



## The $\beta$ -receptor blocker metoprolol alters detoxification processes in the non-target organism *Dreissena polymorpha*

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Evidence for significant physiological changes in an aquatic mollusc due to exposure to a pharmaceutically active compound detected by real-time PCR.

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### ABSTRACT

Due to increasing amounts of pharmaceutically active compounds (PhACs) in the aquatic environment, their largely unknown effects to non-target organisms need to be assessed. This study examined physiological changes in the freshwater mussel *Dreissena polymorpha* exposed to increasing concentrations (0.534, 5.34, 53.4 and 534  $\mu\text{g L}^{-1}$ ) of the  $\beta$ -blocker metoprolol in a flow-through system for seven days. The two lower concentrations represent the environmentally relevant range. Surprisingly, metallothionein mRNA was immediately up-regulated in all treatments. For the two higher concentrations mRNA up-regulation in gills was found for P-glycoprotein after one day, and after four days for pi class glutathione S-transferase, demonstrating elimination and biotransformation processes, respectively. Additionally, catalase and superoxide dismutase were up-regulated in the digestive gland indicating oxidative stress. In all treated mussels a significant up-regulation of heat shock protein mRNA was observed in gills after four days, which suggests protein damage and the requirement for repair processes. Metoprolol was 20-fold bioaccumulated for environmentally relevant concentrations.

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### 1. Introduction

Pharmaceutical drugs, used for treatment of human or animal diseases, are designed to have a specific effect at low doses. They may be partially metabolized in the target organism or excreted unmodified, thus entering the environment as pharmaceutically active compounds (PhACs). In sewage treatment plants (STP) many PhACs are insufficiently degraded and consequently enter the aquatic environment still exhibiting biological activity. Hence the aquatic flora and fauna may be continuously exposed to mixtures of PhACs. The biological effect of even low concentrations of these compounds on organism is so far little explored (Fent et al., 2006; Pomati et al., 2008). For example, it has been reported that bio-active estrogens widely used for contraception are adversely affecting vertebrates through its “feminization” of males (Kloas et al. 2009).

Throughout the last years PhACs have been increasingly detected due to more sensitive methods of measurement, such as mass spectrometry technologies. These detected PhACs include

painkillers,  $\beta$ -blocker, blood lipid regulators, hormones and veterinary pharmaceuticals (Kümmerer, 2009). Concentrations of PhACs are found in surface waters already in the  $\mu\text{g L}^{-1}$  range. The continuous input of these compounds into the environment as well as much higher concentrations directly at the outflow of sewage treatment plants, create an alarming situation for aquatic species, potentially suffering chronic and also acute toxic effects. Furthermore, aquatic organisms are not as efficient as mammals in detoxifying processes, such as biotransformation of PhACs or reducing exposure induced oxidative stress.

It has been shown that standard toxicity tests are less sensitive than selected non-standard tests, which consider the specific mode of action of these substances (Fent et al., 2006). In particular, the effects at extremely low concentrations require sensitive endpoints, such as physiological alterations that have been proven to be suitable and sensitive tools to assess the health status of exposed organisms and provided an insight into pollutant-specific modes of action of the respective substances in their organs. Using zebra fish liver cell line as in vitro test system for mixtures of PhACs at environmental levels, inhibition of cell proliferation has been observed, which was linked to a repression of genes involved in primary metabolism and cell cycle regulation. Furthermore an up-regulation of pathways of kinase signalling and DNA-repair

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mechanisms were observed (Pomati et al., 2007). In mussels, functions of the immune system, of the digestive gland, as well as of hemocytes altered due to the exposure to lipid lowering pharmaceuticals and triclosan, respectively (Canesi et al., 2007a,b). Moreover, general parameters, such as growth of gastropods (Pounds et al., 2008), immunocompetence of bivalves (Gagné et al., 2008), and morphological as well as behavioral changes in cnidarians or amphipods (De Lange et al., 2006; Quinn et al., 2008), may serve as indicators for serious physiological changes. Furthermore, the metabolic cost for maintaining homeostasis for survival and reproduction is an indicative tool of adverse conditions for an organism.

The  $\beta$ -blocker metoprolol used in treatments of diseases of the cardiovascular system has been found in surface waters in concentrations of up to  $2.2 \mu\text{g L}^{-1}$  (Ternes, 1998), and at the outflow of the STP Schoenerlinde, Berlin, Germany even in concentrations of up to  $5 \mu\text{g L}^{-1}$  (personal communication, Duennbier, Berliner Wasserbetriebe). Information on removal rates for  $\beta$ -blockers in STP highly vary between 0 and 96% (Andreozzi et al., 2003; Ternes, 1998). For the non-selective blocker propranolol LOEC in zooplankton and benthic organisms have been shown to be near to maximal measured STP effluent concentration (Fent et al., 2006; Huggett et al., 2002). Acute cytotoxic effects for propranolol have been demonstrated in primary rainbow trout hepatocytes and fish cell lines by the determination of cell viability and induction of cytochrome P450 1A monooxygenase activity (Laville et al., 2004). The impacts of the  $\beta_1$ -selective blocker metoprolol on non-target organisms are less well established, even though persistence in the environment of metoprolol (extrapolated half-life in Central Europe and the U.S. 28–95 days in summer and 190–449 days in winter) has been found to be four to five thousand times higher than persistence of propranolol (Liu and Williams, 2007).

As acute effect metoprolol caused an acceleration of the heart beat rate at low concentrations ( $2.67 \mu\text{g L}^{-1}$ ) in *Daphnia magna*, and a decrease of the heart beat rate at higher concentrations ( $26.7 \text{ mg L}^{-1}$ ) (Villegas-Navarro et al., 2003). Triebkorn et al. (2007) evidenced ultra-structural effects as the collapse of cellular compartmentation, glycogen reduction or vesiculation in liver of rainbow trout already at concentrations as low as  $1 \mu\text{g L}^{-1}$  metoprolol. Kidney and gills of rainbow trouts also displayed small to moderate reactions after 28 days exposure, highlighting the need for chronic tests with potentially affected aquatic species, as well as sensitive parameters in the risk assessment of PhACs.

Therefore, the aim of this study was to detect sublethal effects on the molecular level in the invertebrate species, the zebra mussel *Dreissena polymorpha*, due to exposure to the pharmaceutical metoprolol. A further aim of this study was to explore to which extent metoprolol, being a slightly hydrophobic thus water-soluble drug, can bioaccumulate in animals. The bioconcentration factor (BCF) was assessed for depicted times of exposure since there is no equilibrium for hydrophobic compounds between the concentration in the organisms and the surrounding water, but a tendency to accumulate in the fat tissue of the organism. Acquisition of experimentally derived data concerning bioconcentration are needed as there is no consideration of biotransformation and excretion processes when dealing with theoretical or chemically achieved data on accumulation prediction of chemical compounds for aquatic species (octanol–water partition coefficient).

Compared to metoprolol, elevated bioconcentration in daphnids has been reported for the more hydrophobic beta-blocker propranolol (Clevers, 2005). Possible differences in bioaccumulation depending on exposure concentrations shall be highlighted.

In this study we used the zebra mussel *D. polymorpha* as model organism to detect ecotoxicological effects of metoprolol. This species is widely used for conducting ecotoxicological experiments

(Binelli et al., 2007; Contardo-Jara and Wiegand, 2008; Minier et al., 2006). Since it possesses well functioning mechanisms of oxidative defense, biotransformation and excretion, it exhibits a relatively high resistance to xenobiotics (Contardo-Jara et al., 2009). Due to its ability to survive in contaminated environments, this organism perfectly lends itself to studies of physiological changes.

As molecular markers we used heat shock protein (hsp70) induction as very general ecotoxicological endpoint for protein damage and subsequent protective mechanisms (Lewis et al., 1999). mRNA levels of metallothionein (MT), aryl hydrocarbon receptor (AH-R) and protein phosphatase 2A (PP2A) were examined to give insight into the disturbance of other fundamental cell processes, such as the prevention from damage due to metal and reactive oxygen species exposure, receptor interactions and cell signalling. Furthermore, we measured mRNA expression changes of enzymes essentially involved in the prevention of oxidative stress, since superoxide dismutase (SOD), responsible for the reduction of the superoxide radical to hydrogen peroxide and the catalase (CAT), catalysing the breakdown of hydrogen peroxide to water and oxygen, have been shown to serve as reliable biomarker in environmental risk assessment (van der Oost et al., 2003). mRNA changes of biotransformation enzyme glutathione S-transferase (GST) was supposed to indicate the occurrence of damaging metabolites from phase I metabolism. Of further interest was whether the multi xenobiotic resistance mechanism (MXR), mediated by the trans-membrane P-glycoprotein (P-gp), operates to eliminate the applied substance (Bard, 2000; Smital et al. 2003) or its metabolites derived due to the exposure with the pharmaceutically active compound.

## 2. Materials and methods

### 2.1. Chemicals

( $\pm$ )Metoprolol(+)-tartrate salt (CAS: 56392-17-7) obtained from Sigma–Aldrich (Munich, Germany) with a minimum purity of 99% was dissolved in Milli-Q-grade water and used for exposure stock and HPLC standard preparation.

### 2.2. Maintenance of *D. polymorpha* and exposure scenario

*D. polymorpha* mussels within a size range of 22–25 mm were collected in July 2008 after spawning in 1 m depth from Lake Kuestrin, located in a remote area 150 km north of Berlin Germany. Lake water temperature was  $20^\circ\text{C}$ . During a two week acclimatization period, mussels were kept in 10 L glass aquaria each with approximately hundred individuals at  $20 \pm 0.5^\circ\text{C}$ . Mussels were fed twice daily with freeze dried *Spirulina* sp. powder. The artificial tank water (AFW) was reconstituted from Milli-Q-grade water ( $100 \text{ mg L}^{-1}$  Instant Ocean sea salt,  $200 \text{ mg L}^{-1}$   $\text{CaCl}_2$ ,  $103 \text{ mg L}^{-1}$   $\text{NaHCO}_3$ ) and changed every second day. Photoperiod was set at 12:12 h (light:dark). Two days prior to exposure, mussels were transferred into tanks consisting of 9 L glass aquaria ( $30 \times 20 \times 14.5 \text{ cm}$ ) containing 7 L AFW (70 mussels per tank) of a continuous flow-through system. Nutrition, water, temperature and light conditions were maintained constant during the time of acclimatization and exposure.

Mussels were exposed (3 tanks of 20 mussels for each exposure concentration and the control) for seven days in the continuous flow-through system to increasing concentrations of metoprolol:  $0$ ,  $2 \times 10^{-9}$ ,  $2 \times 10^{-8}$ ,  $2 \times 10^{-7}$  and  $2 \times 10^{-6} \text{ mol L}^{-1}$ , which corresponds to  $0$ ,  $0.534$ ,  $5.34$ ,  $53.4$  and  $534 \mu\text{g L}^{-1}$ , respectively. Each tank contained 7 L exposure medium. The continuous flow-through system operated at a flow rate of at least 50 L exposure medium per tank per day, which is equivalent to a water exchange rate of approximately seven tank volumes per day. One mixing chamber per exposure concentration received continuously filtered ( $0.45 \mu\text{m}$ ), UV-sterilized and temperature conditioned AFW and the according concentrated stock solution. The exposure medium was then split to supply a cluster of four tanks, each receiving  $140 \text{ mL min}^{-1}$  ( $\pm 5\%$ ). Constancy of the applied concentrations was therewith guaranteed, which was further monitored throughout the entire duration of the experiment by HPLC–MS–MS. Waste waters from the exposure were disposed through an activated-charcoal filter.

### 2.3. Tissue sampling

Gill and digestive gland tissue from eight mussels per each exposure were sampled ( $n=8$ ) after 1, 4 and 7 days. After 5 min rinsing in AFW, shells were

Carefully opened and the respective tissue immediately removed. In addition, whole tissue from six mussels ( $n = 6$ ) per concentration was sampled at each time point for analysis of metoprolol tissue content. All samples were taken randomly from the three aquaria of each exposure concentration, immediately shock-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until mRNA-isolation and tissue extraction. Control samples were taken at each time point.

#### 2.4. Metoprolol extraction and analysis

Samples from the exposure medium were acidified (0.1%) with HCOOH and diluted to match with the according calibration (see below).

Tissue samples were weighted and homogenized in 1 mL 0.1% HCOOH, followed by the addition of 30  $\mu\text{L}$  1 N HCl to achieve  $\text{pH} < 2$ . The sample clean up consisted of 3 washing steps with 2 mL diethyl ether, 5 min overhead agitation, 2 min centrifugation at 3000 g and subsequent discharge of the organic phase. The pH was then changed to  $>12$  by addition of 100  $\mu\text{L}$  of 1 N NaOH to the samples. In order to facilitate the separation, 5 mg of NaCl were added before shaking the sample overhead for 30 min with 3 mL ethyl acetate. After centrifugation at 3000 g the organic phase was removed and the extraction process repeated. The organic phases of both extraction steps were then combined and completely evaporated by nitrogen under vacuum. The samples were finally dissolved in 0.1% HCOOH and determined by HPLC-MS-MS using the following equipment and conditions: HPLC Agilent 1200 Series with an Eclipse XDB pre column ( $2.1 \times 12.5$  mm, 5  $\mu\text{m}$  pore size) combined with a Zorbax XDB C18 column ( $2.1 \times 50$  mm, 5  $\mu\text{m}$  pore size) and a furnace temperature of  $30^{\circ}\text{C}$ . Injection volume was 25  $\mu\text{L}$  at a flow rate of 200  $\mu\text{L min}^{-1}$  along a gradient of 97% solvent A ( $\text{H}_2\text{O} + 1\%$  MeOH + 0.1% HCOOH) and 3% solvent B (MeOH + 0.5% HCOOH) for 12 min, followed by 72% solvent A and 28% solvent B for 6 min, 47% solvent A and 53% solvent B for 2 min, and a final return to 97% solvent A and 3% solvent B for 10 min. The metoprolol retention time was 15–16 min.

For the subsequent MS-MS detection (Applied Biosystems 3200 Q Trap, Darmstadt, Germany) the MRM mode (positive mode) was used with a mass transfer of 268.3 (Q1) and 133.1 (Q3, used for calibration) and a detection limit of 0.5  $\mu\text{g L}^{-1}$ . Calibration was linear ( $R^2 = 0.99$ ) between 0.5 and 50  $\mu\text{g L}^{-1}$  and evaluation was conducted with the software package Analyst (Applied Biosystems).

The bioconcentration factor was calculated by dividing the metoprolol tissue concentration by the mean of the weeklong actual measured exposure medium concentration.

#### 2.5. Molecular markers

##### 2.5.1. RNA isolation and reverse transcription

RNA isolation and reverse transcription were conducted as described in Contardo-Jara and Wiegand (2008). Briefly, total RNA was isolated from gill and digestive gland tissue of *D. polymorpha* using the phenolic reagent TRIZOL (Invitrogen, Karlsruhe, Germany) according to the manufacturers' instructions. RNA concentration and purity (260/280 and 230/280 ratio) were assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Schwerte, Germany). Reverse transcription was carried out with M-MLV reverse transcriptase (Promega, Mannheim, Germany).

##### 2.5.2. Real-time PCR

Primers were designed according to the sequences published in the National Center of Biotechnology Information (NCBI). Table 1 summarizes the forward and reverse primer for each sequence analyzed in this study.

Real-time PCR assays for elongation factor 1- $\alpha$  (EF1- $\alpha$ ), hsp70, AH-R, CAT, SOD, MT, piGST, P-gp, and PP2A in *D. polymorpha* were run in a Stratagene Mx3005 p qPCR cyclor. Amplification efficiencies were determined in triplicate with a dilution series of pooled gill and digestive gland cDNA, respectively, and ranged between 1.95 and 2.00 ( $R^2 > 0.99$ , Table 1).

PCR reaction for all gene expressions were carried out with 2  $\mu\text{L}$  diluted cDNA in a 20  $\mu\text{L}$  reaction volume (500 nM each primer, Platinum Taq Buffer, 2 mM  $\text{MgCl}_2$ , 100  $\mu\text{M}$  each dNTP, 200-fold diluted SYBR-Green solution (Invitrogen), ROX reference dye (Invitrogen), and 1 U Platinum Taq polymerase (Invitrogen)) under the following conditions: initial denaturation at  $95^{\circ}\text{C}$  for 7.4 min, followed by 40 cycles of denaturation at  $95^{\circ}\text{C}$  for 17 s, primer annealing at  $62^{\circ}\text{C}$  for 25 s and elongation at  $72^{\circ}\text{C}$  for 25 s. PCR reactions were run in duplicate for all individual samples. Determination of the transcript abundances in individual samples was conducted with the comparative  $C_T$  method ( $\Delta\Delta C_T$ ) in consideration of a calibrator sample of pooled gill or digestive gland cDNA respectively, using corrections with respect to the specific PCR efficiency. Samples were normalized to EF1- $\alpha$  mRNA due to its constant expression. At the end of each reaction a melting curve analysis was carried out to proof assay specificity.

#### 2.6. Statistics

Testing the significance of induced and inhibited mRNA expressions between the different concentrations and the control group was performed using one-way analysis of variance (ANOVA) followed by Duncan's Test,  $p < 0.05$  (StatSoft, Inc. 2000). Log 10 or root transformations were used if ANOVA test criteria of normality

**Table 1**

Summary of primer pairs used. Abbreviation, sequences of the designed primers, NCBI accession numbers of the original sequences of the examined genes, and their efficiencies are presented.

Primer	Sequence	NCBI	Efficiency
EF	F CCACAAAGGCAGCCAAGAG	AJ250733	1.96
	R TGGGACGAGGTCAGCCATAC		
AH-R	F ATCAGCGATGAGCCTCAG	DQ159188	1.96
	R AGACAGCATTGCGAGGTCAC		
CAT	F ACGGCTATGGAAGCCACACG	EF681763	1.97
	R AGGTCGCGCATCGCATAGTC		
SOD	F GACAGCATGGCTTCCATGTG	AY377970	1.98
	R AGATTCTGGGCCAGTCAGAG		
GPx	F TAGACCGCCGCTCGGTAATC	EF194204	1.95
	R CACTCCAGCACCTGGAAC		
piGST	F TCCGCTATATCTGCCTGGAC	EF194203	1.98
	R GCTCCTTCAGACCTGCTTTC		
Hsp70	F TGTCTGCTTGTGGATGTAG	EF526096	1.96
	R CGTGGTGAATGCTCTGTAG		
P-gp	F TGGCAAATGCACATGACTTC	AJ506742	2.00
	R ATGGCAATACGCTGCTCTTG		
PP2A	F GAGGTCCTAATGTGTGAT	AF508223	1.95
	R CAGAGATGTCCTGTCCAAAG		
MT	F GCGTTGAAACCCTGATTGC	U67347	1.96
	R GCCACAACAGTTGGGTTGTC		

and homogeneity of variance failed. In case of further failure, data were analyzed using Dunn's test.

### 3. Results

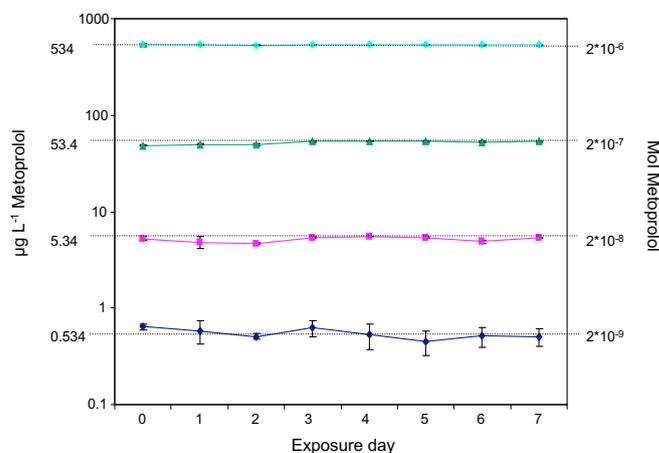
#### 3.1. Exposure medium

During the weeklong exposure, measured treatment concentrations were on average  $0.51 \pm 0.11$ ,  $5.15 \pm 0.17$ ,  $51.89 \pm 0.51$ , and  $537.54 \pm 5.38$   $\mu\text{g L}^{-1}$ , respectively (Fig. 1). For simplification, nominal concentrations are used in the denotation of the particular treatments in results and discussion.

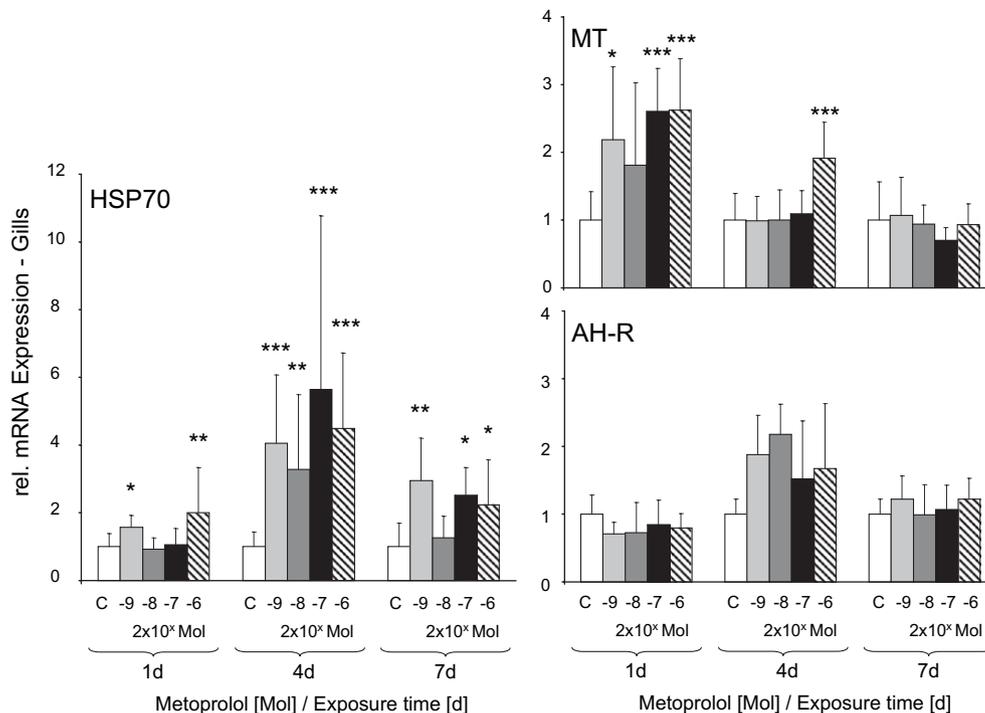
#### 3.2. Gene expression in gills

Strongest changes were found for hsp70, which significantly increased up to four fold in all treatments after four days (Fig. 2). Hsp70 mRNA expression remained up-regulated for one week, indicating protein damage and the requirement for repair processes.

After one day expression of MT mRNA in mussels from all treatments was significantly up to three fold increased compared to



**Fig. 1.** Applied concentration of metoprolol and constancy over exposure time by daily exposure medium measurement via HPLC-MS-MS.



**Fig. 2.** mRNA expression of heat shock protein 70 (hsp70), metallothionein (MT), and aryl hydrocarbon receptor (AH-R) in the gills of *Dreissena polymorpha* after exposure for one, four and seven days to  $2 \times 10^{-9}$ ,  $2 \times 10^{-8}$ ,  $2 \times 10^{-7}$  and  $2 \times 10^{-6}$  M corresponding to 0.534, 5.34, 53.4 and 534  $\mu\text{g L}^{-1}$  metoprolol, respectively. Results (mean  $\pm$  SD) are expressed relative to the control values, normalized to elongation factor 1- $\alpha$  (EF1- $\alpha$ ). Significant differences to the control were tested with one-way ANOVA. Asterisks indicate the significance level, \*\*\* = 0.001, \*\* = 0.01 and \* = 0.05. Log 10 transformation was used if ANOVA test criteria as normality and homogeneity of variances failed. In case of further failure data were analyzed by Dunns test.

control mussels (Fig. 2). After four days, the increase was still significant in mussels exposed to the highest metoprolol concentration. Furthermore, after four days exposure mussels of all treatments displayed higher mean values of AH-R mRNA in their gills. After one week both MT and AH-R mRNA expression returned to control levels.

In gill tissue no up-regulation was observed for the antioxidant enzymes SOD and CAT, which are supposed to ameliorate oxidative stress (Fig. 3). mRNA of piGST was significantly up-regulated after four and seven days at higher concentrations (Fig. 3), indicating biotransformation processes.

After only one day of exposure, a clear mRNA expression increase of the trans-membrane P-gp was observed, indicating an instant regulation to excrete the exposure substance or its metabolites (Fig. 3). In the treatment with the highest metoprolol concentration, P-gp expression was induced up to six times and mRNA levels decreased after longer exposure to control levels.

### 3.3. Gene expression in digestive gland

In digestive gland tissue of mussels exposed to the two higher concentrations hsp70 mRNA was up to five fold up-regulated already after one day, thus not statistically significant due to high variations between specimens. The environmentally relevant concentration of  $2 \times 10^{-8}$  M metoprolol caused a significant hsp70 mRNA up-regulation after four days (Fig. 4).

As in gill tissue, after one day MT mRNA expression increased in all treatments, but returned to the levels of the control group after four days (Fig. 4). A significant down-regulation of MT mRNA was observed in the treatment with  $2 \times 10^{-7}$  M metoprolol after one week. Similar, AH-R mRNA was significantly up-regulated after one day exposure, except at the lowest applied concentration and

decreased to control levels within four days. PP2A mRNA expression increased in all treatments only after four days (Fig. 4).

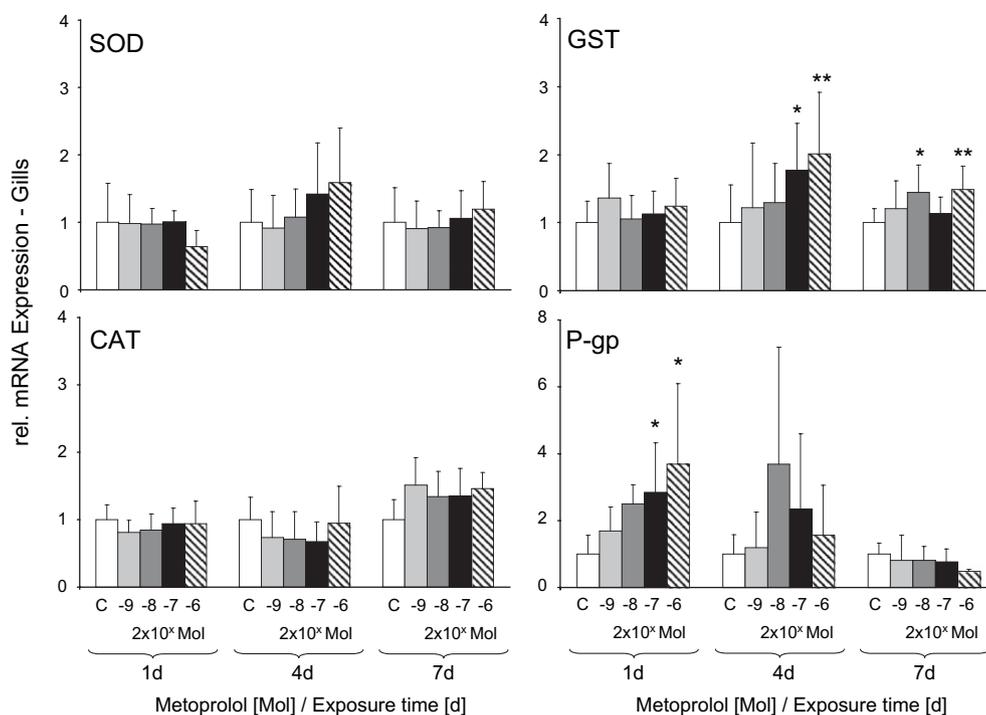
In contrast with gill tissue, digestive gland mRNA of the antioxidant enzymes SOD and CAT were significantly up-regulated after four days in mussels exposed to  $2 \times 10^{-7}$  and  $2 \times 10^{-6}$  M metoprolol, indicating oxidative stress (Fig. 5). mRNA expression of biotransformation enzyme piGST increased slightly in mussels exposed to the two higher concentration levels ( $2 \times 10^{-7}$  and  $2 \times 10^{-6}$  M metoprolol) after four days (Fig. 5). After one week no further alterations in mRNA expression of the selected proteins and enzymes were detectable in digestive gland tissue, indicating adaptation processes.

### 3.4. Bioaccumulation of metoprolol

Mussels exposed to  $2 \times 10^{-9}$  M metoprolol had 20-fold higher concentrations in the tissue compared to the exposure medium after only four days (Fig. 6). The bioconcentration factor (BCF) remained at this level during the one week exposure. In the treatment with  $2 \times 10^{-8}$  M metoprolol animals accumulated the substance four fold within four days and six fold within one week. For the higher applied concentrations  $2 \times 10^{-7}$  and  $2 \times 10^{-6}$  M metoprolol mussels had only two times higher concentrations in the tissue compared to the exposure medium after four days, thus eight times higher concentrations after one week.

## 4. Discussion

Due to increasing amounts of different PhACs in the aquatic environment and only limited understanding of the effects on non-target organisms, the performance of ecotoxicological tests with such organisms is urgently needed.  $EC_{50}$  in *D. magna* and LOEC in



**Fig. 3.** mRNA expression of superoxide dismutase (SOD), catalase (CAT), pi class glutathione S-transferase (GST), and trans-membrane P-glycoprotein (P-gp), in the gills of *Dreissena polymorpha* after exposure for one, four and seven days to  $2 \times 10^{-9}$ ,  $2 \times 10^{-8}$ ,  $2 \times 10^{-7}$  and  $2 \times 10^{-6}$  M corresponding to 0.534, 5.34, 53.4 and 534  $\mu\text{g L}^{-1}$  metoprolol, respectively. Results (mean  $\pm$  SD) are expressed relative to the control values, normalized to elongation factor 1- $\alpha$  (EF1- $\alpha$ ). Significant differences to the control were tested with one-way ANOVA. Asterisks indicate the significance level, \*\*\* = 0.001, \*\* = 0.01 and \* = 0.05. Dunns test was used if ANOVA test criteria as normality and homogeneity of variances failed.

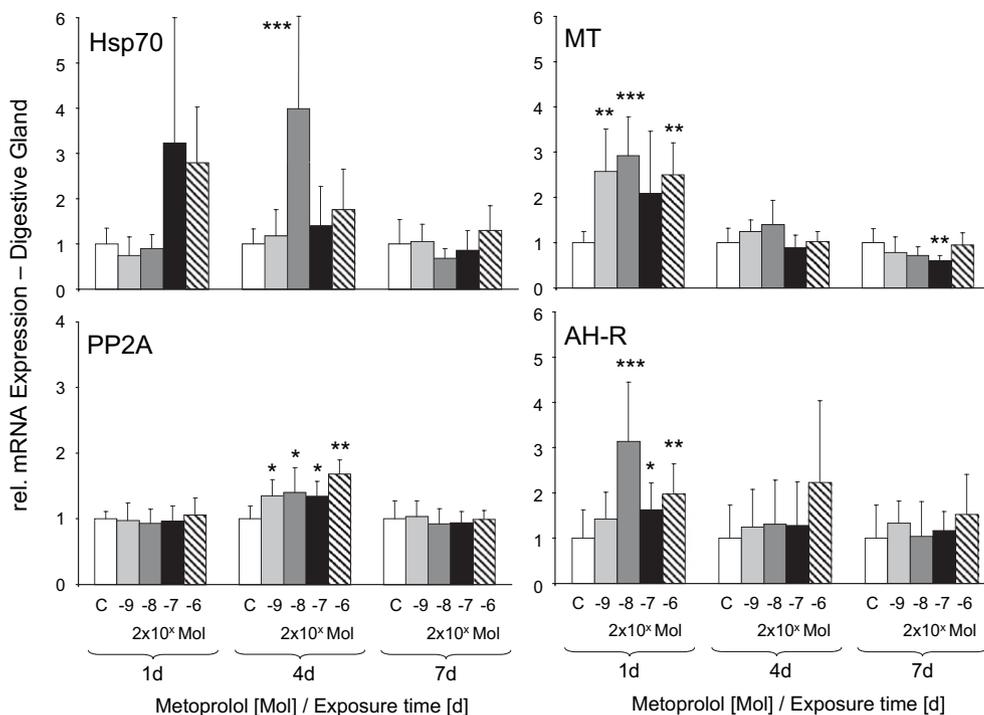
fish for the  $\beta$ -blocker metoprolol are in the environmentally relevant range (Cleuvers, 2005; Triebkorn et al., 2007) justifying toxicological research with organisms from other trophic levels, such as mussels. Mussels in particular are constantly exposed to xenobiotics due to their continuous filtering activity and therefore lend themselves to this type of research.

Since exposure to xenobiotics in general alters the stress status of organisms, provoking an imbalance in the homeostasis, hsp70 expression changes can serve as indicator for the requirement of protein repair, transport or protective processes. Hsp70 mRNA up-regulation in gills was strongest compared to all other alterations measured in this study and remained for one week. In digestive gland tissue of mussels exposed to the two highest concentrations hsp70 mRNA expression was highly induced after just one day. Interestingly, even in the treatment with the ambient relevant concentrations of  $2 \times 10^{-8}$  mol hsp70 mRNA expression was up-regulated although only after four days. In many previous studies induction of hsp70 has been used as a general stress biomarker in a range of algae, invertebrates, fish, and higher vertebrates as they react to protein damage caused by a variety of pollutants (Lewis et al., 1999). Studies with bivalves in particular confirm the rapid up-regulation of proteins of the hsp70 family as a result of thermal stress or exposure to heavy metals (Franzellitti and Fabbri, 2005; Piano et al., 2002, 2004). Only very few studies exist on the induction of hsp70 in invertebrates due to pharmaceutical products. For *D. magna* exposed to diclofenac hsp70 induction has been proven at high concentration in the  $\text{mg L}^{-1}$  range, which is beyond environmental relevance (Haap et al., 2008). We applied hsp70 as a marker of unspecific stress, as molecular mechanisms of metoprolol in invertebrates are not yet described. This revealed that already very low concentrations of xenobiotics, including PhACs, can modulate the mussels' hsp system.

For metoprolol it is known, that the detoxification in humans occurs via CYP2D6, a subfamily of CYP450, mainly by O-demethylation, and to a lesser extent by  $\alpha$ -hydroxylation and N-dealkylation (Belpaire et al., 1998). As the AH-R in turn, once present as a receptor–ligand-complex, serves directly as a transcription factor for enzymes of the CYP450 family, we assume from the observed up-regulation in both investigated tissues that metabolism of metoprolol occurs via CYP450 enzymes in mussels as well.

Furthermore, we examined mRNA changes of SOD and CAT in gills and the digestive gland as they are considered to be reliable biomarkers for oxidative stress derived from a contaminant-stimulated reactive oxygen species production in aquatic organisms (Livingstone, 2001). In contrast with gill tissue with no significant changes in mRNA levels of the antioxidant enzymes SOD and CAT, digestive gland mRNA of both enzymes was significantly up-regulated after four days in mussels exposed to  $2 \times 10^{-7}$  and  $2 \times 10^{-6}$  M metoprolol, indicating oxidative stress. In general, biotransformation processes are expected to be more pronounced in the digestive gland than in gill tissue, increased oxidative stress and reactive oxygen species production arise accordingly stronger in the digestive gland. In our study oxidative stress was presumably generated as a side-effect of phase I biotransformation. Gills, due to being respiratory organs, are continuously in contact with  $\text{O}_2$  and rather adapted to oxidative stress.

Moreover, we found a significant mRNA expression increase of up to three times for MT in all treatments after one day. After four days the increase was still significant in gills of mussels exposed to the highest metoprolol concentration. MT can avoid the damage due to metal exposure by binding metals with different affinity. Furthermore they also play an important role in the prevention of oxidative stress (Amiard et al., 2006; Coyle et al., 2002). The high cysteine content in MTs can also serve as scavenger of the hydroxyl radical derived from detoxifying processes, such as phase I of the



**Fig. 4.** mRNA expression of heat shock protein 70 (hsp70), protein phosphatase 2A (PP2A), metallothionein (MT), and aryl hydrocarbon receptor (AH-R) in the digestive gland of *Dreissena polymorpha* after exposure for one, four and seven days to  $2 \times 10^{-9}$ ,  $2 \times 10^{-8}$ ,  $2 \times 10^{-7}$  and  $2 \times 10^{-6}$  M corresponding to 0.534, 5.34, 53.4 and 534  $\mu\text{g L}^{-1}$  metoprolol, respectively. Results (mean  $\pm$  SD) are expressed relative to the control values, normalized to elongation factor 1- $\alpha$  (EF1- $\alpha$ ). Significant differences to the control were tested with one-way ANOVA. Asterisks indicate the significance level, \*\*\* = 0.001, \*\* = 0.01 and \* = 0.05. Log 10 or root transformation was used if ANOVA test criteria as normality and homogeneity of variances failed. In case of further failure data were analyzed by Dunns test.

biotransformation. Hence, the induction of MT mRNA can also indirectly indicate the oxidative stress status of an organism (Viarengo et al., 2000).

The increase in piGST mRNA expression after four and seven days in the exposure at  $2 \times 10^{-7}$  and  $2 \times 10^{-6}$  M metoprolol proves that also phase II biotransformation processes have occurred. Presumably, the CYP450 activated metoprolol, as indirectly shown by AH-receptor induction, is further metabolized via GST. A further explanation for the observed up-regulation in gills might be the enhanced requirement for biotransformation of waste products, derived from potential cellular damage due to the metoprolol exposure. The isoform piGST in particular inactivates lipoperoxidation products, such as lipid hydroperoxides and their derivatives as well as reactive oxygen species by conjugation to glutathione (Doyen et al., 2005). Martin-Diaz et al. (2009) used GST enzyme activity increase, besides lipid peroxidation and DNA damage in the mussel *Eliphtio complanata*, as ecotoxicological endpoint for the effects of PhACs and personal care products. However they applied on average 500-fold higher concentrations compared to our study. Concluding, mRNA induction of piGST seems to be a sensitive tool for detecting exposure to ambient relevant concentrations (low  $\mu\text{g L}^{-1}$  range) of PhACs and originating lipid peroxidation products, accordingly.

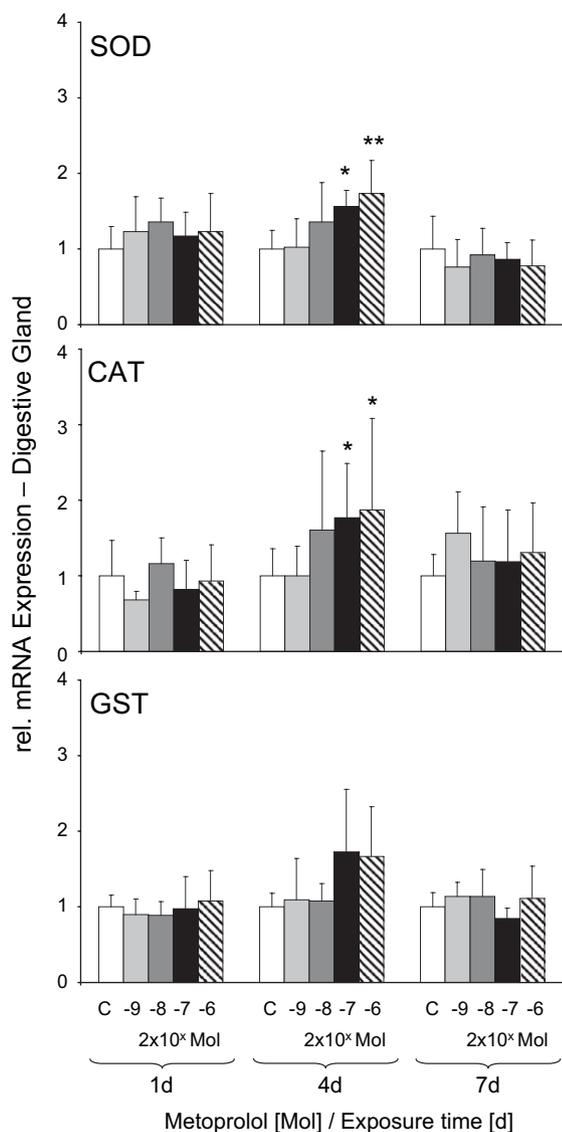
An immediate effort to excrete the applied substance was indicated by a significant up-regulation of the trans-membrane P-gp in gills. The concentration of  $2 \times 10^{-8}$  M metoprolol, which is twice that of environmentally relevant concentrations in German or French river waters (Miège et al., 2006; Ternes, 1998), provoked a P-gp mRNA increase after just four days. Activity and mRNA level changes of the P-gp can be used as an indicator for xenobiotic exposure as this excretion mechanism operates relatively nonspecifically, exporting not only non-metabolized parent compounds,

but also metabolites from the biotransformation as well as waste products derived from potential cell damage (Smital et al., 2003). As the MXR mechanism mediated by the P-gp is ATP dependent, the augmented energy requirement may impair the growth and reproduction in exposed organisms.

The  $\beta$ -blocker propranolol was tested at environmentally relevant concentrations (0.3 and 30  $\mu\text{g L}^{-1}$ ) for possible toxicity in the marine mussels *Mytilus galloprovincialis* (Fabbri et al., 2009). This revealed a similar significant up-regulation of the P-gp after exposure for one week, and further lowered lysosome membrane stability of mussel hemocytes, as well as a protein kinase A activity increase in the mantle. In contrast, Caminada et al. (2008) showed that in fish cell line PLHC-1 metoprolol and propranolol, the latter being a substrate of human P-gp (Yang et al., 2000) had no or an inhibitory effect on the P-gp, respectively.

After one week no further up- or down-regulation of the selected proteins and enzymes was detectable, except from piGST and hsp70 in gills, indicating adaptation processes to cope with the xenobiotic exposure. Nevertheless, the detected mRNA expression increases of the studied proteins and enzymes imply post-translational modifications, which were indicated by an mRNA up-regulation of PP2A. PP2A is responsible for protein phosphorylation, as the most common and important form of reversible protein modification. Hence, enhanced energetic costs due to new synthesis on one hand and regulatory processes on the other hand can cause the reduction in the health status of to PhACs exposed organisms.

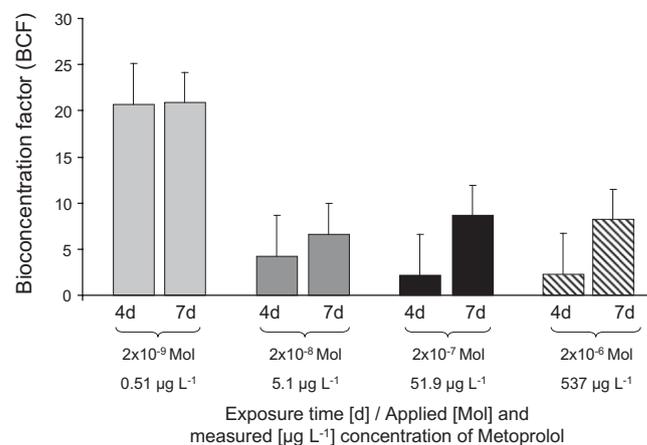
So far, there are only a few studies dealing with physiological alterations in algae and invertebrates due to  $\beta$ -blocker exposure. Cleuvers (2005) established EC<sub>50</sub>, PEC and PNEC for the  $\beta$ -blocker propranolol, metoprolol and atenolol in *D. magna*, *Desmodesmus subspicatus* and *Lemna minor*. He concluded that the established



**Fig. 5.** mRNA expression of superoxide dismutase (SOD), catalase (CAT), and pi class glutathione S-transferase (GST) in the digestive gland of *Dreissena polymorpha* after exposure for one, four and seven days to  $2 \times 10^{-9}$ ,  $2 \times 10^{-8}$ ,  $2 \times 10^{-7}$  and  $2 \times 10^{-6}$  M corresponding to 0.534, 5.34, 53.4 and  $534 \mu\text{g L}^{-1}$  metoprolol, respectively. Results (mean  $\pm$  SD) are expressed relative to the control values, normalized to elongation factor 1- $\alpha$  (EF1- $\alpha$ ). Significant differences to the control were tested with one-way ANOVA. Asterisks indicate the significance level, \*\*\* = 0.001, \*\* = 0.01 and \* = 0.05. Log10 transformation was used if ANOVA test criteria as normality and homogeneity of variances failed.

concentrations were of relatively low risk for these species. Nevertheless, he stresses the point of including chronic biotests with other aquatic organisms, bearing in mind the contribution of each single compound to the overall toxic potential of the mixture of substances present in the aquatic environment. For  $\beta$ -blocker, the toxicity classification can change from “not harmful” to “very toxic” if the substance is applied in single exposure or in a mixture (Hernando et al., 2004). Escher et al. (2006) demonstrated the effects of concentration addition in mixture experiments on the inhibition of the photosynthesis efficiency in green algae.

In our study, after just four days exposure we detected a 20 times higher concentration of metoprolol in mussel tissue compared to the exposure medium of  $2 \times 10^{-9}$  M metoprolol. For the higher applied concentrations  $2 \times 10^{-8}$ ,  $2 \times 10^{-7}$  and  $2 \times 10^{-6}$  M metoprolol, the animals accumulated the substance to a lesser extent, on average six



**Fig. 6.** Bioconcentration factor (BCF) of metoprolol (mean  $\pm$  SE) in *Dreissena polymorpha* after exposure for four and seven days to  $2 \times 10^{-9}$ ,  $2 \times 10^{-8}$ ,  $2 \times 10^{-7}$  and  $2 \times 10^{-6}$  M corresponding to 0.534, 5.34, 53.4 and  $534 \mu\text{g L}^{-1}$  metoprolol, respectively.

to eight times within one week. The predicted  $K_{ow}$  of 75 for metoprolol (Bendz et al., 2005) is much higher than determined in the present study. Presumably biotransformation and excretion processes, as observed in the exposed mussels, are not considered in the theoretical calculation. Furthermore, there is still uncertainty about the cellular up-take of metoprolol. If metoprolol is taken up actively, and thus carrier mediated such as propranolol (Dobson and Kell, 2008), the decreasing BCF with increasing metoprolol concentrations suggests a regulation of the uptake due to a competition for binding sites at the carrier itself, hence an inhibition of influx. This would mean that in mussels exposed to the lowest concentration of  $2 \times 10^{-9}$  M metoprolol no inhibition took place. If the up-take occurs passively by membrane permeation (Hayeshi et al., 2008), no regulation at low concentrations but a concentration dependent enhancement of biotransformation and excretion processes at higher concentrations, as clearly indicated in our study by mRNA expression changes, can be assumed. More understanding about up-take and elimination processes has to be established in future studies by quantification of metabolisation rates.

## 5. Conclusion

In conclusion, with this study we demonstrated the occurrence of physiological changes in a freshwater mussel due to metoprolol exposure at environmentally relevant concentrations. Naturally occurring continuous contact with xenobiotics can be simulated properly through exposure in flow-through systems, as medium concentrations are kept constant throughout the entire experiment.

The consideration of various physiological endpoints, such as biotransformation, antioxidant, elimination or cell protective processes, in this study inferred from gene expression patterns, provides a helpful tool to assess possible effects of PhACs to non-target organism. The observed changes in gene expression suggest a cellular effect of metoprolol on mussels. Whether this will also impact growth and reproduction has yet to be tested in further studies. Even though metoprolol is a water-soluble compound, this study proves that it can accumulate in mussels especially at low and environmentally relevant concentrations.

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## Appendix. Supplementary information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envpol.2010.03.012.

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