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PAH body burden and biomarker responses in mussels (*Mytilus edulis*) exposed to produced water from a North Sea oil field: Laboratory and field assessments

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ABSTRACT

In order to study the impact of produced water (PW) from a North Sea oil field on blue mussels (*Mytilus edulis*), chemical and biological markers were selected. A laboratory exposure (0.125%, 0.25% and 0.5% of PW) and a field study (6 stations 0.2–2 km from a PW discharge point) were conducted. In the laboratory study, PAH bioaccumulation increased in mussel soft tissue even at the lowest exposure dose. Micronuclei frequency demonstrated a dose–response pattern, whereas lysosomal membrane stability showed tendency towards a dose–response pattern. The same markers were assessed in the field study, biomarker analyses were consistent with the contamination level, as evaluated by mussel polycyclic aromatic hydrocarbons body burden. Overall, obtained results confirmed the value of an ecotoxicological approach for a scientifically sound characterisation of biological effects induced by offshore oilfield operational discharges.

1. Introduction

Operation of oil and gas production platforms in the North Sea result in considerable discharges of produced water (PW). This consists of a mixture of formation water contained naturally in the reservoir, injected water used for secondary oil recovery and treatment chemicals added during production (Røe Utvik et al., 1999). The chemical composition varies both between reservoirs and within a reservoir as production proceeds. A chemical characterisation of four offshore oil production platforms in the North Sea showed that the major organic components were BTEX (benzene, toluene, ethylbenzene and xylene), NPD (naphtalenes, phenanthrenes and dibenzothiophenes), polycyclic aromatic hydrocarbons (PAHs), organic acids, alkylphenols (APs) and phenols (Røe Utvik, 1999). Some of the present organic chemicals have the potential to bioaccumulate and may be toxic for organisms living in the vicinity of platforms (Tollefsen et al., 1998; Aas et al., 2000; Gorbi et al., 2008; Hannam et al., 2009; Farmen et al., 2010; Perez-Casanova et al., 2010). As a chronic source of pollution, PW is also a source of concern with respect to possible long term impact on the environment (Neff et al., 2006). Estimates of the PW discharge volume predict an increase on the Norwegian shelf until 2010–2014, reaching about 200 million L/year (OLF, 2007). There is therefore a need for adequate exposure and effect markers used in biomonitoring of offshore discharges (Hylland et al., 2008; Farmen et al., 2010).

PAHs in particular are known to induce toxic effects at the individual level (van der Oost et al., 2003; Bellas et al., 2008). Therefore, integration of chemical analyses with biomarker responses in organisms has been recommended for monitoring offshore exploitation activities (Hylland et al., 2008). Previous studies have indicated that toxic compounds are detectable several kilometres away from a North Sea oil production platform using *in vitro* bioassays and biomarkers (Hylland et al., 2006b). Although there is reason to assume that many PW related chemicals may produce biological responses, the ability to assess the potential for adverse effects is limited by the lack of sufficient *in situ* biomonitoring data and realistic laboratory exposures. A two-tier approach has been proposed to make the biomonitoring tools suitable for application purposes, aiming to build a cost-effective strategy. It contains a tier 1 screening level with sensitive low-cost biomarkers (e.g. lysosomal membrane stability (LMS)) and a tier 2 stress syndrome assessment level applying a battery of biomarkers when an environmental alteration is recorded at tier 1 (Viarengo et al., 2007). For standardisation purposes, the use of caged animals (mussel and/or fish) is also suggested (Cajaraville et al., 2000; Handy et al., 2003; Hylland et al., 2008). Mussels (*Mytilus* sp.) have been widely used since the 90s, and have been shown to be one of the most successful

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model organisms for time-integrated responses to complex mixtures of pollutants (UNEP/RAMOGE, 1999, MedPol, OSPAR). The most appropriate caging duration has been a matter of discussion and an approximate time of one month has proved to be long enough to allow physiological parameters not to be affected by non pollution related changes (i.e. reproductive cycle, food availability etc.) (Viarengo et al., 2007; Gorbi et al., 2008). Under these conditions, observed changes in biomarkers are believed to be related to the effect of toxic chemicals present in the water.

The objectives of the present study were first to evaluate the selected chemical (PAH bioaccumulation) and biological (LMS and micronuclei (MN) assays) markers for studying the ecotoxicological impact of offshore PW discharges. Secondly we aimed to assess the extent to which operational discharges from an oil production platform affect organisms in the water column. To fulfil these objectives a controlled laboratory experiment was used for exposing mussels to PW and subsequently a field survey was performed using caged animals in the vicinity of the Ekofisk oil field. The Ekofisk field was chosen as the study area for several reasons, one of the main reasons is that due to the long production history of the field, discharge of PW is high (approximately 8×10^6 L/day in 2006). The combination of laboratory and field exposures allowed validation of the selected markers and improved knowledge about their potential and limitations, as previously demonstrated in other studies (Zorita et al., 2005; Fang et al., 2008). For a detailed description of the selected biomarkers, LMS determination using neutral red retention time and the micronuclei assay, see Viarengo et al. (2007). Parallel studies were conducted with Atlantic cod, *Gadus morhua*, the other species routinely used in Norwegian offshore water column monitoring (WCM) and results from these are reported in Sundt et al. (2009).

2. Materials and methods

2.1. Laboratory exposure set up

Blue mussels (*Mytilus edulis*, 5–6 cm length) were collected from a reference site at Tysvær, Norway (59°19′28″N, 5°27′36″E, WGS 84), transported to Akvamiljø research facility in Stavanger and allowed to acclimatise in holding tanks (600 L) for 1 week before the exposure. PW from the North Sea Ekofisk field was tapped in a 3 m^3 steel tank at the platform, air flushed, transported to the laboratory, frozen in 20 L aliquot batches in closed polyethylene cans, and kept frozen at −10 °C until used, to minimise chemical degradation. A detailed description of the exposure set up is given in Sundt et al. (2009). During the exposure period, frozen PW aliquots were thawed (with stirring) according to daily consumption. The thawed PW was kept below 9 °C in a mixing tank and diluted with clean sea water (salinity 34‰ and temp 11 ± 1 °C) in the ratio 1:800, 1:400 and 1:200 to obtain the final exposure concentrations of: 0.125%, 0.25% and 0.5% PW, respectively. By means of the DREAM model (Reed et al., 2001) the maximum concentration of total PAHs over a one month period was simulated using May 1990 current data. Concentrations of 0.125% PW was indicated to be reached along most transects, while the 0.5% PW was indicated to occur only in a limited part of the studied transects. The experiment was carried out for 29 days (13th July–11th August 2005). Eight tanks of 600 L containing approximately 100 mussels were used for the exposure. A flow-through exposure system was used to ensure renew of seawater (10 L/min/tank, theoretical exchange rate 1 h) and presence of food particles. Each exposure concentration including the control was run in parallel as backup. Mussels were not additionally fed and no mortality was observed during this exposure.

2.2. Field study using caged mussels

The field investigation was conducted at the Ekofisk oil field in the central North Sea as a part of the Norwegian WCM program. Mussels (5–6 cm length) were obtained from a shellfish farm at Rissa (Trondheim, Norway), transported refrigerated by airplane to Stavanger and then shipped to the platform area in tanks with a continuous flow of seawater. Water samples (10 L, 5 replicates) were taken from the carriers holding tank to check for unintended petrogenic contamination of the mussels. The seawater used for transport of mussels to offshore field sites contained trace amounts of naphthalene (results not shown), whereas other PAHs were below the detection limit (0.005 μg/L). A total of six caging rigs were deployed for 48 days (4th April–22nd May 2006) along the prevailing current direction of the PW outfall, from approximately 200 m from the discharge up to 2000 m from the installations (stations 1–6). In addition two rigs were placed at assumedly clean locations NE of Ekofisk, they were considered as equivalent and treated as one reference station (Fig. 1). Locations of the stations were selected based on previous current measurements at the site and mussels were deployed at approximately 20 m depth. The design of the caging rigs is described in Hylland et al. (2006a). Technical and environmental challenges (wind, wave, safety precautions, etc.) through the field study are important factors in determining experimental design and should be taken into account. Current information was obtained during the exposure period using Aanderaa RCM9 MkII current meters fitted on the rigs of station 2 and 5. Vertical profiles of temperature and conductivity in the water column under the Ekofisk 2/4C installation were recorded on 6 occasions with STD/CTD model SD204. At the end of the experiment caged mussels were collected and utilised for chemical and biological marker analyses. No mussel mortality was observed during this study.

2.3. Chemical analyses

Water samples for PAH analysis were taken 4 times during the laboratory exposure (day 5, 19, 24 and 28). Twenty-four PAH compounds (or groups of compounds) were analysed by gas chromatography mass spectrometry (GC–MS) at IRIS as described by Jonsson et al. (2004). In addition samples for AP analyses were taken 4 times (day 22, 23, 24 and 25) results are reported in Sundt et al. (2009).

2.4. Chemical markers

Body burden of PAHs was analysed in pooled soft tissue samples, consisting of 15 mussels each (3 replicates per experimental point). A total of 24 different PAHs and dibenzothiophene compounds were analysed, 14 of which are included in the list of 16 priority PAHs suggested by the US EPA (1987). Data are presented as μg/kg of wet weight. The method description is reported in Hylland et al. (2008).

2.5. Biological markers

LMS was evaluated by the neutral red retention time (NRRT) assay (Lowe et al., 1995). Briefly, 0.4 ml of haemolymph was obtained from the posterior adductor muscle of each mussel and the cell suspension was spread on a slide, and transferred to a lightproof humidity chamber for 15 min to allow cells to attach. Then 35 μL of the neutral red probe (final concentration 0.1 μg μL^{-1}) was added to the cell monolayer. After a 15 min incubation period, slides were monitored under a light microscope repeatedly at 15, 30, 60, 90, 120, 150 and 180 min of incubation with neutral red. The time period between the dye application and the appearance

of the first evidence of dye loss from the lysosomes to the cytosol in at least 50% of the examined granular haemocytes represents the NRRT for the individual. The mean retention time of 15–16 mussels corresponded to the NRRT for the sampling point. All analyses were preformed blind.

The MN analyses were performed in the haemocytes according to the method described in Baršienė et al. (2004). Haemolymph was withdrawn in physiological saline solution (1/1 v/v). The obtained cell suspensions were spread on slides, air-dried, fixed in methanol for 15 min, and shipped to the Institute of Ecology, Nature Research Center, Lithuania for scoring. Cells were stained with 5% Giemsa and examined under Olympus BX51 microscope (1000 × magnification). Between 12 and 16 mussels were analysed from each exposure group or field station and 2000 agranular haemocytes per mussel were examined. Blind scoring was performed on coded slides. Briefly, MN were scored using the following criteria (Fenech et al., 2003): (i) round or ovoid-shaped non-refractory particles in the cytoplasm, (ii) structure and colour intensity of MN was the same that as the nucleus, (iii) the diameter of the MN was equal or smaller than 1/3 of the nucleus, (iv) MN were completely detached from the nucleus, (v) cells with multiple MNs were not scored.

2.6. Data analysis

Chemical and biological marker results from individual mussels were compared using statistical analysis (Sokal and Rohlf, 1981). Since the biomarker data were not normally distributed, the group comparisons were made by a non-parametric test (Wilcoxon rank-sum). The level of significance to reject H_0 hypothesis (no difference between groups) was set as $p < 0.05$. As regards the chemical analyses and bioaccumulation results, statistical evaluation was not performed due to the low number of replicates ($n = 3$). Statistical analysis was performed using JMP software (Windows, ver. 5.1, SAS Institute, Cary, NC, USA).

3. Results

3.1. Laboratory exposure results

The PAH measurements in the mixing tanks and in the exposure tanks confirmed that the dilution of the PW in the experiment was as planned (Table 1). The mean concentration of the total PAHs in exposure tank water samples from the 0.125%, 0.25% and 0.5% of PW dilutions was 0.10, 0.26 and 0.78 μg/L, respectively. Naphthalenes (C_0, C_1, C_2 and C_3 naphthalene) were the prevalent compounds detected, as they represented above 80% of the total PAHs in the water. In the 0.125% and 0.25% PW exposures, the remaining PAHs were mainly alkylated phenanthrenes (3 membered rings). In the highest PW concentration (0.5%), approximately 3% alkylated dibenzothiophenes were quantified.

Bioaccumulation of PAHs in mussel tissue showed a clear dose response with increased PAH contamination from the PW exposure (Table 1). Mean values of total PAHs were 0.032, 0.101, 0.263 and 0.780 for control, 0.125%, 0.25% and 0.5% PW, respectively.

As regards the LMS assay, the results were not statistically different in the haemocytes of exposed mussels compared to the control animals. The mean value in mussels from the control tank was 102 min (median 90 min, Fig. 2).

The frequency of MN in the haemocytes increased with increasing concentration of PW and was significantly different in the highest concentration of PW (0.5%) compared to the control (Fig. 3). Presence of MN in the haemolymph increased with increasing concentration of PW.

3.2. Field study results

Locations of the stations in the field study (Fig. 1) were based on the local current pattern. At the caging depth, the salinity was generally stable at 35‰ through the deployment, whereas the temperature increased from 5.5 to 9.5 °C at both measuring points (depth

Table 1
Mean concentration of polycyclic aromatic hydrocarbons (PAHs) in exposure tanks of the laboratory study, Limit of Quantification (LOQ) = 0.005 μg/L for single compounds, $n = 8$ for each group, values are reported as μg/L; and mean concentrations of PAHs detected in mussel soft tissues in laboratory exposure and field study (laboratory exposure $n = 4$, field study $n = 3$). Results are reported as μg/kg, LOQ = 0.005 μg/L for single compounds.

PAHs	Laboratory exposure study								Field study						
	Water concentrations				Mussel concentrations				Mussel concentrations						
	Control	0.125%	0.25%	0.5%	Control	0.125%	0.25%	0.5%	Ref	St 1	St 2	St 3	St 4	St 5	St 6
Naphthalene	0.011	0.022	0.057	0.168	1.30	9.44	21.79	45.11	0.71	1.42	2.26	2.56	1.36	1.43	1.44
C_1-Naphthalene	0.011	0.024	0.059	0.173	3.38	74.11	185.56	343.21	0.29	3.54	6.50	8.25	3.89	3.98	3.39
C_2-Naphthalene	0.006	0.024	0.055	0.170	4.83	355.68	749.16	1373.02	0.00	12.06	20.20	26.21	12.79	10.77	8.49
C_3-Naphthalene	<LOQ	0.025	0.050	0.134	12.01	768.16	1541.96	2881.89	0.00	23.49	37.78	48.48	27.62	19.42	13.03
Fluorene	<LOQ	<LOQ	<LOQ	0.007	0.23	12.48	25.76	49.30	0.34	1.04	1.29	1.64	1.11	0.91	0.85
Phenanthrene	<LOQ	<LOQ	0.005	0.017	0.88	51.59	101.16	190.42	0.82	3.79	5.20	5.87	4.21	3.44	2.95
Anthracene	<LOQ	<LOQ	<LOQ	<LOQ	0.10	1.43	0.90	3.22	0.07	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
C_1-Phen/Anthr	<LOQ	<LOQ	0.016	0.042	1.43	344.87	619.58	1156.91	0.66	14.23	23.01	26.74	19.57	12.21	8.77
C_2-Phen/Anthr	<LOQ	0.006	0.022	0.052	1.98	707.77	1200.52	2284.43	0.69	28.84	47.27	54.31	43.71	24.41	17.62
Dibenzothiophene	<LOQ	<LOQ	<LOQ	<LOQ	0.05	7.04	13.71	26.16	0.08	0.44	0.59	0.66	0.49	0.40	0.34
C_1-Dibenzothiophene	<LOQ	<LOQ	<LOQ	0.006	0.90	63.92	118.38	216.42	0.31	3.12	4.87	5.39	4.23	2.62	1.97
C_2-Dibenzothiophene	<LOQ	<LOQ	<LOQ	0.010	1.03	184.38	306.77	576.26	0.52	7.73	12.51	14.25	12.10	6.94	4.90
Fluoranthene	<LOQ	<LOQ	<LOQ	<LOQ	0.95	5.64	8.71	14.30	1.21	2.11	2.25	2.08	2.41	1.72	1.72
Pyrene	<LOQ	<LOQ	<LOQ	<LOQ	0.63	12.66	21.55	39.23	0.45	1.15	1.34	1.41	1.13	0.71	0.66
Benzo(a)anthracene	<LOQ	<LOQ	<LOQ	<LOQ	0.73	4.69	7.80	15.02	0.02	<LOQ	<LOQ	0.05	4.69	<LOQ	<LOQ
Chrysene/Triphenylene	<LOQ	<LOQ	<LOQ	<LOQ	1.18	53.49	80.37	162.97	0.54	2.41	3.74	4.13	4.09	2.12	1.69
C_1-Chrysene	<LOQ	<LOQ	<LOQ	<LOQ	0.58	136.65	217.39	477.59	0.07	2.31	4.09	4.85	4.57	2.01	1.29
C_2-Chrysene	<LOQ	<LOQ	<LOQ	<LOQ	0.40	147.95	247.51	531.03	<LOQ	1.91	3.13	3.61	3.35	1.67	0.98
Benzo(b.j)fluoranthene	<LOQ	<LOQ	<LOQ	<LOQ	0.65	6.52	10.09	20.80	0.14	0.21	0.35	0.35	0.40	0.17	0.12
Benzo(k)fluoranthene	<LOQ	<LOQ	<LOQ	<LOQ	0.03	0.05	0.10	0.23	<LOQ	<LOQ	0.13	0.15	0.17	<LOQ	<LOQ
Benzo(a)pyrene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.18	0.18	0.68	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Indeno(1.2.3-cd)pyrene	<LOQ	<LOQ	<LOQ	<LOQ	0.05	0.23	0.48	0.85	0.08	0.07	<LOQ	0.06	0.08	0.03	<LOQ
Benzo(g.h.i)perylene	<LOQ	<LOQ	<LOQ	<LOQ	0.38	2.13	3.03	5.78	0.10	0.16	0.14	0.14	0.14	0.10	0.09
Dibenzo(a.h)anthracene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.63	1.15	2.48	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Total PAHs	0.03	0.10	0.26	0.78	33.64	2951.63	5483.59	10417.27	7.09	110.04	176.63	211.18	152.13	95.08	70.30

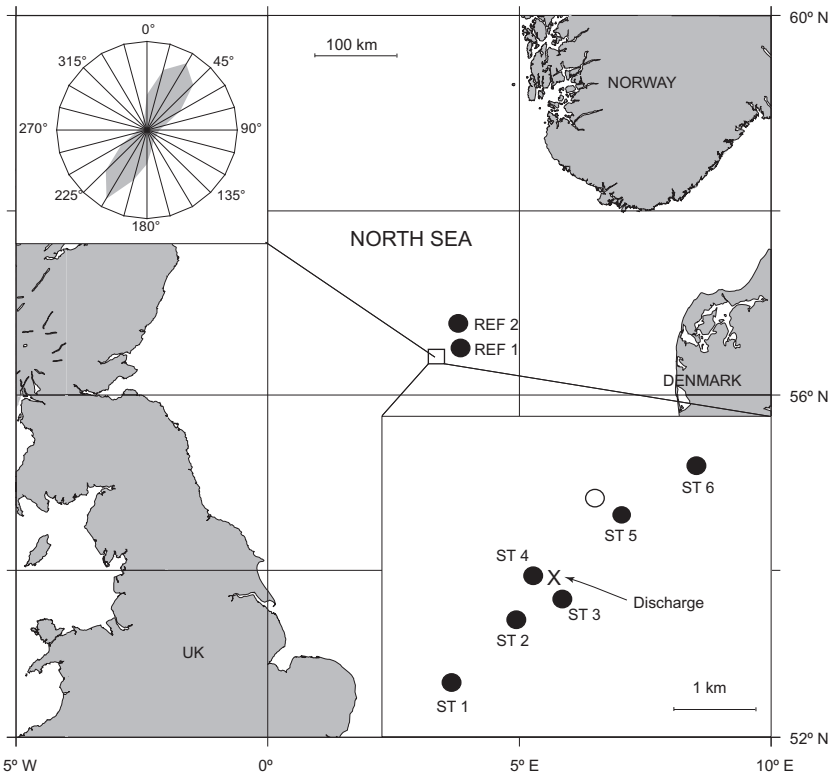

Fig. 1. Map showing location of produced water discharge in relation to exposure gradient (stations 1–6 = ST 1–ST 6) and unexposed reference sites (REF 1 and REF 2). As shown top left, the predominant current axis indicates the oscillating, tidal movement of water in the area, being predominantly either SW or NE. (Discharge position N 56° 32′ 58″ E 03° 12′ 45" – ED 50).

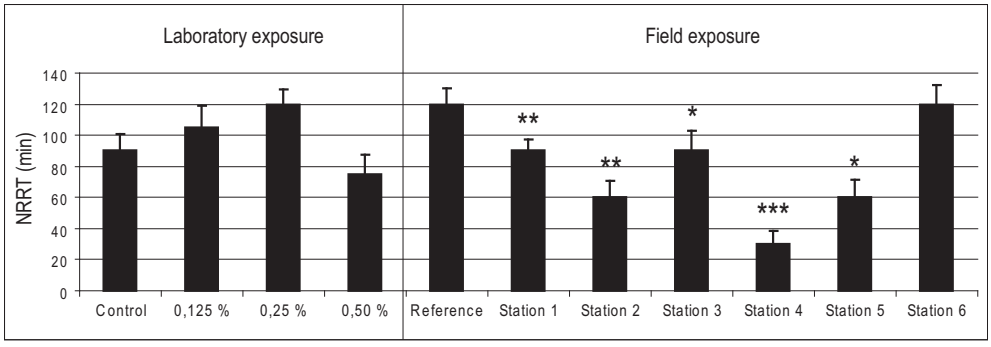

Fig. 2. Lysosomal membrane stability in mussel haemocytes as neutral red retention time (NRRT). Results are presented as median + standard error ($n = 16$ for laboratory exposure, $n = 15$ for field study). Data from exposed groups were compared with the control in the laboratory exposure and results from mussels from different stations were compared with the reference one (Reference) in the field study (Wilcoxon rank-sum $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

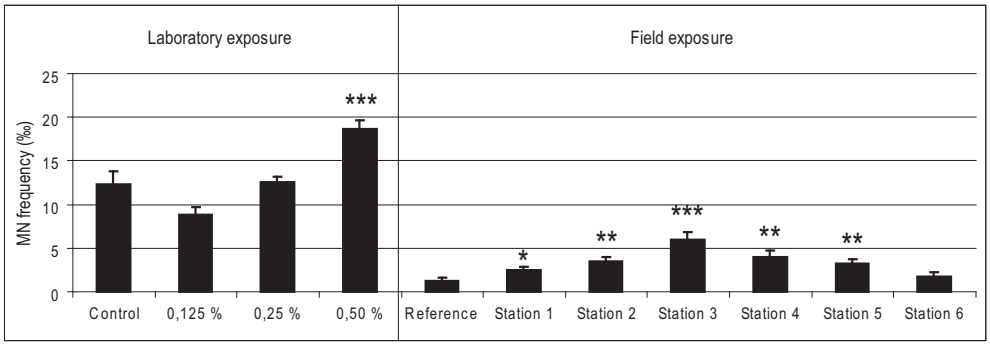

Fig. 3. Micronuclei (MN) frequency in mussel haemocytes. Results are presented as median + standard error ($n = 15–16$ for laboratory exposure, $n = 12–14$ for field study). Data from exposed groups were compared with the control in the laboratory exposure and results from mussels from different stations were compared with the reference one (Reference) in the field study (Wilcoxon rank-sum, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

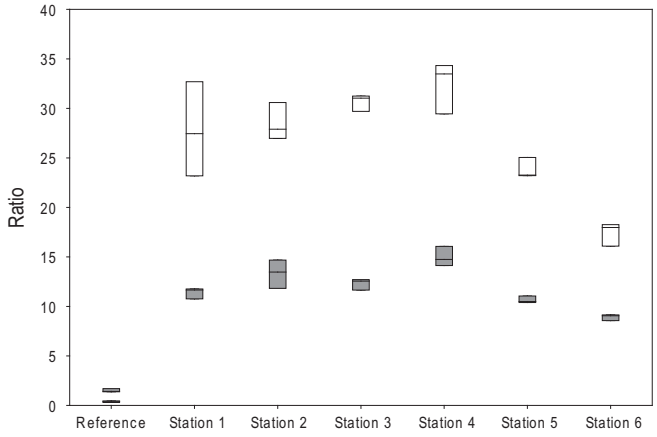

Fig. 4. The ratio of alkylated (C_1, C_2 and C_3; the latter only for naphthalenes) over non-alkylated (parent) polycyclic aromatic hydrocarbons in blue mussels caged at the indicated field stations. Open boxes: naphthalenes. Filled boxes: phenanthrenes/anthracenes. The graph displays median and range $n = 3$.

19 and 17.5 m at station 2 and 5, respectively), showing a typical North Sea spring situation. Measurements of the current during the exposure confirmed a predominantly tidal driven current running along an axis stretching SW–NE and that the cages were deployed in the path of the discharge plume.

As regards to bioaccumulation data, 16 PAHs showed increased values due to the exposure, while 8 PAHs were below the quantification limit (Table 1). All mussels caged in the platform area had higher levels of PAHs in the soft tissue compared to mussels caged at the reference station. The highest values were found in mussels from stations 2, 3 and 4, the closest to the PW discharge. In general, the PAH-level in mussels from the field exposure were lower than the ones from the laboratory exposure. The PAH body burdens in mussels from the laboratory exposure and the field study are both reported in Table 1.

The ratio between alkylated-compounds and parent compounds was calculated to confirm the chemical nature of the pollution source. A higher ratio of alkylated- to parent-compound was found in mussels caged close to the produced water discharge (most evident in stations 2, 3 and 4, Fig. 4).

The observed LMS response in mussel haemocytes from the reference station was within the range of values usually observed for animals in unexposed areas (mean value 120 min). Mean values recorded in mussels caged at stations 1, 2, 4 and 5 were significantly lower than values from reference mussels (Fig. 2). A mathematical correlation (polynomial regression of second degree) between LMS and body burden is shown in Fig. 5, a polynomial regression was found with $R^2 = 0.91$ (station 4 mean was not considered). The relationship between LMS and PAH body burden was calculated without taking into account the results obtained in mussels from the closest station to the PW discharge (station 4). Station 4 was clearly an outlier (as calculated by the Cook D test, data not reported). Including this value, the polynomial regression resulted in a $R^2 = 0.70$.

The frequency of MN in the reference station was 1.24‰, and 50% of specimens did not possess micronucleated haemocytes (Fig. 3). All groups from the Ekofisk area (except station 6, 2 km from the discharge) showed a more than 2-fold increase in MN level compared to mussels from the reference station. Moreover, all mussels caged in the proximity of the platform had a frequency significantly higher than the one in mussels from the reference station. In addition the stations situated closest to the discharge (stations 2, 3 and 4) differed from other groups by their heterogeneity in responses. The frequency of micronuclei also showed a gradient with distance to the discharge similar to the levels of PAH measured in mussel soft tissues. The linear correlation between these two parameters is shown in Fig. 5 ($R^2 = 0.84$).

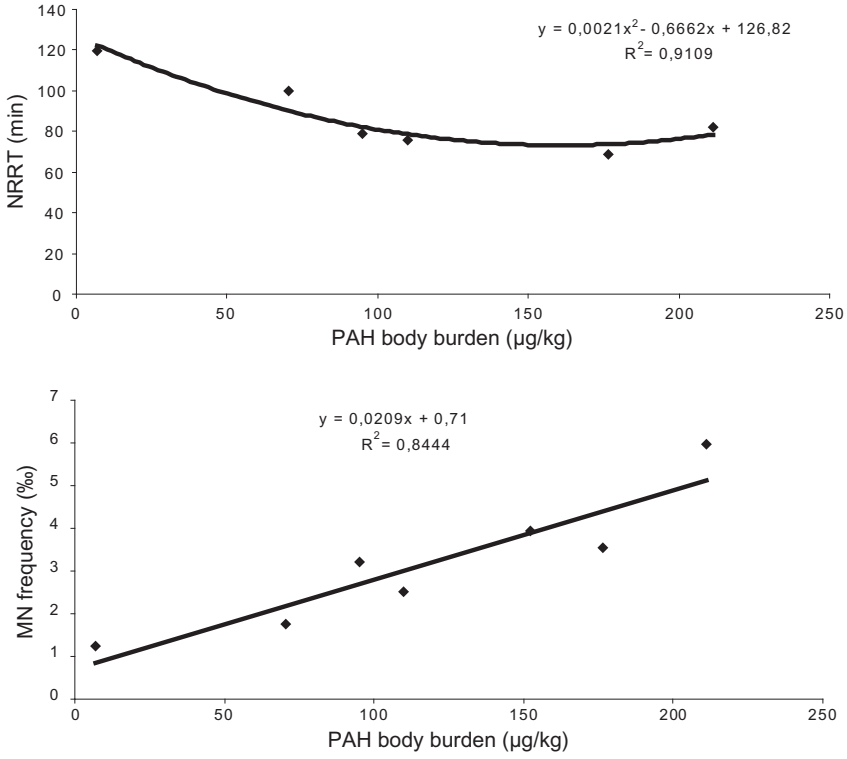

Fig. 5. Correlation between chemical (bioaccumulation) and biological (lysosomal membrane stability assay (LMS) and micronuclei (MN) assay) markers in the field validation study.

4. Discussion

The first objective of the present study was to perform a field-realistic laboratory PW exposure with mussels. PW is a chemically instable mixture due to evaporation of volatile compounds, and chemical and microbial degradation of labile fractions makes mimicking field exposure conditions challenging. The logistics necessary for transportation and storage that secures low degradation of compounds in the relatively large amounts of PW needed for a flow-through exposure system is also demanding. PW dilutions used in the laboratory study were selected based on results from modelling discharge chemistry and dilution. In the present study, measured concentrations of PAH in the exposure tanks were lower than predicted from the modelling. Several factors e.g. the actual availability of the organic pollutants may vary significantly between lab and field. One such factor may be differences in the density of micro algae between laboratory and field conditions affecting the bioavailability of lipophilic compounds (Schrap, 1991). Another obvious difference between the laboratory- and field studies is the continuous exposure concentration the mussels experience in laboratory and the fluctuating exposure in the field. In addition the complex horizontal and vertical discharge distribution pattern may be significantly influenced by changes in local oceanographic conditions. Compared to the current situation at many other offshore platform sites in the North Sea, the circumpolar current system in the Ekofisk area counteracts efficient advection of the discharge out of the area. This situation increases the suitability of the area for biomarker assessment because the probability that the discharge plume will actually hit the cages is high.

The generally very high recipient dilution factor at offshore sites reduces high-concentration-effects of toxic chemicals oil production areas. Nevertheless, research continues in an effort to detect possible ecological risks related to PW discharges (Gorbi et al., 2008). In Norway, environmental monitoring programs have been implemented. Such programs address and follow up documentation of putative "no negative impacts" of PW regarding the biological health and long term sustainability of the offshore marine environment. In the Norwegian WCM, both blue mussels and fish have been used for transplant caging studies in the mixing plume of major PW discharges in the North Sea (Hylland et al., 2008).

Mussels are suitable for caging studies for a wide range of reasons. They endure transportation and are efficient filter feeders, self-sufficient with food during an exposure. Since mussels have the potential to accumulate large amounts of pollutants both as a result of filter feeding behaviour and the relatively low metabolic degradation of organic pollutants, bioaccumulation (as body burden in soft tissue) represents a good marker of PAH exposure in monitoring programs (Baumard et al., 1999; Baussant et al., 2009).

4.1. Chemical markers

The laboratory exposure results showed the expected dose-dependent accumulation of PAHs in mussel soft body tissue. The observed PAH composition was different in seawater and biota samples as expected, due to extensive differences in the different compound's physiochemical properties and therefore bioaccumulation potential. Levels of some of the high molecular weight PAHs (compounds with 5 and 6-membered rings and high octanol:water partition coefficients; K_{OW}s) were not detected in the water samples, but found in quantifiable amounts in the biota samples.

The bioaccumulation of PAHs in mussel soft tissue confirmed that caging stations were exposed to the discharge plume. Total PAH content was higher in mussels caged around the PW discharge compared to the control, and the accumulation pattern followed a gradient with distance to the discharge, with higher values at the closest stations (stations 2, 3 and 4). The total amounts of EPA 16-PAHs varied between 9.52 μg/kg (station 6) and 20.48 μg/kg (station 3), similar values have been found in mussels caged in the vicinity of other Norwegian platforms (Røe Utvik et al., 1999; Hylland et al., 2008).

PAH compounds in a petrogenic exposure often contain one or more methyl- ethyl- or butyl-(and sometimes higher alkyl-) groups on one or more of the aromatic carbons (Neff, 2002). Generally, the abundance of these alkyl substituted PAHs is higher than for the parent compounds (the non-alkylated homologues). Furthermore, the lighter (especially naphthalenes and phenanthrenes) are more abundant than the heavier (more rings) compounds (Latimer and Zheng, 2003). The ratio between alkyl-compounds and the corresponding parent compounds was therefore used to confirm the nature of the pollution source in the field study. It has previously been shown that mussels caged down-stream of PW discharges from oil platforms accumulate higher concentrations of alkylnaphthalenes, alkylphenanthrenes and alkyldibenzothiophenes, than their respective parent compounds (Ruus et al., 2006). In the present field study, the ratio of alkylated over non-alkylated PAHs indicated a diffuse petrogenic contamination in the Ekofisk area (up to 2000 m from PW fallout); a higher ratio was found in mussels caged close to the PW discharge. A possible explanation for that situation may be that the most volatile compounds escape to the atmosphere before the discharge reaches the cages.

The present study shows that the chemical markers are useful for confirming exposure of the animals to PW. Particularly 2- and 3-ring PAH components present in small amounts in the seawater are bioaccumulated following the expected gradient with distance to the discharge along the current axis in the area. This demonstrates the effectiveness of mussels as sentinel organisms also in offshore PW monitoring. A petrogenic source of exposure was verified, allowing the correlation between presence of pollutants and biomaker results.

4.2. Biological markers

In addition to the traditional chemical approach, inclusion of a battery of biomarkers is recommended in environmental monitoring programs (Cajaraville et al., 2000; Moore et al., 2004a; Viarengo et al., 2007). Biomarkers are becoming part of the health assessment and management of aquatic ecosystems. Defining protocols and standard procedures for monitoring programs is an ongoing process and should be based on previous experience (Gorbi et al., 2008; Hylland et al., 2008).

At the cellular level, LMS (assayed by NRRT) is an easy to perform and low cost test to detect impairments of the functional integrity of cells (Livingstone et al., 2000; Moore et al., 2006). Biomarker based risk assessment is often limited by a lack of connection between marker levels and effect data. However, for LMS (as NRRT assay) threshold levels have been defined (ICES/OSPAR, 2009). Mussels are considered to be healthy if the NRRT value is above 120 min, stressed but capable of compensating it if the value is between 120 min and 50 min and severely stressed and probably exhibiting pathology if the value is below 50 min. In the laboratory exposure study, there were no statistical differences between values of control and exposed mussels. The mean value recorded for laboratory control animals was 102 min. This unexpectedly low value indicates a stress condition in mussels, according to the ICES/OSPAR threshold levels, even if the particular source of stress in this laboratory exposure was not identified. Control values above 120 min were reported in previous studies, even if no difference between control and treated groups was found in mussels exposed in the laboratory to mechanically dispersed crude oil (Baussant et al., 2009).

The suitability of this assay towards operational discharges from oilfields was elucidated in the field study. LMS values were comparable in the field and in the laboratory study, median values ranging from 30 to 120 min. Mussels from four out of six stations had LMS values significantly lower than the control animals, showing an effect of PW on the general health status of mussels caged in the platform near field, as previously demonstrated (Gorbi et al., 2008). A gradient was also identified with the lowest values in mussels from stations closest to the platform. According to the ICES/OSPAR threshold levels (ICES/OSPAR, 2009), mussels from the reference station did not display severe health effects; all animals caged around the PW discharge showed a stress condition but were probably capable of compensating, while mussels from only one station (station 4) were severely stressed.

These results are not in agreement with previous data from other platform surveys, where the use of NRRT was considered not adequate for North Sea offshore monitoring (Hylland et al., 2008). These could possibly be due to different chemical composition/concentration of PW in the previous studies, showing the challenges in this type of field study. However, present results support the use of LMS assay in the future WCM.

Alternative techniques for scoring LMS that may have several advantages particularly under field conditions have been suggested (Dondero et al., 2006; Sforzini et al., 2008). The histochemical procedure for LMS determination in frozen samples of mussel digestive gland (Moore et al., 2004b) is also an alternative for offshore monitoring programs, where the field conditions such as a moving vessel may be a limiting factor.

LMS has also been positively correlated with some markers (TOSC, protein synthesis and scope for growth) and negatively with others (MN frequency, lysosomal swelling, PAH and metal body burden; Moore et al., 2006). A significant negative correlation between LMS and PAH body burden in mussel soft tissues was observed, in agreement with previous studies (Moore et al., 2006).

The frequency of MN is regarded as an important tool for *in situ* monitoring of genotoxicity. This assay has been used for various organic and inorganic pollutants in laboratory studies as well as in field studies (Bolognesi et al., 1996; Koukouzika and Dimitriadis, 2005; Baršienė et al., 2006) and it has been recommended for offshore biomonitoring (Baršienė et al., 2006; Gorbi et al., 2008). The test involves scoring of cells containing one or more cytoplasmatic micronuclei in addition to the main nucleus. This biomarker shows a continuously increasing trend in animals exposed to increasing concentrations of pollutant and exposure times (Viarengo et al., 2007). In the present laboratory exposure study, increasing values were found following the increased concentration of PW in the exposure tanks and significantly higher MN frequency was seen in mussels exposed to 0.5% PW. The MN level in the control group was considerably higher than the level observed in the field reference. The reason for this difference is not fully understood, but physical or biological stress in the tank environment or differences in the two mussel batches physiological condition could be an explanation (Gorbi et al., 2008). Mussels in the laboratory exposure were not fed during the exposure. The deep water used (from 80 m depth) most likely did not contain as many food particles as the water closer to the surface where mussels were caged. Availability of food and spawning status may affect baseline levels of the biomarkers (Gorbi et al., 2008).

In the field study, a significant increase in MN frequency was seen in mussels caged at all stations except one (station 6) in the PW discharge area. The highest value was found in mussels caged at the closest station (station 3); these animals also showed the highest accumulated levels of PAHs. A correlation between MN frequency and PAH contamination has previously been observed (Bolognesi et al., 1996; Baršienė et al., 2006) and it has been demonstrated that mussels caged in a PAH contaminated harbour site displayed a continuous increase in MN frequency reaching a plateau after 1 month of exposure (Bolognesi et al., 1996). Genotoxicity parameters were linked with presence of contaminants as well as behavioural and physiological biomarkers (Canty et al., 2009; Rybakovas et al., 2009), in the present study a positive linear correlation was found between MN frequency and PAH body burden.

The data are also in agreement with a previous WCM survey from 2004, and levels of MN frequencies are comparable (Hylland et al., 2008). In addition, this parameter was the only one out of 18 biomarkers altered in a previous investigation of impact of an offshore platform in the Adriatic Sea (Gorbi et al., 2008). The analysis of genotoxicity is considered important since it provides a fundamental early warning sign of adverse long-term effects of contaminants at population and furthermore at ecosystem levels (Rybakovas et al., 2009).

Genotoxicity of contaminants from the petroleum industry has been shown earlier in mussels (Bolognesi et al., 1996; Baršienė et al., 2006; Gorbi et al., 2008; Hylland et al., 2008; Rybakovas et al., 2009). The results of the present study on validation of genotoxicity for the PW assessment in laboratory and in field conditions especially emphasized the positive correlation between the response and most of 16 PAH body burden levels. Thus MN was proven to be very sensitive as biomarker of stress in mussels and is recommended for detecting genotoxicity in offshore monitoring activity.

5. Conclusions

The combination of laboratory and field studies allowed assessment of the selected chemical (PAH body burden) and biological (LMS and MN) markers. PAH body burden analyses were useful to identify mussels exposed to PW in both experiments and provide additional information as compared to the more traditional chemical analysis of the seawater (especially for DBTs and 4, 5 and 6 rings PAHs). Moreover, the quantification of PAHs allowed a correlation between the presence of pollutants and the biomarker responses.

Both LMS and MN were capable of identifying a gradient from the PW discharge in the field study, demonstrating them to be sensitive tools for studying the effect of PW on mussels.

This study supports the concept of a 2-tier approach that it is therefore suggested for future offshore biomonitoring programs. A relatively wide screening of stations around the platform area can be performed using LMS assay as a base evaluator of mussel health condition. In the reference area the use of PAH bioaccumulation data and LMS is suggested to ensure good reference values. In the stations characterised by low level of LMS, the application of a full battery of biomarkers (6–8 biomarkers) is suggested, including MN which represents an important endpoint to evaluate the potential genotoxic effect of the PW discharge.

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