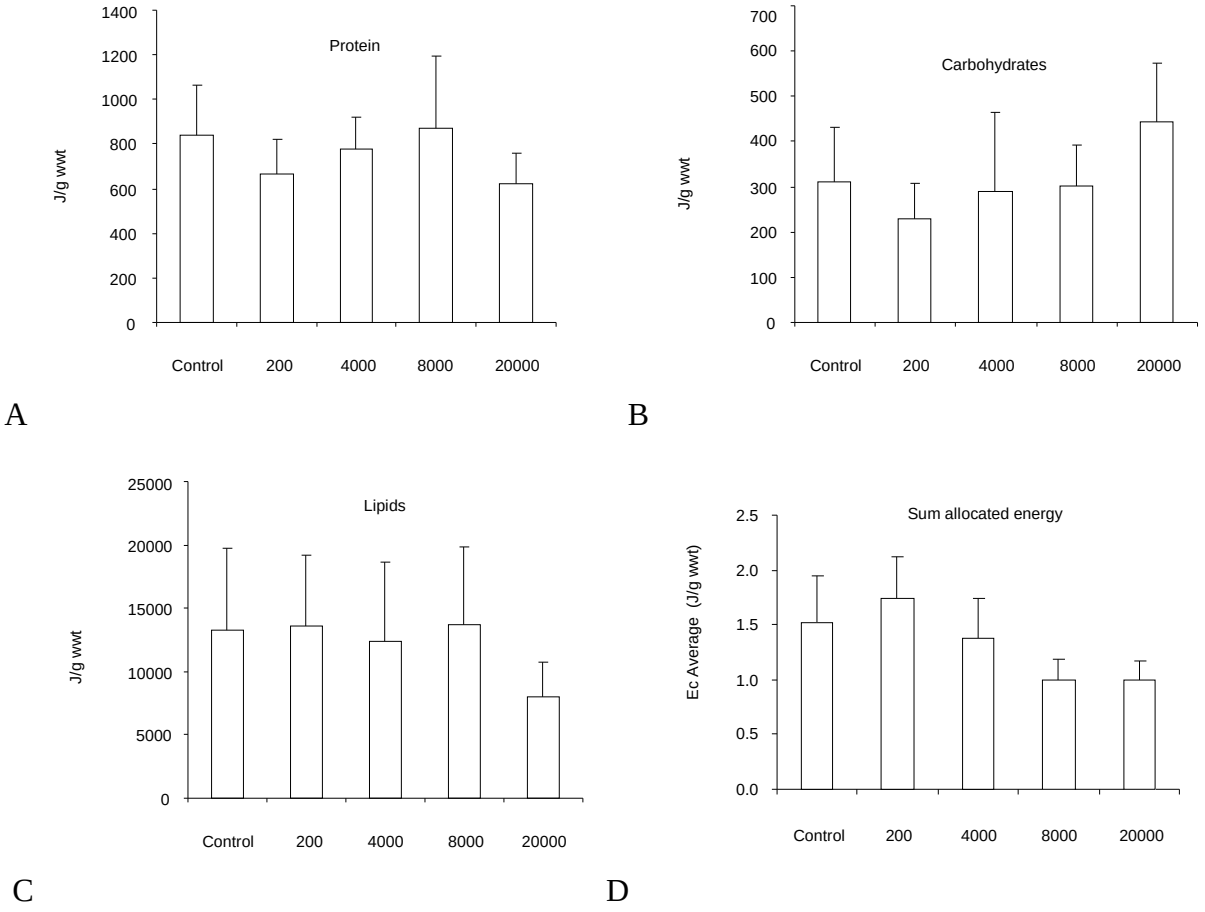
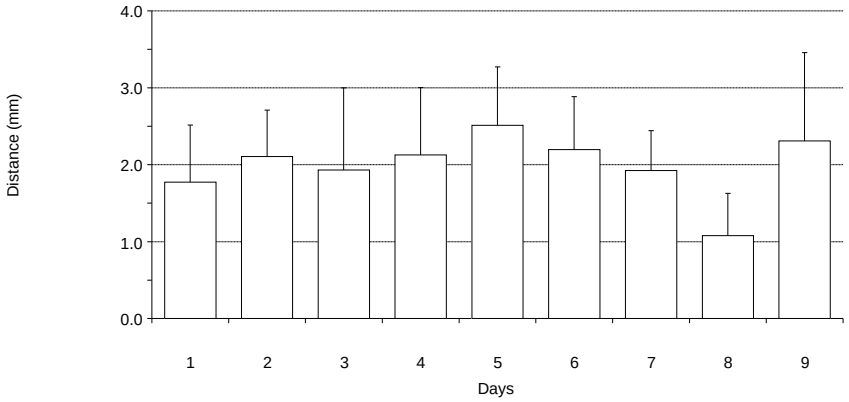
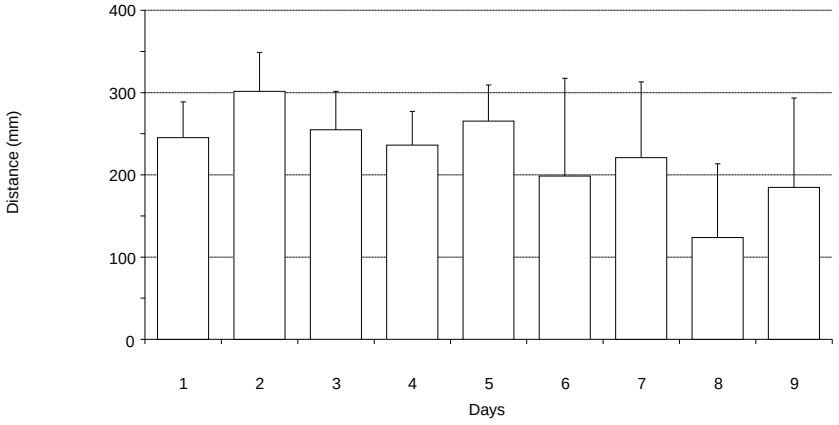


Figure 5: Energy reserves and cellular energy allocation assessed in whole mussels, measured in terms of: A: protein, B: carbohydrates, C: lipids and D: sum allocated energy from protein, carbohydrates and lipids. Data bars represent the mean value (*n*=5) per treatment group with one standard deviation.

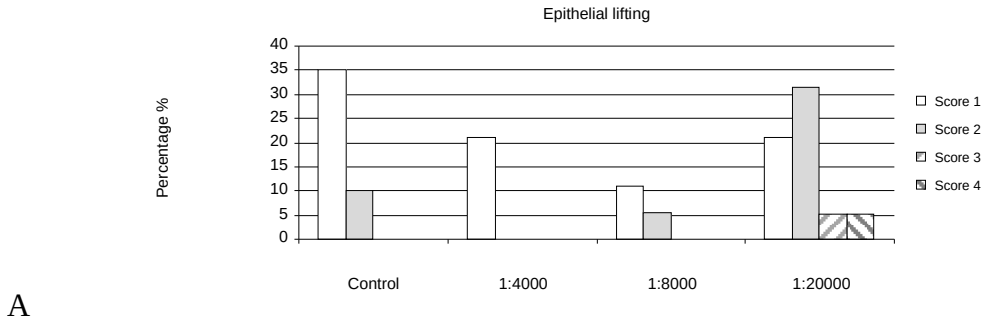


A



B

Figure 6: Shell opening activity in a test group of mussels (*n*=8). The treatment was as follows: Days 1-3 clean seawater, days 4-6 wastewater exposure (200x dilution), days 7-9 clean seawater (recovery). The shell opening of each individual mussel was measured continuously throughout the 9 days test period. A: Mean valve gape over 24 hours (mm) +/- 2 SEM. B: Total mean valve movement distance over 24 hours +/- 2 SEM.



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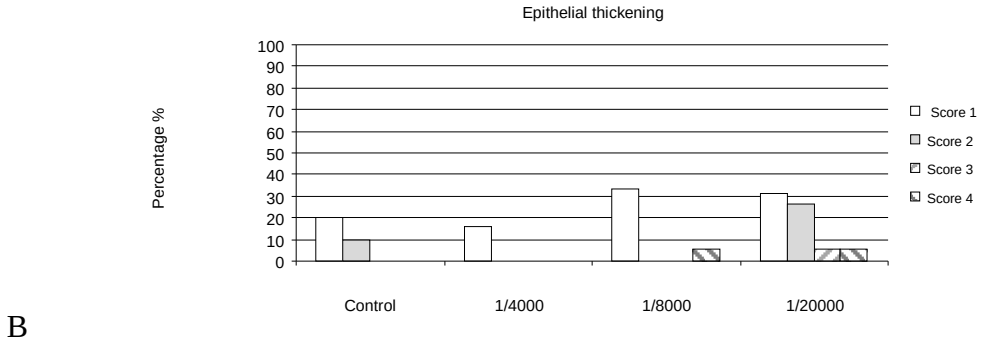

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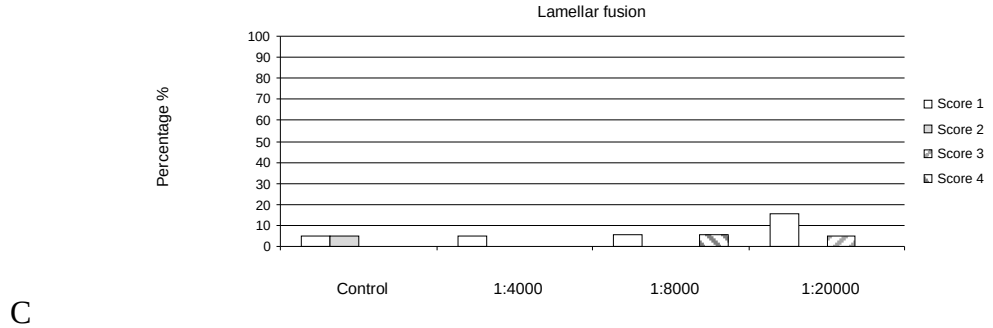

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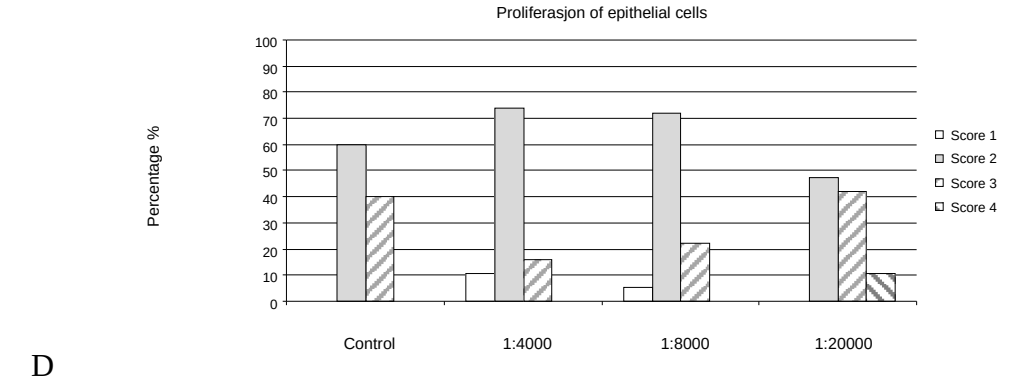

D

Figure 7: Histopathology traits in gills of cod after four weeks WW exposure. The presence of each pathology in 18-20 individual cod per treatment was scored as: 0: absent (data not shown); 1: mild; 2: mild to moderate; 3: moderate; or 4: severe. The bars show the percentage of each WW treatment group scoring in the categories 1-4.

Table 1: Concentration of PAH in blue mussel batch samples of groups caged at Seiland and at the Melkøya location close to the wastewater discharge location.

ng/g (ww)	0-group, Seiland	Group 1, Seiland	Group 2, Seiland	Group 3 Melkøya	Group 4 Melkøya
Naphthalene	< 0.093	0.05	0.05	0.05	0.05
C1-naphthalene	< 0.084	< 0.084	< 0.084	< 0.084	< 0.084
C2-naphthalene	< 0.048	0.25	0.19	0.07	< 0.048
C3-naphthalene	< 0.045	< 0.045	< 0.045	0.52	0.74
Phenanthrene	0.28	0.25	0.24	0.29	0.35
Anthracene	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
C1-phenanth/anthracene	< 0.056	0.18	< 0.056	0.26	0.45
C2-phenanth/anthracene	< 0.111	< 0.111	0.27	0.23	0.17
C3-phenanth/anthracene	< 0.044	< 0.044	< 0.044	0.43	0.32
Dibenzothiophene	< 0.017	< 0.017	< 0.017	< 0.017	< 0.017
C1-dibenzothiophene	< 0.029	< 0.029	< 0.029	< 0.029	< 0.029
C2- dibenzothiophene	< 0.122	< 0.122	< 0.122	0.45	0.29
C3- dibenzothiophene	< 0.064	< 0.064	< 0.064	< 0.064	0.07
Acenaphthylene	0.02	0.02	0.03	0.02	0.01
Acenaphthene	0.04	0.02	0.04	0.04	0.07
Fluorene	0.13	0.08	0.11	0.10	< 0.008
Fluoranthene	0.27	0.20	0.16	0.44	0.28
Pyrene	0.16	0.10	0.07	0.19	0.08
Benzo[a]anthracene	0.02	0.01	< 0.003	< 0.003	< 0.003
Chrysene	0.16	0.16	0.12	0.27	0.21
Benzo[b]fluoranthene	0.15	0.22	0.11	0.19	0.06
Benzo[k]fluoranthene	0.09	0.12	0.07	0.10	0.03
Benzo[e]pyrene	0.08	0.10	0.06	0.12	0.05
Benzo[a]pyrene	0.01	< 0.006	< 0.006	< 0.006	< 0.006
Perylene	0.03	< 0.014	0.02	< 0.014	< 0.014
Indeno[1.2.3-cd]pyrene	0.14	0.15	0.10	0.10	0.04
Benzo[ghi]perylene	< 0.019	< 0.019	< 0.019	< 0.019	< 0.019
Dibenzo[a.h]anthracene	< 0.019	< 0.019	< 0.019	< 0.019	< 0.019
SUM NPD	0.67	1.02	1.01	2.40	2.53
SUM 16 EPA	1.54	1.41	1.13	1.81	1.20

Table 2: Concentration of metals and methyl mercury in blue mussel batch samples of groups caged at Seiland and at the Melkøya location close to the wastewater discharge location.

		0-group, Seiland	Group 1, Seiland	Group 2, Seiland	Group 3 Melkøya	Group 4 Melkøya
Dry matter (DM)	%	17.2	15.2	15	15.1	13.9
As	mg/kg d.w.	7.65	10.7	10.1	9.58	10.2
Cd	mg/kg d.w.	1.88	1.94	1.67	2.1	1.88
Co	mg/kg d.w.	0.25	0.34	0.40	0.41	0.37
Cr	mg/kg d.w.	0.49	21.4	47.3	4.79	1.32
Cu	mg/kg d.w.	6.39	6.88	10.4	6.24	11.4
Hg	mg/kg d.w.	0.170	0.046	0.046	0.048	0.042
Mn	mg/kg d.w.	2.52	3.37	3.15	4.16	4.02
Ni	mg/kg d.w.	0.50	1.81	4.1	1.16	1.37
Pb	mg/kg d.w.	0.60	0.68	0.87	0.86	0.82
Zn	mg/kg d.w.	90.9	87.1	85.2	124	98.4
Methyl mercury	ng/g d.w.	2.3	2.0	1.6	1.7	1.2

Table 3: Concentration of naphthalene and alkylated naphthalenes in five aliquot samples of treated wastewater obtained at the Hammerfest LNG plant.

WW sample	1	2	3	4	5
	ng/g	ng/g	ng/g	ng/g	ng/g
Naphthalene	0,02	0,04	0,03	nd	0,03
C1-Naphthalene	0,03	0,11	0,05	nd	0,04
C2-Naphthalene	0,03	0,20	0,05	nd	0,03
C3-Naphthalene	0,03	0,17	0,06	nd	0,04

nd = below limit of detection

Table 4: Metabolites of PAH and alkylated PAH compounds measured in four individual bile samples from cod in the positive control experiment with Melkøya production water.

Fish individual no.	06	11	22	34
Treatment	Control	Control	1:4000	1:4000
Compound	ng/g	ng/g	ng/g	ng/g
1-OH-Naphthalene	nd	nd	nd	nd
2-OH-Naphthalene	nd	nd	nd	nd
C1-OH-Naphthalene	nd	nd	nd	nd
C2-OH-Naphthalene	nd	nd	nd	nd
C3-OH-Naphthalene	*<	*<	*<	1598
1-OH-Phenanthrene	nd	nd	nd	nd
C1-OH-Phenanthrene	nd	nd	*<	nd
C2-OH-Phenanthrene	*<	*<	*<	1592
1-OH-Pyrene	*<	*<	*<	*<

nd = under limit of quantification

Environmental harm assessment of a wastewater discharge from Hammerfest LNG: A study with biomarkers in blue mussels (*Mytilus sp.*) and Atlantic cod (*Gadus morhua*)

J. Beyer, N. Aarab, A.H. Tandberg, A. Ingvarsdottir, S. Bamber, J.F. Børseth, L. Camus & R. Velvin

Highlights

- The ecotoxicity of a LNG plant wastewater (WW) was assessed in mussels and fish.
- Test animals were exposed by *in situ* caging (mussels) and laboratory exposures.
- A suite of contaminant responsive markers were measured in the test animals.
- Significant responses were not observed in WW exposed test animals.
- The WW discharge is most likely not harmful for the local marine environment.