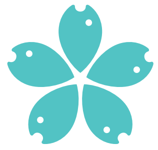

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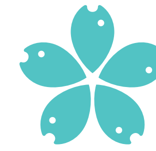
Pharmaceuticals in the aquatic environment and their effects in fish

Viktoriia Burkina



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Farmaka ve vodním prostředí a jejich vliv na ryby

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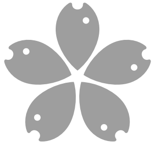

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LIST OF ABBREVIATIONS

4MBC	4-methyl-benzylidene camphor
ACN	Acetonitrile
AhR	Aryl hydrocarbon receptor
AMT	Alanine aminotransferase
ANOVA	Analysis of variance
ATE	Atenolol
BCF	Bioconcentration factor
BFC	7-benzyloxy-4-trifluoromethylcoumarin
BFCOD	7-benzyloxy-4-trifluoromethylcoumarin O-debenzylase
BP-3	2-hydroxy-4-methoxybenzophenone
BP-4	5-benzoyl-4-hydroxy-2-methoxy-benzenesulfonic acid
CAT	Catalase
CCB	Calcium channel blocker
CDNB	1-chloro-2,4-dinitrobenzene
CEC	Critical environmental concentration
CF	Condition factor
CK	Creatine kinase
CLO	Clotrimazole
COH	Coumarin 7-hydroxylase
CSI	Cardiosomatic index
CYP450	Cytochrome P450
DEX	Dexamethasone
DMSO	Dimethylsulfoxid
DNPH	2,4-dinitrophenylhydrazine
EDC	Endocrine disrupting chemical
EDTA	Ethylenediaminetetraacetic acid
EHMC	2-ethyl-hexyl-4-trimethoxycinnamate
ER	Ethoxyresorufin
EROD	7-ethoxyresorufin-O-deethylase
GLU	Glucose
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSI	Gonadosomatic index
GSSG	Glutathione disulphide
GST	Glutathione-S-transferase
Hb	Haemoglobin
Hc	Haematocrit
HFC	7-hydroxy-4-trifluoromethylcoumarin
HPLC	High-performance liquid chromatography
HSDB	Hazardous Substances Data Bank
HSI	Hepato-somatic index
HTPC	Human therapeutic plasma concentration
IC_{50}	Half maximal inhibitory concentration
K_m	Michaelis constant
LAC	Lactate
LC/MS	Liquid chromatography-mass spectrometry
LC_{50}	50% lethal concentration

LDH	Lactatedehydrogenase
Leuko	Leukocyte count
LOQ	Limit of quantification
LPO	Lipid peroxidation
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MDA	Malondialdehyde
MeOH	Methanol
MROD	7-methoxyresorufin-O-demethylase
MXR	Mitoxantrone resistance-associated protein
NADPH	Nicotinamide adenine dinucleotide phosphate
NADPH	β-nicotinamide adenine dinucleotide
NBT	Nitrotetrazolium blue chloride
OC	Octocrylene
PAH	Polycyclic aromatic hydrocarbon
PBSA	2-phenylbenzimidazole-5-sulfonic acid
PCB	Polychlorobiphenyl
PCDD	Polychlorinated dibenzo-p-dioxin
PCV	Haematocrit
PhAC	Pharmaceutical active compound
PLHC	Poeciliopsis lucida hepatocellular carcinoma
PMS	Phenazine methosulfate
PNP	P-nitrophenol
PNPH	P-nitrophenol hydroxylase
PPCP	Pharmaceuticals and personal care products
PR	Pethoxyresorufin
PROD	7-pethoxyresorufin-O-depentylase
PXR	Pregnane X receptor
RBC	Erythrocyte count
ROS	Reactive oxygen species
RSD	Relative standard deviation
SGR	Specific growth rate
SOD	Superoxide dismutase
STP	Sewage treatment plant
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TB-H	Tolbutamide hydroxylase
TCA	Trichloroacetic acid
TP	Total protein
TRIG	Triacylglycerols
TRIS	2-amino-2-hydroxymethyl-propane-1,3-diol
UV	Ultra violet
V_{max}	Maximal velocity
VRP	Verapamil
VTG	Vitellogenin
WBC	White blood cell
WWTP	Waste water treatment plant
β-AR	β-adrenergic receptor

CHAPTER 1

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

According to our hypothesis, emerging anthropogenic pollutants present in wastewater treatment plant (WWTP) effluents represent a specific risk for aquatic organisms and have the potential to affect them from the molecular to the ecosystem level. In this thesis, the impact of atenolol, verapamil, dexamethasone, clotrimazole, and PBSA on the physiological condition and health of rainbow trout (*Oncorhynchus mykiss*) were studied *in vivo* and *in vitro*. These experiments used a biomarker approach to test this hypothesis at the molecular and cellular level by examining the effects of pharmaceuticals on rainbow trout.

1.1. PHARMACEUTICALS AND PERSONAL CARE PRODUCTS: EMERGING ENVIRONMENTAL CONTAMINANTS

The occurrence and fate of micro-pollutants in the aquatic environment have become a research topic in recent decades. Micro-pollutants, also termed emerging contaminants, include pharmaceuticals and personal care products (PPCPs). Due to the extensive consumption of the vast array of PPCPs reaching bodies of water, they are one of the major inputs into the environment. Pharmaceutically active compounds (PhACs) are a group of substances broadly used to treat or prevent diseases in humans and animals. Approximately 3000 pharmaceutical products are available for human use (Fent et al., 2006), and several thousand are in the process of development. Personal care products, fragrances, disinfectants, UV filters, and insect repellents, among others, encompass an even wider range of compounds. From hundreds to thousands of tons of pharmaceuticals are consumed each year (Daughton and Ternes, 1999). A large portion of these compounds and their metabolites are then discharged into wastewater.

Today, India is one of the top emerging markets in the global pharmaceutical scene. Their healthcare sector grew three times in size during the period of 2002–2012, and according to the India Brand Equity Foundation (2013), the Indian pharmaceutical market will be the sixth largest in the world by 2020. In the United States, total prescription drug sales increased from $308.6 billion in 2010 to $319.9 billion in 2011 (Lindsley, 2012). These numbers indicate an increased number of medication prescriptions; therefore, increased consumption must be expected due to the expanding population and the continued growth of chronic therapies.

Due to growing concerns regarding the presence of PPCPs in the aquatic environment, new fast and sensitive multi-residue analytical methods are under development (Grabic et al., 2012; Kasprzyk-Hordern et al., 2007; Petrovic et al., 2014).

1.1.1. Sources and occurrence

To date, more than 150 PhACs have been identified in the aquatic environment, and the list is expanding. There are six major sources of these pollutants in the aquatic environment, including landfills, animal and freshwater aquaculture waste, and hospital, domestic and industrial waste. The most common therapeutic classes of drugs introduced into the aquatic environment are pain controllers, psychiatric drugs, antihypertensive drugs, antibiotics, and sex hormones. Common WWTPs do not effectively remove all of these compounds. Today, the presence of pharmaceuticals in the environment is being reported worldwide, in WWTP effluents (Anderson et al., 2013; Kostich et al., 2014; Ryu et al., 2014), hospital wastewater (Verlicchi et al., 2012; Verlicchi et al., 2010), surface waters (Ascar et al., 2013; Silveira et al., 2013), groundwater (Lapworth et al., 2012), and the soil (Li, 2014). Moreover, the concentrations of the PPCPs can vary with the season (Collado et al., 2014; Golovko et al.,

2014; Kosma et al., 2014). The concentrations measured in environmental samples have ranged from the low ng · L^{-1} up to a few µg · L^{-1} in WWTP effluents. Drug production facilities are another potential way for drugs to enter the environment. The concentrations measured in WWTP effluent differ by three orders of magnitude in comparison to the environmental water samples and are in the µg–mg · L^{-1} range (Larsson et al., 2007; Gilbert et al., 2011). Moreover, some PPCPs are also present in drinking water (Leung et al., 2013; Rodil et al., 2012). The presence of PhACs in drinking water has raised concerns over the potential risks to human health (WHO, 2012).

It is beyond the scope of this dissertation to discuss the global data from several hundred published papers. The level of pharmaceuticals in the aquatic environment that were used in this study are presented in Table 1.

Table 1. *Occurrence of atenolol, verapamil, dexamethasone, clotrimazole, and 2-phenylbenzimidazole-5-sulfonic acid (PBSA) in different aquatic environments around the world.*

Compound	Sampling site	Sample	Concentration (ng · L^{-1})	Ref.
Atenolol	America (South and North) and Canada	WWTP effluent	120–960	1
		Surface water	35	1
		Hospital wastewater	N.F.	
	Europe and UK	WWTP effluent	411–782	2
		Surface water	< LOQ–36	3
		Hospital wastewater	172–1171	2
	Asia	WWTP effluent	411	4
		Surface water	N.D.–690	5
		Hospital wastewater	1607	4
Verapamil	America (South and North) and Canada	WWTP effluent	12–340	6
		Surface water	N.D.	1
		Hospital wastewater	N.F.	
	Europe and UK	WWTP effluent	1.2–3.0	2
		Surface water	N.F.	
		Hospital wastewater	4.1–12	2
	Asia	WWTP effluent	N.F.	
		Surface water	N.F.	
		Hospital wastewater	N.F.	
Dexamethasone	America (South and North) and Canada	WWTP effluent	N.F.	
		Surface water	< LOQ	7
		Hospital wastewater	N.F.	

Table 1. *Occurrence of atenolol, verapamil, dexamethasone, clotrimazole, and 2-phenylbenzimidazole-5-sulfonic acid (PBSA) in different aquatic environments around the world (Continued).*

Compound	Sampling site	Sample	Concentration ($ng \cdot L^{-1}$)	Ref.
Dexamethasone	Europe and UK	WWTP effluent	N.D.	2
		Surface water	< 0.01–0.06	8
		Hospital wastewater	< LOQ–62	2
	Asia	WWTP effluent	2	9
		Surface water	N.D.	10
		Hospital wastewater	N.F.	
Clotrimazole	America (South and North) and Canada	WWTP effluent	N.F.	
		Surface water	N.F.	
		Hospital wastewater	N.F.	
	Europe and UK	WWTP effluent	10–100	11
		Surface water	6–34	12
		Hospital wastewater	N.F.	
	Asia	WWTP effluent	8	13
		Surface water	4	13
		Hospital wastewater	N.F.	
PBSA	America (South and North) and Canada	WWTP effluent	N.F.	
		Surface water	N.F.	
		Hospital wastewater	N.F.	
	Europe and UK	WWTP effluent	240	14
		Surface water	5.1–13000	15
		Hospital wastewater	N.F.	
	Asia	WWTP effluent	N.F.	
		Surface water	N.F.	
		Hospital wastewater	N.F.	

< LOQ, below the limit of quantification; N.D., not detected; N.F., not found in the literature; WWTP, wastewater treatment plant.

1, (Batt et al., 2008); 2, (Santos et al., 2013); 3, (Petrović et al., 2014); 4, (Lin et al., 2009); 5, (Kim et al., 2009); 6, (Kostich et al., 2014); 7, (Sengupta et al., 2014); 8, (Tölgyesi et al., 2010); 9, (Chang et al., 2007); 10 (Liu et al., 2011); 11, (Kahle et al., 2008); 12, (Roberts and Thomas, 2006); 13, (Huang et al., 2010); 14, (Rodil et al., 2012); 15, (Grabicova et al., 2013).

1.1.2. Potential pharmaceutical threats for fish

The presence of the emerging contaminants of human and veterinary pharmaceuticals in aquatic environments has increasingly raised concerns over their potential ecotoxicological effects. In general, because these substances are designed to act through drug target molecules found in humans, pharmaceuticals are suspected to affect non-target species

through those same target molecules. Thus, given adequate exposure, pharmaceuticals may induce potentially adverse effects in other living organisms, including fish. The predictive models are very important for the first-tier prioritisation of pharmaceutical products in an environmental risk assessment. In total, nine prioritization and ranking schemes have thus far been proposed for further ecotoxicity testing and the environmental monitoring of pharmaceutical substances (Roos et al., 2012). These schemes are based on different types of data and are often combined with expert judgment and/or a case-by-case assessment. The pharmaceuticals of interest were chosen based on their sales statistics data (atenolol), pharmacological potency (verapamil and dexamethasone), water concentration (PBSA), and logP rank (clotrimazole).

The human therapeutic plasma concentration (HTPC) is an important parameter for prioritizing drugs during an environmental risk assessment. To assume similar pharmacological effects in mammals and fish, 'effect ratios' are used. The effect ratio is the ratio between the HTPC after treatment with a therapeutic dose of the pharmaceutical and the estimated plasma concentration in fish at a given water concentration. If the effect ratio is ≤ 1, it means that the tested drug represents a possible pharmacological response (adverse or not) in fish and therefore needs to be prioritized for the environmental risk assessment (Hugget et al., 2003). Fick et al. (2010) used the HTPCs for 500 pharmaceuticals to predict the critical environmental concentrations that would induce pharmacological effects in fish.

Due to the constant presence of pharmaceuticals at a low concentration in the aquatic environment, they are most likely to induce chronic rather than acute toxic effects on aquatic non-target organisms. There are still a limited number of studies reporting the effects of PPCPs on aquatic organisms that refer to the environmentally relevant concentrations. Many pharmaceuticals need more investigation regarding their potential long-term ecotoxicological effects for a better understanding of their possible effects on aquatic life.

Despite the anatomical differences, many physiological and biochemical processes in fish are often remarkably similar to those in humans. A summary of the fish and human target system similarities and receptor identities in fish and mammalian organisms is presented in Table 2, with special attention paid to the drugs selected for study in this thesis.

Table 2. *A summary of the receptor/enzyme identities (%) in fish compared to those present in mammalian species in relation to the pharmaceuticals selected for this study.*

Drug	Therapeutic use	Target system	Target receptor/enzyme (Identity in fish)
Atenolol	Antihypertensive	Heart and smooth muscle	Beta-blocker (63%)
Verapamil	Antihypertensive	Heart and smooth muscle	Calcium channel blocker (98%)
Dexamethasone	Anti-inflammatory, immunosuppressant	Multiple systems	Glucocorticoid receptors (79%)
Clotrimazole	Aromatase inhibitor	Multiple systems	CYP450 (50%)

Huggett et al., 2003; Gunnarsson et al., 2008 and Bury et al., 2003.

The following section of the thesis focuses on the effects that may occur after fish are exposed to different drug categories, namely beta-blockers, calcium channel blockers, glucocorticosteroids, antifungals, and one personal care product, UV filters.

Anti-hypertensive medications are frequently detected in the aquatic environment, and beta-blockers and calcium channel blockers are common representatives of these drugs.

Beta-blockers

Atenolol is a selective antagonist of the β1-adrenergic receptors and is used primarily in the treatment of coronary heart disease, arrhythmias, and chest pain. The mode of action of beta-blockers is based on binding to β-adrenergic receptors and thus blocking the binding of catecholamines, adrenaline, and noradrenaline to these receptors (Reid et al., 1998). The concentration of beta-blockers such as atenolol in WWTP effluents is typically at 1 $\mu g \cdot L^{-1}$ level. The presence of beta-adrenergic receptors have been described in various fish tissues and cells. The physiological processes regulated by β-adrenergic receptors in fish, such as blood flow, blood pressure, homeostasis, and immunocompetence, may be affected by beta-blockers (Owen et al., 2007; Fabbri et al., 1995, Reid et al., 1992).

The homology of the human and fish beta receptors varies between fish species. For example, it is 52–56% for zebrafish and 50% for rainbow trout (Christen et al., 2010). Atenolol was reported to be non-toxic to medaka (*Oryzias latipes*) in an acute toxicity test (Kim et al., 2009) and was low toxic to fathead minnows in a short-term toxicity test (Winter et al., 2008). Sub-chronic exposure to atenolol (11 $\mu g \cdot L^{-1}$) caused changes in the gene expression in the brains of Atlantic salmon (*Salmo salar*) (Hampel et al., 2010). In addition, an *in vitro* study showed that environmentally relevant concentrations of atenolol disrupt the liver capacity for glucose production in rainbow trout (Ings et al., 2012). The experimental details and main effects of atenolol on fish are presented in Table 3. Lipophilic beta-blockers are metabolized by C-oxidative pathways and glucuronidation (Smith, 1985). Atenolol has a low lipophilicity, with only 10% metabolized by the liver and the remainder excreted as the parent compound (Oven et al., 2007). Unfortunately, the metabolite profile and effects of atenolol on fish CYP450 enzyme activities have not yet been reported. The effects of atenolol on fish CYP450-mediated reactions are a part of this thesis.

Calcium channel blockers

Verapamil represents an important drug in the calcium channel blocker (CCB) group and is used to reduce the heart rate in humans. Newly published data suggest that verapamil might also be used for reducing seizure frequency in drug-resistant epilepsy (Nicita et al., 2014). CCBs inhibit calcium influx through the slow channels of vascular smooth muscle and cardiac cell membranes. The transmembrane flow of calcium is an important modulator of cell excitability and muscle contraction through the response of the actin–myosin complex. A decrease in the level of intracellular free calcium causes coronary and peripheral vasodilation (Grossman and Messerli, 2004; Whyte et al., 2012). The measured levels of verapamil in the aquatic environment were in the $ng \cdot L^{-1}$ range in a number of effluents from WWTPs around the world.

The eventual effects of verapamil in fish have included decreased growth, a reduced heart rate, histological abnormalities, physiological stress, and responses in antioxidant enzyme activity (detailed in Table 3). Rottbauer et al. (2001) showed that the L-type calcium channel subunit can regulate heart growth and heart contraction functions in zebrafish. Sub-chronic exposure of rainbow trout to verapamil showed multiple responses in the brain, gill, liver, muscle, and intestines, indicating physiological stress of the fish (Li et al., 2011a). The 50% lethal concentrations of verapamil (LC_{50}) after short-term exposure have been determined at early life stages for juvenile rainbow trout (2700 $\mu g \cdot L^{-1}$; Li et al., 2010f), fathead minnow larvae (600 $\mu g \cdot L^{-1}$; Overturf et al., 2012), common carp embryos (16400 $\mu g \cdot L^{-1}$; Overturf et al., 2012), and common carp larvae (7300 $\mu g \cdot L^{-1}$; Steinbach et al., 2013).

In mammals, the contribution of the different cytochrome P450 enzymes (e.g., CYP1A2, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP3A3, and CYP3A5) to verapamil oxidative metabolism has been studied extensively (Ha et al., 2006; Kroemer et al., 1993; Opie, 2000; Pauli-Magnus

et al., 2000). Metabolic transformations were also studied in fish by Alderton et al. (2010), who identified 12 verapamil metabolites in zebrafish larvae that are also formed in mammals. However, there is no information on particular CYP450 isoforms involved in VRP metabolism in fish.

Glucocorticosteroids

Dexamethasone is a potent synthetic member of the glucocorticoid class. Glucocorticosteroids are corticosteroid hormones produced by the adrenal cortex. Due to their potent anti-inflammatory and immunosuppressive actions, glucocorticosteroids are commonly used for treating rheumatoid arthritis and asthma or as direct chemotherapeutic agents, but adverse effects such as impaired memory, attention deficits, increased skin fragility, and muscular atrophy may also occur. Glucocorticosteroids have inhibitory actions on the inflammatory mediators of both the cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism (Vane and Botting, 1987). High concentrations of dexamethasone in river water near a French pharmaceutical factory was reported to be 10 $\mu g \cdot L^{-1}$ (Gilbert, 2011), while concentrations commonly measured in surface waters are in the ng–$\mu g \cdot L^{-1}$ range. Dexamethasone has an intermediate ecotoxicity in hospital wastewaters (Frédéric and Yves, 2014). There are four dexamethasone metabolites in human liver microsomes (Gentile et al., 1996). The formation of two metabolites, 6 beta-hydroxydexamethasone and 6 alpha-hydroxydexamethasone, were catalysed by CYP3A4. Dexamethasone also induces CYP2B, CYP2A, CYP3A, and probably CYP2C11 activity in rats (Ringel et al., 2002). At present, the information on dexamethasone metabolism in fish is limited. Only a few studies have been done that focused on the effects of dexamethasone on the fish CYP450 system. Dexamethasone induces a slight increase of CYP3A activity in the PLHC-1 fish cell line (Wassmur et al., 2013) and induces of CYP1A2 activity in trout (Smith and Wilson, 2010) and grass carp (*Ctenopharyngodon idellus*) (Li et al., 2008). Recently, LaLone et al. (2012) reported effects in fathead minnows after chronic exposure to 500 $\mu g \cdot L^{-1}$ dexamethasone (Table 3). Despite the fact that dexamethasone was not designed to bind to oestrogen receptors, it may elevate the vitellogenin (VTG) protein levels in female fish (LaLone et al., 2012).

Antifungals

Clotrimazole is a specific inhibitor of Ca^{2+}-activated K^{+} channels (Wu et al., 1999) and is used as an aromatase inhibitor for the topical treatment of fungal, dermatophyte, and yeast infections. Antifungal drugs inhibit CYP51, which regulates the biosynthesis of ergosterol and is essential for the developmental growth of fungal membranes (Rozman and Waterman, 1998). Clotrimazole has been detected in wastewater and surface waters in Europe (Kahle et al., 2008; Roberts and Thomas, 2006) and Asia (Huang et al., 2010), ranging up to several $ng \cdot L^{-1}$. Escher et al. (2011) assigned clotrimazole to a list of compounds that have ecotoxicological risk quotients in hospital wastewater. These data are supported by Frédéric and Yves (2014), who highlighted the strong ecotoxicity potency of clotrimazole in hospital wastewater. The metabolite profile of clotrimazole has been studied in mammals (Fazlul, 2007; Shah et al., 2001, Rittenhouse et al., 1997; Brugnara et al., 1995), and at least 3 major non-active clotrimazole metabolites have been found. At the present, the information on clotrimazole interaction with the oxidative metabolism of fish is limited. Generally, a number of studies have evaluated the capability of clotrimazole to interact with CYP17, a key enzyme for androgen biosynthesis (Baudiffier et al., 2013; Baudiffier et al., 2012; Hinfray et al., 2011), and CYP19, the key enzyme regulating the local and systemic levels of oestrogen (Monod et al., 1993) in

aquatic organisms (detailed in Table 3). Recent reports also show that clotrimazole is a potent *in situ* inhibitor of CYP1A in the PLHC-1 cell line (Wassmur et al., 2013) and induces CYP1A expression in rainbow trout hepatocytes (Navas et al., 2004).

Currently, information about the effects of clotrimazole on fish at environmentally relevant concentrations is lacking.

Sun screen: 2-phenylbenzimidazole-5-sulfonic acid (PBSA)

PBSA is an organic UV filter that is listed among common personal care products. UV filters are used in cosmetic products, mainly to protect the skin from sunburn. Their presence has been detected in wastewater and tap water at the $\mu g \cdot L^{-1}$ and $ng \cdot L^{-1}$ levels, respectively (Silvia Diaz-Cruz et al., 2012, Rodil at al., 2012). The highest PBSA concentration was detected in outdoor swimming pools, at 13 $\mu g \cdot L^{-1}$(Grabicova et al., 2013). Photo-degradation is the main PBSA transformation pathway in natural waters. The half-life of PBSA in surface waters has been estimated to be from 3 days (from June to August) to 35 days (December) (Zhang et al., 2012). Lipophilic UV filters accumulate in the biota. Thus, recent studies have indicated that 4-methylbenzylidene camphor (4MBC), octocrylene (OC), and PBSA are able to accumulate in fish at concentrations up to 1800 $ng \cdot g^{-1}$, 2400 $ng \cdot g^{-1}$ and 83 $ng \cdot g^{-1}$, respectively (Buser et al., 2006; Grabicova et al., 2013). To the best of our knowledge, there is no information on the role of particular CYP450 isoforms involved in PBSA metabolism in fish. Generally, the studies have concentrated on the ability of UV filters to bind to oestrogenic receptors or to activate/inhibit hormonal activity. *In vitro* assays were used to test the effects of 18 of 27 commercially available UV filters on hormonal activity in fish. The UV filters displayed oestrogenic (9 compounds), anti-oestrogenic (14 compounds), androgenic (6 compounds), and anti-androgenic (16 compounds) activities *in vitro* (Kunz and Fent, 2006). *In vivo* testing of UV filters have shown increased levels of vitellogenin in fathead minnows, rainbow trout, and male medaka, suggesting oestrogenic activity for the sun screens that have been studied in fish (Kunz et al., 2006; Holbech et al., 2002; Inui et al., 2003). The importance of knowing how UV filters can affect key detoxification systems is to obtain a better picture of the cellular detoxification mechanisms in fish and predict potential health problems. To date, PBSA is a rarely studied UV filter, and there is no information available on the interaction of PBSA with the endocrine or CYP450 systems.

Table 3. *Effects of atenolol, verapamil, dexamethasone, and clotrimazole on fish.*

Drug	CEC (ng · L^{-1})	BCF	Fish species	Exposure concentration (µg · L^{-1})	Duration (days)	Effect
ATE	792332	0–0.27 (1)	Fathead minnows	100, 320, 1000, 3200, 10000	21	Had low chronic toxicity on the reproductive system (> 10 mg · L^{-1}) (2)
			Medaka (*Oryzias latipes*)	100000	4	96h LC_{50} > 100 mg · L^{-1} (3)
			in vitro study on trout hepatocytes	0.0027, 0.0266, 0.266, 2.66, 26.63, 266.34	1	Disrupted epinephrine-induced glucose production (4)
			Atlantic salmon	11	5	Modified the expression of 480 candidate genes; induced carbohydrate, energy, and nucleotide metabolism, genetic information processing, and cellular processes (5)
VRP	24	6.6–16.6 (6)	Rainbow trout	0.5, 27, 270	21, 42	At higher concentrations, induced physiological stress; influenced several blood indices (7)
			Rainbow trout	500, 1000, 2000, 5000, 10000	4	Induced environmental stress (8)
			Common carp embryos	1000, 2000, 5000, 10000, 20000	31	Significantly reduced heart rate; histological abnormalities (6)
			Common carp larvae	1000, 2000, 5000, 10000, 15000, 20000	31	Significantly reduced heart rate; histological abnormalities (6)
			Fathead minnow larvae in early life-stage	38, 75, 150, 300, 600	7	Growth reduction (600 µg · L^{-1}) (9)
DEX	21776	N.F.	Eel	20000 µg	10	Ultrastructural changes (10)
			Fathead minnow	0.1, 50, 500	21	Reduced fecundity and plasma oestradiol concentrations; increased plasma vitellogenin protein levels in females; hatched fry with abnormalities (11)
			Fathead minnow embryo–larvae	0.1, 50, 500	29	Reduced weight and length; increased deformed gill opercula (11)
			Fathead minnow	60, 119, 254, 57, 1160	7	Decreased survival (577 µg · L^{-1}) (9)

Table 3. *Effects of atenolol, verapamil, dexamethasone, and clotrimazole on fish. (Continued).*

Drug	CEC ($ng \cdot L^{-1}$)	BCF	Fish species	Exposure concentration ($\mu g \cdot L^{-1}$)	Duration (days)	Effect
CLO	N.F	610 (13)	Rainbow trout	1000000 μg in food	14	Reduced aromatase activity (12)
			Zebrafish	71, 159, 258	7	Elevated expression of steroidogenesis-related genes in testicular tissue (14)
			Zebrafish	50, 100, 250, 500	7	Affected testicular steroidogenesis *in vivo* and *ex vivo* (15)
			Rainbow trout	50000 $\mu g \cdot kg^{-1}$	1	Inhibition of cytochrome P450 activity (16)

ATE, atenolol; VRP, verapamil; DEX, dexamethasone; CLO, clotrimazole; CEC, critical environmental concentration (Fick et al., 2010); BCF, bioconcentration factor.
(1) Steinbach et al., 2014; (2) Winter et al., 2008; (3) Kim et al., 2009; (4) Ings et al., 2012; (5) Hampel et al., 2010; (6) Steinbach et al., 2013; (7) Li et al., 2011a; (8) Li et al., 2010f; (9) Overturf et al., 2012; (10) Bhattacharyya and Butler, 1980; (11) LaLone et al., 2012; (12) Shilling et al., 1999; (13) OSPAR, 2013; (14) Baudiffier et al., 2012; (15) Hinfray et al., 2011; (16) Levine and Oris, 1999.

1.2. BIOMARKERS AS TOOLS TO ASSESS ENVIRONMENTAL EXPOSURE TO PHARMACEUTICALS

In the aquatic environment, the exposure of living organisms to xenobiotics leads to interactions between the chemical and the biological system, which may give rise to biochemical disturbances and/or adaptive responses. A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to the exposure to environmental chemicals (van der Oost et al., 2003). Biomarkers represent key tools to estimate the ecological consequences of the anthropogenic pollution of water ecosystems. However, there is no single biomarker that can provide a complete diagnosis of the effects of exposure to pollutants on an organism. Consequently, the use of a battery of complementary biomarkers (multi-biochemical responses) is recommended to gain an understanding of how an organism responds to the total pollution load in an area.

Biomarkers can serve as early warning signals of exposure to pollutants, before there are any adverse effects on the individual animals or populations. Because of the ability of fish to metabolise, concentrate, and accumulate PhACs, fish have been used as a bioindicator for the presence of PhACs in aquatic environments.

A suite of fish biomarkers was listed in a review by van der Oost et al. (2003): biotransformation markers (phases I and II), biotransformation products (metabolites), stress proteins, metallothioneins, MXR proteins, haematological parameters, immunological parameters, reproductive and endocrine parameters, genotoxic parameters, neuromuscular parameters, and physiological, histological, and morphological parameters.

Biomarkers have been successfully used worldwide in environmental monitoring programs and for environmental risk assessments to detect the exposure and effects of chemicals.

1.2.1. Markers of biotransformation

PhACs undergo pharmacokinetic processes in aquatic organisms, including absorption, distribution, biotransformation, and excretion. Biotransformation is a specific term used for the biochemical transformation of chemicals in a living organism for protection against possible toxic effects of a xenobiotic. The drug metabolism reactions have been divided into two categories: phase I reactions (oxidative, reductive, and hydrolytic biotransformations) and phase II reactions (conjugations), followed by excretion.

Phase I reactions usually add a functional polar group(s) (-OH, $-NH_2$, -SH, -COOH) to a foreign molecule, which enables the phase II reaction to take place. It is catalysed by the cytochrome P450 (CYP450) group of enzymes and other enzymes associated with the smooth endoplasmic reticulum (oxygenases, enzymes that scavenge reduced oxygen, hydrolytic enzymes, and others). If the metabolites of the phase I reactions are sufficiently water soluble, they can be readily excreted at this point.

The phase II products are more water soluble than the original compound, are readily excreted, and are generally devoid of pharmacological activity and toxicity in the organisms. Phase II detoxification typically involves biochemical conjugation, in which various enzymes in the liver attach small chemical moieties such as glucuronic acid, sulphate, glycine, and other amino acids to the toxin. The conjugation reaction neutralizes the toxins and reactive intermediates left over from the phase I detoxification. Phase II pathways such as methylation and acetylation terminate or attenuate the biological activity, whereas glutathione (GSH) conjugation protects the body against chemically reactive compounds or metabolites.

Cytochrome P450 mono-oxygenases constitute a multigenic superfamily of enzymes (Hasler et al., 1999; Nelson et al., 1996). These enzymes form an extremely important metabolic system due to their involvement in regulating the titres of endogenous compounds such as hormones, fatty acids, and steroids. The identification and localization of CYP isoforms can provide scientists with insights into the potential interaction between pharmaceuticals and physiological functions (Gao et al., 2011). Today, 57 variations of CYP450 have been characterized in humans (Lewis, 2004). The function of mammalian CYPs has been well studied and documented due to their role in drug-drug interactions. CYP450s in fish have received less attention. Uno et al. (2012) reviewed the identification of 18 CYP gene families in fish (CYP1, CYP2, CYP3, CYP4, CYP5, CYP7, CYP8, CYP11, CYP17, CYP19, CYP20, CYP21, CYP24, CYP26, CYP27, CYP39, CYP46, and CYP51). The first three subfamilies are closely associated with drug metabolism. The expression of CYP genes can be induced by xenobiotics that bind to receptor proteins, which in turn results in an increase in protein synthesis and related enzyme activity. However, the affinity of a certain compound to bind to a specific receptor, and consequently induce or repress the respective CYP enzyme, is determined by its chemical structure and properties.

In fish, the CYP1 family contains four subfamilies, CYP1A, CYP1B, CYP1C, and CYP1D. The number of CYP1 genes differs between fish species, e.g. zebrafish have 5 (CYP1A, CYP1B1, CYP1C1, CYP1C2, and CYP1D1) (Jönsson et al., 2007), while rainbow trout have at least 6 (CYP1A1, CYP1A3, CYP1B1, CYP1C1, CYP1C2, and CYP1C3) (Jonsson et al., 2010). All CYP1 genes, with the exception of CYP1D, are regulated by the aryl hydrocarbon receptor (AhR) (Buhler and Wang-Buhler, 1998; Jönsson et al., 2007; Kubota et al., 2011; Sarasquete and Segner, 2000; Scornaienchi et al., 2010). CYP1A is the most studied fish enzyme. A popular assay for CYP1A activity is for 7-ethoxyresorufin O-deethylase (EROD), and it is often used as a biomarker to determine the presence of organic pollutants (Mandal, 2005). The EROD response might be affected by the presence of PPCPs. Studies on fish hepatocytes *in vitro* show that the drugs diclofenac, erythromycin, fluoxetine carbamazepine, sulphamethoxazole,

fenofibrate, clofibrate, nocodazole, clotrimazole, and ketoconazole inhibit CYP1A activity (Laville et al., 2004; Navas et al., 2004; Smith et al., 2012; Wassmur et al., 2013).

The CYP2 gene family is the largest and most complex of the 18 CYP gene families in vertebrates. In zebrafish, 47 CYP2 genes have been identified (Goldstone et al., 2010). Recently, 10 new genes in the new subfamily CYP2AA have been identified, which are not orthologous to any human or other mammalian genes (Kubota et al., 2013). In mammals, the constitutive androstane receptor (CAR) and pregnane X receptor (PXR) are involved in regulating CYP2 genes, but CAR is lacking in fish (Reschly and Krasowski, 2006). The fish regulatory mechanism for CYP2 is not yet well understood, but some CYP2 genes can be induced by PXR agonists (Kubota et al., 2013). At this time, there is only evidence that CYP2A-like, 2B-like, and 2E1-like proteins are present in fish (Celander et al., 1996; Geter et al., 2003). The catalytic activity of those CYP450-like enzymes can be measured by the reactions in fish to human probes, which in mammals are metabolised by CYP2A, CYP2B, and CYP2E1. Kaipainen et al. (1985) reported that rainbow trout microsomes catalyse coumarin 7-hydroxylation (COH) activity, a typical marker of mammalian CYP2A (Juvonen et al., 1988). CYP2B-like protein is expressed in fish, but unlike mammals, it is not induced by phenobarbitals. The regulatory mechanisms of CYP2B expression are a major difference between mammals and fish. The activity of 7-pentoxyresorufin O-depentylase (PROD), which is specific for the mammalian CYP2B forms, has been shown to be an indicator of a phenobarbital-type of induction in fish (Lindström-Seppä and Oikari, 1989). In mammals, p-nitrophenol hydroxylase (PNPH) activity is used to estimate the activity of CYP2E1. In fish, the isoform responsible for this reaction is not yet known, although a kinetic analysis and an *in vitro* inhibition study suggested that this isoform in fish may have traits in common with mammalian CYP2E1 (Geter et al., 2003; Zamaratskaia and Zlabek, 2011). Collectively, the CYP2 enzymes metabolize a variety of pharmacologically and toxicologically significant compounds.

In fish, CYP3 contains the CYP3A, CYP3B, and CYP3C subfamilies. The CYP3A subfamily is the dominant CYP450 form expressed in the fish liver. The induction of CYP3A expression in fish is probably regulated by the PXR (Bresolin et al., 2005; Wassmur et al., 2010). Similar to CYP2B-like protein, fish CYP3A-like activity was measured using reactions to human probes. CYP3A activity was measured as a transformation of the substrate 7-benzyloxy-4-trifluoromethylcoumarin into the product 7-OH-4-trifluoromethylcoumarin. Fish CYP3A catalytic activity can be inhibited or activated by various pharmaceuticals and other environmental pollutants. For example, ketoconazole inhibits CYP3A catalytic activity in rainbow trout *in vivo* by 60% to 80%, in killifish hepatic microsomes by up to 90% (Hegelund et al., 2004), and in juvenile Atlantic cod by 54% (Hasselberg et al., 2005). Antidepressants (fluoxetine, fluvoxamine, and paroxetine) inhibited carp CYP3A-like activity by more than 69% (Thibaut et al., 2006). The incubation details for the enzyme activity assays in rainbow trout liver microsomes are presented elsewhere (Burkina et al., 2012).

1.2.2. Biomarkers of oxidative stress

Oxidative stress is a normal phenomenon in the body, and various enzyme systems are involved in maintaining *in vivo* redox homeostasis. On the other hand, endogenous and exogenous substances can alter biochemical homeostasis, thus continuously raising reactive oxygen species (ROS) levels much higher than the detoxifying (antioxidant enzymes or non-enzymatic scavengers) capability of the local tissues. Therefore, oxidative stress is often defined as an imbalance of pro-oxidants and antioxidants. The term 'reactive oxygen species' includes both non-radical ROS such as hydrogen peroxide and free radicals such as superoxide and hydroxyl radicals, among others. The excessive free radicals then interact with

other molecules within the cells and cause oxidative damage to the nucleic acids, proteins, lipids, membranes, genes, and other cellular components. The mitochondrion is one of the major cell organelles responsible for ROS production, resulting from the generation of ATP through a series of oxidative phosphorylation processes. Other sources of ROS may be lipid peroxidation products and reactions where CYP450 enzyme, peroxisomal oxidase, NADH and NADPH oxidase, or xanthine oxidase kinetics are involved.

A number of oxidation-related biomarkers have been used as early warning signals of disturbances to fish health in environmental monitoring programs.

Defence against reactive oxygen species: Antioxidants

Organisms use both enzymatic (superoxide dismutases, catalase, and peroxidases) and non-enzymatic (e.g. vitamins and glutathione) antioxidants as well as some supporting enzymes to counteract the toxic effects of ROS. Antioxidants neutralize free radicals by donating one of their own electrons, thereby ending the electron-'stealing' reaction.

Superoxide dismutase (SOD) eliminates the free radical superoxide (O^{2-}) by converting it to peroxide that can in turn be destroyed by catalase (CAT) or glutathione peroxidase (GPx) reactions.

Glutathione is a low-molecular-weight antioxidant in cells and is a major non-protein thiol that plays detoxification, regulation, and immune roles in the cell. Glutathione is found in two forms: the reduced glutathione (GSH) monomer and the dimer, glutathione disulphide (GSSG).

The ratio between GSH and GSSG can therefore be used as an indicator of the oxidative stress status of the organism, because this ratio is determined by a combination of the rates of H_2O_2 removal by GPx, which catalyses the oxidation of GSH to GSSG. GSSG is then reduced to GSH by glutathione reductase (GR) at the expense of NADPH, which is recycled by the pentose phosphate pathway.

Glutathione-S-transferase is a phase II conjugation enzyme during biotransformation. It effectively protect cells against an array of hydrophobic and electrophilic compounds, including peroxidized lipids and xenobiotics, through the -SH group. In the process of glutathione conjugating the final product, mercapturic acids are formed and subsequently excreted.

The alterations of each of these enzymes during antioxidant defence are used as biomarkers in response to oxidative stress. A number of studies have evaluated SOD, CAT, GR, GPx, and GST in aquatic organisms, particularly in fish, as sensitive markers of physiological disorders.

Lipid peroxidation

Cell membranes are sensitive to ROS damage. The polyunsaturated fatty acids and cholesterols follow the same oxidation patterns, producing relevant hydroperoxides due to the availability of bis-allylic methylene groups. Bis-allylic hydrogens are very reactive toward oxygen radicals and readily oxidize to the primary products of lipid peroxidation. The primary oxidation products (hydroperoxides) are unstable and susceptible to decomposition. Secondary oxidation products demonstrate a high reactivity with proteins and DNA, and they include aldehydes, ketones, alcohols, and hydrocarbons. The biomarkers of lipid peroxidation have been reviewed by Niki (2014). An indirect TBARS (thiobarbituric acid-reactive substances) assay can be used to estimate lipid peroxidation. In this assay, the end-product of lipid peroxidation, malondialdehyde (MDA), reacts with thiobarbituric acid (TBA) to form a pink MDA-TBA complex that is measured spectrophotometrically (Ohkawa et al., 1979).

Protein carbonylation

Protein carbonylation is another marker of oxidative stress; however, it is not used in the experiments included in this thesis. ROS can oxidize amino groups or cleave the protein backbone, leading to numerous modifications or even the denaturation of proteins and loss of enzymatic activity. After carbonylation, the proteins cannot be repaired by cellular enzymes; they tend to aggregate, and if not eliminated, result in cell death. Carbonyl stress can occur when reactive carbonylated species accumulate in the cell. An assay for the detection of protein carbonyls involves the derivatisation of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH), which leads to the formation of a stable 2,4-dinitrophenyl (Levine et al., 1990).

1.2.3. Biomarkers of endocrine disruption

Many environmental pollutants, including PhACs, are ranked as endocrine disrupting chemicals (EDCs). Endocrine disruptors have the potential to cause reproductive problems and may effect sexual differentiation, growth, and development. Secondary sexual characteristics are under endocrine control. An important group of pollutants includes hormones such as the natural oestrone and 17β-oestradiol, and the synthetic 17α-ethinyloestradiol. To date, the analysis of endocrine disrupting effects has focused primarily on their target organs, the gonads (Dang, 2014). A wide spectrum of potential biomarkers could be applied to the study of endocrine disruption in the aquatic environment. The integrated use of biomarkers, such as plasma steroid hormones (17β-oestradiol and testosterone in females; and testosterone and predominantly 11-ketotestosterone in males), VTG, CYP450 activity, protein levels (i.e. zona radiata proteins), the gonadosomatic index (GSI), and gonad histology, has advanced the understanding of fish reproductive toxicology. VTG has the highest ranking among the biomarkers of endocrine disruption in fish (Marin and Matozzo, 2004). The production of VTG is normally initiated by natural oestrogen hormones. Females, therefore, have different levels of the protein depending on their hormonal cycles, while males and juveniles have very low levels throughout the year. When males and juveniles are exposed to xeno-oestrogens, however, they begin to produce VTG just like females. Therefore, the induction of VTG is used as a biomarker in both laboratory and field studies.

1.2.4. Haematological blood parameters

Fish exposed to environmental pollutants display a variety of physiological responses that include the blood biochemical profile, ion transmembrane transport, decreased oxygen uptake, and transport inhibition. The basic haematological parameters, erythrocyte morphology, and changes in the proportions of various leukocyte populations are used in fish to diagnose disease and as indicators of environmental stress (Velisek et al., 2011). The fish immune system is similar to that of other vertebrates and consists of various types of cells and chemical mediators. Fish haematological blood parameters are widely used in toxicological research for the evaluation of the immunotoxic effects of heavy metals (Svobodova and Pecena, 1988; Witeska et al., 2010). Published data show that a haematological response during exposure to PPCPs can be observed in fish. For example, the haemoglobin concentration, haematocrit, and red blood cells were decreased in rainbow trout after exposure to verapamil (270 $\mu g \cdot L^{-1}$), indicating normocytic anaemia in the exposed fish (Li et al., 2011a). Another study on rainbow trout chronically exposed to propiconazole (500 $\mu g \cdot L^{-1}$) showed the same changes in erythrocyte morphology, but with increased mean cell haemoglobin and mean cell haemoglobin concentration values, indicating macrocytic anaemia in the exposed fish (Li et al., 2011b). The haematological blood parameters can be measured using methods described by Svobodova et al. (1991).

1.3. DIRECTION OF THE PRESENT STUDY

This work investigated the effects of pharmaceuticals and one personal care product on the rainbow trout by using a wide spectrum of biomarkers. Two approaches have been applied:

- **Sub chronic *in vivo* studies**, where rainbow trout were exposed to verapamil, atenolol, clotrimazole, and the personal care product (2-phenylbenzimidazole-5-sulfonic acid). The enzymatic activity of the antioxidant defence system and hepatic CYP450 were estimated. Moreover, a detailed analysis of the effects of clotrimazole were measured among experimental groups for growth rate and condition factors, the hepatosomatic index, energy and oxidative stress status, basic haematological and biochemical blood parameters, and the distribution and bioaccumulation of the tested substance.
- ***In vitro* studies using fish hepatic microsomes** to evaluate the inhibition of CYP450 activity by clotrimazole and dexamethasone.

List of original articles and the author's contribution to the articles:

Paper 1.
Steinbach, C., Burkina, V., Fedorova, G., Grabicova, K., Stara, A., Velisek, J., Zlabek, V., Schmidt-Posthaus, H., Grabic, R., Kocour Kroupova, H., 2014. The sub-lethal effects and bioconcentration of the human pharmaceutical atenolol in rainbow trout (*Oncorhynchus mykiss*). Science of the Total Environment. 497–498: 209–218. (IF 2013 = 3.163)
Burkina Viktoriia participated in the research design, managed the exposure period, assisted in the sampling and sample preparation, and participated in the biomarker measurements, data processing, and discussed the data obtained with the author.

Paper 2.
Burkina, V., Zamaratskaia, G., Randak, T., Li, Z.H., Fedorova, G., Pickova, J., Zlabek, V., 2012. Verapamil does not modify catalytic activity of CYP450 in rainbow trout after long-term exposure. Ecotoxicology and Environmental Safety 79: 148–152. (IF 2011 = 2.340)
Burkina Viktoriia was directly involved in sample preparation, measure of the cytochrome enzyme activity, data processing, statistics, and preparation of the manuscript.

Paper 3.
Grabicova, K., Fedorova, G., Burkina, V., Steinbach, C., Schmidt-Posthaus, H., Zlabek, V., Kocour Kroupova, H., Grabic, R., Randak, T., 2013. Presence of UV filters in surface water and the effects of phenylbenzimidazole sulfonic acid on rainbow trout (*Oncorhynchus mykiss*) following a chronic toxicity test. Ecotoxicology and Environmental Safety 96: 41–47. (IF 2012 = 2.294)
Burkina Viktoriia participated in the research design, assisted in the experiments, care of the fish during the exposure period, numerous sampling events, sample preparation, biomarker measurements, and data processing, and discussed the data obtained with the author.

Paper 4.
Burkina, V., Fedorova, G., Grabicova, K., Grabic, R., Golovko, O., Randak, T., Sakalli, S., Zlabek, V., 2014. Clotrimazole in rainbow trout I: distribution, bioaccumulation, depuration and response of cytochrome system after long-term exposure. Manuscript.
Burkina Viktoriia participated in the research design, performing the experiments, caring for the fish during the exposure period, numerous sampling events, sample preparation,

biomarker measurements, data processing, and statistics; and prepared and finalized the manuscript.

Paper 5.
Burkina, V., Oliveira, R., Schmidt-Posthaus, H., Domingues, I., Fedorova, G., Steinbach, C., Randak, T., Zlabek, V., 2014. Clotrimazole in rainbow trout II: biological and structural effects after long-term exposure. Manuscript.
Burkina Viktoriia participated in the research design, performing the experiments, sampling, sample preparation; measured the biomarkers; processed the data; and prepared the manuscript.

Paper 6.
Burkina, V., Zlabek, V., Zamaratskaia, G., 2013. Clotrimazole, but not dexamethasone, is a potent *in vitro* inhibitor of cytochrome P450 isoforms CYP1A and CYP3A in rainbow trout. Chemosphere 92: 1099–1104. (IF 2012 = 3.206)
Burkina Viktoriia participated in the research design, performed the experiments, sampling, sample preparation, and cytochrome P450 inhibition measurements; processed the data; and prepared the manuscript.

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CHAPTER 2

EFFECTS OF PHARMACEUTICALS AND PERSONAL CARE PRODUCTS ON RAINBOW TROUT

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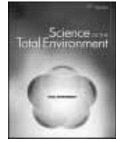

The sub-lethal effects and tissue concentration of the human pharmaceutical atenolol in rainbow trout (*Oncorhynchus mykiss*)

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HIGHLIGHTS

- Atenolol affects the haematological and biochemical profile of trout.
- Atenolol has an effect on the vascular system of trout.
- Atenolol doesn't affect the activities of the studied CYP450 isoforms in the liver.
- Atenolol was found to have a very low bioconcentration factor (reaching 0.27).

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ABSTRACT

Atenolol is a highly prescribed anti-hypertensive pharmaceutical and a member of the group of β-blockers. It has been detected at concentrations ranging from ng L^{-1} to low μg L^{-1} in waste and surface waters. The present study aimed to assess the sub-lethal effects of atenolol on rainbow trout (*Oncorhynchus mykiss*) and to determine its tissue-specific bioconcentration. Juvenile rainbow trout were exposed for 21 and 42 days to three concentration levels of atenolol (1 μg L^{-1} — environmentally relevant concentration, 10 μg L^{-1}, and 1000 μg L^{-1}). The fish exposed to 1 μg L^{-1} atenolol exhibited a higher lactate content in the blood plasma and a reduced haemoglobin content compared with the control. The results show that exposure to atenolol at concentrations greater than or equal to 10 μg L^{-1} significantly reduces both the haematocrit value and the glucose concentration in the blood plasma. The activities of the studied antioxidant enzymes (catalase and superoxide dismutase) were not significantly affected by atenolol exposure, and only the highest tested concentration of atenolol significantly reduced the activity of glutathione reductase. The activities of selected CYP450 enzymes were not affected by atenolol exposure. The histological changes indicate that atenolol has an effect on the vascular system, as evidenced by the observed liver congestion and changes in the pericardium and myocardium. Atenolol was found to have a very low bioconcentration factor (the highest value found was 0.27). The bioconcentration levels followed the order liver > kidney > muscle. The concentration of atenolol in the blood plasma was below the limit of quantification (2.0 ng g^{-1}). The bioconcentration factors and the activities of selected CYP450 enzymes suggest that atenolol is not metabolised in the liver and may be excreted unchanged.

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1. Introduction

Over the last few years, various pharmaceuticals have been widely detected in aquatic environments at concentrations ranging from ng L^{-1} to low μg L^{-1} (Fent et al., 2006). However, because pharmaceuticals are designed to be effective even at low doses, these low concentrations may affect aquatic organisms (Gunnarsson et al., 2008). In general, pharmaceuticals have the potential to affect non-target species because many drug targets are evolutionarily conserved across vertebrate species (Gunnarsson et al., 2008; Corcoran et al., 2010). This fact raises a concern regarding the long-term influence of pharmaceuticals on aquatic organisms (Fent et al., 2006).

Arterial hypertension is a highly prevalent chronic disease in humans. The estimated total number of adults with hypertension worldwide was 972 million in the year 2000 (Kearney et al., 2005). Therefore, it is not surprising that anti-hypertensive and cardiovascular drugs are one of the most commonly prescribed classes of human pharmaceuticals (Jjemba, 2008; Jones et al., 2002). For instance, in the United States, 601 million prescriptions of antihypertensive and

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cardiovascular pharmaceuticals were dispensed in the year 2005 (Jjemba, 2008). As far as atenolol is concerned, Jones et al. (2002) reported the prescription of 29 tons of this drug in England in the year 2000. In the Czech Republic, atenolol prescription for treatment of cardiovascular diseases was 2.7 tons in the year 2011 (The State Institute for Drug Control (Czech Republic); http://www.sukl.eu).

β-blockers are one of the classes of cardiovascular drugs that are used not only to treat arterial hypertonic diseases but also in the treatment of angina pectoris, heart failure, and glaucoma (Owen et al., 2007). As their name suggests, β-blockers block β-adrenergic receptors (β-ARs), which are mainly found in the heart, blood vessels, and the lungs in the human body and can be stimulated by binding with catecholamines (e.g., adrenaline and noradrenaline) (Frishman, 2003; Owen et al., 2007). For instance, β-AR stimulation leads to an increase in the heart rate, heart muscle contraction, blood pressure, and relaxation of smooth muscle in the bronchial tubes in the lungs (Frishman, 2003). The administration of β-blockers to patients blocks the access of catecholamines to their receptors, which results in reductions in the heart rate and blood pressure (Frishman, 2003; Prichard et al., 2001). Atenolol, which belongs to the β-blocker class of pharmaceuticals, is a selective antagonist of the β_1-AR that exhibits a greater binding affinity for the β_1 than for the β_2-AR (Mehvar and Brocks, 2001; Owen et al., 2007).

β-ARs are relatively conserved among vertebrates (Owen et al., 2007). For example, the transmembrane sequence of the rainbow trout β_1 and β_2-ARs share 57 and 63% identity with the corresponding mammalian β-ARs (Nickerson et al., 2001). β-ARs can be found in several fish organs (heart, gills, liver, brain, spleen, head kidney, and muscles) and cells (erythrocytes; Owen et al., 2007). Thus, the physiological processes regulated by β-ARs in fish, such as blood flow, blood pressure, homeostasis, and immunocompetence, may be affected by β-blockers (Nickerson et al., 2002; Owen et al., 2007, 2009; Massarsky et al., 2011).

Atenolol has been widely detected in concentrations ranging from ng L^{-1} up to μg L^{-1} in waste and surface waters. It can be traced in surface water since it is not fully eliminated in STPs, e.g. Vieno et al. (2007) and Golovko et al. (2014) reported for atenolol an elimination rate of 10–61% depending on season. For example, Maurer et al. (2007) reported an atenolol concentration of 1.54 μg L^{-1} in untreated wastewater. In effluent from SPT in Ceske Budejovice (Czech Republic), atenolol concentration was ranging between 0.39 and 1.69 μg L^{-1} during one year monitoring study (Golovko et al., 2014). Zuccato et al. (2006) found 0.17 μg L^{-1} atenolol in surface water. Vieno et al. (2006) and Daneshvar et al. (2010) reported atenolol in concentration of 0.01 to 0.11 μg L^{-1} in river water in Sweden and Finland, respectively. Moreover, Jones et al. (2002) predicted an atenolol concentration of 0.89 μg L^{-1} in English rivers. In addition, atenolol is expected to be present in aquatic environment because it is relatively stable to photolysis by sunlight (half-life = 77 to 730 h), its microbial biodegradation is slow (half-life = 340 to 2900 h), and it has a low affinity for absorption to sediments (Jones et al., 2002; Yamamoto et al., 2009; Küster et al., 2010).

At present, there is limited information available on the effect of atenolol on fish. Atenolol has been found to have low acute toxicity (96hLC_{50} > 100 mg L^{-1}) to medaka (*Oryzias latipes*; Kim et al., 2009). Hampel et al. (2010) reported that a short-term exposure (5 days) to atenolol (11 μg L^{-1}) alters the gene expression patterns in the brain of Atlantic salmon (*Salmo salar*). Winter et al. (2008), who conducted a short-term reproduction study (21 days) and early life stage test (28 days) on fathead minnow exposed to atenolol, found that atenolol affected the studied endpoints at high concentrations. At the concentration of 3.2 mg L^{-1} atenolol caused an increase in condition index of male fish only and it reduced the growth rate of fathead minnow larvae at 10 mg L^{-1} (Winter et al., 2008). In addition, a few in vitro studies have reported an effect of atenolol on fish hepatocytes and gonad cells (Ings et al., 2012; Fernandez et al., 2013). However, long-term exposure studies with environmentally relevant concentrations are lacking.

In mammals, pharmaceuticals are mainly metabolised by the cytochrome P450 (CYP450) superfamily, particularly the members of the CYP1–CYP4 families, which are predominantly expressed in the liver (Hasler et al., 1999). The hepatic CYP3A4, which contributes to the metabolism of 28% of the drugs in human, is one of the most important isoforms associated with this process (Lewis, 2004). CYP1A (EROD) is the most studied isoform in connection with the metabolism of various substances, mainly xenobiotics, in fish and is therefore widely used as a biomarker of environmental contamination (Uno et al., 2012). For example, propranolol, a member of the β-blocker group, induces the activity of CYP1A in rainbow trout primary hepatocytes (Laville et al., 2004). Furthermore, Corcoran et al. (2012) demonstrated that propranolol and other pharmaceuticals are able to induce the expression of CYP450 enzymes in carp primary hepatocytes through their interaction with pregnane X receptors (PXR). In contrast, atenolol does not induce the activity of CYP1A in rainbow trout gonad cells (Fernandez et al., 2013). *In vivo* studies on CYP450 enzyme activities in fish exposed to atenolol are missing.

During the insertion of oxygen into a substrate, a reaction that is catalysed by the CYP450 enzyme system, the uncoupling of the catalytic turnover from substrate oxidation can result in the production of superoxide or H_2O_2, which may lead to oxidative stress (Richard et al., 2008; Lawrence and Hemingway, 2003). Moreover, oxidative stress can be a response to general stress conditions (Lushchak, 2011). Exposure to pollutants is known to enhance the production of reactive oxygen species (ROS), leading to a deficiency in the antioxidant system and thereby resulting in an imbalance of the antioxidant enzyme activities (Morel and Barouki, 1999; van der Oost et al., 2003; Sieroslawska et al., 2012). The activity of antioxidant enzymes has also been used as a biomarker of oxidative stress due to pharmaceuticals, namely diazepam, clofibric acid, and clofibrate (Nunes et al., 2008). At present, only one *in vitro* study on rainbow trout gonad cells exposed to atenolol is available, and the results showed no effect of this substance on ROS production (Fernandez et al., 2013). Information on the antioxidant response of fish exposed to atenolol *in vivo* is missing.

Owen et al. (2007) suggested to study the histological changes in fish exposed to β-blockers to determine an organ-specific response; however, the effect of atenolol on the histological features of fish tissues, particularly the heart and liver, had not been assessed prior to the present study.

The objective of the present study was to assess the sub-chronic effects of the β-blocker atenolol at sub-lethal concentrations on juvenile rainbow trout (*Oncorhynchus mykiss*). Specifically, the present study aimed to 1) investigate the impact of atenolol exposure on the haematological and biochemical profiles of the blood plasma and the histology of the liver and heart, 2) measure the enzymatic activity of the enzymes involved in the detoxification and oxidative stress response in the liver and gills, and 3) analyse the tissue-specific bioconcentration of atenolol.

2. Materials and methods

2.1. Chemicals

Atenolol ((+)-4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy] benzeneacetamide, CAS No.: 56715-13-0, purity 99%), resorufin, 7-ethoxyresorufin (ER), 7-methoxyresorufin (MR), 7-pentoxyresorufin (PR), 7-benzyloxy-4-trifluoromethylcoumarin (BFC), nicotinamide adenine dinucleotide phosphate (NADPH), glutathione disulphide (GSSG), ethylenediaminetetraacetic acid (EDTA), reduced dipotassium salt of β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitrotetrazolium blue chloride (NBT), and 2-amino-2-hydroxymethyl-propane-1,3-diol (TRIS) were obtained from Sigma-Aldrich (Steinheim, Germany). The slides used to determine the biochemical parameters of blood plasma were obtained from IDEXX Laboratories Inc. (Westbrook, ME, USA). LC–MS-grade methanol and acetonitrile (LiChrosolv Hypergrade) were purchased from Merck (Darmstadt,

Germany). Formic acid, which was used to acidify the mobile phases, was purchased from Labicom (Olomouc, Czech Republic). The Aqua-MAX-Ultra System (Younglin, Kyounggi-do, Korea) was used for the preparation of ultrapure water. The internal standard trimethoprim (2,4-diamino-5-(3,4,5-trimethoxybenzyl) pyrimidine; CAS No.: 738-70-5, purity 99%) was obtained from Sigma-Aldrich (Steinheim, Germany).

2.2. Experimental design

Juvenile rainbow trout (*O. mykiss*; mean body length: 23.4 ± 1.3 cm, mean body weight: 145 ± 22 g) were obtained from a local commercial hatchery (Husinec, Czech Republic). Before the start of the experiment, the fish were acclimated for 14 days to the laboratory conditions. The animals were maintained in aquaria with 200 L of fresh water, and 20 fish were randomly distributed in each of eight aquaria. The aquaria were set up with continuous aeration, a constant temperature of 15.8 ± 1.2 °C, and a 12-h light/12-h dark photoperiod. The dissolved oxygen concentration and pH were measured before bath exchange on a daily basis. The dissolved oxygen concentration and pH were 8.3 ± 0.8 mg L^{-1} and 7.43 ± 0.31, respectively. During the acclimation and experimental periods, the fish were fed commercial fish food (Bio Mar, Denmark) at a ratio of 1% of body weight on a daily basis.

The experimental set up followed the OECD guideline 305 (OECD, 2012) with some modifications, as follows. The fish were exposed to atenolol at three different concentration levels under semi-static conditions for 42 days: 1 μg L^{-1} (environmentally relevant concentration), 10 μg L^{-1}, and 1000 μg L^{-1} (sub-lethal concentrations). In addition, a control group of fish maintained in atenolol-free water was included. All of the experimental treatments were conducted in duplicate. One hundred and fifty litres of the solution used for the atenolol treatments and control were refreshed daily with new atenolol-fortified or untreated water, respectively. The mortality during the experimental and acclimation periods was recorded. This study was performed in accordance with the principals of the EU-harmonised Animal Welfare Act of the Czech Republic.

2.3. Fish sampling

At the start of the experiment and after 21 and 42 days of exposure, eight fish from each group (including the replicate groups) were individually sampled. First, blood samples were collected from the *vena caudalis* and stabilised with 40 IU of sodium heparin per 1 mL of blood. Subsequently, the fish were euthanised in buffered ethyl 3-aminobenzoate methanesulfonic acid (MS 222®; Argent Chemical Laboratories, Redmont, WA, USA), and the spinal cord of the animals was severed. The total length and the body, liver, heart, and gonad weights were recorded. The condition factor (FCF), mean specific growth rate (SGR), hepatosomatic index (HSI), cardiosomatic index (CSI), and gonadosomatic index (GSI) were calculated as described by Machova et al. (2009), Velisek et al. (2010), and Powell et al. (2011). Furthermore, to determine the concentration of atenolol, samples of the blood plasma, kidney, liver, and muscle were collected and stored at −20 °C. To determine the enzymatic activity, the liver and gill tissues were sampled and stored at −80 °C. In addition, on the last sampling day (after 42 days of exposure), samples of the liver and heart from the fish exposed to the highest concentration (1000 μg L^{-1}) and the control group were collected and fixed in 10% buffered formalin for histopathological examination.

2.4. Analysis of atenolol in water and fish tissues

Water samples were collected from each aquarium immediately after the water was exchanged and 24 h after the exchange and stored at −18 °C. The extraction of the tissues was conducted in acetonitrile according to the method described by Grabicova et al. (2013) with some modifications. Briefly, samples of 0.5 g of tissue and 200 μL of plasma were used for the determination of the atenolol concentration and were spiked with 20 ng and 50 ng of the internal standard (trimethoprim), respectively. Matrix-matched standards were used for each matrix (tissues or water) to correct possible matrix effects, which can cause the differences from response factor derived from the calibration curve. The QA/QC samples were prepared and analysed to ensure the quality of the results. Blank samples were prepared with each series of processed samples. The accuracy and precision of the method were tested using duplicates and fortified samples: every tenth sample was duplicated, and every tenth sample of fish from the control group was spiked with the target compound at a concentration of 100 ng g^{-1}. The LOQs were based on peak area corresponding to the lowest calibration point fitting the criterion of response factor linearity. This value of peak area was set to the calculation formula for each sample taken into consideration IS peak area, sample amount, and response factor.

The atenolol in the water samples was analysed using on-line solid-phase extraction (SPE) liquid chromatography with tandem mass spectrometry for lower concentrations and direct injection for the highest concentration. The details of the on-line SPE of the MS/MS are described by Grabic et al. (2012) and Khan et al. (2012). The tissue extracts were analysed using the LC–MS/MS method (Grabic et al., 2012; Grabicova et al., 2013). The analyses were performed using TSQ Ultra MS/MS (Thermo Fisher Scientific, San Jose, CA, USA) coupled with Accela 1250 and Accela 600 HPLC pumps (Thermo Fisher Scientific) and a HTS-XT autosampler (CTC Analytics AG, Zwingen, Switzerland). A Cogent Bidentate column (50-mm length, 2.1-mm ID, and 4-μm particle size; MicroSolv Technology Corporation, NJ, USA) was used as the analytical column. A Hypersil Gold column (20 mm × 2.1-mm ID, 12-μm particles; Thermo Fisher Scientific) was used as the pre-concentration column for the on-line SPE. The LC–LC and LC gradients and MS/MS transitions are reported in Supplements 1, 2, and 3, respectively.

The bioconcentration factor (BCF) was calculated according to the OECD Guideline No. 305 (OECD, 2012). For each experimental group (and each tissue), the mean concentration of atenolol at each sampling time was divided by the mean concentration of atenolol in the water of the respective group.

2.5. Haematological and biochemical profile of the blood

The haematological profile of the blood, i.e., the erythrocyte count (RBC), haematocrit (Hc), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), leukocyte count (Leuko), and differential leukocyte count, was determined as described by Svobodova et al. (1991). For the biochemical analysis, the plasma was separated from the blood by centrifugation (10 min at 12,000 g) at 4 °C. Until analysis, the plasma samples were stored at −80 °C. The biochemical analysis of the blood plasma was conducted using a VETTEST 8008 analyser (IDEXX Laboratories Inc., USA). The following indices were measured: glucose (GLU), total protein (TP), triacylglycerols (TRIG), alanine aminotransferase (AMT), creatine kinase (CK), and lactate (LAC).

2.6. Analysis of enzyme activities in the liver and gills

2.6.1. Preparation of post-mitochondrial supernatant and microsomal fraction

The microsome fraction was prepared by differential centrifugation (Burkina et al., 2013) and used to measure the CYP450-mediated reactions. The analysis of the antioxidant parameters was performed using the post-mitochondrial supernatant of the liver tissue, as described by Stara et al. (2012).

2.6.2. Protein analysis

The protein levels were estimated spectrophotometrically through the method described by Smith et al. (1985) using bovine serum albumin as the standard. The microsomes were diluted to obtain a protein

concentration of 4 mg mL^{-1}, and the post-mitochondrial supernatant was diluted to obtain a protein concentration of 10 mg mL^{-1}.

2.6.3. Total CYP450 content

The total CYP450 content in the microsomes was determined from the sodium dithionite-reduced carbon monoxide difference spectrum with an extinction coefficient of 91 mM^{-1} cm^{-1} (Omura and Sato, 1964).

2.6.4. Microsomal enzyme activities

The ethoxyresorufin-O-deethylase (EROD), methoxyresorufin-O-demethylase (MROD), and pentoxyresorufin-O-depethylase (PROD) assays were performed based on the protocol described by Kennedy and Jones (1994). The 7-benzyloxy-4-trifluoromethylcoumarin O-debenzylation (BFCOD) assay was conducted according to the method described by Renwick et al. (2000). The reaction incubations were performed as described by Grabicova et al. (2013).

The alkyl-resorufin reactions were assessed based on the rates of the transformations of ethoxyresorufin (ER), methoxyresorufin (MR), and pentoxyresorufin (PR) to resorufin, and the activity of BFCOD was estimated based on the rate of the transformation of 7-benzyloxy-4-trifluoromethylcoumarin (BFC) to 7-hydroxy-4-trifluoromethylcoumarin (HFC). An Infinite 200 spectrophotometer (TECAN) was used for the analysis of the concentrations of resorufin (excitation/emission: 544/590 nm) and HFC (excitation/emission: 410/538 nm). The enzymatic activities were expressed as pmol of resorufin or HFC produced per min and mg of microsomal proteins (the detection limits for resorufin and HFC were 2 and 1 pmol, respectively).

2.6.5. Activity of antioxidant enzymes

The total superoxide dismutase (SOD) activity was determined using the method developed by Ewing and Janero (1995) with slight modifications. The reaction mixtures in a final volume of 250 μL were added to each well of a 96-well plate. The activity determinations were made for the complete reaction system, which included 25 μL of post-mitochondrial supernatant, 200 μL of reaction solution (48 μM NBT and 80 μM NADPH), and 25 μL of PMS (3.5 μM) in phosphate buffer (50 mM with 0.1 mM EDTA, pH 7.4). This assay was conducted in triplicate for each separate sample. The SOD activity was assessed spectrophotometrically at 560 nm using NBT as the substrate and is expressed as the amount of enzyme per milligramme of protein.

The catalase (CAT) activity was determined using the method developed by Aebi (1984) with slight modifications. The CAT activity was determined by measuring the decrease in hydrogen peroxide in cuvettes. The post-mitochondrial supernatant was diluted 1:50 in phosphate buffer (50 mM with 1 mM EDTA, pH 7.0). Hydrogen peroxide was diluted to a concentration of 0.09% in TRIS buffer (50 mM with 0.1 mM EDTA, pH 7.2). To measure the enzymatic activity, 100 μL of the diluted post-mitochondrial supernatant was mixed with 500 μL of the diluted hydrogen peroxide. The decomposition of the substrate (H_2O_2) was recorded at 240 nm. The calculations were made using a molar extinction coefficient of 40 M^{-1} cm^{-1}.

The glutathione reductase (GR) activity was determined using the method developed by Carlberg and Mannervik (1975) with some modifications. The reaction mixtures in a final volume of 200 μL were added to each well of a 96-well plate. The activity determinations were made for the complete reaction system, which consisted of 50 μL of the post-mitochondrial supernatant, 20 μL of GSSG (1 mM), 20 μL of NADPH (0.1 mM), 30 μL of cold distilled water, and 80 μL of phosphate buffer (0.1 mM with 0.5 mM EDTA, pH 7.0). The assay was performed in triplicate for each sample. The GR activity was assayed spectrophotometrically at 340 nm to measure the level of NADPH oxidation.

An Infinite 200 spectrophotometer (TECAN) was used to measure the absorbances in 96-well plates, and a Specord 210 spectrophotometer (Analytic Jena) was used for the measurement of the absorbance of solutions in cuvettes. The enzymatic activities were determined for three replicates and are expressed as nanomoles or micromoles of substrate hydrolysed per minute per mg of protein.

2.7. Histopathological analysis

Fixed samples of the liver and heart were paraffin-embedded and routinely processed for histological examination. Sections with a thickness of 3 μm were cut, stained with haematoxylin-eosin (H&E), and examined using a light microscope. The histopathological changes were graded as 0 (no), 1 (scattered), 2 (mild), 3 (mild to moderate), 4 (moderate), 5 (moderate to severe), or 6 (severe) (Schmidt-Posthaus et al., 2013; Bettge et al., 2009; Grabicova et al., 2013). The liver tissue was additionally stained with periodic acid-Schiff (PAS) to determine the glycogen content in hepatocytes.

2.8. Statistical analysis

The statistical software STATISTICA (version 8.0 for Windows, StatSoft, Czech Republic) was used to compare the differences between the tested groups, including the control group. The normality and homoscedasticity of the variance were checked by the Kolmogorov–Smirnov test and Bartlett's test, respectively. If the conditions of normal distribution and homogeneity were satisfied, one-way analysis of variance (ANOVA) was employed to determine whether there were significant differences in the measured variables between the experimental groups. Subsequently, Dunnett's multiple range test was applied. If the conditions for ANOVA were not satisfied, a nonparametric test (Kruskal–Wallis) was utilised. A p value of less than 0.05 was chosen as a valid significance level.

3. Results

3.1. Concentration of atenolol in water and fish tissues

For the on line SPE method and direct injection LC–MS/MS the limits of quantification (LOQs) of atenolol in water samples ranged from 0.0014 to 0.0021 μg L^{-1} and from 0.9 to 1.1 μg L^{-1}, respectively (footnotes of Table 1). On average the LOQs of atenolol in water were for on line SPE and direct injection LC–MS/MS 0.0017 μg L^{-1} and 0.98 μg L^{-1}, respectively. The limits of quantification (LOQs) of atenolol in fish tissues ranged from 0.34 to 4.3 ng g^{-1} with the average of 1.3 ng g^{-1}. The tissue specific LOQs are listed as footnotes of Table 2. The differences in the LOQs of atenolol in water and tissues were a result of the different methods of detection as well as different weights of the samples. The average recoveries (trueness of the method) of atenolol from fortified matrices were 95% (relative standard deviation (RSD) of ten replicates: 6%), 107% (RSD of four replicates: 13%) and 102% (RSD of ten replicates: 4%) in the fortified samples of fish tissues, blood plasma, and the water samples,

Table 1
Concentrations of atenolol in the water used in the sub-chronic toxicity test. The concentrations were measured immediately after the water exchange (0 h) and 24 h after the exchange. The values are expressed as the means ± S.D. (n = 6). LOQ = limit of quantification.

Group	Sample time (h)	Water concentration (μg L^{-1})	Difference to the nominal concentration (%)	Min–Max
Control	0	<LOQ		
	24	<LOQ		
1 μg L^{-1}	0	1.6 ± 0.3	60	1.3–2.1
	24	1.6 ± 0.4	60	1.2–2.3
10 μg L^{-1}	0	12.2 ± 1.9	22	8.7–14.2
	24	12.0 ± 1.6	20	9.5–13.4
1000 μg L^{-1}	0	1120 ± 93	12	1040–1240
	24	1070 ± 103	7	975–1218

On-line SPE LC–MS/MS, LOQ (1–10 μg L^{-1}) = 0.0014–0.0021 μg L^{-1}; Direct injection LC–MS/MS LOQ (1000 μg L^{-1}) = 0.9–1.1 μg L^{-1}.

Table 2
Concentrations of atenolol, number of atenolol positive samples and bioconcentration factors (BCFs) of atenolol in the blood plasma, liver, kidney, and muscle of rainbow trout sub-chronically exposed to atenolol. The values are expressed as the means ± S.D. (n = 8). LOQ = limit of quantification.

Tissue	Exposure time (days)	Control	1 μg L^{-1}	10 μg L^{-1}	1000 μg L^{-1}
Plasma	21	<LOQ (0/8)	<LOQ (0/8)	<LOQ (0/8)	<LOQ (0/8)
	42	<LOQ (0/8)	<LOQ (0/8)	<LOQ (0/8)	<LOQ (0/8)
Liver	21	<LOQ (0/8)	<LOQ (0/8)	<LOQ (0/8)	300 ± 260; (8/8); BCF = 0.27
	42	<LOQ (0/8)	<LOQ (0/8)	2.40 ± 0.39; (2/8); BCF = 0.20	230 ± 210; (8/8); BCF = 0.21
Kidney	21	<LOQ (0/8)	<LOQ (0/8)	<LOQ (0/8)	161 ± 220; (8/8); BCF = 0.15
	42	<LOQ (0/8)	<LOQ (0/8)	<LOQ (0/8)	110 ± 24; (8/8); BCF = 0.10
Muscle	21	<LOQ (0/8)	<LOQ (0/8)	<LOQ (0/8)	3.6 ± 1.4; (8/8); BCF = 0.003
	42	<LOQ (0/8)	<LOQ (0/8)	<LOQ (0/8)	2.3 ± 1.4; (8/8); BCF = 0.002

Tissue specific LOQ (average and range) of on-line SPE LC–MS/MS: plasma = 2.00 ng g^{-1}; (1.0–4.3 ng g^{-1}), liver = 1.2 ng g^{-1} (0.68–4.0 ng g^{-1}), kidney = 1.2 ng g^{-1} (0.70–2.0 ng g^{-1}), muscle = 0.89 ng g^{-1} (0.34–2.0 ng g^{-1}).

respectively. It was not possible to prepare fortified plasma samples due to the insufficient amount of these samples. Therefore, the average recovery of atenolol in blood plasma was assessed using samples collected from fish not involved in the experiment.

The concentration of atenolol in the water samples from the control group was below the LOQ. The starting atenolol concentration and the concentration of atenolol 24 h after exposure in the testing aquaria are reported as an average of three samplings within the experimental period (1st, 12th, and 25th days). The measured atenolol water concentrations with nominal values for all of the treatment groups are shown in Table 1.

The concentrations of atenolol in the control fish during the experimental period and in the fish before the start of the exposure were below the LOQ (Table 2). After 21 days of exposure, atenolol was detected only in the fish that were exposed to the highest tested concentration. After 42 days of exposure, atenolol was detected in the fish exposed to the intermediate and the highest tested concentrations. The bioconcentration factor (BCF) of atenolol in the different tissues ranged from 0.002 to 0.27 and is given in Table 2. The bioconcentration of atenolol followed the order liver > kidney > muscle. The atenolol concentrations in the liver and kidney were significantly higher compared to those in muscle. In the plasma, the detected concentrations were below the LOQ.

3.2. Survival and morphological parameters

During the acclimation and experimental periods, no mortality was detected in any of the treatment groups, including the control.

The atenolol-exposed fish were not significantly different in their total length, body weight, CF, HSI, GSI, and CSI compared with the control (Supplement 4). The SGRs calculated at the end of the exposure were 9.52, 8.39, 9.45, and 9.31 in the control group and in the fish exposed to 1, 10, and 1000 μg L^{-1} atenolol, respectively.

3.3. Haematological and biochemical parameters of the blood

The haemoglobin concentration was significantly reduced in the fish exposed to atenolol for 21 days compared with the control. However, the haemoglobin concentration was not additionally affected by continued exposure to atenolol. The haematocrit value was significantly reduced in fish exposed to atenolol at concentrations of 10 and 1000 μg L^{-1} for 21 days compared with the control (Table 3). After 42 days, the haematocrit value was significant reduced in fish exposed to 10 μg L^{-1} atenolol compared with the control (Table 4). The remaining haematological indices (erythrocyte and leucocyte counts, MCV, MCHC, and differential leucocyte count) did not differ significantly between the groups (Table 3 and Supplement 5).

After 21 days, all of the atenolol-exposed fish exhibited a significantly enhanced lactate concentration in the blood plasma compared with the control. After 42 days, only the fish exposed to the highest tested concentration of atenolol (1000 μg L^{-1}) presented a significantly enhanced lactate concentration compared with the control (Table 4). The concentration of glucose was significantly lower in fish exposed to 10 μg L^{-1} atenolol for 21 days compared with the control (Table 4). The concentrations of total protein and triacylglycerols and the activities of aminotransferase and creatine kinase were not affected by atenolol exposure, regardless of the duration (Table 4).

Table 3
Haematological parameters in rainbow trout sub-chronically exposed to atenolol. Hc — haematocrit, Hb — haemoglobin, WBC — white blood cell (leukocyte) count, MCV — mean corpuscular volume, MHC — mean corpuscular haemoglobin, MCHC — mean corpuscular haemoglobin concentration. The values are expressed as the means ± S.D. (n = 8). The asterisks indicate a significant difference compared with the control: *p < 0.05 (ANOVA).

Indices	Exposure time (d)	Control	1 μg L^{-1}	10 μg L^{-1}	1000 μg L^{-1}
Hc (%)	21	44.4 ± 7.3	39.3 ± 4.2	33.3 ± 4.5*	35.9 ± 7.9*
	42	43.8 ± 6.3	40.4 ± 6.7	33.3 ± 5.9*	40.3 ± 4.3
Hb (g L^{-1})	21	82.1 ± 14.6	63.5 ± 5.9*	55.8 ± 5.8*	66.5 ± 13.1*
	42	84.9 ± 15.1	82.8 ± 11.9	72.8 ± 15.1	81.5 ± 8.8
Erythrocytes (T L^{-1})	21	1.2 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.2
	42	1.2 ± 0.2	1.1 ± 0.2	1.0 ± 0.1	1.2 ± 0.2
WBC (g L^{-1})	21	51.5 ± 12.1	68.5 ± 27.6	65.8 ± 38.4	59.5 ± 9.2
	42	53.7 ± 17.7	53.7 ± 9.9	64.4 ± 22.71	59.8 ± 29.2
MCV (fl)	21	378 ± 57	388 ± 58	371 ± 71	369 ± 54
	42	381 ± 79	390 ± 68	370 ± 95	428 ± 105
MCH (pg)	21	69.5 ± 18.0	62.6 ± 8.9	62.1 ± 12.0	68.4 ± 9.0
	42	71.3 ± 6.4	74.4 ± 11.3	71.9 ± 9.5	71.5 ± 7.7
MCHC (g L^{-1})	21	188 ± 29	162 ± 8	168 ± 13	186 ± 10
	42	198 ± 41	206 ± 20	218 ± 14	203 ± 19

3.4. Activity of selected CYP450 enzymes in the liver

The catalytic activity of EROD (CYP1A1/2), MROD (CYP1A1/2), PROD (CYP2B), and BFCOD (CYP3A) and the total CYP450 content were not significantly affected by atenolol exposure (Supplement 6).

Table 4
Biochemical parameters of the blood plasma of rainbow trout sub-chronically exposed to atenolol. GLU — glucose, TP — total protein, TRIG — triacylglycerols, AMT — aminotransferase, CK — creatine kinase, LAC — lactate. The values are expressed as the means ± S.D. (n = 8). The asterisks indicate a significant difference compared with the control: *p < 0.05 (ANOVA).

Indices	Exposure time (d)	Control	1 μg L^{-1}	10 μg L^{-1}	1000 μg L^{-1}
GLU (mmol L^{-1})	21	4.0 ± 0.3	4.2 ± 0.5	3.4 ± 1.1*	4.5 ± 0.5
	42	4.1 ± 0.9	3.9 ± 0.7	3.7 ± 0.7	3.4 ± 0.8
LAC (mmol L^{-1})	21	0.4 ± 0.3	0.6 ± 0.2*	0.7 ± 0.1*	0.7 ± 0.3*
	42	0.61 ± 0.1	0.7 ± 0.1	0.9 ± 0.4	1.6 ± 0.6*
TP (g L^{-1})	21	35.4 ± 4.94	36.0 ± 3.6	37.7 ± 6.3	28.9 ± 13.3
	42	44.6 ± 6.1	43.0 ± 5.0	37.1 ± 5.6	40.9 ± 3.6
TRIG (mol L^{-1})	21	0.9 ± 0.6	0.7 ± 0.2	0.7 ± 0.1	0.7 ± 0.3
	42	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.7 ± 0.2
AMT (μkat L^{-1})	21	4.9 ± 3.0	5.3 ± 3.2	3.8 ± 2.5	2.5 ± 2.1
	42	11.8 ± 4.0	10.3 ± 7.4	7.7 ± 3.9	10.0 ± 5.6
CK (μkat L^{-1})	21	831 ± 524	379 ± 408	555 ± 557	487 ± 360
	42	1113 ± 604	1537 ± 313	1060 ± 305	1815 ± 1219

Table 5
Activity of antioxidant enzymes in the liver of rainbow trout sub-chronically exposed to atenolol. SOD — superoxide dismutase, CAT — catalase, GR — glutathione reductase. The values are expressed as the means ± S.D. (n = 8). The asterisks indicate a significant difference compared with the control: *p < 0.05 (ANOVA).

Indices	Exposure time (d)	Control	1 μg L^{-1}	10 μg L^{-1}	1000 μg L^{-1}
CAT	21	0.04 ± 0.02	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
μmol (min^{-1} mg^{-1})	42	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.00	0.03 ± 0.01
GR	21	0.57 ± 0.21	0.63 ± 0.20	0.59 ± 0.14	0.47 ± 0.10
nmol (min^{-1} mg^{-1})	42	0.60 ± 0.09	0.45 ± 0.09	0.49 ± 0.16	0.29 ± 0.19*
SOD	21	0.24 ± 0.18	0.24 ± 0.13	0.28 ± 0.21	0.28 ± 0.07
nmol (min^{-1} mg^{-1})	42	0.09 ± 0.05	0.10 ± 0.05	0.13 ± 0.08	0.09 ± 0.03

3.5. Activity of antioxidant enzymes

In the liver, the activities of CAT and SOD in the atenolol-exposed fish at any of the sampling times were not significantly different compared with those of the control group (Table 5). The activity of GR was significantly reduced only with the highest tested concentration of atenolol after 42 days of exposure compared with the control (Table 5).

In the gills, the activities of CAT, SOD, and GR were not significantly changed in fish exposed to atenolol compared with the control (Supplement 7).

3.6. Histological changes

In the liver, the control and atenolol-exposed fish showed varying amounts of fat vacuoles in hepatocytes, and the cytoplasm showed mild to moderate granulation. The glycogen content in hepatocytes was not affected by treatment with atenolol (data not shown). In contrast to the controls, the fish exposed to the highest tested concentration of atenolol (1000 μg L^{-1}) displayed mild to severe sinusoidal congestion (Fig. 1 and Supplement 8). The analysis of the heart of atenolol-exposed fish showed that their pericardium exhibited more pronounced signs of inflammation compared with the control (Fig. 2 and Supplement 8). In addition, ranges of scattered to moderate histopathological alterations were observed in the heart of atenolol-exposed fish, e.g., edematous pericardial fibrous tissue, infiltration in the sub-endocardial space, and myocardial edema (Fig. 2 and Supplement 8). One of the atenolol-exposed trout presented multiple vessel walls that were thickened and edematous, and its endothelial cells were occasionally disrupted. The same animal exhibited obvious sub-endocardial infiltration of inflammatory cells in the atrium. The histopathological changes are summarised in Supplement 8. Most of the histopathological alterations caused by atenolol ranged from mild to moderate. Hence, no severe histopathological alterations in the heart and liver were expected in response to exposure to the lower tested concentrations of atenolol. Therefore, the fish exposed to the lower tested concentrations were not examined.

4. Discussion

4.1. Mortality and morphological parameters

The present study showed that long-term exposure to atenolol at a concentration in the range of 1 to 1000 μg L^{-1} did not cause mortality in rainbow trout. This finding is in line with the very low acute toxicity of atenolol ($96hLC_{50}$ > 100 mg L^{-1}) that was found for medaka (*Oryzias latipes*, Kim et al., 2009). The present study revealed that atenolol has no effect on the studied morphological parameters (length, weight, FCF, HSI, and CSI) and no effect on fish fitness and growth. In an embryo–larval study (28 day) on fathead minnow (*Pimephales promelas*), atenolol reduced the growth rate of the exposed larvae; however, the concentration of atenolol used was 10-fold higher than the highest concentration used in the present study (LOEC growth = 10 mg L^{-1}, Winter et al., 2008).

4.2. Bioconcentration in fish

Due to the low lipophilicity (log P = 0.16; EPI Suite™ v4.11 — US EPA, 2012) atenolol is expected to be present mainly in the water phase. Consequently the uptake via the gills in fish can be hypothesised with only negligible contribution of uptake via food (Owen et al., 2007). For the same reason, atenolol is not expected to accumulate to a high extent in fish bodies. In accordance with this assumption, the bioconcentration factors (BCFs) of atenolol in different rainbow trout tissues calculated in the present study were very low, ranging from 0 to 0.27. To the best of our knowledge, this study provides the first demonstration of the tissue-specific bioconcentration of atenolol in fish. In western mosquitofish (*Gambusia affinis*) that were exposed for 96 h to atenolol at concentrations of 100 and 1000 μg L^{-1}, the BCFs in the fish homogenates were estimated to be 0.13 and 0.08, respectively (Valdes

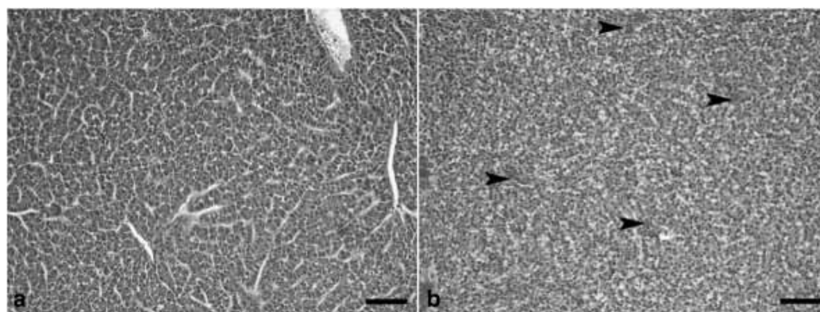

Fig. 1. Histopathological findings in the liver of rainbow trout: a — control fish with normal liver structure; b — fish exposed to 1000 μg L^{-1} atenolol. The exposed fish present small blood vessels and congested sinusoids (arrowheads), as determined through H&E staining; scale bar = 50 μm.

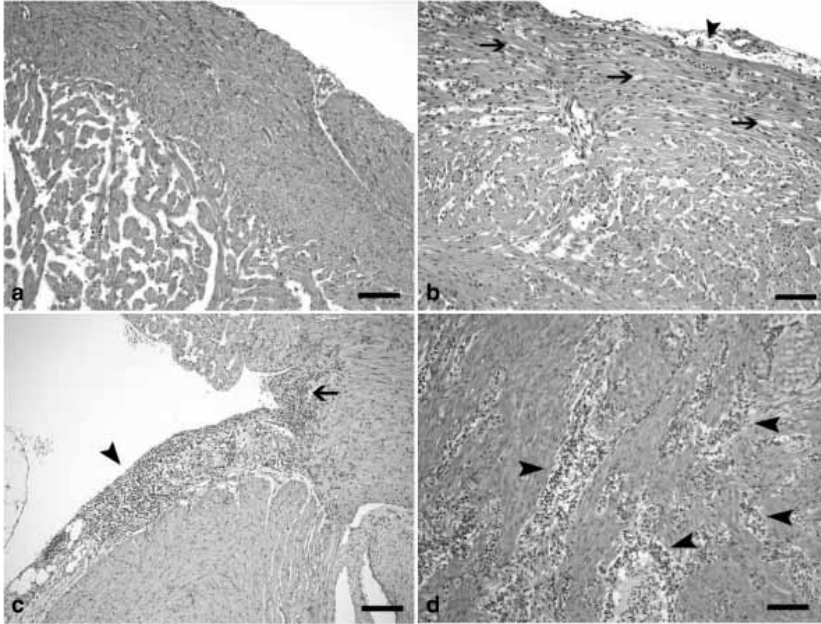

Fig. 2. Histopathological findings in the heart of rainbow trout: a — control fish with normal heart structure; b to d — fish exposed to 1000 μg L^{-1} atenolol. b — pericardial edema (arrowhead) and myocardial edema (arrows), c — subepicardial infiltration with lymphocytes, plasma cells, and macrophages (arrowhead), which multifocally infiltrate into the myocardial tissue (arrow), d — subendocardial infiltration with lymphocytes, plasma cells, and macrophages (arrowheads). The findings are shown through H&E staining; scale bars = 50 μm (a–c) and 25 μm (d).

et al., 2014). These values are comparable to those calculated for rainbow trout tissues in the present study. Furthermore, the atenolol concentration did not reach the LOQ (1.3 ng mL^{-1}) even in the blood plasma of rainbow trout exposed to an atenolol concentration as high as 1000 μg L^{-1}. Winter et al. (2008) detected an atenolol concentration of 48.6 μg L^{-1} in a pooled plasma sample of adult male fathead minnow exposed to atenolol at a concentration of 1000 μg L^{-1}. This value is close to the predicted value presented by Fick et al. (2010), who calculated that atenolol would reach the human therapeutic blood plasma level of 100 μg L^{-1} (Schulz and Schmoldt, 2003) in fish if the animals were exposed to an atenolol concentration of 792.3 μg L^{-1}. However, similarly to the juvenile rainbow trout analysed in the present study, the plasma concentration in adult females of fathead minnow exposed to the same concentration of atenolol did not reach the LOQ (0.5 ng mL^{-1}) (Winter et al. (2008). The differences in the atenolol concentration in the blood plasma among fish of different sexes and ages may be a result of their different elimination rates of atenolol. For instance, Parks and LeBlanc (1998), who exposed fathead minnow to testosterone, discovered that adult females eliminate some testosterone metabolites at a significantly greater rate than males and that juvenile fish eliminate nearly all testosterone metabolites at greater weight-normalized rates than the adults. Similarly, Wiegand et al. (2000) observed that the activity of enzymes involved in detoxification and excretion had higher activity in juvenile compared to adult fish.

In addition, at the pH level used in our experimental protocol (pH 8.3), atenolol can be expected to be present mainly in its protonated positively charged form (pKa = 9.6). Because charged substances are much less membrane-permeable than uncharged ones (Owen et al., 2009; http://pubchem.ncbi.nlm, 2013), this finding may explain why the blood plasma concentration of atenolol in rainbow trout was below the LOQ and why the BCFs in the tissues were lower than the predicted value for the whole fish.

4.3. Haematological and biochemical profiles of the blood plasma

In response to pollutants, the haematological and biochemical profiles of the blood of fish can provide valuable information regarding their internal environment (Velisek et al., 2010). In the present study, atenolol (at the environmentally relevant concentration) induced haematological and biochemical changes in the blood of rainbow trout. The present study verified that changes within the haeme pathway are sensitive biomarkers of pollutants at their environmental concentrations, as was also demonstrated for, e.g., cadmium (van der Oost et al., 2003).

The changes in the haematocrit value and haemoglobin concentration of rainbow trout in response to atenolol observed in the present study may indicate that this substance affects the β-ARs in the organs associated with erythropoiesis, i.e., head kidney and spleen. The presence of β_1-ARs in these organs has been documented in salmonid fish (Owen et al., 2007). However, we cannot rule out the possibility that these changes are part of a general stress response. In the present study, as a consequence of the reduction in the haemoglobin concentration caused by atenolol, the oxygen supply may have been reduced, which may have led to the observed increase in the lactate concentration.

Furthermore, in the present study, a decrease was observed in the glucose concentration in fish exposed to atenolol, indicating an effect of this substance on the carbohydrate and energy metabolisms. Similarly, in the brain of Atlantic salmon (*Salmo salar*) exposed to 11 μg L^{-1} atenolol for five days, Hampel et al. (2010) observed changes in the mRNA expression of genes related to the carbohydrate and energy metabolisms. For example, phosphoenolpyruvate carboxykinase, which is a key

enzyme in gluconeogenesis, presented the most reduced mRNA level. In rainbow trout hepatocytes, Ings et al. (2012) reported that atenolol (2.7 ng L^{-1}) abolishes the epinephrine-induced glucose production. Additionally, van Den Thillart et al. (2001) reported that atenolol reduces the glucose content in the blood plasma of isoproterenol (non-selective β-agonist)-treated common carp. The reduction in the glucose concentration in fish exposed to β-blockers may be a consequence of the blockage of β-ARs, which are then not accessible to catecholamines (e.g., noradrenaline and adrenaline), because catecholamines lead to increases in the blood plasma glucose content (Evans, 1997).

4.4. Activity of enzymes in the liver and gills

Atenolol did not cause any changes in the total CYP450 content and the activities of the selected isoforms of CYP450 enzymes in the liver, which indicates that it is not detoxified/metabolised but most likely directly eliminated in rainbow trout. Similarly, in the human body, atenolol is only partly metabolised (10%) and is mainly excreted unchanged via the kidney (Reeves et al., 1978; Wander et al., 2009). Although atenolol is not significantly metabolised in the human body, pharmaceuticals with log Kow values as low as that of atenolol are substrates for CYP450 enzymes (Lewis, 2000). Therefore, to accurately describe the metabolism of atenolol in fish, further studies on the presence of atenolol metabolites in tissues and excretes are required.

The activities of the antioxidant enzymes SOD and CAT were also not affected by atenolol exposure. In contrast, the activity of another antioxidant enzyme, GR, was decreased in the liver of fish exposed to the highest tested concentration of atenolol for 42 days. GR maintains the redox balance of glutathione, an antioxidant that prevents damage to cells caused by reactive oxygen species, by catalysing the reduction of glutathione disulphide to glutathione (van der Oost et al., 2003; Richard et al., 2008). Therefore, the inhibition of GR activity can cause a decrease in the content of glutathione (Lushchak, 2011; Srikanth et al., 2013). In accordance, atenolol reduces the glutathione content in human blood plasma (Komala et al., 2013). Oxidative stress occurs when the redox homoeostasis within cells is altered by a deficiency of the antioxidant system (Komala et al., 2013). Therefore, fish exposed to atenolol at a concentration of 1000 μg L^{-1} may be at higher risk of oxidative stress.

4.5. Histological changes

The results of the present study demonstrate an absence of structural changes in hepatocytes and no effect on the glycogen level in atenolol-exposed fish. This finding demonstrates that atenolol does not appear to be hepatotoxic for rainbow trout. Similarly, atenolol was not found to have a cytotoxic effect on rat primary hepatocytes (Bandyopadhyay et al., 1990). In humans, β-blockers are also not expected to be hepatotoxic, and in fact, only a very few cases of hepatotoxicity caused by atenolol have been reported (Boillot and Scoazec, 2009). These results further support the hypothesis that, similarly to humans (Flockhart and Tanus-Santos, 2002), atenolol is mainly excreted by the kidney with no obvious metabolism in the liver in fish.

However, the results of the present study show an effect of atenolol on the cardiovascular system of exposed fish. Namely, atenolol causes degenerative changes in the blood vessels of the pericardium and an inflammatory reaction in the pericardium and subendocardium. In line with our findings, an increased incidence of atrial degeneration of the heart has been described in rats fed a very high dosage of atenolol (300 mg atenolol/kg/day) (RxList, 2013). Fish exposed to atenolol also show congestion of the sinusoids (sinusoidal blood vessels), indicating circulatory changes (Henrion, 2012). Because the liver of rainbow trout is rich in β_2-ARs (Owen et al., 2007), it can be assumed that atenolol blocks the β_2-ARs and thereby causes vasodilation. The congestion of the sinusoids may be a consequence of this effect. The congestion may also be related to a reduced oxygen supply due to the observed reduction in the haemoglobin concentration. In this case, the congestion may be regarded as a compensational reaction due to hypoxia in the tissues (Henrion, 2012).

5. Conclusions

In conclusion, the bioconcentration of atenolol in rainbow trout exposed to sub-lethal levels (1–1000 μg L^{-1}) of atenolol is very low: the blood plasma concentration was found to be below the LOQ (2.0 ng g^{-1}), and the maximum BCF in the tissues was found to be 0.27. Furthermore, the absence of structural changes in the hepatocytes, the lack of an effect on the glycogen level, and the unchanged activity of the CYP450 enzymes in the liver indicate that atenolol is not metabolised but rather directly excreted by fish. Similarly, in humans, atenolol is only insignificantly metabolised in the liver and is excreted mainly unchanged by the kidney (Flockhart and Tanus-Santos, 2002).

Contrary to the read-across concept (Huggett et al., 2003; Rand-Weaver et al., 2013), which is based on the hypothesis that a pharmaceutical affects fish if the plasma concentration in the fish is similar to the human therapeutic plasma concentration, atenolol did affect the exposed fish in the present study. At the environmentally relevant concentration, atenolol exposure leads to a decrease in the haemoglobin concentration and an increase in the lactate content of blood plasma, suggesting a reduction in the oxygen supply. Some effects caused by atenolol, mainly at the higher concentrations tested, in fish can be considered to be similar to those observed in humans, particularly the effects on the vascular system of the liver and heart. These changes may be mediated by β-ARs because β-ARs in fish and humans are supposed to have high structural similarities (as demonstrated, e.g., for the β_2-AR, Nickerson et al., 2001; Owen et al., 2009). Taken together, the data demonstrate that atenolol exhibits potential for the induction of sublethal effects on non-target organisms, such as fish, in aquatic environments.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2014.07.111.

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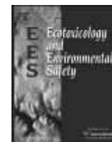

Verapamil does not modify catalytic activity of CYP450 in rainbow trout after long-term exposure

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ABSTRACT

Little is known about the effects of the cardiovascular drug verapamil (VRP) on metabolic processes in fish. Most calcium channel blockers including VRP are metabolized by cytochrome P450 (CYP450) enzymes. In this study we investigated the *in vivo* effect of VRP on some CYP450-mediated reactions in juvenile rainbow trout (*Oncorhynchus mykiss*). Fish were exposed to sublethal concentrations of VRP (0.5, 27 and 270 $\mu g\,l^{-1}$) for 0, 21, and 42 day. The following CYP450-mediated reactions were studied in hepatic microsomes: O-dealkylation of ethoxyresorufin, methoxyresorufin, and pentoxyresorufin, hydroxylation of coumarin, tolbutamide, and p-nitrophenol, and O-debenzylation of 7-benzyloxy-4-trifluoromethylcoumarin. The amounts of products of these reactions did not differ among fish exposed to different levels of VRP and control fish. This suggests that the levels of VPR used did not alter catalytic activity of the selected CYP450 enzymes. In conclusion, none of the investigated CYP450-mediated reactions has potential as a biomarker to monitor VRP contamination of the aquatic environment.

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1. Introduction

Pharmaceutical compounds (PhACs) have a significant effect on biological systems. The pharmaceutical industry is rapidly developing; consequently increasing amounts of PhACs are being released into the aquatic environment (Corcoran et al., 2010). Pharmaceutical compounds and their metabolites are detected in surface and underground waters and in drinking water (Herberer, 2002; Fent et al., 2006).

Verapamil (VRP) is a calcium channel blocker and is among the most frequently prescribed cardiovascular drugs. Clinical uses of VRP include treatment for arrhythmias, systemic hypertension, myocardial ischemia, and hypertrophic cardiomyopathy (Leslie and Tardiff, 1995). It consists of a phenylalkylamine derivative that inhibits calcium influx through the slow channels of vascular smooth muscle and cardiac cell membranes. The transmembrane flow of calcium is an important modulator of cell excitability and response of the actin–myosin complex and muscular contraction. Decrease in the level of intracellular free calcium causes coronary and peripheral vasodilation (Whyte et al., 2003; Grossman and Messerli, 2004).

The presence of VRP has been detected in waste water at concentrations of 0.050–0.068 $\mu g\,l^{-1}$ (Khan, 2002). In the influent and effluent of sewage treatment plants, Verapamil was reported at concentrations of 3.10 $\mu g\,l^{-1}$ and 0.51 $\mu g\,l^{-1}$, respectively (Trautwein et al., 2008). Currently there is limited information about the toxicity of VRP in the aquatic environment and the eventual effects on physiological processes in fish.

In mammals, PhACs are mainly metabolized by the cytochrome p450 (CYP450) enzyme system, particularly by members of the CYP1–CYP3 families, which are predominantly expressed in the liver (Hasler et al., 1999). Verapamil metabolism in humans is mediated by CYP450 with O- and N-demethylation being two major metabolic pathways (Wang et al., 2004). Metabolic pathways of VRP have been intensively studied *in vitro* using human liver microsomes as a model (Kroemer et al., 1993; Lionel et al., 2000; Pauli-Magnus et al., 2000; Ha et al., 2006). Various isoforms have been shown to be involved in VRP metabolism, including CYP1A2, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP3A3, and CYP3A5. The metabolic pathways of VRP in fish have not been reported. The function of at least some isoforms involved in VRP metabolism in mammals is questionable in fish. However, it is generally believed that CYP gene families in fish are similar to those in mammals. CYP3A activity has been detected in Atlantic cod (*Gadus morhua L.*) (Husoy et al., 1994), fathead minnow (*Pimephales promelas*) (Christen et al., 2010), Atlantic salmon (*Salmo salar*) (Zlabek and Zamaratskaia, in press), and other species. CYP2E1-like activity was

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detected in medaka (*Oryzias latipes*) (Geter et al., 2003) and winter flounder (*Pleuronectes americanus*) (Wall and Crivello, 1998). It is not known whether the activity of these isoforms can be altered by the presence of VRP in water. Generally, there are a limited number of studies on the effect of VRP on enzymatic activity of CYP450 in aquatic organisms, including fish. Activity of 7-ethoxyresorufin-O-deethylase (EROD), which is often used as a biomarker in aquatic pollution monitoring, was not altered in the presence of VRP in cultured epithelium cells from rainbow trout gills (Carlsson and Pärt, 2001).

To assess how fish are affected by effluents containing VRP, we investigated its potential to alter the rate of some CYP450-mediated reactions in rainbow trout (*Oncorhynchus mykiss*), a widely used model in aquatic toxicology. Activity of the following enzymes was measured: ethoxyresorufin (EROD), methoxyresorufin (MROD), and pentoxyresorufin O-dealkylase (PROD) as well as p-nitrophenol (PNPH), coumarin (COH), tolbutamide hydroxylase (TB-H), and 7-benzyloxy-4-trifluoromethylcoumarin O-debenzylase (BFC-OD).

2. Materials and methods

2.1. Chemicals

Verapamil ((RS)-2-(3,4-dimethoxyphenyl)-5-[2-(3,4-dimethoxyphenyl)ethyl]-(methyl)amin-2-prop-2-ylpentanenitrile) was obtained from Sigma-Aldrich Corporation (USA). The VRP was dissolved in pure distilled water to make a stock solution at a concentration of 0.05 mg l^{-1}. Resorufin, 7-ethoxyresorufin (ER), 7-methoxyresorufin (MR), 7-pentoxyresorufin (PR), p-nitrophenol (PNP), COH, 7-benzyloxy-4-trifluoromethylcoumarin (BFC), 7-hydroxy-7-benzyloxy-triflouromethylcoumarine (HFC), tolbutamide, 4-hydroxytolbutamide (TB-OH), and nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Sigma-Aldrich (Steinheim, Germany). Aliquots of those solutions were stored at -20 °C. High-performance liquid chromatography (HPLC) grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany).

2.2. Fish and treatment

Juvenile rainbow trout weighing 40.43 ± 2.55 g (mean $\pm$ standard deviation) were purchased from a local commercial hatchery (Husinec, Czech Republic). Fish were transferred to aquaria containing 250 l of freshwater (temperature 15 ± 1 °C, pH 7.4 ± 0.2, dissolved oxygen 7.5–8.0 mg l^{-1}) and subjected to a 12:12 L:D photoperiod. Fish were allowed to acclimatize for 14 days before the beginning of the experiment and were fed commercial fish food (BioMar, Denmark) during this period. Fish were not fed for 24 h prior to sampling to avoid prandial effects during the assay.

Forty fish were randomly distributed into each of eight aquaria containing 200 l of water with a semi-static system. Prior to experimental exposure, 8 additional fish were sampled (group C0) for comparison to all other groups. The test fish were exposed to sublethal concentrations of VRP for 21 or 42 day, with an unexposed control group. Based on reports on acute toxicity of VRP on rainbow trout (Li et al., 2010), the nominal concentrations of VRP used were 0.5 μg l^{-1} (E1 group, environmentally relevant concentration), 27 μg l^{-1} (E2 group, 1% 96 h LC50 of VRP in rainbow trout), and 270 μg l^{-1} (E3 group, 10% 96 h LC50 of VRP in rainbow trout), and the control group in freshwater only. Each experimental condition was duplicated. The treatment solution was renewed each day to maintain water quality and the appropriate VRP concentration. The concentration of VRP in the water was measured three times by LC–MS/MS during the experimental period. Water was sampled on day 1, 20, and 41, twice in every 24 h, immediately before and after the bath change. The average concentration of VRP measured in water samples varied within 20% of the intended concentrations and corresponded to 0.47 ± 0.05, 26.18 ± 1.36, and 251.33 ± 19.81 μg l^{-1}, which in turn corresponds to the nominal concentrations of 0.5, 27, and 270 μg l^{-1}. Details of the experimental system and conditions are given by Li et al. (2011a).

Experimental animals were handled in accordance with national and institutional guidelines for the protection of human subjects and animal welfare.

2.3. Sampling and microsome preparation

At the end of the exposure period, the fish were killed and the liver samples (0.5 g) were collected and stored at -80 °C until required for microsome preparation. The microsome fraction was prepared by differential centrifugation (Li et al., 2011b).

Protein levels were estimated spectrophotometrically by the method of Smith et al. (1985) using bovine serum albumin as a standard. The microsomes were diluted to a protein content of 4 mg ml^{-1}.

2.4. Microsomal enzyme activity

The catalytic activity of EROD, MROD, PROD, COH, PNPH, TB-H, and BFC-OD was estimated. The EROD, MROD, and PROD activity was determined as the rate of transformation of ER, MR, and PR to resorufin (Zamaratskaia and Zlabek, 2009). Activity of COH was measured as described for porcine microsomes (Zamaratskaia et al., 2009) with slight modifications for fish microsomes with respect to incubation time and temperature and protein content. Activity of PNPH was measured as described by Zamaratskaia and Zlabek (2011), and activity of BFC-OD as described by Zlabek and Zamaratskaia (in press). Activity of TB-H was estimated with an HPLC-based method developed and validated for analyses of TB-OH formation in fish hepatic microsomes. The incubation mixtures (0.25 ml) contained 0.2 mg of microsomal protein in an incubation medium of 50 mM Tris-HCl, 10 mM $MgCl_2$, and 0.1 EDTA buffer (pH 7.4). The reaction was initiated with NADPH (1 mM) and was allowed to proceed for 60 min (25 °C in a water bath). The reaction was terminated by the addition of 0.25 ml of 100% cold acetonintrile, vortexed, and centrifuged for 5 min at 7500 × g. The supernatant was transferred to HPLC vials, and the concentration of tolbumatide metabolite TB-OH was measured using a fluorescence detector (excitation, 226 nm; emission, 318 nm). Details on concentration of substrate, incubation time, and protein are given in Table 1. The enzymatic activity was expressed in pmol of product formed per mg protein per minute.

All HPLC analyses were performed on a system comprising a pump (L-7100), autosampler (L-7200), a fluorescence detector (L-7485), or UV–vis detector (L-4250), and D-7000 HPLC Manager software (Merck, Hitachi, Tokyo, Japan). A Lichrospher RP-18 column (5 μm, 250 × 4 mm^2) equipped with a guard column was used to quantify TB-H, and Hypersil ODS (3 μM, 60 × 4.6 mm^2) with a guard column for quantification of resorufin, p-nitrocatechol, and 7-hydroxycoumarin.

Table 1
Experimental conditions for enzyme activity assays in rainbow trout liver microsomes.

Isoform in mammals	Labeled in the text as	Substrate	Substrate concentration in final incubation volume (μM)	Microsomal protein amount (mg)	NADPH (mM)	Incubation time (min)	Terminating reagent
CYP1A1/1A2	EROD	7-ethoxyresorufin	2	0.2	1.0	5	MeOH
CYP1A1/1A2	MROD	7-methoxyresorufin	2	0.2	1.0	15	MeOH
CYP2B	PROD	7-pentoxyresorufin	10	0.2	0.5	10	MeOH
CYP3A	BFC-OD	7-benzyloxyresorufin	200	0.2	0.5	10	MeOH
CYP2A6	COH	coumarin	8	0.2	1.25	30	40% TCA
CYP2E1	PNPH	p-nitrophenol	400	0.5	0.5	30	40% TCA
CYP2C9	TB-H	tolbutamide	100	0.2	1.0	60	ACN

EROD—7-ethoxyresorufin O-deethylase; MROD—7-methoxyresorufin O-demethylase; PROD—7-pentoxyresorufin O-depentylase; BFC—7-benzyloxy-4-trifluoromethylcoumarin; COH—coumarin hydroxylase; PNPH—p-nitrophenol hydroxylase, TB-H—tolbutamide hydroxylase; MeOH—methanol; TCA—trichloroacetic acid; ACN—acetonitrile.

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2.5. Statistical analysis

Statistical analysis was performed with Statistical Analysis System, version 9.1 (SAS Institute, Cary, NC, USA). Fish were divided into nine groups according to the exposure time and VRP concentration. The normality of the distribution of measured variables was tested using the Kolmogorov–Smirnov test. A logarithmic transformation was applied to normalize data. One-way ANOVA, with treatment as the fixed factor and tank as the random factor followed by the Student-Newman–Keuls test was used to determine differences between the mean values of nine groups. The data are presented as means after back transformation per group of fish, followed by their respective 95% confidence intervals. Results were considered significant when $P < 0.05$.

3. Results and discussion

The aim of this study was to identify CYP450-mediated reactions in fish that might be useful as biomarkers for monitoring VRP contamination of the aquatic environment.

In mammals and fish, pharmaceuticals are mainly metabolized by CYP450 enzymes. Thus, CYP450 activity is a crucial factor determining the detoxification ability of organisms. Pharmaceuticals can affect CYP450 activity through various mechanisms. Many pharmaceuticals are able to induce CYP450 enzymes by interaction with the receptors and activation of CYP450 gene expression. Alternatively, pharmaceuticals, including VRP, can directly affect CYP450 activity by acting as a competitive or mechanism-based inhibitor. For example, Wang et al. (2004) demonstrated the potential of VRP to irreversibly inhibit CYP3A in human liver microsomes. Both induction and inhibitory effects may alter the metabolic clearance and detoxification ability of fish. Altered CYP450 activity can be used as a marker of the presence and/or action of a xenobiotic. However caution is required, since it is difficult to establish that a specific response of CYP450 is due solely to a particular compound. The presence of a compound in the aquatic environment must be confirmed by analytical methods, which can be time-consuming and costly. Another limitation of using CYP450 activity as a biomarker is the natural variability in its activity and rapid response to environmental changes. Existing data on the use and limitations of, and procedural considerations for, fish CYP activity as a biomarker of chemical exposure have been summarized by Sarasquete and Segner (2000) and Corcoran et al. (2010).

The identification of novel biomarkers for contamination of the aquatic environment is important. Among these biomarkers, CYP450-mediated activity, either singly or in combination, is a promising tool for detecting fish exposure to environmental pollutants. Previous studies of CYP450 in fish were focused mainly on a limited number of CYP450-mediated reactions. In our study, to identify a candidate biomarker, we investigated seven CYP450-mediated reactions. This enabled more accurate determination of the CYP450 response to VRP and the selection of a reaction, or combination of reactions, that could be examined as candidate biomarkers of exposure to VRP.

Statistical analysis revealed that treatment had significant overall effect on EROD, COH, and PNPH ($P < 0.001$ for all) activities (Tables 2 and 3). However, the Student Newman–Keuls test demonstrated that no experimental group exposed to VRP differed from its corresponding controls.

In environmental monitoring, the measure of EROD activity is the most widely used biomarker to estimate CYP1A induction (van der Oost et al., 2003; Široka and Drastichová, 2004; Havelková et al., 2007). CYP1A and corresponding EROD activity is induced in the presence of numerous pharmaceuticals and other environmental contaminants such as polycyclic aromatic hydrocarbons (PAHs), dioxins and dioxin-like polychlorobiphenyls (PCBs), and dioxins (PCDDs). This induction is likely due to activation of the aryl hydrocarbon receptor (AhR). Although activity of CYP1A is commonly estimated using EROD, it is likely that MROD also reflects CYP1A activity. In the current study, no significant effect of VRP on either EROD or MROD activity was observed, suggesting that CYP1A is not modified by the presence of environmentally relevant concentrations of VRP.

Pentoxyresorufin O-dealkylase is used as a probe reaction for mammalian microsomal CYP2B (Lubet et al., 1995) and BFC-OD for mammalian CYP3A activity (Mingoia et al., 2007). CYP2B- and CYP3A-like proteins were detected in rainbow trout (Celander et al., 1996). Whether PROD activity is related to CYP2B-like protein in fish is unknown. Pentoxyresorufin O-dealkylase activity is generally very low in fish liver but can be induced by environmental pollutants (Parente et al., 2004). Our study suggested that VRP does not modify CYP2B-like activity in rainbow trout (Table 2).

7-benzyloxy-4-trifluoromethylcoumarin is a commonly used substrate to estimate CYP3A activity in fish (Christen et al., 2010) and VRP is a known inhibitor of BFC metabolism (Stresser et al., 2000; Wang et al., 2004). Study on mammals suggested that VRP is mainly metabolized by CYP3A4 (Kroemer et al., 1993). Even that the isoforms responsible for VRP metabolism in fish are not known, we expected that VRP would alter BFC metabolism. However, no significant effect of VRP on BFC metabolism was observed in the present study.

Coumarin 7-hydroxylation is catalyzed by CYP2A in many animal species (Juvonen et al., 1988). Microsomes from rainbow trout also expressed COH activity, although the activity was lower compared to mammals (Kaipainen et al., 1985). In this study, COH activity in VRP-treated groups significantly differed from that in controls at time 0, but did not differ from the corresponding controls at 21 and 42 day of exposure to VRP. Thus, COH activity cannot be used as a biomarker for monitoring pollution with VRP.

Table 2
EROD, MROD, and PROD activity in hepatic microsomes of rainbow trout.

Time exposure of VRP Day(s)	Group	EROD pmol (min^{-1} mg^{-1})	MROD pmol (min^{-1} mg^{-1})	PROD pmol (min^{-1} mg^{-1})
0	C	11.3 ^{abc} (8.2–15.5)	2.6 (1.9–3.7)	1.1 (0.9–1.2)
21	C1	13.9 ^{ab} (10.6–18.3)	3.4 (2.6–4.3)	1.2 (1.1. – 1.3)
	E1	14.8 ^{ab} (11.5–19.0)	4.4 (3.2–6.0)	1.6 (1.3–1.9)
	E2	15.8 ^{ab} (11.1–22.5)	4.4 (3.2–6.1)	1.4 (1.2–1.6)
	E3	20.2 ^{a} (16.1–25.5)	5.3 (4.1–6.8)	1.4 (1.3–1.5)
42	C2	9.5 ^{bc} (5.9–15.3)	2.4 (1.3–4.3)	1.3 (1.0–1.7)
	E1	7.1 ^{c} (4.9–10.4)	3.5 (2.4–5.0)	1.4 (1.2–1.6)
	E2	10.6 ^{abc} (7.3–15.4)	4.0 (2.9–5.4)	1.4 (1.2–1.7)
	E3	11.9 ^{abc} (7.7–18.5)	3.1 (2.1 -4.6)	1.3 (1.1 -1.6)
P-value		<0.001	0.061	0.559

Data are back-transformed means and 95% confidence interval within brackets.EROD—7-ethoxyresorufin O-deethylase; MROD—7-methoxyresorufin O-demethylase; PROD—7-pentoxyresorufin O-depentylase; C—control group (no exposure); E1, E2, E3—groups of fish exposed to 0.5 $\mu g\ l^{-1}$, 27 $\mu g\ l^{-1}$, 270 $\mu g\ l^{-1}$ concentrations of VRP, respectively. Means with different superscripts within column differ ($P < 0.05$).

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Table 3
BFC, COH, and PNPH activity in hepatic microsomes of rainbow trout.

Time exposure of VRP Day(s)	Group	BFC-OD pmol (min^{-1} mg^{-1})	COH pmol (min^{-1} mg^{-1})	PNPH pmol (min^{-1} mg^{-1})
0	C	362.7 (309.6–425.0)	0.25 [a] (0.17–0.35)	40.1 [abc] (32.8–49.1)
21	C1	279.3 (211.0–369.8)	0.10 [bc] (0.09–0.11)	44.4 [abc] (33.1–59.5)
	E1	395.1 (259.9–600.7)	0.11 [b] (0.09–0.13)	47.8 [abc] (39.7–57.7)
	E2	310.9 (250.4–386.0)	0.10 [bc] (0.09–0.11)	38.7 [bc] (34.0–44.1)
	E3	293.5 (233.3–369.1)	0.09 [bc] (0.08–0.11)	34.7 [c] (31.5–38.3)
42	C2	363.1 (266.5–494.7)	0.08 [bc] (0.07–0.10)	52.7 [ab] (39.4–70.5)
	E1	328.8 (258.9–161.6)	0.08 [bc] (0.07–0.09)	57.1 [ab] (48.0–68.0)
	E2	384.7 (338.1–437.7)	0.08 [c] (0.07–0.08)	58.4 [a] (50.9–66.9)
	E3	267.0 (200.6–355.5)	0.08 [bc] (0.07–0.10)	49.9 [abc] (40.9–60.9)
P-value		0.229	0.001	0.001

Data are back-transformed means and 95% confidence interval within brackets.BFC—7-benzyloxy-4-trifluoromethylcoumarin; COH—coumarin hydroxylase; PNPH—p-nitrophenol hydroxylase; C—control group (no exposure); E1, E2, E3—groups of fish exposed to 0.5 $\mu g\, l^{-1}$, 27 $\mu g\, l^{-1}$, 270 $\mu g\, l^{-1}$ concentrations of VRP, respectively. Means with different superscripts within column differ ($P<0.05$).

P-nitrophenol hydroxylation is a marker of CYP2E1-catalyzed reaction mammals and possibly in fish (Geter et al., 2003; Zamaratskaia and Zlabek, 2011). P-nitrophenol hydroxylation activity in medaka is inducible by mammalian CYP2E1 inducers ethanol and acetone, as well as by the toxic compounds 3-chloro-4-(dichloromethyl)-5-hydroxy-2w5Hx-furanone and methylazoxymethanol acetate (Geter et al., 2003). The present study demonstrated that PNPH activity in rainbow trout is not affected by VRP.

Formation of TB-OH from tolbutamide was undetectable in all samples, suggesting that juvenile rainbow trout do not possess tolbutamide-metabolizing activity.

This study had certain limitations. First, the concentration of VRP in the liver and other tissues was not measured; so the amount taken up from the surrounding water was not known. However, we have recently observed that carp larvae experimentally exposed to VRP take up the chemical from water and maintain a consistent concentration in whole body homogenates (Steinbach et al., unpublished observation). Another limitation of the study was the lack of data on the half-life of VRP in the system. However, repeated measurement of VRP concentration in water confirmed that VRP was stable, and we addressed this limitation by renewing the treatment solution twice daily to maintain the appropriate concentration of VRP.

Generally, *in vivo* effects of pharmaceuticals on fish CYP450 are evaluated through investigation of EROD activity and alterations in gene expression of, for example, AhR or AhR nuclear translocator. However, measurement of enzymatic activity is a more appropriate marker to assess the physiological effects of pharmaceuticals as it better reflects fish ability to detoxify. Additionally, investigations on pharmaceuticals and environmental pollutants should not be limited to *in vivo* experiments but should also involve *in vitro* models. Ideally, both enzymatic activity and gene expression should be measured in such studies. Given the unexpected lack of VRP effect on CYP450s activity in the present study, it would be useful to identify metabolic pathway(s) of VRP in fish. In our study, gene expression was not measured, because no alterations in CYP450-mediated reactions were observed.

4. Conclusion

This is the first report of CYP450-mediated reactions in liver of juvenile rainbow trout after long term exposure to VRP. The catalytic activities of EROD, MROD, PROD, COH, PNPH, TB-H, and BFC-OD showed no affect by VRP exposure. Thus, none of the investigated CYP450-mediated reactions can be used as a biomarker in exposed fish to monitor VRP pollution of aquatic environment.

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Presence of UV filters in surface water and the effects of phenylbenzimidazole sulfonic acid on rainbow trout (*Oncorhynchus mykiss*) following a chronic toxicity test

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ABSTRACT

UV filters belong to a group of compounds that are used by humans and are present in municipal waste-waters, effluents from sewage treatment plants and surface waters. Current information regarding UV filters and their effects on fish is limited. In this study, the occurrence of three commonly used UV filters – 2-phenylbenzimidazole-5-sulfonic acid (PBSA), 2-hydroxy-4-methoxybenzophenone (benzophenone-3, BP-3) and 5-benzoyl-4-hydroxy-2-methoxy-benzenesulfonic acid (benzophenone-4, BP-4) – in South Bohemia (Czech Republic) surface waters is presented. PBSA concentrations (up to 13 $\mu g\ L^{-1}$) were significantly greater than BP-3 or BP-4 concentrations (up to 620 and 390 ng L^{-1}, respectively). On the basis of these results, PBSA was selected for use in a toxicity test utilizing the common model organism rainbow trout (*Oncorhynchus mykiss*). Fish were exposed to three concentrations of PBSA (1, 10 and 1000 $\mu g\ L^{-1}$) for 21 and 42 days. The PBSA concentrations in the fish plasma, liver and kidneys were elevated after 21 and 42 days of exposure. PBSA increased activity of certain P450 cytochromes. Exposure to PBSA also changed various biochemical parameters and enzyme activities in the fish plasma. However, no pathological changes were obvious in the liver or gonads.

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1. Introduction

Municipal waste-waters contain numerous compounds that are used or produced by humans, including pharmaceuticals and personal care products (PPCPs), pesticides and bactericides, heavy metals, and nutritive components such as sweeteners, which are used in large quantities around the world. Most sewage treatment plants (STPs) are not designed to remove these substances. Thus, many of these compounds are not removed during treatment processes in STPs and can enter the aquatic environment from sewage effluents (Petrovic et al., 2003; Verlicchi et al., 2012).

UV filters (also called sunscreens) are a member of the PPCPs family. These filters are often used to protect skin from chronic (skin cancer) or acute (sunburn, photo-ageing) exposure to ultraviolet radiation from sunlight (Gasparro, 2000). Two general types of sunscreen have been developed: organic and inorganic (Gasparro et al., 1998). Octocrylene (OC), 2-phenylbenzimidazole-5-sulfonic acid (PBSA), homosalate, 2,4-dihydroxybenzophenone, 2,2′,4,4′-tetrahydroxybenzophenone, oxybenzone, 2-hydroxy-4-methoxybenzophenone (benzophenone-3 = BP-3), 5-benzoyl-4-hydroxy-2-methoxy-benzenesulfonic acid (benzophenone-4 = BP-4), 4-methyl-benzylidene camphor (4MBC), 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC), and ethyl-4-aminobenzoate are the most commonly used organic UV filters. Individual organic UV filters have a relatively narrow absorption spectrum. Therefore, they are usually combined to obtain protection against the entire UV-radiation spectrum. A list of UV filters that are allowed in cosmetic products is given in EU Regulation No. 1223/2009, Annex VI (Regulation, 2009). UV filters can be added in concentrations up to ten percent in sunscreen products (Schlumpf et al., 2001).

UV filters enter the environment by two pathways (Balmer et al., 2005). The first pathway is by direct input. Direct input occurs when UV filters enter surface waters as they are washed off

Abbreviations: PBSA, 2-phenylbenzimidazole-5-sulfonic acid; BP-3, benzophenone-3; BP-4, benzophenone-4; 4MBC, 4-methyl-benzylidene camphor; EHMC, 2-ethyl-hexyl-4-trimethoxycinnamate; OC, octocrylene; PPCPs, pharmaceuticals and personal care products.

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of individuals while those individuals bathe or swim. The second pathway is by indirect input. Indirect input occurs when UV filters are washed off individuals when those individuals shower or are released from towels and clothing during laundering. Thus, these UV filters are transported to sewage waters. Consequently, UV filters enter surface waters and are only partly eliminated in STPs. For example, the mean removal of PBSA from water by STPs is only 21 percent, and the mean removal of BP-3 and BP-4 by STPs is approximately 60 percent (Rodil et al., 2012). According to Leal et al. (2010), PBSA is not removed from water by any of the biological treatment systems. The highest concentrations of UV filters in water are measured during the summer (up to 2.7 μg L^{-1}) (Balmer et al., 2005; Poiger et al., 2004).

Moreover, some UV filters have been detected in semipermeable membrane devices, which indicates that these UV filters are potentially bioaccumulated (Poiger et al., 2004). Some UV filters have been detected in aquatic organisms (Buser et al., 2006; Nagtegaal et al., 1997; Poiger et al., 2004). A summary of UV filters that accumulate in fish has been presented by Fent et al. (2008) and more recently by Gago-Ferrero and Diaz-Cruz (2012). 4MBC and OC are the most bioaccumulated UV filters in fish from Swiss lakes and rivers (Balmer et al., 2005; Buser et al., 2006). Several UV filters cause estrogenic activity in fish (Fent et al., 2008). BP-3 and BP-4 interfere with the sex hormone system and can affect the reproduction of fish (Bluthgen et al., 2012; Gago-Ferrero and Diaz-Cruz, 2012; Zucchi et al., 2011).

The cytochromes P450 (CYP450s), mainly the first three families – CYP1, CYP2, and CYP3 – are involved in the metabolism of xenobiotics, such as pharmaceuticals and environmental pollutants, in mammals (Daughton and Ternes, 1999; Hasler et al., 1999; Meunier et al., 2004). These enzymes are also present in fish (Uno et al., 2012). Altered cytochrome 1A (CYP1A) activity has been used as a marker for the impact of xenobiotics on fish for more than 15 years (Bucheli and Fent, 1995; Burkina et al., 2012; Fent, 2003). However, to date, no information is available on the effects of polar UV filters on CYP enzymes. The activity of CYP1A is traditionally measured as the catalytic activity of 7-ethoxyresorufin *O*-deethylase (EROD). The induction of CYP1A is generally mediated via the aryl hydrocarbon receptor (AhR) pathway (Nilsen et al., 1998). However, whether UV filters are involved in the activation of AhR is not clear.

Metabolic transformation of xenobiotics may lead to increased risk of oxidative stress. The protective functions of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) have been studied in fish (Stara et al., 2012).

Despite of detection of PBSA in aquatic environment, little information is available about its biological effects and mode of action in aquatic biota. Even if PBSA has a low potential to bioaccumulate ($\log K_{OW(PBSA)}$ in range from −0.16 (Leal et al., 2010) to 1.6 (Rodil et al., 2008) have been reported), the relatively high concentration present in water may negatively affects aquatic organisms. Therefore, basic information on molecular responses in organisms exposed to environmental concentrations should be acquired. The PBSA effects were assessed on the basis of liver microsomal enzymes activities, oxidative stress parameters, biochemical and hematological parameters, and histology. Moreover, the concentrations of PBSA in fish tissues and plasma were analyzed.

The aim of this study was to (1) determine environmental concentrations of the most commonly used UV filters, (2) observe the effects of long-term PBSA exposure on fish using biomarkers and (3) study bioaccumulation and biodegradation of PBSA in fish tissues. PBSA, BP-4 and BP-3 were chosen as representative UV filters because they are the most common UV filters detected in German surface waters (Wick et al., 2010). A laboratory experiment was conducted with the PBSA UV filter on rainbow trout (*Oncorhynchus mykiss*).

2. Materials and methods

2.1. Chemicals

PBSA (2-phenylbenzimidazole-5-sulfonic acid, CAS 27503-81-7; purity 96 percent), benzophenone-3 (2-hydroxy-4-methoxybenzophenon e, CAS 131-57-7; purity 98 percent) and benzophenone-4 (5-benzoyl-4-hydroxy-2-methoxy-benzenesulfonic acid, CAS 4065-45-6, purity >97 percent) were obtained from Sigma-Aldrich (Steinheim, Germany). The PBSA was dissolved in tap water to prepare a 150 mg L^{-1} stock solution for aquarium experiments. Stock solutions of PBSA, BP-3 and BP-4 were prepared in methanol at a concentration of 1 mg mL^{-1} for analytical purposes.

Liquid chromatography–mass spectrometry (LC/MS) grade acetonitrile and methanol (Lichrosolv, Hypergrade) were obtained from Merck (Darmstadt, Germany). Formic acid (LC/MS grade) was obtained from Fisher Scientific (USA). Trimetoprim was obtained from Sigma Aldrich (purchased from Labicom, Olomouc, Czech Republic, purity 98.5 percent) and was used as an internal standard.

The chemicals used for analyses of the activities of microsomal enzymes and antioxidant enzymes are detailed elsewhere (Burkina et al., 2012; Paskova et al., 2008, respectively).

Slides to determine glucose (GLU), triglycerides (TRIG), alanine aminotransferase (ALT), creatine kinase (CK), lactate (LAC) and total proteins (TP) were obtained from IDEXX Laboratories (Westbrook, USA).

A transformation solution (0.1 g potassium ferricyanide, 0.025 g potassium cyanide, and 0.07 g potassium dihydrogenphosphate diluted to 0.5 L with distilled water) was used for hemoglobin determination (Svobodova et al., 1991).

2.2. Analytical system

The analytical system included a triple-stage quadrupole MS/MS TSQ Quantum Ultra (Thermo Fisher Scientific, San Jose, CA, USA) equipped with Accela 1250 LC and Accela 600 LC pumps (Thermo Fisher Scientific) and a HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland). This system was equipped with a Hypersil GOLD Phenyl column (50 mm × 2.1 mm i.d., 3-μm particle size) and a Hypersil GOLD Phenyl guard column (10 mm × 2.1 mm i.d., 3-μm particle size) from Thermo Fisher Scientific for all LC–MS/MS determinations. In-line solid phase extraction LC–MS/MS (SPE-LC–MS/MS) extraction was performed on Hypersil GOLD column (20 mm × 2.1 mm i.d., 12-μm particle size) from Thermo Fisher Scientific.

2.3. Water sampling and sunscreen determination by LC/LC–MS/MS

Grab subsurface water samples were collected in the South Bohemia region during one week of the peak summer season (mid of July). Samples were collected at depths of approximately 0.3 m, transported to the laboratory on ice and stored at −20 °C until analysis. The longest time between sampling and sample storage was 8 h. The most popular summer bathing locations, including outdoor swimming pools (five localities), recreational ponds (six localities) and rivers downstream of contamination sources (holiday camps, STPs; eight localities) were classified as highly exposed waters. The upper part of the sampled river and production ponds that were not used for bathing were used as background localities (four locations). The target UV filters (PBSA, BP-3 and BP-4) in the water samples were determined by two-dimensional liquid chromatography tandem mass spectrometry (LC/LC–MS/MS). For LC/LC–MS/MS determination, samples were filtered through 0.45 μm regenerated cellulose filters (Labicom, Olomouc, Czech Republic) and spiked with an internal standard of 5 ng trimethoprim per 10 mL of sample. The method parameters are documented in Supplementary material 1). The average relative recoveries for PBSA, BP-3 and BP-4 in water were 95 percent with RSD (relative standard deviation) 13 percent, 87 percent with RSD ten percent and 97 percent with RSD fourteen percent, respectively at a concentration level of 100 ng L^{-1} in tap water. The limits of quantification (LOQs) for PBSA, BP-3 and BP-4 in the water samples were 2.3, 3.9 and 1.8 ng L^{-1}, respectively.

2.4. Fish exposure experiment

Juvenile rainbow trout (*O. mykiss*) were purchased from a local commercial hatchery (Husinec, Czech Republic). These fish were transferred to fresh water aquariums (200 L; temperature 16.6 ± 0.7 °C; pH 7.5 ± 0.2; dissolved oxygen 8.2 ± 0.9 mg L^{-1}) and allowed to acclimate for 14 days before the experiment was started. The fish were fed with commercial fish pellets (BioMar, Denmark; one percent of the total body weight day^{-1}).

One hundred and sixty fish with weights of 121 ± 12 g and total lengths of 22.1 ± 1.2 cm (mean ± standard deviation, SD) were randomly placed into eight aquariums with semi-static systems (three-quarters of the water was changed every day and refilled with fresh PBSA-fortified water). Fish were exposed to PBSA at concentrations of 1 (environmentally relevant concentration; S1), 10 and 1000 μg L^{-1} (10 and 1000 times greater concentrations, respectively; S2 and S3) for 21 or 42 days. The control group was kept in PBSA-free water. All concentrations were tested in duplicate. Six fish from both aquariums with the highest tested

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concentrations were placed into PBSA-free water after 42 days and kept there for 16 days (58 days from the start of the experiment) to determine the half-life of PBSA in the fish.

Experimental animals were handled in accordance with the national and institutional guidelines for the protection of human subjects and animal welfare.

2.5. Fish and water sampling

Fish were sampled 24 h after feeding to reduce prandial effects. Blood samples were collected from eight fish from each group after 21 and 42 days of exposure. Blood samples were centrifuged (837 × g, 10 min, 4 °C) to obtain plasma samples. Next, the fish were sacrificed, and the liver, kidney and muscle were collected and stored at −20 °C (for PBSA analysis) or −80 °C (for microsomal preparation and for determination of biochemical and antioxidant parameters). Plasma samples from recovered fish were collected after 44, 51 and 58 days (from the start of the experiment), and muscle and liver tissues were collected after 58 days. Liver and gonad pieces from eight control and S3-group fish after 42 days of exposure were fixed in ten percent buffered formalin for histopathological examination.

In addition, the PBSA concentrations in water sampled from each aquarium were determined. Water samples were collected three times from each aquarium during the experiment, after the water was changed each time (time 0) and before the water was changed the next day each time (time 24). For the LC/LC and LC injections, water samples were filtered (0.45 μm regenerated cellulose filters, Labicom, Olomouc, Czech Republic) and spiked with an internal standard of 5 (control, S1 and S2 groups) or 5000 ng (S3 group) trimethoprim for each 10 mL of sample, respectively. The method parameters are documented in Supplementary materials 1 and 2.

2.6. Tissue extraction and LC–MS/MS PBSA determination

Liver, kidney and muscle samples were cut into small pieces. The method for determining pharmaceuticals (Fedorova et al., in press) was modified and validated for PBSA. Briefly, acetonitrile with 0.1 percent formic acid and the internal standard (trimethoprim) were added to an Eppendorf-tube that contained the tissue. The tissue was homogenized at 30,000/min for 10 min (homogenator TissueLyser II, Quiagen, Germany) and then centrifuged at 9500 × g for 10 min (Micro 200R centrifuge, Hettich Zentrifugen, Germany). The supernatant solution was filtered (0.45 μm regenerated cellulose filters, Labicom, Olomouc, Czech Republic) and allowed to evaporate overnight. Preconcentrated samples after the evaporation were analyzed by liquid chromatography with tandem mass spectrometry (see Supplementary material 3). Initially, the method recovery was determined from analysis of fortified fish muscles. The average recovery for fish muscle fortified with PBSA to a concentration of 100 ng g^{-1} was 98 percent with six percent RSD. The QA/QC samples were analyzed in duplicate with each batch of processed samples, and process blanks, and after every fifth sample. In addition, the fortified control samples were analyzed after every tenth sample (see results in Supplementary material 4). The LOQs for fish samples are given in Supplementary material 4.

2.7. Liver microsome preparation and microsomal enzyme activity analysis

The microsome fraction from the liver was prepared by differential centrifugation (Li et al., 2011). The total protein concentration was determined by UV–vis spectrophotometry using bovine serum albumin as a standard (Smith et al., 1985). The microsomal samples were diluted to a protein content of 4 mg mL^{-1}.

The catalytic activities of EROD, 7-methoxyresorufin-*O*-deethylase (MROD), 7-penthoxyresorufin-*O*-deethylase (PROD) and 7-benzyloxy-4-trifluoromethylcoumarine-*O*-debenzylase (BFCOD) were determined as the conversion rates of 7-ethoxyresorufin, 7-methoxyresorufin and 7-penthoxyresorufin to resorufin and of 7-benzyloxy-4-trifluoromethylcoumarine (BFC) to 7-benzyloxyresorufin, respectively (Jonsson et al., 2006; Kennedy and Jones, 1994) on a 96-well plate. Briefly, microsomal protein mixtures, the potassium buffer (50 mM; pH 7.8) and the substrate (8 μM 7-ethoxyresorufin, 7-methoxyresorufin or 7-penthoxyresorufin, 25 μM BFC) were added to the well plate. The reaction was started by the addition of NADPH (1.2 mM and 0.6 mM for BFCOD). Fluorescence intensity (resorufin: excitation/emission 544/590 nm, 7-benzyloxyresorufin: excitation/emission 410/538 nm) was measured with a microplate reader (Infinite M200, Tecan, Mannedorf, Switzerland). Enzyme activity was expressed as pmol min^{-1} mg^{-1}.

Total cytochrome concentrations were determined according to the method of Omura and Sato (1964) using a molar extinction coefficient of 91 cm^{-1} mM^{-1}.

2.8. Antioxidant parameters

Antioxidant parameters, including superoxide dismutase [SOD; (Ewing and Janero, 1995)], glutathione reductase (GR, Carlberg and Mannervik, 1975) and CAT (Paskova et al., 2011) were measured spectrophotometrically (Specord 210 BU, Analytik Jena, Germany) in fish liver tissues from the control and the S3 group. Enzymatic SOD activity was expressed as the amount of product that was produced per min/mg protein. Enzymatic GR activity was defined as the decrease in the amount of NADPH per min mg^{-1} protein, and enzymatic CAT activity was defined as the decrease in the amount of hydrogen peroxide per min mg^{-1} protein.

2.9. Biochemical and hematological parameters

The following biochemical parameters were analyzed in fish plasma: LAC and GLU as stress indicators, TRIG and ALT for monitoring the permeability and integrity of cell membranes, CK as an indicator of skeletal musculature disorder and TP as an indicator of condition. These parameters were analyzed by a VETTEST-analyzer (Velisek and Svobodova, 2004).

The amount of leukocytes and erythrocytes, the hemoglobin concentration and the hematocrit values were determined according to the method of Svobodova et al. (1991).

2.10. Histology

Fixed samples were embedded in paraffin and routinely processed for histological examinations. Sections of 3 μm thickness were cut. These sections were stained with hematoxylin–eosin (H&E) and examined by light microscopy. Histopathological changes in the liver and gonads were graded as 0 (none), 1 (scattered), 2 (mild), 3 (mild to moderate), 4 (moderate), 5 (moderate to severe) or 6 (severe).

2.11. Statistical analyses

The mean ± SD of the data is presented. Statistical analysis of the data was conducted with the STATISTICA v.10 software for Windows (StatSoft, Czech Republic). Normally distributed and homoscedastic data were assessed by a one-way ANOVA (followed by post hoc Tukey HSD test). When data were not normally distributed and homoscedastic, the non-parametric method (Kruskal–Wallis test) of data analysis was used. Differences were considered statistically significant at $p < 0.05$.

3. Results and discussion

3.1. The presence of UV filters in surface waters

The concentration of selected UV filters was determined in water samples from popular recreational areas in South Bohemia in the Czech Republic (ponds, rivers and outdoor swimming pools). As reported in Table 1, the concentrations of PBSA and BP-3 in recreational areas were higher than those in the background localities ($p < 0.05$). A low concentration of BP-3 is consistent with results from BP-3 determinations in sewage sludge, where BP-3 was rarely detected but its two major degradation products (4,4′-dihydroxybenzophenone and 4-hydroxybenzophenone, both endocrine disruptors) were found (Gago-Ferrero et al., 2011). The BP-4 concentrations were not significantly different between the recreational and background sites.

The PBSA concentration was the highest among all of the measured UV filters (Table 1). These results differed from Spanish data, where BP-4 and 4MBC concentrations were higher than PBSA concentrations in surface waters (Rodil et al., 2012). The concentration of PBSA was higher than the concentrations of BP-3 and BP-4 in German surface waters (Wick et al., 2010); these results are similar to our results obtained in the Czech Republic.

On the basis of the results, we decided to study the effects of PBSA (as the predominant UV filter in Czech surface water) on fish.

3.2. Accumulation of PBSA in fish

To ensure that the fish were exposed to the tested concentrations of PBSA, we determined the concentration of PBSA in the aquarium water (Supplementary material 5). The measured PBSA concentrations equaled the expected concentrations within the measurement uncertainty (twenty percent).

UV filters EHMC and BP-3 have been detected in aquatic organisms (Fent et al., 2010). However, to the best of our knowledge, the

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Table 1
Occurrence of sunscreens in surface waters during summer 2011.

UV filters	Outdoor swimming pools $n=5$	Recreational ponds $n=6$	Rivers under the source of pollution $n=8$	**Background localities** $n=4$
PBSA (ng L^{-1})	240–13 000	24–930	11–500	5.1–48
BP-4 (ng L^{-1})	3.3–35	4.0–46	4.6–390	3.4–37
BP-3 (ng L^{-1})	26–620	21–550	12–67	14–20

PBSA, 2-phenylbenzimidazole-5-sulfonic acid; BP-4, 5-benzoyl-4-hydroxy-2-methoxy-benzenesulfonic acid; BP-3, 2-hydroxy-4-methoxybenzophenone.
The concentrations of PBSA, BP-3 and BP-4 in blank samples were less than 2 ng L^{-1}.
Outdoor swimming pools – Vodnany (GPS 49°8′35.729″N, 14°10′32.100″E, 5000 m^3, approx. 100 persons in sampling day), Prachatice (GPS 49°0′32.184″N, 13°59′57.510″E, 1000 m^3, approx. 300 persons), Hluboka and Vltavou (GPS 49°2′59.142″N, 14°26′41.485″E, 1500 m^3, 250 persons), Small pool and big pool in Olesnik (GPS 49°6′22.726″N, 14°22′1.839″E, 2400 m^3, 400 persons).
Recreational ponds – Lipno dam (GPS 48°64′13.169″N, 14°21′96.739″E, 48.7 km^2), Zahorsky pond (GPS 49°8′50.812″N, 14°7′32.627″E, 0.33 km^2), Podrouzek pond (GPS 49°2′8.727″N, 14°10′50.938″E, 0.30 m^2), Mydlovarsky pond (GPS 49°4′26.394″N, 14°21′30.858″E, 0.34 km^2), Stilec pond (GPS 49°4′26.394″N, 14°21′30.858″E, 0.12 km^2), Lipno dam (GPS 48°44′22.658″N, 14°6′27.928″E, 48.7 km^2).
Rivers under the source of pollution – Vltava (GPS 49°0′5.275″N, 14°27′30.080″E, flow 28 m^3 s^{-1}, source of pollution camps and STPs along the river), Zivny stream (GPS 49°1′40.124″N, 14°0′49.704″E, 0.3 m^3 s^{-1}, STP), Vltava river (GPS 48°51′17.118″N, 14°21′54.995″E, 16 m^3 s^{-1}, camps), Bezdrevsky stream (GPS 49°3′22.920″N, 14°12′18.956″E, 0.5 m^3 s^{-1}, STP), Blanice (GPS 49°9′51.788″N, 14°12′9.725″E, 4 m^3 s^{-1}, STP), Vltava river (GPS 48.6573417N, 14.3646083E, 13 m^3 s^{-1}, camp), Vltava river (GPS 48°55′2.617″N, 14°25′31.492″E, 20 m^3 s^{-1}, camps) town, Volarsky stream (GPS 48.8891694N, 13.8911939E, 0.4 m^3 s^{-1}, STP).
Background localities – pond No. 65 at model part of Faculty of Fisheries and Protection of Waters (GPS 49°9′19.106″N, 14°10′0.324″E, 0.002 km^2), Blanice (GPS 49°1′57.863″N, 13°58′27.704″E, 2 m^3 s^{-1}), Zivny river (GPS 48°59′29.140″N, 14°1′33.185″E, 0.1 m^3 s^{-1}), Vltava river (48°38′13.081″N, 14°17′22.211″E, 13 m^3 s^{-1}).

literature contains no data about the fate, distribution or effects of PBSA on exposed aquatic organisms.

The PBSA concentrations that were measured in the fish tissues are shown in Table 2. The PBSA concentrations in the fish plasma and tissues were near the LOQ and were highly variable for most exposure levels and times. Even the relatively high PBSA concentrations that were found in the fish livers exhibited a high SD. In addition, for tissues fortified with 100 ng of PBSA, an uncertainty of approximately twenty percent (RSD) was found. The variability within groups was much higher than the analytical method uncertainty. The intragroup variability was too high for the results to be evaluated with statistical software. More than 85 percent of the measured tissue and plasma samples were positive in the S3 group for both sampling days. The PBSA concentrations in more than 50 percent of the liver samples in the S2 group were above the LOQ on both days and in the S1 group after 42 days of exposure. PBSA was found in the fish plasma, liver, muscle and kidney at low concentrations whereas it was not present in fish plasma and tissues after recovery in PBSA-free water. This finding indicates that PBSA does not accumulate in fish but can enter the fish and can therefore induce a response in the liver (see Section 3.3).

3.3. Cytochrome activity in fish liver

Activities of EROD (CYP1A), MROD (CYP1A), PROD (CYP2B) and BFCOD (CYP3A) are presented in Table 3 and in Fig. 1. The EROD, MROD and PROD activities significantly increased in the tested groups (relative to their activities in the control) after 21 days of exposure to PBSA. This result indicates that environmentally

Table 2
Concentration of PBSA (ng g^{-1}) in rainbow trout tissues.

Tissues	Exposure (days)	Tested groups		
		S1	S2	S3
Plasma	21	0 ± 0 (0/8)	0.11 ± 0.32 (1/8)	2.5 ± 3.5 (8/8)
	42	0.33 ± 0.64 (2/8)	0 ± 0 (0/8)	0.87 ± 0.40 (8/8)
	44	–	–	0 ± 0 (0/12)
	51	–	–	0.75 ± 1.7 (3/12)
	58	–	–	0 ± 0 (0/12)
Liver	21	0 ± 0 (0/7)	6.8 ± 12 (5/7)	26 ± 29 (6/7)
	42	83 ± 100 (5/8)	81 ± 140 (4/8)	20 ± 16 (8/8)
	58	–	–	0.085 ± 0.31 (1/8)
Kidney	21	0 ± 0 (0/8)	0 ± 0 (0/8)	9.3 ± 13 (8/8)
	42	0 ± 0 (0/7)	0 ± 0 (0/7)	4.7 ± 1.7 (6/6)
Muscle	21	4.9 ± 1.6 (8/8)	4.2 ± 1.4 (8/8)	7.0 ± 5.0 (8/8)
	42	2.2 ± 1.5 (6/8)	1.5 ± 0.64 (6/8)	2.1 ± 1.3 (7/8)
	58	–	–	0.33 ± 0.67 (3/8)

Data are average ± S.D. Values in brackets are number of positive samples/number of analyzed samples.

relevant concentrations of PBSA affect CYP activity even after 21 days of exposure. The mode of interaction causing increased activity of CYP by PBSA is yet to be discovered. Because AhR is mainly responsible for induction of CYP1 enzymes (Nebert et al., 2004) investigation into interaction with intracellular receptors might be of interest. No significant change occurred between the control and the PBSA-treated groups after 42 days of exposure. However, in each group, the EROD, MROD and PROD activities were higher after 42 days of exposure than after 21 days (statistically significant increases in MROD and EROD activities in the control, S1 and S3 groups, and in the PROD activity in the S1 and S3 groups were observed). This general unexpected increase including control may be explained by age related development of metabolism. The highest observed 6-fold increase of EROD activity compared to control does not reach the response to the strongest AhR inducers. However, such increase should be noticed as relevant and trigger our attention.

No statistically significant changes in BFCOD activity were found among the control and the PBSA-treated groups for either exposure period.

Total microsomal proteins were not significantly altered in any of the exposed groups.

3.4. Oxidative stress in fish liver after exposure to PBSA

The anti-oxidative stress enzyme activity results are shown in Table 4. The CAT and SOD activities were not significantly different between the control group and the group that was exposed to the highest concentration of PBSA (S3 group). The GR activity after 42 days of exposure to PBSA was significantly lower ($p < 0.05$) than the GR activity in the control. GR catalyzes the reduction of glutathione (from disulfide to sulfhydryl) in the presence of NADPH to maintain its intracellular levels. This decreased activity of GR might be caused by the impairment of the enzyme by ROS or toxic aldehydes (e.g., Ochi, 1990; Rousar et al., 2010; Vessey and Lee, 1993) or by the diminished availability of NADPH (Li et al., 2010a).

3.5. Biochemical and hematological parameters

The biochemical parameters of the plasma from rainbow trout that were exposed to PBSA are given in Table 5. The GLU level was significantly higher ($p < 0.05$) in the S2 and S3 groups than in the control group on both sampling days. The GLU content was significantly higher ($p < 0.05$) in the S1 group after 42 days of

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Table 3
Activity of CYP450 enzymes in rainbow trout liver.

Parameters	Units	Exposure (days)	Tested groups			
			Control	S1	S2	S3
EROD	$pmol min^{-1} mg^{-1}$	21	4.7 ± 1.9	20 ± 8.5*	31 ± 10*	18 ± 6.7*
		42	19 ± 10	40 ± 17	33 ± 13	41 ± 21
PROD	$pmol min^{-1} mg^{-1}$	21	0.49 ± 0.068	0.68 ± 0.10*	0.79 ± 0.13*	0.61 ± 0.10*
		42	0.63 ± 0.19	0.84 ± 0.14	0.78 ± 0.18	0.86 ± 0.16
BFCOD	$pmol min^{-1} mg^{-1}$	21	68 ± 9.4	57 ± 15	67 ± 17	57 ± 17
		42	47 ± 5.5	57 ± 17	58 ± 11	58 ± 15
total CYP	$uM mg^{-1}$	21	0.10 ± 0.019	0.14 ± 0.018	0.11 ± 0.035	0.10 ± 0.046
		42	0.082 ± 0.066	0.15 ± 0.12	0.11 ± 0.058	0.12 ± 0.049

EROD – 7-ethoxyresorufin-*O*-deethylase; PROD – 7-penthoxyresorufin-*O*-deethylase; BFCOD – 7-benzyloxy-4-trifluoromethylcoumarine-*O*-debenzylase; CYP – cytochromes. Data are average ± S.D., $n=8$.

* Significant differences ($p < 0.05$) in exposed group compared with the control.

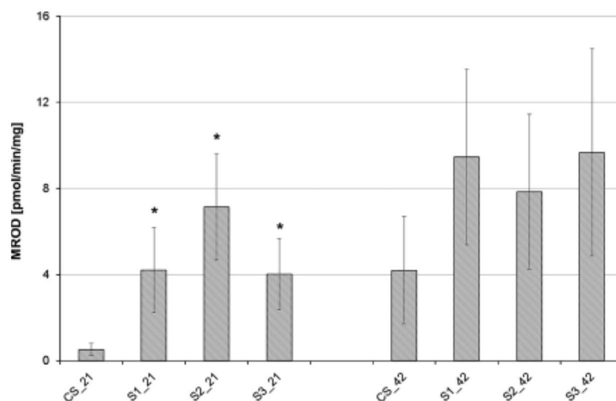

Fig. 1. Activity of 7-methoxyresorufin-*O*-deethylase (MROD) [pmol/min/mg]. CS – control group; S1 – concentration of PBSA 1 $\mu g L^{-1}$; S2 – concentration of PBSA 10 $\mu g L^{-1}$; S3 – concentration of PBSA 1000 $\mu g L^{-1}$; 21 – sampling after 21 days; 42 – sampling after 42 days. Significant differences ($^*p < 0.05$) were observed in the tested group compared with the control for each day of exposure.

Table 4
Activity of anti-oxidative enzymes in rainbow trout liver after 42 days of exposure.

Enzymes	Units	Tested groups	
		Control	S3
CAT	$\mu mol H_2O_2 min^{-1} mg^{-1}$	0.035 ± 0.0055	0.041 ± 0.0077
SOD	$nmol NBT min^{-1} mg^{-1}$	0.0074 ± 0.0021	0.0069 ± 0.0012
GR	$nmol NADPH min^{-1} mg^{-1}$	0.040 ± 0.010	0.027 ± 0.0082*

CAT – catalase, SOD – superoxide dismutase, GR – glutathione reductase, S3 – concentration of PBSA 1000 $\mu g L^{-1}$. Data are mean ± S.D., $n=8$.

* Significant differences ($p < 0.05$) in exposed group compared with the control.

exposure than in the control. Higher concentrations of GLU in blood indicate metabolic stress (Portz et al., 2006). During the entire experiment, no significant change ($p > 0.05$) in the TRIG level of PBSA-treated groups occurred relative to the control. ALT and CK in plasma are markers of environmental stress (El-Sayed et al., 2007; Li et al., 2010b). Although the CK activity increased with increasing PBSA concentration (Table 5), the ALT activity did not. The LAC concentration in the S3 group decreased after 42 days relative to the control. The TP levels in the S2 and S3 groups were higher than those in the control after 42 days of exposure.

No differences in hematological parameters occurred between the control and PBSA-treated groups for both sampling days (number of leukocytes and erythrocytes, hematocrit and concentration of hemoglobin).

3.6. *Histology*

Pathological changes, which were encountered in the liver of all of the examined groups, included mild sinusoidal congestion, mild infiltration with lymphocytes, and plasma cells. In one control animal, macrophages in perivascular and randomly in parenchyma were discovered in the liver. The content of the fat vacuoles in the hepatocytes was variable. The hepatocytes cytoplasm was mild to moderately granulated (Supplementary material 6).

The gonads were well developed with testes that contained germ cells up to the developmental stage of the spermatids and with ovaries that contained germ cells up to the perinucleolar stage of oocytes. One animal in the S3 group had a slightly higher amount of intracytoplasmic vacuoles in the oocytes. No significant difference between the control and the PBSA-exposed animals was

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Table 5
Biochemical parameters of blood plasma in rainbow trout affected by chronic exposure to PBSA.

Parameters	Units	Exposure (days)	Tested groups			
			Control	S1	S2	S3
LAC	$mmolL^{-1}$	21	1.2 ± 0.57	1.5 ± 1.2	1.5 ± 0.57	2.1 ± 1.9
		42	1.2 ± 0.31	1.9 ± 1.2	1.2 ± 0.46	0.67 ± 0.13*
GLU	$mmolL^{-1}$	21	2.5 ± 1.0	4.1 ± 1.3	4.7 ± 0.7*	5.2 ± 0.4*
		42	4.2 ± 0.6	5.6 ± 0.5*	6.4 ± 0.5*	4.8 ± 0.6
TRIG	$mmolL^{-1}$	21	0.55 ± 0.34	0.50 ± 0.08	0.77 ± 0.19	0.61 ± 0.14
		42	0.86 ± 0.27	1.1 ± 0.16	0.91 ± 0.08	0.79 ± 0.19
ALT	UL^{-1}	21	11 ± 4.6	11 ± 3.6	21 ± 10	9.4 ± 3.6
		42	15 ± 13	11 ± 5.4	11 ± 3.2	10 ± 3.6
CK	UL^{-1}	21	1230 ± 549	1448 ± 450	2457 ± 320*	2886 ± 329*
		42	1163 ± 411	1581 ± 223	2384 ± 305*	2799 ± 366*
TP	gL^{-1}	21	48 ± 7.9	44 ± 2.4	54 ± 6.2	57 ± 5.6*
		42	37 ± 4.1	37 ± 5.0	47 ± 2.7*	50 ± 3.0*

CS – control group, S1 – concentration of PBSA 1 $\mu g\ L^{-1}$, S2 – concentration of PBSA 10 $\mu g\ L^{-1}$, S3 – concentration of PBSA 1000 $\mu g\ L^{-1}$. Data are average ± S.D., $n=8$.
* Significant differences ($p < 0.05$) in tested group compare with the control.

detected. In addition, signs of ovo-testis or degenerative changes in the gonads were not detected.

4. Conclusions

The present study demonstrates the occurrence of three most commonly used UV filters in surface waters. The PBSA surface water concentrations were substantially greater than the BP-3 and BP-4 concentrations in South Bohemia. A chronic toxicity test with PBSA followed the pilot screening of UV filters in surface waters. Even if the accumulation of PBSA in fish tissues was not confirmed in the aquarium experiment, we did find low concentrations of PBSA in the plasma, liver and kidney of fish exposed to all of the tested concentrations of PBSA, including the environmentally relevant concentration.

The interaction of PBSA with intracellular receptors should be expected, as indicated by the elevated activities of CYP1A and CYP2B. Therefore, studies on the interaction of PBSA with AhR are necessary. PBSA could also cause oxidative stress in fish, as indicated by decreased activity of GR. Moreover, PBSA affected the concentrations of several biochemical parameters in the blood plasma (LAC, GLU, CK, and TP). No adverse effects on histologically studied tissues were observed. In conclusion, the present data show that environmentally relevant PBSA concentrations might influence the biological processes of exposed organisms.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2013.06.022.

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CLOTRIMAZOLE IN RAINBOW TROUT I: DISTRIBUTION, BIOACCUMULATION, DEPURATION, AND RESPONSE OF THE CYTOCHROME SYSTEM AFTER LONG-TERM EXPOSURE

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Abstract

Clotrimazole (CLO) behaviour and its effects on rainbow trout were investigated following exposure up to 42 days at concentrations of 0.01 (environmentally relevant), 1.0, and 10 $\mu g \cdot L^{-1}$ in a semi-static system. Based on water and fish clotrimazole concentration, the bioconcentration factors were estimated for plasma (132 to 169) and muscle (58 to 550). The half-life of CLO was estimated to be 72, 159, and 682 h in liver, muscle, and kidney, respectively. The CLO concentration in faeces was low compared to the amount of CLO in water (3 to 7%). Fish exposed to 1.0 and 10 $\mu g \cdot L^{-1}$ concentrations showed significantly higher hepatosomatic indices after 42 days, which may indicate detoxification in the liver. Ethoxyresorufin-O-deethylase (EROD, CYP1A1) activity in hepatic microsomes was significantly higher in exposed groups at 42 days compared to control groups. 7-benzyloxy-4-trifluoromethylcoumarin O-debenzylase (BFCOD, CYP3A) was significantly lower after 21 days in the group exposed to 10 $\mu g \cdot L^{-1}$ CLO. After 42 days exposure, CYP3A activity was reduced in the 0.01 $\mu g \cdot L^{-1}$ group and increased in the 10 $\mu g \cdot L^{-1}$ group. Clotrimazole rapidly adsorbed in the aquarium water-fish system. There were significant differences between tested systems with and without fish. The nominal 10 $\mu g \cdot L^{-1}$ concentration of CLO in aquarium water without fish decreased to 4.9 $\mu g \cdot L^{-1}$ over 24 h and, in water containing fish, decreased to 3–77% over 24 h. Clotrimazole has a long half-life in fish compared to humans, suggesting that interactions between CLO and biological systems may induce biochemical disturbances in fish.

Key words: *Bioconcentration, Cytochrome P450, Fish, Half-life, Metabolism, Pharmaceutical*

Highlights

- Environmental pollution with clotrimazole is an important issue
- Chronic toxicity test was carried out with clotrimazole on rainbow trout.
- The muscle bioconcentration factor of clotrimazole ranged from 58 to 550.
- The half-life of CLO was estimated to be 72, 159, and 682 h in liver, muscle, and kidney, respectively.
- We found clotrimazole affects CYP450-mediated reactions at environmentally relevant concentration

1. INTRODUCTION

Clotrimazole (CLO), bis-phenyl-2-chlorophenyl-1-imidazolyl methane, is an antifungal agent widely used in human and veterinary medicine for treatment of dermatological and gynaecological fungal infections (Rochette et al., 2003; Vanden Bossche et al., 2003). It is available in a variety of formulations and is presumed to be introduced into the aquatic environment mainly through domestic (Thomas and Hilton, 2004) and hospital wastewater discharge (Escher et al., 2011; Frédéric and Yves, 2014). Clotrimazole has been detected in river water at 6 to 71 $ng \cdot L^{-1}$ (Huang et al., 2010; Thomas and Hilton; 2004; Roberts and Thomas, 2006) and in waste water at 0.6 to 111.0 $ng \cdot L^{-1}$ (Huang et al., 2010; Loos et al., 2013). Clotrimazole has recently attracted the interest of the research community for its toxicity to aquatic organisms (OSPAR, 2013; Porsbring et al., 2009) and a potential water pollutant.

Clotrimazole is weakly basic and nearly insoluble in water (0.49 $mg \cdot L^{-1}$) with little or no biodegradability and strong adsorption to activated sludge (Kahle et al., 2008). It belongs to a group of medications containing the imidazole ring that can affect the activity of cytochrome P450 (CYP450). Members of this family of heme-containing enzymes are involved in metabolism of exogenous and endogenous compounds (Verras and de Montellano, 2006). Clotrimazole has been shown to be a potent modulator of many mammalian and fish CYP450-mediated reactions, including sterol 14α-demethylation (CYP51; Ford 2004), ethoxyresorufin-O-deethylation (CYP1A; Burkina et al., 2013), 7-benzyloxy-4-trifluoro-methylcoumarin (BFC) debenzylation (CYP3A; Burkina et al., 2013), and others (Hinfray et al., 2006; Ronis et al., 1998; Wassmur et al., 2013; Zhang et al., 2002). In addition, CLO modulates cellular Ca^{2+} homeostasis in mammals (Klokouzas et al., 2002) and steroidogenesis in aquatic organisms (Baudiffier et al., 2012, Baudiffier et al., 2013) These are considered negative effects that may potentially lead to undesirable alterations in fish endocrine and reproductive systems.

Sub-chronic toxicology tests can provide useful information for understanding possible ecotoxicological effects of human pharmaceuticals on aquatic organisms. The aim of the study was to investigate the effects of CLO on juvenile rainbow trout, *Oncorhynchus mykiss*, by determination of concentration level of the compound in liver, kidney, muscle, blood plasma, and faeces. The effect of CLO on the activity of ethoxyresorufin-O-deethylase (EROD) and BFC debenzylase (BFCOD) was also studied.

2. MATERIALS AND METHODS

2.1. CHEMICALS

Clotrimazole (CAS Number 23593-75-1, $C_{22}H_{17}ClN_2$) was purchased from Voight (USA). Econazole was obtained from Sigma Aldrich as nitrate salt (purity 85.83%) and was used as an internal standard. Liquid chromatography–mass spectrometry (LC/MS) grade acetonitrile and methanol (Lichrosolv, Hypergrade) were obtained from Merck (Darmstadt, Germany). Formic acid (LC/MS grade) was obtained from Fisher Scientific (USA). The chemicals used for analyses of microsomal enzyme activity are detailed in Burkina et al. (2012).

2.2. FISH

Juvenile rainbow trout (n = 200), weighing 220 ± 40 g (mean ± SD), were obtained from a local commercial hatchery. Fish were transferred to aquaria containing 250 L of freshwater

(temperature 15.6 ± 0.9 °C, pH 7.2 ± 0.15, dissolved oxygen 7.5–8.5 $mg \cdot L^{-1}$) under a 12:12 L:D photoperiod. These conditions were maintained during acclimation and the experimental period. Fish were fed during the acclimation (14 days) and experimental periods with commercial fish food (BioMar, Denmark; 1% of the total body weight/day). Experimental animals were handled in accordance with national and institutional guidelines for the protection of human subjects and animal welfare.

2.3. EXPOSURE TO CLO AND WATER CHEMICAL ANALYSIS

Fish were randomly divided into groups of 20 placed in each of ten aquaria containing 200 L of water or test solution in a semi-static system. A stock solution of clotrimazole was prepared in DMSO (2 $mg \cdot ml^{-1}$). Three working solutions in water (2, 200, and 2000 μg) were diluted from DMSO stock. Test solutions were prepared from these working solutions daily. Fish were exposed to concentrations of CLO of 0.01 (environmentally relevant concentration), 1.0, and 10 $\mu g \cdot L^{-1}$ for 21 and 42 days. A control group was exposed to clean freshwater and a solvent control (SC) group was exposed to the volume of DMSO (v/v, 0.01%) used in the CLO exposed concentration.

Prior to the experiment, stability of the stock solution with the highest concentration of CLO was evaluated under the experimental conditions, and was confirmed to be stable for one week. Stock stored at room temperature and protected from light was used for test solution preparation within this period.

Test solutions were renewed each day to maintain water quality and the appropriate CLO concentration. The concentration of CLO in the water was measured 6 times during the experimental period, immediately after solution renewal (0 h) and after 24 h, on days 1, 20, and 41. Two additional aquaria containing 10 L of water with 10 $\mu g \cdot L^{-1}$ CLO were used to assess the adsorption level of CLO in conditions without fish. The water sampling regime was as described above for the experimental period. Water samples from each exposure aquarium were analyzed separately in duplicate. Differences in measurements of water samples from two aquaria of the same group were within the analytical uncertainty of the method used (< 20% relative standard deviation).

The water samples were analyzed using in-line SPE liquid chromatography with tandem mass spectrometry (LC/LC-MS/MS). The analysis was performed with a TSQ Ultra MS/MS (Thermo Fisher Scientific, San Jose, CA, USA) coupled with Accela 1250 and Accela 600 HPLC pumps (Thermo Fisher Scientific) and a HTS-XT autosampler (CTC Analytics AG, Zwingen, Switzerland). MS/MS settings used for chemical analyses of CLO in water samples were described in Grabic et al. (2012). The methods are documented in Supplementary Material 1.

For the LC/LC injections, water samples were filtered (0.45 μm regenerated cellulose filters, Labicom, Olomouc, Czech Republic) and spiked with an internal standard (10 ng of econazole to 10 ml of sample).

2.4. COLLECTION AND PREPARATION OF SAMPLES

After 21 and 42 days of exposure to CLO, four fish from each duplicate aquarium were killed and combined into a single group of 8. Blood samples and tissue were quickly removed in the following order: blood, liver, kidney, and white muscle.

Blood was taken by caudal vein puncture using a heparinized syringe. An aqueous solution of heparin sodium salt (5000 $IU \cdot ml^{-1}$) at 0.01 $ml \cdot ml^{-1}$ of blood, was used to stabilize the samples. Blood plasma for chemical analysis was obtained from cooled centrifuged blood (10 min, 837 × g, 4 °C) and stored at -20 °C until analysis. Liver tissue was divided into two

samples for preparation of microsomal fraction (approximately 0.4 g) and chemical analyses and stored at -80 °C and -20 °C, respectively. Kidney and muscle tissue was stored at -20°C. On day 20 of exposure, fish faeces were collected over a 24 h period in all experimental aquaria, and CLO concentrations were measured.

Fork length (FL), body weight (BW), and liver weight (LW) were recorded. Condition factor (CF) and hepatosomatic index (HSI) was calculated according to White and Fletcher (1985):

$CF = BW (g)/FL^3 (cm) \times 100$

$HSI = LW (g)/BW (g) \times 100$

After 42 days exposure, fish exposed to CLO at 1.0 $\mu g \cdot L^{-1}$ were placed into CLO-free water. The depuration of CLO from fish tissue was determined after 7 days (6 fish) and 13 days (8 fish) (49 and 55 days from the start of the experiment). First-order kinetics was assumed for the depuration rate estimation. The half-life (50% depuration, t_{50}) of CLO in fish was calculated from the value of the slope (k) obtained from linear regression of the natural logarithm (ln) of the detected concentration vs. depuration time (t_{50}) = 0.693/k.

2.5. ANALYSIS OF CLOTRIMAZOLE IN FISH TISSUE AND FAECES

A modified extraction procedure (Fedorova et al., 2013) was used to extract CLO from fish tissue and faeces. Briefly, samples of liver, kidney, muscle, and faeces (approximately 3 g), internal standard (econazole, 50 ng per sample), and 1 ml of extraction solvent (acetonitrile : isopropanol, 1 : 1) were homogenized at 30 000 rpm for 10 min (TissueLyser II, Quiagen, Germany) and centrifuged at 9500 × g for 10 min (Micro 200R, Hettich Zentrifugen, Germany). The supernatant was filtered (0.45 µm regenerated cellulose filters) and evaporated to 0.5 ml. Faeces samples were assayed immediately after extraction. The extract was diluted with water (1 : 3) for LC-MS/HRMS analysis.

The bioconcentration factor (BCF) was calculated for blood plasma and for white muscle as the mean concentration of CLO in the fish sampled after 21 and 42 days, divided by the mean concentration of CLO in water at time 0 (first day of experiment) in the respective group (Table 1).

CLO was quantified using hybrid quadrupole/Orbitrap mass spectrometer QExactive (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Accela 1250 LC pump (Thermo Fisher Scientific) and a HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland). This system was equipped with a Hypersil GOLD analytical column (50 mm × 2.1 mm i.d., 3 µm particle size) and a Hypersil GOLD guard column (10 mm × 2.1 mm i.d., 3-µm particle size) (Thermo Fisher Scientific). Targeted MS/HRMS analysis after electrospray ionization was performed using the mass inclusion list and expected retention times of the target analytes, with a 1 min time window. The first quadrupole was operated at 0.7 full width at half maximum (FWHM) resolution, and the Orbitrap spectrometer was operated at 17 500 FWHM resolution. AGC target was set to 1 000 000, with maximum AGC injection time of 50 ms. Collision energy values were optimized to 35 volts. The method details are documented in Supplementary Material 2.

Quality assurance/quality control (QA/QC) of the analysis included blank samples to ensure that the target compound is not introduced by sample handling, replicates (every tenth sample was duplicated), and fortified samples (every tenth sample from the control group was spiked with the target compound at the concentration 100 $ng \cdot g^{-1}$ to validate the method). Matrix-matched standards (control fish extracted without internal standard and spiked with both target compound and internal standard) were used for each tissue to correct response factor of the calibration curve prepared in methanol.

2.6. VALIDATION OF METHOD FOR QUANTIFICATION OF CLOTRIMAZOLE IN FISH TISSUE

The analytical method was validated with respect to linearity, repeatability, limit of quantification (LOQ), and recovery. The method was found to be linear over the range of 1 to 500 ng · g^{-1} ($R^2 = 0.994$). Method repeatability was tested for ten replicates; relative standard deviation (RSD) for each concentration in replicates was 8%. Recovery of CLO from fish tissue was evaluated by spiking "clean" fish samples with the target compound before the extraction procedure. Average recovery of CLO was 98% with an RSD of ten replicates of 9%. The LOQ of CLO was 6.2, 0.97, 9.8, and 2.6 ng · g^{-1} in plasma, muscle, liver, and kidney, respectively.

2.7. MICROSOMAL FRACTION AND PROTEIN CONTENT ESTIMATION

The microsomal fraction was prepared from liver homogenate by differential centrifugation (Burkina et al., 2013). As a final step, the samples were diluted in glycerol buffer (0.1 mM EDTA, 20% glycerol, 50 mM Tris, and 10 mM potassium phosphate, pH 7.4) to a volume of 1 ml and homogenized (UltraTurrax; Ika, Germany). All steps were carried out on ice. Microsomal fractions were immediately frozen and stored at -80 °C until analysis. Levels of microsomal protein were estimated spectrophotometrically at 562 nm using bovine serum albumin as a standard (Smith et al., 1985). The samples were diluted to a protein content of 4 mg · ml^{-1}.

2.8. DETERMINATION OF CYP450 CONTENT AND MICROSOMAL ENZYME ACTIVITY

CYP450 content was determined by the spectrophotometric method using a molar extinction coefficient of 91 $mM^{-1} \cdot cm^{-1}$ (Omura and Sato, 1964a, b).

CYP450 activity was estimated using 7-ethoxyresorufin (ER) and 7-benzyloxy-4-trifluoromethylcoumarin (BFC) as substrates.

The catalytic activity of EROD was determined as the transformation rate of ER to resorufin (Kennedy and Jones, 1994) with some modifications. The reaction was performed in a black 96-well plate. Reaction mixture consisted of potassium buffer (50 mM, pH 7.8), ER (8 μM), and microsomal protein (0.2 mg). The reaction was initiated with addition of NADPH (1.4 mM) to total volume of 260 μl in each well.

Activity of BFCOD was estimated as the transformation rate of 7-benzyloxy-4-trifluoromethylcoumarin (BFC) to 7-hydroxy-4-trifluoromethylcoumarin (HFC) (Renwick et al., 2001) with some modification. Incubations were conducted in a 250 μl volume in 96-well plate. For BFCOD reaction, 0.2 mg of microsomal protein mixture in potassium buffer (50 mM, pH 7.8) and BFC (25 μM) were placed in each well. The reaction was initiated with addition of NADPH (0.6 mM).

Fluorescence detector (Infinite M200, Photometer TECAN, Mannedorf, Switzerland) was used for detection of resorufin (excitation/emission 544/590 nm) and HFC (excitation/emission 410/538 nm).

Enzyme activity was expressed as pmol of resorufin or HFC formed per min per mg of microsomal protein (limit of detection for resorufin was 2 pmol and 1 pmol for HFC).

2.9. STATISTICAL ANALYSIS

All statistical analyses were conducted using STATISTICA (v. 10.0 for Windows, StatSoft). One-way analysis of variance (ANOVA) was employed to determine whether there were significant differences in measured variables among experimental groups after fulfilling criteria of

normality and variance homoscedasticity tests. For comparison of control and solvent control, the t-test was used. Differences between treatment and control fish in biometric features were determined using the Dunnett *post hoc* test. For assessment of biomarker responses of exposed groups, the control and solvent control groups were combined into a single control group for 21 and 42 days exposure. A square root transformation was applied to normalize data (EROD, BFCOD, and CYP450 content). Differences between treatments and control were determined using Tukey's *post hoc* test. Statistical significance was set at $p < 0.05$. The data are presented as means after back transformation with 95% confidence interval.

3. RESULTS AND DISCUSSION

During the experiment, control and exposed groups showed normal growth and feeding behaviour. No mortalities occurred during the 42 days in control and clotrimazole exposed groups. For all biochemical variables, there were no significant differences between the control and solvent control groups.

3.1. CLOTRIMAZOLE CONCENTRATIONS IN AQUARIUM WATER

Water samples for LC/LC–MS/MS analysis of chemical concentrations were collected on days 1, 20, and 41 of exposure at 0 h and 24 h after renewing the test solution (Table 1).

Control water samples were all below the LOQ (< 0.009 $\mu g \cdot L^{-1}$) for CLO.

Measured concentration of CLO at the environmentally relevant level (0.01 $\mu g\ L^{-1}$) was within ± 30% of the nominal concentration during the experimental period.

At the beginning of the trial, the concentration of CLO in freshly renewed solutions (0 h) in aquaria containing fish dramatically decreased to 25% and 27% of the nominal concentration of 1.0 and 10 $\mu g \cdot L^{-1}$, respectively. After 24 h, measured CLO concentration was 5% and 3% of the nominal concentrations 1.0 and 10 $\mu g \cdot L^{-1}$, respectively. Decrease of CLO concentration over 24 h was less pronounced on day 20 and lowest on day 41. The smaller decrease of CLO concentration observed on day 20 may have been due to saturation of the system (glass walls, plastic tubing, fish, faeces). Less decreased concentration of CLO at 41 days to 69% of the nominal concentration 1.0 $\mu g \cdot L^{-1}$ at exposure time 0 and 77% after 24 h and 31% of the concentration 10 $\mu g \cdot L^{-1}$ at time 0 and 30% after 24 h might be associated with reduction in the biomass of fish owing to sampling on day 20. No significant fluctuation of CLO at the environmental concentration was observed during the experiment, probably due to proximity of environmental concentration to LOQ (uncertainty of the assessment method was 30–40% at LOQ).

In the aquarium system without fish containing 10 $\mu g \cdot L^{-1}$ CLO, no adsorption in freshly prepared solution was observed. Significant decrease of 10 $\mu g \cdot L^{-1}$ CLO concentration to 4.9 $\mu g \cdot L^{-1}$ was seen after 24 h. Adsorption behaviour of CLO was also reported by Peschka et al. (2007).

The significantly different adsorption of CLO in aquaria systems containing fish from that without fish may suggest that, under experimental conditions, CLO may have been partly adsorbed by aquaria surfaces and partly taken up by fish.

3.2. BIOMETRIC INDICES

Body weight and FL of rainbow trout did not significantly differ among treatments ($p > 0.05$) on day 21 (Table 2). Significantly increased ($p < 0.05$) body and liver weight were

observed among the groups, exposed to 1.0 and 10 $\mu g \cdot L^{-1}$ at 42 days. Differences observed in morphological indices between fish in the two treatment groups compared to the SC group at the end of the experiment may indicate that CLO-treated groups showed increased growth. This finding might be connected with a prophylactic effect of CLO which reduces the incidence of fungal infection.

The HSI is associated with liver energy reserves and metabolic activity, while CF is used to assess the general condition of fish. The effect of human pharmaceuticals on HSI and CF has been described in fish species (Li et al., 2010c; Xu and Jing, 2012). No significant differences (p > 0.05) in CF were detected in treatment groups compared to the SC group after 42 days of exposure. In this study, significantly higher HSI was observed in the 1.0 and 10 $\mu g \cdot L^{-1}$ exposure groups. This increase of liver/body ratio might be associated with increased hepatic detoxification activity, showing response to the presence of an exogenous compound in liver. This result is supported by observations of Burkina et al. (manuscript) in an investigation of the effect of CLO on the energy biomarker lactate dehydrogenase. Our data showed that energetic pathways in liver were significantly induced in all exposed groups after 42 days exposure.

3.3 CLOTRIMAZOLE IN FISH TISSUE AND FAECES

According to the OECD, the main criterion for accumulation is partition coefficients (log K_{ow}) value. If log K_{ow} > 3, substances have a tendency to accumulate. The partition coefficient of CLO is 4.1 (OSPAR, 2013). According to United States Environmental Protection Agency, compounds are considered not bio-accumulative when BCF is ≤ 1000, while criteria of Organization for Economic Cooperation and Development OECD (2001) for identification of low level bioconcentration is ≤ 500. Bioconcentration factor calculated by quantitative structure relationships (QSARs) of CLO for fish is 610 (OSPAR, 2013). No experimental results of bio-accumulation of CLO in fish tissue are available.

We detected no CLO in any tissue from control or solvent control groups. More than 89% of the analyzed samples in CLO-exposed groups were positive at both sampling times. The concentrations of CLO in fish tissue and blood plasma for the exposure periods are presented in Table 3.

The differing concentrations of CLO in tissues can be attributed to the lipid content as well as to fish physiology. In the present study, the highest concentrations were found in kidney, followed by liver and muscle. CLO was not detectable in blood plasma (< 6.2 $ng \cdot g^{-1}$) in 0.01 and 1.0 $\mu g \cdot L^{-1}$ groups and was detected in 10 $\mu g \cdot L^{-1}$ groups at both sampling times. Exposure for 21 days to the environmentally relevant concentration of CLO was associated with CLO concentrations of 2.6, 51, and 106 $ng \cdot g^{-1}$ in muscle, liver, and kidney, respectively, and at 42 days exposure with 5.5, 251, and 184 $ng \cdot g^{-1}$ in muscle, liver, and kidney, respectively (wet weight).

On day 42, CLO concentrations were higher in kidney than in liver. This distribution can be explained by CLO's rapid liver-detoxification before being excreted. The observed level of activity of CYP3A, one of the most important isoforms of the CYP450 family responsible for drug metabolism, supports these results.

The actual concentrations of CLO in water on day 1 were used to calculate the BCFs: 0.01, 0.26, and 2.68 $\mu g \cdot L^{-1}$ corresponded to the test concentrations of 0.01, 1.0, and 10 $\mu g \cdot L^{-1}$, respectively.

Bioconcentration factor varied with CLO concentration. The BCFs calculated for muscle were 260 and 550 for 0.01 $\mu g \cdot L^{-1}$, 58 and 108 for 1.0 $\mu g \cdot L^{-1}$, and 184 and 415 for 10 $\mu g \cdot L^{-1}$ after 21 and 42 days exposure, respectively. In blood plasma, CLO concentration in 0.01 $\mu g \cdot L^{-1}$

and 1.0 $\mu g \cdot L^{-1}$ groups was below limits of quantification, which precluded calculation of BCF values. For the 10 $\mu g \cdot L^{-1}$ group, BCFs were 132 and 169 after 21 and 42 days. According to our results, the environmentally relevant concentration of CLO may present a moderate bioconcentration potential for aquatic organisms.

Faeces were collected from the experimental aquaria on day 21 (Table 4), as this means of excretion may be important (Escher et al., 2011). The level of CLO in faeces ranged from 3 to 7% of the total amount in the 200 L aquaria (2, 200, and 2000 µg of CLO). The level of CLO excretion via faeces cannot be confirmed, since sorption of CLO from water might contribute to faecal CLO.

3.4. DEPURATION OF CLO FROM FISH TISSUE

The depuration rate of CLO was measured in tissues of fish exposed to 1.0 $\mu g \cdot L^{-1}$ of CLO (Table 5).

The detection of measurable levels of the test compound in the tissues of fish resided in clean aquaria for 13 days indicates that CLO may not get rapidly cleared from the tissues. In the present study the depuration of CLO differed among tissues. Kidneys had the highest CLO concentration during whole depuration phase. The estimated half-life of CLO was 72 h in liver, 159 h in muscle, and 682 h in kidney. It was not possible to calculate half-life of CLO in blood plasma due to concentrations below the LOQ (6.2 $ng \cdot g^{-1}$). Clotrimazole had a longer elimination half-life in all analyzed tissues than that reported for human blood (3.5-5.5 h with 1.5 g oral dose (Reynolds and Prasad, 1982). Similar results were obtained for verapamil (Steinbach et al., 2013), with half-life in exposed carp higher than in humans. The short half-life of CLO in liver, compare to other tissues may suggest that liver actively eliminates CLO. Relatively high half-life in fish kidney, almost 28.4 days, implies greater potential for adverse effects in non-target species. This finding is significant in view of our recent study indicating that sub-lethal levels of CLO exposure in fish results in structural changes in the kidney (Burkina et al., manuscript).

Our results reveal much slower elimination of CLO in fish after waterborne exposure, compared to human oral exposure. According to half-life of CLO in the analyzed tissues, the elimination rate in fish organs follows the order liver < muscle < kidney.

3.5. TOTAL CYTOCHROME P450 CONTENT, EROD AND BFCOD ACTIVITY

The CYP450 content was not affected by treatment (Figure 1). The activity of EROD was significantly higher in 0.01 and 10 $\mu g \cdot L^{-1}$ groups on day 42 compared to the corresponding control (Figure 2). EROD activity in control fish on day 42 was significantly lower than on day 21 ($p < 0.05$), indicating a decrease in EROD activity in control rather than an increase in exposed fish. Variations in EROD activity in exposed fish (3.6 and 2.4-fold differences in 0.01 and 10 $\mu g \cdot L^{-1}$ groups compared to control group at day 42, respectively) were statistically significant, but not concentration-dependent. This might indicate that EROD activity is slightly increased by the presence of CLO in water, but is mainly related to fish age and weight differences between 21 and 42 days.

Numerous *in vitro* and *in vivo* studies of CLO effect on fish hepatic CYP450 have demonstrated inhibition of CYP450 activity. The antifungals clotrimazole and ketoconazole have been shown to act as potent inhibitors of CYP1A activity in fish liver microsomes (Burkina et al., 2013; Hasselberg et al., 2008; Hegelund et al., 2004; Wassmur et al., 2010). In addition, they can induce CYP1A expression in rainbow trout hepatocytes (Navas et al., 2004) via aryl hydrocarbon receptor activation.

The studied inhibitory mechanism indicates that ketoconazole is a potent non-competitive inhibitor of CYP1A activity in juvenile Atlantic cod (Hasselberg et al., 2005), while CLO is non-competitive in hepatic microsomes of juvenile rainbow trout (Burkina et al., 2013). Clotrimazole has also been reported to have endocrine disrupting potency via interactions with steroidogenic enzymes CYP17 and CYP19 (Baudiffier et al., 2013; Baudiffier et al., 2012; Hinfray et al., 2011, Monod et al., 1993). The difference in our results from those previously published may be due to the CLO concentrations used and/or different *in vitro* and *in vivo* models.

BFCOD activity in the exposed fish at day 21 differed from control only for the highest CLO concentration (Fig. 3). Longer duration of exposure was associated with the inhibition in the 0.01 $\mu g \cdot L^{-1}$ concentration group and induction by 10 $\mu g \cdot L^{-1}$ of CLO. This biphasic variation in CYP450 activity might be due to differential release of CLO and/or its metabolites from fish. The metabolite profile of CLO (Brugnara et al., 1995; Fazlul, 2007; Rittenhouse et al., 1997; Shah et al., 2001) includes at least 3 major metabolites (2-chlorophenyl-4-hydroxyphenyl-phenylmethane, 2-chlorophenyl-bis-phenyl-methane and 2-chiorophenyl-bis-phenyl-methanol). The nature of these variations and its relevance to fish physiology require further investigations.

This study has several limitations. Results of water analysis suggested adsorption of CLO, which makes it difficult to control its concentration in aquaria. In addition, the effect of pollutants on fish might vary due to the complex mixture of chemicals present in aquatic ecosystems. Our results therefore need to be interpreted with caution. The long-term ecological and physiological consequences of CLO in the aquatic environment require further investigation.

In conclusion, we show that clotrimazole is a highly adsorbing compound, accumulated and detoxified by rainbow trout in sub-chronic conditions.

Clotrimazole was detected in all fish samples after all exposure times using 3 concentrations of CLO. Rainbow trout were found to take up CLO from water and maintain a consistent concentration in muscle, liver, and kidney after exposure to environmentally relevant concentrations. The half life indicated that CLO residence times were longer in tissues compared to blood plasma. The half-life was long (the longest period in kidney) compared to that in humans, indicating there might be greater potential for CLO to adversely effect this organ.

The BCF levels in muscle tissue suggest moderate bioaccumulation of CLO at environmentally relevant concentrations. Clotrimazole did not alter the total CYP450 concentration, but affected BFCOD activity in a biphasic manner depending on concentration with longer exposure. EROD activity was slightly increased after 42 days of exposure, possibly due to effects unrelated to induction of cytochrome P450. According to our results and other reports, CLO can interfere with CYP450-mediated reactions, which are associated with drug detoxification. However, observed effects could be influenced by a number of PPCPs present in the aquatic environment.

Acknowledgement

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Table 1. *Mean clotrimazole concentrations (μg · L^{-1}) and percent of nominal concentration (%) in aquarium water. n = 12 and 2 for aquaria water with fish and without fish, respectively.*

Clotrimazole (target concentration) μg · L^{-1}	Water analysis μg · L^{-1}						Without fish	
	day 1		day 20		day 41		day 20	
	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h
0.010	0.010	0.010	0.012	0.010	0.013	0.010	–	–
	100%	100%	120%	100%	130%	100%	–	–
1.0	0.26	0.05	0.38	0.17	0.69	0.31	–	–
	25%	5%	37%	16%	69%	31%	–	–
10	2.8	0.35	2.4	0.80	7.7	3.0	10	4.9
	27%	3%	24%	8%	77%	30%	101%	49%

Detected limit was < 0.009 μg · L^{-1}.

Table 2. *Effect of clotrimazole on biometric characteristics of rainbow trout. Significant differences compared with solvent control (SC), P < 0.05 (*, ANOVA). Data are means ± S.D., n = 8.*

Indices	Unit	Exposure (days)	Test groups				
			C	SC	0.01 µg · L⁻¹	1.0 µg · L⁻¹	10 µg · L⁻¹
FL	cm	21	25 ± 1.1	24 ± 1.3	23 ± 0.68	24 ± 1.8	25 ± 2.3
		42	26 ± 0.63	26 ± 0.85	26 ± 1.0	27 ± 0.88*	28 ± 1.1*
BW	g	21	218 ± 31	183 ± 25	175 ± 17	188 ± 36	203 ± 45
		42	225 ± 18	230 ± 32	218 ± 26	256 ± 25*	257 ± 18*
LW	g	21	2.0 ± 0.43	1.6 ± 0.68	1.6 ± 0.29	1.7 ± 0.35	1.8 ± 0.35
		42	1.7 ± 0.20	1.7 ± 0.35	1.7 ± 0.19	2.2 ± 0.45*	2.2 ± 0.20*
CF	g/cm^3	21	1.3 ± 0.13	1.3 ± 0.17	1.4 ± 0.13	1.4 ± 0.27	1.4 ± 0.21
		42	1.3 ± 0.09	1.3 ± 0.14	1.2 ± 0.05	1.2 ± 0.05	1.2 ± 0.10
HSI	%	21	0.92 ±0.11	0.87 ± 0.34	0.91 ± 0.15	0.92 ± 0.13	0.92 ± 0.16
		42	0.78 ±0.08	0.75 ± 0.12	0.78 ± 0.05	0.87 ± 0.11*	0.87 ± 0.06*

FL, fork length; BW, body weight; LW, liver weight; CF, condition factor; HSI, hepatosomatic index.

Table 3. *Concentration of clotrimazole ($ng \cdot g^{-1}$) and bioconcentration factor (BCF) in rainbow trout. Groups exposed to clotrimazole 0.01, 1, and 10 $\mu g \cdot L^{-1}$. Data are means ± S.D., n = 8.*

Groups	Exposure (days)	Tissue					
		Plasma	BCF	Muscle	BCF	Liver	Kidney
0.01 µg · L⁻¹	21	≤ LOQ		2.6 ± 0.56(8/8)	260	51 ± 26 (8/8)	16 ± 12 (8/8)
	42	≤ LOQ		5.5 ± 2.6 (8/8)	550	251 ±130(8/8)	180 ± 70 (8/8)
1.0 µg · L⁻¹	21	≤ LOQ		15 ± 8.9 (7/8)	58	1140 ± 700(8/8)	110 ± 40 (8/8)
	42	≤ LOQ		28 ± 4.3 (8/8)	108	1300 ± 350(8/8)	240 ± 120 (8/8)
10 µg · L⁻¹	21	350 ± 40(8/8)	132	490 ± 200 (8/8)	184	2960 ± 1780(8/8)	1260 ± 310 (8/8)
	42	450 ± 50(8/8)	169	1110 ± 260(8/8)	415	3710 ± 1470(8/8)	4280 ± 1980 (8/8)

LOQ – limit of quantification (plasma = 6.2 $ng \cdot g^{-1}$; muscle = 0.97 $ng \cdot g^{-1}$; liver = 9.8 $ng \cdot g^{-1}$, kidney = 2.6 $ng \cdot g^{-1}$). Values in brackets are number of positive samples/number of analysed samples. BCF was calculated as measured clotrimazole concentration in fish tissue/measured exposure concentration in water at start of experiment (second column in table1, time 0).

Table 4. *Concentration of clotrimazole in faeces collected after 24 h of exposure in experimental aquaria (n = 2).*

Clotrimazole (nominal concentration) (μg · L^{-1})	Total amount of CLO in 200 L (μg/aquaria)	Faeces analysis (μg · gww^{-1}), RSD (%)	Amount of CLO in faeces collected after 24 h (μg/aquaria)	Faeces/water [CLO in faeces/ nominal CLO aq.] × 100 (%)
0.01	2.0	0.037 (15)	0.14	7
1.0	200	2.3 (29)	6.3	3
10	2000	29 (9)	83	4

LOQ for faeces = 5.6 ng · g^{-1}.

Table 5. *Depuration and half-life of clotrimazole in fish tissue. Group exposed to CLO 1 μg · L^{-1} 7 (n = 6) and 13 (n = 8) days after cessation of exposure. Data are means ± S.D. Units ng · g^{-1}.*

Groups	Post exposure period (days)	Tissue			
		Plasma	Muscle	Liver	Kidney
1.0 μg · L^{-1}	7	≤ LOQ	6.9 ± 4.7 (3/6)	125 ± 109 (6/6)	1010 ± 820(6/6)
	13	≤ LOQ	30 (1/8)	57 ± 40 (8/8)	920 ± 380 (8/8)
Half-life (h)		ND	159	72	682

LOQ – limit of quantification (plasma = 6.2 ng · g^{-1}; muscle = 0.97 ng · g^{-1}; liver = 9.8 ng · g^{-1}, kidney = 2.6 ng · g^{-1}). Values in brackets are number of positive samples/number of analyzed samples. Half-life (mean) calculated as t_{50} = 0.693/k. ND – not detectable.

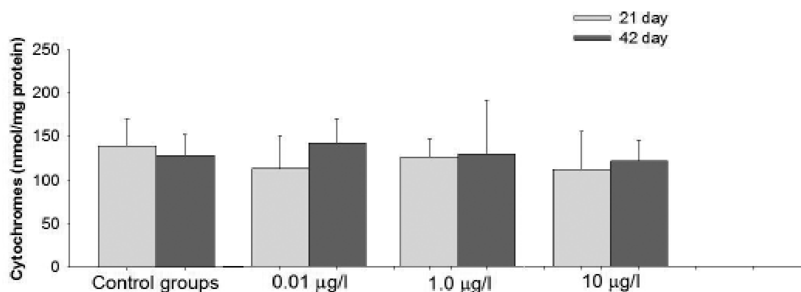

Figure 1. *Effect of CLO on CYP P450 content in the microsomes in rainbow trout tissue. Significant differences compared with solvent control (SC), p < 0.05 (ANOVA). Data are back-transformed means with 95% confidence interval, n = 8.*

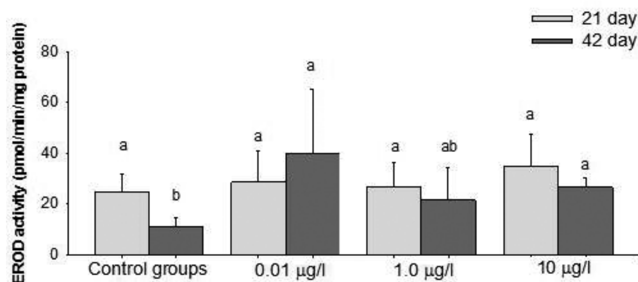

Figure 2. *Effect of CLO on hepatic ethoxyresorufin-O-deethylase (EROD) activity in rainbow trout tissue. Significant differences compared with solvent control (SC), p < 0.05 (*, ANOVA). Data are back-transformed means with 95% confidence interval, n = 8.*

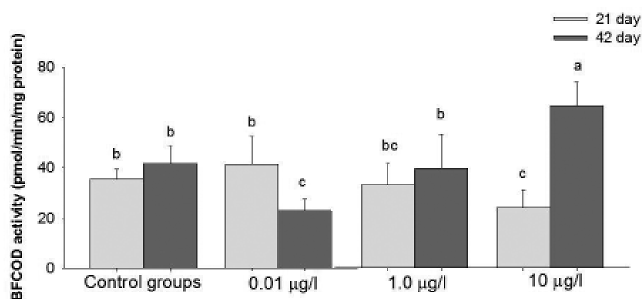

Figure 3. *Effect of CLO on hepatic 7-benzyloxy-4-trifluoromethylcoumarin O-debenzylase (BFCOD) activity in rainbow trout tissue. Significant differences compared with solvent control (SC), p < 0.05 (ANOVA). Data are back-transformed means with 95% confidence interval, n = 8.*

Supplement 1. *LC-LC gradient for the elution of target compounds (water samples, in-line-SPE).*

Analytical pump				***High-flow pump for in-line extraction***				
Time, min	**A, %**	**C, %**	**Flow, µl · min^{-1}**	**Time, min**	**A, %**	**B, %**	**C, %**	**Flow, µl · min^{-1}**
0	90	10	300	0	100	0	0	1200
1.05	90	10	300	1.05	100	0	0	1200
6.00	60	40	350	1.07	0	100	0	50
9.00	20	80	400	11.10	0	100	0	50
10.00	0	100	400	12.00	0	50	50	2000
13.00	0	100	400	12.01	100	0	0	1000
13.01	90	10	300	16.00	100	0	0	1000
16.00	90	10	300					

A – water (with 0.1% FA); B – methanol (with 0.1% FA); C – ACN (with 0.1% FA). 1 min – loading of the sample; 5.6 min – elution of target compounds.

Supplement 2. *LC gradient for the elution of target compounds (fish tissue and faeces extracts).*

Time, min	A, %	B, %	Flow, $\mu l \cdot min^{-1}$
0.00	95	5	300
1.00	95	5	300
3.00	75	25	300
5.00	0	100	350
7.00	0	100	350
7.01	95	5	300
10.00	95	5	300

A – water (with 0.1% FA); B – methanol (with 0.1% FA).

CLOTRIMAZOLE IN RAINBOW TROUT II. BIOLOGICAL AND STRUCTURAL EFFECTS AFTER LONG-TERM EXPOSURE

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Abstract

The aim of this study was to characterize enzyme, haematological, and structural changes in juvenile rainbow trout exposed to 0.01, 1.0, and 10 $\mu g \cdot L^{-1}$ of the fungicide clotrimazole (CLO) for 42 days. At days 21 and 42, glutathione-S-transferase, catalase, glutathione reductase, glutathione peroxidase, thiobarbituric acid reactive substances, and lactate dehydrogenase were measured in liver, gill, and brain. Histology of gill, liver, kidney, and gonad along with haematological indices were assessed. Glutathione-S-transferase showed high levels in liver and gill possibly associated with detoxification of CLO. Antioxidant defence enzymes, which responded to CLO exposure, changed the oxidative stress status of tissue cells, but no differences were observed in lipids. Induction of hepatic LDH activity suggested that oxidative stress has a high cost to the cell when energetic pathways are activated. Differences in haematological variables and differential leukocyte counts possibly may reflect a stress response of the immune system. Histopathology was most pronounced in kidney and testis in the group exposed to 10 $\mu g \cdot L^{-1}$. Structural changes in the kidney included tubulonephrosis and hyaline droplet degeneration in the tubular epithelial cells. The relative proportions of germ cells in testis changed; numbers of spermatozoa were reduced, while spermatogonia and spermatocytes were increased compared to the solvent control group.

Clotrimazole was associated with histological effects at the highest tested concentration and intracellular events in fish organs even at an environmentally relevant concentration.The presence of CLO in the environment is of concern with respect to its impact on health status of fish.

Key words: *Clotrimazole, biomarkers, oxidative stress, histopathology, haematology, rainbow trout*

Highlights

- Juvenile rainbow trout were sub-chronically exposed to clotrimazole
- Exposure to clotrimazole changed a number of enzymatic activities
- Clotrimazole could induce adverse impacts on fish blood parameters
- Histological changes were most obvious in kidney and testis

1. INTRODUCTION

Residues and metabolites of pharmaceuticals that reach aquatic systems can exert adverse effects on non-target organisms (Baumann et al., 2013; Kasprzyk-Hordern et al., 2008; Brausch and Rand, 2011; Verlicchi et al., 2012).Clotrimazole (CLO), 1-[(2-Chlorophenyl) diphenylmethyl]-1H-imidazole, a specific inhibitor of Ca^{2+}-activated K^+ channels (Wu et al., 1999), is used for topical treatment of fungal, dermatophyte, and yeast infections.The antifungal action is through the inhibition of cytochrome P450-dependent 14α-demethylase, which is critical to ergosterol biosynthesis (Rupp et al., 2005).

Medicinal CLO reaches waste water treatment plants through domestic sewage. Peschka et al. (2007) measured the level of CLO in three German rivers/streams and in the river Tyne, UK, where CLO was present in nanograms per litre range. Since CLO is a lipophilic compound, over 80% of the molecules will adsorb to activated sludge (Kahle et al., 2008) and is easily degraded, resulting in an increase of CLO concentration in waste water effluent and eventually in natural surface waters. Roberts and Thomas (2006) reported surface water CLO concentration at 6 locations in the River Tyne, UK ranging from 6 to 34 $ng \cdot L^{-1}$ with a median of 21 $ng \cdot L^{-1}$. The calculated environmental input of CLO (2 g per day) was not sufficient to maintain the mean water concentration, suggesting an additional unknown source of CLO in the river.

Clotrimazole is toxic to aquatic organisms (OSPAR, 2013). Studies on fish as well as on human cell lines have shown azole fungicides to behave as endocrine disruptors. Observations include inhibition of human placental aromatase activity due to ketoconazole (Ayub and Levell, 1987) and inhibition of brain and ovarian aromatase activity in fathead minnows *Pimephales promelas* exposed to fadrozole (Ankley et al., 2002; Villeneuve et al., 2006) or prochloraz (Ankley et al., 2005) and in rainbow trout *Oncorhynchus mykiss* exposed to clotrimazole (Monod et al., 1993; Hinfray et al., 2006). Clotrimazole has shown an impact on steroidogenesis in adult zebrafish *Danio rerio* (Hinfray et al., 2011; Baudiffier et al., 2012). Prochloraz at 0.3-4.8 $mg \cdot L^{-1}$ has been shown to be associated with effects of cholinesterase, lactate dehydrogenase, and glutathione S-transferase activity in several life stages of zebrafish (Domingues et al., 2013). Oxidative stress has gained considerable interest in the field of ecotoxicology. Many compounds may induce their toxic effect through the formation of reactive oxygen species (ROS) thus causing oxidative stress (Li et al., 2010a, 2011a). The antioxidant protection system, the function of which is to remove ROS, thus protecting organisms from oxidative stress, is considered a sensitive target for pharmaceuticals, not only in mammals but also in aquatic organisms (Ashtiani et al., 2011).

Due to its structural and physiological characteristics, gill tissue is highly sensitive to the pollutants in the aquatic system. The brain, an organ in which homeostasis must be strictly maintained, has high affinity to attacks of free radicals induced by pharmaceuticals in the aquatic environment and a weak antioxidant defence system.

Blood is involved in elimination of drugs from the body and gives information about the general state of health. Thus, haematological and blood plasma biochemical variables are useful for indicating physiological status and predicting possible chemical toxicity in humans (Flanagan and Dunk, 2008) and aquatic organisms (Li et al., 2011b).

Structural changes in fish gill, liver, kidney, and gonad tissue can be biomarkers of environmental pollution (Triebskorn et al., 2004). Ovaries of female fathead minnows *Pimephales promelas* exposed to fadrozole showed a decrease in mature oocytes and an increase in preovulatory atretic follicles (Ankley et al., 2002), while CLO-exposed testis of male zebrafish showed increasing numbers of type A spermatogonia (Baudiffier et al., 2013).

In vitro and short-term effects of CLO are well documented (Baudiffier et al., 2013; Burkina et al., 2013; Wassmur et al., 2013); however, there is little information on the effects of

chronic exposure to CLO in fish. The goal of the study was to assess the effects of chronic exposure to CLO on the fish antioxidant defence system and haematological at 21 and 42 days and histological variables of kidney, liver, gill, and gonads at 42 days.

2. MATERIALS AND METHODS

2.1. CHEMICALS

Clotrimazole (CAS Number 23593-75-1, $C_{22}H_{17}ClN_2$) was purchased from Voigt Global Distribution Inc (USA). All other chemicals used were obtained from Sigma Aldrich Europe.

2.2. FISH

Juvenile rainbow trout (n = 200), weighing 215.31 ± 4.28 g (mean ± SE), were obtained from a local commercial hatchery. Fish were transferred to aquaria containing 250 L fresh water: 15.6 ± 0.9 °C, pH 7.2 ± 0.15, dissolved oxygen 7.5–8.5 $mg \cdot L^{-1}$, 12 : 12 L : D photoperiod. These conditions were maintained during 14 days acclimation and the experiment periods. The fish were fed commercial fish food (BioMar, Denmark) at 1% total body weight/day. Experimental animals were handled in accordance with national and institutional guidelines for the protection of human subjects and animal welfare.

2.3. EXPOSURE TO CLOTRIMAZOLE AND WATER CHEMICAL ANALYSES

The experimental conditions are described elsewhere (Burkina et al., manuscript). In brief, clotrimazole was dissolved in 2000 $\mu g \cdot ml^{-1}$ DMSO and 3 stock solutions were prepared through successive dilutions (2, 200 and 2000 $\mu g \cdot ml^{-1}$). One millilitre of CLO solutions were added to aquaria containing 200 L water. Fish were exposed to concentrations of CLO of 0.01 (environmentally relevant concentration), 1.0 and 10 $\mu g \cdot L^{-1}$ for 21 and 42 days. A control group of fish was exposed to clean fresh water and a solvent control group (SC) was exposed to DMSO at a level equal to that of the highest CLO concentration (v/v, 0.01%). Fish were randomly separated into groups of 20 and placed in ten aquaria containing 200 L water or test solution in a semi-static system (5 treatments in duplicate).

Before experimentation, the stock solution with the highest CLO concentration was tested for stability under the same conditions as the chronic test. Stock solution was stable for one week; thus stock solutions were renewed weekly. Solutions used to renew the test media were stored at room temperature and protected from light.

Test solutions were renewed each day to maintain water quality and the intended CLO concentration. Water samples were collected from each exposure aquarium and directly analyzed on days 1, 20, and 41 immediately after (0 h) and after (24 h) solution renewal. To assess agreement between nominal and actual compound concentrations in the aquaria, water samples were analyzed by SPE liquid chromatography with tandem mass spectrometry (LC/LC-MS/MS).The sorption effect for the two highest concentrations of CLO was observed. The average percentage of nominal concentration of 1.0 and 10 $\mu g \cdot L^{-1}$ was 26% (0 h) and 4% (24 h) on day 1; 31% (0 h) and 12% (24 h) on day 20; 73% (0 h) and 31% (24 h) on day 41 of the experiment. Relative standard deviation of replicates ranged from 0 to 30%. No significant fluctuation of CLO at environmental concentration was evident during whole experiment probably due to proximity of environmental concentration to LOQ (uncertainty of the used method, 30-40%).

Details of LC/LC/MS/MS analytical methods and discussion of water chemical analyses have been described elsewhere (Burkina et al., manuscript).

2.4. TISSUE SAMPLES

The fish were not fed for 24 h prior to sampling to avoid prandial effects. Blood samples were collected from eight fish from each group at 21 and 42 days post-exposure. Blood samples were centrifuged (837 x g, 10 min, 4 °C) to obtain plasma, which was stored at -80 °C for determination of biochemical catalase and glutathione reductase activity. A small volume of blood was immediately used for determination of haematological variables and differential leukocyte counts(Svobodova et al., 1991). Fish were killed by severing the spinal cord, and liver (approx. 0.5 g), whole brain, and both gills were removed and stored at -80°C for biochemical analysis.

At 42 days post-exposure, samples of kidney, liver, gill, and gonads of fish in the SC and 10 $\mu g \cdot L^{-1}$ groups were fixed in 10% formalin and sent to the Centre for Fish and Wildlife Health (FIWI), Switzerland, for histological examination.

2.5. HAEMATOLOGICAL VARIABLES

Transformation solution (0.1 g potassium ferricyanide, 0.025 g potassium cyanide, 0.07 g potassium dihydrogenphosphate, up to 0.5 L distilled water) was used for determination of haemoglobin concentration.

The following haematological indices were measured: haemoglobin concentration (Hb), red blood cells count (RBC), haematocrit (PCV), mean corpuscular erythrocyte volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), leukocyte count (Leuko), and granulocyte count. The level of haemoglobin was determined using a spectrophotometer (Helios Epsilon, UNICAM). The MCV and MCHC values were obtained from blood count analysis as conventional biomarkers. Procedures were based on unified methods for haematological examination of fish (Svobodova et al., 1991).

2.6. BIOCHEMICAL ASSAYS

2.6.1. Post-mitochondrial supernatant

The post-mitochondrial supernatant (PMS) was obtained as described by Howcroft et al. (2009). Samples were homogenized in 1.5 ml K-phosphate 0.1 M buffer, pH 7.4 using an ultrasonic homogenizer (Ystral GmbH D-7801; Dottingen, Germany). From the homogenate, 100 µl were separated for lipid peroxidation (LPO) determination. The remaining tissue homogenate (1400 µl) was centrifuged at 10 000 x g for 20 min at 4 °C to isolate the PMS.

2.6.2. Glutathione S-transferase

Glutathione S-transferase (GST) activity was determined using 1-chloro-2,4-dinitrobenzene as substrate according to the method of Habig et al. (1974) adapted for the microplate reader by Frasco and Guilhermino (2002). The incubation mixture (0.3 ml) contained 100 µl PMS (approx. 0.04 mg of protein/ml) and reaction solution. The reaction solution was a mixture of 2.55 ml 1-chloro-2,4-dinitrobenzene (CDNB), 10 mM, 15.30 ml reduced glutathione (GSH), 10 mM, and 84.5 ml K-phosphate buffer 0.1 M, pH 6.5. The CDNB conjugate was measured as increase in absorbance at 340 nm. Calculations were made using a molar extinction coefficient of 9.6 $mM^{-1} \cdot cm^{-1}$.

2.6.3. Catalase

Catalase (CAT) activity was determined by the method of Clairborne (1985), by measuring the decrease in hydrogen peroxide in a 96-well flat-bottom UV-transparent microtitre plate. We mixed 15 µl of PMS with 100 µl H_2O_2 0.03 M and 185 µl K-phosphate 0.05 M (pH 7.0). The decomposition of the substrate (H_2O_2) was recorded at 240 nm. Calculations were made using a molar extinction coefficient of 40 $M^{-1}·cm^{-1}$.

2.6.4. Glutathione reductase

Glutathione reductase(GR) activity was determined by the method of Cribb et al. (1989) with some modifications using 50 µl of PMS (approx. 0.2 $mg·ml^{-1}$) and 150 µl of reaction solution. The reaction solution was a mixture of NADPH (0.4 mM), L-glutathione oxidized (GSSG) (2 mM), and diethylenetriaminepentaacetic acid (DTPA) (1 mM) in 50 ml K-phosphate buffer 0.05 M (pH 7.0). The enzyme activity was quantified by measuring the disappearance of NADPH at 340 nm. Calculations were made using a molar extinction coefficient of 6220 $M^{-1}·cm^{-1}$.

2.6.5. Glutathione peroxidase

Glutathione peridoxase (GPx) activity was measured by the method of Mohandas et al. (1984). The incubation mixture (0.3 ml) contained 15 µl PMS (approx. 0.2 $mg·ml^{-1}$), 252 µl K-phosphate buffer 0.05 M (pH 7.0), in EDTA 1 mM solution, sodium azide 1 mM and glutathione reductase from baker›s yeast (7.5 ml from stock containing 1 $U·ml^{-1}$). 30 µl reduced GSH 4 mM and NADPH 0.8 mM was added as substrate to the solution. The reaction was started by the addition of 0.5 mM of H_2O_2. Oxidation of NADPH was recorded at 340 nm. Calculations were made using a molar extinction coefficient of 6220 $M^{-1}·cm^{-1}$.

2.6.6. Lipid peroxidation

Oxidative damage was assessed by determining the level of lipid peroxidation (LPO) following the methodology of Ohkawa (1979). Briefly, 100 µl homogenate was mixed with 333 µl of cold trichloroacetic acid (12%), 200 µl of 60 mM Tris-HCl with 0.1 mM DTPA, and 333 µl of 2-thiobarbituric acid (0.73%). Tubes were held 1 h at 100 ºC. The absorbance of the supernatant was determined at 535 nm after centrifugation at 3000 x g for 5 min at 25 ºC. Calculations were made using a molar extinction coefficient: ε = 156 000 $M^{-1}·cm^{-1}$.

2.6.7. Lactate dehydrogenase

Lactate deydrogenase (LDH) activity was determined by the method by Vassault (1983) adapted for microplate, as described in Diamantino et al. (2001). Incubations used 15 µl PMS (approx. 0.02 $mg·ml^{-1}$), 35 µl Tris-NaCl buffer (0.1 M, pH 7.2), 250 µl Tris-NaCl-NADH (0.24 mM), and 40 µl Tris-NaCl-pyruvate (10 mM). Calculations were made using a molar extinction coefficient of 6,300 $M^{-1}·cm^{-1}$.

All assays were performed spectrophotometrically in quadruplicate using a 96-well microplate reader (Labsystem Multiscann EX). Samples were held on ice and measurements made at 25 ºC. The variation in absorbance at each reaction well was linear over time ($R^2 > 0.8$). Enzyme activity was expressed as units of substrate hydrolyzed per minute per mg protein. For LPO the oxidative damage was expressed as the mmol of thiobarbituric acid-reactive substances (TBARS) per wet weight of tissue. The protein concentration in

PMS was calculated by the Bradford method (1976), using a wavelength of 595 nm and γ-globulin as standard.

2.7. HISTOLOGY

Fixed samples of kidney, liver, gill, and gonads of SC fish and groups treated with 10 $\mu g \cdot L^{-1}$ of CLO were paraffin-embedded and cut into 3 μm sections for histology. Sections were stained with haematoxylin-eosin (H&E) and examined by light microscopy. Pathological changes were graded as 0 (none), 1 (minimal), 2 (mild), 3 (mild to moderate), 4 (moderate), 5 (moderate to severe) or 6 (severe).

2.8. STATISTICAL ANALYSIS

Statistical analyses were conducted using STATISTICA v. 10.0 for Windows (StatSoft). Data are presented as mean ± standard error (SE). Data was checked for normality and homoscedasticity of variance. If those conditions were satisfied, one-way analysis of variance (ANOVA) was used to assess effects of CLO concentrations on each variable.

Differences between treatments and control were determined using the Dunnett post hoc test. When data were not normally distributed and homoscedastic, the non-parametric method (Kruskal-Wallis test) of data analysis was used.

The t-test was used for comparison between values of control and SC.

Two-way ANOVA was used to assess interactions between CLO concentrations and exposure times. Haematological indices and biomarkers data were the dependent variables, with CLO concentrations and exposure time as fixed factors.

All statistical analyses based on a significance level of $P < 0.05$.

3. RESULTS

No mortality was observed in treatment or control groups. There were no significant differences between the control and SC groups in any measured parameter.

3.1. BIOCHEMICAL EFFECTS

No differences from SC in CAT levels were observed in liverat any tested concentration. At d 21, GR activity in the 0.01 $\mu g \cdot L^{-1}$ group and LDH in the 1.0 and 10 $\mu g \cdot L^{-1}$ groups was significantly higher than in SC, while GPx in the 0.01 $\mu g \cdot L^{-1}$ group was significantly lower. At 42 days, GST activity in the 0.01 and 1.0 $\mu g \cdot L^{-1}$groups, GR in the 0.01 and 10 $\mu g \cdot L^{-1}$ groups, and LDH in the 0.01, 1.0, and 10 $\mu g \cdot L^{-1}$groups was significantly higher than in SC, while the activity of GPx of the 10 $\mu g \cdot L^{-1}$ groups was significantly lower.

At 21 days, gill tissue showed a significantly higher level of CAT activity, and GPx a significantly lower level in the 1.0 and 10 $\mu g \cdot L^{-1}$ groups compared to SC. At 42 days, GPx and GST activity was significantly higher than SC in the 1.0 $\mu g \cdot L^{-1}$ group.

In brain, activity of GST in the 1.0 $\mu g \cdot L^{-1}$group and of GR in the 0.01 and 1.0 $\mu g \cdot L^{-1}$ groups was significantly lower after 21 days exposure compared to SC. At day 42, CAT activity in the 1.0 and 10 $\mu g \cdot L^{-1}$ groups was significantly higher than in SC, while LDH activity was significantly lower at CLO concentration of 1.0 $\mu g \cdot L^{-1}$.

In blood plasma, the GR level was significantly higher than SC in the 1.0 and 10 $\mu g \cdot L^{-1}$ groups after 21 days of exposure. No differences from SC were observed at 42 days.

In all studied tissues, LPO levels showed no significant differences from the controls group at any time.

3.2. HAEMATOLOGICAL PROFILES

Erythrocyte and leukocyte profiles of rainbow trout exposed to CLO are shown in Table 1. At 21 days, MCH was significantly lower than controls in the 0.01 $\mu g \cdot L^{-1}$ group at 13% while PBC and Hb were at 23 and 17%, respectively, not significantly greater than SC. At 42 days, higher values of granulocytes in the 0.01 and 10 $\mu g \cdot L^{-1}$ groups were observed, along with significantly lower levels of MCHC in the 0.01 and 1.0 $\mu g \cdot L^{-1}$ groups at 9 and 8%, respectively, than found in SC.

3.3. TWO-WAY ANOVA ANALYSIS

The CLO concentration-dependent variables included haematological profile (RBC, PCV, MCH, and MCHC) and xenobiotic biotransformation biomarkers in liver (GR, and LDH), gill (CAT), brain (TBARS, CAT, GST, and LDH), and plasma (CAT and GR).

The exposure time-dependent variables included haematological profiles (RBC, PCV, MCH, MCHC, and Leuko) and xenobiotic biotransformation biomarkers in liver (GR and GPx), gill (TBARS, CAT, GR, GPx, and GST), brain (TBARS, CAT, GST, and LDH), and plasma (LDH).

The variables significantly affected by interactions between CLO concentration and exposure time included haematological profile (RBC), xenobiotic biotransformation biomarkers in liver (CAT, GR, and GPx), in gill (CAT and GPx), in brain (CAT and GR), and blood plasma (CAT).

3.4. HISTOPATHOLOGICAL CHANGES

3.4.1. Gill

The SC and 10 $\mu g \cdot L^{-1}$ CLO treatments showed similar results in gill after 42 days exposure. Pathologies were characterized by mild to moderate epithelial cell hyperplasia and hypertrophy, multifocal epithelial cell detachment, and capillary aneurisms (Table 3).

3.4.2. Kidney

Besides mild vacuolation and granulation of tubular epithelial cell cytoplasm, no major histopathological changes were found in the SC group. In solvent control animals, 4 of 8 fish showed mild cytoplasmic vacuolation or granulation of tubular epithelial cells. In the 10 $\mu g \cdot L^{-1}$ CLO treated group after 42 days exposure, 4 of 8 fish showed mild alterations of tubules, such as vacuolation or granulation of tubular epithelial cells, often accompanied by karyopycnosis (tubulonephrosis) and deposition of eosinophilic homogenous material in the cytoplasm of tubular epithelial cells (hyaline droplet degeneration). In two fish, an increased level of tubuloneogenesis (newly formed tubules) was recorded. Three fish showed dilation of the Bowman's capsule, in one fish this included deposition of eosinophilic granular material (Figure 7).

3.4.3. Liver

Similar structural changes were observed in the SC and 10 $\mu g \cdot L^{-1}$ CLO groups, including mild to moderate lipid vacuolation of hepatocytes and granulation of cytoplasm (Table 3).

3.4.4. Gonads

In the ovaries, no differences were observed between the SC and 10 μg · L^{-1} CLO treatment group. In each of the groups, two fish were revealed to be male. Composition of testicular germ cells differed from that of controls after 42 days exposure. Compared to controls, fewer spermatozoa were present, whereas the number of spermatogonia and spermatocytes was increased (Figure 8).

4. DISCUSSION

The results of the present work demonstrate the effects a widely used fungicide, CLO, on the activity of enzymes from biotransformation, antioxidation, and energy metabolism. Effects on haematological indices and mild histopathological alterations of organ tissue were observed.

4.1. EFFECTS ON BIOTRANSFORMATION SYSTEM

The study examined the status of the protective antioxidant system in rainbow trout under chronic CLO exposure. Many studies have demonstrated the adverse effects of pharmaceuticals on fish, including triggering the production of ROS, which may be scavenged by the antioxidant defence system (Sanchez-Muros et al., 2013). Enzyme activity can decrease as negative feedback either from excess of substrate or damage induced by oxidative modification (Lushchak, 2011). However, the role of CLO in biotransformation, oxidative stress, and energetic biomarkers is unclear.

The metabolites formed by phase I biotransformation are conjugated via phase II enzymes (e.g., GST) before excretion. GST is an effective protective mechanism against an array of hydrophobic and electrophilic compounds, including peroxidized lipids, secondary metabolites of LPO, through the –SH group (Bastos et al., 2013). The GST response varies with species, organ, and the type of toxicant (Song et al., 2006). A clear inverted U-shaped GST response was eliminated at 42 days with exposure to 0.01 and 1.0 μg · L^{-1} in liver and to 1.0 μg · L^{-1} in gill indicating that GST detoxifying enzyme was inhibited in presence 10 μg · L^{-1} of CLO, resulting in decreased capacity of phase II glutathione conjugation to xenobioticsubstrates. In contrast, brain showed a lower GST after 21 days at 1.0 μg · L^{-1}. Molecular modelling of the metabolism of CLO suggests that it can react with cellular nucleophiles such as glutathione, causing its depletion (Klokouzas et al., 2001; Fazlul, 2007).

Catalase is considered the first line of defence against oxygen toxicity in oxidative stress (Dorval and Hontela, 2003). Its high activity implies the presence of a large amount of H_2O_2 in the system. Fungicides can induce inhibition or induction of CAT activity in rainbow trout (Li et al., 2010b,c,d) and three-spined stickleback *Gasterosteus aculeatus* (Sanchez et al., 2008) depending on the intensity and the duration of the stress. In the current study as well as in Toni et al. (2011) no effect on liver CAT activity was observed, but it was induced in gill (after 21 days) and brain (after 42 days). The sensitivity of fish organs that are intensively involved in detoxification of H_2O_2 follow the order of gill > brain > liver.

The antioxidant enzymes expressed by GR and GPx activity can be induced by an adaptive mechanism to low levels of oxidative stress. The detoxification of ROS and hydroperoxides implies the oxidation of GSH to GSSG by GPx (Ahmad, 1995). GSSG is then reduced to GSH by GR at the expenses of NADPH, which is recycled by the pentose phosphate pathway. Decreased GR activity may lead to GSH depletion and increased GSSG, if its loss cannot be compensated for by the synthesis of new glutathione molecules (Srikanth et al., 2013). In

the current study, no alteration in gill GR activity was observed. However, GR was inhibited in brain but increased in liver and blood plasma, probably due to differences in the availability of NADPH. The same trend in GR activity in liver and brain was observed by Li et al. (2010c,e).

Glutathione peroxidase catalyzes the reduction of both hydrogen peroxide and lipid peroxide. Reduction in GPx prevents the formation of radical intermediates by oxygen reduction mechanisms (Srikanth et al., 2013). No alteration in brain GPx activity was observed. In liver, GPx activity was higher in fish exposed for 21 days to CLO at a 0.01 $\mu g \cdot L^{-1}$ than in other exposed groups, and at 42 days the level of the 10 $\mu g \cdot L^{-1}$ exposure group was 65% that of the SC group). Lower GPx activity was also observed in gill after 21 days. At 42 days, gill GPx, catalytic activity was significantly higher at 1.0 $\mu g \cdot L^{-1}$. Such shift could indicate the generation of ROS and the adaptive response to oxidative stress.

Glutathione reductase and GPx activity did not show a dose-dependent response. This could be interpreted as an adaptive response of the detoxification mechanism to possible oxidative stress. More studies are recommended to better characterize the role of these enzymes in the mitigation CLO toxic effects.

Lactate dehydrogenase is a cytoplasmic enzyme, and commonly reflects the energy metabolic capacity of tissues after acute (Rao, 2006) or long-term (Osman et al., 2010) exposure to a contaminated environment. It is involved in the final step of anaerobic glycolysis to regulate the pyruvate-lactate conversion under stress conditions, thus involved in the production of energy. In liver, ratio of NADH to NAD^+ is lower than in muscle, and the LDH reaction is reversed; lactate is transported back to the liver where it is converted into pyruvate by the Cori cycle. LDH is a useful marker for diagnosis of cardiac problems (Reis et al., 1988) and is commonly measured in blood plasma (Li et al., 2010a, 2011b), liver and muscle (Quintaneiro et al., 2008) to diagnose cell and tissue damage. In the present study, LDH activity in gill and brain did not differ from controls. The levels of SC in blood plasma were consistent over the course of the experiment, but exposed groups showed responses according to exposure period and concentrations, with increasing CLO concentration the LDH activity showed increasing/ decreasing trends at 21 and 42 days, respectively. This could be explained by depletion of LDH and failure to metabolize pyruvate in blood plasma induced by CLO. Liver was the most sensitive organ with respect to LDH response at both sampling points, suggesting that oxidative stress has high a cost to the cell when energetic pathways are induced.

Accumulation of lipid peroxide is believed to be the primary manifestation of the loss of cell function under oxidative stress (Almroth et al., 2005). Toni et al. (2011) investigated the action of tebuconazol, a triazole fungicide, on TBARS levels in liver, brain, and muscle of common carp *Cyprinus carpio* under two experimental conditions over 7 days. In a pond trial, TBARS levels were found to increase in all investigated tissues at 31.95 $\mu g \cdot L^{-1}$ tebuconazol and, in laboratory exposure, in liver at 33.47 and 36.23 $\mu g \cdot L^{-1}$ and muscle at 33.47 $\mu g \cdot L^{-1}$. Li et al. (2010b,c) exposed rainbow trout to 0.2–500 $\mu g \cdot L^{-1}$ propiconazol over 30 days and found a significant increase of TBARS levels in brain and intestine. Sanchez et al. (2008) reported unchanged TBARS level in three-spined stickleback after 7 days exposure to 10–500 $\mu g \cdot L^{-1}$ prochloraz. Our results showed that chronic exposure to CLO did not lead to oxidative damage in the investigated tissues as indicated by TBARS levels similar to controls (Figure 1). The induction of activity of several antioxidant enzymes indicates that CLO may, however, cause oxidative stress in the organism.

4.2. HAEMATOLOGICAL VARIABLES

The observation of haematological variables allows the most rapid assessment of fish health status. Blood variables can be used to assess physiological disturbances in stressed fish and provide information about level of damage (Li et al., 2011b).

MCH level was lower in fish exposed for 21 days to CLO at 0.01 $\mu g \cdot L^{-1}$ than in other exposed groups. Our prolonged CLO exposure affected MCHC index at 0.01 and 1.0 $\mu g \cdot L^{-1}$. The conclusive statements of such results are difficult due to the fact that calculation formula includes Hb, RBC, and PCV levels were not significantly affected during the chronic test. On the other hand, changes observed in MCH and MCHC might be attributed to possible disruption of haemoglobin synthesis.

With respect to leukocytes, only the number of granulocytes showed increase. Granulocytes act to protect against infection by surrounding and destroying invading bacteria and viruses, and lymphocytes aid in the immune defence system. Changes in differential leukocyte count are recognized as a sensitive indicator of environmental stress response (Li et al., 2011c). In this study, such changes may relate to a stress response of the immune system.

4.3. HISTOLOGY

Histological changes were most obvious in kidney and testis, while gill, liver and ovaries of exposed animals showed no differences from controls. Kidney degeneration, including tubulonephrosis and hyaline droplet degeneration were most prominent in the tubular epithelial cells. These results indicate that CLO might be excreted by the fish kidney, inducing degenerative changes in the tubular epithelial cells. An increase in the tubuloneogenesis was observed in some exposed fish. Regeneration in kidney has been reported in fish species (Reimschuessel et al., 1990, 1996; Reimschuessel, 2001; Salice et al., 2001; Watanabe et al., 2009; Diep et al., 2011) and can be interpreted as a response to CLO exposure.

Changes in the gonads were visible only in the two males, making conclusions difficult. However, in animals exposed to CLO, a clear decrease in numbers of spermatozoa and an increase in spermatogonia and spermatocytes was observed. These findings are in accordance with the increase in spermatogonia reported by Baudiffier et al. (2013) in zebrafish exposed to 197 $\mu g \cdot L^{-1}$ of CLO for 21 days. These effects may be due to disruption of steroidogenesis by CLO in fish (a known EDC and priority hazardous substance), capable of inhibiting P450-steroidogenic enzymes (Ankley et al., 2007; Brown et al., 2011). Studies of a greater number of males are needed.

5. CONCLUSIONS

This study provided important results for the evaluation of long-term impact and influence of CLO on non-target organisms, including concentrations measured in surface waters.

Long-term exposure to CLO caused biochemical effects in rainbow trout, changes in haematological indices and differential leukocyte count, as well as pathological alterations. There were no significant changes in oxidative stress status measured in fish exposed to CLO, indicating the adaptive responses to environmental stress. The immune system of fish was affected mainly atenvironmentally relevant concentration of CLO with exception of granulocyte count increase at the highest tested concentration. The antioxidant defence enzymes, which responded to CLO exposure, showed effects at the environmentally relevant concentration. Oxidative stress imposes a high cost on the cell when the energy pathways are

induced. Molecular mechanisms of these biochemical responses are unclear and need further investigation with higher CLO concentrations to obtain a clear picture of imbalance between pro-oxidant and antioxidant defence mechanisms related to the depletion of antioxidants or excessive accumulation of ROS. With prolonged exposure to high concentrations of CLO, pathological changes in testis indicate a disruption of steroidogenesis in fish by CLO. A more detailed study is needed on a greater number of males.

Our data provide an indication of sublethal impacts of pharmaceuticals in the aquatic environment. The presence of CLO in the environment might have consequences for fish health.

Acknowledgements

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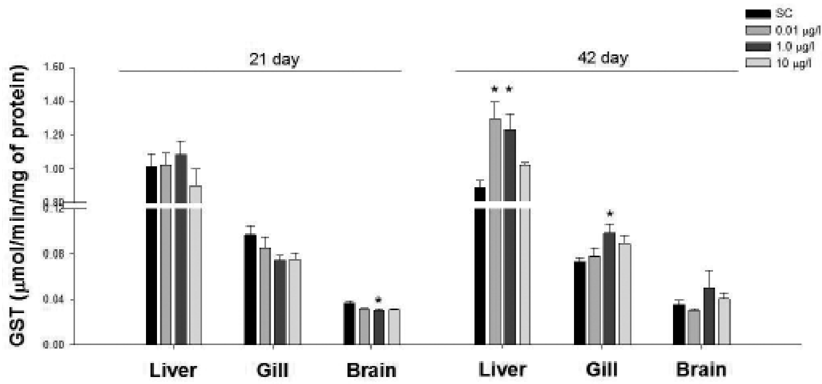

Figure 1. *Effect of CLO on glutathione S-transferase (GST) activity in rainbow trout tissues. Significant differences compared with solvent control (SC), P < 0.05 (*, ANOVA). Data are means ± S.E., n = 8.*

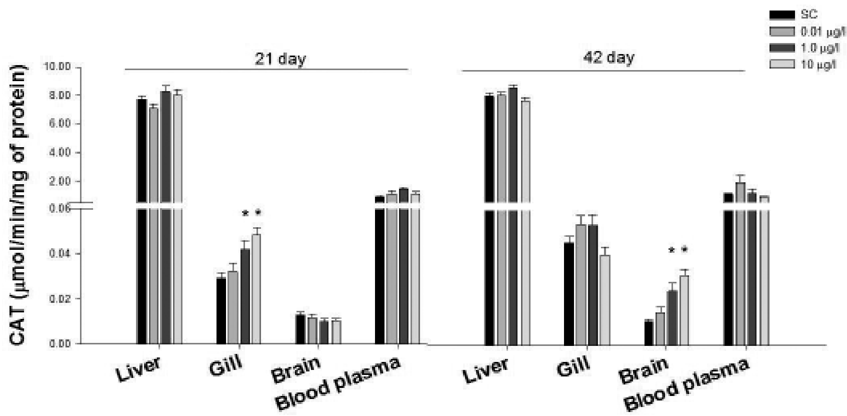

Figure 2. *Effect of CLO on catalase (CAT) activity in rainbow trout tissues and blood plasma. Significant differences compared with solvent control (SC), P< 0.05 (*, ANOVA). Data are means ± S.E., n = 8.*

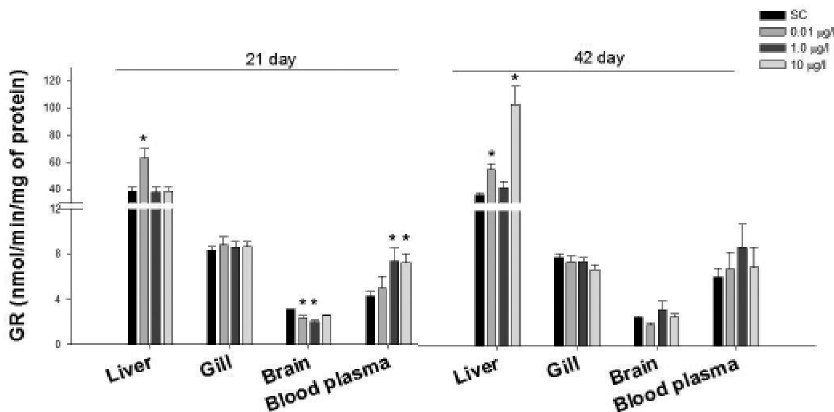

Figure 3. *Effect of CLO on glutathione reductase (GR) activity in rainbow trout tissues and blood plasma. Significant differences compared with solvent control (SC), P< 0.05 (*, ANOVA). Data are means ± S.E., n = 8.*

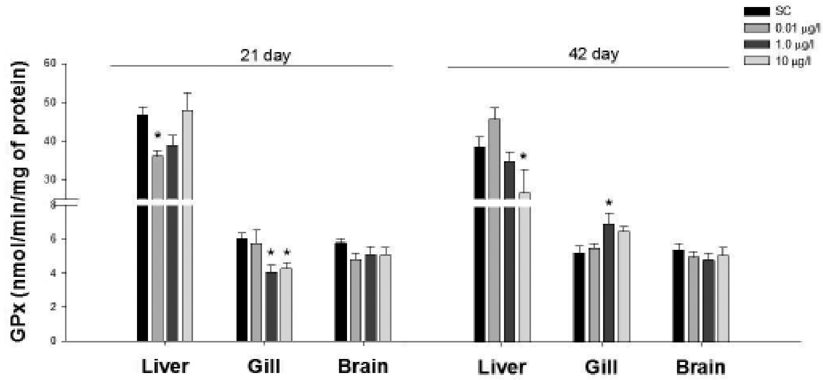

Figure 4. *Effect of CLO on glutathione peroxidase (GPx) activity in rainbow trout tissues. Significant differences compared with solvent control (SC), P< 0.05 (*, ANOVA). Data are means ± S.E., n = 8.*

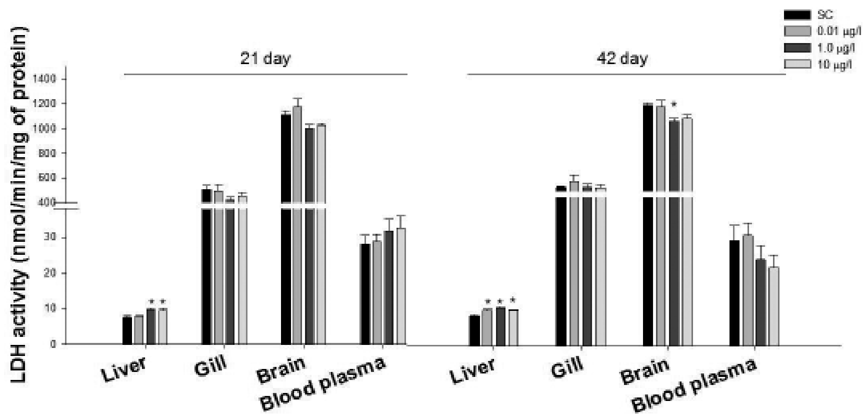

Figure 5. *Effect of CLO on lactate dehydrogenase (LDH) activity in rainbow trout tissues and blood plasma. Significant differences compared with solvent control (SC), P< 0.05 (*, ANOVA). Data are means ± S.E., n = 8.*

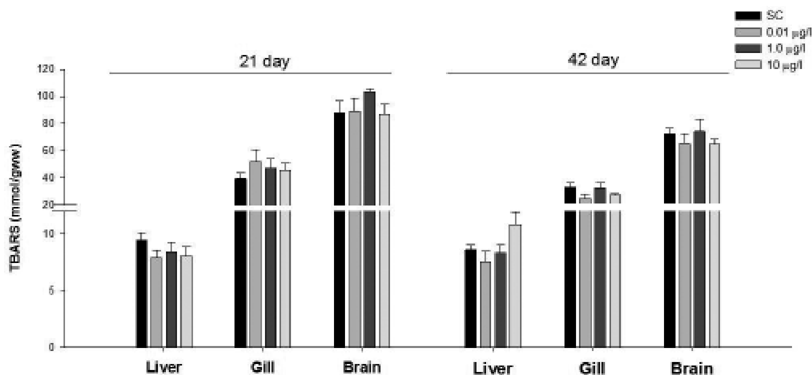

Figure 6. *Effect of CLO on level of thiobarbituric acid reactive substances (TBARS) in rainbow trout tissues. Significant differences compared with solvent control (SC), P< 0.05. Data are means ± S.E., n = 8.*

Table 1. *Haematological parameters and leukocyte differential count in rainbow trout chronicaly exposed to clotrimazole. Significant differences compared with solvent control (SC), P< 0.05 (*, ANOVA). Data are means ± S.E., n = 8.*

Indices	Unit	Exposure (days)	Test groups				
			C	SC	0.01 μg · L^{-1}	1.0 μg · L^{-1}	10 μg · L^{-1}
RBC	T · L^{-1}	21	1.3 ± .12	1.1 ± .07	1.2 ± .05	1.2 ± .06	1.1 ± .06
		42	1.4 ± .07	1.4 ± .13	1.7 ± .09	1.3 ± .08	1.2 ± .11
Hb	g · L^{-1}	21	78 ± 5.0	75 ± 3.2	72 ± 3.4	78 ± 1.9	86 ± 12
		42	81 ± 1.9	77 ± 3.8	83 ± 2.3	76 ± 4.2	75 ± 3.9
PCV	l · l^{-1}	21	0.38 ± .03	0.39 ± .02	0.39 ± .02	0.41 ± .02	0.37 ± .03
		42	0.43 ± .01	0.42 ± .02	0.49 ± .02	0.45 ± .03	0.41 ± .02
MCV	fl	21	294 ± 27	358 ± 10	329 ± 12	335 ± 17	329 ± 24
		42	327 ± 17	319 ± 16	305 ± 20	361 ± 16	360 ± 16
MCH	pg	21	60 ± 2.3	69 ± 2.2	61 ± 2.2*	63 ± 1.7	76 ± 7.9
		42	62 ± 3.5	59 ± 3.0	51 ± 3.0	62 ± 3.2	67 ± 3.3
MCHC	g · L^{-1}	21	214 ± 21	194 ± 4.5	185 ± 4.8	192 ± 9.3	236 ± 21
		42	188 ± 2.9	185 ± 3.8	168 ± 5.4*	171 ± 2.4*	186 ± 3.1
Leuko	G · L^{-1}	21	96 ± 14	62 ± 10	91 ± 19	83 ± 15	73 ± 11
		42	106 ± 12	131 ± 26	128 ± 11	124 ± 15	93 ± 7.2
Lymphocytes small	%	21	94 ± 2.1	92 ± 3.3	96 ± 1.8	87 ± 5.7	96 ± 1.7
		42	97 ± .61	97 ± .56	94 ± 2.6	98 ± .61	95 ± 1.1
Monocytes	%	21	2.6 ± 1.0	3.3 ± 1.2	3.3 ± 1.7	8.0 ± 3.8	3.0 ± 1.4
		42	1.9 ± .35	1.4 ± .21	2.1 ± .56	0.69 ± 0.19	1.3 ± .41
Granulocytes	%	21	2.1 ± .68	4.0 ± 1.8	.61 ± .29	3.6 ± 1.5	.75 ± .33
		42	1.3 ± .52	1.1 ± .33	3.5 ± 1.8*	1.1 ± .46	3.1 ± .52*

Note: PCV, haematocrit; Hb, haemoglobin concentration; RBC, red blood cells; Leuko, leukocyte count; MCH, mean erythrocyte haemoglobin; MCV, mean erythrocyte volume; MCHC, mean corpuscular haemoglobin concentration.

Table 2. *Two-way ANOVA for the effects of CLO concentrations and time exposure on parameters measured in rainbow trout.*

Indices	Experimental conditions			Indices	Experimental conditions		
	Time	**CLO**	**Time x CLO**		**Time**	**CLO**	**Time x CLO**
RBC	0.007	0.017	0.041				
Hb	0.918	0.866	0.156	TBARS_L	0.793	0.212	0.139
PCV	< 0.001	0.049	0.389	CAT_L	0.242	0.072	0.036
MCV	0.542	0.243	0.389	GR_L	0.001	< 0.001	< 0.001
MCH	0.013	< 0.001	0.6	GPx_L	0.012	0.19	< 0.001
MCHC	< 0.001	0.005	0.288	GST_L	0.453	0.151	0.078
Leuko	0.001	0.337	0.874	LDH_L	0.753	< 0.001	0.484
				TBARS_G	< 0.001	0.884	0.127
				CAT_G	< 0.001	0.03	0.002
				GR_G	< 0.001	0.834	0.403
				GPx_G	0.003	0.953	< 0.001
				GST_G	< 0.001	0.869	0.232
				LDH_G	0.101	0.332	0.456
				TBARS_B	0.002	0.048	0.84
				CAT_B	< 0.001	< 0.001	< 0.001
				GR_B	0.874	0.088	0.012
				GPx_B	0.854	0.142	0.883
				GST_B	0.003	0.044	0.826
				LDH_B	< 0.001	< 0.001	0.619
				CAT_P	0.5	0.027	0.044
				GR_P	0.149	0.048	0.771
				LDH_P	0.011	0.933	0.18

Notes: Data expressed as p-value. L, liver; G, gill; B, brain; P, blood plasma.

Table 3. *Results of histological investigation. 0 = no changes, 1 = minimal changes, 2 = mild changes, 3 = mild to moderate changes, 4 = moderate changes.*

		Gill					Kidney				Liver		Gonads	
Treatment	Animal	Epithelial cell hypertrophy	Epithelial cell hyperplasia	Epithlial cell detachment	Capillary aneurisms	Lymphocytic infiltration	Tubular epithelial cell vacuolation and granulation	Hyalin droplet degeneration	Increased tubuloneogenesis	Dilation of Bowman´s space	Fat vacuoles	Hepatocyte cytoplasm granulation	Sex	Decreased numbers of spermatozoa
Solvent control	1	2	0	0	0	0	0	0	0	0	0	4	m[a]	0
	2	2	2	0	2	0	0	0	0	0	3	0	ng[b]	0
	3	2	2	0	2	0	2	0	0	0	3	0	ng	0
	4	2	2	0	2	0	0	0	0	0	2	2	f[c]	0
	5	2	2	0	4	0	2	0	0	0	0	4	m	0
	6	2	2	4	0	0	0	0	0	0	0	0	ng	0
	7	2	0	0	0	3	2	0	0	0	2	2	f	0
	8	2	2	4	0	2	2	0	0	0	3	0	f	0
Clotrimazole 10 µg·L^{-1}	1	0	0	0	0	0	0	0	0	0	4	0	m	4
	2	3	3	2	2	0	0	0	0	0	2	2	m	4
	3	2	2	2	0	0	2	2	0	2	2	4	f	0
	4	2	2	2	0	0	2	0	0	2	2	4	f	0
	5	2	2	0	1	2	2	0	2	0	0	4	ng	0
	6	2	2	0	0	0	2	2	2	2	2	2	f	0
	7	2	2	0	0	0	0	0	0	0	3	2	f	0
	8	3	3	0	0	0	0	0	0	0	2	2	f	0

m – male
f – female
ng – no gonads/not sufficiently developed

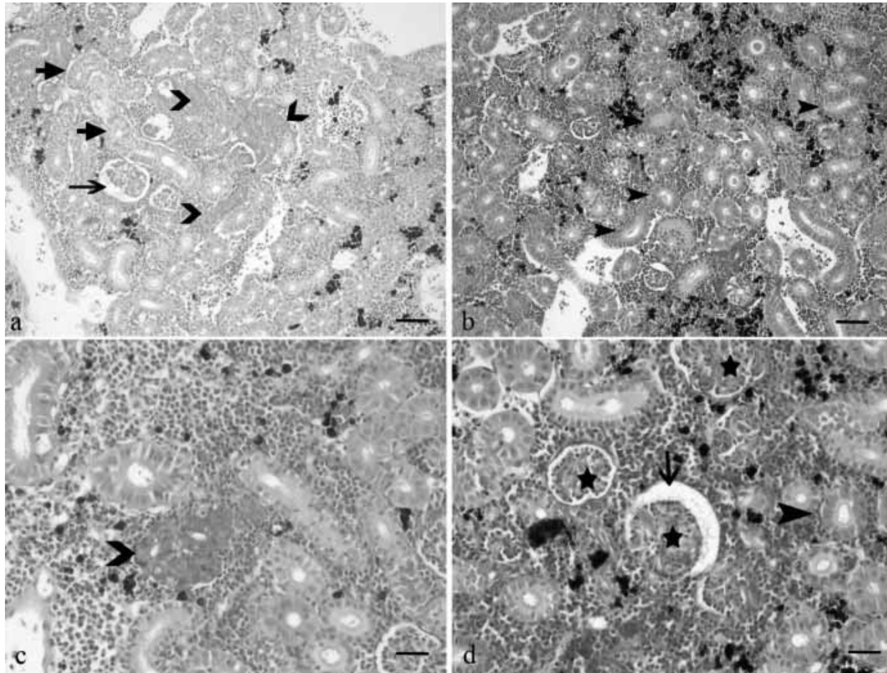

Figure 7. *Histopathological changes to the kidney. a. Animals exposed to 10 μg·L $^{-1}$ of CLO for 42 days showed vacuolation of tubular epithelial cells together with kyryopycnosis (tubulonephrosis) (closed arrows), increased amount of tubuloneogenesis (open arrowheads) and dilation of Bowman's space (open arrow), bar = 100 μm; b. Hyaline droplet degeneration in tubular epithelial cells (closed arrowheads), bar = 50 μm; c. Higher magnification of tubuloneogenesis (newly formed tubules), bar = 25 μm; d. Higher magnification of dilation of Bowman's space (open arrow), hyaline droplet degeneration (closed arrowhead), congestion in glomerular loops (stars), bar = 25 μm. HE stain.*

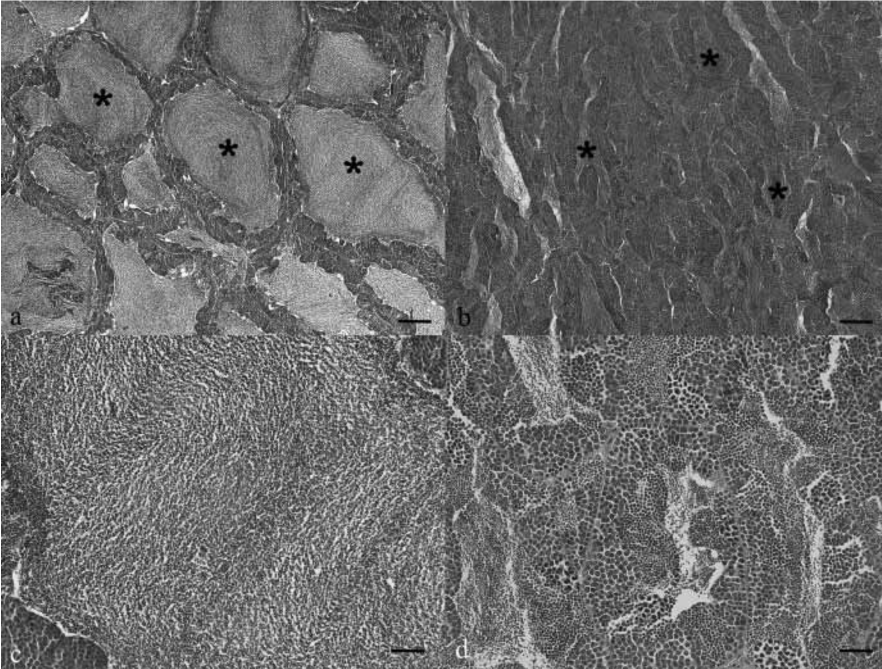

Figure 8. *Histopathology of testis. a, c: testis of control group, exposed to DMSO. b, d: testis of fish exposed to 10 μg·L^{-1} of CLO for 42 days, showing decreased amount of spermatozoa and increased amount of spermatogonia and spermatocytes. Stars = tubular lumen filled with spermatozoa; a, b: bar = 100 μm, c, d: bar = 25 μm. HE stain.*

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Clotrimazole, but not dexamethasone, is a potent *in vitro* inhibitor of cytochrome P450 isoforms CYP1A and CYP3A in rainbow trout

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HIGHLIGHTS

- ▶ We analyzed CLO and DEX inhibitory potency on rainbow trout liver microsomes.
- ▶ Three CYP450 mediated reaction were measured: EROD, BFCOD, PNPH.
- ▶ CLO exhibits non-competitive inhibition of EROD and competitive inhibition of BFCOD.
- ▶ DEX showed no inhibitory potency on any of investigated reactions.

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ABSTRACT

The effects of clotrimazole (CLO) and dexamethasone (DEX), both detected in the aquatic environment, were assessed on inhibition of cytochrome P450 (CYP450) in hepatic microsomes of rainbow trout. Activity of three CYP450 isoforms: ethoxyresorufin O-deethylase (EROD; CYP1A), 7-benzyloxy-4-trifluoromethylcoumarin O-debenzylase (BFCOD; CYP3A) and p-nitrophenol hydroxylase (PNPH; CYP2E1-like protein) was investigated in the presence of four concentrations of CLO and DEX. Clotrimazole in a concentration range of 1–100 μM decreased the activity of EROD and BFCOD. The inhibition was reversible, as pre-incubation of the microsomes with CLO, before addition of the substrate, had no effect. EROD activity was non-competitively inhibited with a K_i of 0.5 μM, and BFCOD activity revealed competitive inhibition with a K_i of 0.04 μM. The relatively low K_i for CLO inhibition of EROD and BFCOD activity may indicate that the ability of CYP1A and CYP3A to metabolize xenobiotics is reduced in the presence of CLO. PNPH activity was not affected by CLO. DEX showed no inhibitory potency on any investigated reaction. CLO, but not DEX, inhibited EROD and BFCOD activity by different mechanisms.

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1. Introduction

Over the past decade, a wide range of pharmaceutical compounds for human use, agriculture, and aquaculture have been detected in the aquatic environment (Beausse, 2004; Fent et al., 2006). Pharmaceutical compounds and their derivatives have effects on non-target aquatic organisms, including fish, even at the low concentrations typical of aquatic systems (Schwaiger et al., 2004; Corcoran et al., 2010; Hirakawa et al., 2012). The majority of detoxification occurs in the liver via a multitude of enzyme systems. Phase I, usually reactions of oxidation, reduction, or hydrolysis and phase II conjugation reactions biochemically transform toxic substances into more water-soluble, and therefore excretable, substances (Liska et al., 2006). Phase I enzymes is primarily cytochrome P450 (CYP450), which is a superfamily of heme-containing proteins involved in the biotransformation of many endogenous compounds and exogenous substrates. The proteins belonging to the first three families of CYP450: CYP1, CYP2, and CYP3 are closely associated with drug activation and metabolism (Uno et al., 2012).

CYP450 contributes to the metabolism of more than 90% of currently available drugs (Shapiro and Shear, 2002). Currently, CYP1A is the most studied xenobiotic-metabolizing isoform in fish and is often used as a biomarker for early effects of pollutants in aquatic

Abbreviations: BFC, 7-benzyloxy-4-trifluoromethylcoumarin; BFCOD, 7-benzyloxy-4-trifluoromethylcoumarin O-debenzylase; BQ, 7-benzyloxyquinoline; BzRes, benzyloxyresorufin; CLO, clotrimazole; DBF, dibenzylfluorescein; DEX, dexamethasone; EROD, ethoxyresorufin O-deethylase; P450, cytochrome P450; PNP, p-nitrophenol; PNPH, p-nitrophenol hydroxylase.

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organisms (Havelkova et al., 2007). The CYP3A enzymes represent the family responsible for metabolism of 50% of currently prescribed drugs (Hasler et al., 1999). Presence of a CYP3A protein has been detected in Atlantic cod (*Gadus morhua l.*) (Husoy et al., 1994) and fathead minnow (*Pimephales promelas*) (Christen et al., 2010). Other isoforms may play a major role in metabolism of pharmaceutical compounds. The ethanol-inducible CYP2E1 contributes to oxidative and/or reductive metabolism of a broad range of toxicologically important compounds (Caro and Cederbaum, 2004). P-nitrophenol hydroxylation (PNPH) is a CYP2E1-catalyzed reaction in mammals and often used as a marker to estimate mammalian CYP2E1 activity. P-nitrophenol hydroxylation activity has also been detected in fish (Geter et al., 2003; Zlabek and Zamaratskaia, 2012).

The presence of pharmaceuticals in the aquatic environment can induce and/or inhibit CYP450 activity in fish and thus modify enzymatic pathways mediated by CYP450 and cause unfavorable physiological effects and toxicity (Laville et al., 2004). Clotrimazole (CLO) and dexamethasone (DEX) have been detected in the aquatic environment at ng L^{-1} level (Peschka et al., 2007; Liu et al., 2011; Kugathas et al., 2012). Fish can serve as sensitive bioindicators for exposure of aquatic organisms to chemical pollutants (van der Oost et al., 2003).

Clotrimazole belongs to the imidazole group, often used for treatment of fungal infections. It decreases fungal growth by inhibiting CYP51 conversion of lanosterol to ergosterol, an essential component of fungal cell membranes (Rozman and Waterman, 1998). As CLO acts via the inhibition of the CYP51-mediated sterol 14α-demethylase activity, there is a potential for interaction with other crucial CYP mediated reactions. For example, CLO has unwanted side effects through the inhibition of aromatase activity (CYP19), a key steroidal enzyme involved in the synthesis of androgens to estrogens, as shown *in vitro* in humans (Trosken et al., 2006) and in fish (Monod et al., 1993; Hinfray et al., 2006; Cheshenko et al., 2008).

Recent studies have indicated that short-term exposure of zebrafish to CLO results in induction of several steroidogenic enzymes and increased expression of testicular genes related to steroidogenesis (Hinfray et al., 2011; Baudiffier et al., 2012). Other reports on fish show that CLO acts as an inhibitor of CYP1A (Levine et al., 1999), CYP2K1, CYP3A27 (Miranda et al., 1998) and as inducer of CYP1A (Navas et al., 2004).

Dexamethasone is a potent synthetic member of the glucocorticoid class of steroid drugs (Hockey et al., 2009). Glucocorticosteroids have inhibitory action on inflammatory mediators of both the cyclo-oxygenase and lipoxygenase pathways of arachidonic acid metabolism (Vane and Botting, 1987).

The metabolite profile of DEX in human liver microsomes has been described by Tomlinson et al. (1997). DEX influences induction of CYP2B, CYP2A, CYP3A, and probably also CYP2C11 activity in rat (Ringel et al., 2002) and induction of CYP3A in human (Lu and Li, 2001) primary hepatocytes. There is limited information about the elimination pathway of DEX in fish. Studies on trout have suggested DEX induction of 3-cyano-7-ethoxycoumarin metabolism, a reaction used to estimate mammalian CYP1A2 activity, but have not shown it to significantly affect CYP450-mediated reactions in killifish (*Fundulus heteroclitus*) (Smith and Wilson, 2010) and grass carp (*Ctenopharyngodon idellus*) (Li et al., 2008). These results were explained by significant differences in the regulation of CYP induction via the pregnane X receptor in mammals and fish.

This study was undertaken to evaluate the inhibitory potential of CLO and DEX on three CYP450-mediated reactions in fish: ethoxyresorufin O-deethylation (EROD, CYP1A), 7-benzyloxy-4-trifluoromethylcoumarin O-debenzylation (BFC-OD, CYP3A), and p-nitrophenol hydroxylation (PNPH, CYP2E1). For this purpose, hepatic microsomes of rainbow trout were co-incubated with the clotrimazole and dexamethasone and specific substrates for CYP1A, CYP3A and CYP2E1-like protein.

2. Materials and methods

2.1. Chemicals

The test substances, CLO (CAS Number 23593-75-1, $C_{22}H_{17}ClN_2$) and DEX (CAS Number 2392-39-4, $C_{22}H_{28}FNa_2O_8P$), were obtained from Sigma–Aldrich (Czech Republic). Resorufin, 7-ethoxyresorufin (ER), 7-benzyloxy-4-trifluoromethylcoumarin (BFC), p-nitrophenol (PNP), p-nitrocatechol, reduced β-nicotinamide adenine dinucleotide phosphate (NADPH), dimethylsulfoxide (DMSO), and trichloroacetic acid (TCA) were obtained from Sigma–Aldrich (Steinheim, Germany). Acetonitrile (ACN) and methanol (MeOH) of HPLC grade were purchased from Merck (Darmstadt, Germany). Stock solutions (25 mM) of CLO and DEX were prepared in DMSO and MeOH, respectively. Stock solutions were further diluted from 25 mM to 0.25 mM to obtain several concentrations of the inhibitor (from 1 to 100 μM).

2.2. Fish

Juvenile rainbow trout of both sexes (body length 23.9 ± 1.29 cm (mean ± SD), weight 154 ± 31.69 g (mean ± SD)) were obtained from a commercial hatchery (Husinec, Czech Republic). They were maintained according to conditions described previously by Burkina et al. (2012). Fish were not fed for 24 h prior to sampling to avoid prandial effects during the assay. Liver samples were collected from 16 fish, immediately frozen in liquid nitrogen, and stored at −80 °C until use for microsome preparation.

Experimental animals were handled in accordance with national and institutional guidelines for the protection of human subjects and animal welfare.

2.3. Preparation of hepatic microsomes and protein estimation

The microsome fraction was prepared by differential centrifugation as described by Li et al. (2011). Briefly, approximately 0.5 g liver tissue was minced in Tris-sucrose buffer (10 mM Tris–HCl, 250 mM sucrose, pH 7.4) and homogenized (Schuett Homogen, Germany) for 3 × 10 s with 10 s intervals. After 15 min centrifugation (Beckman Coulter Optima™L-90 K ultracentrifuge) at 10000*g* at 4 °C, the supernatant (crude homogenate) was separated from sediment tissue for further centrifugation at 36000*g* for 60 min at 4 °C. As a final step the samples were diluted in the appropriate buffer (Tris–sucrose) to a volume of 1 mL and homogenized (UltraTurrax; Ika, Germany). All steps were carried out on ice.

Protein levels were estimated spectrophotometrically by the method of Smith et al. (1985) using bovine serum albumin as a standard. The microsomes were diluted to a protein content of 10 mg mL^{-1}.

2.4. Microsomal enzyme activity

Three CYP450-mediated reactions were investigated; EROD, BFC-OD, and PNPH. EROD activity was determined according to Zamaratskaia and Zlabek (2009) with slight modifications. The incubation mixtures (0.5 mL) contained 0.2 mg of microsomal protein in an incubation medium of 50 mM potassium phosphate buffer (pH 7.4) and 7-ethoxyresorufin (0.5 μM). The reaction was started by the addition of 0.5 mM of NADPH, followed by incubation in a water bath at 25 °C for 5 min and stopped by addition

of 500 μL of ice-cold 100% methanol. BFCOD activity was measured as described by Zlabek and Zamaratskaia (2012) using the same incubation mixture but with the addition of 180 μM BFC. The reaction was started by the addition of 0.5 mM NADPH, incubated in a water bath at 25 °C for 10 min and stopped by the addition of 500 μL ice-cold 100% methanol. The CYP1A and CYP3A catalytic activity was measured as EROD and BFCOD activity, using resorufin and 7-hydroxy-4-trifluoromethylcoumarin (HFC) as standards. Resorufin and HFC were detected with a fluorescence detector (L-7480). The detection wavelengths were 560 nm and 586 nm (excitation and emission) for resorufin, and 410 nm and 538 nm for HFC. The HPLC system comprised a pump (L-7100), autosampler (L-7200), fluorescence detector (L-7485), and D-7000 HPLC Manager software (Merck, Hitachi, Tokyo, Japan). A reverse-phase LiChrospher RP-18 column (5 μm) equipped with a guard column was used.

Activity of PNPH was measured as described by Zamaratskaia and Zlabek (2011). The incubation mixtures (0.25 mL) contained 0.5 mg microsomal protein in an incubation medium of 50 mM Tris–HCl, 10 mM $MgCl_2$, 0.1 EDTA buffer (pH 7.4), and p-nitrophenol (0.2 mM). The reaction was started by the addition of 1 mM of NADPH and incubated in a water bath at 25 °C for 30 min. The reaction was stopped by the addition of 10 μL of ice-cold 40% TCA. The concentrations of 4-nitrocatechol were analyzed by HPLC comprising a pump (L-6200A), autosampler (AS 2000), a UV–VIS detector (L-4250), and D-6000 HPLC Manager software (Merck, Hitachi, Tokyo, Japan). The UV–VIS detector was set at 345 nm. A Hypersil ODS (5 μM, 60 × 4.6 mm) with a guard column was used to separate p-nitrophenol and 4-nitrocatechol.

The differences between duplicates did not exceed 12% in any assay. The incubation time and the amount of microsomal protein were in the linear range for the rate of metabolite formation. The assays were conducted in duplicate, and the means used for statistical analysis. Enzyme activity was expressed as pmol of the reaction product formed per min per mg of microsomal protein.

2.5. Inhibition study

Inhibition studies were conducted on individual microsomes from three fish using two approaches. First, to evaluate the possibility of a reversible inhibition effect of CLO and DEX on the rate of selected CYP450-mediated reactions, assays were conducted in the presence of 1, 10, 50, and 100 μM concentrations of CLO and DEX. An equal volume of solvent (DMSO or MeOH) was used for control reactions without pharmaceuticals. Second, the possibility of irreversible inhibition (NADPH-dependent metabolite formation) was investigated by pre-incubation of pharmaceuticals in the presence of NADPH prior to the addition of a probe substrate. NADPH was added only once at the beginning of a pre-incubation period, because our pilot experiment demonstrated that NADPH is not completely consumed or exhausted during the incubation (up to 40 min). The choice of pre-incubation times of 5 min (EROD and BFCOD) or 10 min (PNPH) was based on preliminary experiments and did not affect final activity in the absence of the inhibitor. The rates of metabolite formation from substrate probes in the presence of either CLO or DEX were compared with those for controls.

If the pharmaceutical exhibited a reversible mode of inhibition (no increase in degree of inhibition after inclusion of the pre-incubation step), the kinetic pattern of inhibition (competitive or non-competitive) was further determined using six substrate concentrations, from 0.05 to 2 μM of ER and from 0.001 to 1.20 mM of BFC. The final concentrations of CLO used in the microsomal incubation were 1 and 10 μM. These studies were performed using two or three individual microsomes. The incubation mixtures were processed as described above without the pre-incubation step.

2.6. Data analyses

A non-linear regression analysis (GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego California, USA) was used to estimate the concentration of inhibitor causing 50% inhibition of the control enzyme activity (IC_{50}) with and without a pre-incubation period. Michaelis–Menten parameters (K_m and V_{max} values), the kinetic pattern of inhibition (competitive and non-competitive), and inhibition constants (K_i) were also determined by this program. Goodness-of-fit criteria (r^2 and Akaike's information criterion) were used to determine the inhibition pattern that best described the data.

3. Results

3.1. Clotrimazole

Both EROD and BFCOD activity was inhibited by CLO in a concentration-dependent manner (Fig. 1). Inclusion of a pre-incubation step did not enhance the degree of inhibition. Consequently, no decrease in IC_{50} was detected (0.1 μM vs. 0.6 μM for EROD, and 2.0 μM vs. 1.8 μM for BFCOD with and without pre-incubation, respectively). Further Michaelis–Menten kinetic analyses were used to determine the nature of the inhibition. When CLO was added to the incubations, EROD activity was inhibited in a non-competitive manner, with K_i value of 0.5 μM, while BFCOD activity was inhibited competitively, with K_i value of 0.04 μM (Fig. 2). PNPH activity was reduced only by the highest concentration of CLO, and this reduction did not exceed 50%. No further analysis was performed for PNPH.

3.2. Dexamethasone

As demonstrated in Fig. 3, PNPH activity was somewhat reduced in the presence of DEX when a pre-incubation step was included. However, this inhibition did not exceed 50% with the highest DEX concentration tested, and kinetic analysis failed to fit the data to concentration–response curves and calculate IC_{50}.

4. Discussion

There are a large number of studies of the inhibition of CYP450 by pharmaceuticals in mammalian *in vitro* models. In contrast, limited information is available for fish CYP450. This study evaluated *in vitro* inhibition potency of human drugs CLO and DEX on selected CYP450-mediated reactions in hepatic microsomes from juvenile rainbow trout. Both CLO and DEX are known to inhibit mammalian CYP450 (Ayub and Levell, 1988; Relling et al., 1993).

The most significant finding was the inhibition of EROD and BFCOD activity by CLO. Inhibition of EROD and BFCOD were reversible, as pre-incubation did not enhance CLO inhibitory potency, and no reductions in IC_{50} values were observed. We further characterized the kinetic pattern of inhibition using six substrate concentrations and found that EROD activity was non-competitively inhibited by CLO (decreased V_{max}, similar K_m). Most studies investigating the inhibitory mechanism of imidazole fungicides such as propiconazole, prochloraz, and CLO on CYP1A1 in fish have suggested differing inhibiting mechanisms, such as non-competitive (Snegaroff and Bach, 1989; Levine et al., 1999), competitive (Snegaroff and Bach, 1989; Miranda et al., 1998), and non-competitive mixed type mechanisms (Levine and Oris, 1999). This variability might be due to the variation in substrate and inhibitor concentrations used in the studies and the fact that the imidazoles have different modes of inhibition. The role of inter-species and individual

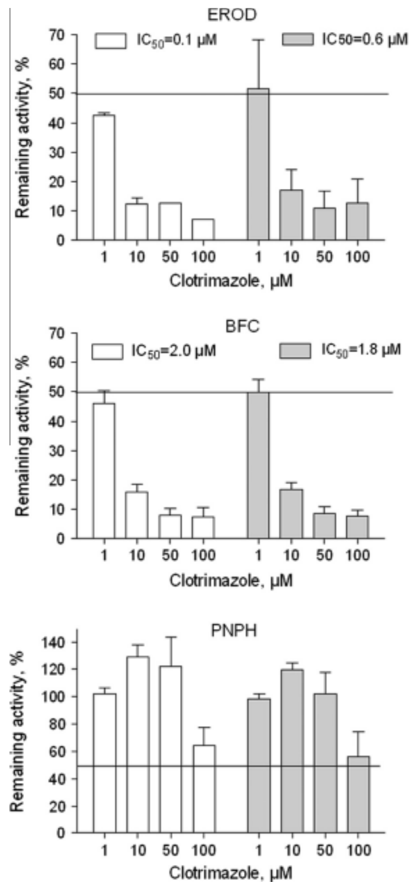

Fig. 1. *In vitro* inhibition of EROD, BFCOD and PNPH activity in hepatic microsomes of rainbow trout by clotrimazole. An inhibition study was conducted using a single concentration of substrate (1 μM of ER, 0.18 mM of BFC or 0.2 mM of PNP) and four CLO concentrations from 1 to 100 μM with (white bars) and without (grey bars) a pre-incubation step. Data represent percent control values (mean ± standard deviation) obtained from the microsome incubations of three fish. IC_{50} were estimated with a non-linear regression analysis (GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego California, USA).

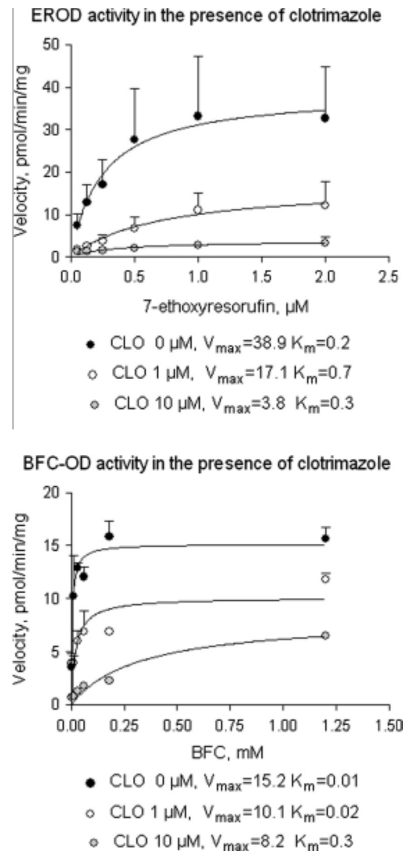

Fig. 2. EROD and BFCOD activity in hepatic microsomes of rainbow trout with and without addition of CLO. Each point represents the mean ± standard error of the incubations on three (EROD) or two (BFCOD) fish. V_{max} and K_m values were estimated with a non-linear regression analysis (GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego California, USA).

variations in the response to the inhibitor should also be considered.

Another family of CYP450 that is responsible for the biotransformation of a large number of xenobiotics in fish liver is CYP3A (Hegelund and Celander, 2003). According to Stresser et al. (2000), CLO is a submicromolar inhibitor of CYP3A in microsomal protein prepared from wild-type baculovirus infected insect cells using 7-benzyloxy-4-trifluoromethylcoumarin (BFC) as a substrate. We demonstrated a similar interaction between CLO and product formation from BFC. Kinetic analysis showed a competitive mechanism of inhibition (similar V_{max}, increased K_m), suggesting that both CLO and BFC are metabolized by the same isoform. It is possible that both inhibitors used in the present study are metabolized by the investigated enzymes, especially CYP3A. Thus, it is likely that the observed decrease in specific substrate metabolism is due to competition between CLO and the substrate for the active site of the enzyme and is not indicative of inhibition of overall specific CYP450 activity. The K_i values for CLO inhibition of EROD and BFCOD activity were relatively low, suggesting that ability of CYP1A and CYP3A to metabolize xenobiotics may be reduced in the presence of CLO.

To our knowledge, neither *in vivo* or *in vitro* inhibition potential of CLO and DEX on PNPH activity in fish has been studied, whereas studies on human cell lines have demonstrated that CLO is a uncompetitive inhibitor of CYP2E1 (Tassaneeyakul et al., 1998). Unlike EROD and BFCOD, which were both inhibited by CLO at concentrations ranging from 1 to 100 μM, PNPH activity was reduced only in the presence of the highest tested CLO concentration. Although this inhibition did not exceed 50%, there is a possibility that this is sufficient to be of physiological significance. Thus, the potential effects of this inhibition require further research.

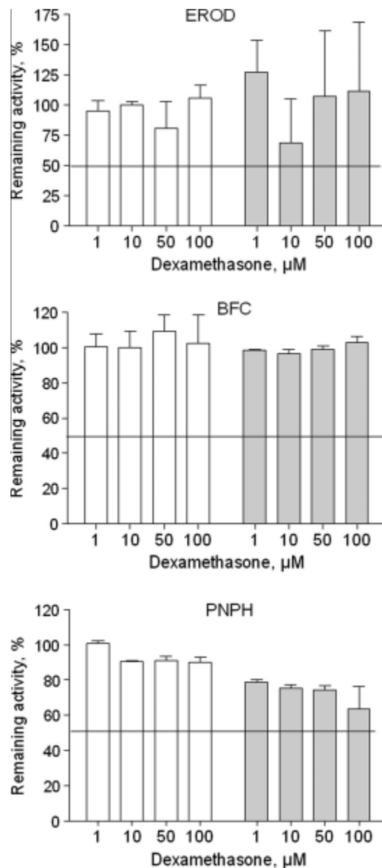

Fig. 3. *In vitro* inhibition of EROD, BFCOD and PNPH activity in hepatic microsomes of rainbow trout by dexamethasone. An inhibition study was conducted using one concentration of substrate (1 μM of ER, 0.18 mM of BFC or 0.2 mM of PNP) and four DEX concentrations from 1 to 100 μM with (white bars) and without (grey bars) a pre-incubation step. Data represent percent control values (mean ± standard deviation) obtained from the microsome incubations of three fish. IC_{50} were estimated with a non-linear regression analysis (GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego California, USA).

A possible limitation of our study was that two concentrations of the inhibitor CLO used for the kinetic analyses were above the calculated K_i values. These concentrations were chosen from a preliminary study using several concentrations of CLO to reduce enzyme activity by 50% for EROD and by 80–90% for BFCOD. It could be argued that these conditions may not be sufficient to determine precise K_i values, yet, we believe that this set of experiments allows for accurate determination of inhibition mode.

This is the first demonstration of non-competitive inhibition of EROD activity by CLO in hepatic microsomes of rainbow trout. BFCOD activity was inhibited by CLO competitively. Therefore, a controlled *in vivo* study is warranted to further investigate the effect of CLO on activity of these enzymes. The presence of DEX in the incubations did not affect either EROD or BFCOD activity. Neither CLO nor DEX affected PNPH activity in hepatic microsomes from rainbow trout.

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CHAPTER 3

GENERAL DISCUSSION
ENGLISH SUMMARY
CZECH SUMMARY
ACKNOWLEDGEMENTS
LIST OF PUBLICATIONS
TRAINING AND SUPERVISION PLAN DURING STUDY
CURRICULUM VITAE

GENERAL DISCUSSION

Due to the massive use of pharmaceuticals and personal care products (PPCPs), water pollution from PPCPs has been growing in recent decades. Fish can be affected by the concentrations found in surface waters (Brodin et al., 2013; Mehinto et al., 2010). These effects include behavioural responses, morphological changes, and changes to physiological and biochemical indices. In this respect, it is important to recognize drug-specific modes of action and the effects that can occur in fish after exposure. In aquatic toxicological research, both *in vitro* and *in vivo* tests have the potential to reveal the biological response of the organism to toxic actions of the chemical. In this thesis, specific biomarker approaches have been applied to assess the *in vivo* (laboratory experiments) and *in vitro* (microsomal fractions) effects on rainbow trout exposed to different levels of PPCPs.

The experiments discussed in this thesis were performed within a project of the Czech Science Foundation. In general, the project aimed to assess the specific effects of individual PPCPs (atenolol, verapamil, PBSA, clotrimazole, dexamethasone, carbamazepine, and diltiazem) on fish after long-term exposure. Several experimental settings and a wide range of endpoints were investigated to achieve the goals of the project. Numerous articles referring to the performed experiments have already been published by Li et al. (2011a; 2010a; 2010b; 2010c; 2009), Grabicova et al. (2013), and Steinbach et al. (2013). It should be noted that the discussion in this thesis covers portions of the above mentioned experiments, with a special focus on the effects of atenolol, verapamil, PBSA, dexamethasone, and clotrimazole on the CYP450 system and oxidative status of the rainbow trout.

Biotransformation and antioxidant enzymes in fish exposed to atenolol, verapamil, and the UV filter 2-phenylbenzimidazole-5-sulfonic acid (PBSA)

PPCPs are designed to be active for long periods, and can thus still be active when they reach different bodies of water and affect the aquatic organisms. Acute toxicity data have been reported for several different PPCPs thus far. However, such data alone may not be suitable to specifically address questions regarding the environmental effects. Sub-chronic toxicological tests can provide more useful information to understand the possible ecotoxicological effects of human pharmaceuticals on aquatic organisms.

The effects of individual PPCPs were studied using *in vivo* tests, where the fish were exposed to selected compounds under controlled laboratory conditions (Steinbach et al., 2014; Burkina et al., 2012; Grabicova et al., 2013, chapters 2.1, 2.2, and 2.3).

Atenolol, verapamil, and PBSA are PPCPs used worldwide and are frequently detected in the aquatic environment. In our sub-chronic exposure tests, juvenile rainbow trout were exposed for 21 or 42 days to three concentrations of atenolol, PBSA (both at 1.0 $\mu g \cdot L^{-1}$, the environmentally relevant concentration; 10 $\mu g \cdot L^{-1}$; and 1000 $\mu g \cdot L^{-1}$), and verapamil (0.5 $\mu g \cdot L^{-1}$, the environmentally relevant concentration; 27 $\mu g \cdot L^{-1}$; and 270 $\mu g \cdot L^{-1}$). The CYP450 family is particularly important in the metabolism of xenobiotics, and it has been well established as a useful tool for environmental contamination biomonitoring. However, little attention has been paid to the use of biomarkers for predicting drug interactions in fish. The CYP450 family members are enzymes, catalysing the conversion of lipophilic xenobiotics into entities that are more water-soluble and readily excreted. Thus, the adequate activity of CYP450s is crucial for the detoxification process.

There is limited knowledge on the effects of atenolol on fish CYP450 activities. In our study, the sub-chronic exposure of juvenile rainbow trout to atenolol did not alter CYP450-mediated

reactions, with the EROD, MROD, PROD, and BFCOD activity levels remaining unchanged and the total CYP450 content remaining similar to that of the control groups (Steinbach et al., 2014). These results suggest that the activity of the analysed CYP450-mediated reactions cannot be used as biomarkers for the presence or effects of atenolol in the aquatic environment.

A number of UV filters are known to elicit hormonal activity in fish (Kunz et al., 2006a; Kunz et al., 2006b; Kunz et al., 2009; Fent et al., 2008). A relatively high concentration of PBSA in surface water has been detected, and concern has increased regarding its potential effects on fish. For the first time, our study shows an induction of EROD, MROD, and PROD in all of the exposed groups after 21 days of exposure. The treatment with PBSA resulted in a 6-fold increase in CYP1A-mediated EROD activity and a 1.4-fold increase in CYP2B-like protein-mediated PROD activity. It is known that CYP1A is regulated by the aryl hydrocarbon receptor, but the regulatory mechanism for CYP2B-like protein is not yet understood. We have speculated that PBSA can interact with intracellular receptors, even at environmentally relevant concentrations.

Oxidative stress may occur due to the metabolic transformation of xenobiotics. It is well known that oxidative stress is caused by the formation of free radicals (e.g. the hydroxyl radical, superoxide anion radical, and hydrogen peroxide), primarily as the by-products of oxidative metabolism. To avoid oxidative stress, the organism activates antioxidant defence systems. In this study, the activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR) were measured. In our study, GR activity was inhibited by atenolol and PBSA after 42 days at the highest concentrations tested (1000 $\mu g \cdot L^{-1}$). This inhibition might be caused by a deficiency of NADPH. GR maintains the redox balance of glutathione, an antioxidant that prevents damage to cells caused by ROS, by catalysing the reduction of glutathione disulphide to glutathione (van der Oost et al., 2003; Richard et al., 2008). Decreased GR activity can cause a decrease in the glutathione content (Srikanth et al., 2013). Therefore, fish exposed to atenolol or PBSA at concentrations of 1000 $\mu g \cdot L^{-1}$ may be at a higher risk of damage caused by ROS due to the reduction of GR activity. However, such a large concentration of the tested compound is unlikely to occur in the aquatic environment.

Similarly to atenolol, the sub-chronic exposure of rainbow trout to verapamil did not affect CYP450 activity. Verapamil did not modify any of the measured CYP450-mediated reactions, namely EROD, MROD, PROD (CYP2B-like protein), BFCOD, COH (CYP2A-like protein), and PNPH (CYP2E1-like protein). Moreover, our study suggested that juvenile rainbow trout do not possess tolbutamide-metabolizing activity, as the formation of tolbutamide metabolites was not detected. Alderton et al. (2010) studied the metabolite profile of verapamil in zebrafish homogenates. The metabolic products of verapamil in the zebrafish larvae were similar to those of humans, indicating similarities in their metabolism. In mammals, the biotransformation of verapamil includes two major metabolic pathways known as O- and N-demethylation, and it has been shown that verapamil inhibits CYP3A (Wang et al., 2004). Thus, the fact that CYP3A (BFCOD) was not influenced by verapamil in fish is surprising and suggests further research is yet needed to determine the metabolic pathway(s).

These studies demonstrate that *in vivo* tests are valuable systems for evaluating the effects that PPCPs can have on fish at environmentally relevant concentrations. The main advantage of such tests is the possibility of taking into account the factor of cell compensatory mechanisms (Fent at al., 2001). Cells contain various types of regulatory mechanisms, which are triggered by a multitude of intracellular signalling events that can influence the relevance of the cellular toxicological response. However, there are some limitations to the *in vivo* laboratory exposures. For example, the use of experimental fish is controversial, and it is problematic to

extrapolate to real environmental conditions where mixture effects may influence the overall harmful action of the individual chemicals.

The inhibition of hepatic CYP1A and CYP3A activity in microsomes exposed *in vitro* to dexamethasone and clotrimazole

In vitro methods are highly simplified models of i*n vivo* processes and are ideal when there is a specific receptor-mediated mechanism or specific toxicity end point to be determined. However, *in vitro* experiments also have several limitations, because they do not take into account the biokinetics, tissue distribution, and biotransformation that may occur *in vivo*.

In the next study (Burkina et al., 2013, chapter 2.6), the *in vitro* inhibition potency of the human drugs clotrimazole and dexamethasone on the activities of CYP1A1, CYP3A, and CYP2E1 in hepatic microsomes from juvenile rainbow trout were evaluated.

The presence of pharmaceuticals in the effluents from WWTPs may influence the CYP450 system in fish living in the waters receiving those effluents. Clotrimazole and dexamethasone have been detected in the aquatic environment at the $ng \cdot L^{-1}$ level.

Recent reports show that clotrimazole is a potent endocrine disruptor due to interactions with the steroidogenic enzymes CYP17 and CYP19 (Baudiffier et al., 2013; Baudiffier et al., 2012; Hinfray et al., 2011, Monod et al., 1993). In addition, clotrimazole has been shown to interact with different cytoplasmic or nuclear receptors such as the aryl hydrocarbon receptor (Navas et al., 2004), pregnane X receptor (Wassmur et al., 2010), and androgen receptor, and it can therefore interfere with a broad range of physiological processes.

The *in vitro* inhibition of EROD, BFCOD, and PNPH activity in rainbow trout hepatic microsomes was investigated using four concentrations of clotrimazole and dexamethasone (1, 10, 50 and 100 μM) as well as a single concentration of other substrates (1 μM ER, 0.18 mM BFC, or 0.2 mM PNP).

Clotrimazole inhibited both EROD and BFCOD activity. We further characterized the kinetic pattern of inhibition and found that EROD activity was non-competitively inhibited by clotrimazole, while BFCOD activity was competitively inhibited. It should be noted that the inhibitory mechanisms of imidazole fungicides, such as propiconazole, prochloraz, ketoconazole, and clotrimazole, on CYP1A1 and CYP3A might significantly differ. The following three types of inhibition have been identified for fish CYP1A enzyme activity: competitive (Snegaroff and Bach, 1989; Miranda et al., 1998), non-competitive mixed (Levine and Oris, 1999), and non-competitive (Snegaroff and Bach, 1989; Levine et al., 1999). Hasselberg et al. (2005) showed that ketoconazole is a non-competitive inhibitor of the CYP3A enzyme. This variability might be due to the variation in substrate and inhibitor concentrations used in the studies and the fact that the imidazoles have different modes of inhibition.

Studies on trout have suggested that dexamethasone induces 3-cyano-7-ethoxycoumarin metabolism, a reaction used to estimate mammalian CYP1A2 activity (Smith and Wilson, 2010). In grass carp, CYP3A activity was slightly induced after incubation with dexamethasone (Li et al., 2008). In our study, dexamethasone did not inhibit any of the reactions investigated. To our knowledge, neither an *in vivo* nor *in vitro* inhibition potential for clotrimazole or dexamethasone on PNPH activity has been found in fish. A slight reduction of PNPH activity was observed in the presence of the highest tested concentration of clotrimazole, although this inhibition did not exceed 50%. Yet, the possibility that this reduction is physiologically significant needs to be considered.

Fate and effects of clotrimazole in rainbow trout

Due to the growing concerns over the potential chronic toxic effects of pharmaceuticals, especially on aquatic species, there has been a steady increase in the number of long-term exposure studies that have measured multiple biomarker responses. Antifungals such as clotrimazole could have undesirable side effects through the inhibition of CYP19, the key enzyme regulating the local and systemic oestrogen levels in the body, as has been shown *in vitro* in both humans (Trosken et al., 2006) and fish (Cheshenko et al., 2008; Hinfray et al., 2006; Monod et al., 1993). Our *in vitro* study provided further evidence of clotrimazole interacting with the hepatic CYP450 system, which triggered an *in vivo* investigation.

Juvenile rainbow trout were exposed to sub-lethal concentrations of clotrimazole (0.01 $\mu g \cdot L^{-1}$, the environmentally relevant concentration; 1.0 $\mu g \cdot L^{-1}$; and 10 $\mu g \cdot L^{-1}$) for 21 and 42 days. To the best of our knowledge, our *in vivo* experiment is the first report on the experimentally determined distribution, bioaccumulation, depuration, and responses of a wide spectrum of biochemical markers as well as the structural changes in different fish tissues after sub-chronic exposure to clotrimazole.

The adsorption behaviour of clotrimazole in the aquaria-water and aquaria-water-fish systems showed that it is a highly adsorbing compound, a result that agrees with a previous observation (Kahle et al., 2008).

The present study has shown that fish can take up the clotrimazole from the water, with the uptake by various tissues having the following rank order: kidney > liver > muscle > blood plasma. The distribution of clotrimazole in the different tissues can be attributed to their lipid content. The bioconcentration factors (BCFs) were calculated based on the water concentration and uptake levels of clotrimazole in the blood plasma and muscle tissue. According to the United States Environmental Protection Agency, a chemical is considered 'bioaccumulative' if the BCF is greater than 1000. The tissue-specific BCFs (wet weight) varied between 58 and 550.

A commonly detected concentration of clotrimazole in an aquatic environment may present a risk for aquatic organisms due to a moderate potential to bioconcentrate. The estimated half-life of clotrimazole is 72 h in the liver, 159 h in the muscle, and 682 h in the kidney, which is relatively long compared to that of humans, which is 3.5–5.5 h according to the Hazardous Substances Data Bank. Results similar to ours were obtained for verapamil (Steinbach et al., 2013), where the half-life in exposed carp larvae was also longer than it is in humans. It should be emphasized that mammals may have more potent metabolic pathways for rapidly clearing the drugs from the body in comparison to non-mammalian aquatic vertebrates. In addition, different exposure scenarios, such as the exposure concentration, route of exposure, and differences in the extent of receptor responses, could dictate the overall clearance rates of pharmaceuticals. The short half-life of clotrimazole in the liver in comparison to the other tissues suggests that the liver might actively eliminate clotrimazole. A long depuration period for clotrimazole in the kidneys (half-life = 28.4 days) may imply a greater potential for adverse effects in non-target species. In addition, the histological alterations of the fish kidneys support these results, where degeneration, including tubulonephrosis and hyaline droplet degeneration, was found in the presence of the highest clotrimazole concentration. Furthermore, the relative proportions of germ cells in the testis were also altered. The number of spermatozoa was reduced, while spermatogonia and the number of spermatocytes increased relative to the control group. These findings are in agreement with Baudiffier et al. (2013), who found that clotrimazole inhibited steroidogenesis in zebrafish.

The clotrimazole taken up from the surrounding water was able to induce several responses in the liver tissue of the exposed fish. The hepatosomatic index, an indicator of hepatic

detoxification activity, was elevated. Changes in EROD and BFCOD activities were also detected. Specifically, the activity of EROD was higher in the exposed fish at day 42 in comparison to the control group. BFCOD was significantly inhibited by clotrimazole after 21 days in the group exposed to 10 $\mu g \cdot L^{-1}$, and biphasic variations were obtained after 42 days of exposure, where expression was reduced in the group at the environmentally relevant concentration and increased in the 10 $\mu g \cdot L^{-1}$ group. The reason for the biphasic dose–response pattern is not clear, but the results suggest that the environmentally relevant concentration could negatively interact with the CYP450 system in fish.

Antioxidant stress enzyme (CAT, GR, and GPx) activity, which prevents the adverse effects of oxidative stress in cells, was investigated in the liver, gill, brain, and blood plasma (only CAT and GR). The antioxidant defence enzyme activity in response to the clotrimazole exposure had different effects in the fish tissue at the environmentally relevant concentrations. The accumulation of lipid peroxide is believed to be the primary manifestation of the loss of cell function under oxidative stress (Almroth et al., 2005). Our results showed that chronic exposure to clotrimazole did not lead to oxidative damage in the fish tissues investigated, as indicated by the unchanged TBARS levels during the experiment. The results for LDH, a key enzyme in the anaerobic pathway for energy production, showed that oxidative stress has a high energetic cost to the cell when the energetic pathways in the liver and brain tissues are changed. The induction of the activity of several antioxidant enzymes may indicate that the fish displayed adaptation mechanisms during exposure to clotrimazole. Glutathione S-transferase, an important component of cellular defence mechanisms, showed high levels in the liver and gill, possibly associated with the detoxification of clotrimazole. Thus, our results are in agreement with previous modelling observations, where clotrimazole was suggested to be able to react with cellular nucleophiles such as glutathione, causing its depletion (Fazlul, 2007).

Haematological parameters, including the differential leukocyte count, are important for toxicological research, and they are useful for predicting possible chemical toxicity in aquatic organisms (Li et al., 2011b). In the sub-chronic clotrimazole study, the main haematological response of the rainbow trout was a significantly lower MCH in the 0.01 $\mu g \cdot L^{-1}$ group exposed for 21 days and a lower MCHC in the 0.01 and 1.0 $\mu g \cdot L^{-1}$ groups exposed for 42 days. The changes observed in MCH and MCHC might be attributed to the possible disruption of haemoglobin synthesis. With respect to leukocytes, only the number of granulocytes increased. Granulocytes act to protect against infection by surrounding and destroying invading bacteria and viruses, and lymphocytes aid in the immune defence system. In this study, such changes in the leukocyte populations may be related to a stress response of the immune system.

From this sub-chronic test, it is clear that the presence of clotrimazole in the aquatic environment represents a risk for fish and can affect them even at the environmentally relevant concentration. Those effects may be influenced by a number of PPCPs that are present during real exposures in the wild.

It should be stressed, that the clotrimazole concentration was not stable during the 24 h period of bath renewal. A high degree of adsorption was observed and described in detail. Verifying the actual exposure under laboratory conditions is crucial for compounds with a high K_{ow} such as clotrimazole.

Conclusions and future perspectives

The toxicity of PPCPs to rainbow trout was evaluated by *in vitro* and sub-chronic *in vivo* tests. The *in vitro* experiments indicated possible effects and specific modes of action for the tested PhACs in non-target organisms, i.e. fish. On the other hand, the *in vivo* experiments provided

valuable data on the actual effects of the PhACs, including possible indirect or compensatory effects relevant at the organism level. Both *in vitro* and *in vivo* studies confirmed that clotrimazole can affect the enzyme activity of CYP3A and CYP1A in rainbow trout. Sub-chronic exposure to PBSA or clotrimazole at the levels present in freshwater environments affected the biochemical processes in rainbow trout. The concentrations of atenolol and verapamil that were tested did not affect selected CYP450 activity in the fish; however, atenolol did affect the fish redox status.

Further studies are needed to investigate the effects of other drugs included in the drug prioritization list for environmental risk assessments. The challenge of environmental toxicology is to characterize the effects of a cocktail of toxins present in the environment amid the possible involvement of receptor crosstalk. Thus, observations under laboratory conditions must be carefully evaluated and extrapolated with caution to real environmental conditions.

Despite the comprehensive research on pollution, there is a continuous need for the investigation and selection of new suitable biomarkers to better understand the possible effects of PPCPs on aquatic life. For example, advanced software in combination with new analytical methods might offer new insights into the effects of PPCPs in organisms at the molecular level. Advanced processing software for metabolomic data may provide a powerful tool for assessing the metabolic fate of bioactive compounds, including pharmaceuticals, within a reasonable time frame. Consequently, investigations into possible effects of PPCPs should not be limited only to biochemical endpoints. Attention should also be paid to the ecological impact, for example, behavioural responses that could have serious consequences for the whole population of aquatic organisms. As such, investigating the biological interactions of emerging contaminants within aquatic organisms remains a challenge for the aquatic toxicologist.

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ENGLISH SUMMARY

Pharmaceuticals in the aquatic environment and their effects in fish

Viktoriia Burkina

Pharmaceuticals and personal care products have significant effects on biological systems. The presence of PPCPs in the aquatic environment have been detected at concentrations from the low $ng \cdot L^{-1}$ to the $\mu g \cdot L^{-1}$ level. The effects of PPCPs have received considerable attention in recent years, and several PPCPs have been identified as emerging environmental pollutants. However, there is still a limited number of studies reporting the effects of environmentally relevant concentrations of PPCPs on aquatic organisms.

In this thesis, the impact of atenolol, verapamil, dexamethasone, clotrimazole, and PBSA on the physiological condition and health of rainbow trout (*Oncorhynchus mykiss*) were studied *in vivo* and *in vitro*.

Atenolol is a human pharmaceutical used in medicine as a selective beta-blocker. The sub-chronic test of atenolol, including at the environmentally relevant concentration, demonstrated that only the fish exposed to atenolol at a concentration of 1000 $\mu g \cdot L^{-1}$ may be at a higher risk of oxidative stress.

The second pharmaceutical compound studied was an L-type calcium channel blocker, verapamil. Despite the relatively high concentration of verapamil included in the experiments, the rates of seven hepatic CYP450-mediated reactions (EROD, MROD, PROD, BFCOD, COH, and PNPH) were unchanged in the exposed rainbow trout. This indicates that the activities of the selected CYP450 enzymes were not affected. Unfortunately, knowledge of verapamil metabolism in fish is lacking, and further investigation is needed to determine the involved CYP450 isoforms.

Chemical compounds such as UV filters have recently been recognized as a group of emerging pollutants in the aquatic environments; therefore, the effect on fish by the UV filter PBSA was tested in a sub-chronic test. Exposure to the highest concentration of PBSA (1000 $\mu g \cdot L^{-1}$) led to significantly inhibited glutathione reductase activity. The activities of the EROD, MROD, and PROD enzymes gradually increased, beginning at the environmentally relevant concentrations. We suggest that long-term exposure to PBSA could cause oxidative stress in fish, as indicated by the decreased activity of GR. Moreover, PBSA could interact with intracellular receptors due to the elevated activities of CYP1A (EROD and MROD) and CYP2B (PROD).

Clotrimazole is an antifungal pharmaceutical widely used in human and veterinary medicine for the treatment of dermatological and gynaecological fungal infections. Rainbow trout showed multiple responses after exposure to clotrimazole. Changes in the haematological parameters in the exposed rainbow trout suggested that the immune system and haemoglobin synthesis were affected. The antioxidant defence enzymes that responded to the clotrimazole exposure influenced the oxidative stress status of the fish. Sub-chronic exposure to clotrimazole did not lead to oxidative stress in any of the fish tissues studied, as indicated by the unchanged TBARS levels. Hepatic energetic pathways were activated in the tested groups after 42 days of exposure to clotrimazole. High levels of glutathione S-transferase were seen in the liver and gill tissues, possibly associated with the detoxification of clotrimazole. The interaction of clotrimazole with intracellular receptors can be expected due to the changes in the catalytic activities of CYP1A1 and CYP3A. The BCF levels in the muscle tissue suggest a moderate bioaccumulation of CLO. The relatively long elimination period for CLO in the fish kidney (half-life = 29 days) may imply a greater potential for adverse effects in this organ. This finding is significant in view of the structural disturbances that appeared in the kidney and testis. The

changes observed in the kidney were most prominent in the tubular epithelial cells. Changes in the gonads were only visible in the male specimens, and the pathological changes in the testis may indicate a disruption of steroidogenesis.

The potential of clotrimazole and dexamethasone to inhibit CYP450 enzyme activity in rainbow trout hepatic microsomes was investigated *in vitro*. Clotrimazole non-competitively inhibited EROD activity in the hepatic microsomes, while BFCOD activity was competitively inhibited. PNPH activity was only slightly reduced in the presence of clotrimazole, indicating that it does not significantly interact with CYP2E1. The presence of dexamethasone in the incubations did not affect any of the CYP450 enzymes investigated.

Everything considered, the results of the study confirmed that the presence of clotrimazole in the environment is of concern with respect to its impact on the health status of fish.

In this thesis, the data of the actual effects of several PPCPs on fish were discussed. Based on the response of selected molecular endpoints, the cardiovascular and anti-inflammatory drugs studied do not present a significant risk for fish, while the antifungal drug and UV-screening agent that were tested may disrupt several physiological processes in fish.

CZECH SUMMARY

Farmaka ve vodním prostředí a jejich účinky na ryby

Viktoriia Burkina

Farmaka a přípravky pro osobní péči (PPCP) jsou ve vodním prostředí detekovány v koncentracích od ng L^{-1} až po µg L^{-1}. Látky patřící mezi PPCP mohou mít významný vliv na biologické systémy. Účinky PPCP na vodní organizmy získaly v nedávné době značnou pozornost a některé PPCP byly identifikovány jako závažné polutanty znečišťující životní prostředí. Nicméně v současné době existuje pouze omezené množství studií zabývajících se účinky PPCP na vodní organizmy v koncentracích běžně se vyskytujících ve vodním prostředí. Tato práce se zabývá *in vivo* a *in vitro* studiem vlivu atenololu, verapamilu, dexamethasonu, clotrimazolu a PBSA na fyziologický stav a zdraví pstruha duhového (*Oncorhynchus mykiss*).

Atenolol je farmakum používané v humánní medicíně jako selektivní beta-blokátor. Subchronický test zahrnující environmentálně relevantní koncentrace atenololu prokázal, že vyšší riziko vzniku oxidativního stresu je pouze u ryb vystavených působení atenololu v koncentraci minimálně 1 000 $\mu g \cdot L^{-1}$.

Druhým studovaným léčivem byl verapamil, blokátor kalciových kanálů typu L. Přes relativně vysoké koncentrace verapamilu zahrnuté do experimentu nebyla u exponovaných pstruhů duhových zjištěna změna úrovně žádné ze sedmi jaterních reakcí zprostředkovaných enzymatickým systémem CYP450 (EROD, MROD, PROD, BFCOD, COH a PNPH). Tyto výsledky naznačují, že aktivita vybraných CYP450 nebyla verapamilem ovlivněna. Metabolizmus verapamilu u ryb zatím nebyl popsán a identifikace izoforem CYP450 účastnících se metabolické transformace verapamilu bude vyžadovat další pozornost.

Chemické látky používané jako UV filtry byly nedávno identifikovány jako skupina nových znečišťujících látek vodního prostředí. Vliv PBSA ze skupiny UV filtrů byl na rybách testován v sub-chronickém testu. Expozice nejvyšší koncentraci PBSA (1 000 $\mu g \cdot L^{-1}$) vedla k významné inhibici aktivity glutathionreduktasy (GR). Aktivita EROD, MROD, PROD se zvyšovala v závislosti na koncentraci PBSA postupně od environmentálně relevantních koncentrací. Snížená aktivita GR naznačuje, že dlouhodobé působení PBSA může u ryb zvýšit oxidativní stres. Na základě zjištění zvýšené aktivity CYP1A (EROD a MROD) a CYP2B (PROD) je možné předpokládat interakce PBSA s intracelulárními receptory.

Clotrimazol je fungicidní léčivo široce používané v humánní i veterinární medicíně pro léčbu dermatologických a gynekologických plísňových infekcí. U pstruha duhového byla zjištěna po vystavení clotrimazolu řada fyziologických poruch. Změny hematologických parametrů exponovaných ryb naznačují, že u pstruha duhového došlo k ovlivnění imunitního systému a poruchám syntézy hemoglobinu. Antioxidační obranné enzymy také reagovaly na expozici clotrimazolu a tím ovlivnily oxidativní stav exponovaných ryb. Nezměněná úroveň TBARS ve všech studovaných tkáních ryb sub-chronicky exponovaných clotrimazolu nepotvrdila oxidativní poškození lipidů. U ryb exponovaných 42 dnů clotrimazolu byla zjištěna aktivace jaterních energetických drah. Vysoká aktivita glutathion-S-transferasy byla zjištěna v játrech a žábrách, tedy v orgánech pravděpodobně spojených s detoxikačními procesy clotrimazolu. Na základě zjištěných změn v katalytické aktivitě CYP1A1 a CYP3A lze očekávat interakce clotrimazolu s intracelulárními receptory. Úroveň biokoncentračního faktoru (BCF) ve svalové tkáni naznačuje mírný bioakumulační potenciál clotrimazolu. Relativně vysoký poločas rozpadu clotrimazolu v rybích ledvinách, téměř 29 dnů, představuje větší potenciál pro nepříznivé působení na tento orgán. Toto zjištění je důležité s ohledem na strukturální změny zjištěné histologickým vyšetřením ledvin a varlat. Změny pozorované v ledvinách byly nejvýraznější

u tubulárních epitelových buněk. Histologické změny gonád byly viditelné pouze u samců, přičemž patologické změny varlat mohou způsobit narušení steroidogeneze.

Potenciál clotrimazolu a dexamethasonu inhibovat činnost enzymatické skupiny CYP450 v jaterních mikrozomech byl u pstruha duhového zkoumán také pomocí *in vitro* experimentu. Clotrimazol způsobil v jaterních mikrozomech pstruha duhového nekompetitivní inhibici aktivity EROD. BFCOD aktivita byla clotrimazolem inhibována kompetitivně. PNPH aktivita byla za přítomnosti clotrimazolu snížena jen mírně, což naznačuje fyziologicky nevýznamnou interakci clotrimazolu s CYP2E1. Přítomnost dexamethasonu neovlivnila žádné studované enzymy CYP450. V souhrnu výsledky této studie potvrdily, že přítomnost clotrimazolu v životním prostředí je znepokojující, pokud jde o jeho dopad na zdravotní stav ryb.

Tato práce shrnuje aktuální údaje o účincích některých PPCP na ryby. Studovaná kardiovaskulární a protizánětlivá léčiva nepředstavují na základě odezvy vybraných molekulárních markerů významné riziko pro ryby, zatímco testované fungicidní léčivo a UV filtr mohou u ryb narušit řadu fyziologických procesů.

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TRAINING AND SUPERVISION PLAN DURING STUDY

Name	Viktoriia Burkina
Research department	2010–2014 Laboratory of Environmental Chemistry and Biochemistry of FFPW
Daily supervisor	
Supervisor	Assoc. Prof. Dipl.-Ing. Vladimír Žlábek, Ph.D.
Period	1st October 2010 until 30th September 2014

Ph.D. courses	**Year**
Pond aquaculture	2011
Basic of scientific communication	2011
Applied Hydrobiology	2011
Ichthyology	2012
English Language	2013

Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	2011 2012
Environmental Chemistry and Ecotoxicology (Masaryk University, Brno)	2012
Seminar days of RIFCH and FFPW	2012
Basic principles of diagnostic pathology (FFPW, Vodňany)	2013
Seminar days of RIFCH and FFPW	2014

International conferences	**Year**
Burkina, V., Zamaratskaia, G., Randak, T., Li, Z.H., Fedorova, G., Pickova, J., Zlabek, V. Verapamil does not modify catalitic activites of CYP rainbow trout after long term exposure. 15th International conference on diseases of fish and shellfish, Split, Croatia, 12–16 September 2011, Abstract book, p. 248. (Poster presentation).	2011
Burkina, V., Zamaratskaia, G., Randak, T., Li, Z.H., Fedorova, G., Pickova, J., Zlabek, V. Cardiovascular drug verapamil has no significant effect on activity of CYP1-3 representatives in liver of rainbow trout. AQUA, Prague, the Czech Republic, 1-5 September 2012, Abstract book, p. 174. (Poster presentation).	2012
Burkina, V., Zlabek, V., Zamaratskaia, G. *In vitro* inhibition of hepatic cytochrome P450-mediated reaction by human pharmaceuticals in rainbow trout microsomes. DIFA II, Vodnany, Czech Republic, 24–26 September 2013, Abstract book, p. 48. (Oral presentation).	2013
Burkina, V., Oliveira, R., Schmidt-Posthaus, H., Domingues, I., Fedorova, G., Steinbach, C., Sakalli, S., Randak, T., Zlabek, V. Biological and structural changes in rainbow trout (*Oncorhynchus mykiss*) after long-term exposure to clotrimazole. SETAC Europe 24th Annual Meeting, Basel, Switzerland, 11-15 May 2014, Abstract book, TU 132. (Poster presentation).	2014

Foreign stays during Ph.D. study at RIFCH and FFPW	**Year**
Assoc. Prof. Galia Zamaratskaia, Ph.D., Department of Food Science, Swedish University of Agricultural Sciences, Uppsala, Sweden (2 weeks)	2011
Assoc. Prof. Galia Zamaratskaia, Ph.D., Department of Food Science, Swedish University of Agricultural Sciences, Uppsala, Sweden (2 weeks)	2012
Assoc. Prof. Ines Domingues, Ph.D., CESAM & Department of Biology, University of Aveiro, Portugal (1 month)	2012
Assoc. Prof. Ines Domingues, Ph.D., CESAM & Department of Biology, University of Aveiro, Portugal (2 months)	2013
Assoc. Prof. Galia Zamaratskaia, Ph.D., Department of Food Science, Swedish University of Agricultural Sciences, Uppsala, Sweden (1 month)	2014

CURRICULUM VITAE

Surname: **Burkina**
First name: **Viktoriia**
Title: **Master of Science (M.Sc.)**
Born: 10th September, 1987
Nationality: Ukranian
Marital status: Single

EDUCATION

2010–present Ph.D. student, University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, Laboratory of Environmental Chemistry and Biochemistry
2008–2009 M.Sc., V.N.Karazin Kharkov National University, Ukraine (Biophysics)
2004–2008 B.Sc., V.N.Karazin Kharkov National University, Ukraine (Biophysics)

PH.D. COURSES

Pond Aquaculture, Basic of Scientific Communication, Applied Hydrobiology, Ichthyology, English Language
Environmental Chemistry and Ecotoxicology (Masaryk University, Brno), Basic Principles of Diagnostic Pathology (University of South Bohemia, Vodňany)

RESEARCH INTEREST

Evaluation of potential impacts of pharmaceutical compounds discharged into the aquatic environment, focusing on possible biological effects in fish by suitable biochemical markers

RESPONSIBLE LEADER OF PROJECTS

International Summer School 2013: Pharmaceuticals in the aquatic environment and their effects in fish. Responses of antioxidant defence system in liver of rainbow trout (*Oncorhynchus mykiss*), chronically treated with diltiazem
International Summer School 2014: Pharmaceuticals in the aquatic environment and their effects in fish. Responses of antioxidant defence system in liver and gill of rainbow trout (*Oncorhynchus mykiss*), chronically treated with dexamethasone

FOREIGN STAYS DURING PH.D. STUDY AT RIFCH AND FFPW

Assoc. Prof. Galia Zamaratskaia, Ph.D., Department of Food Science, Swedish University of Agricultural Sciences, Uppsala, Sweden (2 months)
Assoc. Prof. Ines Domingues, Ph.D., CESAM & Department of Biology, University of Aveiro, Portugal (3 months)