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

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General introduction

During the past 30 years, interest in environmental problems caused by pollution with xenobiotics has grown rapidly. Because of this interest, a new field in science has been developed, ecotoxicology. Xenobiotics are chemicals that are foreign, useless or even hazardous to biological systems and include industrial chemicals and pesticides. Natural compounds may also be toxic to aquatic life when too large quantities are introduced into the environment by disturbances of the ecological equilibrium. Politicians are continuously engaged to create a legislative and administrative body with the intention to protect the environment against increasing pollution by human activities. However, industries are generally some strides ahead of this legislative intention, and every year new chemicals appear in the environment with unknown effects on marine life. Facing this situation, the environmental manager needs rapid answers with respect to potential environmental impacts of a broad range of chemicals.

A particularly difficult problem is the analysis of effects of thousands of chemicals in a complex environment and their interaction at different levels of the biological organisation. The introduction and application of biomarkers is a development in ecotoxicology which has the potential to solve at least some of the problems of the environmental manager. Biomarkers are defined as tools that are employed either to measure exposure to pollutants or to measure deleterious effects of environmental contamination in biological samples. Measurement of characteristic cellular responses in an organism to a specific change in its environment can provide the evidence necessary to identify the presence or absence of such a change. This approach is progressing rapidly because of the availability of novel techniques in molecular biology, cell biology and biochemistry to measure deleterious effects of chemicals in challenged marine biotopes.

The presence of toxic compounds in the environment can be detected by chemical analysis of water and sediment samples, but this approach gives little information about the bioavailability and the effects on biological systems of these toxic chemicals. Analytical chemistry of water or sediments does not provide information on concentrations of pollutants in organisms. The use of biomarkers is a strategy that enables the detection of alterations in the metabolism of organisms in relation with accumulation of pollutants in the whole organism, its tissues and cells. The impact of this approach is also based on the fact that cells are an intermediate level of complexity in-between molecular events and events at the level of
the organism. Consequently, changes in cells may reflect what occurs at higher levels of biological organisation.

Metabolic changes in cells are considered to be early biomarkers of pollution (Shugart et al. 1990, Shugart et al. 1992). Two types of biomarkers are distinguished: biomarkers of exposure and biomarkers of effects. Biomarkers of exposure indicate changes at the cellular or molecular level that reflect exposure of the organism to contaminants such as induction of biotransformation enzymes to protect the cells. Biomarkers of effects indicate changes at the cellular or molecular level that reflect pathological responses of the organism to a toxic contaminant. These biomarkers can both be specific (specific for one chemical) or general (non-specific within a range of chemicals). An important feature of these biomarkers is that they can be employed in a prognostic way, thus allowing the development of strategies before irreversible environmental damage takes place with ecological consequences at higher levels of the biological organisation (population, ecosystem). Therefore, validation of biomarkers under experimental conditions in the laboratory and in the field are necessary to define the impact of pollutants.

The selection of appropriate sentinel species as bioindicators is of utmost importance in ecotoxicology. A sentinel species is an organism that accumulates pollutants in its tissues. At best the bioindicator is able to survive in its polluted habitat. Moreover, it should occupy an important position in an ecosystem and thus, it has to integrate the effects of contaminants on that ecosystem.

Marine mussels are now widely used as sentinel organisms in the so-called “Mussel-Watch” biomonitoring programmes due to their ability to accumulate pollutants. Bivalves (oysters, clams and in particular mussels) have been found to be useful as indicators and integrators of exposure and effects of contaminants because of their:

1.- wide geographic distribution,
2.- presence in coastal and estuarine communities,
3.- role in accumulating pollutants in their cells and tissues as filter-feeder,
4.- ability to respond to environmental pollution whereas stress due to handling of bivalves can be easily controlled
5.- economic importance as a protein source for humans,
6.- sessile behaviour, reflecting directly the contaminant situation of their habitat,
7.- high population density, and
8.- ease to be transplanted and maintained in cages at sites of interest.
‘White mouse’ of the sea, the blue mussel.

*Mytilus edulis*, the blue mussel, is often considered as the “white mouse” of marine biologists. Its geographic distribution includes the Mediterranean Sea, the North Pacific, the North Atlantic Ocean up to the Arctic. It is common in littoral to sublittoral zones down to 100 m depth but it is also found in deeper zones down to a depth of 500 m. It can easily be collected and maintained in the laboratory. The blue mussel is an active suspension feeder as planktotrophic larva and as adult, filtering mainly phytoplankton from the water. The blue mussel predominantly inhabits shores and estuarine environments. These habitats are the most complex habitats of all marine systems with respect to variations in temperature, salinity, duration of exposure to air and food supply. To cope with these stress factors, the blue mussel has developed a series of behavioural, physiological and metabolic adaptations. Blue mussels tolerate low temperatures for extended periods, for example, they are frozen in ice for up to 8 months each year in Labrador (Seed 1976). The upper temperature limit is approx. 29 °C with a LD$_{50}$ of 350 h. The blue mussel is an euryhaline species that can handle successfully both oceanic salinity (34 ppt) and mesohaline estuarine conditions (2-18 ppt). Blue mussels are osmoconforming, that means that the osmotic pressure of the intracellular fluids is kept iso-osmotic with the environment by adjustment of the intracellular concentration of free amino acids (Bayne et al. 1976a, Bayne et al. 1976b, Bayne & Livingstone 1977, Bayne et al. 1977). They lack oxygen-transporting pigments in their blood. During periods of shell closure, tissues of the animal are subject to hypoxic conditions and energy is supplied by anaerobic metabolic pathways (Gabbott 1976). Joergensen has described the mussel as an autonomous unit, incapable of regulation of its metabolism, so that temporal variations of ‘automatized’ physiological processes occurs solely in direct response to environmental factors (Joergensen 1990). However, a compelling body of evidence confirms that mussels act as homeostatic systems, responding to environmental changes by modulating their metabolism, physiology and/or morphology in order to compensate for reduction in performance (Shakhmatova et al. 1991, Gainey 1992, Sukhotin & Poertner 2001).

In the present study, we focus on four tissues of the blue mussel: the mantle, gills, the posterior adductor muscle and the digestive gland (Fig. 1 and 2). The mantle is multifunctional. The mantle contains the larger part of the gonads and the ventral mantle margin comprises the inner, middle and outer folds. The outer and middle folds are small, the outer fold is responsible for the production of shell and periostracum, the middle fold is
muscular and consists mainly of the pallial retractor muscle, that retracts the mantle margin when the shell valves are closed. The inner fold consists of mucus-secreting cells and the larger part of the gonads. In our studies, we focused on the large inner mantle fold.

The gills, more correctly referred to as ctenidia, are typical of Mytiloideae. They are flat, homorhabdic, nonplicate and filibranch. The ctenidia function in both respiration and feeding. Ctenidia comprise of a pair of demibranches, an inner and an outer branch, which divide the pallial cavity into inhalant and exhalant chambers.

The posterior adductor muscle enables the mussel to close the shell as a defence mechanism against predation and to withstand exposure to air during tidal cycles, to low salinities and to toxicant exposure. The muscle consists of ‘fast’ striated and ‘slow’ nonstriated fibres for phasic and sustained contraction.

The digestive gland or digestive diverticula is the site of intracellular digestion. There are four openings of the digestive diverticula at the right side of the stomach, all separated from each other. In the stomach, food particles are selected for intracellular digestion in the digestive diverticula. Three ciliated sorting areas are located in the left pouch of the stomach. The food-sorting caecum and the apertures of the digestive diverticula at the right side of the stomach form the gateways for food for intracellular digestion.

Fig. 1. Blue mussel *M. edulis*. The posterior adductor muscle has been dissected to open the shells.
Molecular biology applied to unravel cellular processes in marine species.

Molecular biology is a ‘novel’ tool, that may provide a new dimension to ecotoxicology. Molecular biology techniques offer the possibility to investigate the structure, functional organisation, and regulation of cellular processes and expression of nucleic acid and proteins. Molecular biology techniques enable characterisation of a species and delineates it from other species on the basis of its molecular adaptation to a specific environment. With the use of molecular biology, marine scientists can address questions such as:

Do two or more morphologically-similar organisms belong to the same species, to different species or represent ecotypes? Does an organism contain genes encoding enzymes that are important to mediate biochemical transformation or detoxification of particular compounds? If so, are the genes constitutively expressed or are they inducible, and if the latter is the case, under which conditions?

The pathway leading to the synthesis of a gene product is a multi-step process with many potential points of control, of which transcription rate has been viewed as the primary mechanism in both pro- and eukaryotes. Therefore, the reverse transcription-polymerase chain reaction (RT-PCR) technique has been used in the studies described in this thesis to detect relative levels of a set of mRNA species occurring in tissue samples. Despite variation in RNA degradation during sample handling and storage, relative amounts of these templates can be expected to remain fairly constant. Homologous mRNA templates are likely to be subjected to similar variations in efficiency of RNA extraction and reverse transcription reactions. Thus, the ratio of cDNA templates in PCR reactions reflects that of mRNAs in samples analysed. This ratio can be accurately estimated as PCR products during exponential amplification cycles. Therefore, a standard curve should be part of every experiment.
Additionally, when using multiplex PCRs independence of every amplification step has to be proven. Results can be controlled by Northern blotting experiments. Using this technique, relative mRNA levels of a series of genes can be accurately determined even in tissue samples containing a heterogeneous mixture of cells of various origin and without knowledge of the exact amount of analysed cells.

Established biomarkers.
There are two major approaches in the application of biomarkers in ecotoxicology. The first is the analysis of individual organisms that live in a specific environment or at specific contaminated sites. Biomarkers used for this purpose are mainly of biochemical or physiological nature. The second approach is the analysis of the health of the ecosystem as a whole in which the organisms live. In the present thesis individuals are studied and therefore we will focus on biomarkers to analyse the condition of individuals.

The most frequently applied and intercalibrated biomarkers are lysosomal membrane instability, induction of the cytochrome P450 (CYP450) system, induction of metallothioneins (MTs), peroxisomal proliferation, inhibition of acetylcholine esterase (AChE), occurrence of imposex, and various pollutant-specific and unspecific protein alterations (for review, see Shugart et al. 1992).

Biomarkers of exposure.
Metals are major contaminants in the marine environment. In the late 1950s, MTs were detected in mammals by Margoshes & Vallee (1957). In *M. edulis*, MTs were found to act like cadmium-binding proteins (Noel-Lambot 1976). MTs in molluscs have a high glycine content, whereas mammalian MTs contain especially cysteine. MTs have a low molecular weight, are heat stable and have a strong affinity for binding 6-12 class B metals such as silver, cadmium, copper, mercury and zinc. MTs are mainly present in the cytosol but have also been detected in the nucleus and lysosomes. MTs bind excess of cations such as metals and thus protect the organism against toxicity by limiting the availability of these cations at undesirable sites. Different methods are available to quantify the amount of MTs at the protein level or as a function of metals bound to MTs (differential pulse polarography, spectrophotometry, ELISA, and radioimmunoassays) (Roesijadi 1986, Roesijadi et al. 1988). Basal MT levels in *M. edulis* and *M. galloprovincialis* are in the range of 2-3 mg/g dry weight in soft tissues and levels of this biomarker appear to be comparable over a wide geographical range from the northern hemisphere to the mediterranean. Induction of MTs is most
significant in gills of mussels (Lemoine et al. 2000). MT concentrations have been positively correlated with concentrations of metals in many scientific studies (Roesijadi & Fellingham 1987).

Inhibition of AChE is directly linked with the toxic action of organophosphorus and carbamate insecticides by direct binding to the catalytic site of the enzyme. AChE hydrolyses acetylcholine into choline and acetic acid in nerve synapses after signal transduction. Only few studies deal with the effects of organophosphate insecticides in aquatic organisms (Ozretic & Krajnovic-Ozretic 1992). Field work has been carried out on mussels in the Ebro Delta (Spain) where these insecticides have been widely used. Seasonal variations in mussel gill AChE were found and the variations were correlated with the local use of insecticides in agricultural activities.

CYP1A, an inducible member of the CYP450 family of monooxygenases, is widely used in fish as biomarker of exposure in biomonitoring programmes because of its role in biotransformation of many foreign compounds such as dioxins, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and furans (Lindstrom-Seppa et al. 1994, Elliott et al. 1996, Amcoff et al. 1998, Fiedler et al. 1998, Aas et al. 2000). CYP1A catalyses the conversion of lipophilic xenobiotics to more water-soluble compounds and is thus considered as a first phase (phase I) of detoxification and biotransformation. However, some of its metabolites are highly reactive and may be even more toxic than the mother compound (Liu et al. 1995). Many studies have been carried out in fish where 7-ethoxyresorufin O-deethylase (EROD) activities or CYP1A protein levels are correlated with environmental levels of CYP1A-inducing chemicals such as PAHs or PCBs (Beyer 1996, Beyer et al. 1996). Yet, CYP1A can only be used as biomarker of exposure in concert with other biomarkers of effect such as lysosomal instability. Furthermore, CYP1A levels in mussels are low. Therefore, this biomarker can not be applied to mussels.

Another biomarker that has been used in the recent past is the diagnosis of ‘imposex’. The imposition of male characteristics on female marine organisms known as ‘imposex’ has been identified in many aquatic invertebrates, but mainly in gastropods (Stewart et al. 1992, De Mora & Pelletier 1997). The organotin tributyltin (TBT), which is used in antifoulings, as stabilisers in plastic and as pesticides, has been identified as the main triggering factor of imposex in marine environments. Ecological and economical consequences of the use of organotin compounds as antifouling agents were first reported in the late 1970s after a sharp decline in oyster production in the Basin d’Arcachon, France (Alzieu 1986, Alzieu et al. 1989, Chagot et al. 1990). Biological alterations that have been ascribed to organotins are death of
mollusk larvae (Horiguchi et al. 1998), (Axiak et al. 1995) and the occurrence of male sexual characteristics in female gastropods (Bryan et al. 1987, Gibbs et al. 1987, Ellis & Pattisina 1990, Oehlmann et al. 1991). The latter effect was named imposex. Impossex was induced in gastropod species both in field studies and in laboratory studies at very low concentrations of TBT. These effects suggested that TBT and other organotins may also act as endocrine disruptors (Horiguchi et al. 1995, Bettin et al. 1996, Liu & Suen 1996, Oehlmann et al. 1996, Matthiessen & Gibbs 1998).

Biomarkers of effect.

Lysosomal enlargement in molluscan digestive cells and liver cells of fish is widely accepted as a marker of general stress (Lowe et al. 1993, Lowe & Moore 1993, Etxeberria et al. 1994, Cajaraville et al. 1995, Broeg et al. 1999) since it is induced in response to a variety of stressors including persistent organic and metal pollutants. Lysosomes contain acid hydrolases that are capable of degrading most biomolecules to low-molecular-weight products that can be used again in the synthesis of new macromolecules. Lysosomes in molluscan digestive cells accumulate both metal and organic contaminants that can not be degraded and provoke significant alterations in these organelles (Moore et al. 1980a, Moore et al. 1980b, Nott et al. 1985, Viarengo et al. 1985, Sarasquete et al. 1992, Cajaraville et al. 1995). In general, contaminants of the environment cause a significant increase in size and number of lysosomes (Marigomez et al. 1989, Regoli et al. 1998). These alterations are often associated with a reduction in the stability of lysosomal membranes, which is a risk for the health of cells or individual organisms when hydrolytic enzymes are released into the cytoplasm by causing intracellular damage. Instability of lysosomal membranes may also induce apoptosis (Neuzil et al. 2002).

Peroxisomal proliferation has been proposed as a specific marker of pollution by organic chemicals such as PAHs. Peroxisomes are ubiquitous cytoplasmic organelles involved in the metabolism of long-chain fatty acids and several metabolic processes involving oxidases. Peroxisomes in liver of rodents proliferate when treated with certain environmentally-relevant organic xenobiotics such as hypolipidemic drugs, phthalate ester plasticisers, petroleum products including PAHs, organic solvents and pesticides (Nemali et al. 1988, Nemali et al. 1989, Espandiari et al. 1995, Holme & Dybing 2002). Peroxisomal proliferation is defined as an increase in volume and number of peroxisomes, which is often but not always accompanied by induction of peroxisomal enzymes, particularly those of the fatty acid beta-oxidation pathway. Peroxisomes of marine organisms including bivalve molluscs and fish are
able to proliferate under experimental exposure to organic xenobiotics such as PAHs. Peroxisomal proliferation has been used recently in field studies to assess environmental pollution in coastal systems at the Iberian Peninsula (Regoli et al. 1998) and the Pacific coast of North America (Krishnakumar et al. 1995).

The activities of enzymes other than those mentioned above have been used as indicators of toxicity. For example, aminolevulinic acid dehydratase (ALAD) activity is induced by the specific toxic metal lead (Hodson et al. 1980).

Pollution-enhanced generation of oxyradicals or reactive oxygen intermediates (ROI) is often involved in cytotoxicity and carcinogenesis. Antioxidant defences, that are present in all aerobic organisms, include oxyradical scavengers (catalase, glutathione peroxidase, superoxide dismutase and vitamines) protecting organisms from oxidative stress. These antioxidant enzymes are most interesting because their inhibition has potential use as biomarker of pollution (Walker et al. 2000).

MXR in aquatic organisms.

The term MXR, multixenobiotic resistance, is used in marine ecotoxicology and is derived from the life science term MDR, multidrug resistance. MDR equips cancer cells with intrinsic or acquired cross resistance to diverse chemotherapeutic agents and hampers the effective treatment of cancer. Clinical drug resistance of tumors may be caused by different molecular mechanisms. Drug-modifying and efflux-mediating proteins are among the most-intensively studied proteins. Transmembrane efflux proteins, often in combination with drug-modifying or drug-tagging proteins, reduce intracellular accumulation or intratumoral retention of cytotoxic drugs below therapeutic thresholds. Moreover, these transmembrane efflux proteins may also prevent accumulation of drugs in specific organelles within cells, such as the nucleus to prevent DNA damage.

Many aquatic species are able to survive in habitats which are endangered by high levels of multiple natural toxins or anthropogenic pollutants. Whereas intensive investigations in the medical sector aim to circumvent the MDR mechanism by resistance modulators to improve the therapeutic efficiency of cytotoxic drugs, resistance to cytotoxic substances is an essential cellular function in marine organisms to survive and successfully reproduce in contaminated habitats.

Efflux of fluorescent dyes such as rhodamine and calcein AM from cells and organs of marine organisms has always been linked to P-glycoprotein (P-gp) activity. Additionally, immunohistological localisation of P-gp-like proteins was successful in many aquatic
organisms with the crossreacting human antibody C-219. However, other transporters may be involved as well. For example, clams freshly collected from a polluted Rhine river site accumulated 41% less vincristine than control clams held in clean aquaria for 6 weeks. Western blot analysis detected no difference in P-gp protein levels, suggesting that other transport systems may be responsible for the observed efflux of vincristine (Kurelec et al. 1996). Because MDR/MXR prevents accumulation of xenobiotics inside cells, it is considered as the initial phase (phase 0) of detoxification and biotransformation and regarded as first line of defence.

The most intensively studied proteins involved in MDR/MXR are P-gp, glutathione S-transferase pi (GSTpi), heat shock proteins (HSP), lung resistance protein (LRP) which is the major vault protein (MVP) and the multidrug resistance-related (or associated) protein (MRP). Aquatic organisms that express one or more elements of MDR/MXR are listed in Table 1. For example, membrane vesicles isolated from the freshwater mussel A. cygnea (Kurelec & Pivcevic 1989), the clam C. fluminea (Waldmann et al. 1995), the marine mussel M. galloprovincialis (Kurelec & Pivcevic 1991), or the sponges T. aurantium (Kurelec & Pivcevic 1992), G. cydonium and V. aerophoba (Kurelec et al. 1992) possess a verapamil-sensitive binding activity for xenobiotics such as 2-acetylaminofluorene and vincristine in a similar manner as that described for membrane vesicles isolated from male bovine adrenal cortex cells known to contain P-gp. Western blot studies of the sponges G. cydonium and V. aerophoba revealed that polyclonal antibodies raised against hamster P-gp crossreact with a sponge protein of Mr 125,000. A protein reacting with antibodies against mammalian MDR protein was identified as well in C. fluminea (Waldmann et al. 1995), embryos of the marine worm U. caupo (Toomey & Epel 1993), bile canaliculi and liver cancer in dab (Koehler et al. 1992) and flounder (Kohler et al. 1998), phagocytic blood cells in mussels (Minier et al. 1993) and in lysosomes of hepatocytes of C. maenas (Koehler et al. 1998). In addition, exposure of these organisms to different xenobiotics in the presence of verapamil induced accumulation of the xenobiotics in their organs (Minier & Moore 1996). These observations are considered to be an indication that an MDR-like system, called MXR, may be functional in these organisms in vivo as well. These observations were also made in specimens collected from pristine areas, i.e. specimens that have not been exposed previously to pollutants. This argues strongly that protective MDR-like mechanisms are inherent in these species and that its expression does not require induction.
<table>
<thead>
<tr>
<th>Species</th>
<th>Common name</th>
<th>Scientific name</th>
<th>Tissue</th>
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Table 1: Marine and freshwater organisms that have been shown to express MDR/MXR mechanisms as detected in specific tissues using immunostaining, P-gp-like transport activity of model substrates, or genetic data (Bard 2000).

P-glycoprotein (P-gp).

P-gp belongs to the ABC superfamily of transporters and acts as an ATP-dependent efflux pump. It has been shown to reduce intracellular accumulation of drugs with unrelated chemical structure and different mechanisms of action (Juranka et al. 1989, Bradley & Ling 1994, Sauna et al. 2001).

The isolation of mammalian cDNAs encoding P-gp enabled the analysis of the structure of the polypeptide. The sequences predicted that P-gps and related transporters are integral membrane glycoproteins with remarkably similar structures (Fig. 3). The length of the mammalian P-gps vary between 1276 and 1281 amino acids predicting a molecular weight of
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approximately 140 kDa. This is consistent with the molecular weight of 130-180 kDa of purified P-gp. Approx. 10-15 kDa of the observed molecular weight is accounted for by N-linked glycosylation (Greenberger et al. 1988). Indeed, the amino acid structure of P-gp indicates 7-10 potential N-linked glycosylation sites. Insights into the structure of P-gp have recently been obtained by electron microscopy, single particle imaging and Fourier projection of 2D crystalline arrays. The data suggest that P-gp is a cylinder of approximately 10 nm in diameter with one half of the molecule inserted in biomembranes, the so-called membrane spanning domains (MSDs), whereas the remainder of the molecule is present intra- and extracellularly. A large central pore of approx. 5 nm in diameter is created by a roughly hexagonal array of the membrane-spanning segment. The pore is hydrophilic and larger than is required for the passage of known substrates. It is closed at the cytoplasmic side by two intracellular domains which contain nucleotide-binding domains (NBDs) (Croop 1993). Binding of drugs and ATP to P-gp induces conformational changes in the protein, and the drug-binding site is conformationally coupled to the NBDs. Evidence has accumulated that suggests that the transporter interacts directly with lipophilic substrates in the environment of the lipid bilayer, and may act as a drug flippase, moving drugs from the inner to the outer leaflet of the bilayer. Chemosensitizers that block the action of P-gp have been proposed to act as alternative substrates, that spontaneously flip-flop across the membrane at a high rate resulting in futile cycling of the transporter and thus in inhibition of the transport of other molecules (Cai & Gros 2003).
Fig. 3. Schematic diagrams of ABC transporters as they are inserted in biological membranes. (A) Typical four-domain core structure (MSD1–NBD1–MSD2–NBD2) of P-gp. (B) Structure of members of the ABC subfamily that contain the core structure with an extra N-terminal cytoplasmic region, such as MRP4, MRP5, and cystic fibrosis transporter (CFTR). The regulatory "R" domain of CFTR is shown as dashed line. (C) Membrane topology of MRP1 and closely related transpoters contain the additional N-terminal MSD domain. The orientation of the lipid membrane bilayer is indicated (Cai & Gros 2003).

Major vault protein (MVP).

MVP is a 110-kDa protein that forms the major part of the vault particle, a ribonucleoprotein complex. MVP is overexpressed in a wide variety of P-gp-negative multidrug-resistant cancer cell lines including MRP-negative cell lines (Izquierdo et al. 1996a, Izquierdo et al. 1996b). Vaults have an octagonal barrel-shaped structure with protruding caps and an invaginated waist. These structures are highly conserved among eukaryotes, suggesting that they are essential for cell function (Kedersha et al. 1990). Vaults are located in the cytoplasm, nuclear envelope and nuclear pore complexes. They mediate bidirectional nucleocytoplasmic
transport of a wide range of substrates, including cytotoxic drugs. Multidrug-resistant cell lines overexpressing \textit{mvp} show decreased ratios of nuclear to cytoplasmic drug levels or show increased levels of drugs in cytoplasmic vesicles. The mechanism of resistance is complex and may be due to the redistribution of drugs within cells, thereby reducing exposure of the nucleus to cytotoxic agents (Izquierdo et al. 1996a, Izquierdo et al. 1996b).

**Multidrug resistance-related or associated protein (MRP).**

MRP, which is another transmembrane ABC transporter, with a molecular weight of 190 kDa, is overexpressed in most multidrug-resistance cell lines not expressing P-gp. MRPI is a polypeptide of approx. 170 kDa which is consistent with the molecular mass of 177 kDa predicted from the \textit{mrp} cDNA sequence. This immature form of the protein is rapidly processed posttranscriptionally to the larger 190 kDa mature form by N-linked glycosylation. MRPI contains five, six and four transmembrane segments in MSD1, MSD2 and MSD3, respectively (Ishikawa et al. 1997, Keppler et al. 1997, Hipfner et al. 1999). Activity of MRPI (sometimes referred to as glutathione-X pump) is considered to be responsible for so-called phase III elimination of conjugated organic anions produced by phase I and phase II metabolism of many endo- and xenobiotics (Ishikawa 1992). Most organic anion substrates are conjugated with reduced glutathione (GSH), glucuronide, or sulfate in phase II of detoxification and biotransformation. MRPs mainly transport GSH-conjugated drugs and only for a small part untagged organic ions. Therefore, MRPs are linked with glutathione S-transferases (GSTs) which catalyse the conjugation of GSH to electrophilic substances. MRP-related transport of lipophilic drug is rather an enigma but it is somehow linked with the transport of free GSH (Ishikawa et al. 1996).

**Glutathione S-transferases (GSTs).**

GSTs form a group of isoenzymes that are involved in cellular detoxification of both xenobiotics and endobiotic metabolites. GSTs are divided into a number of subclasses, alpha, mu, pi, and theta. Classification is made on the basis of sequence similarity and immunological crossreactivity. GST isoenzymes represents approx. 1% of the total cellular protein content and are found in most aerobic eukaryotes. X-ray crystallography and site-directed mutagenesis studies have elucidated structure and function of GSTs. Catalysis occurs by conjugation of hydrophobic compounds with GSH via a nucleophilic addition reaction. The less toxic and more hydrophilic products are partially metabolised and excreted. Levels of expression of different isoforms are tissue specific. GST expression is usually increased in
cancer cells after chemotherapy, and especially expression of GSTs of the pi class (Gajewska & Szczypka 1992, Daniel 1993, Shen et al. 1997).

Heat shock protein 70 (HSP).
HSPs are present in both prokaryotes and eukaryotes. Their highly-conserved structure suggests that they play a fundamental role in cellular processes. As the name suggests, HSPs are induced in cells exposed to heat. Heat is not the only stimulus that can induce expression of HSPs. Exposure of cells to amino acid analogs, heavy metals, sodium arsenite, microbial infections, hormones and antibodies can also induce expression of HSPs. Increased expression of HSPs has been shown to be protective in many cultured cells and animal tissues. Protection is likely due to the fact that HSPs can function as molecular chaperones to prevent inappropriate protein aggregation and to mediate transport of immature proteins to target organelles for final packaging, degradation, or repair (Fuqua et al. 1994, Kiang & Tsokos 1998, Jaattela 1999).

Fig. 2. Cellular localisation of proteins investigated in this thesis.
There are only few studies that characterise expression patterns and functional aspects of MXR-related proteins in wild populations of aquatic organisms especially those studies that determine how these genes may interact with xenobiotics, other detoxification enzymes or repair systems and/or metabolic pathways. Elucidation of induction and regulation mechanism in the expression of these genes in aquatic invertebrates may throw light onto their functions and the complex interactions with other detoxification systems. Identification of inducers and ‘chemosensitisers’ of gene regulation of MXR-transporters may provide useful information for risk assessment in aquatic ecosystems since activation or inhibition of these proteins may be decisive whether a harmful chemical accumulates in critical concentrations in cells or organelles with fatal outcome for the organism.
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