Multi-xenobiotic resistance (MXR) transporters and biotransformation enzymes in the blue mussel Mytilus edulis
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Citation for published version (APA):
Chapter II

Scope of the thesis

Introduction.
Accumulation of toxic chemical contaminants in marine organisms, including those consumed by humans, is a well-documented fact. The monitoring of hazardous levels of contaminants and variations in their geographic and temporal distribution is relevant for assessing and maintaining the health of the marine environment and the safety of seafood consumption. Chemical analysis in biota, water and sediments is sensitive and specific, but is expensive and provides little information on the biological effects of contaminants and their interactions. Furthermore, protocols of chemical analysis are limited to known chemicals. Newly-introduced chemicals often remain undetected. Biomonitoring, on the other hand, can be used to establish effects of contaminants in marine animals irrespective of their identity. Therefore, biomonitoring has potential for routine monitoring of effects of contaminants on marine biotopes. Biomonitoring needs sentinel species as is described in Chapter I and the blue mussel is an excellent species for this purpose.

Selection of relevant genes for biomonitoring.
There is a need for new biomarkers to assess environmental quality. Commonly-used biomarkers such as lysosomal membrane stability, induction of CYP450 enzymes, MT levels, peroxisomal proliferation, inhibition of AChE, occurrence of imposex and altered levels of pollutant-specific and -unspecific proteins applied to mussels often show a narrow specificity for a class of pollutants such as MTs that indicate exposure to metal contaminants only. Imposex is not observed in mussels yet and levels of the members of CYP450 family (phase II of detoxification) are too low to be used as a biomarker in mussels. Lysosomal enlargement and destabilisation is a well-established biomarker in mussels but is an integrative marker of general cell dysfunction and pathology and is therefore only applied in concert with contamination-specific biomarkers.

Implementation of new techniques may broaden the spectrum of the biomarker approach. Toxicogenomics, for example, could be a promising tool to improve assessment of potential toxicity at the genetic level. However, only few studies to date have used gene expression profiling in the field of marine invertebrate toxicology and were mainly focussed on the CYP450 system and MTs (Hahn et al. 1993, Wootton et al. 1996, Barsyte et al. 1999), the mussel byssal adhesive system (Coyne & Waite 1425, Inoue & Odo 1994, Coyne & Waite 2000, Watabe et al. 2000, Waite & Qin 2001) or the nervous system (Favrel & Mathieu 1996, 24
Several groups of genes encoding proteins such as DNA-repair proteins, heat shock proteins or proteins with physiological key functions may be used for a toxicogenetic approach. DNA microarrays for parallel detection of different genes can circumvent some of the conventional biomarker problems, but sequence data for the blue mussel are still rare. Therefore, the general practicability of toxicogenetics in the field of marine biomonitoring is doubtful before sequencing projects have been started.

In the present thesis, studies of first-line-of-defense proteins are described. These so-called phase 0 proteins have a fundamental role in the protection of cells from accumulation of xenobiotics. Detoxification systems are highly conserved within the animal kingdom. In general, they consist of 4 phases. Phase 0 proteins hamper their substrates (contaminants) from accumulation in cells or organelles such as the nucleus. Phase I proteins that mainly consist of the CYP450 proteins chemically reduce their substrates. Reduction of lipophilic xenobiotics by CYP450 results either in more polar metabolites, which are easily excreted directly or converted into a chemically more reactive molecule which is a better substrate for phase II enzymes. Phase II enzyme activities catalyse the conjugation of xenobiotics to endogenous substrates such as GSH. The most prominent members of phase II proteins are GSTs. Subsequently, the conjugated compounds are substrate for phase III proteins that transports them out of the cells. Following this definition P-gp and MVP can be considered as phase 0 enzymes while MRP is part of both phase 0 and phase III detoxification. GST \( \pi \) is a member of phase II of detoxification.

Products of the genes investigated in the present thesis protect cells against effects of different classes of chemicals before they can be destructive in cells. In contrast to repair enzymes, these proteins act before damage to the cell occurs. In other words, they provide an active barrier function such as for example the blood-brain barrier. Therefore, these proteins have a fundamental function in the homoeostasis of cellular metabolism. Like many other detoxification proteins and repair proteins, they are highly conserved, not only in their amino acid sequence but also with respect to their substrate specificity. For example, identical substrates and inhibitors are used in clinical chemistry and ecotoxicology for assays of the activity of these enzymes. This is not surprising since these proteins have maintained their function at the cellular level from bacteria to man. The present thesis describes a molecular biology approach to analyse expression of members of detoxification proteins in the blue mussel, \( M. \text{edulis} \). \( M. \text{edulis} \) is considered to be a good model organism for ecotoxicological studies as described in Chapter 1 not only because marine molluscs are the second species-
rich phylum after the arthropods but they can be used as well as sentinel for other marine phyla. Since mussels are located centrally in the marine food web, toxic substances that accumulate in mussel tissue also accumulate in higher organisms such as fish and birds. Therefore, the detoxification capacity of mussels is likely to have impact on accumulation of toxins in predator organisms including human consumers.

Toxicogenetics as a new tool in ecotoxicology.

The principle of toxicogenomics is based on the fact that patterns of gene expression are characteristic for particular classes of toxicants, for example PAHs versus peroxisomal proliferators. The approach of toxicogenomics is based on the assumption that responses to toxic chemicals are reflected by changes in gene expression. Thus, the analysis of altered gene expression in response to a toxicant may be a direct measure of toxicity. There are at least 80,000 compounds that are currently used commercially such as drugs, cosmetics and other chemicals, but only a minor part has been tested for toxicity so far. This deficiency is due to the high costs and the time-consuming procedures of animal testing. Toxicogenetics may accelerate investigations of the biological effects of chemicals and keep up with the invention and release of new chemicals. The first step in this novel approach is to determine whether chemicals with unknown biological effects affect gene expression patterns in a similar way as known classes of toxicants.

To investigate whether toxicogenetics is useful in marine ecotoxicological studies, we need to estimate the feasibility of toxicogenetic methods. Standards must be well-defined with respect to environmental factors and experimental conditions. Moreover, species-specific gene expression in the different tissues of an organism, sex-specific gene regulation, interfering effects such as reproductive cycles, environmental parameters and site-specific adaptations must be known because all these factors may interfere with the effects of pollutants or modify the effects at the level of gene expression.

The aim of the present thesis was to determine whether toxicogenetics can play a role in biomarkers research in the future. *M. edulis* was used as a model organism and the study was focused in particular on yet unknown phase 0, I, II and III biotransformation proteins. Limitations of the toxicogenetic approach are described as well and recommendations are given for the direction of future research. Moreover, the usefulness of primary cell cultures in the toxicogenetic approach is discussed.
Chapter II

The approach to address the aim of the study was structured in the following way:

1. Selection of an indicator organism (sentinel species) suitable for the toxicogenetic approach: Chapter 1
2. Assessment of genes that are involved in detoxification and biotransformation processes in marine invertebrates: Chapters 1, 2
3. Identification of genes of detoxification and biotransformation proteins that are transcriptionally active in *M. edulis*: Chapters 3, 4
4. Verification of the identity and characterisation of relevant genes: Chapters 3, 4
5. Determination of tissue-specific expression of the relevant genes and assembly of a multiplex RT-PCR: Chapters 3, 4
6. Optimisation of total RNA isolations from different tissues of *M. edulis*: Chapters 3, 4, 5, 6
7. Determination of seasonal differences in expression of relevant genes: Chapters 3, 4
8. Analysis of gene expression patterns in relation to the reproductive cycle: Chapter 3
9. Use of explants as an alternative for cell culture-based applications: Chapter 3
10. Determination of environmental parameters that may effect gene expression: Chapter 5
11. Analysis of site-specific expression of genes in laboratory experiments and under field conditions: Chapter 6
12. Analysis of the usefulness of toxicogenetics in environmental research using *M. edulis* as intact organism: Chapter 7

Detailed presentation of the chapters.

Bivalves are widely used as sentinels for monitoring effects of pollution of the environment. These sessile filter-feeding organisms take up and concentrate contaminants to levels well above those present in the environment, thus providing information on the specific local pollution situation. In 1975, a monitoring strategy, 'The Mussel Watch' programme, was proposed for four types of pollutants that challenge the quality of marine waters: artificial radionuclides, petroleum hydrocarbons, chlorinated hydrocarbons and metals (Goldberg 1975). Since then, toxicological studies have focused on the blue mussel *M. edulis* and their subspecies. Since *M. edulis* is the best-investigated marine invertebrate to date, we have selected *M. edulis* as subject for our studies.
A large number of genes are involved or related to detoxification and biotransformation processes. Therefore, we focus on MXR-related genes because these genes are well studied in mammals due to their importance in the treatment of cancer. Furthermore, there are indications that these genes are involved in detoxification and biotransformation processes in aquatic organisms. The aim of the thesis was to identify at least one member of each class of MXR-related genes that is inducible and therefore its expression may be regulated in response to exposure to xenobiotics. We selected 5 genes on the basis of reports in the literature: P-gp, MVP, MRP, GST pi and CYP450 4A. Additionally, we selected actin, TOPOII and HSP 70 as putative internal standards. To identify transcripts of the genes in *M. edulis*, we decided to apply a degenerated primer-based approach. Total RNA was isolated and different primer combinations were investigated with respect to their ability to amplify a defined fragment of the genes. At least one member of each gene family was identified by this experimental design. Followed by a first characterisation of the genes in *silico*, Northern blots were used to evaluate the total length of the putative mRNAs. Afterwards, tissue-specific expression was investigated in muscle, gill, mantle tissue and digestive gland of *M. edulis*

Furthermore, we investigated the inducibility of expression of these genes since only genes which expression is regulated by toxicants can be used in gene-expression profiling related to toxicant exposure. Since a major aim of the present study was to set up a system that can also be used under field conditions, tissue-specific expression was determined and its variation in relation to sex as well as seasonal variations in the reproductive cycle (Chapters 3 and 4). We also analysed the option to replace animals by tissue explants in experiments in Chapter 3.

After characterisation of gene expression under standardised laboratory conditions, we analysed the effects of natural environmental factors (salinity, temperature and anaerobiosis) on gene expression which have to be taken into consideration before field studies can be performed. The open sea is a relatively stable system but considerable changes in temperature, salinity and oxygen levels occur especially in coastal regions as a result of the tidal cycle and seasonal changes. Previous studies that focussed on protein levels and their activity suggested that these parameters had effects on P-gp (Eufemia 2000, Keppler & Ringwood 2001), GST (Suteau et al. 1985, Vidal et al. 2002) and the CYP450 system (Sole et al. 1995). We limited our study to differences based on sampling sites rather than differences based on seasonal changes (Chapter 5).

In addition to environmental parameters, the metabolic condition of mussels in a certain habitat may be the result of specific adaptations (Dietz & Somero 1992, Goldspink 1995,
Bucher et al. 1996, Lau et al. 2001). Especially, sessile organisms like the blue mussel are known to express a ‘habitat-defined metabolic adaptation’ to site-specific conditions such as mechanical stress due to their exposure to the impact of waves or anaerobiosis during the low tide (Sukhotin & Poertner 1999). To investigate possible toxic/environmental effects at the level of gene expression in response to chemical contaminants, we collected mussels from different sites that were exposed to the same water current but differed in the exposure to tidal cycles. Mussels were sampled in the so-called ‘Felswatt’, a typical intertidal rocky shore habitat around the German off-shore island Helgoland. Moreover, mussels were collected from traffic buoys around the island, which were positioned within 2 km from the ‘Felswatt’ sampling site. In contrast to mussels from the ‘Felswatt’, traffic buoy mussels are not exposed to anaerobic conditions and the impact of waves is low as the buoys follow water movements. Differences in inducibility of MRP-related protein and P-gp expression were investigated in these two habitats (Chapter 6).

Furthermore, we performed field sampling at sites in a Norway Fjord system that addresses the question of the practicability of gene expression analysis in field samples. Aim of this approach was to investigate whether differences in expression levels of the genes that we selected were detectable at the level of individuals as a result of a specific contamination situation. Therefore, sampling sites were selected on the basis of known contaminant impact and uncontaminated sites as reference sites. Sites were strongly influenced by copper from closed mines or by actual input from an aluminium smelter and kelp factory (PAHs and formaldehyde). Gill and digestive gland were investigated for their use in field studies (Chapter 6).

Finally, in Chapter 7 results are summarised and discussed with respect to the usefulness of MXR-related gene expression studies as a biomarker of environmental pollution. Based on our data, recommendations are given for future studies.
Cited literature


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