Multi-xenobiotic resistance (MXR) transporters and biotransformation enzymes in the blue mussel Mytilus edulis

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Site-specific expression of detoxification genes related to multi-xenobiotic resistance (MXR) in *Mytilus edulis*

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ABSTRACT

Multixenobiotic resistance (MXR) -related proteins form a first line of defense in marine organisms (phase 0 detoxification) by the elimination of xenobiotics to prevent their accumulation in cytoplasm and organelles such as the nucleus. Expression of MXR-related genes were examined in the blue mussel, *Mytilus edulis*, in habitats of different structure (rocky shore, free water column) to analyse whether differences in ecotypes affect gene-expression responses to hazardous substances (benzo[a]pyrene, cycloheximide, rifampicine and 2-acetylaminofluorene). Furthermore, the value of MXR-related gene expression as biomarker of pollution effects was investigated in various fjord sites in Norway. Expression of P-glycoprotein, major vault protein and multidrug resistance-related protein as phase 0 proteins, heat shock protein 70 as general stress protein and topoisomerase II as indicator of cell proliferation were analysed in gill and digestive gland at the transcriptional level. The phase 0/III genes showed specific inducibility by chemicals during experimental exposure in relation to habitat structure. Mussels from the rocky shore appeared to be less responsive as reflected by low inducibility of MXR transcripts than mussels attached to buoys living under more constant conditions. During the Norwegian field campaign, site-specific differences in the expression of MXR-related genes were detected. It appeared that in non-tidal habitats, contamination with polyaromatic hydrocarbons inhibited MXR-related protection in digestive glands whereas contamination with copper induced MXR-related mechanisms. Our study indicates that regulation patterns of MXR-related gene expression, either inhibition or induction, can be used as biomarker of pollution effects. However, sampling strategies for biomonitoring programs must take into account differences in sensitivity of mussel populations in relation to habitat-specific adaptations to obtain comparable sets of data.

KEYWORDS: Habitat-specific gene expression, multixenobiotic resistance (MXR), P-glycoprotein (P-gp), major vault protein (MVP), multidrug resistance-related protein (MRP), heat shock protein 70 (HSP70), *Mytilus edulis*

INTRODUCTION

Blue mussel (*M. edulis*) and other marine filter-feeding molluscs are widely used in laboratory and field experiments as sensitive indicators for anthropogenic contamination. On the basis of their capacity to accumulate xenobiotics and toxic compounds and their wide geographic distribution over coastal marine environments, mussels are useful sentinel organisms for biomonitoring effects of chemical contamination. Moreover, mussels are commercially-important organisms as wild catches and in aquafarming. Mussels living in habitats of different ecological conditions display distinct physiological adaptations. Some physiological parameters, such as growth rate, respiration, and clearance rate (Dickie et al. 1984, Stirling & Okumus 1994, Sukhotin & Maximovich 1994, Labarta et al. 1997, Reimer & Harms-Ringdahl 2001) are determined by specific environmental conditions and are reversed when these environmental conditions change. Other adaptations, such as assimilation efficiency, energy partitioning and ammonia excretion (Dickie et al. 1984, Rodhouse et al. 1984, Mallet et al. 1990, Stirling & Okumus 1994, Labarta et al. 1997) are maintained for prolonged periods after changes in the environment. This suggests genetic differences between populations or an 'ecological memory' of individuals with respect to pre-experimental conditions driven by circadian rhythms, for example.

The aim of the present study was to investigate whether site- and habitat-specific differences are detectable by RT-PCR at the level of gene expression in tissues of *M. edulis*. On the basis of these results, recommendations for the application of RT-PCR studies in biological effect monitoring are made. We selected 3 genes for our study, P-glycoprotein (P-gp), an ATP-driven transmembrane efflux pump of 170 kDa, the multidrug resistance-related protein (MRP), a 190 kDa protein, and the lung resistance protein (LRP) which is the major vault protein (MVP), that has a molecular weight of 110 kDa. P-gp is a transmembrane protein mainly present in the plasma membrane, with a secondary localisation in the Golgi apparatus (Molinari et al. 2002). It is involved in excretion of many types of lipophilic compounds across biological membranes. P-gp acts in normal tissues as a protective mechanism against xenobiotics and as transporter of endogenous substrates.

MRP contains 2 ATP-binding domains and 3 membrane-spanning domains. It is expressed at a basal level in a wide variety of tissues, including epithelial cells and hematopoietic cells, suggesting a function common to most cell types. As plasma-membrane drug-efflux pump,

MRP contributes to a multidrug resistance (MDR) phenotype (Zaman et al. 1994, Barrand et al. 1997).

MVP is associated with 2 high-molecular weight proteins p240 and p193 around a small 140 nucleotides-long RNA species located in the cytoplasm in nucleoprotein complexes, called vaults. A small fraction (approx. 5%) is located in the nuclear envelope and nuclear pores. Vaults mediate bidirectional nucleocytoplasmic transport of a wide range of substrates, including cytotoxic drugs. Vaults may be involved in both vesicular and cytosolic transport of molecules (Izquierdo et al. 1996).

These 3 proteins are members of the phase 0 or phase III detoxification system. Besides their capacity to export conjugated metabolites (phase III), these transporters can act as a first line of defence by preventing drugs and xenobiotics from accumulation in the cytoplasm or nucleus (phase 0). The proteins have been identified in cancer cells, where they provide resistance to a broad spectrum of structurally- and functionally-unrelated drugs, thus causing the phenomenon known as multidrug resistance (MDR) (Endicott & Ling 1989, Gottesman et al. 1996). A MDR-like system called MXR (multixenobiotic resistance) has been identified in marine invertebrates which protects organisms against a large variety of xenobiotics (Galgani et al. 1996, Eufemia & Epel 1998, Britvic & Kurelec 1999, Bard 2000, Eufemia 2000, Smital et al. 2000) thus enabling survival in polluted environments.

In the present study, we investigated the effects of habitat structure and life history of mussel populations on MXR-related gene expression induced by anthropogenic chemicals. In a series of experiments, we compared regulation of transcription of MXR-related genes in mussels obtained from different habitats, a rocky shore and traffic buoys, to investigate the effects of prolonged exposure to variations in environmental factors related to the tide such as oxygen supply, nutrient supply, temperature fluctuations, wave impacts and predator pressure. On the basis of the results of the habitat-specific responses, sampling protocols were defined. Mussels were also investigated in Norwegian fjords at differently-contaminated sites and at reference sites. On the basis of these experiments, the usefulness of MXR-related gene expression as biomarker was established.

MATERIAL AND METHODS

Animal handling.

Mussels of the species *M. edulis* were collected on or near the German off-shore island Helgoland and along the Norwegian south west coast near Stavanger (Fig. 1). All mussels were approx. 5 cm in shell length. Mussels from Helgoland were kept in a seawater circuit at

15°C for 3 days without feeding before RNA extraction. Tissues of Norwegian mussels were dissected directly after harvesting the mussels, frozen in liquid nitrogen and stored at –80°C until RNA extraction.

Isolation of total RNA.

The digestive gland and gills were isolated from the mussels and total RNA was prepared from all samples immediately after isolation using 1 ml of RNApure reagent (peqlab, Erlangen, Germany) according to the manufacturer's specification. An additional purification step with the High Pure RNA Tissue Kit (Roche, Mannheim, Germany) was performed to remove any remaining contamination of polysaccharides and to improve RNA quality. The amount of total RNA was determined by UV spectroscopy. Contamination with polysaccharides or proteins was detected on the basis of absorbance values at 260, 280 and 320 nm. Integrity of RNA was investigated on 1.5% agarose gels.

RT-PCR.

Total RNA concentrations were adjusted to 0.5 µg/µl followed by an additional quantification control using non-denaturing gel electrophoresis. For RT-PCR, reverse transcription was performed with 1 µg of DNase-treated total RNA with 20 U mMulv reverse transcriptase (Life Technologies, Karlsruhe, Germany), 50 pmol oligo-dT primer and 1mM of dATP, dGTP, dCTP and dTTP (dNTPs) (Sigma, Taufkirchen, Germany) each in a total volume of 30 µl.

PCR samples (10 µl) were analysed at cycle 26, 28 and 30 by gel electrophoresis to ensure that all mRNAs investigated were still in the exponential phase of amplification. Amplification was performed for 28 sequential cycles at 95 °C for 30 sec, at 60 °C for 60 sec, at 72 °C for 60 sec followed by a final 3-min extension at 72 °C. Equal aliquots of each PCR reaction were separated on 2% agarose gels using Tris-borate buffer and photographed after ethidium bromide staining. Gels were analysed with Digital Science 1D Image analysis software (Kodak, Rochester NY, USA). Band net intensity was measured for gene expression level analysis. For semi-quantitative RT-PCR, the following primer pairs were used: ***mrp2*** sense AAA GAC GGA CTG GAT CAC CA and antisense AAA TTG GTC GGT GAG TCG AA, 296 bp, concentrations in PCR, 0.1 µM each; ***pgp*** sense CAG AGG TTC TAT GAC CCA GAT GCA G and antisense GTT CTC ACT CTC AGA GTC TAA TGC AG, 381 bp, concentrations in PCR, 0.1 µM each; ***topoII*** sense CTT CTC TGA TAT GGA CAA ACA TAA GAT TCC and antisense GGA CTG TGG GAC AAC AGG ACA ATA C, 664 bp,

concentrations in PCR, 0.2 µM each; ***mvp (lrp)*** sense ACA GGT TGT AAC TCC CTT G and antisense CTT CAT GAT GAC CTC GAC C, 818 bp, concentrations in PCR, 0.1 µM each; ***hsp70*** sense GAC TTG GGT GGT GGA AC and antisense GGC TAC AGC TTC ATC AGG G, 516 bp, concentrations in the PCR, 0.1 µM each.

We selected genes that did not express a difference between control and test experiments or exposed sites and reference sites as internal controls in the gels. The histograms are based on absorbance measurements of net band intensity. Bars were calculated as derivation from the average value of control animals.

Sampling at Helgoland.

Site A: traffic buoys -Hogstean, -Sellebrun-W, -Nathurn-N; sampling date: 15.01.03. Mussels from these three buoys were pooled in the experiments. Site B: tidal area called 'Felswatt', sampling date: 15.01.03. Salinity (33 ppt) and water temperature (3.2°C) was similar at all sites.

Biometric data.

Thickness, length, width and height of the shells were measured using a 0.1 mm precision calliper. After the biometric measurements, the wet weight of meat and shells were determined as a measure of fitness of mussels (MWW and SWW, respectively). Condition index was calculated as: CI = (MWW/SWW) x 10.

Habitat-specific gene induction.

Exposure to a mixture of 3 chemicals.

Benzo[a]pyrene (B(a)P) is a polycyclic aromatic hydrocarbon (PAH) and occurs generally as a complex mixture and not as a single compound. PAHs are primarily by-products of incomplete combustion. B(a)P, along with other PAHs, is suspected to cause cancer in humans and rodents. Because of its lipophilicity and volatility it is bioaccumulative, it is not degraded easily in the environment, and can be spread by air (Sticha et al. 2002, Boysen & Hecht 2003).

Cycloheximide is an odorless white crystalline powder used in hospitals and research laboratories as antibiotic, inhibitor of protein synthesis, or plant-growth regulator. Furthermore, cycloheximide has had a broad agricultural use as fungicide, but this has been prohibited because cycloheximide cause birth defects in animal (Morgan 1982).

Rifampicin is an antibiotic that is used primarily for prophylaxis or treatment of tuberculosis. However, it interacts with contraceptive steroids. Irregular bleeding and pregnancies have

been reported among women concurrently using rifampicin and oral contraceptives (Bolt et al. 1974, Bolt et al. 1977).

Exposure to single inducers.

2-Acetylaminofluorene (AAF) is frequently used by biochemists as a positive control in the study of liver detoxifying enzymes and in studies of carcinogenic and mutagenic effects of aromatic amines (Sparfel et al. 2002). Exposure of rats to AAF in their diet induces tumors in the liver, bladder, zymbal gland (in the ear canal), colon, lung, pancreas, and testis.

Experimental conditions of induction experiments.

In the first experiment, dimethylsulfoxide (DMSO) was used as solvent for the mixture of chemicals whereas AAF was dissolved in vegetable oil since its solubility in DMSO was too low. The exposure time was 3 days at a temperature of 15°C in seawater without water exchange. Mussels from both sites were incubated together in one aquarium with constant air supply. Total concentrations of the chemicals were 0.25 mM B[a]P, 25 µM cycloheximide, 20 µM rifampicine and 200 µM AAF.

Sites and dates of sampling in fjords near Stavanger (Norway).

Mussels for the field study in Norwegian fjords were sampled at non-tidal sites similar to the mussels collected from buoys near Helgoland. For a geographical map of the sampling sites, see Fig. 1. Site **NA**: Salvøy (reference), 17.09.01; site **NB**: Visnes (metal), 18.09.01; site **NC**: Førlandsfjorden (reference), 20.09.01; site **ND**: Alvestad (reference), 19.09.01; site **NE**: Høgevarde (PAH), 21.09.01; site **NF**: Bukkøv (organic), 22.09.01. Salinity at all sites was 29.70-33.05 ppt and water temperature ranged between 14.2°C and 15.4°C.

Description of sampling sites.

NA (Salvøy) is an outer reference site on the west side of Karmøy. The site has a very good water circulation and exchange with the North Atlantic. The site is slightly affected by small fishing boat traffic, and there is a small cod farm close to the site. **NB** (Visnes) is a very exposed site on the west side of Karmøy. The site is affected by an old copper mine which was in production from 1865 to 1894, and was reopened for a few years until 1965. Both, tailings and slag was dumped into the sea. Mainly copper, zinc and iron were introduced to the seawater and sediment from fillings and the run off from land. No sign of marine life was evident in areas close to the old mine. **NC** (Førlandfjorden) is an extremely sheltered fjord in

the inner part of the fjord system. There is hardly any human activity in the fjord, except for some minor boat traffic and some small farms that drain to the fjord. However, there is a mussel farm located in the fjord, and the production of mussels was high along the shores of the fjord. **ND** (Alvestad) is a reference site in the exposed part of the fjord system with appropriate water exchange. It is not affected directly, but ship traffic and salmon farms are present at distance of the site. **NE** (Høgevarde) is the site just north of the discharge point from an aluminium smelter in Karmsund. Karmsund is a sheltered straight with appropriate water exchange. Karmsund is affected by heavy ship traffic and it has been exposed to tributyltin (TBT). The volume of PAHs discharges from the aluminium smelter into the sea was 0.6 tons in 2001. The factory also discharges 19 tons per year of biocide (C-Treat 6 = N-trimethylendiamine acetate) used in the seawater pipe system to prevent fouling. **NF** (Bukkøy) is north of Høgevarde in Karmsund and is close to a factory that produces alginates from kelp. The site has the same general environmental conditions as Høgevarde, and is seriously affected by PAHs. The factory discharged into the sea 6700 tons of kelp residues and 90 tons of formaldehyde in 2001.

RESULTS

Induction experiments.

Mussels from buoys showed a distinctly-different morphological phenotype than mussels from the rocky shore. Mussels from the rocky shore 'Felswatt' had a 7-fold thicker shell than mussels from the buoys. The condition index of buoy mussels was 4.5±1.1 and that of mussels from the 'Felswatt' 2.7±0.9 (Table 1). Mussels from the rocky shore had a 58% higher meat content but due to a 20% increased height it results in a similar free intra-shell volume in the mussels from both sites.

During the first induction experiment, we used a mixture of 3 chemicals, B[a]P (0.25 mM), cycloheximide (25 µM) and rifampicine (20 µM) as a single dosage. After a 3-day incubation period, *pgp* gene expression was induced in digestive glands of mussels from buoys whereas mussels from the rocky shore showed no response under these experimental conditions (Fig. 2). Alterations in gene expression levels of the other genes *(mrp2, hsp70* and *topoII*) were not detected.

In a second experiment, a single dosage of 200 µM AAF was used. Again, site-specific inducibility of gene expression was observed. *Mrp2* expression was induced in mussels from the buoys. They showed induction of *mrp2* gene expression in digestive glands by a factor of 2.5 and in gill by a factor of 3 following incubation with AAF, whereas *mrp2* gene expression was not significantly induced in digestive gland and gill of mussels from the rocky shore.

Alterations in gene expression levels of the other genes *(pgp, hsp70* and *topoII)* were not detected. In gill, inter-individual variance in *mrp2* gene expression levels was high in mussels from the rocky shore, as compared with mussels from buoys (Fig. 3).

As marker of mitotic activity, *topoII* gene expression was compared in control mussels from both sites. Expression of *topoII* gene was significantly different in the two sites. Mussels from the traffic buoys showed a higher *topoII* expression and a lower inter-individual variance than mussels from the rocky shore (Fig. 4).

Gene expression in mussels in differently-contaminated fjord sites.

Site-specific expression of 3 MXR-related genes *pgp*, *mvp* and *mrp2* was studied in mussels collected in Norway. Sampling sites were within an area of 30 km^2 where salinity and water temperature were similar (Fig. 1). The mussels were not exposed to the tide and experienced water exchange as mussels collected from buoys in the induction experiment. The sites were selected because of their specific point source contamination. The 3 genes investigated showed site-specific expression patterns at 3 sites. Expression levels of *pgp* in digestive gland were virtually abolished at sites Høgevarde (PAH) and Bukkøv (organic) (Fig. 5). *Mvp* expression was reduced in digestive gland at the same sites (Fig. 6). Alterations in *pgp* and *mvp* expression levels were not found in gill tissue in these sites. *Mrp2* expression was increased by a factor 2 in digestive gland tissue of mussels sampled at sites Visnes (metal) and Alvestad (reference) (Fig. 7). In gill, *mrp2* expression was elevated in Visnes by a factor 2 but not in Alvestad (data not shown). In reference sites Salvøy and Førlandsfjorden, expression of the investigated genes was similar in both gills and digestive glands.

As control for the specificity of gene induction, we investigated *hsp70* as general marker of cellular stress. No significant induction or repression of expression of *hsp70* was detectable in any site. Expression of *hsp70* showed a low inter-individual variation that ranged between 14% and 38% within individuals and sites (data not shown).

DISCUSSION

Within any particular region, mussel patch structure and dynamics differ among habitat types because of environmental differences influencing the intensity and outcome of biological interactions and metabolic pathways. The aim of the present study was to evaluate the use of the expression of MXR-related genes as biomarker in field studies. Habitat conditions are well-known as a factor which influence physiological responses like assimilation efficiency, mortality rate, energy partitioning, ammonia excretion, respiration rate, clearance rate, growth

rate and anaerobiosis (Seed & Suchanek 1992, Sukhotin & Poertner 1999). In this context, information is essential whether differences in expression levels of genes in mussel populations at different sampling sites are primarily modulated by habitat conditions such as substrate availability, food acquisition and periodical anaerobiosis or whether these expression levels are exclusively responses to acute contamination in the habitat of mussels.

In order to answer the question whether inducibility of gene expression is an effect of site-specific environmental parameters, we investigated the inducibility of genes encoding the major drug transporter proteins, *pgp* and *mrp2*, in mussels from two habitats near the German island Helgoland. These experiments demonstrate that levels of transcriptional activity of 'first line of defense' genes of the investigated mussel population in response to specific chemicals may strongly depend on the environmental conditions of a specific habitat. For identification of these habitat-specific populations in future experiments, we investigated morphological data of the mussels. These data showed that mussels from the rocky shore develop a different phenotype as compared with buoy mussels. Rocky shore mussels develop a stronger shell and have a higher meat content. Although mussels from the rocky shore display an increased mantle volume, intra-shell free volume is identical in both populations due to a different shape of the shell. Free intra-shell volume is an important issue since this volume serves as a reservoir for oxygen and for metabolic end-products during shell closure. In the field, mussels of these two populations can easily be identified on the basis of their morphological parameters.

Exposure of the two morphologically-different ecotypes to various inducers of xenobiotic transport resulted in habitat-specific transcriptional activation of MXR-genes. *Pgp* gene expression in mussels from the buoy habitat was inducible in digestive gland by a mixture of 3 chemicals within 3 days of exposure whereas mussels from the tidal habitat showed no induction. A comparable result of *mrp2* gene expression was obtained with AAF. AAF exposure induced gene expression in gill tissue and digestive gland, especially in buoy mussels. In order to analyse the observed effect in more detail a general marker of mitotic activity, the expression of *topoII,* was measured in the digestive gland of control mussels from both habitats. Mussels from buoys showed significantly higher expression of this gene as compared with animals that were exposed to the tide.

The habitat-specific inducibility of gene expression may be caused by the availability of ATP in mussels from these habitats. In mussels, ATP generation during anaerobiosis is reduced to 10% as compared with aerobic conditions. Protein synthesis which forms the basis of metabolic adaptations is energetically expensive. Mussels from a non-tidal habitat are able to

generate ATP constantly since prolonged shell closure is not necessary. Therefore, they can afford to respond more sensitively to changes in the environment because of the availability of ATP (Branch et al. 1979). Mussels in a tidal habitat express a reduced cellular metabolism and protein turnover because of the limited availability of ATP (Brown et al. 1976, Hawkins et al. 1989, Hawkins & Day 1996). These physiological conditions may result in a limited ability to adapt to environmental changes. Extended periods of anaerobic metabolism, the increased need for byssus threats to cope with wave impacts (Raubenheimer & Cook 1990), elevated ATP demands for muscle exercise (foot movement, shell closure) and the need for a thicker shell due to predator pressure may have induced these metabolic adaptations. Mechanisms like this may affect ecological adaptation or selection processes by differences in transcriptional activity in response to external stimuli as observed in the two ecotypes of mussel in the present study. Whether this is also the case in other habitats such as the Wadden Sea or in estuaries has to be investigated in further studies. Results from our induction experiments indicate that sampling in identical habitats only allows comparison of the effects of contamination at the level of gene expression. For valid analysis of habitat-specific adaptation or selection, and therefore site-specific gene expression responses to pollution in different habitat types, a strategy using additionally caged mussels rather than using local catches from the wild population alone is advised.

The observed differences in transcriptional activity of genes related with detoxification and/or biotransformation may have consequences when mussels at different habitats are exposed to toxic compounds. In mussels with low inducibility of MXR genes, chemicals may accumulate in tissues and induce damage to cells such as DNA damage or protein inactivation before the barrier function of MXR-related proteins is effective.

On the basis of the data obtained with the use of mussels from buoys and rocky shore, the strategy for a sampling expedition in Norway was defined. All mussels tested in the field study in Norway were not affected by the tide or the impact of waves and, therefore, are considered to be responsive with respect to gene expression of the MXR system. In the context of biomonitoring, these mussels are in general recommended as better indicators of pollution for gene expression-based studies. The applicability of the RT-PCR-based analysis of gene expression was investigated in a field study at differently-contaminated sites and reference sites within the south-west Norway fjords. Our results demonstrate that expression levels of the investigated MXR-related genes are detectable. We found reduced levels of *pgp* gene expression in digestive gland at contaminated sites Høgevarde (PAH) and Bukkøv (organic). Reduced *mvp* expression was also found in digestive gland at these sites. Both sites

are exposed to comparable contamination as they are located in the same area and water exchange occur between the 2 sites. Therefore, it is not surprising that gene expression at these sites is affected in a similar manner. Extracts of river water from polluted sites were found to inhibit P-gp activity at the protein level in a culture of NIH 3T3 mouse fibroblasts and in some marine invertebrates (Smital & Kurelec 1997, 1998). Besides posttranslational down-regulation, reduced phase 0 activity can be the consequence of inhibitory effects at the transcriptional level. Chemicals like PAHs causing such an effect are known as 'chemosensitizers' because they inhibit the defence mechanisms of marine organisms against a broad spectrum of chemicals (Kurelec 1995).

The digestive gland is the main site of biotransformation in *M. edulis* and hepatic metabolic activation of carcinogens, such as B[a]P, lead to increased toxcicity and the production of reactive metabolites (Prescott 1983). This might explain the tissue-specific gene expression pattern of *pgp* and *mvp* at sites Høgevarde and Bukkøv. In contrast to the inhibition of *pgp* and *mvp* at the PAH-affected site, induction of *mrp2* gene expression was detectable at the sites Visnes (metal) and Alvestad (reference). Alvestad was considered originally as a reference site and data of a specific contamination are not available but during sampling a thin oil layer on the water surface was observed in this site. Therefore, Alvestad cannot be considered as reference site. Visnes is highly contaminated by heavy metals as described in material and methods part. A protective effect of a *mrp* homologue to metals has been described in the literature in the nematode *C. elegans* (Broeks et al. 1996). In this study, targeted inactivation of *mrp-1* in *C. elegans* resulted in increased sensitivity to heavy metal ions to which wild-type worms are highly tolerant. The most pronounced effect of the *mrp-1* mutation affects the ability of animals to recover from temporary exposure to high concentrations of heavy metals (Broeks et al. 1996). Whether this mechanism also accounts for *M. edulis* has to be verified in further experiments. *Mrp2* gene induction was tissue specific in the two sites. Elevated levels of *mrp2* mRNA were detected in both tissues investigated at the site Visnes, but gene expression only occurred in digestive gland at the site Alvestad. This implies two different ways of action of the toxic chemicals. At Visnes (heavy metals), the regulatory effect may be exerted directly by the contaminant whereas at Alvestad (doubted reference site) hepatic biotransformation may occur in digestive gland that produces reactive metabolites causing the observed effects.

On the basis of our data, we conclude that toxicogenetic analysis is practicable in principle in field studies. This conclusion is made because, first, gene expression was detectable with our approach, and some genes showed similar expression levels within all sites, second, two

reference sites showed average expression of the genes investigated and, third, gene expression of *pgp*, *mvp* and *mrp* was significantly altered at some of the point source contaminated sites whereas sites with similar contamination showed similar gene expression patterns.

Our findings of pollution-related responses of expression levels of MXR-related genes at different sampling sites with individual contamination status demonstrate the future applications of gene expression arrays in the assessment of biological effects of pollution even in marine field situations.

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Biometric data	Rocky shore	Traffic buoys
Shell wet weight (g)	7.2	2.5
Meat wet weight (g)	1.9	1.2
Length (cm)	4.3	4.3
Width (cm)	2	2.2
Height (cm)	2	1.65
Volume (ml)	4.4	4.4
Shell thickness (mm)	1.4	0.2
Condition index	2.7±0.9	4.5±1.1

Table. 1. Biometric data of mussels collected in two habitats at the German off-shore island Helgoland, rocky shore and traffic buoys. Mussels from the rocky shore underwent tidal variations (n=20) and those from the traffic buoys (n=20) did not. Inter-individual variation in the two groups was less than 20%. The condition index is a measure of meat weight versus shell weight.

Fig. 1

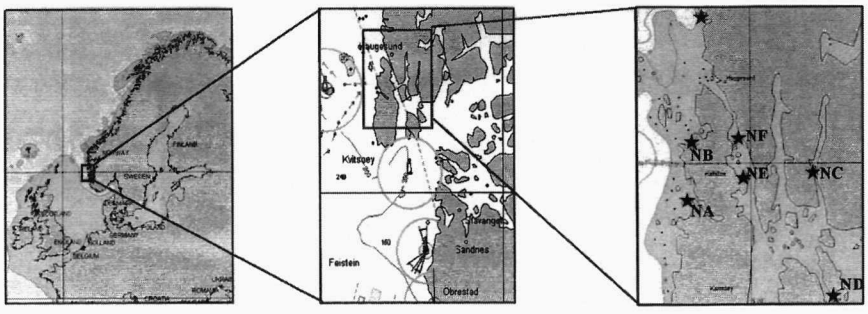


Fig. 2

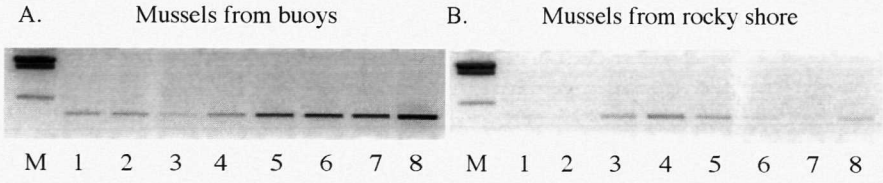

A.
Mussels from buoys
B.
Mussels from rocky shore
M 1 2 3 4 5 6 7 8
M 1 2 3 4 5 6 7 8

Fig. 3

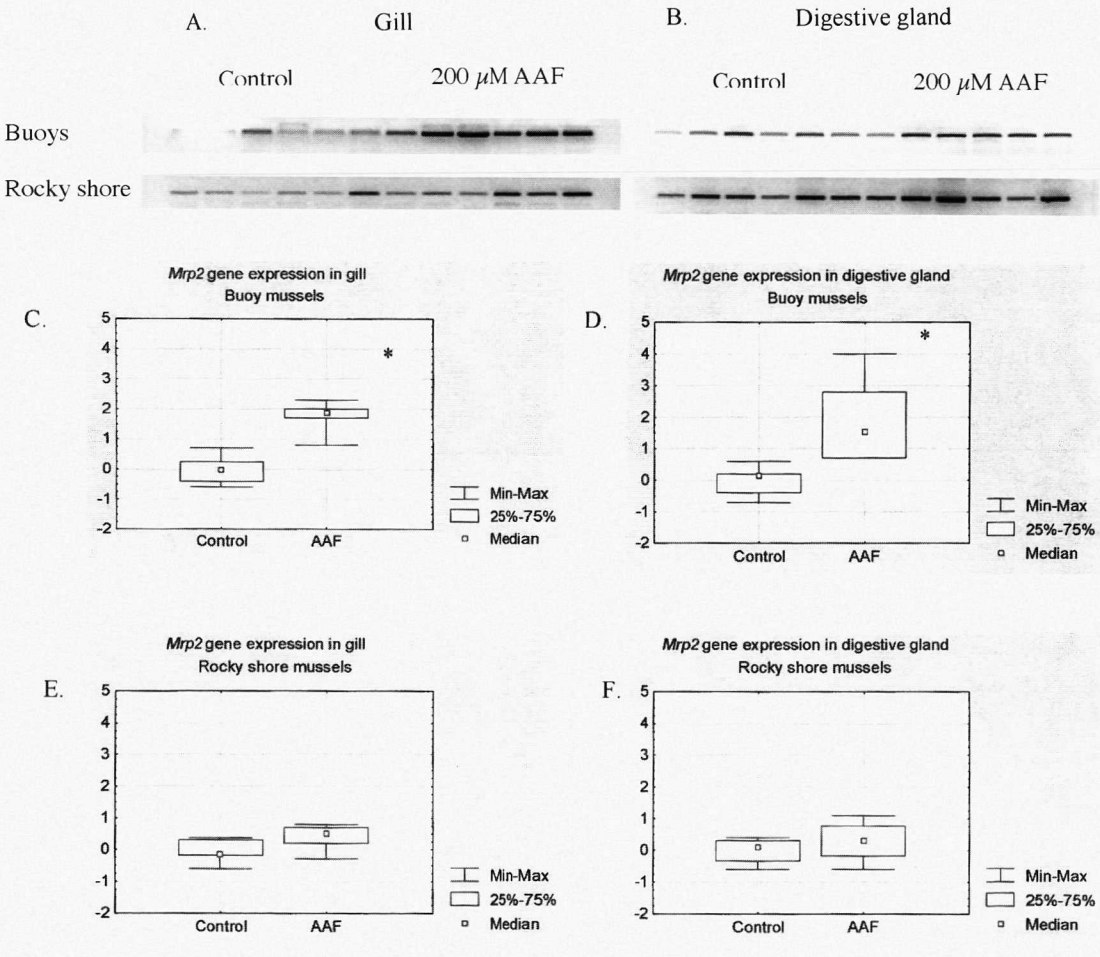

A.
Gill
B.
Digestive gland
Control
200 μM AAF
Control
200 μM AAF
Buoys
Rocky shore
C.
Mrp2 gene expression in gill
Buoy mussels
D.
Mrp2 gene expression in digestive gland
Buoy mussels
E.
Mrp2 gene expression in gill
Rocky shore mussels
F.
Mrp2 gene expression in digestive gland
Rocky shore mussels
Control
AAF
Min-Max
25%-75%
Median

Fig. 4

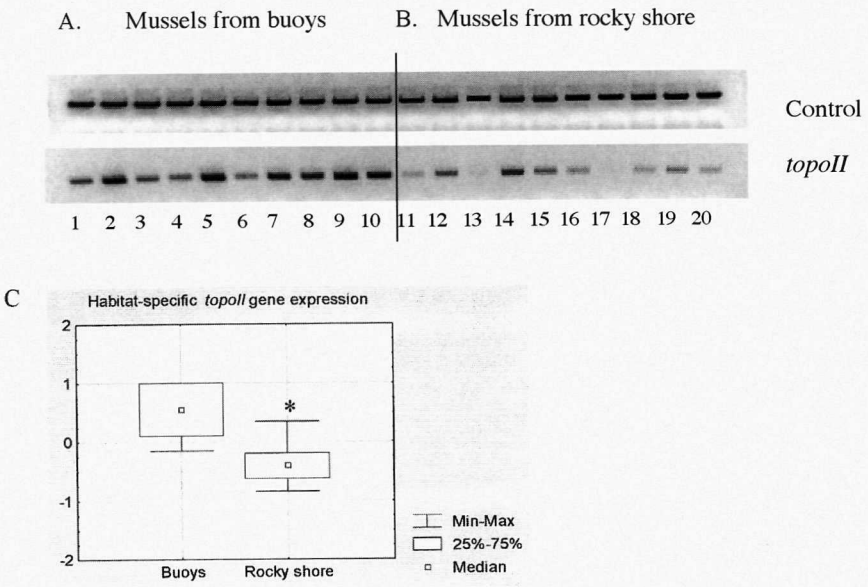

A. Mussels from buoys
B. Mussels from rocky shore
Control
topoII
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
C
Habitat-specific topoII gene expression
2
1
0
-1
-2
Buoys
Rocky shore
Min-Max
25%-75%
Median

Fig. 5

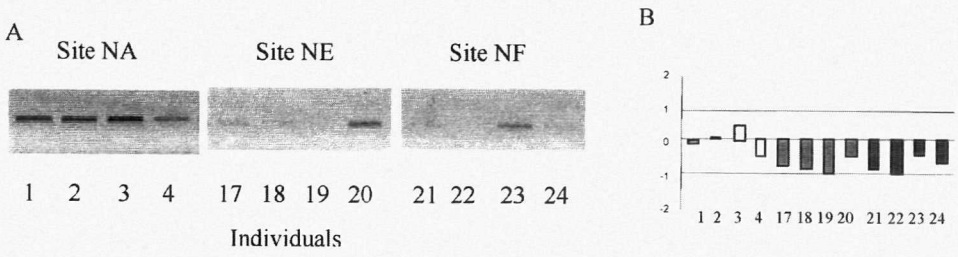


Fig. 6

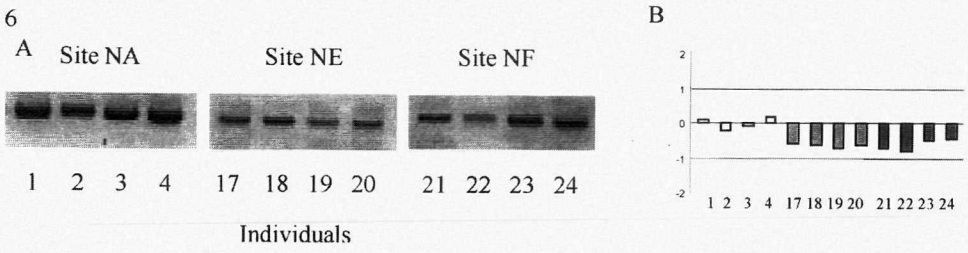
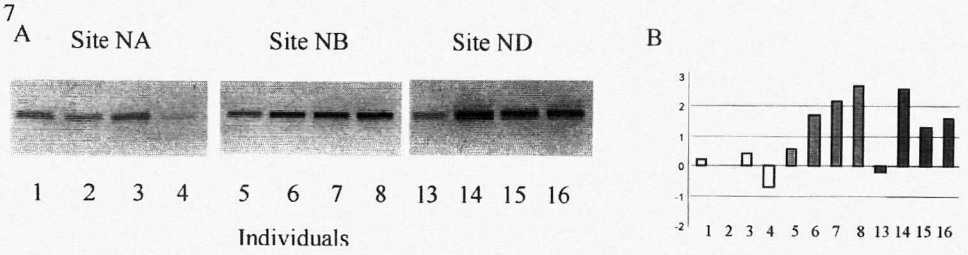


Fig. 7



Legends

Fig. 1. Geographical map of sampling sites of *M. edulis* in the field study in Norwegian fjords.

Fig. 2. Habitat-specific inducibility of *pgp* gene expression in digestive gland of *M. edulis*. Mussels were sampled from buoys (A) or rocky shore (B). Numbers indicate individual mussels within the two habitats (sampling code). 1-4, Control, 5-8, mussels exposed to a mixture of 3 chemicals. M, molecular weight marker.

Fig. 3. Habitat-specific inducibility of *mrp2* gene expression in gill (A) and digestive gland (B) of *M. edulis*. Mussels were sampled from buoys or rocky shore. Box-wisker graphs (C-F) were calculated on the basis of deviations in individual gene expression levels from the mean value of control animals. Significant differences with $p<0.05$ are marked with *.

Fig. 4. Expression of *topoII* gene in control mussels from buoys (A) and rocky shore (B). As control, *hsp70* gene expression was selected. Numbers indicate individual mussels: 1-10, from buoys thus not affected by tidal changes and 11-20, mussels from rocky shore affected by tidal changes. Box-wisker graphs (C) were calculated on the basis of deviations in individual gene expression levels from the mean value of control animals. Significant differences with $p<0.05$ are marked with *.

Fig 5. Site-specific expression of the *pgp* gene. A. Site NA: Salvøy (reference); site NE: Høgevarde (PAH); site NF: Bukkøv (organic). Numbers represent individual mussels (sampling code). B. Diagram showing deviations in gene expression in individual mussels from the mean value of control animals.

Fig. 6. Site-specific expression of the *mvp* gene. A. Site NA: Salvøy (reference); site NE: Høgevarde (PAH); site NF: Bukkøv (organic). Numbers represent individual mussels (sampling code). B. Diagram showing deviations in gene expression in individual mussels from the mean value of control animals.

Fig. 7. Site-specific expression of *mrp2* gene. A. Site NA: Salvøy (reference); site NB: Visnes (metal); site ND: Alvestad ('reference'). Numbers represent individual mussels (sampling

code). B. Diagram showing deviations in gene expression in individual mussels from the mean value of control animals.