Echinoderm coelomocytes as a cellular model in toxicity testing and biomonitoring

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Abbreviations

AB	Alamar Blue™, dye used in metabolic activity assay
CFDA-AM	5-carboxyfluorescein diacetate acetoxymethyl ester
HBCD	Hexabromocyclododecane
MDR	Multidrug resistance
MRP	Multidrug resistance- associated protein
MXR	Multixenobiotic resistance
PBS	Phosphate buffered saline
PFNA	Perfluorononononaic acid
PFOS	Perfluorooctane sulfonate
P-gp	Poly-glycoprotein, common mediator of multixenobiotic resistance
TBBPA	Tetrabromobisphenol-A
TBT	Tri-n-butyltin

Abstract

In the studies described in this manuscript, a new cellular model system has been developed and two fluorometric assays have been assessed for their applicability in biomonitoring and in vitro *toxicity testing. Echinoderm coelomocytes were chosen as the cellular system because of their ease of sampling, and because their immunofunction makes effects on these cells likely to cause adverse effects on the host organism. Primary cell cultures of coelomocytes were established in the 96-well microtiter plate format by removal of coelomic fluid, dilution to suitable cell density in culture medium phosphate buffered saline and application in wells without further processing. The 96-well format is suitable for high sample number and small sample size, thus allowing the high throughput screening that is desirable in biomonitoring and toxicity testing. Two fluorometric assays, the alamar Blue™ and CFDA-AM cytotoxicity assay and the multixenobiotic resistance (MXR) accumulation assay, were optimised and applied on the cultured coelomocytes. MXR is believed to serve as a cellular first line of defence against numerous substances, and is therefore expected to be highly relevant for cell viability and function. The biomonitoring study was conducted in Kaštela Bay, Croatia, on coelomocytes from the sea cucumber* Holothuria tubulosa. *Cells taken from individuals collected at a heavily polluted site was compared to cells taken from individuals at a relatively pristine site. In the toxicity testing study,* Asterias rubens *coelomocytes in culture were exposed to different toxicants for 96 hours, before the assays were run. Both assays provided significant results in biomonitoring and toxicity testing.*

Used in combination, coelomocyte primary culture and the two fluorometric assays may constitute a rapid, cost-effective, easily performed procedure for biomonitoring and toxicity testing.

1 Introduction

Since the early 1960s, the presence and potentially harmful effects of man-made chemicals in the environment has recieved increasing attention both in the scientific community and the general public. After years of using and releasing such substances, surprisingly little is known about many of them. It is not known how they are spread and transformed in the environment or how they are taken up by living organsisms. Nor is the effects of the substances on man or other biota known. For instance, up until the implementation of the new EU chemicals legislation (The European Commission 2001), 100106 different chemicals have been used in Europe without any demands for testing of their effects on man or the environment (ECB). At the same time the number and amounts of chemicals used is steadily increasing.

When released, the chemicals may be transported (some across large distances) and many of them with lakes and oceans as the final recipient, where they can be taken up by aquatic organisms. The presence of a specific chemical in the environment or in biota is not in itself indicative of adverse effects of that chemical. Therefore a number of techniques have been established to measure and quantify the responses to chemicals in organisms. A biomarker may be defined as a biological response related to exposure to or toxic effects of environmental contaminants at the sub-individual level (Van Gestel and Van Brummelen 1996). This study aimed to develop new biomarker techniques. The techniques were later applied in a biomonitoring study and in toxicity tests of different well known contaminants, to assess their value in these contexts.

Certain criteria have been proposed for good biomarkers. First, the assay to quantify the biomarker should be robust, relatively cheap and easy to perform and the biomarker response should be sensitive to pollutant exposure in order to serve as an early warning parameter. Baseline data of biomarker should be well defined in order to distinguish between natural variability (noise) and contaminant induced stress. The impacts of confounding factors to the biomarker response and the toxicological significance of the biomarker should be known. Finally, the underlying mechanism of the relationship between biomarker response and pollutant exposure should be well established (van der Oost et al. 2003). It has also been suggested that the technique applied should be non-invasive or non-destructive (Fossi et al. 1997). Using a non-invasive sampling technique will allow for the same individuals to be sampled repeatedly and decrease the number of animals required in the assay and allow the monitoring to continue over a period of time. It also allows for biomonitoring in protected species.

Biological monitoring is a repetitive observation for defined purposes, of one or more chemical or biological elements according to a prearranged schedule over time and space, using comparable and standardized methods (definition by the United Nations Environmental Program (UNEP)). When biomarkers are used in biological monitoring, the respons to the total environmental stress experienced by feral or caged organisms is assessed. Biomarkers are sensitive indicators of the presence and toxic effects of contaminants at a critical target. This is because the primary interaction between a toxic chemical and biota occurs at the cellular (or sub-cellular) level. Implicitly, one can expect changes at the cellular level to occur before changes in a tissue or organism (Fent 2003). Thus, the use of biomarkers in monitoring have the advantage of being early warning signals towards more deliterious effects (e.g. individual death or eradication of a population) (Bucheli and Fent 1995).

Another approach towards identifying toxic effects is the use of laboratory assays, with subsequent extrapolation to field conditions. Toxicity testing in laboratories may provide information about the potential toxicity of substances and about their mode of action. *In vitro* techniques for toxicity testing are valuable because they can allow assessment of tissue and target specific effects. They are also well suited for analysis of mechanisms and dose and time dependencies. It should be noted, however, that the complexity of toxicokinetics and interactions between different chemicals inside an organism are factors that are not accounted for in *in vitro* toxicity testing (nor are general effects of the cells being removed from their host organisms).

The echinoderms is a group of animals that include, among others, the starfish (Asteroidea), sea urchins (Holothuroidea) and sea cucumbers (Echinoidea). They have a free-swimming (pelagic) larval stage, but the adult forms are sedentary. They are therefore expected to experience relatively high exposure to the many environmental contaminants that tend to accumulate in sediments. Many echinoderms also have a diet that probably make them exposed to high levels of contaminants, for instance as deposit-feeders (such as the sea cucumber *Holothuria tubulosa*) or as predators on mussels and other filter-feeders that are known to accumulate contaminants (such as the starfish *Asterias rubens*). Additionally echinoderms are invertebrate deuterostomes, they thus represent the phylogenetic link between chordates and invertebrates, and may provide information regarding the relationships and differences between these two groups. The sea urchins are used today for toxicity testing (sea urchin fertilisation test), but other groups of echinoderms have rarely been used or assessed in toxicity testing or biomonitoring perspectives.

Coelomocytes are circulating cells in the fluid filled body cavity of echinoderms. They have been ascribed a number of different functions, including digestion, storage and transport of food materials, oxygen transport, pigment biosynthesis and excretion (Boolootian 1966). Several morphogically different types of coelomocytes may be found in an echinoderm species, but in starfish the phagocytic amoebocytes are predominant, and these cells are also common in the sea cucumber coelomic fluid (Boolootian 1966). The phagocytes are the main immune effector cells in echinoderms (Smith and Davidson 1992; Gross et al. 1999), effects on phagocytes therefore may predict harmful effects on the immune system and therefore impairment in the organisms resistance to deseases.

Multixenobiotic resistance (MXR) is analogous to the multidrug resistance (MDR) described for human cancer cells and different pathogens (Kessel et al. 1968; Borst and Ouellette 1995; George 1996). MDR is caused by energy dependent pumping of drugs out of target cells (Endicott and Ling 1989), and is mediated by different ATP-dependent transmembrane proteins with an unusually broad substrate specificity (Juliano and Ling 1976; Cole et al. 1992). It has proven a major obstacle in cancer chemotherapy, therefore several agents (chemosensitisers) have been developed to reverse or inhibit the mechanism. MXR was first described in the freshwater clam *Anodonta cygnea* by Kurelec and Pivcevic (1989), and has later been identified in numerous aquatic organisms. MXR has been shown to lower intracellular concentrations of many toxins (as shown by e.g. Galgani et al. (1996) and Toomey et al.(1993)), it has therefore been proposed that MXR corresponds to a widespread defence mechanism to lower intracellular concentrations of environmental stressors (Epel 1998). MXR is found inherently, but may be induced or inhibited upon exposure to different agents (Minier et al. 1993; Kurelec 1995; Kurelec et al. 1996; Toomey et al. 1996). Because of the assumed protective role of MXR, agents that are able to inhibit the resistance are of great concern. Inhibition causes increased accumulation of several other toxicants, which then may exert effects at environmental levels that are not expected to be harmful (Kurelec 1992; Kurelec et al. 1992; Waldmann et al. 1995).

In the studies herein, an accumulation assay based on the procedure described by Smital and Kurelec (1997) is applied. The principle for this assay is fluorometric measurements of the accumulation of a fluorescent dye and MXR-substrate inside cells.

Cytotoxicity may easily be measured by using the alamar Blue^{TM} (AB) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) assays. These assays involve the addition of a non-fluorescent dye to cells in culture. The dyes are enzymatically converted

to highly fluorescent metabolites, and the extent of this conversion, which can be quantified by the metabolite's fluorescence, is a reflection of metabolic activity (AB) or membrane stability (CFDA-AM) (Schirmer et al. 1997). Compared to other classical cell viability assays (such as reduction of tetrazolium compounds or staining with fluorescent DNA-specific dyes) such fluorometric assays are simple to use. They are non-toxic to the user, they do not require any special handling or disposal methods since no radioactive nor toxic materials are used (which also makes them less costly). Finally, the assays are homogeneous in nature and can thus be adapted for large scale in vitro screening (Nakayama et al. 1997). In this study the AB assay and the CFDA-AM assay were combined by the adding of both dyes simultaneously to the same cells as described by (Schirmer et al. 1997). The combination of the two gives the possibility of assessing the connection between metabolic activity and membrane integrity of a cell, and could give a clearer picture of the cells' overall health/ viability. Additionally the time and cost of the assays are reduced.

Fluorometric assays performed with the 96-well microtiter plate format allows for rapid screening of numerous samples and may therefore be particularly useful in biomonitoring and toxicity testing. The AB/CFDA-AM cytotoxicity assay and the MXR- assay were therefore examined for compatability with coelomocyte primary cultures in 96-well microtiter plates.

The model cell system and the above mentioned assays were evaluated in a toxicity testing perspective. In order to do so a range of different (but relevant) substances were chosen as model toxicants. These toxicants include two representative brominated flame retardants (HBCD and TBBPA), two perfluorinated substances (PFNA and PFOS) and TBT. Additionaly two environmental samples, with previously described toxic potentials were applied.

The curious reader is referred to chapter 2 (Background) for a more comprehensive account of echinoderm coelomocytes, the MXR- and cytotoxicity assays and the substances tested in the toxicity tests.

This study aimed to develop and assess the suitability of a novel *in vitro* cell system for use in biomonitoring and toxicity testing. The objectives was therefore to establish a protocol for echinoderm coelomocyte sampling and conditions for coelomocyte primary cultures, to adapt cell-based bioassays with MXR activity and cell viability as endpoints for echinoderm coelomocytes, to assess the value of the methods in a biomonitoring and a toxicity testing context, and finally; to enhance the understanding of echinoderm coelomocyte responses to certain model toxicants

The work described in this manuscript was separated into three parts. First, several experiments that aimed to develop techniques for establishing coelomocyte cell cultures and preliminary screening for the applicability of two different assays were conducted. Second, the techniques and assays were applied in a field study to assess their value as tools in biomonitoring. Finally, *in vitro* toxicity tests on various well-known toxicants were conducted using the same techniques. The method development studies are described in this manuscript, while the biomonitoring and toxicity testing descriptions can be found in the article manuscripts.

2 Background

2.1 Echinoderms and coelomocytes

Many echinoderms have characteristics that are wanted for toxicity testing and biomonitoring organisms. They are geographically widespread, easily sampled and identified, relatively hardy against sampling, and they have a suitable size for laboratory maintenance. As biomonitoring species they have the additional advantages of being longlived, having sedentary behaviours and a sufficient adult size for analysis of tissue samples. The starfish *Asterias rubens* and the sea cucumber *Holothuria tubulosa* are considered key species in several communities (Zavodnik 1971; den Besten et al. 1990; Coulon 1992; Temara et al. 1996). As bottom-dwellers and suspension feeders or predators on filter-feeding organisms, many echinoderms would be expected to experience relatively high exposures to hydrophobic contaminants. Echinoderms are deuterostomes, thus they represent the phylogenetic link between chordates and evertebrates. Echinoderm responses may be a useful in elucidating the relationship between responses in the two respective groups. Despite of the above mentioned advantages, the starfish (Asteroidea) and sea cucumbers (Holothuroidea) have rarely been utilised in an ecotoxicological perspective.

Asterias rubens has been demonstrated cabable of accumulating several contaminants, among them polychlorinated biphenyls (PCBs) (den Besten et al. 1990; den Besten et al. 2001), metals (den Besten et al. 1989; Sørensen and Bjerregaard 1991; Coteur et al. 2003) polycyclic aromatic hydrocarbons (PAHs) (den Besten et al. 2001) and organochlorine pesticides (den Besten et al. 2001). While the starfish species *Leptasterias polaris*, *Asteria pectinifera* and *Asterias amurensis* accumulated tributyltin (TBT) (Bekri and Pelletier 2004; Shim et al. 2005).

Effects after contaminant exposure that have previously described in echinoderms include alkaline phosphatase activity (Temara et al. 1997), speed and quality of arm regeneration (Temara et al. 1997), effects on early development (Coteur et al. 2003), inhibition of amoebocyte reactive oxygen species (ROS)- production (Coteur et al. 2003), effects on the cytochrome P450 system (den Besten et al. 1991; den Besten et al. 1991; den Besten et al. 1993) and effects on gametogenesis (den Besten et al. 1990).

Coelomocytes are circulating cells in the coelomic fluid of the echinoderm body cavity. The coelomocytes may consist of a mixture several morphologically different types, depending on species. Asteroids have four coelomocyte types, phagocytes, colourless morula cells, small

pigment cells and hyaline plasma cells (Boolootian 1966). However, cell types other than phagocytes are not frequent in the coelomic fluids of starfish (Boolootian 1966). Numerous cell types have been described in the holothuroid coelomic fluid, but it is probable that only four basic types are common to all species; lymphocytes, phagocytes, colourless morula cells and fusiform cells (Boolootian 1966). Four additional coelomocyte types are found in some groups; coloured morula cells, haemocytes, crystal cells and vibratile cells (Boolootian 1966).

The coelomocytes have been ascribed a number of different functions, including digestion, storage and transport of food materials, oxygen transport, pigment biosynthesis and excretion (Boolootian 1966). Main focus have been on the phagocytic ability, and coelomocytes are considered to be the main effector cells of the echinoderm immune system (Wardlaw and Unkles 1978; Smith and Davidson 1992; Gross et al. 1999). Studies of echinodermal immune responses by Ilya Metchnikoff was the beginning of the field of comparative cellular immunology (Gross et al. 1999) and he was awarded the Nobel Prize in 1908 (together with Paul Ehrlich) for his ground breaking work on echinodermal models. Through introduction of rose prickles and glass rods into bipinnaria larvae of starfish, Metchnikoff could observe that mesodermal cells migrated to the injury site and encapsulated the prickle. He also demonstrated that this phenomenon occured when bacteria were introduced into the larva, and the bacteria would be neutralised by phagocytosis (Gross et al. 1999).

Pyloric caeca is the echinoderm digestive system and is the compartment most commonly studied in the existing reports on starfish (e.g. Everaarts (1998), den Besten (2001) and Temara (1998). This compartment may be expected to have higher concentrations of lipophilic xenobiotics than the coelomic fluid because or a higher lipid content. Butyltin (BT) concentrations in coelomic fluid were in fact below detection limits in a study by Bekri and Pelletier (2004). Yet, these authors were able to detect effects on immunotoxicological parameters (phagocytic activity and lysosomal stability in amoebocytes) associated to BT-accumulation in surrounding tissues. For use in biomonitoring and toxicity testing the coelomocytes have some important advantages above other tissues/cell types: They may be sampled by withdrawal of coelomic fluid, which is a very simple sampling technique compared to the dissection necessary to sample other tissues. This sampling is also non-destructive and the same animal may be sampled repeatedly (provided that the animal is given sufficient time to replace removed coelomic fluid and coelomocytes). Because the coelomocytes are the immune effector cells in echinoderms, effects on these cells may be expected to have an adverse influence on the organism.

2.2. Cytotoxicity

Cytotoxicity in these studies was evaluated with the two fluorescent dyes alamar Blue™ and 5-carboxyfluorescein diacetate acetoxymethyl ester.

Resazurin is the functional dye in the commercial reagent alamar Blue ™ (AB). Resazurin is the blue and non-fluorescent oxidised form of the dye, while the reduced form is the pink and highly fluorescent resorufin (Figure 1). The conversion of rezasurin is catalysed by cellular reductases. The location of the responsible reductases is debated. According to some authors, the enzymes catalyzing this reaction are mitochondrial oxyreductases and AB fluorescence is therefore a measure for mitochondrial function (Springer et al. 1998). However, O'Brien et al. (2000) found no evidence for a mitochondrial location of the reduction. Whichever reductase is responsible for the reduction of AB, there is a broad agreement on the applicability of AB as a metabolic activity and cell viability reporter (Page et al. 1993; Nakayama et al. 1997; O'Brien et al. 2000). Herein AB fluorescence will be referred to as a measure of metabolic activity. The AB assay is as sensitive as thymidine and tetrazolium reduction assays (Page et al. 1993; Ansar Ahmed et al. 1994). It should be noted that the assay has been reported to be cytotoxic (Squatrito et al. 1995) and fluorescence intensity is sensitive to temperature (Nakayama et al. 1997).

5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) is a non-fluorescent dye, commonly used in cell membrane integrity assessments. It is hydrolysed by intracellular non-specific esterases, to form the highly fluorescent 5-carboxyfluorescein (CF) (Figure 2). Cells with an intact membrane maintain a cytoplasmic environment that supports esterase activity, and a decline in fluorescence readings is interpreted as a loss of membrane integrity and/or cell viability (Schirmer et al. 1997).

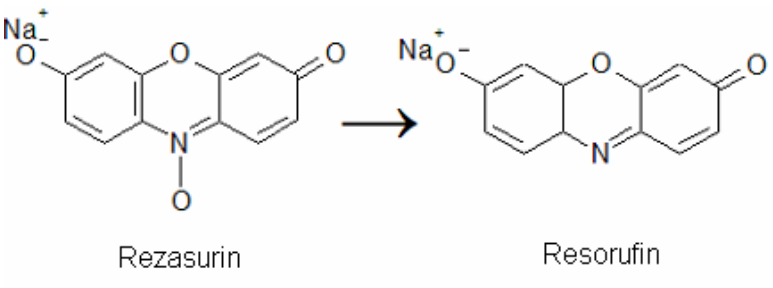

Figure 1. The enzymatic conversion of rezasurin to resorufin. Rezasurin is the functional dye within the commercial dye alamar BlueTM.

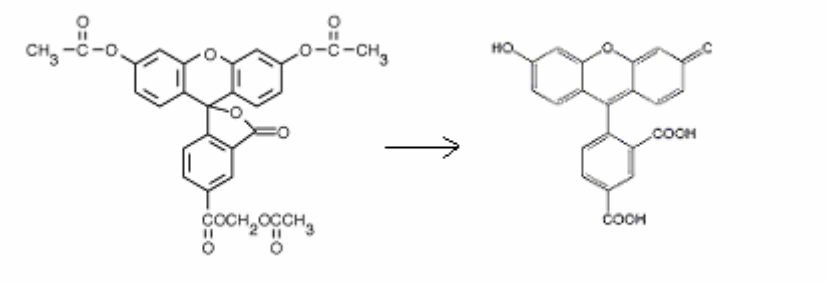

Figure 2. The enzymatic conversion of CFDA-AM to 5-carboxyfluorescein.

2.3. Multixenobiotic resistance

Multidrug resistance (MDR) was first described in mammalian tumor cell lines which were selected for resistance against a single cytotoxic agent, but showed cross resistance against a wide variety of antineoplastic drugs such as anthracyclines, *Vinca* alkaloids, epipodophyllotoxins, taxol and actinomycin D (Kessel et al. 1968; Biedler and Riehm 1970; Kessel and Bosmann 1970). MDR has later been described in numerous pathogens, among them bacteria (George 1996), protozoans (Borst and Ouellette 1995) and fungi (Prasad et al. 1995). Analysis of the drugs in MDR have revealed no common structural features, but they are often positively charged at physiogical pH and have hydrophobic regions (Gottesman and Pastan 1988; Fardel et al. 1996).

MDR was found to be mediated by certain transmembrane proteins, that catalysed an ATP-dependent efflux of the diverse drugs/xenobiotics, thus lowering their intracellular concentrations (Endicott and Ling 1989). The most common of these transport proteins are the P-glycoproteins (Juliano and Ling 1976), which belong to the ABC (ATP-binding cassette) superfamily of traffic ATPases (Doige and Luzzi Ames 1993). The multidrug resistance-assosiated protein (MRP) is another MDR-active ABC superfamily member indentified in mammalian cells (Higgins 1992).

The first evidence of a MDR-like mechanism in aquatic organisms was found in freshwater mussel *Anodonta cygnea* (Kurelec and Pivcevic 1989) and this finding may be concidered the introduction of a new field of research within ecotoxicology. Through their pionering work Kurelec and co-workers were able to define such MDR-like mechanisms in a number of different aquatic species (Appendix 4 provides an overview on observations of MXR features) and concluded that this resistance corresponds to a widespread defence mechanism. Kurelec (1992) proposed the term multixenobiotic resistance (MXR), as the resistance observed in these organisms was not confined to therapeutic agents, but also a range of contaminants.

Several naturally occurring substances are also MXR-substrates and MXR is believed to serve as a first line of defence against environmental stressors in organisms (Epel 1998).

Induction of MXR/MDR has been shown through a range of studies (e.g. Minier et al. (1993), Kurelec et al. (1995), Kurelec et al. (1996) and Minier and Moore (1996)), and may occur via multiple mechamisms including gene amplification (Roninson et al. 1986), transcriptional (Shen et al. 1986) and post-transcriptional controls (Ratnasinghe et al. 1998). The inducers of MXR-activity may or may not be substrates for the MXR-mediating proteins (Chaudhary and Roninson 1993) and MXR induction might in fact be part of a generalized defense mechanism against cell injury or DNA damage caused by cytotoxic xenobiotics (Chaudhary and Roninson 1993; Fardel et al. 1998). MXR has also been shown to increase with increasing stresses from UV radiation (Uchiumi et al. 1993) or heat shock (Chin et al. 1990).

The regulation pathways for MXR-mediating proteins are not fully understood. Deng (2001) and Mathieu (2001) demonstrated the involvement of ROS generation in MXR induction after treatment with 3-methylcholanthrene (3-MC), benzo[*a*]pyrene (BaP) or 2-acetylaminofluorene (2-AAF). The regulatory mechanism in mammals thus seemed to involve ROS generated via CYP1A after exposure to aryl hydrocarbon receptor (AhR) ligands. However, reports are conflicting when it comes to the involvement of AhR (Gant et al. 1991; Teeter et al. 1991; Bard et al. 2002). Several recent studies suggest that Pgp expression may be regulated by protein kinase C (PKC) mediated phosphorylation (Chambers et al. 1990; Ma et al. 1991);(Kurelec 1995; Miller et al. 1998). Both positive and negative correlations between PKC- and MXR activity have been reported. Nishio et al. (2005) presented results suggesting that thyroid hormone induces Pgp expression.

Multidrug resistance has been an important impedement in cancer chemotherapy, and conciderable efforts have been made to battle the phenomenon. Numerous drugs, termed chemosensitisers, have been developed to inhibit the unwanted efflux of chemotherapeutic agents, e.g. verapamil (Tsuruo et al. 1981; Yung et al. 1991), cyclosporin A (Twentyman et al. 1987; Foxwell et al. 1989) and MK-571 (David Chauvier 2002). Inhibition of the efflux of dyes that are MXR substrates (e.g. rhodamine) by these agents has been used in assays as evidence of a MXR mechanism (Figure 3). At high concentrations, the inhibitors have been reported to be cytotoxic.

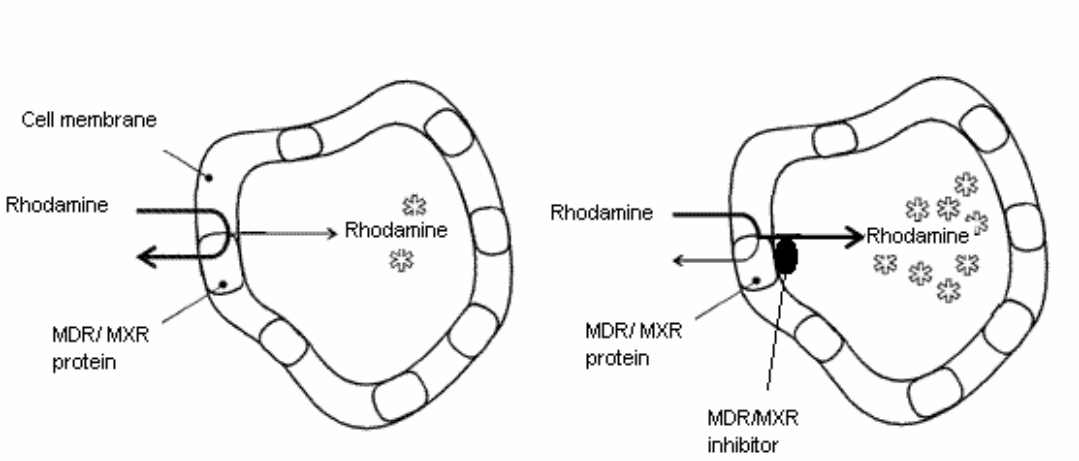

Figure 3: MXR rhodamine accumulation assay. Inhibition of MXR protein causes decreased cellular efflux of rhodamine. Intracellular rhodamine may be quantified fluorometrically, and the difference in fluorescence between non-inhibited (left) and inhibited (right) may serve a measure of MXR activity. Modified from invitrogen.com (2005).

In a rhodamine efflux assay Toomey and Epel (1993) found that bacterial metabolites from the gut of the marine worm *Urechis caupo* were also able to inhibit MXR. MXR-inhibitory potential was later found for different pesticides and 2-AAF (Toomey et al. 1996) at concentrations similar to what animals would be exposed to in the aquatic environment (Cornwall et al. 1995; Galgani et al. 1996). MXR inhibition by polluted sea- or river-water was confirmed by Smital and Kurelec (1997). Kurelec et al. (1998) were able to demonstrate that MXR inhibitory potential in muncipal wastewaters was correlated to dissolved organic carbon, but not to neither mutageneity nor levels of 48 identified polyaromatic hydrocarbons (PAHs) of the extracts. From this study, it may be interpreted that organic substances that are a part of household waste, but are not concidered to be pollutants today, have potential to disrupt MXR. Inhibition may enhance the accumulation of substances that are MXR substrates (Kurelec et al. 1995; Toomey et al. 1996). Several studies have already provided results that indicate possible ecotoxicological effects of MXR inhibitors, in which toxicity of different substances were enhanced by the presence of a MXR inhibitor (Kurelec 1992; Kurelec et al. 1992; Kurelec 1995; Waldmann et al. 1995; Kurelec et al. 1996; Kurelec et al. 1998). In sea urchin embryos, the apoptotic potency of etoposide was enhanced when it was combined with the model MXR inhibitors verapamil or reversine 205 (Smital 2004). As is the case with MXR inducers, the chemosensitisers are not necessarily substrates for MXR proteins. Examples are the PKC inhibitor staurosporine (Kurelec 1995), ATPase inhibitors and membrane fluidizers/permeabilizers (Sharom 1997). Also it is important to keep in mind

that toxic effects of a substance in the presence of a MXR inhibitor would be unexpected, because the levels of the known toxicant could be below what is established threshold values. (Smital 2004)

Assays for studies on MXR may be diveded in two categories, immunochemistry and activity assays (Bard 2000). The most common immunochemistry assay is an immunoblot assay using the murine monoclonal antibody C219 (IgG_{2a}). This antibody recognises a highly conserved amino acid sequence common to all Pgp isoforms whose sequence is known (Kartner et al. 1985; Bard et al. 2002). This implies that it will be impossible to distinguish, not only between different MXR-mediating Pgps, but also between these and Pgps with other functions, such as transport of bile acids. The second category are assays based on measurements of transport of fluorescent dyes that are known to be substrates for MXR-mediating proteins. Kurelec et al. (1996) and Smital et al. (2000) argue that MXR activity assays are superior to the immunoblot assays for several reasons. First, no specific antibodies for Pgp in aquatic organisms are developed (as of 2005). Secondly, the existing Pgp antibodies have a relatively low affinity (Epel 1998) and finally the amount of work required in immunoblots makes less suited for routine application.

The reports on MXR in echinoderms are few and ambiguous. Epel and co-workers investigated eggs of sea urchins (*Strongylocentrotus purpuratus* and *Lytechinus pictus*) and starfish (*Pisaster ochraceous*), but found no evidence for a MXR-mediating protein after activity analysis nor after immunological cross-binding analysis (Epel 1998). These authors did not look at adult tissues. However, Eufemia et al. (2002) showed vinblastine-sensitive effects on cell division in *Lytechinus pictus* embryos. Effects that were enhanced by model MXR inhibitor verapamil. Using a calcein-AM transport assay, and inhibition by the inhibitor MK-571, Smital et al. (2004) were able to detect MXR activity in sea urchin embryos. In mammalian cells calcein-AM and MK 571 are selective for the multidrug resistance-associated proteins (MRPs). In summary, MXR features (proteins, genes and/or activity) have been described in a variety of aquatic organisms, also in echinoderms (Eufemia et al. 2002; Smital et al. 2004), but to the knowledge of this author, never before in starfish or sea cucumber.

2.4. Model contaminants investigated

Brominated flame retardants (Hexabromocyclododecane and tetrabromobisphenol-A)

Tetrabromobisphenol-A (TBBPA; figure 4) and hexabromocyclododecane (HBCD, figure 5) are the two most commonly used brominated flame retardants (BFRs) in Europe, with consumption volumes of 7800 tons (TBBPA)(BSEF 2004) and 9500 tons (HBCD)(BSEF 2003) as of 2002 . Recently, the use of penta- and octamixtures of polybrominated diphenyl ethers (PBDEs) has been restricted in Europe, and there are indications that HBCD is being used as a replacement for these BFRs (Janak et al. 2005).

The majority of adverse effects of TBBPA have been found *in vitro*. TBBPA is a halogenated phenolic substance, and effects on biological membranes have been found (Inouye et al. 1979; IPCS 1995; Birnbaum and Staskal 2004). In one study TBBPA exposure resulted in haemolysis of human erythrocytes and uncoupling of the oxidative phosphorylation in rat mitochondria (Inouye et al. 1979; IPCS 1995). Also the structure of TBBPA closely resembles the structure of thyroxin (Figure 6) and endocrine effects have been described by Kitamura et al. (2002; 2005) and Meerts et al. (2000).

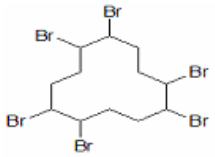

Figure 4. Hexabromocyclododecane (HBCD)

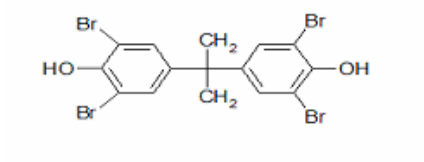

Figure 5. Tetrabromobisphenol-A (TBBPA)

Yamada-Okabe et al. (2005) found an enhancement of thyroxin receptor-mediated gene transcription and suggested that HBCD can act as an endocrine disrupter in humans and other animals, despite of lack of structural relationship with thyroxin. Mariussen and Fonnum (2003) showed inhibition of glutamate and dopamine uptake in rat brain synaptosomes after in vitro exposure to HBCD at 1 μM (significant) and 4±1 μM (IC50), proposed a neurotoxic effect for both HBCD and TBBPA. Dopamine is readily oxidised in the cytoplasm and may give rise to increased oxidative damage (Cubells et al. 1994; Mariussen and Fonnum 2003). Pullen et al. (2003) found a NOAEL of 3 μM TBBPA on cytotoxicity in splenocytes from female rats, using MTT colorimetric assay.

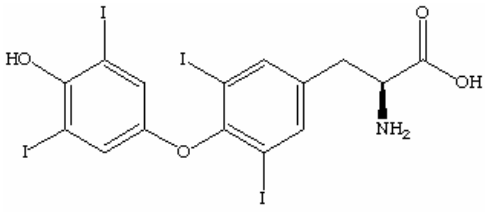

Figure 6. Thyroxin

Perfluorinated substances (Perfluorononanoic acid and perfluorooctane sulfonate)

In contrast to most contaminants, which tend to accumulate in the fatty tissues of biota, previous studies have shown that PFOS (figure 7) and related fluorochemicals (e.g. PFNA, figure 8) concentrate in both liver and in blood. (Kannan et al. 2001). Perfluorinated acids have no known route of biotic or abiotic degradation in the environment, and they are bioaccumulative when the perfluorinated chain reaches a length of between 6 and 7 carbons (Martin et al. 2003).

Certain perfluorinated acids inhibit of gap-junction intercellular communication (Upham et al. 1998) and some are tumour promoters (Biegel et al. 2001). Two other effects that are proposed to contribute to PFOS' toxicity is induction of membranous damage and disruption of the equilibrium between DNA damage and its repair processes (Hoff et al. 2003). Matsubara et al. (Matsubara et al. 2006) found effects from both PFOS and PFNA on backward swimming in *Paramecium caudatum*. This model system is considered to be sufficient for predicting the potential toxicities on ion channels. PFOS is a peroxisomal proliferator (Sohlenius et al. 1993; Berthiaume and Wallace 2002), and may interfere with the thyroid hormone economy (Thibodeaux et al. 2003).

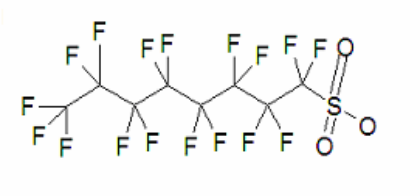

Figure 7. Perfluorooctanoic sulfonate (PFOS)

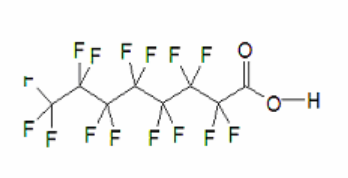

Figure 8. Perfluorononanoic acid (PFNA)

Tributyltin

Tributyltin (TBT) is a well-known endocrine disrupter (see da Silva de Assis et al. (2005) for a review on this subject), and is considered to be the main effector for the imposex observed in several marine invertebrates (Gibbs and Bryan 1986). It is suggested that TBT-induced imposex is caused by an inhibition of the CYP dependent aromatase system leading to an increase in the androgen (testosterone) level in the gastropods (Folsviksrk et al. 1999).

TBT may induce apoptosis, possibly through opening of the permeability transition pore with subsequent releasing of cytochrome *c* (Nishikimi et al. 2001), as well as necrosis (da Silva de Assis et al. 2005). However, the cytotoxic effect seems to occur at relatively high doses. Cell viability in sponge cells, was unaffected after 12 h incubation with 0.5 µg/mL TBT (Fafandel et al. 2003). TBT was found to induce apoptosis in blue mussel (*Mytilus galloprovencialis*) gills after *in vivo* exposure to a single dose 3 µg/g, but not 1 µg/g, TBT (Micic et al. 2001). In trout hepatocytes, apoptosis was not detected after exposure to 1 µM TBT for 90 minutes (Reader et al. 1999)

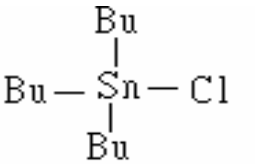

Figure 9: Tributyltin

For an overwiew of the environmental occurance and toxic effects of the different model toxicants, the reader is referred to appendix 2 and 3.

3 Materials and methods

These experiments have focused on the use of cells from echinoderms in the toxicological applications toxicity testing and biomonitoring. Two different assays, both based on fluorescence measurements on primary cell cultures, have been used in the studies. Neither of the assays has, to the knowledge of this author, been performed previously on starfish or sea cucumbers. Therefore a series of experiments that aimed to develop techniques for non-invasive cell sampling, to establish primary cell cultures of coelomocytes and to accommodate the assays to the coelomocytes from these species were performed. The method development and toxicity testing experiments took place in Norway, with coelomocytes from the starfish species *Asterias rubens*. The biomonitoring experiments were conducted in Kaštela Bay, Croatia. Here coelomocytes from the sea cucumber *Holothuria tubulosa* were used. An optimalisation for the multixenobiotic resistance- (MXR-) assay was performed for this species, while cell culture conditions (cell density and culture medium) were adopted from the pilot studies on *Asterias rubens*. Both species are native and quite common in the respective study areas.

3.1 Collection of animals and sampling areas

Oslofjord

Method development experiments were performed in November 2004 (water temperature at 5 meters depth was between 8.8°C and 11.3°C) on cells from starfish collected in September that year. Individuals of the starfish species *Asteria rubens* were hand picked at 1-2 meters depth. Toxicity testing experiments took place in June 2005 (water temperature between 7.0°C and 7.5°C), on animals hand picked by divers between 1 and 10 meters depths in February and March the same year. The animals were kept at NIVAs marine research station in Solbergstrand in 300 litre tanks with circulating water taken from the sampling area at 5 m depth. During this period the animals were fed ad libitum on mussels (*Mytilus edulis*). In both studies specimens between 5 and 10 cm measured from the base of one arm to the arm tip were used and sampling area was the same; along the shore close to Drøbak, Norway. The MXR activity level in other species has been shown to be induced with increased environmental stress. The water in which the animals were collected and kept has a relatively low pollution load. The coelomocytes from the starfish were therefore assumed to express a low, inherent level of MXR activity at the beginning of the study.

Kaštela Bay

The biomonitoring experiments took place in October 2004 in and around Kaštela Bay (total area 60 km^2, average depth 23 meters (Ujevic et al. 2000)), near Split on the coast of southern Croatia. The area of the Kaštela Bay is known as one of the most polluted areas along the eastern Adriatic coast. The environmental pollution is a consequence of fast industrialisation and urbanisation without development of appropriate urban infrastructure, in particular of a wastewater collection and disposal system (Margeta and Baric 2001). According to estimates, the highly eutrophic Kaštela Bay annually receives 32 million m^3 of untreated municipal wastewater and 20 million m^3 of partially treated industrial wastewater. (Ujevic et al. 2000). The bay is contaminated by heavy metals, particularly mercury (Margeta and Baric 2001) but also lead, cadmium and manganese (Ujevic et al. 2000).The study period is outside the *H. tubulosa* spawning season (Despalatovic et al. 2004). While the water inside the bay is heavily polluted, as described above, the area around the island Šolta outside the bay is regarded to have a relatively low pollution load and served as a control site in the experiment. The collection of animals at Šolta station was done by bottom trawling between 40 and 50 m depths, while trawling inside the bay was between 30 and 40 m depths. Upon collection, the animals were put in tanks with aerated sea surface water from the sampling site and kept there until sampling of the coelomic fluid. Sampling of coelomic fluid took place within 24 hours from capture of the animals.

3.2 Sample preparations

Coelomic fluid sampling technique and primary cell culture establishment was similar for all experiments, also for the biomonitoring experiments using sea cucumbers. The sample preparations are described below, with specifications for the different studies where necessary. Individuals that were suspected to be in bad condition, e.g. individuals that had low tube foot activity upon examination, were excluded from the study.

Coelomic fluid was extracted from the animals, using a 1 mL syringe with a 23 gauge needle. In starfish, the needle was inserted into the distal third of one arm into the coelomic cavity, an extraction technique previously described by (Bekri and Pelletier 2004). In sea cucumbers the needle was inserted directly into the body cavity at the anterior end of the animal. Both needle and syringe were pre-treated with cold phosphate buffered saline (PBS - pH=7.8; 0.1 M; 8.5 mL NaH_2PO_4 (stock 0.2 M dissolved in distilled water); 91.5 mL Na_2HPO_4 (stock 0.2 M dissolved in distilled water) in 100 mL water and adjusted for the salinity in the sampling area

with 2.4% or 3.6% w/v NaCl) as an anticoagulant buffer. The volume of fluid extracted was adjusted for individual size of the animal. Typically 0.5 mL was taken from smaller starfish and 1 mL was taken from larger starfish. 3-5 mL coelomic fluid was extracted from sea cucumbers. The extracts from each individual were mixed with cold culture medium (PBS or alternatively cold Leibowitz's L-15 medium for the culture medium experiment) by a ratio of about 1:3 (coelomic fluid:PBS) in a glass tube (all method development and optimalisation was performed on pooled samples). To further prevent aggregation of the coelomocytes, the tube was kept on ice at all times and gently turned every few minutes.

The cell density in diluted *A. rubens* samples was determined by counting particles between 11 µm and 20µm in a Coulter counter® (Multisizer™ 3, from Beckman Coulter™). The extract then diluted in a culture medium (PBS or alternatively L-15) to obtain the final wanted cell densities for the experiment. The diluted *H. tubulosa* samples were directly applied to the wells of a microtiter plate. Cell density was not established for each sample, however, in four samples cell density was determined by microscopy counting, assuring that the cell density did not exceed the interval that yielded linear fluorescence results as determined for starfish coelomocytes. The cell content was later quantified by protein measurements, using the Bradford reagent and procedure.

Two hundred µL of the cell suspension was applied to each well of a 96-well microtiter plate and the plate was incubated in the dark 24 hours (at 15°C for experiments conducted in Norway, and at room temperature in the field study in Croatia). This would allow for the cells to sink and form a confluent monolayer at the bottom of the well.

3.3 Cytotoxicity

The cytotoxicity assay was applied both during establishment of cell culture conditions (method development) and for the biomonitoring and toxicity testing studies. The procedure was the same for all experiments and is described below.

The principle of the assays is the intracellular conversion of the dyes into fluorescent products. The conversion of alamar Blue™ (AB) is catalysed by reductases, and the rate of the conversion is commonly used as a measure of metabolic activity in the cell. 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) is hydrolysed by intracellular esterases to 5-carboxyfluorescein (CF). Cells with an intact membrane maintain a cytoplasmic environment that supports esterase activity. CF-fluorescence is therefore interpreted as a measure of membrane integrity and/or cell viability (Schirmer et al. 1997).

The protocol is adapted from (Schirmer et al. 1998): Dye working solutions were prepared by adding 11.6 µL CFDA-AM stock solution (4 mM in DMSO) and 579 µL AB to every 11 mL PBS. Cell suspension was carefully removed from each well, before 100 µL of dye working solution was added. The microtiter plate was incubated for 30 minutes in room temperature on an orbital shaker set at 100 rpm. Fluorometric measurements were made on a microtiter plate reader (Cytofluor™ 2300, Millipore). Excitation and emission wavelengths were 485 nm and 530 nm respectively for CF and 530 nm and 590 nm respectively for AB. The dyes are light sensitive, and every step of the protocol was carried out in the dark.

Cell density and culture medium

Leibowitz's L-15 medium has been widely used to culture fish cells as well as cells from other aquatic species such as mussel (*Mytilus galloprovencialis*) (Takeuchi et al. 1999), shrimp (*Penaeus monodon*) (Jiraporn and Raewat 1999), and Dublin Bay prawn (*Nephrops norvegicus*) (Mulford and Austin 1998). In order to determine whether a modified L-15 medium or PBS should be used for the coelomocyte primary cultures and to define a range of cell densities in which fluorescence yield would be linear to cell density, the following experiment was performed.

A pooled coelomocyte sample was divided in two groups. One group was diluted in PBS, the other in modified L-15 medium (L-glutamine; penicillin; streptomycin; fungizone; $NaHCO_3$), as describe above, to densities of (10^4 - $2*10^4$ - $4*10^4$ - $8*10^4$) cells/200 µL with each density in 21 replicates. However, a large part of the L-15 diluted suspension had later to be discarded, and final replicate number for this sample is therefore lower and different for the different cell densities (5, 3, 3 and 7 respectively).

The microtiter plates with the cells were incubated in the dark and 15˚C for 7 days. After the incubation period, cell viability was assessed using the AB/ CFDA-AM assay described above.

Results are expressed as fluorescence units and as fluorescence divided by cell density (fluorescence per cell).

Time series

One experiment was performed to follow cell viability over a period, so as to assure that the application of a 4-day exposure period in the toxicity testing was appropriate. Using the combined AB/CFDA-AM assay, coelomocyte viability was assessed regularly over 11 days.

The sample preparations were as described above, using PBS as the culture medium and a cell density of 40000 cells/200 µL. The assay was conducted by the procedure described above.

Results were expressed as fluorescence units.

Cytotoxicity pilot study

Cytotoxicity is often expressed relative to a standard curve derived from exposure to a known cytotoxic agent such as phenol. To assess the applicability of a phenol standard curve for cytotoxicity in coelomocytes, the cells were exposed to phenol in 8 different concentrations, before a cytotoxicity assay was run.

The exposure to phenol was semi-static and performed as follows: Cells in a pooled sample were applied to the wells of a 96-well microtiter plate as described above, at a density of 40000 cells/200 µL. A phenol standard was prepared by diluting phenol stock solutions (dissolved in dimethyl sulfoxide DMSO) in PBS to final concentrations between 200 mM and 0.06 mM. 75 µL of cell suspension was removed from each well of the microtiter plate. 125 µL of each of the concentrations of phenol was applied to designated wells, each concentration in 5 replicates, to obtain final phenolic exposure concentrations of (30 – 100 – 300 – 10^3 – 3*10^3 – 10^4 – 3*10^4 – 10^5) µM in the wells. Some samples were later discarded and final replicate number was 3-5.

The plates were then incubated in the dark and 15˚C for 48 hours. After incubation, the cells were re-exposed by removing 125 µL from each well, adding 125 µL of the same phenol concentration as previously, and incubated for another 48 hours under the same conditions.

Results were expressed as fluorescence units.

3.4 Multixenobiotic resistance

The multixenobiotic resistance (MXR) assay was used to examine the existence of an MXR mechanism in coelomocytes, and later optimalised for both species used. Finally the optimalised assay was applied in biomonitoring and toxicity testing experiments. The procedure was similar for all experiments, and the procedure for the MXR pilot study is described below. Specifications for the two optimising experiments are described in the following paragraphs.

MXR pilot study

The competitive inhibition of rhodamine efflux in a model cellular system by the addition of verapamil is considered one evidence of MXR (Bard 2000). The MXR pilot study was

performed to investigate whether or not an inherent MXR mechanism exists in *Asterias rubens*, and if so, it can be measured using an assay based on rhodamine accumulation inside cells.

The principle for the assay is fluorescence measurements of intracellular concentrations of a MXR substrate (e.g. rhodamine 123). In cells with MXR activity, a certain amount of substrate will be transported out of the cells. When this transport is inhibited by verapamil (or other model MXR inhibitors), the intracellular concentration of substrate increases. The difference in rhodamine concentration when MXR activity is intact and when it is inhibited, will provide a quantitative measure for MXR activity in the cells. The procedure is based on a method described by Smital and Kurelec (1997). The original procedure described an assay for accumulation of dye in whole specimens of animals, and had to be modified for *in vitro* accumulation of dyes. Rhodamine 123 working solution was prepared by dilution of stock solution (5 mM in DMSO) in PBS, to concentrations of 0.08, 0.4 and 2 µM. Verapamil working solution concentrations were 2, 10 and 20 µM (prepared from a 5 mM stock solution in distilled water). Cell suspension was carefully removed from the wells, leaving the cell layer at the bottom. One hundred µL rhodamine 123 working solution and 100 µL verapamil (alternatively PBS for control cells) working solution in different concentrations was added to designated wells. The cells were incubated for one hour in the dark at room temperature on an orbital shaker set at 100 rpm. After incubation, extracellular rhodamine 123 was removed from the wells by the careful removal 100 µL incubation media from each well and adding 100 µL of PBS. This washing step was repeated once. Subsequently all liquid was removed from all wells and 100 µL triton x-100 (0.1% in PBS) was added to lyse the cells. Fluorescence was measured in the microtiter plate reader Cytofluor™ 2300 (Millipore). Excitation and emission wavelengths were 485 nm and 530 nm, respectively.

This pilot study was performed on a pooled coelomocyte sample, applied to a microtiter plate with a cell density of 40000 cells/200 µL. For each concentration of rhodamine 123, each concentration of inhibitor was tested twice. Controls were in 6 replicates.

Results were expressed as fluorescence units.

Optimisation of MXR for *Holothuria tubulosa*

A number of different reagents have previously been proved useful for the MXR accumulation assay for different species. Both the fluorescent substrates and the MXR inhibitors are expected to have species-specific properties, and in mammals they have been

shown to respond differently to different MXR-mediating proteins. Before the biomonitoring experiment was started, a preliminary test was conducted to determine which of the MXR reagents available, and at which concentrations, would provide the clearer results on coelomocytes from *Holothuria tubulosa*. Only animals from the reference station were included in this test.

The two dyes rhodamine B and calcein AM and the three inhibitors verapamil (non-specific), cyclosporine A (selective inhibitor of MDR-like proteins in mammals) and MK-571 (selective inhibitor of MRP-like proteins in mammals) were included in this test. Stock solutions were diluted in PBS to final concentrations of 0.1 µM for both dyes and 0, 0.1, 1 and 10 µM for all inhibitors. The optimisation was performed on a pooled sample with coelomic fluid from about 10 animals. The sample was applied to two different microtiter plates, in 36 wells on each plate. Thus the two dyes were kept on different plates. The MXR assay procedure was similar to the one described above, but with one modification: One hundred µL of incubation suspension was removed from the 72 wells, 50 µL of dye and 50 µL of inhibitor was added. Also the fluorometric readings were performed with a different microtiter plate reader, the Fluorolite 1000 (Dynatech) with excitation and emission wavelengths at 535 nm and 590 nm, respectively, for rhodamine B and 485 nm and 530 nm for calcein AM.

Every combination of dye and inhibitor was tested in triplicate. This would leave a total of 18 wells without inhibitor (9 wells for each dye). Fifty µL PBS was added to these wells, so that they served as controls.

Results were expressed as fluorescence units.

Optimisation of MXR-assay for *Asterias rubens*

In November 2004 coelomic fluid was extracted, pooled and seeded onto microtiter plates as described for the method development experiments.

The dyes rhodamine B and rhodamine 123 and the inhibitors verapamil, MK-571 and cyclosporine A were tested in the optimising study. Concentrations were as follows: Rhodamine B and rhodamine 123 0.1 µM. Verapamil, MK-571 and cyclosporine A each in 0.1, 1 and 10 µM. The procedure for the assay was as described above, with one modification: One hundred µL of the cell suspension in the well was removed and 50 µL of dye and 50 µL of the chosen inhibitor added to achieve the wanted final concentrations in the designated wells. The fluorescent substrates were added to 48 wells each. Each inhibitor was added to 12 of the 48 wells, with the three different concentrations in 4 replicates. Wells with only dye

and no inhibitor (50µL of PBS added) was in 12 replicates per dye. Excitation and emission wavelengths were 485 nm and 530 nm, respectively for rhodamine 123 and 535 nm and 590 nm, respectively for rhodamine B.
Results were expressed as fluorescence units.

3.5 Data/statistical analysis

Statistical analyses were performed with Statistica software (version 6.1; Statsoft Inc.).

Data from the MXR optimisations were checked for homogeneity of variances using Levene's test and for normality of error in a normal-probability plot before ANOVA was applied. Dunnett's test was applied as the post-hoc test. In the case of non-homogeneity of variances, data were log-transformed and Levene's test and further analyses run on the log-transformed data.
Significance level was set at $p<0.05$.

4 Results

4.1 Cytotoxicity

Cell density and culture medium

Only very small differences could be observed between cells cultured in PBS and cells in L-15 medium. This was true for both membrane stability (CF fluorescence) (Figure 10) and metabolic integrity (AB fluorescence) (Figure 11) at all the tested cell densities. At the cell density of 40000 cells/200 µL, CF fluorescence was slightly higher in the L-15 group. Regardless of culture media used, both CF and AB fluorescence were evaluated to increase in a linear manner when cell density increased in the interval 10000 to 40000 cells/200 µL. Fluorescence per cell declined in a linear manner with increasing cell density up to 40000cells/200 µL (Figure 12 and 13).

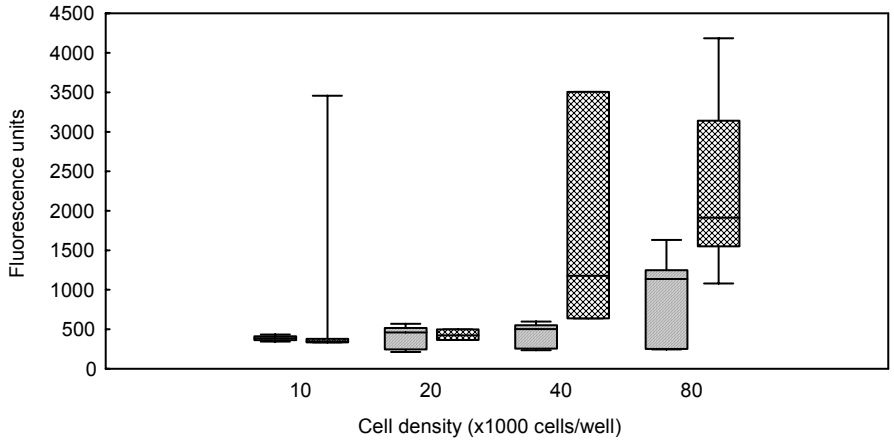

Figure10. CF fluorescence in coelomocytes cultured at different cell densities in PBS (hatched bars) or modified L-15 medium (cross-hatched bars). Depicted in plot: Median, quartiles, 10 and 90 percentiles.

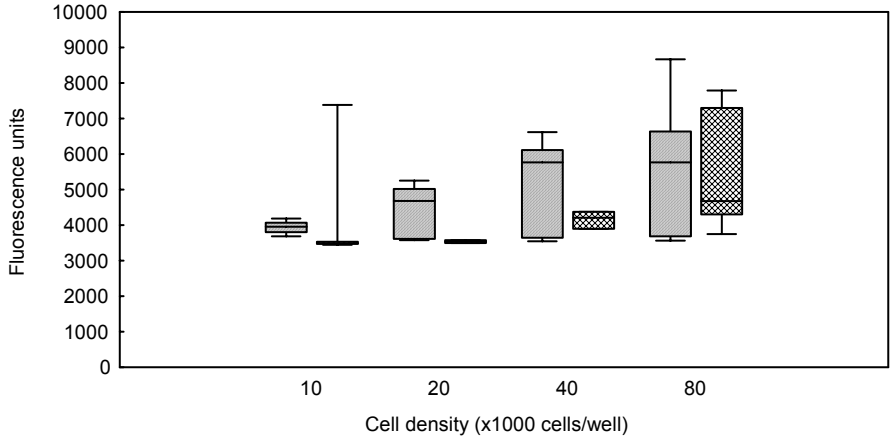

Figure 11. AB fluorescence in coelomocytes cultured at different cell densities and in PBS (hatched bars) or modified L-15 medium (cross-hatched bars). Median, quartiles, 10 and 90 percentiles.

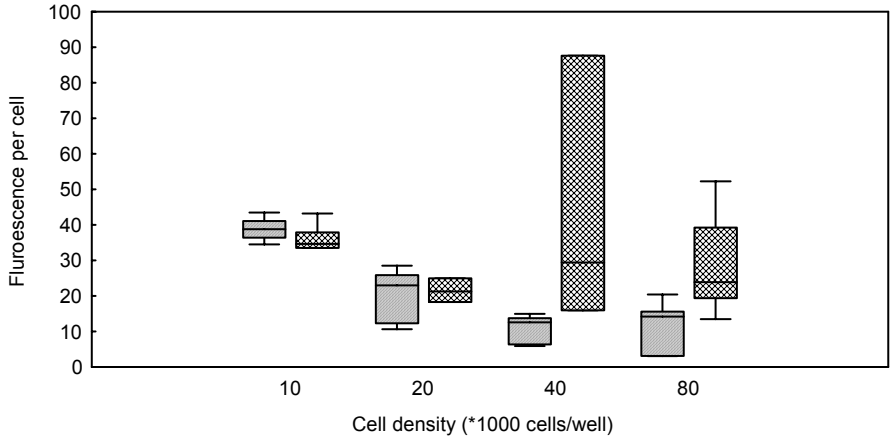

Figure 12. CF fluorescence per cell in coelomocytes cultured at different cell densities and in PBS (hatched bars) or in modified L-15 medium (cross-hatched bars). Median, quartiles, 10 and 90 percentiles.

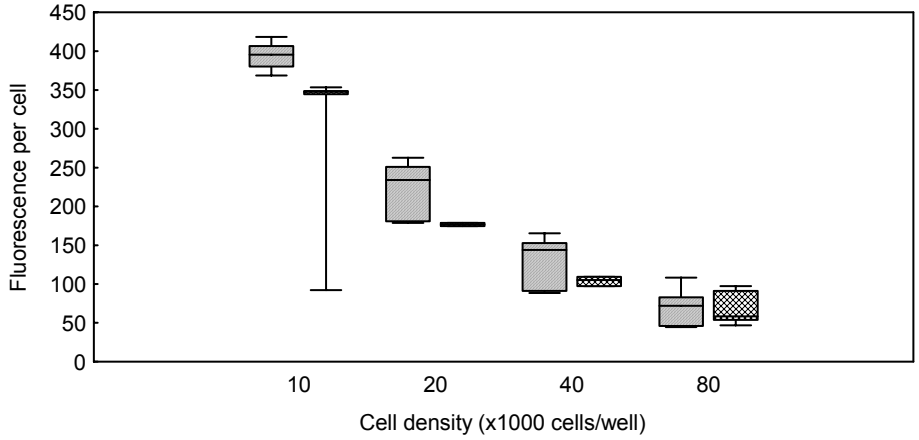

Figure 13. AB fluorescence per cell in coelomocytes cultured at different cell densities and in PBS (hatched bars) or in modified L-15 medium (cross-hatched bars). Median, quartiles, 10 and 90 percentiles.

Time series

Cell viability measured as metabolic activity and membrane integrity, was time-dependent (Figure 14). The decrease in membrane integrity was evaluated to be stable in the time interval 1-11 days. The metabolic activity had a decrease between day 1 and day 2, and was then stable through day 6. On day 11 metabolic activity increased, and was quite similar to day 1.

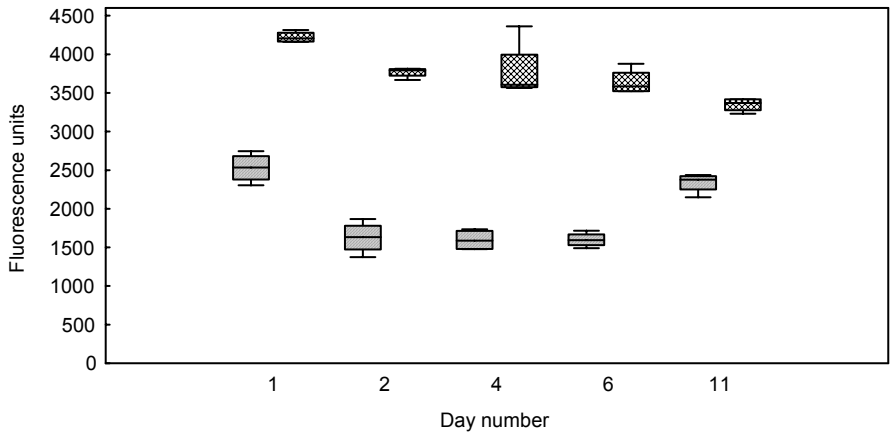

Figure 14. AB (hatched bars) and CF (cross-hatched bars) fluorescence in coelomocytes cultured for different periods of time. Depicted in plot: median, quartiles and 10 and 90 percentiles.

Cytotoxicity pilot study

Cell viability seemed negatively affected by phenol exposure. Both membrane integrity (Figure 15) and metabolic activity (Figure 16) decreased after exposure to the highest concentration of phenol applied. The other concentrations had less effect on metabolic activity, while membrane integrity was lower than control also for these cells.

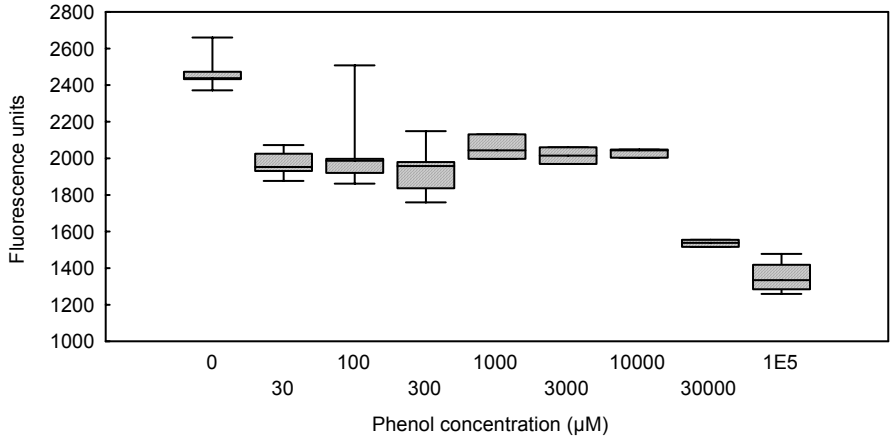

Figure 15. CF fluorescence in coelomocytes after exposure to different concentrations of phenol for 2*48 hours. Median, quartiles and 10 and 90 percentiles. Note scale break from 0 to 1000 on y-axis.

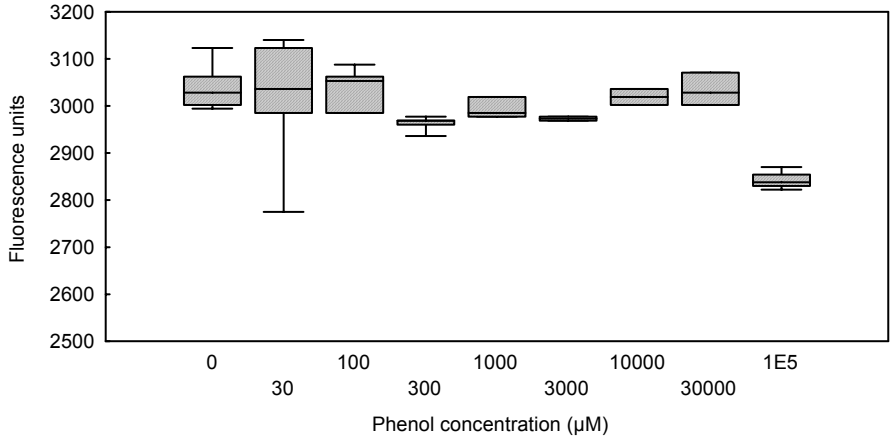

Figure 16. AB fluorescence in coelomocytes after exposure to different concentrations of phenol for 2*48 hours. Median, quartiles and 10 and 90 percentiles. Note scale break from 0 to 2500 on y-axis.

4.2 Multixenobiotic resistance

MXR pilot study

Intracellular rhodamine 123, measured as fluorescence, was not detectable after incubation with 0.04 µM dye (rhodamine 123). Cells incubated with 0.2 µM or 1 µM dye had rhodamine 123 fluorescence larger than control. However, fluorescence in the 0.2 µM incubated group, was not higher when cells were co-incubated with MXR-inhibitor (verapamil). Cells co-incubated with 1 µM dye and either 1 µM or 5 µM verapamil, had the expected higher fluorescence compared to cells incubated with 1 µM dye alone. Cells co-incubated with 10 µM verapamil had a lower fluorescence (comparable to control cells).

Optimisation of MXR-assay for *Holothuria tubulosa* coelomocytes

There was a slight, but not significant increase in rhodamine B accumulation when cells were incubated with lower concentrations of the MXR-inhibitors verapamil or cyclosporine A (Figure 17). At higher concentrations, fluorescence decreased. When calcein AM was used as the fluorescent dye, results were highly variable and the model MXR-inhibitors used in this test did not seem to affect calcein-AM accumulation (Figure 18).

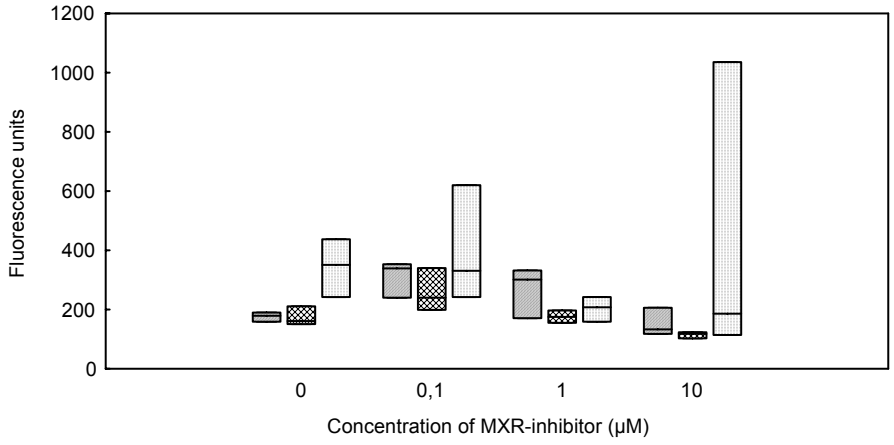

Figure 17. Rhodamine B (0.1 µM) accumulation in coelomocytes after co-incubation with the model inhibitors verapamil (hatched bars), cyclosporine A (cross-hatched bars) or MK-571 (dotted bars). Median, quartiles and 10 and 90 percentiles.

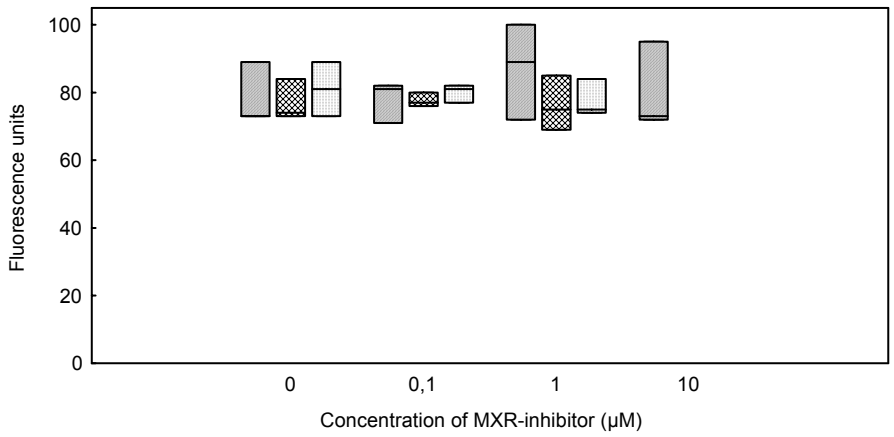

Figure 18. Calcein AM (0.1 µM) accumulation after co-incubation with the model inhibitors verapamil (hatched bars), cyclosporine A (cross-hatched bars) or MK-571 (dotted bars). Shown in plot: Median, quartiles and 10 and 90 percentiles.

Optimisation of MXR-assay for *Asterias rubens* coelomocytes

Comparing the two dyes, rhodamine B fluorescence was less variable (Figure 19) than rhodamine 123 fluorescence (Figure 20). When cells were incubated with 0.1 µM of either cyclosporine A or MK-571, rhodamine B accumulation was significantly higher then in cells that had not been incubated with MXR-inhibitor (ANOVA, $p<0.05$). Other differences were not significant.

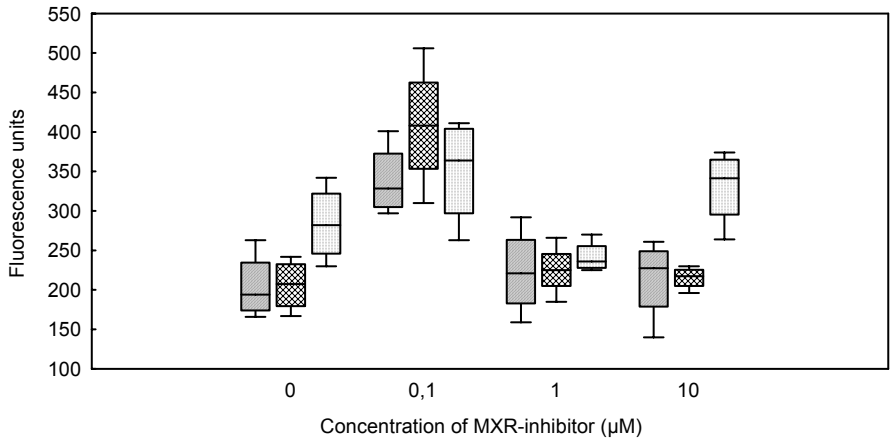

Figure 19. Rhodamine B (0.1 µM) accumulation after co-incubation with the model inhibitors cyclosporine A (hatched bars), MK-571 (cross-hatched bars) or verapamil (dotted bars). Median, quartiles and 10 and 90 percentiles.

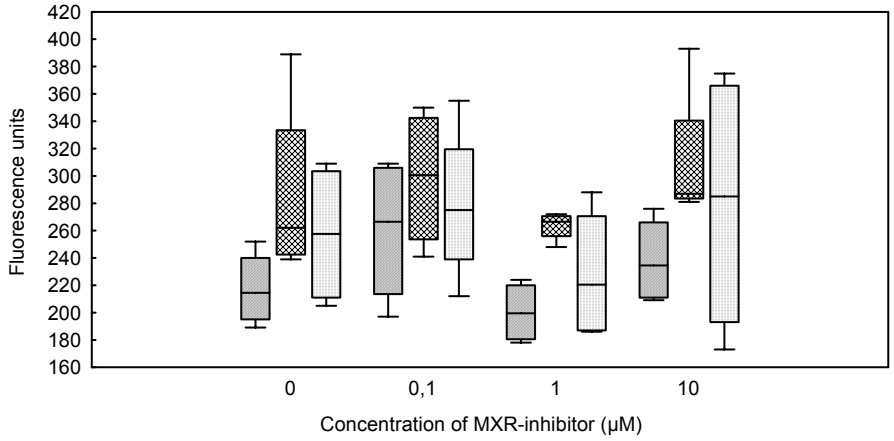

Figure 20. Rhodamine 123 (0.1µM) accumulation after co-incubation with the model inhibitors cyclosporine A (hatched bars), MK-571 (cross-hatched bars) or verapamil (dotted bars). Median, quartiles and 10 and 90 percentiles.

5 Discussion

From the preliminary studies it was concluded that coelomocytes may be cultured in microtiter plate wells and that coelomocytes respond to the AB and CFDA-AM cytotoxicity assay. Furthermore, MXR activity was found in both echinoderm species in these studies. It is, to the knowledge of this author, the first time MXR activity is shown for *A. rubens* and *H. tubulosa*, or for any starfish or sea cucumber species.

CF fluorescence was slightly higher in the L-15 group at the cell density of 40000 cells/well. Nevertheless it was decided to culture the cells in PBS. This medium provided satisfactory results in the cytotoxicity assay, and was chosen for its simplicity and low cost. Although fluorescence per cell decreased, a higher cell density yielded higher total fluorescent values, which is desirable because it is expected to increase the sensitivity of the test. The subsequent cytotoxicity development was therefore conducted with a cell density of 40000 cells/200 µL, which provided the maximal total fluorescence within the linear area of the fluorescence graph.

Up until day 6, cell viability followed the expected pattern. On day 11, an unexpected increase in metabolic activity occurred. The reason for this increase is unknown, but one possibility is a high occurrence of apoptotic cells with high metabolic activity. Regardless of reason for the increase, it may not be recommended to use coelomocyte primary cultures older than 6 days for future assessments.

Cell viability was negatively affected by phenol exposure. However, because of the poor dose-dependency for the intermediate phenol concentrations, it was decided not to use this phenolic standard curve to quantify cytotoxicity, but to apply the highest (10^5 µM), the lowest (30 µM) and one intermediate phenol concentration (300 µM) as quality assessment. Viability would then be expressed as fluorescence relative to control cells.

It was indicated in the preliminary MXR study that coelomocytes *Asterias rubens* possess an inherent MXR activity, and that this activity may be measured using a rhodamine accumulation assay. Verapamil is a non-specific MDR inhibitor (chemosensitiser) in mammalian cells, and is expected to affect all MXR mediating proteins similarly. The nature of the MXR mediating proteins that are present in echinoderms is therefore not indicated in this test. The decrease in intracellular rhodamine 123 at higher concentrations of inhibitor is interpreted as a cytotoxic effect of the inhibitors.

It was decided to use rhodamine B as the dye in MXR assay of coelomocytes from *Holothuria tubulosa*, and to apply the dye in combination with the inhibitor 0.1 µM verapamil. MXR activity in *H.tubulosa* was sensitive to both verapamil and cyclosporine A, indicating the existence of MDR-like proteins in this species. The lack of sensitivity to MK-571 and the calcein AM data is an indication that this species do not possess MRP-like proteins, or that they have low levels of this protein (/these proteins) compared to other MXR mediating proteins. The decrease in intracellular rhodamine B at higher concentrations of inhibitor is most likely due to cytotoxic effects of the inhibitors, as proposed above.

It was decided to use rhodamine B in combination with the inhibitor 0.1 µM MK-571 for the MXR assays on coelomocytes from *A. rubens*. All three model inhibitors were able to alter rhodamine B accumulation in this species, indicating the presence of both MDR-like and MRP-like proteins in *A. rubens* coelomocytes The decrease when higher concentrations of MXR inhibitor was used, is most likely due to cytotoxic effects.

In the toxicity testing experiments, MXR activity was altered after treatment with any of the six test substances. Cytotoxicity parameters were altered for three (metabolic activity) and two (membrane stability) substances. Although direct comparisons are difficult to make, the assay seems to be as sensitive as reported for other cell systems. A notable exception is for perfluorinated substances, which induced alterations at concentration lower than previously reported.

MXR-activity was significantly higher in cells from polluted sites in the biomonitoring experiment. Metabolic activity in mussel haemocytes was lower at contaminated sites, while membrane integrity was less sensitive; one of the contaminated sites had lower haemocyte membrane integrity. The results for sea cucumber coelomocytes were probably influenced by different trawling times during collection of animals; apparently cells from the reference site had lower cell viability.

In summary, the method development, toxicity testing and field studies demonstrated the applicability of the coelomocytes for use in cell based bioassays. The cytotoxicity and MXR assays may be rapid, inexpensive and easily performed alternatives in biomonitoring and toxicity testing. Both assays have provided results in a biomonitoring setting and in toxicity testing, but generally the MXR assay seems to be the more sensitive assay. However, the cytotoxicity assay may provide important information on the test system as a whole, and the importance of applying several endpoints in toxicity tests is emphasised by these authors

6 Future directions

The results presented here (including those described in the article manuscripts) holds promise for the coelomocyte model system and the cytotoxicity and MXR activity assays as tools in toxicity testing and biomonitoring. However, these studies are preliminary in nature and further caracterisations of both cells responses and methods are needed before such implementation may take place.

Firstly, the coelomocytes need to be characterised by their baseline levels and variability of MXR activity, and the proteins mediating the resistance should be identified. Although induction of activity have been described numerous times for individuals in polluted areas, no attempts have been made (to the knowlegde of this author) to relate the activity to pollution gradients. Also dose-dependencies after in vitro exposure should be assessed.

It is recommended to use inhibitor/non-inhibitor ratios when applying MXR-assays, as alterations in metabolic activity or membrane permeability may be confounding variables. One also avoids the possibility of reduced intracellular rhodamine being simply due to lower numbers of viable cells. Even when the ratio of MXR activity is applied (so that the number of viable cells at the end of the assay/incubation period is accounted for), the combination with a cytotoxicity assay offers the advantage of a more complete picture of the toxic effects on the cells.

Finally, the findings of possible adverse effects of PFNA and PFOS at extremely low (and environmentally exceeded) concentrations should be an incentive for further reasearch on perfluorinated substances.

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Article 1 Field study

Cytotoxicity and multixenobiotic resistance in haemocytes from mussels and coelomocytes from echinoderms; a field study in Kaštela Bay, Croatia

Cytotoxicity and multixenobiotic resistance in haemocytes from mussels and coelomocytes from echinoderms; a field study in Kaštela Bay, Croatia

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Abstract

Within biomonitoring the common practice is to analyze the concentrations of pollutants in the organisms. However, focus is shifting towards an approach which combines such chemical analyses with measurements of effects of the contaminants in the organisms. In this study, the objective has been to develop methods for such monitoring of effects in cells from two aquatic organisms, the blue mussel Mytilus edulis *and the sea cucumber* Holothuria tubulosa*. Both species are sedentary, common and widely distributed, but differ in habitat and feeding habits. The blue mussel inhabitates rocky bottoms and is a filter-feeder, while the sea cucumber often is found in muddy/sandy areas and is a deposit-feeder. The blue mussel is commonly used in environmental monitoring. The sea cucumber has not previously been used in a monitoring perspective. Haemocytes and coelomocytes from these species were used as biomarkers on pollution. Cell viability and MXR-activity bioassays were adapted and evaluated for use in this cell model in a field study in Kaštela Bay, Croatia. The cell viability was assessed with the two parameters metabolic activity (measured as alamar Blue-reduction capacity) and membrane integrity (measured as CFDA-AM-conversion capacity). MXR-activity is measured as capacity to extrude a fluorescent model substrate from the cell. Pollution load has previously been shown to change MXR-activity and reduce cell viability in many aquatic organisms, although the assays have not yet been incorporated in environmental monitoring practice. The study area is known to be one of the most polluted areas along the eastern Adriatic coast. Organisms in this area are expected to experience severe environmental stress from a range of different pollution categories, such as industrial waste, pesticides, heavy metals and untreated sewage. Both assays gave significant results, but the MXR-activity assay appeared to be more sensitive. However, the importance of measuring several parameters simultaneously is emphasised, as this increases the understanding of the model system as a whole.*

Introduction

Biomonitoring is a collective term for techniques that use living organisms to produce information about biotic and abiotic components of an environment. The basis for environmental monitoring today is the determination or prediction of contaminant levels in the environment, in animals and in their organs, and a comparison of these levels with threshold values and their known effects. This will provide information about which pollutants are present in the environment/ indicator species, at which concentrations and where it can be found. But the presence of pollutants in an aquatic ecosystem does not by itself indicate deleterious effects on living organisms. Measurement of biological responses to an environmental chemical at individual level or below might provide such information (van der Oost et al. 2003). A response to a chemical at this level has been defined as a biomarker by Walker et al. (2001). The notion that chemical approaches should be combined with an effect-based biomarker approach, has been promoted for more than 25 years (Alabaster and Lloyd 1980; Sarakinos et al. 2000). Yet, this has still not been fully implemented.

As the interest in an effect-based approach has increased, so have efforts to develop new and reliable biomarkers. There are certain criteria which are important when choosing a biomarker. First, the assay to quantify the biomarker should be robust, relatively cheap and easy to perform and the biomarker response should be sensitive to pollutant exposure and effects in order to serve as an early warning parameter. Baseline data of biomarker should be well defined in order to distinguish between natural variability (noise) and contaminant induced stress. The impacts of confounding factors to the biomarker response, underlying mechanism of the relationship between biomarker response and pollutant exposure and toxicological significance of the biomarker and organism should be well established (van der Oost et al. 2003). It has also been suggested that the biomarker should be non-invasive or non-destructive to allow or facilitate biological monitoring in protected or endangered species (Fossi et al. 1997). Using a non-invasive sampling technique will allow for the same individuals to be sampled repeatedly and decrease the number of animals required in the assay and allow the monitoring to continue over a period of time.

When biomarkers are used in risk assessment, ideally the same biomarker should be measured in different species to obtain insight in the variation in sensitivity (den Besten 1998). Therefore, the implementation of biomarkers in environmental monitoring requires the selection of vertebrate and invertabrate species which can be regarded as representative of the

diversity in life strategies that are found in nature (den Besten 1998). In addition to this, the species should be relatively stationary, robust against handling and culture keeping and well characterized in terms of biology and physiology so that the pattern of exposure is known and sources of uncontrolled variation can be minimized.

Bivalves are dominant members of coastal and estuarine communities and have a widespread geographical distribution. They are sedentary and relative tolerant to a wide range of environmental conditions. The populations are large and can bear repeated sampling, and they can easily be transplanted into areas of interest. Mussels are also filter feeders that pump large volumes of water and concentrate pollutant in their tissues and make it available to other trophic level in the aquatic community. They play an important part of the food supply in the predator community (Widdows and Donkin 1992).

Holothuria tubulosa inhabitates muddy, corse and fine sands and detritic bottoms, and dominates the soft-bottom community in shallow areas in the Adriatic (Zavodnik, 1971). *H. tubulosa* is a deposit feeder and by its occurence in coastal and estuarine waters it is directly exposed to anthropogenic contaminants. The contaminant exposure is expected to differ from the exposure in blue mussels: while the mussels will be exposed to the water-fraction and the dissolved organic matter (DOM)-fraction of contaminants, sea cucumbers will to a greater degree be exposed to the sediment fraction (note that this fraction also will contain DOM from the water column).

For the cell based assays in this study, hemocytes and coelomocytes were chosen as model cells. Haemocytes and coelomocytes represent the most important internal defence mechanism in marine bivalves (Gosling 2003) and echinoderms (Smith and Davidson 1992), respectively. Haemocytes are not confined to the hemolymph system, but moves freely in and out of the sinus into surroundings connective tissues, mantle cavity and gut lumen. The hemolymph also plays an important role in gas exchange, osmoregulation, nutrient distribution and elimination of wastes. The cells react to foreign substances and by phagocytosis or encapsulation, and infection is generally accompanied by intense proliferation of haemocytes. It is suggested that the intensity of the proliferation could be used as a quantifiable determination of the immunodefence response to physiological or pathological stress. Various pollutants are known to exert adverse effect on the immunity, and this can affect resistance to infection and thus influencing survival (Gosling 2003). Coelomocytes are circulating cells in the echinoderm body cavity. Sea urchin coelomocytes have been shown to respond to stress conditions (Matranga *et al.* 2000) and has also been used as indicators of

pollution (Matranga *et al*. 2000). To our knowledge, coelomocytes from holothurians have not previously been used as models for contamination effects.

Assays of cell viability assays have a broad application both within cell culturing and in the use of *in vitro* toxicology. In this experiment, the two molecular probes alamar Blue™ and CFDA-AM were used in a cell viability assay. Alamar Blue™ is reduced by cellular reductases, possibly by diaphorases or by NADH dehydrogenase (O'Brien et al. 2000) to form a fluorescent product. The reduction has been believed to take place on the mitochondria of the cells, but no evidence for this was found by O'Brien et al (2000). The probe is water soluble and can diffuse freely along the concentration gradient in both reduced and oxidized form. Herein, alamar Blue™ fluorescence will be referred to as the metabolic activity of the cells. The esterase activity is measured by the molecular probe 5-carboxyfluorescein diacetate, acetoxymethyl ester, CFDA-AM. This probe is converted by non-specific esterases in living cells to a non-polar, fluorescent dye. The substrate thus diffuses rapidly into the cell while the product diffuses slowly out of the cells and the amount of product can be read fluorometrically. The fluorescence readings will reflect the membrane integrity of the cells. This is either because of reduced esterase activity when the cytoplasmic environment is disturbed in cells with lower membrane stability, or because metabolised CFDA-AM diffuses more rapidly from cells with lower membrane integrity. Because the two probes are measured at different emission wavelengths both dyes can be added together to perform the assay in one single step, and provide a measure for cell viability. This will both reduce time and cost spent on the assay. The probes do not have to be removed from the wells, and the assay may be conducted immediately after the incubation without further procedures (Ganassin 2000).

The ability of aquatic organism to live and reproduce in polluted areas, and hold cell and tissue levels of contaminants below observed concentration in the surrounding environment indicates a well working defence system. It has been shown that aquatic organisms possess a mechanism similar to the multidrug resistance phenomenon observed in tumour cells resistant to anti-cancer drug (Kurelec and Pivcevic 1991; Kurelec 1992; Minier and Galgani 1995; Keppler and Ringwood 2001). Induction of this mechanism, named multixenobiotic resistance (MXR), has been reported in numerous studies of aquatic organism after exposure in laboratory or in environment by anthropogenic contaminants or natural stress (Kurelec et al. 1995; Minier and Moore 1996; Eufemia 2000; Minier 2000; Smital et al. 2003). If the cells have a mechanism to remove harmful compounds from their environment it might prevent injurious effects. MXR is caused by energy-dependent pumping of substances out of the cells.

This removal is conducted by certain transmembrane proteins, probably can several different proteins act at the same time in one cell and the substrates are both endogenous chemicals and xenobiotics. The accumulation and toxic effects of substances in the cells, can thus be prevented (Kurelec 1992). The protective role of the defence appears to be fragile: As opposed to MXR induction, it is also demonstrated that there are many classes of chemicals which are capable of inhibiting the MXR function. These are referred to as chemosensitisers, and can be environmentally hazardous chemicals, because they may lead to accumulation of xenobiotics and elevate internal levels of toxins in organisms (Smital and Kurelec 1998). Elevated MXR-activity might occur via multiple mechanisms, and the factors to explain it are not fully understood (Bard 2000). The induction or inhibition of MXR has not yet been established as a biomarker on environmental pollution (van der Oost et al. 2003).

The objectives of this study were to develop non-invasive biomonitoring techniques, using coelomocytes from echinoderms and haemocytes from bivalve molluscs as models and to adapt cell-based bioassays with MXR-activity and cell viability as endpoints.

Materials and methods

Study area

The study took place in October 2004 in and around Kaštela Bay (Figure 1). Kaštela Bay is the largest bay in the central part of Croatian coast with a total surface area of 60 km^2 and an average depth 23 meters Annual mean salinity is around 34‰ in the surface layer and 37‰ in bottom layers and the water renewal period of the entire bay is 1 month (Ujevic et al. 2000). The bay is known as one of the most polluted areas of the eastern Adriatic coast, largely as a consequence of fast industrialization and urbanization without development of appropriate urban infrastructure, in particular of a wastewater collection and disposal system (Margeta and Baric 2001). Today approximately 32 million m^3 of untreated municipal waste water and 20 million m^3 of partially treated industrial waste waters are discharged into the bay annually (Ujevic et al. 2000). Major industries located along the coast are shipbuilding, cement production, chemical factories and metal processing. In the towns of Kaštela and Trogir only 25% of the inhabitants are served by municipal sewage treatment works. The bay is contaminated by heavy metals, particularly mercury (Margeta and Baric 2001), but also by lead, cadmium and manganese (Ujevic et al. 2000). After chemical analysis of the tissues from *M. galloprovincialis* from the bay, high concentrations of heavy metals, PCBs and PAHs were found (Ruus, personal

communication). While the water inside the bay is heavily polluted, the area around Šolta is viewed as having a relatively low pollution load and served as a control site for the study.

Collection of blue mussels and sea cucumbers

Mytilus galloprovincialis were collected at five different locations inside and outside the Kaštela Bay (figure 1). The shipbuilding yard in Trogir, Adriavinil, which is close to an old factory believed to be a source of mercury, and Vranjic, close to the main sewage discharge from Split. All three stations are located inside the bay. Mussels from these places would be exposed to a range of pollutants such as mercury and lead, antifouling containing organic tin compounds, pharmaceuticals and halogenated hydrocarbons. Outside the bay, cultured mussels from a shellfish farm and from the island of Šolta were used. Ten mussels were sampled from each station. At the shipbuilding yard in Trogir only five were sampled because of high mortality among the caged animals. The study period was outside the spawning season for *M. galloprovincialis*. The sizes of the mussels were 4-8 cm. The mussels were kept in big tanks with aerated surface water from the Šolta sampling site overnight.

Sea cucumbers (*Holothuria tubulosa*) were collected by bottom trawling at two locations, one inside Kaštela Bay and one close to the island Šolta (Figure 1). The study period is outside the *H. tubulosa* spawning season (Despalatovic et al. 2004). The collection of animals at Šolta station was done between 40 and 50 m depth. In Kaštela Bay, collection was done at 30-40 meters. The animals were kept in tanks with aerated surface water from the respective sites until sampling of the coelomic fluid.

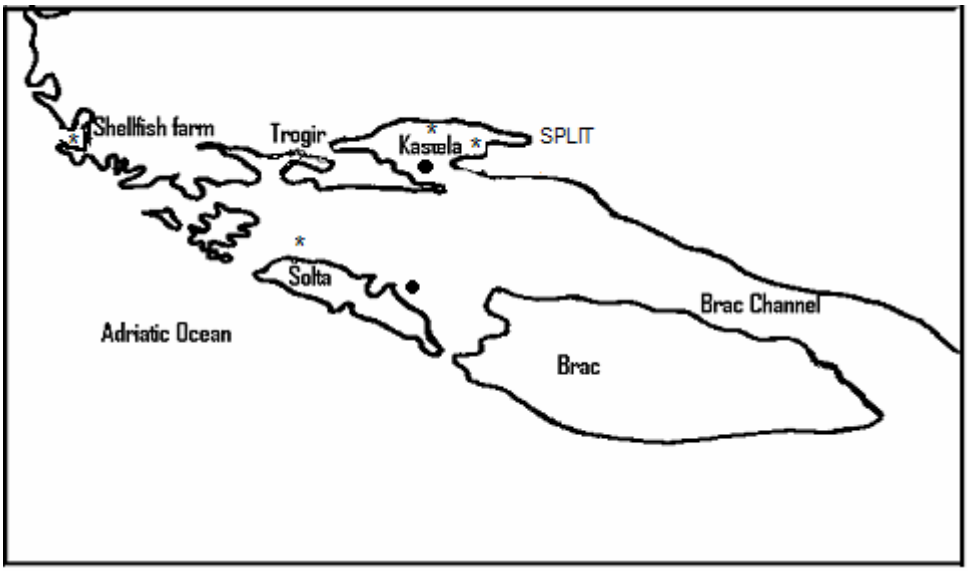

Figure 4: Kaštela Bay and surroundings, Croatia; sampling locations are indicated by stars (mussels) and circles (sea cucumbers).

Sample preparations

Sampling of hemolymph from blue mussels and coelomic fluid from sea cucumbers took place within 24 hours from collection of the animals. The sampling was done using a 1 ml syringe with a 23 gauge needle, both pre-treated with cold PBS (36‰ w/v NaCl). Hemolymph from the blue mussels was withdrawn from the posterior adductor muscle and put in individual glass tubes containing 1 ml PBS (36‰ w/v NaCl) and the sample was further diluted with an equal amount of PBS (36‰ w/v NaCl). From the sea cucumbers, 3-5 mL of coelomic fluid was withdrawn from each individual and the coelomic fluid diluted 1:4 in cold PBS in separate glass tubes. Cell suspension from each individual was seeded into the wells of 96 well microtiter plates, with 200 µl pr well, in 8 replicates pr. individual. The cells in the microtiter plates were then incubated in the dark at room temperature for 24 h to achieve a confluent monolayer at the bottom of each well.

Quantification of haemocytes

Each individual sample was fixed in paraformaldehyde (50:150 µl) and stored in 1.5 mL eppendorf tubes. Quantification of the haemocytes in this fixed cell suspension was done using a Multisizer^{TM} 3 Coulter counter with size settings ranging from 3-15 µm.

Quantification of coelomocytes

Coelomocytes were quantified using the commercial kit Quick Start™ Bradford protein assay from Biorad using Bovine serum albumin standards (Bradford 1976).

Cytotoxicity assay

Cell viability was assessed by the assay simultaneously using alamar Blue™ for metabolic integrity and CFDA-AM for membrane integrity. The protocol is adapted from (Schirmer et al. 1998): After 72 hours the media was carefully removed from the wells and 100 µl PBS (36‰ w/v NaCl) containing 4µM CFDA-AM and 5% (v/v) alamar Blue™ was added to each well. The microtiter plates were incubated for 30 minutes in room temperature in the dark. The fluorometric readings were performed on the plate reader Fluorolite 1000 Dynatech. Excitation and emission wavelengths were 485nm and 530nm respectively for CFDA-AM and 530 nm and 590 nm respectively for alamar Blue™. The dyes are light sensitive, and every step of the protocol was carried out in the dark.

Results were expressed separately for the two probes, and on the mean of replicates for each individual. Different parameters for membrane integrity and metabolic integrity can be derived from the fluorometric readings. For blue mussels the parameters used are fluorescence values relative to the median of the fluorescence values for cells from the control site Šolta. For sea cucumbers the parameters used are fluorescence relative to protein content of the individual samples.

Quality control of the cytotoxicity assay

An internal standard followed all microtiter plates for the cytotoxicity assay, so that variation between different plates could be monitored.

Multixenobiotic resistance assay

In order to find the most suitable MXR reagents among the reagents available for the species in this study, as well as the best suited concentration of inhibitor, a preliminary test was conducted. The three dyes rhodamine B, rhodamine 123 and calcein AM and the three MXR-inhibitors verapamil, cyclosporine A and MK571 were included in this test. Final concentrations were 0.1 µM for all three dyes and 0.1 µM, 1 µM and 5 µM, respectively, for all three MXR-inhibitors. The test was conducted on pooled hemolymph from the shellfish farm and pooled coelomic fluid from sampling site Šolta. All combinations of dye and inhibitor were tested in triplicates.

The preliminary tests lead to the decision to use different procedures for the MXR-assay for the two model species. For blue mussels preliminary testing resulted in the use of rhodamine123 (0.1 µM) as a dye, and no MXR-inhibitor. The assay was performed on individual samples. 20 µl media from each well was removed and replaced with 20 µL rhodamine 123 to obtain final wanted concentrations in the wells. After 1 hour incubation in the dark at room temperature, the cells were washed by removing 100 µL solution from each well and replace it with 100 µL PBS (36 ‰ w/v NaCl). This washing step was repeated. After the second washing step, all solution was carefully removed from each well, and 100 µL triton X-100 (0.1% v/v in PBS) was added. Fluorescence measurements were performed on the fluorescence plate reader, with excitation and emission wavelengths of 485 nm and 530 nm, respectively. MXR-activity was expressed relative to the reference site Šolta, which was done by dividing the median of fluorescence values for individuals by a random chosen value originating from the control site Šolta.

For sea cucumbers the assay on was performed on individual samples, using 0.1µM rhodamine B and 0.1 µM cyclosporine A. One hundred µL of the cell suspension in the well was removed and 50 µl of dye and 50 µl of the chosen inhibitor were added to achieve the wanted final concentrations in the designated wells. The rinsing and fluorometric readings were as described for blue mussels. Fluorescence measurements were adjusted for protein content in the respective sample. Some samples had to be discarded and the final number of individuals was 10 from Šolta and 5 from Kaštela. Each individual was analysed in four replicates, and analyses are performed on the mean of these replicates. MXR-activity is expressed as the ratio of fluorescence measurements when no model Pgp-inhibitor is present and measurements with the model Pgp-inhibitor cyclosporine A present.

Quality control for the MXR-assay

An internal standard followed all microtiter plates for the MXR-assays, so that variation between different plates could be monitored.

Statistical analysis

Statistical analyses were performed with Statistica 6.0 from StatSoft, Inc.

H_{0a}: “There are no differences between cells originating from blue mussels sampled at the different stations”. The data were analysed with Kruskall-Wallis analysis. Significant level for the rejection of H_0 was set to $p \leq 0.012$ according to Bonferroni correction with n equals 5 (Fisher and van Belle 1993) .

H_{0b}: "There are no differences between cells originating from sea cucumbers collected near Šolta and in Kaštela". Level of significance for the rejection of H_0 was set to $p<0.05$. Normality of distribution for each sampling station was assessed in a normal-probability plot. Data that were found to be normally distributed were analysed using t-test. Data that were found not to be normally distributed were log-transformed and log-transformed data assessed for normality of distribution prior to t-test.

Results

Quantification of haemocytes

All results for haemocyte content and shell size were expressed relative to values from randomly chosen individuals collected at the reference site Šolta. The cultured mussels from the shellfish farm had a significantly higher concentration of haemocytes in their hemolymph compared to Šolta mussels (Kruskall-Wallis test, p=0.0009) and the highest median concentration of all stations (figure 3). These mussels also had a significantly larger shell size (figure 2) (Kruskall-Wallis test, p= 0.0006). The animals from Adriavinil were significantly smaller than the Šolta mussels (Kruskall-Wallis test, p=0.0013) Concentration of haemocytes in the hemolymph did not show any significant differences. Animals from Vranjic had a significantly higher concentration of haemocytes (Kruskall-Wallis test, p=0.0012). The size was not significantly different from the reference. From the shipbuilding yard in Trogir no significant differences in size or concentration compared to reference were found.

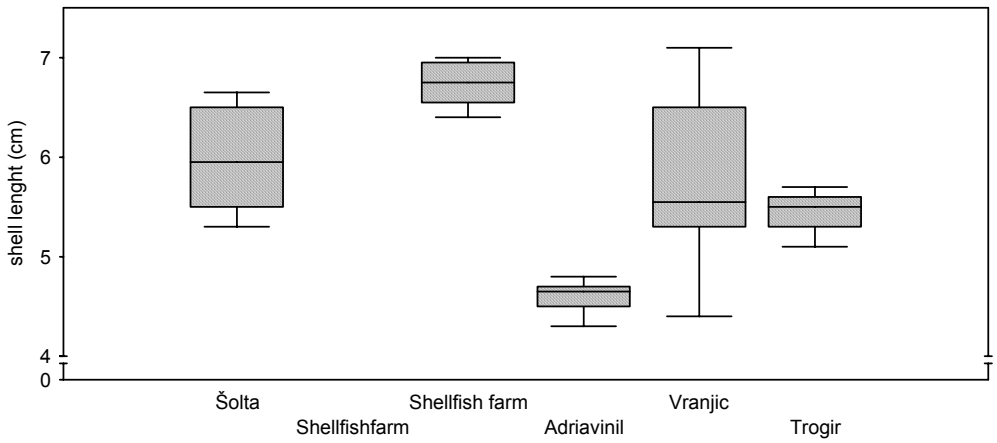

Figure 5. Length of the shell from mussels (*Mytilus edulis*) collected at five different sampling sites (n=10, except from Trogir station: n=5). Lines are median length, boxes are quartiles and whiskers are 10 and 90 percentiles. Scale break from 0.5 to 4.0 cm on the Y-axis.

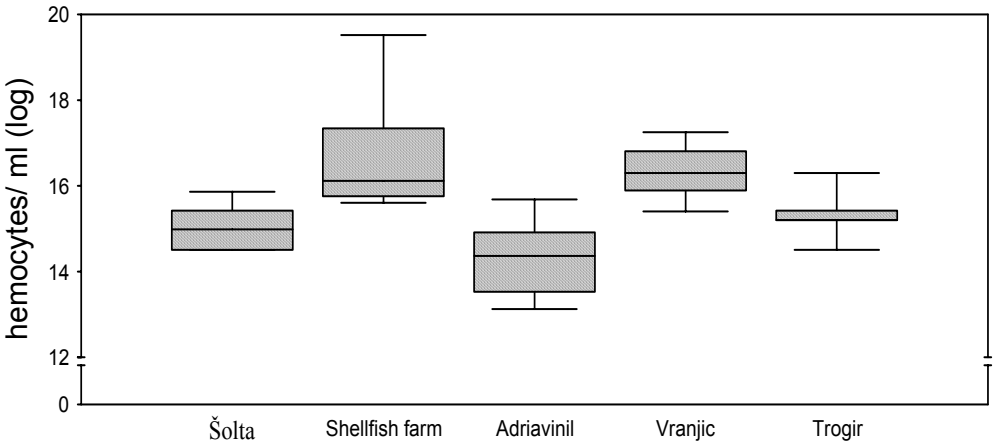

Figure 6. Concentration of haemocytes in the hemolymph in animals from five different sampling sites. Lines are median concentration, boxes are quartiles and whiskers are 10 and 90 percentiles. Scale break from 1 to 12 cm on the Y-axis.

Quantification of coelomocytes

No significant differences between stations in protein content in the samples were found in this experiment (figure 4).

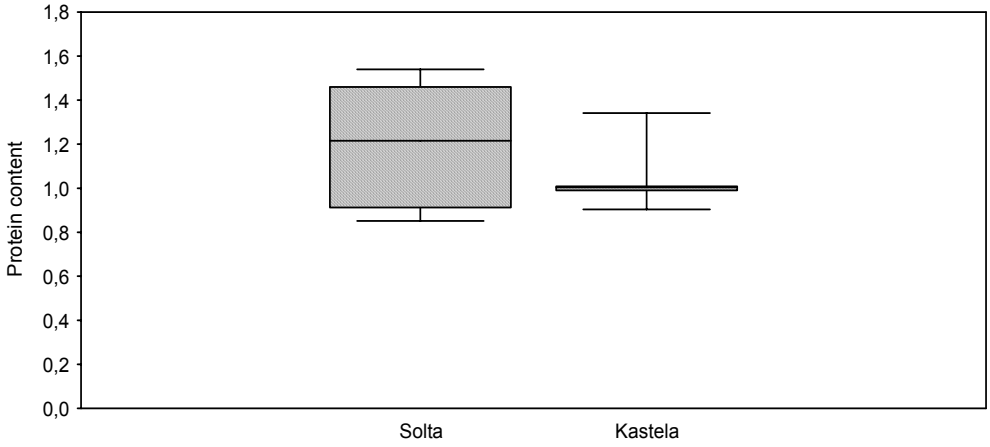

Figure 7: Protein content in sea cucumber (*Holothuria tubulosa*) coelomic fluid samples taken from individuals at sampling station Šolta (n=10) and sampling station Kaštela (n=5). Lines are medians, boxes are quartiles and whiskers are 10 and 90 percentiles.

Cell viability assay

Results for the cell viability assay on mussels are expressed relative to the median values from the reference site Šolta. Haemocytes from the mussels collected at the island of Šolta (figure 5) had the highest metabolic integrity, and haemocytes from animals collected at Adriavinil showed the lowest activity with 0.6 of the activity compared to Šolta (Significantly different, Kruskall-Wallis test, p=0.0002). From the shipbuilding yard in Trogir haemocytes had 0.7 metabolic integrity compared to Šolta (Kruskall-Wallis test, p=0.0002) At the site Vranjic the haemocytes had an activity at 0.8 (Significantly different, Kruskall-Wallis test, p=0.0001).There were not found any significant differences in metabolism were between haemocytes from cultured mussels from the shellfish farm and mussels from Šolta.

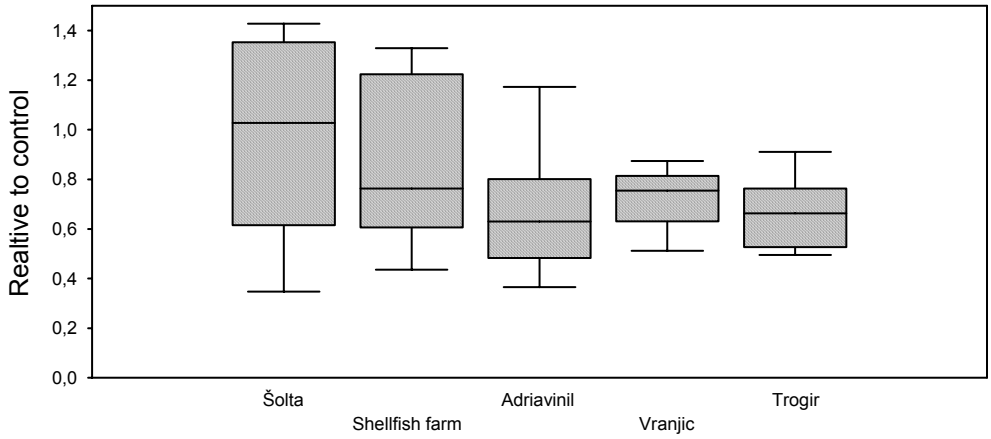

Figure 8: Metabolic integrity measured at five different sampling sites. Metabolic integrity in haemocytes was measured by the molecular probe alamar Blue. Lines are medians, boxes are quartiles and whiskers are 10 and 90 percentiles.

Membrane integrity had a large variation from in the four sites Adriavinil, Šolta, Shellfish farm and Vranjic, but only in the haemocytes sampled from the caged mussels in Trogir had a significant lower integrity compared with integrity in mussels from Šolta (Kruskall-Wallis test, p=0.001) The membrane integrity being 0.2 of the values from Šolta (figure 6).

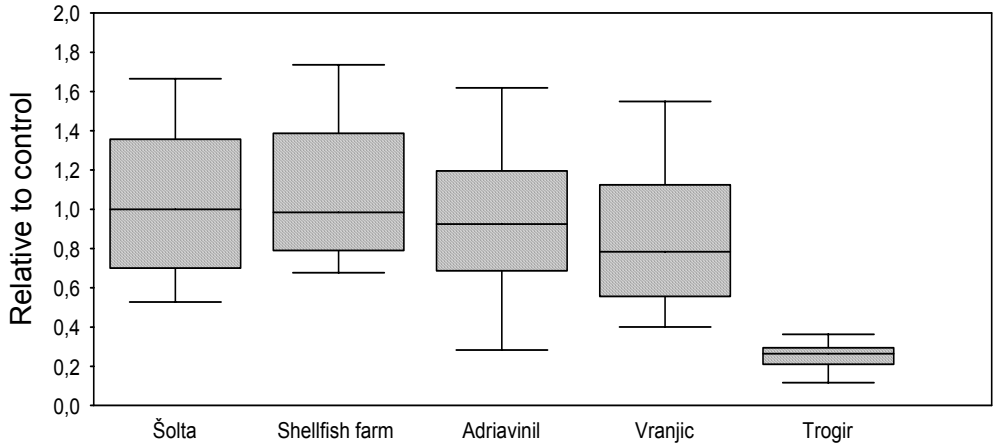

Figure 9: Membrane integrity measured at five different sampling sites. Membrane integrity in haemocytes was measured by the molecular probe CFDA-AM. Lines are medians, boxes are quartiles and whiskers are 10 and 90 percentiles.

Metabolic integrity in sea cucumber coelomocytes have been expressed as alamar Blue^{TM} fluorescence units relative to protein content of the individual samples (figure 7). Metabolic integrity in sea cucumber coelomocytes was found to be normally distributed in a normal-probability plot. Metabolic integrity was significantly higher in Kaštela cucumbers compared to Šolta cucumbers (t-test; p=0.000002).

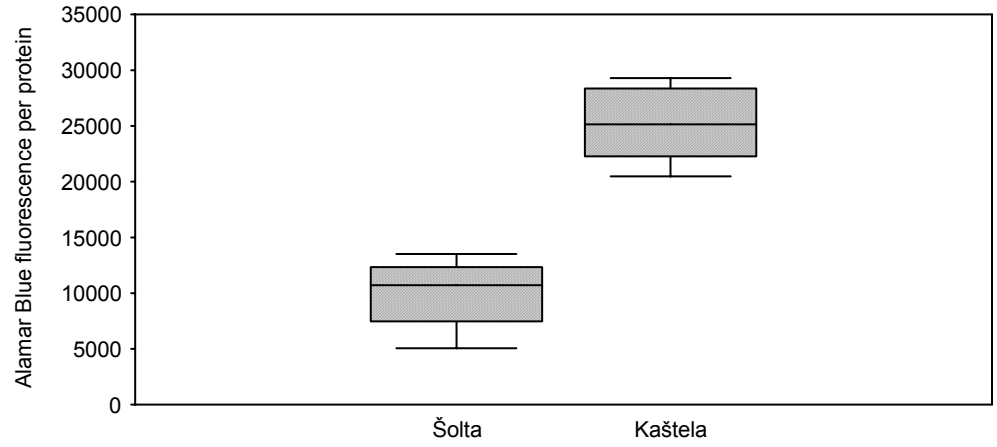

Figure 10: Metabolic integrity measured in sea cucumber (*Holothuria tubulosa*) cells taken from individuals sampling station Šolta (n=10) and sampling station Kaštela (n=5). Metabolic integrity given as alamar Blue fluorescence units per protein content in the sample. Lines are medians, boxes are quartiles and whiskers are 10 and 90 percentiles.

Membrane integrity in sea cucumber coelomocytes is expressed as CFDA-AM fluorescence units relative to protein content of the individual samples (figure 8). Membrane integrity in sea cucumber coelomocytes was found to be normally distributed in a normal-probability plot. Membrane integrity was significantly higher in Kaštela cucumbers (t-test: p=0.000007).

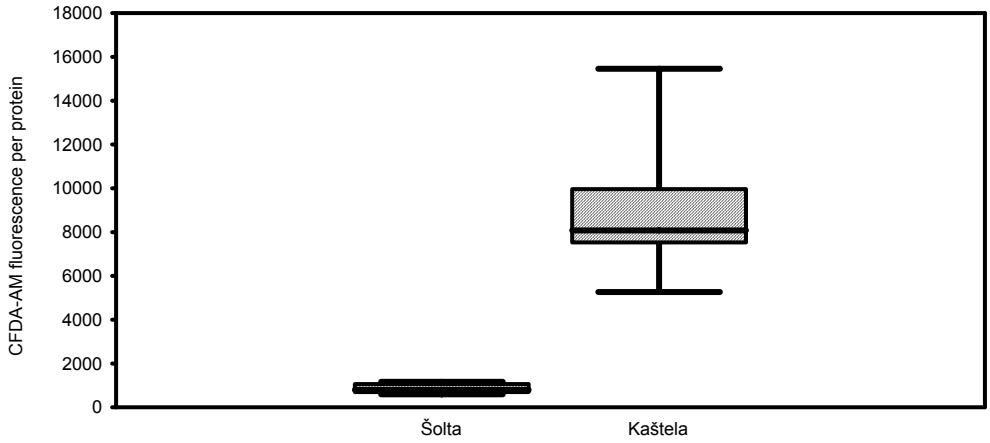

Figure 11: Membrane integrity measured in sea cucumber (*Holothuria tubulosa*) cells taken from individuals sampling station Šolta (n=10) and sampling station Kaštela (n=5). Membrane integrity given as CFDA-AM fluorescence units per protein content in the sample. Lines are medians, boxes are quartiles and whiskers are 10 and 90 percentiles.

Multixenobiotic Resistance Assay

Fluorescence of the accumulated substrate rhodamine 123 in haemocytes is related to MXR activity with high fluorescence in cells with high concentration of substrate rhodamine 123 and low MXR activity (figure 9). Mussels from the unexposed island Šolta had the highest accumulation of the substrate rhodamine 123 in the haemocytes. The mussels from Trogir had accumulated an average about 0.6 of the possible substrate, and was significantly different from control cells (Kruskall-Wallis test, p= 0.001). The cultured shell from the shellfish farm had a MXR activity ratio at 0.4 compared to control (Kruskall-Wallis test, p= 0.001), the mussels from Adriavinil had 0.3 compared to control (Kruskall-Wallis test, p= 0.001) and from Vranjic 0.2 compared with control (Kruskall-Wallis test, p= 0.001). There were no differences in the ratio of substrate in the four sites Shellfish farm, Adriavinil, Vranjic or Trogir.

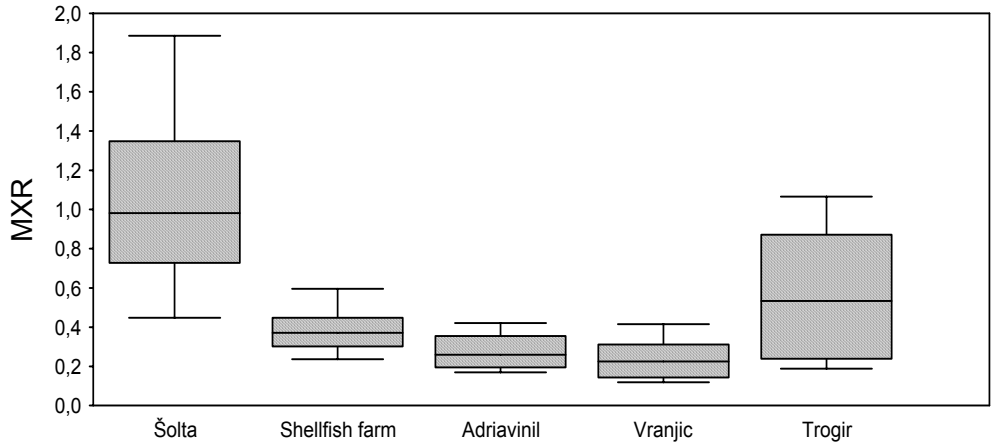

Figure 12: Accumulated rhodamine 123 in mussels from five different sampling sites. Content of rhodamine indicates degree of MXR activity with low content of rhodamine 123 as a response to high MXR activity. The fluorescence from accumulated rhodamine 123 was related to the median accumulated rhodamine 123 in mussels from Šolta. Lines are medians, boxes are quartiles and whiskers are 10 and 90 percentiles.

Significant differences were found in median rhodamine B fluorescence values in sea cucumber coelomocytes between different stations (figure 10). Both fluorescence measurements with inhibitor present and the ratio of the two fluorescence measurements showed significant differences between stations. Fluorescence with inhibitor was significantly higher at the Kaštela station compared to the Šolta station (t-test; p=0.028187). No significant differences were found in fluorescence without Pgp-inhibitor present.

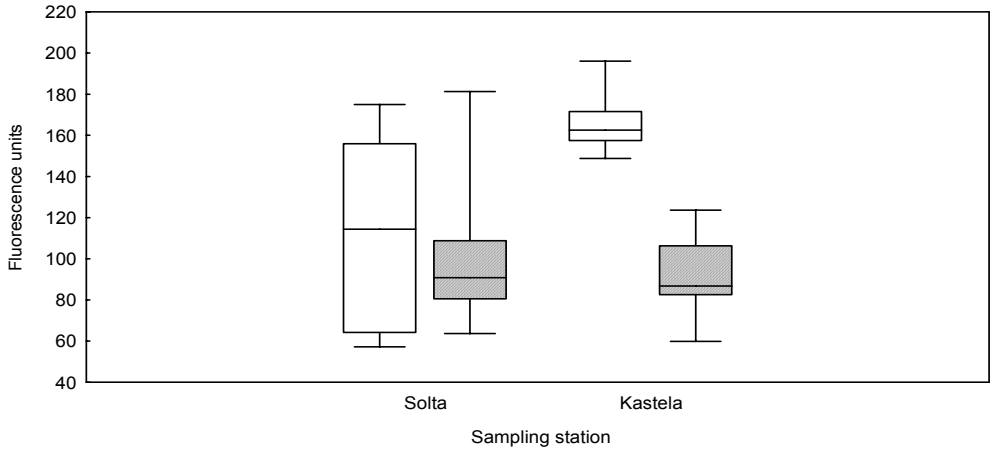

Figure 13: Rhodamine B fluorescence measurements on sea cucumber (*Holothuria tubulosa*) cells taken from individuals sampling station Šolta (n=10) and sampling station Kaštela (n=5). Fluorescence measurements with Pgp-inhibitor present (open boxes) and fluorescence measurements without Pgp-inhibitor present (shaded boxes). Lines are median fluorescence values, boxes are quartiles and whiskers are 10 and 90 percentiles.

The ratio of the two fluorescence measurements (figure 11) was significantly lower at the Kaštela station compared to the Šolta station (t-test; p=0.020010). The inversion of this ratio provides a measure of MXR-activity in the organisms at the stations, this activity thus being higher in the Kaštela station compared to the Šolta station.

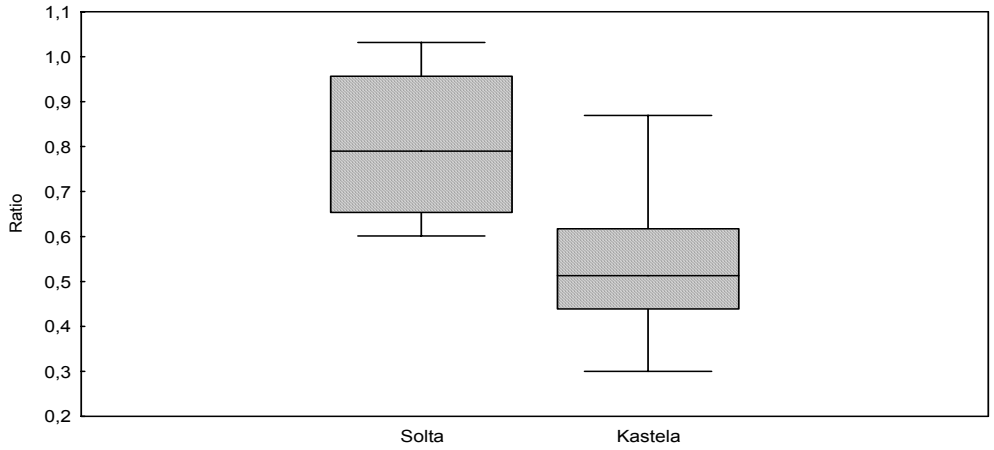

Figure 14: The ratio of Rhodamine B fluorescence measurements made on sea cucumber (Holothuria tubulosa) cells taken from individuals from station Šolta (n=10) and station Kaštela (n=5) without Pgp-inhibitor present and fluorescence measurements with Pgp-inhibitor present, respectively. Lines are median fluorescence values, boxes are quartiles and whiskers are 10 and 90 percentiles.

Discussion

The objective of this study was to develop non-invasive biomonitoring techniques, using coelomocytes from echinoderms and haemocytes from bivalve molluscs as model and to adapt cell-based bioassays with MXR-activity and cell viability as endpoints.

Concentration of cells

Haemocytes have been found to proliferate upon infection (Arala-Chaves and Sequeira 2000). Different pollutants have been known to act cytotoxic, and the haemocytes will undergo necrosis or apoptosis (Sokolova et al. 2004). In the hemolymph samples there were significant differences between the stations in haemocyte concentration. The concentration did not correlate with expected pollution load or mussel size. Data for size and haemocyte concentration did not show any significant correlation, which is in accordance with previous studies (Carballal et al. 1998).

Cell viability measures

Viability measurements of haemocytes from *Mytilus galloprovincialis* showed significant differences. The animals collected from Šolta had a significant higher metabolic activity than the more contaminated sampling sites. This could be due higher degree of environmental stress or lowered metabolic activity in haemocytes from the contaminated areas as enzyme inhibition or increased mortality among haemocytes. Enhanced metabolic activity has been shown in *M. edulis* after exposure to cadmium and in anoxic condition (Zwaan et al. 1995). Other studies have shown decreased metabolic activity after exposure to metals in *Anodonta cygnea* (Mouraa et al. 2000) and decreased phagocyte activity (Cajaraville et al. 1996). Apoptosis in haemocytes are suggested due to inhibition of ATPase and/or mitochondrial ADP/ATP or substrate transport (Sokolova et al. 2004). The results indicates that metabolic integrity might be a sensitive measure in haemocytes as biomarkers as there were significant differences between the non-polluted area and polluted areas. The membrane integrity was lower at the Trogir station only, and there were no significant differences between mussels collected at Šolta and mussels collected at the other stations. At Trogir the mussels were caged and there was high mortality in the group. Chemical analysis has earlier shown high concentrations of contaminants at this site. The mortality could be due to lethal concentrations of these contaminants. The lower integrity did not relate to a lower metabolic activity compared with the other polluted areas. The lack of lowered metabolism compared to the other polluted areas might be

caused by programmed cell death, apoptosis. Apoptosis is an energy-dependent process. Another possibility is higher haemocyte concentration in the haemocytes. This possibility was rejected by the cell counting. Membrane integrity has been shown to be disrupted in *M. edulis* after exposure to copper (Brown et al. 2004). Exposure to high concentration of chemicals may result in increased lysosomal destabilization (Hwang et al. 2002).

Both alamar Blue™ fluorescence and CFDA-AM fluorescence in coelomocytes was higher at the Kaštela sampling station than in the reference site Šolta. CFDA-AM fluorescence is a result of esterase-catalysed biotransformation. It is usually interpreted as the membrane stability of the cells. In this case, the assumption that esterases are in surplus to the amount of CFDA-AM added in the assay is made. This would imply higher membrane stability in the cells from the contaminated site. There are certain possible mechanisms that could cause such an increase in membrane stability, i.e., elevated levels of membrane stabilising factors, such as HSP, estrogenic compounds, cholesterol, Vitamin E or cellular or external antioxidants. Generally, the opposite results have been found with a reduction of membrane stability upon environmental stress measured in different assays (Schirmer et al. 2001). Similarly, alamar Blue™ fluorescence has been interpreted as a measure of metabolic activity (Goegan et al. 1995). When fluorescence measurements are interpreted as metabolic activity and membrane integrity, respectively, and the two taken together as a measure of cell viability, one gets the unexpected result of higher cell viability in cells from the contaminated site. A feasible explanation is that the Šolta individuals experienced more stress during sampling. The trawling period was somewhat longer at this station, approximately 40 minutes at the Šolta station and approximately 20 minutes at the Kaštela station. The stress could further have led to cell mortality during the incubation period and therefore decreasing the total response in each sample. This would also explain the results of rhodamine B fluorescence, which also showed higher levels in Kaštela. Alternatively one could imagine a genetic adaptation of the population of Kaštela to higher environmental stress, with cells that in some respect are hardier to the strain of the handling in the assay. This could, for instance, result in a high detoxification activity in these cells. However, the sea cucumber larvae are part of the pelagic plankton, and disperse outside the parent range. The adult sea cucumber populations in Kaštela and Šolta are most likely not genetically isolated from each other, because of the pelagic larval stage. And again, this would be in contrast to results from previous studies, where environmental stress has been found to decrease cell viability in other models.

MXR-activity

In haemocytes the level of accumulated rhodamine 123 was highest in the haemocytes from reference site, indicating a low MXR activity. The lower accumulated substrate at the other sites could be due to a higher MXR activity which removes the substrate from the haemocytes. If there was lowered membrane integrity at these sites, the lowered accumulation could be a consequence of substrate leaching out because of membrane disruption. Only at Trogir there was significant lowered membrane integrity. As no lowered membrane integrity was detected in this assay, there is more likely to be a higher MXR activity at these sites. A higher MXR activity could be due to genetic adaptation among the mussels at polluted areas with higher levels of these proteins, or induction as a result of pollution. Also blue mussels have a pelagic larval stage which might exclude the possibility of genetic adaptation. Several studies have shown a higher degree of MXR activity in animals living in polluted areas. In transplant experiments a change in MXR activity has been shown after the moving of animals from polluted to unpolluted areas (Smital et al. 2000; Keppler and Ringwood 2001; Smital et al. 2003).

Sea cucumber cells had a cyclosporine A sensitive ability to extrude rhodamine B. To the knowledge of these authors, this is the first time MXR-activity is shown for *H. tubulosa*, or for any holothurians. MXR-activity measurements in coelomocytes showed a significantly higher MXR-activity in cells from individuals sampled at the Kaštela station, compared to individuals sampled at the Šolta station. These results are in accordance with studies on other models, showing induction of MXR-activity in individuals at polluted sites. Notably, the rhodamine B fluorescence was higher for the Kaštela samples (compared to the Šolta samples) when the protein pump was inhibited by cyclosporine A, indicating a higher intracellular concentration of rhodamine B for the Kaštela cells. This could be due to a lower activity of drug transporting membrane proteins other than the one inhibited by cyclosporine A. The presence of several different such proteins are known from other species, through transport-studies, immunolabeling studies etc. This would imply that in this model system one (or several) MXR-active protein in the sea cucumbers was inhibited at the same time as others were induced (as shown by the ratio (R)) in this study. Another option is that cells from Kaštela had higher cell viability than the Šolta cells, which is supported by the cell viability assay. Cell death during the incubation period of the assay, would lead to lower total intracellular rhodamine concentrations at the end. Such cell death would be expected to influence both the fluorescence with MXR-

inhibitor and without MXR-inhibitor similarly, leaving the ratio of these two measurements as an applicable measure of MXR-activity in the cells.

MXR compared to cell viability

Significant differences between groups were found for all biomarkers measured in this study, i.e. differences in membrane integrity, metabolic activity and MXR activity in haemocytes from *M. galloprovincialis* originating from different stations and in coelomocytes from *H. tubulosa* originating from different stations. In haemocytes the metabolic activity was a sensitive measure, and showed large variation between the different stations. MXR activity also differed as predicted according to presumed pollution load. The membrane integrity did not differ to the same extent as the two other parameters, but was useful when considering the MXR activity in the same haemocytes. Of the parameters measured in sea cucumbers, membrane integrity showed the largest difference between locations, with the activity at Kaštela being 10-fold the activity at Šolta. Metabolic activity at Kaštela was 2.5-fold the Šolta activity, and MXR-activity was 0.5 times higher at Kaštela than at Šolta.

Conclusions

Blue mussel haemocyte viability can be impaired by contamination with a lower metabolic activity at contaminates sites. Membrane integrity was lower only at a very contaminated site, in which several animals died. There was significant higher MXR activity at polluted sites than the reference. The sea cucumber *Holothuria tubulosa* possesses a MXR-mechanism, and the MXR-activity can be induced by contamination. The cell viability was lower in cells from the reference site; this was probably due to the sampling technique.

MXR-activity holds promise as a possible biomarker of exposure. High MXR-activity is not in it self a deleterious effect and it should therefore not be used as a biomarker of effect. The mechanisms of induction and inhibition of MXR are not fully understood, nor are the transport-proteins characterised in these species. These points should be clarified before MXR-activity is implemented as a biomarker. With the non-consistent results for cell viability found in this study, there is reason to call for caution when applying this method in future studies. However, the importance of measuring several parameters

simultaneously is emphasised, as this increases the understanding of the model system as a whole.

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Article 2 Toxicity testing

In vitro toxicity testin with echinoderm coelomocytes, with cytotoxicity and multixenobiotic resistance as endpoints.

In vitro toxicity testing with echinoderm coelomocytes, with cytotoxicity and multixenobiotic activity as endpoints.

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Abstract

The use of in vitro *toxicity testing is expected to increase in the future, as the sheer number of chemicals makes* in situ *assessment complex and difficult, and* in vivo *laboratory testing practically (and ethically) challenging. This study describes the applicability of new cellular methods, with echinoderm coelomocytes, applying two fluorometric assays, the alamar Blue™ and CFDA-AM assay to score cell viability and a multixenobiotic resistance (MXR) assay. Coelomocytes were exposed to different well-known toxicants for 96 hours, before the assays were run. Sediment extracts from the contaminated Tromsø harbour was also tested. The toxicants included two brominated flame retardants (hexabromocyclododecane, HBCD, and tetrabromobisphenol-A, TBBPA), two perfluorinated substances (perfluorononanoic acid, PFNA, and perfluorooctanesulfonate, PFOS) and tributyltin (TBT). Whereas all five toxicants induced significant effects on MXR, cell viability parameters/variables were significantly altered after exposure to two of the chemicals (HBCD and TBBPA). The sediment extract significantly altered one cell viability variable (metabolic activity) and MXR. The results demonstrate that the cell viability and MXR fluorescence assays are rapid, inexpensive and easily performed alternatives in toxicity testing. Echinoderm coelomocytes respond in the assays at concentrations similar to, or lower than, concentrations reported for other test systems, and may serve as a cellular model in future toxicity testing.*

Introduction

The number of chemicals used and released into the environment is high (well above 100000 - (The European Commission 2001)) and increasing. The aquatic environment will be the final recipient of many of these chemicals. Still only a small proportion of them have been adequately described in terms of their potential toxicity to aquatic organisms and ecosystems. One approach towards identifying toxicological effects is the use of laboratory assays, with

subsequent extrapolation to field conditions. Toxicity testing in laboratories is useful to assess the potential toxicity of substances and to characterise their mode of action. *In vitro* techniques for toxicity testing are useful because they can provide knowledge about tissue and target specific effects, and they are well suited for analysis of mechanisms and dose and time dependencies. In addition Fent (2003) argued that studies of cellular and biochemical responses are equally important to whole-organism studies in an ecotoxicological perspective. This is because the primary interaction between a toxic chemical and biota occurs at the cellular (or sub-cellular) level. Implicitly, one can expect changes at the cellular level to occur before changes in a tissue or an organism. It should be noted, however, that the complexity of toxicokinetics and interactions between different chemicals inside an organism are factors that are not accounted for in *in vitro* toxicity testing.

In this study the use of a new cell model in *in vitro* toxicity testing, echinoderm coelomocytes has investigated. Echinoderms are deuterostome invertebrates and therefore belong to a phylogenetic group that could provide information about the relationship between invertebrates and vertebrates. Coelomocytes are circulating cells in the echinoderm body cavity. The coelomocytes will be of several morphologically different types depending on species, however, in asteroids (starfish), the phagocytic amoebocytes are predominant (Boolootian 1966). Coelomocytes were chosen as the cell model for several reasons. Firstly, they can easily and repeatedly be sampled by withdrawal of coelomic fluid from an individual. They can also easily be kept in the laboratory. Finally, coelomocytes are the effector cells of the echinoderm immune system (Gross et al. 1999) and are expected to be highly relevant to the health and survival of individuals.

A 96-well microtiter plate format was used for the different assays. Coelomocytes from the starfish species *Asterias rubens* were exposed to the environmental contaminants hexabromocyclododecane (HBCD), tetrabromobisphenol-A (TBBPA), perfluorononanoic acid (PFNA), perfluorooctanesulfonate (PFOS) and tributyltin (TBT). Additionally some cells were exposed to sediment extracts from a contaminated area. Multixenobiotic resistance (MXR) and cytotoxicity bioassays were run on the exposed cells. The cytotoxicity assay was conducted using the two molecular probes alamar Blue™ and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) to assess metabolic activity and membrane integrity, respectively. Resazurin is the functional dye in the commercial reagent alamar Blue™. It is non-toxic and redox-active. Resazurin is the blue and non-fluorescent oxidised form of the dye, while the reduced form is the pink and highly fluorescent resorufin. The conversion can

be monitored by fluorometric measurement, and will herein be referred to as a measure of metabolic activity. CFDA-AM is converted by non-specific esterases in living cells to form 5-carboxyfluorescein (CF). The substrate (CFDA-AM) is non-fluorescent and non-polar, whereas the product (CF) is fluorescent and polar. Cells with an intact membrane maintain a cytoplasmic environment that supports esterase activity, and a decline in fluorescence readings is interpreted as a loss of membrane integrity (Schirmer et al. 1997). Because the two probes are measured at different emission wavelengths both dyes can be added to perform the assay in one single step, and provide a combined measure for cell viability. This will both reduce time and costs spent on the assay. The probes do not have to be removed from the wells of the microtiter plate, and the assay may be conducted immediately after the incubation without further procedures (Ganassin 2000).

Multixenobiotic resistance (MXR) has been identified in a range of aquatic organisms (e.g. mussels (Kurelec and Pivcevic 1989), sponges (Kurelec et al. 1992), oysters (Minier et al. 1993), marine worms (Toomey and Epel 1993) and fish (Chan et al. 1992)). MXR is analogous to the multidrug resistance (MDR) described for cancer cells and various pathogens in medical literature (Kessel et al. 1968; Borst and Ouellette 1995; George 1996). MXR/MDR result in dramatically reduced intracellular concentrations of a range of cytotoxic substances. Therefore it has been proposed that MXR serves as a "first line of defence" in the cells (Epel 1998). The causative agents of MXR/MDR are a variety of transmembrane transport proteins, the most common among them being P-glycoproteins (P-gps) (Bard 2000). The MXR proteins act as energy-dependent pumps and have a broad spectrum of substrates (Ambudkar et al. 1999; Bard 2000). Examples of substrates have been found within both drugs, natural products (Gottesman and Pastan 1988) and anthropogenic compounds (Cornwall et al. 1995; Bain and Leblanc 1996). Although the basis for this low specificity is not known, the substrates seem to share the properties of moderate hydrophobicity, positively charged domains and similar size (see Bard (2000) for review).

The MXR activity may increase or be inhibited in response to different agents. Populations originating from polluted areas have been found to have higher activity (Minier et al. 1993; Kurelec 1995; Kurelec et al. 1995), and both natural products and anthropogenically introduced chemicals (Bard 2000) may induce the activity as shown after *ex situ* exposure. Induction of MXR activity may also be a generalised response to stressful conditions, such as cellular injury (Chaudhary and Roninson 1993). MXR inhibition causes increased bioaccumulation of xenobiotics (Kurelec 1995; Waldmann et al. 1995) and therefore elevated

internal levels of substances that can exert toxic effects at levels not otherwise considered harmful. Evidence for such effects have been provided by Kurelec (1992), Waldmann et al. (1995), Toomey and Epel (1993) and Schröder et al. (1998). Many classes of chemicals, and among them many contaminants, are capable of such inhibition. The induction or inhibition of MXR has not yet been widely established as a biomarker of environmental pollution (van der Oost et al. 2003).

In this study, the MXR activity was measured using the rhodamine B accumulation assay. Rhodamine B is a fluorescent substrate for MXR proteins, its intracellular concentration can therefore be measured fluorometrically. Cells were exposed to different known toxicants, and for the assay incubated with rhodamine B with or without the presence the model MXR inhibitor MK-571. In mammalian cells, MK-571 selectively inhibits the MXR protein multixenobiotic resistance-associated protein (MRP) (Gekeler et al. 1995). The difference between the rhodamine B accumulations in these two treatments will be a measure of the amount of rhodamine B transported out of the cells by MRP-like proteins, i.e. a measure of MRP-mediated MXR in the cells. When the fluorescence in toxicant-treated cells that were not incubated with MK-571, is compared directly to control cells, the total activity of different MXR proteins (including MK-571 insensitive proteins) is indicated. And when cells that were incubated with MK-571 are compared to their respective control cells, the activity of MK-571 insensitive proteins is indicated.

In order to characterise the model system and assays across a range of different substances, cells were exposed to two representative brominated flame retardants (HBCD and TBBPA), two perfluorinated substances (PFNA and PFOS) and TBT, as well as a sediment extract from a contaminated harbour.

The objective of this study was to develop a model system for toxicity testing, by using cell-based assays with *in vitro* exposure, and to clarify effects of selected environmental contaminants.

Materials and methods

Animals

Specimens of *Asterias rubens* between 5 and 10 cm (measured from the base to the tip of the arm), were hand-picked by divers at depths from 1-10 meters. Sampling took place in Norway

from February to March 2005, in an area close to Drøbak. The animals were kept at NIVAs marine research station in Solbergstrand in 300 litre tanks with circulating water taken from the sampling area at 5 m depth and fed ad libitum on mussels (*Mytilus edulis*). The water in the sampling area has a low pollution load. The coelomocytes from the starfish were therefore assumed to express a low inherent level of MXR activity at the beginning of the study.

Coelomocyte preparation

In June 2005 coelomic fluid was extracted from the cultured animals. Specimens that were suspected to be in a bad condition, e.g. individuals that had low tube foot activity upon examination or had recently lost one arm, were excluded from the study. Water temperature at 5 meters depth at the time was between 7.0˚C and 7.5˚C. Coelomic fluid was extracted from the animals using a 1mL syringe with a 23 gauge needle, both pre-treated with cold phosphate buffered saline (PBS, pH 7.8; 0.1 M; 8.5 mL NaH_2PO_4 (stock 0.2 M dissolved in distilled water); 91.5 mL Na_2HPO_4 (stock 0.2 M dissolved in distilled water) in 100 mL water and adjusted for the salinity in the sampling area with 2.4% w/v NaCl). The needle was inserted at the distal third of one arm into the coelomic cavity, an extraction technique previously described by Bekri and Pelletier (2004). The volume of fluid extracted was adjusted for the individual size of the animal. Typically 0.5 mL was taken from smaller individuals and 1 mL was taken from larger individuals. The extracts from each individual were mixed with cold PBS by a ratio of 1:3 in separate glass tubes. The tubes were kept on ice at all times and gently turned every few minutes. The cell density in diluted *A. rubens* samples was determined by counting particles between 11 µm and 20µm in a Coulter counter® (Multisizer™ 3, from Beckman Coulter™). The extract then diluted in PBS to obtain the final wanted cell densities for the experiment. Two hundred µL of the diluted samples to be used in the MXR-assay were seeded into the wells of 96 well microtiter plates (40000 cells/well) in 8 replicates per individual. Those to be used cytotoxicity assay were seeded in 4 replicates on one microtiter plate and in 4 replicates on a separate microtiter plate. The microtiter plates with the cells were then incubated at 15˚C and in the dark for 24 hours to allow the coelomocytes to sink and attach to the bottom of each well.

In vitro exposure

Samples to be used in the MXR assay and samples on one of the microtiter plates for the cytotoxicity assay were exposed to selected environmental contaminants. Cells on the other

microtiter plate for the cytotoxicity assay were exposed to different concentrations of the model cytotoxicant phenol, to serve as a quality control.

Exposure media were prepared by diluting the model toxicants from stock solution in PBS into three different concentrations. Cells from five individuals were assigned to each of the toxicants (except from HBCD cytotoxicity assay, in which n=10). Samples to be used in the MXR assay were in 8 replicates, and each of the toxicant-concentrations was added to two of the replicates, with the last two replicates to serve as controls. The cytotoxicity samples were in four replicates, here each toxicant dilution was added to one replicate, and the last replicate served as control. Phenol was diluted in three concentrations, and was added to wells on the quality control plate in the same manner. The final exposure concentrations for the cells were as described in Table 1. The exposure to the different substances was semi static with a 48 hour incubation period, re-exposure and another 48 hours incubation before the assays were performed.

Table 1. Exposure concentrations for toxicity study. Toxicant stock solutions were prepared in acetone or dimethyl sulfoxide (DMSO).

Toxicant	Stock solution	Exposure concentrations µg/mL (with concentrations in µM given in parenthesis)		
TBBPA	1mg/(mL DMSO)	2.67 (5)	5.35 (10)	10.69 (20)
HBCD	4mM (in DMSO)	3.21 (5)	6.42 (10)	12.83 (20)
PFOS	10mg/(mL DMSO)	$5*10^{-5}$ ($1.9*10^{-6}$)	$5*10^{-4}$ ($1.9*10^{-5}$)	$5*10^{-3}$ ($1.9*10^{-4}$)
PFNA	20mg/(mL acetone)	10^{-6} ($2.2*10^{-6}$)	10^{-5} ($2.2*10^{-5}$)	10^{-4} ($2.2*10^{-4}$)
TBT	2mg/(mL DMSO)	1 (0.0031)	10 (0.031)	100 (0.31)
Phenol	in DMSO	2.823 (30)	28.23 (300)	9410 (100000)

In addition, one group of samples was exposed to organic extracts (extracted by Accelerated Solvent Extraction, ASE) of sediments from a harbour area in Tromsø, Norway (Hylland et al. 2003). Two different extracts were tested, one originating from an area in which sediments had previously been characterised as highly toxic (sample 1043) (Hylland et al. 2003), the other (sample 1045) had uncertain toxicity (Hylland et al. 2003), but with some contamination of benzo[a]pyrene and Indeno[1,2,3-cd]pyrene (Ruus 2005).

Cytotoxicity assay

Cell viability was assessed by the assay simultaneously using alamar Blue™ for metabolic activity and CFDA-AM for membrane integrity. The protocol was adapted from Schirmer et al. (1998): After 72 hours the exposure media was carefully removed from the wells and 100 µl PBS (24‰ w/v NaCl) containing 4 µM CFDA-AM and 5% (v/v) alamar Blue™ was added to each well. The microtiter plates were incubated for 30 minutes in room temperature in the dark, before fluorometric readings were performed on the plate reader Cytofluor™ 2300 (Millipore). Excitation and emission wavelengths were 485 nm and 530 nm respectively for CF (the CFDA-AM metabolite) and 530 nm and 590 nm respectively for alamar Blue™. The dyes are light sensitive, and every step of the protocol was carried out in the dark. The assay was also run on the phenol-exposed cells.

Results are expressed separately for the two probes. Metabolic activity is expressed as the ratio of alamar Blue™ fluorescence in the sample and alamar Blue™ fluorescence in the respective control well (which was set at 1). Membrane integrity is expressed as the ratio of CF fluorescence in the sample and CF fluorescence in the respective control well (set at 1).

MXR assay

The assay was performed using 0.1 µM rhodamine B (Sigma Aldrich, St. Louis, MO, USA) as the model MXR-substrate and dye and 0.1 µM MK-571 (Cayman Chemical, Ann Arbor, MI, USA) as the model MXR-inhibitor, as determined after pilot studies. Sixty µL of the cell suspension in the well was removed and 50 µL of dye was added to achieve the wanted final concentration in the wells. To 4 of the 8 replicates for each individual, 10µL model MK-571 was added so that final concentration in the wells was 0.1 µM. To the remaining replicates, 10 µL PBS was added. After 1 hour incubation in the dark at room temperature, the cells were washed by removing 100 µL solution from each well and replace it with 100 µL PBS. This washing step was repeated. After the second washing step, all solution was carefully removed from each well, and 100 µL triton X-100 (0.1% v/v in PBS) was added. Fluorescence measurements were performed in the fluorescence plate reader, with excitation and emission wavelengths of 485 nm and 530 nm, respectively. MXR activity is expressed as the ratio of rhodamine B fluorescence when no model MXR inhibitor (MK-571) was administered and rhodamine B fluorescence after incubation with MK-571. Rhodamine B fluorescence is also expressed relative to respective controls (set at 1), both for measurements after incubation with MK-571 present, and after incubation without MK-571 present.

Statistical methods

Statistical analyses were performed with Statistica software (version 6.1; Statsoft Inc.).

For measurements of alamar Blue™ fluorescence, CF fluorescence, and rhodamine B fluorescence, student's t-test was applied to detect differences from control group (t-test against the fixed value 1). Normality of data was assessed in a normal-probability plot.

MXR activity was analysed in ANOVA, after Levene's test for homogeneity of variances. Dunnett's test was applied as a post-hoc test to determine which treatment groups that was different from control.

Level of significance was set to $p<0.05$ for all analyses.

Results

Cytotoxicity assay

There were no significant differences in membrane integrity or in metabolic activity for cells exposed to PFNA (Figure 1), PFOS (Figure 2) or TBT (Figure 3) at the concentrations tested in this study.

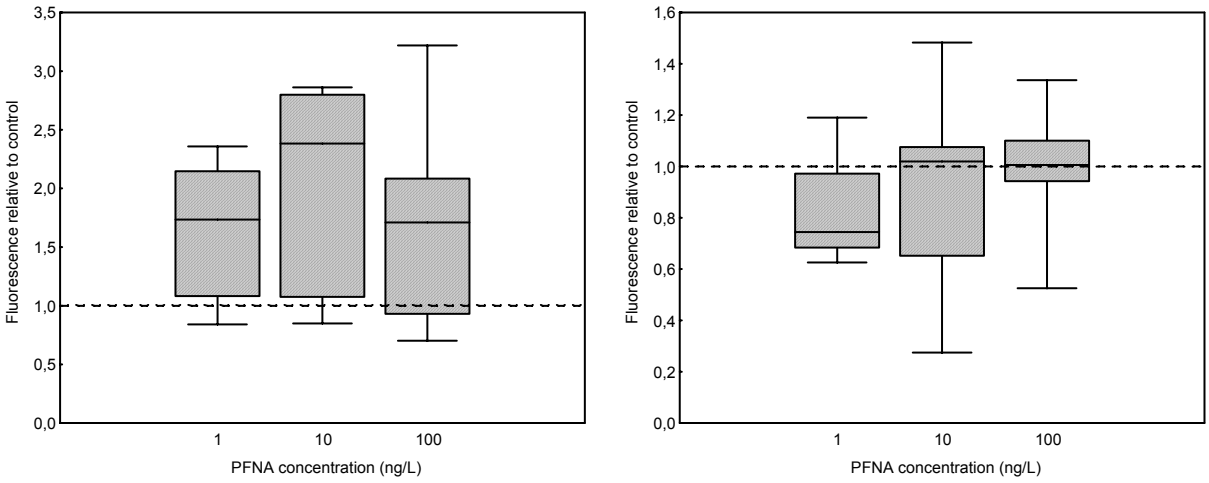

Figure 1. Membrane integrity (left) and metabolic activity (right) in coelomocytes exposed to different concentrations of PFNA in vitro (n=5, note logarithmic scale on x-axes). Fluorescence has been expressed relative to control cells (cells exposed to 0µM PFNA). Presented in plot: Median, quartiles and 10 and 90 percentiles.

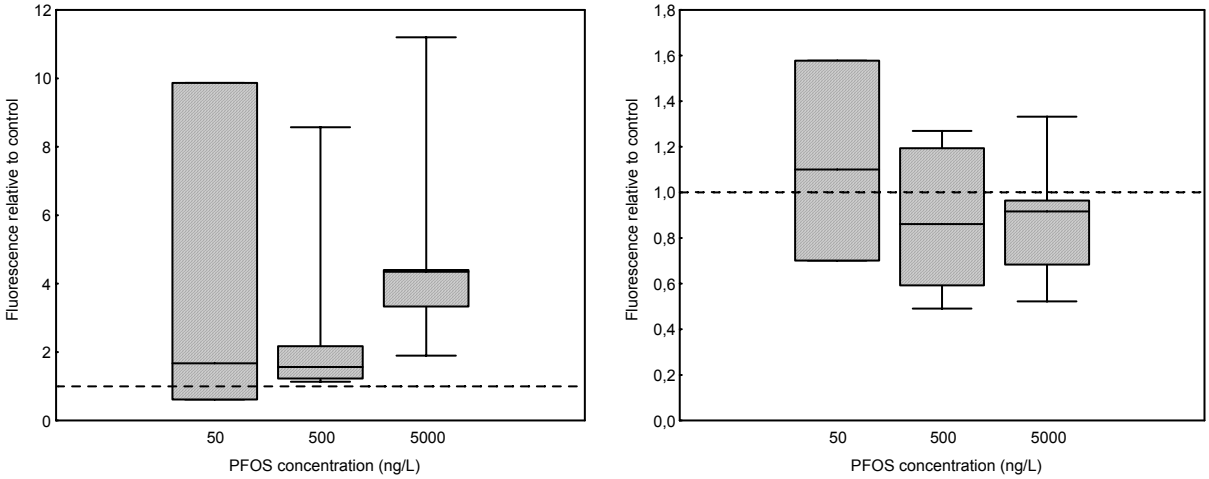

Figure 2. Membrane integrity (left) and metabolic activity (right) in coelomocytes exposed to different concentrations of PFOS in vitro (n=5, note logarithmic scale on x-axes). Fluorescence has been expressed relative to control cells (cells exposed to 0µM PFOS). Median, quartiles and 10 and 90 percentiles.

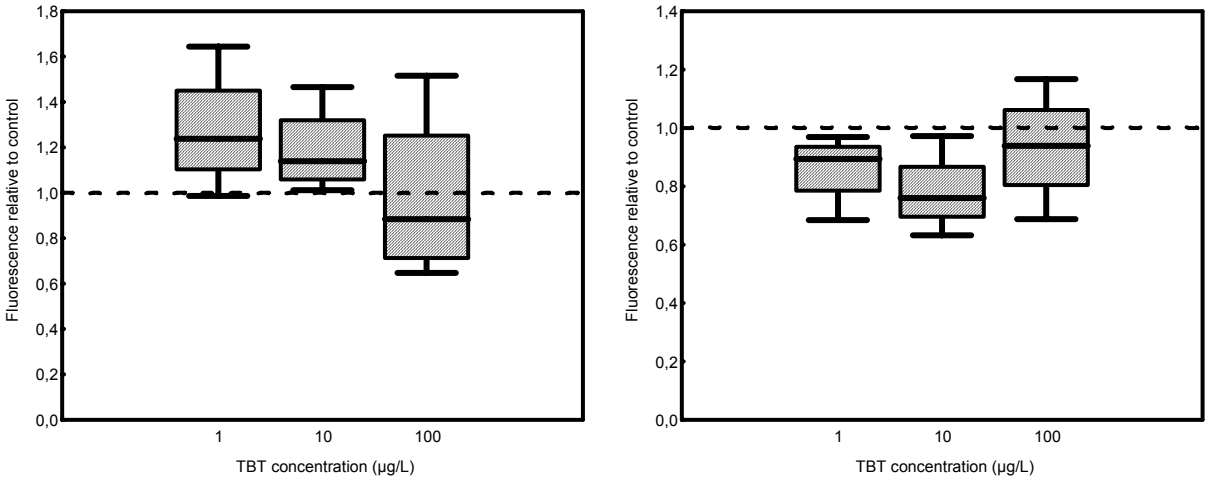

Figure 3. Membrane integrity (left) and metabolic activity (right) in coelomocytes exposed to different concentrations of TBT in vitro (n=5, note logarithmic scale on x-axes). Fluorescence has been expressed relative to control cells (cells exposed to 0µM TBT). Median, quartiles and 10 and 90 percentiles.

Cells exposed to HBCD had a lower metabolic activity compared to control cells (Figure 4). The decrease was dose-dependent and significant for cells exposed to all of the concentrations of HBCD tested in this experiment. A significantly higher membrane integrity was found in all HBCD exposed groups compared to control (Figure 4).

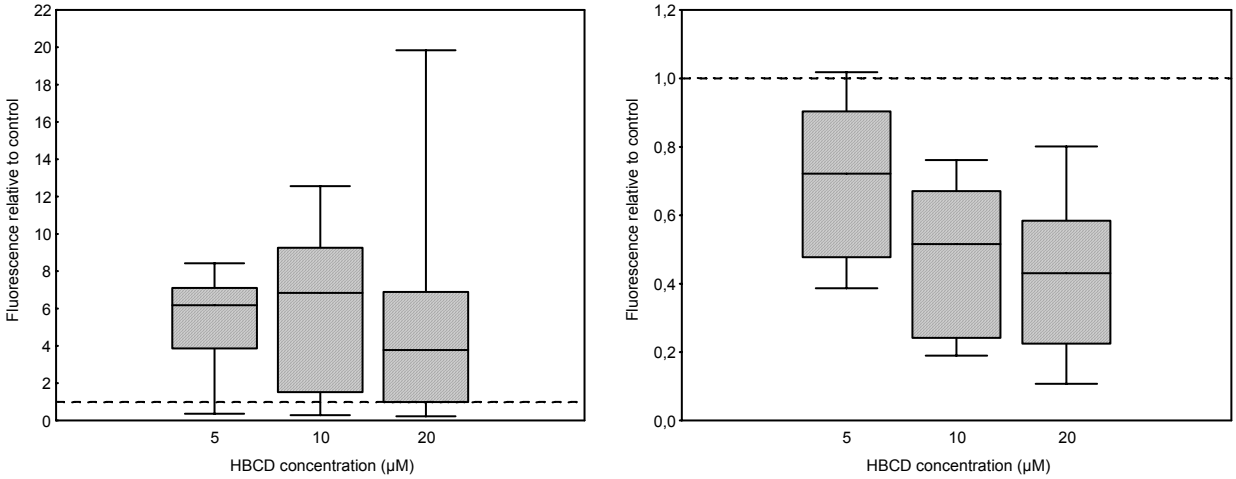

Figure 4. Membrane integrity (left) and metabolic activity (right) in coelomocytes exposed to different concentrations of HBCD in vitro (n=5). Fluorescence has been expressed relative to control cells (cells exposed to 0µM HBCD). Median, quartiles and 10 and 90 percentiles.

Cells exposed to 10 µM and 20 µM TBBPA had significantly lower metabolic activity compared to control cells (Figure 5). The decrease after exposure to 5µM TBBPA was not significant. There were no significant differences in membrane integrity after TBBPA exposure in this study (Figure 5).

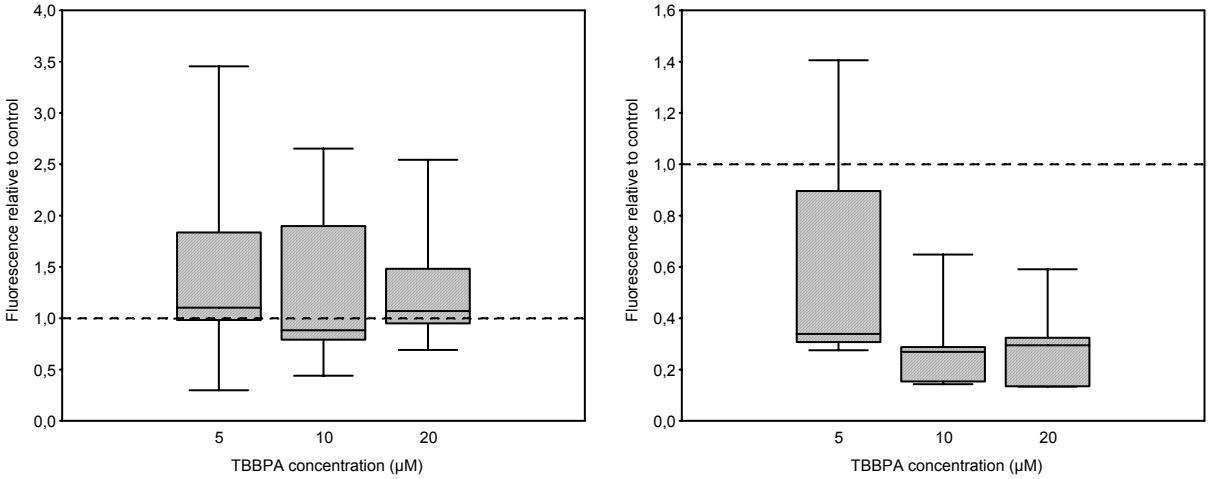

Figure 5. Membrane integrity (left) and metabolic activity (right) in cells exposed to different concentrations of TBBPA *in vitro* (n=5). Fluorescence has been expressed relative to control cells (cells exposed to 0µM TBBPA). Median, quartiles and 10 and 90 percentiles.

Cells exposed to sediment extracts from station 1043 in Tromsø harbour had a significantly lower metabolic activity compared to control cells. The difference in membrane integrity was not significant. No significant differences in metabolic activity or in membrane integrity were found in cells exposed to sediment extracts from station 1045 (Figure 6).

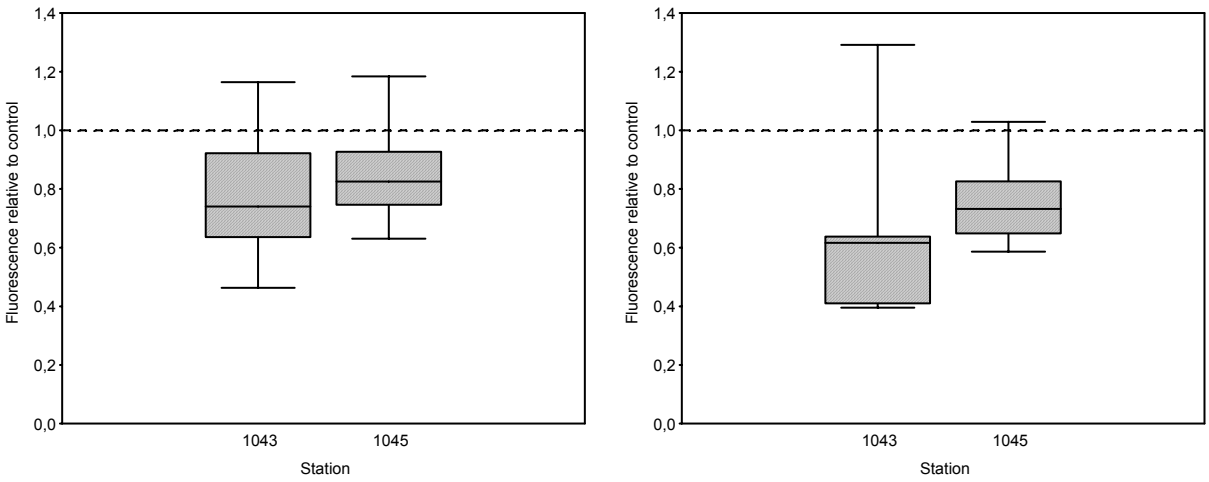

Figure 6. Membrane integrity (left) and metabolic activity (right) in cells exposed to sediment extracts from different stations. Fluorescence has been expressed relative to control cells. Median, quartiles and 10 and 90 percentiles.

MXR assay

Both cells that had not been incubated with MXR inhibitor and cells in which MRP-mediated MXR activity was inhibited by MK-571, had significantly different rhodamine B accumulation compared to control cells (Figure 7). Exposure to 5 µM HBCD (with MK-571) significantly increased accumulation, whereas treatment with 10 µM HBCD lowered accumulation. Cells treated with 5 µM and 10 µM HBCD had significantly higher rhodamine B accumulation than control cells after incubation without MK-571. Also accumulation ratio differed significantly between treatment groups (Figure 8). The accumulation ratio was significantly higher for 10 µM HBCD exposed cells and significantly lower for 20 µM HBCD exposed cells compared to control.

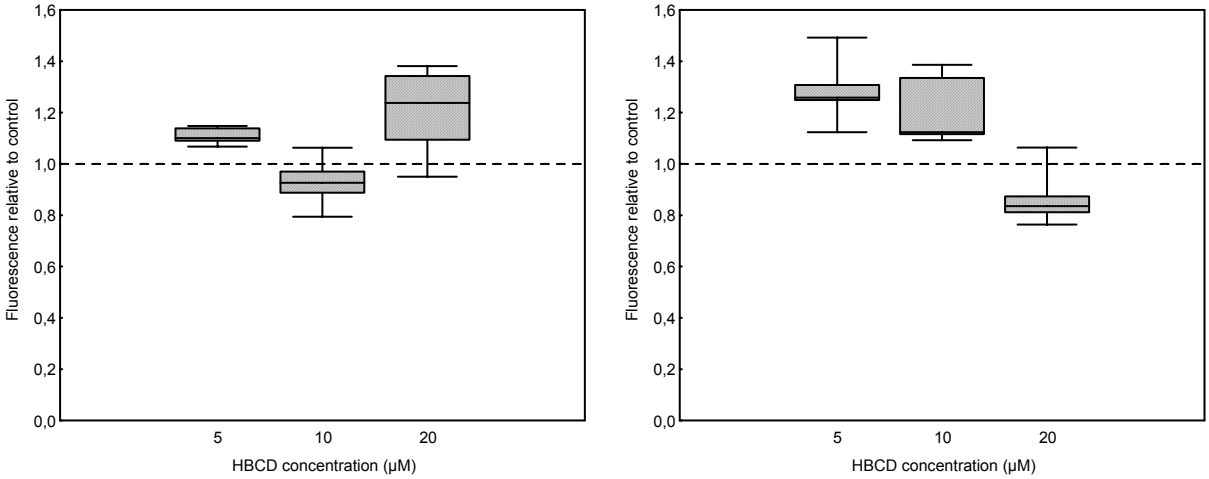

Figure 7. Rhodamine B accumulation relative to control in cells exposed in vitro to HBCD at different concentrations after incubation with (left) and without (right) model MXR-inhibitor MK-571 (n=10). Presented in plot: Median, quartiles and 10 and 90 percentiles.

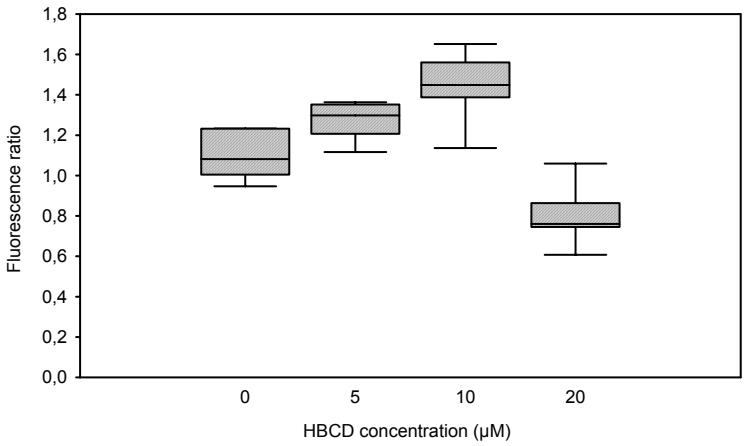

Figure 8. Ratio of rhodamine B accumulation with and without incubation with MK-571 of cells exposed to HBCD at different concentrations (n=10). Median, quartiles and 10 and 90 percentiles.

Treatment with PFNA significantly altered rhodamine B accumulation, both when no MXR inhibitor was administered and when MRP-mediated MXR activity was inhibited by MK-571 (Figure 9). Accumulation after incubation with MK-571 was significantly lower compared to control cells at any of the PFNA concentrations tested. Accumulation without model inhibitor was significantly higher compared to control for cells exposed to 10 ng/L and 100 ng/L PFNA. The accumulation ratio was significantly higher than control for cells exposed to any of the tested concentrations of PFNA, with an apparent dose-dependent increase (Figure 10).

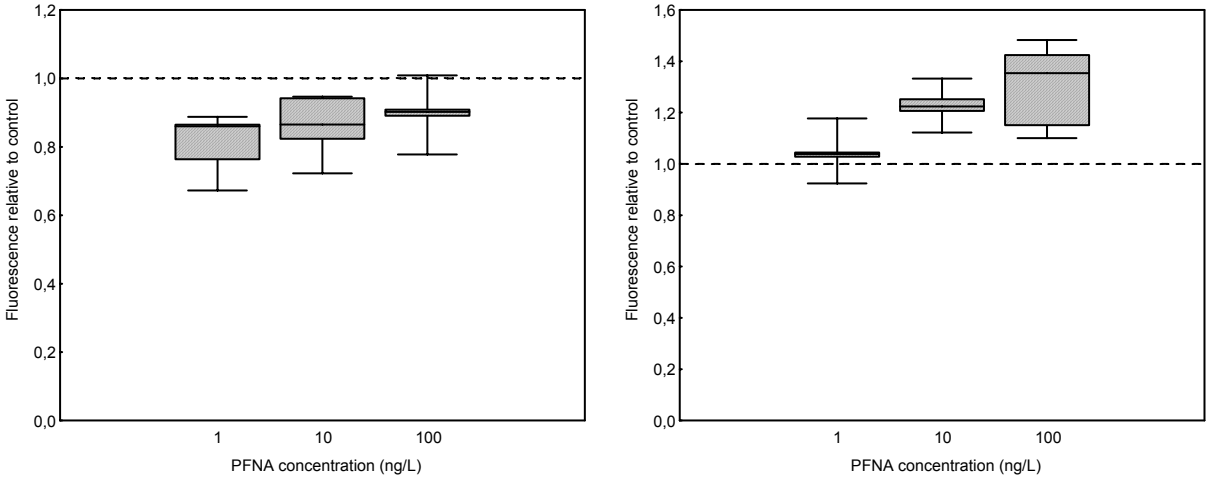

Figure 9. Rhodamine B accumulation relative to control in cells exposed to PFNA at different concentrations after incubation with (left) and without (right) model MXR-inhibitor MK-571 (n=5, note logarithmic scale on x-axes). Median, quartiles and 10 and 90 percentiles.

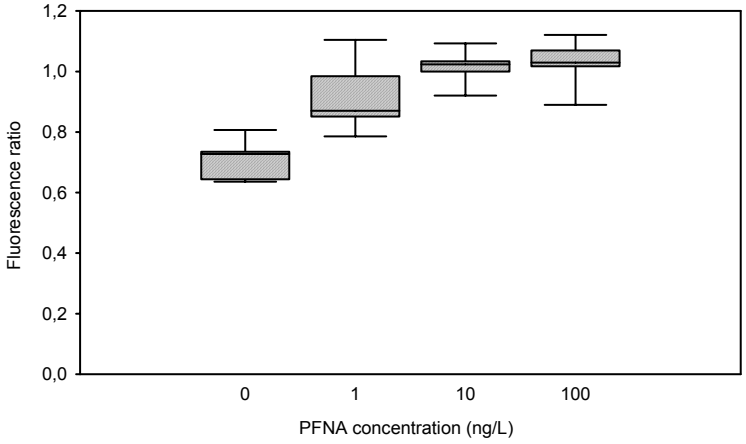

Figure 10. Ratio of rhodamine B accumulation with and without incubation with MK-571 of cells exposed to PFNA at different concentrations (n=5, note logarithmic scale on x-axis). Median, quartiles and 10 and 90 percentiles.

There were significant differences in rhodamine B accumulation in cells exposed to different concentrations of PFOS compared to control cells. Accumulation without incubation with MK-571 was significantly higher for cells exposed to 5000 ng/L PFOS compared to control cells (Figure 11). Accumulation after incubation with MK-571 was higher in cells exposed to any of the tested concentrations of PFOS compared to control cells (Figure 12). No significant differences were found in the accumulation ratio for any of the PFOS concentrations tested in this study (Figure).

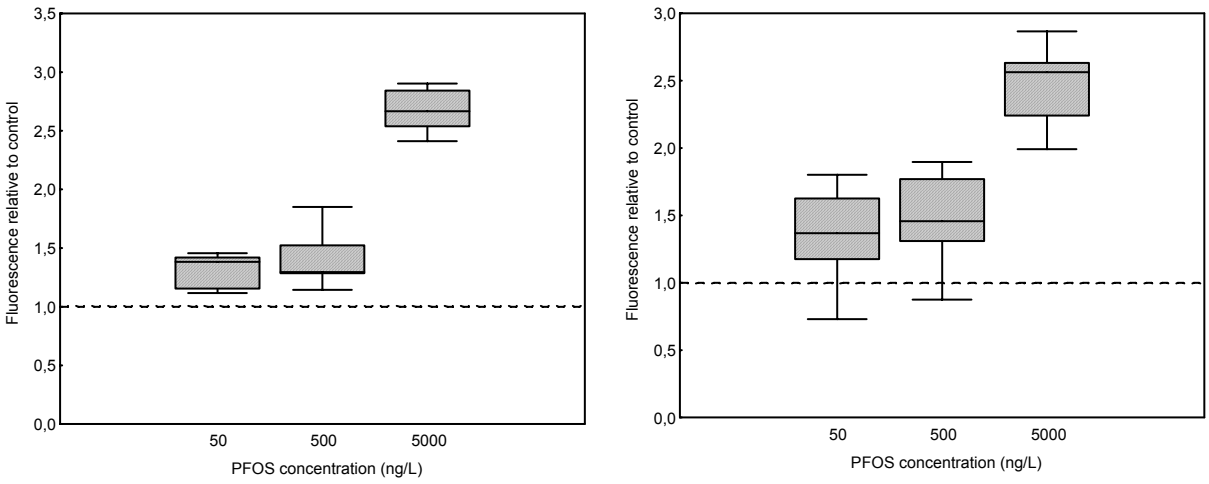

Figure 11. Rhodamine B accumulation relative to control in cells exposed to PFOS at different concentrations after incubation with (left) and without (right) model MXR inhibitor MK-571 (n=5, note logarithmic scale on x-axes). Presented in plot: Median, quartiles and 10 and 90 percentiles.

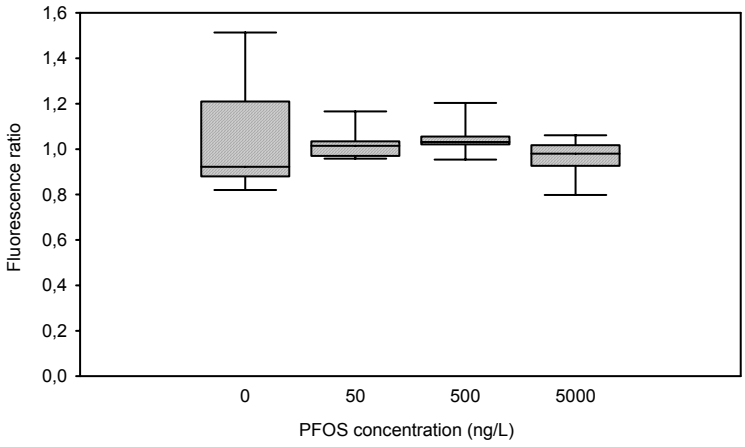

Figure 12. Ratio of rhodamine B accumulation with and without incubation with MK-571 of cells exposed to PFOS at different concentrations (n=5, note logarithmic scale on x-axis). Median, quartiles and 10 and 90 percentiles.

There were significant differences in rhodamine B accumulation between cells exposed to different concentrations of TBBPA compared to control cells. Accumulation with no model

MXR inhibitor present was significantly higher in cells exposed to 10 µM and 20 µM TBBPA (Figure 13). Accumulation in the presence of MK-571 was significantly higher in cells exposed to 20 µM TBBPA (Figure 13). No significant differences in the rhodamine B accumulation ratio in cells exposed to different concentrations of TBBPA were found (Figure 14).

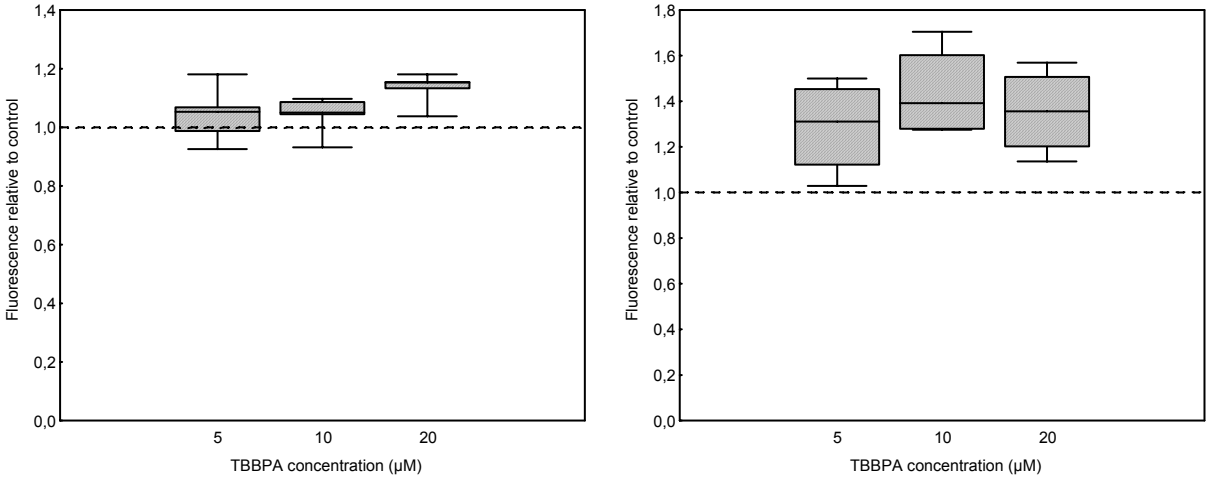

Figure 13. Rhodamine B accumulation relative to control in cells exposed to TBBPA at different concentrations after incubation with (left) and without (right) model MXR inhibitor MK-571 (n=5). Median, quartiles and 10 and 90 percentiles.

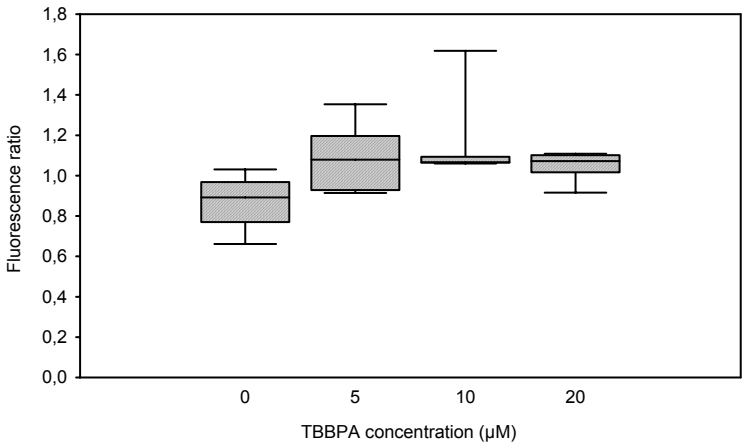

Figure14. Ratio of rhodamine B accumulation with and without incubation with MK-571 of cells exposed to TBBPA at different concentrations (n=5). Median, quartiles and 10 and 90 percentiles.

Cells exposed to TBT had a rhodamine B accumulation significantly different from control cells, both for cells exposed to TBT without administration of model MXR inhibitor and for cells incubated with MK-571 for the assay (Figure 15). Also accumulation ratio differed significantly from control (Figure 16). Rhodamine B accumulation decreased in a dose-

dependent manner when no MXR inhibitor had been administered. In that case, cells exposed to 100 µg/L TBT had significantly lower fluorescence than control cells, whereas exposure to lower concentrations yielded no significant deviations from control. Accumulation after incubation with MK-571 decreased in a dose dependent manner, with a significantly lower fluorescence for all of the tested concentrations of TBT. Accumulation ratio decreased in a dose-dependent manner, the decrease was significant compared to control cells for all of the TBT concentrations tested in this study.

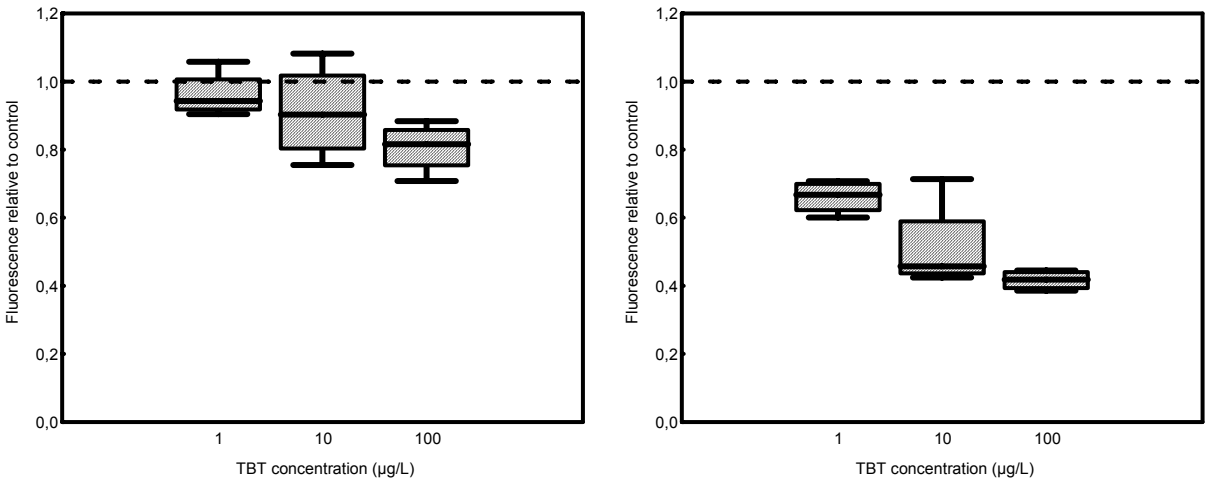

Figure 15. Rhodamine B accumulation relative to control in cells exposed to TBT at different concentrations after incubation with (left) or without (right) model MXR-inhibitor MK-571 (n=5, note logarithmic scale on x-axes). Median, quartiles and 10 and 90 percentiles.

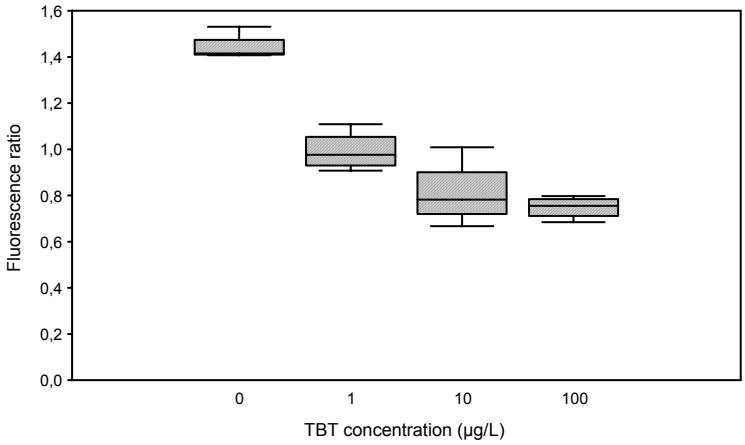

Figure 16. Ratio of rhodamine B accumulation with and without incubation with MK-571 of cells exposed to TBT at different concentrations (n=5, note logarithmic scale on x-axis). Median, quartiles and 10 and 90 percentiles.

In cells exposed to sediment extracts from Tromsø harbour, rhodamine B accumulation was significantly lower than control after incubation with MK-571 (Figure 17). For station 1043, accumulation was higher when no MK-571 had been administered. There were significant differences in MRP-mediated MXR activity in cells exposed to sediment extracts from different stations, compared to control cells (Figure 18).

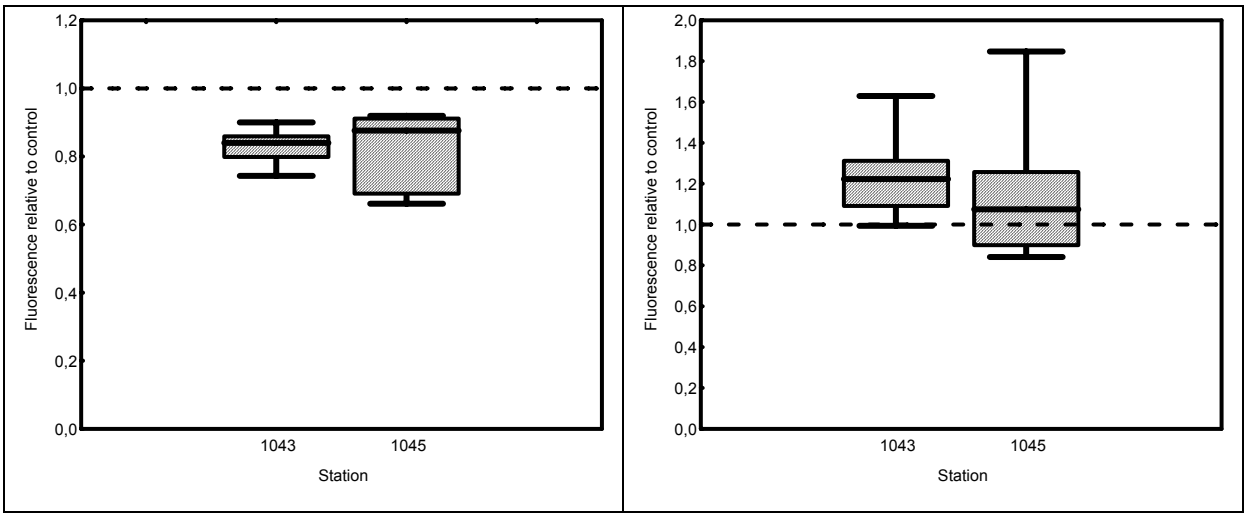

Figure 17. Rhodamine B accumulation relative to control in cells exposed to sediment extracts from different stations after incubation with (left) or without (right) model MXR-inhibitor MK-571 (n=5). Median, quartiles and 10 and 90 percentiles.

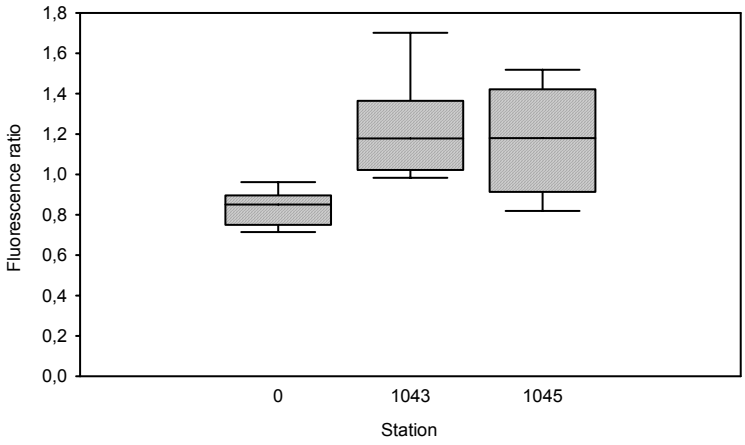

Figure 18. Ratio of rhodamine B accumulation with and without incubation with MK-571 of cells exposed to sediment extracts from different stations (n=5). Median, quartiles and 10 and 90 percentiles.

Discussion

Cytotoxicity

Any concentration of HBCD significantly altered all variables measured in this study. The test concentrations exceed those found in starfish pyloric caeca by Morris et al. (2004), but match lipid contents found in fish and marine invertebrates (Sellstrom et al. 1998; Morris et al. 2004) The membrane integrity appeared to be higher in cells exposed to HBCD, whereas metabolic activity decreased in a dose-dependent matter. It may therefore not be concluded that exposure to HBCD lead to decreased cell viability, but it did affect both membrane integrity and metabolic activity. An increase in CF fluorescence after treatment with triton X-100 at a concentration that proved cytotoxic in other assays was observed by Dayeh et al. (2004), and Zucker et al. (1989) found increases in CF fluorescence after exposure to different alkyltins. In the latter report, it was concluded that the increase was due to alterations in membrane properties (decreased membrane permeability to leakage of CF). An alternative explanation for the increase is the up-regulation of the responsible esterase, as previously shown for carboxyl esterase activity subsequent to exposure to different toxicants (peroxisomal proliferators) (Yan et al. 1995; Parker et al. 1996). However, this experiment was not designed to determine the up- or down-regulation of enzymes, and the increase in CF fluorescence may equally well be due to alterations in membrane properties. As all of the concentrations applied here induced significant effects, lower concentrations of HBCD should also be assessed, and preferably among them concentrations similar to those reported in starfish pyloric caeca. At present, the cytotoxicity assay on coelomocyte seems to be at least as sensitive as that found by Yamada-Okabe et al. (2005) who studied TR-mediated luciferase activity in the transfected HeLaTR cell line.

No significant differences in cell viability parameters were detected after exposure to either of the perfluorinated compounds in this assay. Hu et al. (2003) reported effects on membrane fluidity to occur at 16.5 mg/L in fish leukocytes, and increases in membrane tetrachlorodibenzodioxin (TCDD) permeability for the PLHC-1 cell line when cells were exposed to TCDD in combination with 0.05 mg/L PFOS. Membrane fluidity/permeability alterations seem therefore to occur at higher concentrations of PFOS than applied in this study.

TBBPA has previously been described as a membrane disrupter (Inouye et al. 1979), but at higher concentrations (50µM) than applied in this study, and such effects were not detected

here. Exposure to TBBPA resulted in lower metabolic activity, but did not significantly alter membrane integrity in the coelomocytes. Previous studies have also found a higher sensitivity for the alamar Blue™ assay, than for the CFDA-AM assay (Schirmer et al. 1997; Schirmer et al. 2001; Dayeh et al. 2004; DeWitte-Orr and Bols 2005). Schirmer et al. (1997) concluded that the two probes measured the same damage in their experiment, with the alamar Blue™ assay being a more sensitive indicator. Other possibilities include effects on metabolic activity before any membrane alterations, or because alamar Blue and CFDA-AM may respond differently to different types of cell death, such as apoptosis or autophagy, compared to necrosis. Canesi et al. (2005) studied effects on mussel haemocyte function, and found significant effects after exposure to 5 µM TBBPA, but not after exposure to 1 µM.

No effects on cell viability after exposure to TBT were found in this study, with concentrations similar to those found in some bivalves (Shim et al. 2005) and dog whelk (Folsviksrk et al. 1999) (expressed relative to dry weight), but larger than what was reported in *A. rubens* pyloric caeca (Stronkhorst et al. 2003). In a study by Bekri and Pelletier (2004), no cytotoxic effects on coelomic amoebocytes from TBT were detected in the polar sea star *Leptasterias polaris* after *in vivo* exposure to different butyltins (BTs). TBT body burdens were between 1.26 µg and 2.35µg (body weight 132-247 g) and total BT body burden was between 4.97µg and 7.17µg. These authors did not detect any BTs in coelomic fluid at the end of the experiment, only in gonadal and pyloric caeca tissue. In our study, coelomocytes were exposed to 1 µg/L, 10 µg/L and100 µg/L TBT *in vitro*, most likely exceeding the exposure in Bekri and Pelletiers study. Still at these exposure conditions, no effects on cell viability could be found. Previously TBT has been reported to both induce apoptosis (Micic et al. 2001; Nishikimi et al. 2001) and necrosis (da Silva de Assis et al. 2005) and to decrease CF membrane permeability before a break point interpreted as the viability breakpoint (Zucker et al. 1989). However, the apoptotic/cytotoxic effects seem to occur at higher concentrations of TBT than those used here, and apoptosis may even be inhibited by low concentrations of TBT (Yamanoshita et al. 2000). Membrane alterations on the other hand, were observed down to 0.1µM TBT (Zucker et al. 1988; Girard et al. 1997). Coelomocytes seems therefore to be relatively robust to the membrane destabilising effects of TBT.

While no differences in membrane integrity could be found after exposure to the environmental samples, metabolic activity was significantly lower for cells exposed to sediment extract from station 1043, but not from station 1045. These findings are consistent with the characterisation of 1043 as the highly toxic extract. Previous studies have also found

a higher sensitivity for the alamar Blue™ assay, than for the CFDA-AM assay, as discussed for the results after treatment with TBBPA.

MXR

Total MXR activity in HBCD exposed cells, seemed to increase with increasing doses of HBCD. The MRP-mediated MXR activity was biphasic with HBCD-dose, with an apparent decrease at lower doses, and increase at higher doses. The MXR activity by MK-571 insensitive proteins followed a different pattern, with an increase followed by an inhibition at higher doses. Surprisingly, accumulation of rhodamine in some cells (the 10 µM group) was lower after incubation with MK-571, than it was when all of the MXR-active proteins were active. This indicates that the extrusion of rhodamine was more efficient when part of the MXR proteins (the MRPs) were inhibited. The results for the MXR assay in HBCD-treated cells were complex in total, and may seem conflicting. Combined with an increased membrane stability and reduced metabolic activity, as observed in the cytotoxicity assay in this study, the MXR response may follow a number of different mechanisms. However, the echinoderm MXR proteins have not yet been characterised, and the reasons for the complex MXR response in HBCD-exposed cells may not at this point be fully explained.

The amphiphilic nature of PFNA (and also PFOS) suggests a possible MXR substrate, in which case rhodamine B efflux could be competitively inhibited by PFNA. In fact PFNA exposure appeared to inhibit MRP-mediated MXR activity (higher accumulation ratio) at all concentration tested. The extrusion activity of MK-571 insensitive MXR proteins seemed to increase. Yet, total MXR activity was inhibited. With significant effects down to a concentration of 10^{-6} µg/mL (0.0022 pM), the environmental occurrences of PFNA should be a cause for concern. Even though they have generally been found to be lower than PFOS concentrations by 3- and 20-fold in most animals (Martin et al. 2004), observed PFNA concentrations in biota (Martin et al. 2004; Taniyasu et al. 2005) is well within the concentration range tested in this study. Previously effects on fecundity have been described down to 1 nM PFNA in a four generation study on *Caenorhabditis elegans* (Tominaga et al. 2004). Further studies on MXR with even lower concentrations of PFNA are needed to determine any threshold value for activity.

Both total MXR activity and MXR activity mediated by MK-571 insensitive proteins seemed significantly lower in cells exposed to PFOS, while the ratio of the two did not change significantly. Also, the ratio did not differ significantly from one, meaning that inhibition of

MRP-like proteins had little effect on rhodamine B extrusion from cells in PFOS exposed cells. PFOS did therefore not significantly alter MK-571 sensitive MXR activity, but the inhibition total MXR activity is indicated. Provided that both PFOS (as indicated in this study) and TCDD (not previously assessed) are MXR-substrates, the enhanced effects of TCDD after co-exposure with PFOS (Hu et al. 2003) may be explained by reduced cellular extrusion of TCDD, and not because of increased influx due to reduced membrane stability. The most sensitive study of PFOS today (as of 2005), is to the knowledge of these authors that by Tominaga et al. (2004), who found effects on *Caenorhabditis elegans* fecundity after exposure to 10 pM PFOS. It appears therefore, the sensitivity of the assay described in this article (effects at 0.0019 pM) is higher than to the most sensitive system previously described for PFOS. Also the concentrations applied in this study appear to be lower than levels found in several environmental compartments (Kannan et al. 2002; VandeVijver et al. 2005).

TBBPA seemed to significantly inhibit both total MXR activity and MK-571 insensitive MXR activity, while the decrease in MRP-mediated MXR activity was not significant.

MXR activity appeared to be induced by TBT-exposure at all concentrations tested. These results support the reports on TBT as a potent toxicant with effects down to nanomolar concentrations (Gibbs and Bryan 1986). Tujula at al.(2001) found effects on phenoloxidase activity in tunicate haemocytes after exposure to 1µg/L TBT, and membrane permeability in sea urchin embryos have been reported to increase at an even lower concentration (0.1µM). The fluorescence measurements for control cells show higher fluorescence without MK-571 present, than with MK-571 present. The reason for this result is unclear.

The sediment extracts from Tromsø harbour both apparently inhibited MRP-mediated MXR activity. Similar results after exposure to environmental samples have been provided by Toomey et al. (1996), Smital and Kurelec (1997) and Kurelec et al. (1998) in natural (non-concentrated) polluted seawater. Both sediment extracts seemed to induce MK-571 insensitive proteins, whereas total MXR activity seemed to be inhibited by sediment extracts from station 1043, but not station 1045. Notably, the sediment extracts seemed to have a slight (and with one exception not significant) negative effect on cell viability variables. And an alternative explanation to lower rhodamine B accumulation in MK-571 incubated cells is a therefore a decrease in cell viability in sediment exposed cells.

The data presented here demonstrate the applicability of the coelomocyte cell model for use in cell based bioassays. Using relatively low concentrations, all of the five toxicants and the sediment extracts significantly altered MXR activity. Generally, the MXR activity seems to

be the more sensitive parameter, with cytotoxicity parameters showing significant effects for 3 (metabolic activity) and 2 (membrane stability) of the substances tested here. However the cytotoxicity assay may provide important information on the test system as a whole, and the importance of applying several endpoints in toxicity tests is emphasised by these authors. Because studies of the MXR activity is a relatively new field in ecotoxicology, results are more difficult to interpret (for instance because MXR active proteins in aquatic organisms are not well characterised). In this study, coelomocytes in coelomic fluid were applied in testing without further clean-up or preparation. Combined with the employment of the 96-well microtiter plate format, this model allows for a very simple, high through-put system for toxicity testing.

The MXR assay also appears to be relatively sensitive compared to other assays in previous reports. Effects are noted at similar concentrations in comparable systems for most substances, however for the perfluorinated substances, effects are found at lower concentrations than previously reported. Additionally the effect found is the potential harmful effect of MXR inhibition, and the presence of perfluorinated substances in the environment may already represent a threat to aquatic organisms.

As previously noted coelomocytes are the immune effector cells in echinoderms and would be especially suitable for studies on immunotoxic effects. The induction of MXR activity does not necessarily imply a harmful effect on the cells, whilst the inhibition of MXR activity and decreased cell viability should be considered harmful. However, the effect on immunofunction from changes in immune cell MXR activity has not yet been investigated. Note also that in starfish, the coelomic fluid has been shown to contain lower levels of at least one of the model toxicants in this study (TBT), compared to pyloric caeca (Bekri and Pelletier 2004). Cytotoxic effects on coelomocytes may also be indicative of possible cell damage in other echinoderm cell types.

Despite these considerations, effects on coelomocytes as described in this study can be an early warning signal on the potential toxicity of chemicals, as well as providing knowledge about toxicity mechanisms of different substances.

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Appendix 1
Chemicals and equipment

Beckman CoulterTM
Multisizer^{TM} 3, Coulter counter$_{®}$

BioSource Europe S.A., Nivelles, Belgium
alamar BlueTM

Biowhittaker Inc., Walkerswille, Maryland, USA
L-15 (Leibowitz) medium

Cayman Chemical, Ann Arbor, MI, USA
MK 571

Chiron Corporation, Emeryville, CA , USA
1,2,5,6,9,10-hexabromocyclododecane (HBCD)
Tetrabromobisphenol A (TBBPA)

Dynatech (Chantilly, Virginia, USA)
Fluorolite 1000

Fluka, Buchs SG, Switzerland
Cyclosporin A

Merck, Whitehouse Station, New Jersey, USA
NaCl
$Na_2HPO_4 \cdot 12H_2O$
$NaH_2PO_4 \cdot 12H_2O$

Millipore, Billerica, Massachusetts, USA
CytoFluor^{TM} 2300 Fluorescence Measurement System

Molecular probes, Leiden, the Netherlands
CFDA-AM
Calcein AM
Rhodamine 123

Rathburns Chemicals, Walkerburn, Scotland
Acetone

Sigma Aldrich, St. Lous, Missouri, USA
Tri-n-Butyltin cyanid (TBT)
Heptadecafluorooctanesulfonic acid tetra ethylamonium salt (PFOS)
Triton X-100
Verapamil
Rhodamine B
Phenol

Heptadekafluorononanoic acid (PFNA)
Rhodamine 123

Appendix 2
Selected observations of toxicants

Substance	Place	Compartment	Level	Ref.
HBCD	Viskan river, Sweden	Freshwater fish	4000-8000 ng/g lipid weight (l.w.)	(Sellstrom et al. 1998)
HBCD	Viskan river, Sweden	Sediment	B.D.L-7600 ng/g ignition loss	(Sellstrom et al. 1998)
HBCD	Ebro river, Spain	Sediment	B.D.L.-513,6 ng/g dry weight (d.w.)	(Eljarrat et al. 2004)
HBCD	Ebro river, Spain	Freshwater fish (Barbus graellsi), muscle	B.D.L.-1127 ng/g wetweight (w.w.)	(Eljarrat et al. 2004)
HBCD	Ebro river, Spain	Freshwater fish (Barbus graellsi), liver	B.D.L.-1172 ng/g w.w.	(Eljarrat et al. 2004)
HBCD	Western Scheldt Estuary, the Netherlands	Marine fish (several species), muscle and liver	9-1110 ng/g l.w.	(Janak et al. 2005)
HBCD	Swedish lakes	Eel and pike	65-1808 ng/g l.w.	(Remberger et al. 2004)
HBCD	Western Scheldt Estuary, the Netherlands	Starfish (*Asterias rubens*), digestive system	<30-84 µg/kg l.w.	(Morris et al. 2004)
HBCD	North Sea	Common whelk (*Buccinium undatum)*	29-47 µg/kg l.w.	(Morris et al. 2004)
HBCD	North Sea	Harbour porpoise (*Phocoena phocoena*) (blubber)	440-6800 µg/kg l.w.	(Morris et al. 2004)
HBCD	Western Scheldt Estuary, the Netherlands	Sediment	<0,6-99 µg/kg d.w.	(Morris et al. 2004)
HBCD	Sweden	Peregrine falcon (Falco peregrinus) eggs	34-2400 ng/g l.w.	(Lindberg et al. 2004)
TBBPA	Sweden	Sediment, downstream from plastics industry	430 ng/g ignition loss	(Sellstrom and Jansson 1995)
TBBPA	North Sea	Common whelk	5,0-96 ng/kg l.w.	(Morris et al. 2004)
TBBPA	North Sea	Harbour porpoise (blubber)	0,1-418 ng/kg l.w.	(Morris et al. 2004)
TBBPA	Western Scheldt	Sediment	<0,1-3,2 ng/kg d.w.	(Morris et al. 2004)
TBBPA	England	Sediment, estuarine and riverine	<2,4-9750 ng/kg d.w.	(Morris et al. 2004)
PFOS	Greenland	Polar bear (Ursus maritimus), liver	1245 and 1325 ng/g w.w.	(Bossi et al. 2005)
PFOS	Greenland	Shorthorn sculpin (Myoxocephalus scorpius), liver	nd-18 ng/g w.w.	(Bossi et al. 2005)
PFOS	Michigan waters, USA	Chinook salmon, muscle	7-190 ng/g w.w.	(Giesy and Kannan 2001)
PFOS	Michigan waters, USA	Chinook salmon, liver	33-170 ng/g w.w.	(Giesy and Kannan 2001)
PFOS	Baltic Sea	Atlantic salmon (Salmo salar), liver	<8 ng/g w.w.	(Kannan et al. 2002)
PFOS	Western Scheldt	Plaice (Pleuronectes platessa) , liver	<10-111 ng/g w.w.	(Hoff et al. 2003)
PFOS	Western Scheldt	Bib (Trisopterus luscus), liver	<10-87 ng/g w.w.	(Hoff et al. 2003)
PFOS	Gulf of Mexico and Chesapeake Bay, USA	Oyster (Crassostrea virginica)	<42-1125 ng/g d.w.	(Kannan et al. 2002)
PFOS	Tokyo Bay, Japan	Flatfish (Pleuronectiformes pleuronectidae), liver	158-198 ng/g w.w.	(Taniyasu et al. 2003)

PFOS	Tokyo Bay, Japan	Flatfish (Pleuronectiformes pleuronectidae), blood	74-194 ng/mL	(Taniyasu et al. 2003)
PFOS	Japan waters	Marine fish (several species), liver	3-7900 ng/g w.w.	(Taniyasu et al. 2003)
PFOS	Japan waters	Marine fish (several species), blood	1-834-ng/mL	(Taniyasu et al. 2003)
PFOS	Western Scheldt Estuary, the Netherlands	Starfish (Asterias rubens), soft tissue	9-176 ng/g w.w.	(Van de Vijver et al. 2003)
PFOS	Western Scheldt Estuary, the Netherlands	shrimp (Crangon crangon), soft tissue	19-520 ng/g w.w.	(Van de Vijver et al. 2003)
PFOS	Western Scheldt Estuary, the Netherlands	crab (Carcinus maenas), soft tissue	24-877 ng/g w.w.	(Van de Vijver et al. 2003)
PFOS	Tokyo Bay, Japan	water sample	338-57700 pg/L	(Yamashita et al. 2005)
PFNA	Kuujjuarapik, Canada	white sucker (Catostomus commersoni), liver	0,61-1,7 ng/g	(Martin et al. 2004)
PFNA	Kuujjuarapik, Canada	brook trout (Salvelinus fontinalis), liver	5,9-6,5 ng/g	(Martin et al. 2004)
PFNA	Kuujjuarapik, Canada	northern pike (Esox lucius), liver	<0,5 ng/g	(Martin et al. 2004)
PFNA	Kuujjuarapik, Canada	Arctic sculpin (Myoxocephalus scorpioides), liver	2,2 ng/g	(Martin et al. 2004)
PFNA	Sanikiluaq, Canada	Polar bear (Ursus maritimus), liver	108-230 ng/g	(Martin et al. 2004)
PFNA	Dutch Wadden Sea	Harbour seal (Phoca vitulina), liver	<LOD-14,00 ng/g w.w.	(VandeVijver et al. 2005)
PFNA	Blokkersdijk, The Netherlands	Wood mouse (Apodemus sylvaticus), liver	<LOD-0,27 ng/g w.w.	(Hoff et al. 2004)
PFNA	Tokyo Bay, Japan	water sample	163-71000 pg/L	(Yamashita et al. 2005)
PFNA	New York, USA	Human plasma,	0.1-2 ng/mL	(Kannan et al. 2004)
PFNA	Atlanta, USA	Human serum	1.3-4.4 ng/mL	(Kuklenyik et al. 2004)
PFNA	Poland	Beaver, liver	1,34 ng/g w.w.	(Taniyasu et al. 2005)
PFNA	Poland	Human, blood	3,84 ng/mL	(Taniyasu et al. 2005)
TBT	German Northern Sea	Sediment	80-720 ng/g d.w.	(Biselli et al. 2000)
TBT	German Baltic Sea	Sediment	570-17000 ng/g d.w.	(Biselli et al. 2000)
ΣBT	US, West coast	Sediment	n.a.-1600 ng ΣBTs/g w.w.	(Krone et al. 1996)
ΣBT	US, West coast	Fish, liver	n.a.-1600 ng ΣBTs/g w.w.	(Krone et al. 1996)
ΣBT	US, East coast	Sediment	n.a.-310 ng ΣBTs/g w.w.	(Krone et al. 1996)
ΣBT	US, East coast	Fish, liver	n.a.-970 ng ΣBTs/g w.w.	(Krone et al. 1996)
TBT	Mediterranean Sea, Spain	Sediment	59-6860 ng/g d.w.	(Diez et al. 2002)
TBT	Greenland	Blue mussel (Mytilus edulis)	0,2-1,2 µg(Sn)/kg w.w.	(Jacobsen and Asmund 2000)
TBT	Iceland	Blue mussel (Mytilus edulis)	14,7-122,6 ng/g w.w.	(Skarphedinsdottir et al. 1996)
TBT	Iceland	Dogwhelk (Nucella lapillus)	8,5-61,9 ng/g w.w.	(Skarphedinsdottir et al. 1996)
TBT	Korea	Starfish (Asteria pectinifera)	n.d.-323 ng(Sn)/g d.w.	(Shim et al. 2005)
TBT	Korea	Starfish (Asterias amurensis)	14-685 ng(Sn)/g d.w.	(Shim et al. 2005)

TBT	Korea	Bivalves (Mytilus edulis and Crassostrea gigas)	16-1610 ng(Sn)/g d.w.	(Shim et al. 2005)
TBT	Japan waters	Sea star (Ctenodiscus crispatus),	1,9 ng/g w.w.	(de Brito et al. 2002)
TBT	North Sea	Sea star (Asterias rubens), pyloric caeca	4,7-8,5 ng/g w.w.	(Stronkhorst et al. 2003)
TBT	Norwegian coast	Dogwhelk (Nucella lapillus)	<7-1096 ng/g dryweight	(Folsviksrk et al. 1999)
TBT	Japan waters	Sun star (Solaster uchidai)	22 ng/g w.w.	(Takahashi et al. 1997)
TBT	Japan waters	Goniasterid star (Ceramaster japonicus)	5,5 ng/g w.w.	(Takahashi et al. 1997)
TBT	Japan waters	Echinothuirid sea urchin (Phormosona bursarium)	11 ng/g w.w.	(Takahashi et al. 1997)

Appendix 3
Selected effects observations of toxicants

Substance	Compartment	Effect	Parameter	Level	Notes	Ref.
HBCD	Rat brain synaptosomes	Glutamate uptake	26±9% inhibition	1µM	In vitro, 15 min pre-incubation with HBCD	(Mariussen and Fonnum 2003)
HBCD	Rat brain synaptosomes	Dopamine uptake	IC50	4±1µM	In vitro, 15 min pre-incubation with HBCD	(Mariussen and Fonnum 2003)
HBCD	Rainbow tout, liver	Catalase activity	Sign. increase	50mg/(kg bw)	Intraperitoneal (i.p.) injection, once. 5 days incubation	(Ronisz et al. 2004)
HBCD	Rainbow tout, liver	LSI	Sign. increase	50mg/(kg bw)	I.p. injection, once.28 days incubation	(Ronisz et al. 2004)
HBCD	Rainbow tout, liver	EROD-activity	Sign. decrease	500mg/(kg bw)	I.p. injection, once.28 days incubation	(Ronisz et al. 2004)
HBCD	HeLaTR cells	TR-mediated gene transcription (luciferase activity)	Sign. increase	3,12µM	In vitro, days pre-incubation with HBCD	(Yamada-Okabe et al. 2005)
HBCD	Algae		EC50	0,37-9,3 µg/L		KEMI 1999, OECD 2003, (Birnbaum and Staskal 2004)
HBCD	Daphnia		Life-cycle NOEC	3,1µg/L		OECD 2003, (Birnbaum and Staskal 2004)
HBCD	Rainbow trout		EC50	2,5µg/L		OECD 2003, (Birnbaum and Staskal 2004)
HBCD	Mammalian celline, Sp5 and SPD9	Growth	IC50	0,03mM		(Helleday et al. 1999)
TBBPA	Mouse lymphocytes	Cytotoxicity	NOEC	3µM		(Pullen et al. 2003)
TBBPA	Rat brain synaptosomes	Glutamate uptake	IC50	6±1µM	In vitro, 15 min pre-incubation with HBCD	(Mariussen and Fonnum 2003)
TBBPA	Rat brain synaptosomes	Dopamine uptake	IC50	9±2µM	In vitro, 15 min pre-incubation with HBCD	(Mariussen and Fonnum 2003)
TBBPA	Rat brain synaptosomes	GABA uptake	IC50	16±2µM	In vitro, 15 min pre-incubation with HBCD	(Mariussen and Fonnum 2003)
TBBPA	Rat brain synaptosomes	Membrane potensial (3H+-TPP uptake)	IC50	16±6µM	In vitro, 15 min pre-incubation with HBCD	(Mariussen and Fonnum 2003)
TBBPA	Rat brain vesicles	Dopamine uptake	IC50	3±1µM	In vitro, 15 min pre-incubation	(Mariussen and Fonnum

					with HBCD	2003)
TBBPA	MtT/E-2 cells	Thyroid hormone homeostasis (T3 and TTR binding	Sign. inhibition	10^{-6} - 10^{-4} M	In vitro, 40 min	(Kitamura et al. 2002)
TBBPA	GH3 cells	Thyroid hormone homeostasis (growth increase)	Sign. Induction	10^{-6} - 10^{-4} M	In vitro, one week	(Kitamura et al. 2002)
TBBPA	MtT/E-2 cells	Estrogen homeostasis (Inhibition of ER-binding)	Sign. inhibition	10^{-5} - 10^{-4} M	In vitro, 40 min	(Kitamura et al. 2002)
TBBPA	Rat hepatocytes	Thyroid hormone homeostasis (T4 and TTR binding)	IC50	7,7±0,9 nM	In vitro, overnight	(Meerts et al. 2000)
TBBPA	Mammalian celline, Sp5 and SPD8	Growth inhibition	IC50	0,08-0,09 mM		(Helleday et al. 1999)
TBBPA	Mammalian celline, Sp5 and SPD8	Colony formation	IC50	>0,07-0,18 mM		(Helleday et al. 1999)
TBBPA	Rainbow tout, liver	EROD-activity	Sign. decrease	100mg/kg/bw/day	I.p. injection, once, 4 days	(Ronisz et al. 2004)
TBBPA	Rainbow tout, liver	GR-activity	Sign. increase	100mg/kg/bw/day	I.p. injection, once, 4, 14 days	(Ronisz et al. 2004)
TBBPA	Human neutrophil granulocytes	ROS-production	Sign. increase	2µM	In vitro, 60 min	(Reistad et al. 2005)
TBBPA	Mussel hemocytes	Lysosomal membrane stability	Sign. decrease	5µM	In vitro, 30 min	(Canesi et al. 2005)
TBBPA	Mussel hemocytes	MAPK phosphorylation	Sign. increase	5µM	In vitro, ≤60 min	(Canesi et al. 2005)
TBBPA	Mussel hemocytes	PKC phosphorylation	Sign. increase	5µM	In vitro, ≤60 min	(Canesi et al. 2005)
TBBPA	Mussel hemocytes	Immune function (several parameters)	Sign. effect	5µM	In vitro, ≤60 min	(Canesi et al. 2005)
TBBPA	Freshwater fish (Carassius auratus)	ROS-production	Sign. increase	100mg/kg	I.p. injection, once	(Shi et al. 2005)
TBBPA	MCF-7 cells	Cell growth	Sign. induction	10^{-5} M	6 days	(Olsen et al. 2003)
PFOS	Rat, liver	LSI	Sign. increase	100 mg	I.p. injection, once.	(Berthiaume and Wallace 2002)
PFOS	Rat, liver	Body weight	Sign. decrease	100 mg	I.p. injection, once.	(Berthiaume and Wallace 2002)
PFOS	Rat, liver	Peroxisomal β-oxidation	Sign. increase	100 mg	I.p. injection, once.	(Berthiaume and Wallace 2002)
PFOS	Carp	membrane disrupting potential (serum ALT	Sign. increase	561ng/g liver	I.p. injection, once	(Hoff et al. 2003)

		activity)				
PFOS	Carp	membrane disrupting potential (serum AST activity)	Sign. increase	864ng/g liver	I.p. injection, once	(Hoff et al. 2003)
PFOS	Rat WB-534 cells	Gap junction intercellular communication	NOEL	6,25µM	30 min	(Hu et al. 2002)
PFOS	Dolphin kidney cells	Gap junction intercellular communication	NOEL	6,25µM	31 min	(Hu et al. 2002)
PFOS	Carp leukocytes	Membrane fluidity	Sign. increase	33µM	In vitro, 15 min	(Hu et al. 2003)
PFOS	Carp leukocytes	Mitochondrial membrane potential	Sign. increase	33µM	In vitro, 15 min	(Hu et al. 2003)
PFOS	Rats	L-FABP-DAUDA-binding	IC50	4,9µM		(Luebker et al. 2002)
PFOS	Paramecium caudatum KNZ 82	Backwards swimming	Sign. effect	5µM		(Matsubara et al.)
PFOS	Female pregnant rats	Serum T3 levels	Sign. effect	1mg/kg	In vivo, daily, 18 gestational days	(Thibodeaux et al. 2003)
PFOS	Nematode Caenorhabditis elegans fecundity	Multi-generation, number of worms	Sign. decrease	10pM	Four generations	(Tominaga et al. 2004)
PFOS	Nematode Caenorhabditis elegans acute toxicity	Mobility	EC50	2,22mM	48 hours	(Tominaga et al. 2004)
PFNA	Rat, liver	acyl-CoA elongations (palmotoyl-CoA)	Sign. effect	10mg/kg	In vivo, daily, 5 days	(Toyama et al. 2004)
PFNA	Rat, liver	activity of palmitoyl-CoA	Dose-dependent effect		In vitro	(Toyama et al. 2004)
PFNA	Paramecium caudatum KNZ 82	Backwards swimming	EC50	98,7±20,1µM		(Matsubara et al.)
PFNA	Rat, liver	Peroxisomal β-oxidation	Dose-dependent effect	2,5-20mg/kg	I.p. injection, daily, 5 days	(Kudo et al. 2000)
PFNA	Rat hepatocytes	acyl-CoA oxidase	Sign. increase	100µM	In vitro, 72 hours	(Kudo et al. 2000)
PFNA	Nematode Caenorhabditis elegans fecundity	Multi-generation egg-laying	Sign. decrease	1nM	Four generations	(Tominaga et al. 2004)
TBT	Sea star, Leptasterias polaris	Cell viability	No effect	2,35±0,86 µg(Sn) body burden	TBT-diet, 56 days	(Bekri and Pelletier 2004)
TBT	Sea star,	Lysosomal membrane	Sign. increase	1,41±0,99 µg	TBT-diet, 42 days	(Bekri and Pelletier 2004)

	Leptasterias polaris	stability		(Sn) body burden		
TBT	Sea star, Leptasterias polaris	Phagocytic activity	Sign. decrease	1,41±0,99 µg (Sn) body burden	TBT-diet, 42 days	(Bekri and Pelletier 2004)
TBT	Sea urchin, Parocentrotus lividus	embryonic development	NOEC	0,1µg/L	48 hours	(Bellas et al. 2005)
TBT	Sea urchin, Parocentrotus lividus	embryonic development	LOEC	0,2µg/L	48 hours	(Bellas et al. 2005)
TBT	Ciona intestinalis	embryonic development	NOEC	2µg/L	24 hour	(Bellas et al. 2005)
TBT	Ciona intestinalis	embryonic development	LOEC	4µg/L	24 hour	(Bellas et al. 2005)
TBT	Rat	Hemoglobin level	Sign. decrease	2µg/kg	gavage, weekly, 30 days	(da Silva de Assis et al. 2005)
TBT	Rat	Erythrocyte level	Sign. decrease	2µg/kg	gavage, weekly, 30 days	(da Silva de Assis et al. 2005)
TBT	Rat	Resident mononuclear peritoneal cells	Sign. decrease	2µg/kg	gavage, weekly, 30 days	(da Silva de Assis et al. 2005)
TBT	Rat thymocytes	ROS-production	Sign increase	1µM	5 min	(Gennari et al. 2000)
TBT	Sea urchin, Parocentrotus lividus eggs	Membrane destabilising (Ca2+-permeability)	Sign. increase	>7µM	10 min	(Girard et al. 1997)
TBT	Sea urchin, Parocentrotus lividus eggs	Membrane destabilising (Na+-permeability)	Sign.increase	0,1µM	20 min	(Girard et al. 1997)

Appendix 4

Multixenobiotic resistance in taxons

Taxonomical group	Organism	Reference
Bacteria	Clostridium	(George 1996)
Bacteria	Staphylococcus	(George 1996)
Bacteria	Streptococcus	(George 1996)
Protozoa	Plasmodium spp.	(Borst and Ouellette 1995)
Protozoa	Entamoeba spp.	(Borst and Ouellette 1995)
Protozoa	Leishmania spp.	(Borst and Ouellette 1995)
Fungi	Candida	(Prasad et al. 1995)
Fungi		
Sponge	Geodia cydonium	(Kurelec et al. 1992)
Sponge	Verongia aerophoba	(Kurelec et al. 1992)
Mussel	*Mytilus californianus*	(Cornwall et al. 1995)
	Mytilus galloprovincialis	(Galgani et al. 1996)
	Mytilus edulis	(Minier et al. 1993)
Clam	*Corbicula fluminea*	(Waldmann et al. 1995)
Oyster	*Crassostrea gigas*	(Minier et al. 1993)
	C. virginica	(Keppler and Ringwood 2001)
Marine worm	*Urechis caupo*	(Toomey and Epel 1993)
Marine snails	*Monodonta turbinata*	(Kurelec et al.)
Fish	Pleuronectes americanus	(Chan et al. 1992)
	Scaphthalmus maximus	(Tutundjian et al. 2002)
	Fundulus heteroclitus	(Bard et al. 2002)
Frog	*Xenopus laevis,*	(Bonfanti et al. 1998)