BIOMARKERS, 1999, VOL. 4, NO. 6, 442–454

The multi-xenobiotic resistance phenotype as a tool to biomonitor the environment

CHRISTOPHE MINIER[1]*, NANCY EUFEMIA[2] and DAVID EPEL[2]

[1] Laboratoire d'Écotoxicologie, Université du Havre, 25 rue Philippe Lebon, BP540, F-76058 Le Havre, France

[2] Hopkins Marine Station, Stanford University, Pacific Grove, California 93950, USA

Organisms use a variety of cellular mechanisms to avoid the effects of toxins. These strategies include de-toxification of putative toxins, sequestration of the toxins or the utilization of transport mechanisms to actually prevent the entry and accumulation of toxins in the cells. These toxin avoidance mechanisms, which presumably evolved in response to natural toxins, can also be used to counter the effects of anthropogenic compounds introduced into the environment by the activities of our modern society. In this article we discuss (1) the use of transport mechanism strategies to protect against toxins and (2) the possible use of these mechanisms as biomarkers indicative of exposure to man-made toxins. We will first review the characteristics of these transport mechanisms, including their biology, genetics and molecular properties and then discuss their use as biomarkers.

Keywords: P-glycoprotein, drug resistance, MXR, biomarker, bivalve, mussel.

The multi-drug resistance phenotype

Multi-drug resistance of tumour cells represents a major problem in cancer treatment, often leading to failure of chemotherapy. Cell lines exhibiting this phenotype, derived by *in vitro* selection with a single cytotoxic drug, show cross-resistance to a variety of structurally and functionally unrelated compounds—hence the term multi-drug resistance for this phenotype (Gottesman and Pastan 1993).

Intensive studies on such cancer cell lines have lead to the realization that there could be transport mechanisms to reduce the accumulation of toxins in eucaryotic cells. Indeed, the main characteristic phenotype of multi-drug resistance (or MDR) is decreased intracellular retention of cytotoxic drugs (Danö 1973, Endicott and Ling 1989). Kinetic studies indicate that this decreased accumulation results from impaired influx and increased efflux outside of the cell, such that the drugs are kept below their toxic level (Gottesman and Pastan 1993).

The most common form of this multi-drug resistance arises from the selection of cells with high amounts of a 170 KDa transport protein referred to as P-glycoprotein or P-gp. This is a membrane-spanning ATP-binding protein that transports chemotherapeutic drugs out of the cells (Juliano and Ling 1976). An absence of definitive substrate specificity is the most characteristic and also the most enigmatic aspect of this transporter. Thus, the transporter effluxes a wide variety of organic compounds, the only common structural feature being moderate hydrophobicity. These substrates include many of the drugs used in cancer

* Corresponding author: Christophe Minier, Laboratoire d'Écotoxicologie, Université du Havre, 25 rue Philippe Lebon, BP540, F-76058, Le Havre, France; e-mail: minier@univ-lehavre.fr

chemotherapy, many of which are natural products. The list is long and includes such diverse drugs as antibiotics, *Vinca* alkaloids, alkylating agents, anthracyclines and platinum-containing drugs. Furthermore, this mechanism is sensitive to membrane-activity compounds and other chemicals that can specifically block it. These compounds, such as drugs analogues, calcium antagonists or calmodulin inhibitors, are termed modulators or chemosensibilators (see review by Gottesman and Pastan 1993).

Evidence that this multi-drug resistance phenotype arises from over-expression of the P-glycoprotein was provided by transfection experiments with the *MDR1* gene, which showed that ove-expression of the MDR-protein alone can confer multidrug resistance on an otherwise drug-sensitive cell line (Gross *et al.* 1986, Ueda *et al.* 1987, Guild *et al.* 1988). It is assumed that this protein acts to reduce accumulation of its substrates in the cell, and the mechanism of reduced accumulation involves the ATP-dependent transport of substrates out of the cell. There are several hypotheses to explain this transport, including the idea that the substrates are pumped from the cytosol or directly from the lipid bilayer (Higgins and Gottesman 1992, Homolya *et al*. 1993) or that the reduced content of drug results from perturbation of the plasma membrane electrochemical potential (Simon *et al.* 1994) which could be related to alterations of the pHi of cells and indirect effects of this pH change on substrate accumulation (Roepe 1995).

Non-P-gp mediated multi-drug resistance

A number of tumour cell lines display a multiple resistance associated with impaired drug accumulation without P-gp overexpression. In these cases, several proteins of various molecular weight might account for the observed phenotype (Krishnamachary and Center 1993, Scheper *et al*. 1993, Doyle *et al.* 1996). The 110 kDa lung resistance-associated protein (LRP) is associated with an impaired drug accumulation and unfavourable prognosis outcome (Verovski *et al.* 1996, Michieli *et al.* 1997). The multi-drug resistance-associated protein (MRP), is responsible for a drug-resistant phenotype as shown by transfection with the MRP gene (Grant *et al.* 1994, Kruh *et al.* 1994). This 190 kDa MRP protein, is localized in intracellular membranes but predominantly in the plasma membrane and can be differentially glycosylated (Hipfner *et al.* 1994, Zaman *et al.* 1994). The profile of anticancer drugs resistance expelled in the presence of MRP is similar, but not identical, to that of P-gp. It confers resistance to several lipophilic compounds but, unlike P-gp, MRP is able to transport metallic anions including arsenite, arsenate, trivalent and pentavalent antimonials and glutathione conjugates (Cole *et al.* 1994). It is not fully understood how MRP brings about the decreased intracellular drug retention, but it might involve an ATP and glutathione-dependant efflux (Müller M. *et al.* 1994, Zaman *et al*. 1994).

Other mechanisms can be involved in multiple resistance to toxic compounds and these might be of interest to the environmental toxicologist as providing insights as to the spectrum of mechanisms that can be used to resist pollutants. In the 'atypical' MDR phenotype (At-MDR), a broad resistance spectrum is observed without alteration in drug accumulation (Pommier *et al.* 1986, Beck *et al.* 1987, Danks *et al.* 1987). This form of resistance appears to be the result of an alteration in the enzyme DNA topoisomerase II with loss of drug-stimulating DNA cleavage activity (Fernades *et al.* 1990). Some other cases seem to be due to a sum of

different mechanisms including the glutathione S-transferase activity (Zijlstra *et al.* 1987, Townsend and Cowan 1989, Hoban *et al.* 1992).

The ABC superfamily of transport proteins

Both the MDR and MRP proteins are members of the ATP-binding cassette (ABC) superfamily of transport systems (Hyde *et al.* 1990, Cole *et al.* 1992), also referred to as traffic ATPases (Ames *et al.* 1990). More than 50 members of the family have been identified to date (Higgins 1992). The conserved sequences between all members are short motifs within the ATP-binding domain including the Walker motifs associated with nucleotide binding (Walker *et al.* 1982, Higgins *et al.* 1986) but extend far them, characterizing the ABC domain (Higgins 1992).

Members of the family are widespread among living organisms. In bacteria, transport systems have been found, for example in *Escherischia coli*, in which they can actively import several organic or inorganic nutriments (Zumft *et al.* 1990, Abouhamad *et al.* 1991) and vitamins (Friedrich *et al.* 1986) or export toxins (Felmlee *et al.* 1985, Gilson *et al.* 1990). A large number of other species from different genera, including *Cyanobacteria, Mycoplasma, Rhizobium, Salmonella*, etc. possess a related transporter (reviewed in Higgins 1992). In *Saccharomyces cerevisiae*, the ABC protein is involved in secretion of a pheromone (McGrath and Varshavsky 1989) and several plants have also been shown to possess a member of this family (Ohyama *et al.* 1986, Dudler and Hertig 1992). Among the invertebrates, ABC proteins could be responsible for resistance to several toxic compounds: chloroquine in the malarial parasite *Plasmodium falciparum*(Wilson *et al.* 1989), arsenite (Callahan and Beverley 1991) or drugs in *Leishmania* (Gueiros-Filho *et al.* 1995) and hydrophobic cytotoxic compounds in *Drosophilia melanogaster* (Wu *et al.* 1991). In vertebrates, several MDR genes have been found in numerous mammalian species (Ng *et al.* 1989). In humans, two other linked genes have been discovered. One is associated with transport of peptides into the endoplasmic reticulum for class 1 antigen presentation (Müller *et al.* 1994), and another corresponds to the cystic fibrosis gene (CFTR) (Riordan *et al.* 1989).

MDR-related phenotype in aquatic organisms

Numerous lines of evidence–including isolation of homologous genes, immunological cross-reactivity and activity characteristics—indicate that members of the P-gp (P-glycoprotein) family are present in aquatic organisms.

Winter flounder (*Pleuronectes americanus*) and yellowtail flounder (*Limanda ferrugina*) possess two P-gp genes as indicated by Southern blot analysis (Chan *et al.* 1992) and C-terminal sequences of both genes from *P. americanus* share more than 60% identity to mammalian P-gp genes at the amino acid level. In the sponge *Geodia cydonium*, Northern blot studies using a human cDNA probe revealed the presence of a related 4.2 kb RNA (Kurelec *et al.* 1992). A fragment of 327 bp overlapping the ATP-binding site from *Mytilus edulis* has been found by RT-PCR. Its sequence shows 32% homology with mammalian P-gp gene (Minier 1994). A 650 bp fragment has been sequenced from the marine worm, *Urechis caupo* and shows 60%homology with the human MDR gene (Toomey 1996). Finally, a 130 bp of turbot (*Scophthalmus maximus*) MXR gene shows 80% homology with the human *mdr1* gene (Tutundjian and Minier unpublished results).

An MDR-like protein has also been identified immunologically in a number of aquatic organisms using both polyclonal and monoclonal antibodies. Polyclonal antibodies raised in rabbit against human P-gp reacted with a glycoprotein of 125 kDa in the sponges *G. cydonium* and *Verongia aerophoba* (Kurelec *et al.* 1992) and with a 140 kDa protein from the gastropod *Monodonta turbinata* (Kurelec *et al.* 1995a). The monoclonal antibody C219 has been especially useful since it recognizes a highly conserved region close to the ATP-binding site (Georges *et al.* 1990) and is considered 'a universal probe for the detection of P-glycoprotein' (Endicott and Ling 1989). Using this antibody a 140 kDa protein has been identified in *U. caupo* (Toomey and Epel 1993), two proteins of 220 and 240 kDa in the oyster *Crassostrea gigas* and the blue mussel *M. edulis* (Minier *et al.* 1993), two of 145 and 220–240 kDa in *M. galloprovincialis* (Galgani *et al.* 1995) and one of 170 kDa in *M. californianus* (Cornwall *et al.* 1995). However other reports showed only the 170 kDa protein in *M. edulis* (Minier and Moore 1996). These results could be related to differences in glycosylation or preparation procedures of the protein extracts. Immunohistochemistry has also been used to localize P-gp-related proteins in the shore crab *Carcinus maenas* (Köhler *et al.* 1998a), in the European flounder *Platischthys flesus* (Köhler *et al.* 1998b) and the guppy *Poecilia reticulata* (Hemmer *et al.* 1995).

Photo-affinity analogues have also been used to label the drug transporter. In *U. caupo* oocytes, several proteins could be covalently attached to photoaffinity-labelled forskolin, but only the 140 kDa protein was immunoprecipitated with an antibody to the P-glycoprotein (Toomey and Epel 1993).

MDR-like activity has also been studied in a number of aquatic organisms. The first evidence for an MDR-like activity was drug binding to membrane preparations. Kurelec and coworkers measured the binding of radiolabelled 2-acetylaminofluorene and vincristine to membrane vesicles from molluscs and sponges and found that the extent of labelling was decreased in the absence of ATP, trypsinization of the vesicles or incubation in the presence of a non-radioactive competitive substrate (Kurelec 1992).

Functional evidence for MDR-like activity has come from studies on accumulation of fluorescent substrates such as rhodamine 123, rhodamine B and calcein-AM or with radio-labelled substrates such as vincristine. These studies have found that accumulation of these compounds is enhanced by inhibitors of the MDR-activity (such as verapamil). Examples of such studies include sponge medula (Kurelec 1992, De Flora *et al.* 1995), *Urechis* embryos (Toomey and Epel 1993), *Corbicula* gills (Waldmann *et al.* 1995), mussel gills (Galgani *et al.* 1995) or haemocytes (Minier and Moore 1996) or whole animals (Smital and Kurelec 1997).

The role of the P-glycoprotein in xenobiotic resistance

As indicated above, the most probable role for the P-glycoprotein transport protein is in protection against xenobiotics. This is obviously the case for the drug-resistant phenotype seen in tumour cells in which the over-expression of the P-gp results in protection from the chemotherapeutic drugs. The P-glycoproteins are also expressed in normal tissues, and their tissue distribution [liver, colon, jejunum, kidney (Thiebaut *et al.* 1987, Sugawara *et al.* 1988)] again suggests a role in protection from diet-derived toxins (or endogene metabolites). In most cases this P-gp is on the apical membranes of cells facing an excretory compartment,

suggesting that the protein activity functions to keep xenobiotics out of the organ or the organism. P-glycoprotein is also expressed at lower levels in the capillary endothelial cells of the brain, testis and placenta (Cordon-Cardo *et al.* 1990) and this localized expression may in part relate to the blood–brain, blood–testis or blood–placenta barriers against xenobiotics.

The most direct evidence indicating a role for these transporters in toxin resistance has come from studies with knockout of genes or inhibition of transport activity. Mice with a knockout for the *mdr-1* gene are normal except for their susceptibility to toxins, such as pesticides (Schinkel *et al.* 1997). P-gp activity is present in mouse embryos, and inhibiting this transport activity enhances sensitivity to the anti-mitotic drug colchicine (Elbling *et al.* 1993). Finally, increasing the dosage of an MDR-related protein in *Caenorhabditis elegans* increased the resistance to colchicine (Broeks *et al.* 1995).

Studies on a variety of aquatic organisms similarly indicate that the natural function of this transporter is in toxin defence. As noted above, the transporter is present in a variety of aquatic organisms and this transport activity indeed functions to protect against xenobiotics. Several studies have shown that this mechanism is induced in response to exposure to toxic compounds (Kurelec 1995, Minier and Moore 1996, Smital and Kurelec 1998). In *M. edulis* haemocytes, rhodamine B retention within the cell was shown to be reduced in relation to the total dose of drug administered and duration of the exposure to the toxic compound vincristine (Minier and Moore 1996). Similarly, incubation of *M. californians* in several different substrates of the P-glycoprotein, such as pentachlorphenol, will induce both protein titre and activity in the mussel gills (Eufemia and Epel 1998). Furthermore, Köhler and coworkers showed that the MXR transporter is expressed in the bile canaliculi of hepathocytes of Flounder (1998b) and in the lysosomal membrane of *C. magneas* hepathrocrancreatic cells (1998a). The later results might indicate the role of MXR in uptake of toxins from the cytoplasm into the lysosomal compartment as seen in mussel haemocytes by confocal laser microscopy (Minier and Moore 1996). Indeed this compartment is of particular importance for toxins tolerance in mussels (Moore and Willows 1998).

This protective mechanism is of particular importance for aquatic organisms as its inhibition might lead to dramatic effects. In embryos of *U. caupo*, for example, cell division is not affected by 0.5 μM vinblastine, but if inhibitors of the transporter are present, cell division ceases (Toomey and Epel 1993). Similarly, genotoxic compounds, as far as they are substrates of the MXR-mechanism, will not greatly affect genome integrity in *C. fluminea* cells at low concentrations. But in the presence of an inhibitor of the MXR transporter, the DNA-damage caused by the genotoxin will be enhanced (Waldmann *et al.* 1995, Kurelec *et al.* 1996b).

This property of making cells resistant to a wide variety of xenobiotics thus provides a multixenobiotic resistance (MXR) which represents a first line of defence for aquatic organisms. The occurrence of inhibitors of the MXR system in the environment raises the concern of the enhancement of toxicity of environmental toxins (Kurelec *et al.* 1998).

Substrates of the multi-xenobiotic transporter in the aquatic environment

To understand the role of the transporter in toxin protection, one needs to identify the transporter substrates (although the list might be long and always

incomplete). A useful approach to identify substrates (or inhibitors) of the transporter is based on competitive inhibition of the transport activity when two different substrates are present. The easiest way to do this is to measure the effects of a potential substrate on the accumulation of fluorescent compounds such as rhodamine B. Rhodamine B is a substrate for the transporter and the transporter activity will normally exclude the dye and the cell will exhibit little fluorescence. However, if another substrate is present, it will compete with the transporter for the rhodamine, and the concentration of the fluorescent dye will increase in the cell and the cell will be more fluorescent. An increase in fluorescence then indicates that the test compound is a substrate (or inhibitor) of the transport activity. Alternatively, these transport activities and modulation can also be assessed by direct chemical analysis, by accumulation of radioactivity if the compound can be radiolabelled, or by fluorescence measurements with other fluorescent compounds such as rhodamine 123 and calcein-AM.

These approaches were used to identify the pesticide dachtal (dimethyl-2,3,5,6-tetrachlorterephtalate, DCPA) as a substrate of the transporter in mussel gills. When rhodamine and dachtal were incubated together with mussel gill tissue, the fluorescence of the cells increased, indicating that DCPA might be a substrate. This was confirmed by direct chemical analysis of dachtal in experiments in which mussels were incubated in dachtal in the presence or absence of verapamil (the transporter inhibitor). The concentration of pesticide increased two-fold in the presence of the inhibitor (Cornwall *et al.* 1995).

Nevertheless, interpretation of the results of competitive inhibition tests may be complicated by the occurrence of compounds that do not bind to the transporter but inhibit the transport indirectly. For example, staurosporine inhibits the protein kinase C (PKC) which activity is essential for P-gp-mediated transport. Consequently, this inhibitor is not a substrate although it leads to an increased concentration of MXR-substates (Kurelec 1995). In addition, some results suggest that some substrates can stimulate each other transport by interacting with two cooperative sites within the P-glycoprotein (Shapiro and Ling 1997).

A list of identified substrates (or inhibitors) for the mussel transporter is shown in table 1 which lists substrates that have been identified by (1) competition assays with rhodamine, (2) by direct measurement of accumulation of compounds and enhancement of their uptake by verapamil or (3) by measuring the binding to membrane vesicles prepared from the molluscan tissue. Highly hydrophobic compounds are poor substrates and in *M. californianus*, DDT and Arochlor 1254 did not act as substrates in a rhodamine competition assay but Arochlor (1 ppm) can induce activity and titre of the MXR protein (Eufemia and Epel 1998). In *M. galloprovincialis*, these compounds were weak substrates, showing activity at levels above 3 μM.

Natural waters also contain substrates for the transporter. *In vitro* tests using the rhodamine or calcein-AM competition assay indicated the presence of compounds interacting with the MXR system in pore water from mud flats (Toomey and Epel 1993) and from river and sediments extracts (Kurelec 1995, Kurelec *et al.* 1995b, Smital and Kurelec 1997).

Environmental substrates that are not necessarily toxic can also be present in the environment and can accentuate the effects of more toxic relatives. These less toxic compounds (which could be natural or anthropogenic) could compete with the MXR transport system and saturate the transport mechanism, thus

Table 1. Compounds that interact with the mussel (*M. galloprovincialis* and *M. californianus*) MXR system.

Cytotoxic drugs and modulators	Environmental contaminants
Modulators:	
Verampamil	Dacthal (DCPA)
Forskoline	Chlorbensine
Quinidine	Pentachlorophenol
Trifluoperazine	Sulphate-(*N´*-(2-chloroallyl)-diethyl-dithiocarbamate (CDEC)
Cytotoxic agents:	
Vinblastine	DDT[a]
Vincristine	DDD[a]
Rhodamine	DDE[a]
2-Acetylaminofluorene	PCB: Arochlor 1254[a]
Emetine	Hydrocarbons: diesel-2 oil[a]

[a] *M. galloprovincialis* only.

diminishing the effectiveness of the transporter system against more highly toxic substances. The presence of such substrates in natural waters were shown to accentuate the effects of acetoaminofluorene in clam cells (Waldmann *et al.* 1995, Kurelec *et al.* 1996b).

The MXR phenotype as a biomarker

The above results indicate that the MXR system expressed in aquatic organism provides a first line of defence against xenobiotics. It can be induced in response to toxic compounds exposure, indicating that it is a potential biomarker of the health status of the organism and possibly a biomarker to assess the presence of pollutants in the ambient environment.

Measurements of protein titre by Western blots have been made on organisms in the field and these indicate some correlation between amount of MXR protein and organic pollution. In *M. edulis*, specimens from sites highly contaminated with PAH, PCB and DDT in the Seine Bay showed high levels of MXR proteins in gills and mantle tissue, the most contaminated station showing significant higher expression level ($p < 0.025$) (Minier *et al.* 1993). Similarly, MXR protein content assessment in samples from stations in southern England showed significant variation between contaminated and uncontaminated sites (Minier and Moore 1996). In *M. galloprovincialis*, the MXR activity, as assessed by the radiolabelled-vincristine competitive assay, increased with the concentration of pollutants along a pollution gradient (Kurelec *et al.* 1996a). This trend was correlated with the protein expression level with the remarkable exception of the samples from the most contaminated site which showed a marked decrease in transport protein content. In oysters, *Crassostrea virginica*, collected from polluted and unpolluted sites in Charleston Harbor, SC, USA, no significant relationships between MXR proteins and pollutant levels were detected (A. H. Ringwood, personal comm.). And in *Crassostrea gigas*, no relationships between MXR proteins and pollution level were found along the French south-west coast (Minier *et al.* 1993, Minier 1994). These later results might indicate differences between species or interaction of other factors, as discussed below.

If MXR protein measurements can be validated as a marker, the available immunological reagents, such as the C219 antibody, will prove simple to use. This antibody, directed against mammalian P-gp, apparently works against the P-gp present in a wide variety of organisms. Western blots are tedious but dot blot or slot blot measurements could be carried out on a routine basis if Western blot analysis indicates that there is no cross-reaction with other proteins for the particular test tissue (Galgani *et al.* 1995, Minier unpublished results).

Activity tests should also be done in concert with the above immunological assays but these are more difficult to perform. Simple methods are needed for this purpose. Accordingly, Kurelec and coworkers developed a rhodamine B-efflux method using whole animals. Mussels are exposed to rhodamine-containing water and then transferred to clean water. Rate of efflux can then be assessed in a non-destructive manner (Smital and Kurelec 1997). Another approach is to use calcein-AM as a substrate and to measure its accumulation in hemocytes (Minier and Moore 1998).

Interpreting *in situ* measurements of the MXR phenotype might not be simple, however. One concern is that the MXR phenotype can be induced in response to natural factors that are not necessarily indicative of pollution. For example, the mammalian MDR system is affected by heat shock (Chin *et al.* 1990) and modulated by several hormones (Qian and Beck 1990). In a similar manner, heat shock can induce MXR protein expression in mussels (Eufemia and Epel unpublished) and results of a 1 year study of mussel MXR protein and heat shock protein (HSP70) expression showed that they vary similarly during the year with a peak in August and are correlated with water temperature (Minier *et al.* 1999).

A related and potentially more serious problem is separating out the effects of natural toxins from pollutants. For example, bacteria and plants produce substrates for the MXR transporter, and seasonal variations in levels of these natural products could induce concomitant increases in MXR titre and activity (Toomey *et al.* 1996). Indeed, macroalgal fragments and phytoplankton present in Monterey Bay produce transport substrates (Eufemia and Epel unpublished) and, for example, extracts from the marine algae, *Caulerpa taxifolia*, can reverse the multi-xenobiotic resistance (Smital *et al.* 1996). Variations in content of these substrates could induce the transporter of pollutant concentrations. Indeed, the observed and seasonal variations in the titre activity of the transporter could be related to variations in natural substrates as well as to variations in temperature.

A related problem comes from the finding that there can also be seasonal variation in inducibility of the MXR protein in the gill tissue of *M. californianus* in response to substrates (Eufemia and Epel, unpublished). This inability to induce the MXR response has been particularly seen when the titre is already high, as in the summer months. This could result from a saturation response, in which the amount of transporter protein reaches a maximum such that no further induction can occur. This variation in inducibility has been especially apparent this past year (1997/98), which was during an intense El Nino cycle. The variations could be related to higher water temperature, as noted above. Other factors that could increase the titre could be sporadic algal blooms. An opposite factor that could affect the MXR response could be nutrient availability, with poor or rich nutrient conditions affecting the physiology and response of the mussels. There could also be effects related to the reproductive status of the animals, but these have not yet been studied.

Interaction of natural parameters are common to many biological responses and, in the case of MXR, further work should focus on the possible way(s) to correct or adjust for these sources of variation. Two possible ways of interest can be mentioned in order to circumvent the above problems. One is to screen other organisms to find ones that are not so subject to seasonal variation. In this regard, mussels are typically harvested from intertidal areas, and these might be more susceptible to seasonal stresses than benthic animals. It could be worth surveying a variety of organisms from different habitats to see if there are bivalves (or other test organisms) that normally have a low level of MXR protein but which is inducible in response to xenobiotics.

In vitro tests using animals or cells grown in the laboratory could provide a second way to avoid problems arising from changes in season or reproductive status. One could use these animals or cell lines to measure induction in response to exposure to test water samples. Cell lines of marine invertebrates would be ideal for these types of studies, but good sources of such cells are not available, and this is a subject for future research. Having such lines would considerably simplify testing of waters for their ability to induce the MXR protein. They could also be used to determine the cellular and molecular mechanisms of sensing pollutants and the cell response to these compounds.

A final unanswered question is whether other transport mechanisms besides the MXR strategy is used by aquatic organisms to avoid the effects of toxins. In this paper we mentioned the MRP transporter, which is one example of a newly-discovered transport protein in mammals, that might be of general importance to all organisms. There may be many members of such transport families, and insights into these mechanisms could provide alternative candidates for useful biomarkers.

Conclusions

The multi-xenobiotic resistance system is an important defence mechanism against environmental toxins which has been highly conserved during evolution. This phenotype can be induced by exposure to toxic compounds and significant different expression levels can be assessed between populations living in more or less polluted sites. Accordingly, this phenotype has the potentials of a biomarker which might be useful for environmental diagnosis. However, as for many biological responses, natural factors can interfere with the MXR phenotype. Heat shock and compounds of natural origin can modulate both activity and protein expression and future work should address the important question of finding ways to correct for these sources of variation.

References

ABOUHAMAD, W. N., MANSON, M., GIBSON, M. and HIGGINS, C. F. 1991, Peptide transport and chemotaxis in *Echerischia coli* and *Salmonella typhimurium*: characterization of the dipeptide permease (Dpp) and the dipeptide-binding protein. *Molecular Microbiology* **5**, 1035–1047.

AMES, G. F. L., MIMURA, C. S. and SHYAMALA, V. 1990, Bacterial periplasmic permeases belong to a family of transport proteins operating from *Escherichia coli* to human: traffic ATPases. *FEMS Microbiology Reviews*, **75**, 429–446.

BECK, W. T., CIRTAIN, M. C., DANKS, M. K., FELSTED, R. L., SAFA, A. R., WOLVERTON, J. S., PARKER, SUTTLE, D. and TRENT, J. 1987, Pharmacological, molecular and cytogenetic analysis of 'atypical' multidrug-resistant human leukemic cells. *Cancer Research*, **47**, 5455–5460.

BROEKS, A., JANSSEN, H. W. R. M., CALAFAT, J. and PLASTERK, R. H. A. 1995, A P-glycoprotein protects *Caenorhabditis elegans* against natural toxins. *EMBO Journal*, **14**, 1858–1866.

CALLAHAN, H. L. and BEVERLEY, S. M. 1991, Heavy metal resistance: a new role for P-glycoprotein in *Leishmania*. *Journal of Biological Chemistry* , **266**, 18427–18430.

CHAN, K. M., DAVIES, P. L., CHILDS, S., VEINOT, L. and LING, V. 1992, P-glycoprotein genes in the winter flounder, *Pleuronectes americanus*: isolation of two types of genomic clones carrying 3′ terminal exons. *Biochemica et Biophysica Acta*, **1171**, 65–72.

CHIN, K. V., TANAKA, S., DARLINGTON, G., PASTAN, I. and GOTTESMAN, M. M. 1990, Heat shock and arsenite increase expression of the multidrug-resistance (MDRI) gene in human renal carcinoma cells. *Journal of Biological Chemistry,* **265**, 221–226.

COLE, S. P. C., BHARDWAI, G., GERLACH, J. H., MACKIE, J. E., GRANT, C. E., ALMQUIST, K. C., STEWART, A. J., KURZ, E. U., DUNCAN, A. M. V. and DEELEY, R. G. 1992, Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science*, **258**, 1650–1654.

COLE, S. P. C., SPARKS, K. E., FRASER, K., LOE, D. W., GRAT, C. E., WILSON, G. M. and DEELEY, R. G. 1994, Pharmacological characterisation of multidrug-resistant MRP-transfected human tumor cells. *Cancer Research*, **54**, 5902–5910.

CORDON-CARDO, C., O'BRIEN, J. P., BOCCIA, J., CASALS, D., BERTINO, J. R. and MELAMED, M. R. 1990, Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. *Journal of Histochemistry and Cytochemistry*, **38**, 1277–1287.

CORNWALL, R., TOOMEY, B. H., BARD, S., BACON, C., JARMAN, W. M. and EPEL, D. 1995, Characterization of multixenobiotic/multidrug transport in the gills of the mussel *Mytilus californianus* and identification of environmental substrates. *Aquatic Toxicology*, **31**, 277–296.

DANKS, M. K., YALOWICH, J. C. and BECK, W. T. 1987, Atypical multiple drug resistance in human leukemic cell line selected for resistance to teniposide (VM-26). *Cancer Research*, **47**, 1297–1301.

DANÖ, K. 1973, Active outward transport of daunomycin in resistant Ehrlich ascites tumor cells. *Biochimica et Biophysica Acta,* **323**, 466–483.

DE FLORA, S., BAGNASCO, M., BENNICELLI, C., CAMOIRANO, A., BOJINEMIRSKI, A. and KURELEC, B. 1995, Biotransformation of genotoxic agents in marine sponges. Mechanisms and modulation. *Mutagenesis*, **10**, 357–364.

DOYLE, L. A., YANG, W., RISHI, A. K., GAO, Y. and ROSS, D. D. 1996, H19 gene overexpression in atypical multidrug-resistant cells associated with expression of a 95-kilodalton membrane glycoprotein. *Cancer Research*, **56**, 2904–2907.

DUDLER, R. and HERTIG, C 1992, Structure of an *MDR*-like gene from *Arabidopsis thaliana*. *Journal of Biological Chemistry*, **267**, 5882–5888.

ELBLING, L., BERGER, W., REHBERGER, A., WALDHOR, T. and MICKSCHE, M. 1993, P-glycoprotein regulates chemosensitivity in early developmental stages of the mouse. *FASEB Journal,* **7**, 1499–1506.

ENDICOTT, J. A. and LING, V. 1989, The biochemistry of P-glycoprotein-mediated multidrug resistance. *Annual Review of Biochemistry,* **58**, 137–171.

EUFEMIA, N. A. and EPEL, D. 1998, The multixenobiotic defence mechanism in mussels is induced by substrates: implication for a general stress response. *Marine Environmental Research*, **46**, 401–405.

FELMLEE, T., PELLET, S. and WELCH, R. A. 1985, Nucleotide sequence of an *Escherichia coli* chromosomal hemolysin. *Journal of Bacteriology,* **163**, 94–105.

FERNADES, D. J., DANKS, M. K. and BECK, W. T. 1990, Decreased nuclear matrix DNA topoisomerase II in human leukemia cells resistant to VM-26 and m-AMSA. *Biochemistry*, **29**, 4235–4241.

FRIEDRICH, M. J., DEVEAUX, L. C. and KADNER, R. J. 1986, Nucleotide sequence of the *btuCED* genes involved in vitamin B_{12} transport in *Escherichia coli* and homology with components of periplasmic-binding-protein-dependant transport systems. *Journal of Bacteriology*, **167**, 928–934.

GALGANI, F., CORNWALL, R., TOOMEY, B. H. and EPEL, D. 1995, Interaction of environmental xenobiotics with a multixenobiotic defense mechanism in the bay mussel *Mytilus galloprovincialis* from the coast of California. *Environmental Toxicology and Chemistry*, **15**, 325–331.

GEORGES, E., BRADLEY, G., GARIEPY, J. and LING, V. 1990, Detection of P-glycoprotein isoforms by gene-specific monoclonal antibodies. *Proceedings of the National Academy of Sciences USA*, **87**, 152–156.

GILSON, L., MAHANTY, H. K. and KOLTER, R. 1990, Genetic analysis of an MDR-like export system: the secretion of colicin V. *EMBO Journal,* **7**, 3971–3974.

GOTTESMAN, M. M. and PASTA, I. 1993, Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annual Review of Biochemistry*, **62**, 385–427.

GRANT, C. E., VALDIMARSSON, G., HIPFNER, D. R., ALMQUIST, K. C., COLE, S. P. C. and DEELEY, R. G. 1994, Overexpression of multidrug-resistance-associated protein (MRP) increases resistance to natural product drugs. *Cancer Research*, **54**, 357–361.

GROS, P., BEN NERIAH, Y., CROOP, J. M. and HOUSMAN, D. E. 1986, Isolation and expression of a cDNA (*MDR*) that confers multidrug resistance. *Nature* (London), **323**, 728–731.

GUEIROS-FILHO, F. J., VIOLA, J. P., GOMES, F. C., FARINA, M., LINS, U., BERTHO, A. L., WIRTH, D. F. and LOPES, U. G. A. D. 1995, *Leishmania amazonensis*: multidrug resistance in vinblastine-resistant promastigotes is associated with rhodamine 123 efflux. DNA amplification, and RNA overexpression of a Leishmania MDR1 gene. *Experimental Parasitology*, **81**, 480–490.

GUILD, B. C., MULLIGAN, R. C., GROS, P. and HOUSMAN, D. E. 1988, Retroviral transfer of a murine cDNA for multidrug resistance confers pleiotropic drug resistance to cells without prior drug selection. *Proceedings of the National Academy of Sciences USA*, **85**, 1595–1599.

HEMMER, M. J., COURTNEY, L. A. and ORTEGO, L. S. 1995, Immunohistochemical detection of P-glycoprotein in teleost tissues using mammalian polyclonal and monoclonal antibodies. *Journal of Experimental Zoology*, **272**, 69–77.

HIGGINS, C. E. and GOTTESMAN, M. M. 1992, Is the multidrug transporter a 'flipase'? *Trends in Biochemical Science*, **17**, 18–19.

HIGGINS, C. F. 1992, ABC transporters: from microorganisms to man. *Annual Review of Cell Biology*, **8**, 67–113.

HIGGINS, C. F., HILES, I. D., SALMOND, G. P. C., GILL, D. R., DOWNIE, J. A., EVANS, I. J., HOLLAND, I. B., GRAY, L., BUCKELS, D., BELL, A. W. and HERMODSON, M. A. 1986, A family of related ATP-binding subunits coupled to many distinct biological processes in bacteria. *Nature* (London) **323**, 448–450.

HIPFNER, D. R., GAULDIE, S. D., DEELEY, R. G. and COLE, S. P. C. 1994, Detection of the *Mr* 190,000 multidrug resistance protein, MRP, with monoclonal antibodies. *Cancer Research* **54**, 5788–5792.

HOBAN, P. R., ROBSON, C. N., DAVIES, S. M., HALL, A. G., CATAN, A. R., HICKSON, I. D. and HARRIS, A. L. 1992, Reduced topoisomerase II and elevated class glutathione S-transferase expression in a multidrug resistant CHO cell line highly cross-resistant to mitomycin C. *Biochemical Pharmacology* **43**, 685–693.

HOMOLYA, L., HOLLO, Z., GERMANN, U. A., PASTAN, I., GOTTESMAN, M. M. and SARKADI, B. 1993, *Journal of Biological Chemistry*, **268**, 21493–21496.

HYDE, S. C., EMSLEY, P., HARTSHORN, M., MIMMACK, M. M. and GILEADI, U. 1990, Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. *Nature*, **346**, 362–365.

JULIANO, R. I. and LING, V. 1976, A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochimica et Biophysica* **455**, 152–162.

KÖHLER, A., LAURITZEN, B., JANSEN, D., BOTTCHER, P., TEGULIWA, L., KRÜNER, G. and BROEG, K. 1998a, Detection of P-glycoprotein mediated MDR/MXR in *Carcinus maenas* hepatopancreas by immuno-gold-silver labeling. *Marine Environmental Research*, **46**, 411–414.

KÖHLER, A., LAURITZEN, B., JANSEN, D., BAHNS, S., GEORGE, C. G., FÖRLIN, L. and VAN NOORDEN, C. J. F. 1998b, Clonal adaptation of cancer cells in flatfish liver to environmental contamination by changes in expression of P-gp related MXR, CYP450, GST-A and G6PDH activity. *Marine Environmental Research*, **46**, 191–195.

KRISHNAMACHARY, N. and CENTER, M. S. 1993, The MRP gene associated with a non-P-glycoprotein multidrug resistance encodes a 190-kDa membrane bound glycoprotein. *Cancer Research* **53**, 3658–3661.

KRUH, G. D., CHAN, A., MYERS, K., GAUGHAN, K., MIKI, T. and AARONSON, S. A. 1994, Expression complementary DNA library transfer establishes mrp as a multidrug resistance gene. *Cancer Research*, **54**, 1649–1652.

KURELEC, B. 1992, The multixenobiotic resistance mechanism in aquatic organisms. *Critical Review in Toxicology*, **19**, 291–302.

KURELEC, B. 1995, Reversion of multixenobiotic resistance mechanism in gills of a marine mussel *Mytilus galloprovincialis* by model inhibitor and environmental modulators of P170-glycoprotein. *Aquatic Toxicology*, **33**, 93–103.

KURELEC, B., KRCA, S., PIVCEVIC, B., UGARKOVIC, D., BACHMANN, M., IMSIECKE, G. and MÜLLER, W. E. G. 1992, Expression of P-glycoprotein gene in marine sponges. Identification and characterization of the 125 KDa drug-binding glycoprotein. *Carcinogenesis*, **13**, 69–76.

KURELEC, B., LUCIC, D., PIVCEVIC, B. and KRCA, S. 1995a, Induction and reversion of multixenobiotic resistance in the marine snail *Monodonta turbinata*. *Marine Biology*, **123**, 305–312.

KURELEC, B., PIVCEVIC, B. and MÜLLER, W. E. G. 1995b, Determination of pollutants with multixenobiotic resistance inhibiting properties. *Marine Environmental Research*, **39**, 261–265.

KURELEC, B., KRCA, S. and LUCIC, D. 1996a, Expression of multixenobiotic resistance mechanism in a marine mussel *Mytilus galloprovincialis* as a biomarker of exposure to polluted environment. *Comparative Biochemistry and Physiology*, **113C**, 283–289.

KURELEC, B., WALDMANN, P. and ZAHN, R. K. 1996b, The modulation of protective effects of multixenobiotic resistance mechanism in a clam *Corbicula fluminea*. *Marine Environmental Research*, **42**, 383–387.

KURELEC, B., BRITVIÆ, S., PIVEEVIÆ, B. and SMITAL, T. 1998, Fragility of multixenobiotic resistance in aquatic organisms enhances the complexity of risk assessment. *Marine Environmental Research*, **46**, 415–419.

MCGRATH, J. P. and VARSHAVSKY, A. 1989, The yeast STE6 gene encodes a homologue of the mammalian multidrug resistance P-glycoprotein. *Nature* (London), **340**, 400–404.

MICHIELI, M., DAMIANI, D., ERMACORA, A., RASPADORI, D., MICHELUTTI, A., GRIMAZ, S., FANIN, R., RUSSO, D., LAURIA, F., MASOLINI, P. and BACCARANI, M. 1997, P-glycoprotein (PGP) and lung

resistance-related protein (LRP) expression and function in leukaemic blast cells. *British Journal of Haematology*, **96**, 356–365.

MINIER, C. 1994, Non-specific esterase activity and MXR as biomarkers for the assessment of biological effects in marine organisms. PhD thesis, University of Nantes (France), 153 pp.

MINIER, C. and MOORE, M. N. 1996, Rhodamine B accumulation and mxr protein expression in mussel blood cells: effects of exposure to vincristine. *Marine Ecology Progress Series*, **142**, 165–173.

MINIER, C. and MOORE, M. N. 1998, Calcein accumulation in mussel blood cells. *Marine Environmental Research*, **46**, 425–428.

MINIER, C., AKCHA, F. and GALGANI, F. 1993, P-glycoprotein expression in *Crassostrea gigas* and *Mytilus edulis* in polluted seawater. *Comparative Biochemistry and Physiology*, **106**, 1029–1036.

MINIER, C., BORGHI, V. and PORTE, C. 1999, Seasonal variation of MXR and stress proteins of the common mussel, *Mytilus galloprovincialis*. (In press).

MOORE, M. N. and WILLOWS, R. I. 1998, A model for cellular uptake and intracellular behaviour of particulate-bound micropolluants. *Marine Environmental Research*, **46**, 509–514.

MÜLLER, K. M., EBENSPERGER, C. and TAMPÉ, R. 1994, Nucleotide binding to the hydrophylic c-terminal domain of the transporter associated with antigen processing (TAP). *Journal of Biological Chemistry*, **269**, 14032–14037.

MÜLLER, M., MEIJER, C., ZAMAN, G. J. R., BORST, P., SCHEPER, R. J., MULDER, N. M., DE VRIES, A. G. E. and JANSEN, P. L. M. 1994, Overexpression of the gene encoding the multidrug resistance-associated protein result in increased ATP-dependent glutathione-S-conjugate transport. *Proceedings of the National Academy of Sciences USA*, **91**, 13033–13037.

NG, W. F., SARANGI, F., ZASTAWNY, R. L., VEINOT-DREBOT, L. and LING, V. 1989, Identification of members of the P-glycoprotein multigene family. *Molecular and Cellular Biology*, **9**, 1224–1232.

OHYAMA, K., FURKUZAWA, H., KOCHI, T., SHIRAI, H. and SANO, T. 1986, Chloroplast geneorganization deduced from complete sequence of liverwort. *Marchantia polymorpha* chloroplast DNA. *Nature*, **332**, 572–574.

POMMIER, Y., KERRIGAN, D., SCHWARTZ, R. E., SWACK, J. A. and MCCURDY, A. 1986, Altered DNA topoisomerase II activity in Chinese hamster cells resistant to topoisomerase II inhibitors. *Cancer Research*, **46**, 3075–3080.

QIAN, X. and BECK, W. T. 1990, Progesterone photoaffinity labels P-glycoprotein in multidrug-resistant human leukemic lymphoblasts. *Journal of Biological Chemistry*, **265**, 18753–18756.

RIORDAN, J. R., ROMMENS, J. M., KEREM, B., ALON, N., ROZMAHEL, R., GRZELCZAK, Z., LOK, S., PLAVSIC, N., CHOU, J. L., DRUM, M. L., IANNUZZI, M. C., COLLINS, F. S. and TSUI, L. C. 1989, Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science*, **245**, 1066–1073.

ROEPE, P. D. 1995, The role of the MDR protein in altered drug translocation across tumor cell membranes. *Biochim et Biophysica Acta* **1241**, 385–406.

SCHEPER, R. J., BROXTERMAN, H. J., SCHEFFER, G. L., KAAIJK, P. and DALTON, W. S. 1993, Overexpression of a M(r) 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance. *Cancer Research*, **53**, 1475–1479.

SCHINKEL, A. H., MAYER, U., WAGENAAR, E., MOL, C. A. A. M., VAN DEEMTER, L., SMIT, J. J. M., VAN DER VALK, VOORDOUW, A. C., SPITS, H., VAN TELLINGEN, O., ZIJLMANS, J. M. J. M., FIBBE, W. E. and BORST, P. 1997, Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. *Proceedings of the National Academy of Sciences USA*, **94**, 4028–4033.

SHAPIRO, A. B. B. and LING, V. 1997, Positively cooperative sites for drug transport by P-glycoprotein with distinct drug specificities. *European Journal of Biochemistry*, **250**, 130–137.

SIMON, S., DEBORSHI, R. and SCHINDLER, M. 1994, Intracellular pH and the control of multidrug resistance. *Proceedings of the National Academy of Sciences USA*, **91**, 1128–1132.

SMITAL, T. and KURELEC, B. 1997, Inhibitors of the multixenbiotic resistance mechanism in natural waters: in vivo demonstration of their effects. *Environmental Toxicology and Chemistry*, **16**, 2164–2170.

SMITAL, T. and KURELEC, B. 1998, The activity of multixenbiotic resistance mechanism determined by rhodamine B-efflux method as a biomarker of exposure. *Marine Environmental Research*, **46**, 443–447.

SMITAL, T., PIVCEVIC, B. and KURELEC, B. 1996, Reversal of multidrug resistance by extract from a marine algae *Caulerpa taxifolia. Period. Biol.* **98**, 165–171.

SUGAWARA, I., KATAOKA, I., MORISHITA, Y., HAMADA, H., TSURUO, T., ITOYAMA, S. and MORI, S. 1998, Tissue distribution of P-glycoprotein encoded by a multidrug-resistant gene as revealed by a monoclonal antibody, MRK 16. *Cancer Research,* **48**, 1926–1929.

THIEBAUT, F., TSURUO, T., HAMADA, H., GOTTESMAN, M. M., PASTAN, I. and WILLINGHAM, M. C. 1987, Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proceedings of the National Academy of Sciences USA,* **84**, 7735–7738.

TOOMEY, B. H. 1996, A multixenobiotic defense mechanism in the embryos of the echiuran worm *Urechis caupo.* PhD thesis, Stanford University, 102 pp.

TOOMEY, B. H. and EPEL, D. 1993, Multidrug resistance in *Urechis caupo* embryos: Protection from environmental toxins. *Biological Bulletin,* **185**, 355–364.

TOOMEY, B. H., KAUFMAN, M. R. and EPEL, D. 1996, Marine bacteria produce compounds that modulate multixenobiotic transport activity in *Urechis caupo* embryos. *Marine Environmental Research*, **42**, 393–397.

TOWNSEND, A. J. and COWAN, K. H. 1989, Glutathione *S*-transferases and antineoplastic drug resistance. *Cancer Bulletin*, **41**, 31–37.

UEDA, K., CARDARELLI, C., GOTTESMAN, M. M., and PASTAN, I. 1987, Expression of a full-length cDNA for the human *MDR1* gene confers resistance to colchicine, doxorubicin and vinblastine. *Proceedings of the National Academy of Sciences, USA,* **84**, 3004–3008.

VEROVSKI, V. N., VAN DEN BERGE, D. L., DELVAEYE, M. M., SCHEPER, R. J., DE NEVE, W. J. and STORME, G. A. 1996, Low-level doxorubicin resistance in P-glycoprotein-negative human pancreatic tumour PSN1/ADR cells implicates a brefeldin A-sensitive mechanism of drug extrusion. *British Journal of Cancer*, **73**, 596–602.

WALDMANN, P., PIVCEVIC, B., MÜLLER, W. E. G., ZAHN, R. K. and KURELEC, B. 1995, Increased genotoxicity of acetylaminofluorene by modulators of multixenobiotic resistance mechanism: Studies with the fresh water clam *Corbicula fluminea. Mutation Research* **342**, 113–123.

WALKER, J. E., SARASTE, M., RUNSWICK, M. J. and GAY, N. J. 1982, Distantly related sequences in the α- and β-subunits of ATP synthetase, myosin, kinases and other ATP requiring enzymes and a common nucleotide binding fold. *EMBO J.* **1**: 945–951.

WILSON, G. M., SERRANO, A. E., WASLEY, A., BOGENSCHUTZ, M. P., SHANKAR, A. H. and WIRTH, D. F. 1989, Amplification of a gene related to mammalian MDR genes in drug-resistant *Plasmodium falciparum. Science*, **244**, 1184–1186.

WU, C. T., BUDDING, M., GRIFFIN, M. S. and CROOP, J. M. 1991, Isolation and characterisation of *Drosophila* multidrug resistance gene homologs. *Molecular and Cellular Biology*, **11**, 3940–3948.

ZAMAN, G. J. R., FLENS, M. J., VAN LEUSDEN, M. R., DE HAAS, M., MULDER, H. S., LANKELMA, J., PINEDO, H. M., SCHEPER, R. J., BAAS, F., BROXRTERMAN, H. J. and BORST, P. 1994, The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. *Proceedings of the National Academy of Sciences USA*, **91**, 8822–8826.

ZIJLSTRA, J. G., DE VREIS, E. G. E. and MULDER, N. H. 1987, Multifactorial drug resistance in an adriamycin-resistant human small cell lung carcinoma cell line. *Cancer Research*, **47**, 1780–1784.

ZUMFT, W. G., VIERBROCK-SAMBALE, A. and BRAUN, C. 1990, Nature oxide reductase from denitrifying *Pseudomonas stutzen. European Journal of Biochemistry*, **192**, 591–599.