Fakulta rybářství
a ochrany vod
Faculty of Fisheries
and Protection
of Waters

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

Bioactive compounds in the aquatic environment and their effects on fish – a special focus on piscine cytochrome P450

Bioaktivní látky ve vodním prostředí a jejich vliv na ryby – zaměření na cytochromy P450 v rybách

Sidika Sakalli

Czech Republic, Vodňany, 2018

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Date, hour, and place of Ph.D. defence:
20th September 2018 at 15:30 in USB, FFPW, RIFCH, Vodňany, Czech Republic

Name: Sidika Sakalli
Title of thesis:
Bioactive chemicals in the aquatic environment and their effects on fish – a special focus on piscine Cytochrome P450
Bioaktivní látky ve vodním prostředí a jejich vliv na ryby – zaměření na cytochromy P450 v rybách

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Ph.D. thesis, USB FFPW, RIFCH, Vodňany, Czech Republic, 2018, 114 pages, with the summary in English and Czech.

Graphic design & technical realisation: JENA Šumperk, www.jenasumperk.cz

ISBN 978-80-7514-077-7

CONTENT

LIST OF ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
BFC	7-benzoyloxy-4-trifluoromethyl coumarin
BFCOD	7-benzyloxy-4-trifluoromethylcoumarin O-debenzylase
BQ	7-benzyloxyquinoline
BQOD	7-benzyloxyquinoline O-debenzylase
CAT	Catalase
CF	Condition factor
CI	Combination index
CLO	Clotrimazole
CYP	Cytochrome P450
DEX	Dexamethasone
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
EPA	Eicosapentaenoic acid
ER	7-ethoxyresorufin
EROD	7-ethoxyresorufin O-deethylase
FA	Fatty acids
GPx	Glutathione peridoxase
GR	Glutathione reductase
GST	Glutathione S-transferase
HAHs	Halogenated aromatic hydrocarbons
Hb	Hemoglobin concentration
HFC	7-hydroxy-4-trifluoromethylcoumarin=
HIS	Hepatosomatic index
I3C	Indole-3-carbinol
LDH	Lactate dehydrogenase
Leuko	Leukocyte count
LPO	Lipid peroxidation
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular erythrocyte volume
MeOH	Methanol
MUFA	Monounsaturated fatty acids
NADPH	Reduced β-nicotinamide adenine dinucleotide phosphate
nMDS	Non-metric multidimensional scaling
NSAIDs	Nonsteroidal anti-inflammatory drugs
OECD	Organization for Economic Co-Operation and Development
PAHs	Polycyclic aromatic hydrocarbons
PBC	Polychlorinated biphenyls
PCV	Hematocrit
PNPH	p-nitrophenol hydroxylase
POCIS	Polar Organic Chemical Integrated Sampler
PPCPs	Pharmaceuticals and personal care products
PUFA	Poly unsaturated fatty acids
PXR	Pregnane-X receptor
RBC	Red blood cell count
ROS	Reactive oxygen species
SFA	Saturated fatty acids
STPs	Sewage treatment plants

CHAPTER 1

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

The term "bioactive compounds" is a general term referring to chemicals that affect or trigger a response in living organisms, tissues, or cells. Bioactive compounds encompass a wide range of substances that are produced naturally by microorganisms, plants, and animals or via synthetic means (Fig. 1). It is well known that these compounds may reach aquatic environments and can cause adverse effects on non-target organisms such as fish. Awareness of the existence of these compounds in the aquatic environment has increased after technological methods for their detection have been developed (Kot-Wasik et al., 2007). Development of new analytical methods with lower detection limits has made it possible to detect various compounds that may have adverse effects on fish. This thesis is focused on the effect of several bioactive compounds (such as pharmaceuticals, phytochemicals, or complex mixtures of pollutants) on fish using *in vitro, in vivo*, and *in situ* methods.

Phytochemicals as secondary metabolites of plants are naturally occurring biologically active compounds. Numerous plants synthesize these secondary metabolites as a primary defence mechanism against environmental stress, damage, and insects (Bennett and Wallsgrove, 1994). Phytochemicals have been intensively used in humans for their beneficial health benefits mainly for anti-cancer, antioxidant, free radical scavenging, anti-viral, and/or anti-inflammatory purposes; however, several phytosterols and phytoestrogens can cause adverse effects on fish as non-target species (Jarošová et al., 2015). Moreover, a wide variety of plant leaves, seeds, and extracts are poisonous to fish (Neuwinger, 2004). Plant-originated bioactive compounds are released into the aquatic environment via processing of plant materials (such as pulp and paper mills) or sewage treatment plants or they can occur naturally in different bodies of water. Several cases in which the effluents affected fish reproduction have been with respect to for pulp and paper mill (Hewitt et al., 2008; Munkittrick et al., 2013) or soybean processing effluents (Csaky and Feteke, 2004). Microorganisms such as cyanobacteria produce and release various types of bioactive compounds into surface waters during cyanobacterial blooms that can cause adverse effects on organisms (Bláha et al., 2009). These blooms can cause death, liver failure, respiratory and osmotic balance problems in fish (Wiegand and Pflugmacher, 2005). Biologically active compounds of animal origin can be released into the environment from animal husbandry farms. Even humans excrete hormones that can cause adverse effects in aquatic organisms (Ying et al., 2002). Synthetically produced bioactive compounds are chemicals that include pharmaceuticals, polychlorinated biphenyls (PCBs), dioxins, and pesticides that have with significant biological activities.

Pharmaceuticals and personal care products (PPCPs) such as medications, cosmetics, lotions, soaps, and shampoos represent one of the major groups of compounds that are used for improving the quality of daily life. PPCPs may have either beneficial or adverse effects on target organisms, and these effects depend on their dose, substance, or bioavailability. In the European Union (EU), there are nearly 3000 different chemicals used in medicines such as antibiotics, nonsteroidal anti-inflammatory drugs (NSAIDs), anti-inflammatories, beta-blockers, steroid hormones, and lipid regulators (Fent et al., 2006). PPCPs fate and existence in aquatic environments have been recently recognised as an emerging issue in environmental chemistry due to exposure of non-target organisms (Baker et al., 2013; Heberer, 2002). There are several ways in which PPCPs are released into water. After their intake, PPCPs or their metabolites are excreted by urine and faeces or they may be released directly by disposal of unused drugs into sewage effluent (Andreozzi et al., 2003). PPCPs can enter surface water via wastewater treatment, aquaculture or agricultural systems (Boxall et al., 2012; Ternes, 1998). Sewage treatment plants (STPs) are designed to remove these chemicals using physical, chemical, and biological processes. However, STPs are often insufficient to eliminate some of

these contaminants; therefore, they are considered one of the major sources of PPCPs. PPCPs have been found in drinking, ground, sewage, and waste waters. Their concentrations are generally low as ng L^{-1} or µg L^{-1} (Fedorova et al., 2014; Heberer, 2002; Montes-Grajales et al., 2017; Pascoe et al., 2003). Despite numerous studies involving pharmaceuticals in the aquatic environment, there is still lack of information about their ecotoxicological effects on aquatic organisms and wildlife (Carlsson et al., 2006; Fent et al., 2006).

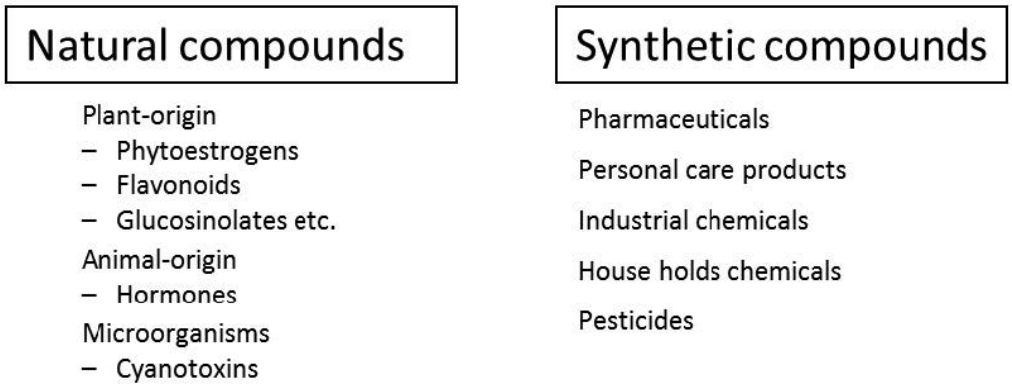

Figure 1. *Sources of bioactive compounds found in the aquatic environment.*

Xenobiotic metabolism in fish via cytochrome P450

Xenobiotic metabolism is the ability of an organism to eliminate foreign compounds that enter their systematic circulation or organs either intentionally or unintentionally (Sinz, 2012). The main xenobiotic elimination pathway is enzymatic biotransformation that generally includes Phase I and II metabolic reactions. Phase I metabolism involves namely three groups of reactions: (1) oxidation; (2) reduction; and/or (3) hydrolyses of the xenobiotics, while phase II metabolism involves conjugation reactions to increase xenobiotic polarity for the purpose of elimination. In some cases, metabolism produce toxic compounds.

The most important group of Phase I metabolic enzymes consists of cytochrome P450 (CYP). CYP indicates the CYP superfamily. These are membrane-associated proteins and contain heme cofactors. CYP metabolism occurs in various organs such as gills, intestines, and liver (Andersson and Förlin, 1992; Bartram et al., 2012; Burkina et al., 2015b; James et al., 2005; Yuen and Au, 2006). Piscine hepatic CYPs, as biomarkers of environmental xenobiotics, have been well studied (Anderson et al., 1996; Arinç et al., 2001; Burkina et al., 2015a; Burkina et al., 2016a; Sakalli et al., 2018; Zlabek et al., 2016). In humans, the intestine has a huge metabolic capacity, and the microflora that are present in the small intestine can also metabolize xenobiotics to some extent as they move along (Sinz, 2012; Xu et al., 2005). This metabolism particularly happens when orally-ingested or intestinally-applied pharmaceuticals are used (Kaminsky and Zhang, 2003; Xie et al., 2016). Similarly, in fish intestines, CYP enzymes are activated after exposure to dietary or orally-administrated xenobiotics (James et al., 2005; Yuen and Au, 2006). Moreover, intestinal CYP activity is indirectly affected by the antibiotics that can alter the intestinal microbiome (Martin et al., 2016; Zhang et al., 2014). Information on intestinal CYPs in fish is still lacking. Intestinal CYP activities were previously investigated in fish exposed to environmental xenobiotics (Doering et al., 2012; Hegelund and Celander, 2003; James et al., 2005; Yuen and Au, 2006). Previous studies have shown that observed intestinal CYPs activities might be significant for first-pass metabolism in addition to ingested xenobiotics.

There are a total of 137 CYP genes that have been identified in fish species (Burkina et al., 2017; Burkina et al., 2015b; Goldstone et al., 2010; Nelson, 2003; Zhang et al., 2014) and 57 CYP genes identified in human (Hasler et al., 1999). The numbers of genes vary between species (Goldstone et al., 2010; Nelson, 2003). Some CYPs can be induced by wide range of xenobiotics (Zanger and Schwab, 2013). CYP families 1, 2, and 3 are mainly associated with xenobiotic metabolism. In this thesis, these three CYPs and their relationships with environmental pollutants in fish are discussed.

Generally, four subfamilies of CYP1 family exist in fish (CYP1A–D). CYP1A has been widely studied as a biomarker in fish that were exposed to environmental contamination. Exposure to polycyclic aromatic hydrocarbons (PAHs) or halogenated aromatic hydrocarbons (HAHs) induces CYP1A. Induction of CYP1A can be measured either at the gene expression or protein levels or as 7-ethoxyresorufin-O-deethylase (EROD) activity, which is a known CYP1A marker reaction (Beijer et al., 2013; Carlos et al., 2013; Oliva et al., 2014). CYP1A gene transcription can be induced through xenobiotics that activate the aryl hydrocarbon receptor (AhR). Dioxin-like compounds (such as PAH or HAHs) evoke their toxicity mainly via the AhR pathway. In addition, other compounds (such as pharmaceuticals) have been reported to affect CYP1A activity in fish (Bartram et al., 2012; Burkina et al., 2016a; Sakalli et al., 2018; Smith et al., 2012). The AhR contains a gene superfamily encoding the transcription factors that senses endogenous and exogenous xenobiotics and transfers this sensing into cellular responses by regulating the expression of these compounds' target genes (Bielefeld et al., 2008; Jönsson et al., 2010; Stevens et al., 2009). Regulation of gene expression at the transcriptional level by AhR plays a crucial role in xenobiotic metabolism and clearance in order to protect an organism from environmental threats (Xu et al., 2005).

The CYP2 family has the highest number of CYP genes in fish, which contain 13 CYP2 subfamilies (Uno et al., 2012). However, information on these genes in fish is limited and not yet well understood. Evidence shows that CYP2A-, CYP2B-, and CYP2E-like proteins are present in fish (Celander et al., 1996b). In mammals, CYP2E1 is associated with ethanol metabolism, and several substrates of CYP2E1 such as ethanol or acetone act as inducing agents of this enzyme. Only a few pharmaceuticals are metabolised via this enzyme (such as paracetamol; Zanger and Schwab, 2013; Zhou et al., 2015). The activity of P-nitrophenol hydroxylase (PNPH) is generally used to estimate mammalian CYP2E1 activity. The presence of such activity in fish suggests the possibility of its involvement in xenobiotic detoxification (Zamaratskaia and Zlabek, 2011). PNPH activity has been studied in fish (Burkina et al., 2013; Geter et al., 2003; Sakalli et al., 2015; Zamaratskaia and Zlabek, 2011).

Mammalian CYP3A is involved in the metabolism of ~50% of currently available drugs (Bachmann, 2009; Stanley, 2017; Zanger and Schwab, 2013). In fish, CYP3A-like activity is usually measured as 7-benzyloxy-4-trifluoromethylcoumarin O-debenzylase (BFCOD). CYP3A gene expression and its regulation have been extensively studied in fish recently (Ding et al., 2016; Felício et al., 2016; Kropf et al., 2016). CYP3A is induced by a variety of compounds, including steroidal chemicals such as glucocorticoids. CYP3A expression is generally associated with pathways regulated by the pregnane-X receptor (PXR), which is predominantly expressed in liver, intestines, and kidneys (Xu et al., 2005).

Numerous cells (such as those derived from fish liver and gills) or subcellular fractions (such as microsomal, cytosolic, and mitochondrial fractions) within each organ contain xenobiotic-metabolizing enzymes (Sinz, 2012). Microsomes are the gold standard for phase I metabolism studies, and their genotype variations are usually minimised by pooling multiple liver samples together. In addition, they can be used for both *in vivo* and *in vitro* studies.

When performing CYP inhibition studies, the incubation medium should contain substrates and potential inhibitors; in this study, aquatic pollutants and dissolved in organic solvents

(such as methanol, acetonitrile, dimethyl sulfoxide) which may affect the CYP activity were used. It is obvious that some organic solvents alter the activity of CYPs in human (Busby et al., 1999; Chauret et al., 1998; Easterbrook et al., 2001; Hickman et al., 1998; Vuppugalla et al., 2007), and rat (Kawalek and Andrews, 1980; Wang et al., 1996). However, effects of these solvents in fish CYP activity is unknown. Therefore, prior to further experiments, a methodical study using several organic solvents was performed to identify the concentrations of solvents that can alter fish CYP activity. The details of this study are described in Chapter 2.1.

Methods

Evaluating and predicting the combined effect of several chemicals on aquatic organisms or ecosystem is a great challenge in ecotoxicology. Biomarkers help us to predict the effects of toxic chemicals in exposed organisms. These biomarkers can be tested via several bioassays which target different levels of molecules, cells, organs, organisms, or populations. Therefore, investigation of bioassays can be performed under laboratory conditions (using *in vitro* or *in vivo* systems) or in the field, under natural condition (*in situ* systems). There have been numerous *in vitro, in vivo, in situ* studies done to identify the effects of contaminants in fish. Their advantages and disadvantages are listed in Table 1. Many studies are limited to the use of one or a few chemical due to laboratory conditions; therefore, natural conditions significantly differ from laboratory data.

In vitro

In vitro systems provide valuable information regarding metabolism of xenobiotics in aquatic organisms. To understand the mode of action of chemicals, *in vitro* assays can be useful tools. In addition, animal rights' concerns are one of the main issues for the risk assessment of environmental pollutants due to the significant number of animals used for fulfilling test requirements. After public concerns increased about research animals' welfare, Russell and Burch (1959) established the concept "3Rs" (replacement, reduction, refinement) for the humane use of testing animals. Therefore, *in vitro* assays are more acceptable testing models (Hecker and Hollert, 2011; van der Burg et al., 2010; Wang et al., 2014). These assays are commonly used to monitor the effects of environmental contaminants (Wang et al., 2014). Fish cell lines (Babin et al., 2005; Celander et al., 1996a; Creusot et al., 2015; Della Torre et al., 2011) and microsomes (Burkina et al., 2016b; Burkina et al., 2013; Sakalli et al., 2018) have been previously used for identifying the effects of xenobiotics on piscine CYPs. Details and description of methods can be found in Chapter 2. Although *in vitro* studies provide useful information on drug metabolism, *in vivo* effects of xenobiotics on the whole organism cannot be fully interpreted from *in vitro* assays. Therefore, to identify the whole organism response to exposure to xenobiotics, *in vivo* studies are more ecologically relevant when compare with *in vitro* studies.

In vivo

In vivo-based bioassays test the effects of single or a mixture of compounds or extracts from contaminated matrixes such as water, sediment, or passive samplers on whole organisms exposed to these compounds. Generally, *in vivo* assays that are conducted in the laboratory have controlled experimental conditions so that environmental stress factors can be eliminated (Connon et al., 2012). *In vivo* systems can provide better understanding of the components that contribute to overall metabolism such as first-pass and/or extrahepatic

metabolism (Sinz, 2012). In addition, several complex biomarkers, overall health status, and/or organism behaviour can be tested. These tests generally follow standardised testing guidelines suggested by the Organisation for Economic Co-Operation and Development (OECD). Several species such as *Daphnia magna, Danio rerio, Pimpephales promelas, Oncorhynchus mykiss,* and *Cyprinus carpio* are used to measure standardised acute or chronic toxicities. Freshwater species are more convenient to use in *in vivo* assays because they are easy to rear in the laboratory or on fish farms and are widely available throughout the year (OCDE, 2012). In this thesis, Chapter 3 includes two *in vivo* experiments performed under laboratory conditions using fish exposed to several concentrations of pharmaceuticals. Because *in vivo* assays ignore the physical and biological stressors that the organisms face in the natural environment, *in situ* assays give us more specific information for understanding the effects of contaminants in real scenarios.

In situ

In situ systems, in which organisms are exposed to various mixed chemical concentrations under natural conditions, are more environmentally meaningful than *in vitro* or *in vivo* tests because *in situ* conditions can reflect the actual effects on wild populations (Connon et al., 2012). Recent developments in analytic chemistry have made it possible to perform *in situ* assays because of the identification of the chemicals that are present in the aquatic environment at low concentrations. *In situ* assays integrate the effects of complex environmental conditions and various chemical-associated exposures. They are generally performed in a caged system (Ings et al., 2011), flow-through system, (Giang et al., 2018) or in a pond (Giang et al., 2017) that receives water from STP discharge. There are no standardised methods for *in situ* studies; therefore, certain issues should be considered during planning. Organisms that will be used in the experiment should not be stressed by their enclosure. For example, cage studies have been applied to give an alternative for field scenarios; however, stress factors of the fish in caging studies must be well thought out in advance of the experiments. Another issue is that chemicals that are present in the experimental system should be monitored in order to identify their possible effects in the organism. In addition, control sites should have similar environmental conditions (without added contaminants) as the experimental site so that different results in the experimental conditions can be linked to the added contaminants.

Table 1. *Advantages and disadvantages of experimental methods.*

	In vitro	*In vivo*	*In situ*
Time consuming [1]	-	+	+
Cost-effective [1, 2, 3]	+	-	-
Understanding the mode of action of chemical [2, 4]	+	-	-
Ideal for specific receptor-mediated mechanism or for specific toxicity endpoint to be determined [2, 5]	+	-	-
Ideal for evaluating the effects of PPCPs on fish at environmentally relevant concentrations [6, 7, 8]	-	+	+
Considering the factor of cell compensatory mechanisms [1]	-	+	+
Extrapolation to environmental conditions where mixture effects may influence the overall harmful action of the individual chemicals [1]	-	+	+
The ability to maintain specific experimental conditions [1, 2, 4]	+	+	-
Possible to explore the entire effect of pollutant without excluding any biochemical pathway [1]	-	+	+
Ethical in the use of animals to fulfil testing requirements [1, 2, 3]	+	-	-

[1](Connon et al., 2012); [2](Smith, 2009); [3](Hecker and Hollert, 2011); [4](Sinz, 2012); [5](Eisenbrand et al., 2002); [6](Burkina et al., 2016a); [7](Giang et al., 2017); [8](Giang et al., 2018)

The hypothesis is that certain bioactive compounds at certain concentrations affect fish as non-target organisms; moreover, combinations of two or more compounds might increase or decrease the effects by acting in a synergistic, antagonistic, and/or additive manner. In addition, performing different experimental studies provides specific information on cellular responses, modes of action, biochemical responses of whole organisms, and effects of environmental stressors.

The aim of this thesis

The aim of this thesis was to identify the effects of selected bioactive compounds on fish CYPs using several methods:

(1) *In vitro* effects of the phytochemicals, naringenin, quercetin, diosmin, and indole-3-carbinol on fish CYP1A and their combined behaviour in the presence of dexamethasone or clotrimazole. In addition, a methodological study was performed to identify the effects of carrier organic solvents on widely used CYP biomarkers;

(2) *In vivo* exposure of fish to dexamethasone and clotrimazole at environmentally relevant concentrations using a wide range of biomarkers; and

(3) *In situ* experiments*:* Effects of complex PPCPs mixtures on fish exposed to STP effluents' discharge water under natural conditions.

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

Paper 1.
Sakalli, S., Burkina, V., Zlabek, V., Zamaratskaia, G., 2015. Effects of acetone, acetonitrile, ethanol, methanol and DMSO on cytochrome P450 in rainbow trout (*Oncorhynchus mykiss*) hepatic microsomes. Toxicology Mechanisms and Methods 25, 501–506.
Sidika Sakalli was directly involved in the experimental design, sample preparation, measurement of the cytochrome enzyme activity, data processing and the preparation of the manuscript.

Paper 2.
Sakalli, S., Burkina, V., Pilipenko, N., Zlabek, V., Zamaratskaia, G., 2018. *In vitro* effects of diosmin, naringenin, quercetin and indole-3-carbinol on fish hepatic CYP1A1 in the presence of clotrimazole and dexamethasone. Chemosphere 192, 105–112.
Sidika Sakalli was directly involved in the experimental design, sample preparation, measurement of the cytochrome enzyme activity, data processing, statistics and the preparation of the manuscript.

Paper 3.
Burkina, V., Sakalli, S., Rasmussen, M.K., Zamaratskaia, G., Koba, O., Thai, G.P., Grabic, R., Randak, T., Zlabek, V., 2015. Does dexamethasone affect hepatic CYP450 system of fish? Semi-static *in vivo* experiment on juvenile rainbow trout. Chemosphere 139, 155–162.
Sidika Sakalli participated in the research design, took care of the fish during the exposure period, participated in three sampling events, preparation of microsomal fractions and manuscript preparation.

Paper 4.
Burkina, V., Zamaratskaia, G., Oliveira, R., Fedorova, G., Grabicova, K., Schmidt-Posthaus, H., Steinbach, C., Domingues, I., Golovko, O., Sakalli, S., Grabic, R., Randak, T., Zlabek, V., 2016. Sub-lethal effects and bioconcentration of the human pharmaceutical clotrimazole in rainbow trout (*Oncorhynchus mykiss*). Chemosphere 159, 10–22.
Sidika Sakalli was involved in discussion of received data, their interpretation and in the manuscript preparation.

Paper 5.
Sakalli, S., Giang, P.T., Burkina, V., Zamaratskaia, G., Rasmussen, M.K., Khalili, T.S., Bakal, T., Sampels, S., Kolarova, J., Grabic, R., Turek, J., Randak, Tomas., Zlabek, V., 2018. The effects of sewage treatment plant effluents on hepatic and intestinal biomarkers in common carp (*Cyprinus carpio*). Science of the Total Environment 635, 1160–1169.
Sidika Sakalli participated in the sampling, sample preparation for fatty acid composition analysis, measured intestinal and hepatic cytochrome P450 enzyme activities, data analysis, statistics and the preparation of the manuscript.

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CHAPTER 2

IN VITRO EXPERIMENTS: EFFECTS OF SELECTED BIOACTIVE COMPOUNDS AND CARRIER SOLVENTS ON CYTOCHROME P450 ACTIVITY USING PISCINE MICROSOMES

Sakalli, S., Burkina, V., Zlabek, V., Zamaratskaia, G., 2015. Effects of acetone, acetonitrile, ethanol, methanol and DMSO on cytochrome P450 in rainbow trout (*Oncorhynchus mykiss*) hepatic microsomes. Toxicology Mechanisms and Methods 25, 501–506.

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Toxicology Mechanisms and Methods

http://informahealthcare.com/txm
ISSN: 1537-6516 (print), 1537-6524 (electronic)

Toxicol Mech Methods, 2015; 25(6): 501–506
 DOI: 10.3109/15376516.2015.1074326

Taylor & Francis
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RESEARCH ARTICLE

Effects of acetone, acetonitrile, ethanol, methanol and DMSO on cytochrome P450 in rainbow trout (*Oncorhynchus mykiss*) hepatic microsomes

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Abstract

In vitro impacts of five organic solvents on cytochrome P450 (CYP450) enzyme activity were investigated using hepatic microsomes of rainbow trout. The rates of several CYP450-mediated reactions were investigated at solvent concentrations ranging from 0.01% to 3%. The solvents greatly affected all tested reactions. In at least 0.8% ethanol, 2% methanol or acetone, 1% acetonitrile or 3% dimethyl sulfoxide (DMSO), 7-ethoxyresorufin-O-deethylase (EROD) activity decreased and at 3% acetonitrile or ethanol, it was undetected. At 3%, all tested solvents except methanol reduced 7-benzyloxy-4-trifluoromethylcoumarin-*O*-debenzylase (BFCOD) activity, but at low concentrations of ethanol (2% and lower) or DMSO (1% and lower), it was induced. This was not seen with the inclusion of a pre-incubation step. *p*-Nitrophenolhydroxylase (PNPH) activity was not affected at concentrations below 1% DMSO, and at 2% acetonitrile it was reduced, as it was above 1% methanol or 0.5% ethanol. Acetone did not affect PNPH activity with or without a pre-incubation step. In general, the degree of inhibition was similar with and without the pre-incubation step. We conclude that the concentration of organic solvent for solubilizing the substrate and inhibitor in *in vitro* microsomal studies should be minimized.

Keywords

CYP1A1, CYP2E1, CYP3A, enzyme activity, organic solvent

History

Received 10 March 2015
Revised 11 July 2015
Accepted 14 July 2015
Published online 14 August 2015

Introduction

Cytochrome P450 (CYP450) enzymes play important roles in the metabolism of a large number of xenobiotic and endogenous compounds. They are found primarily in the membranes of the endoplasmic reticulum. Mammalian hepatic and non-hepatic microsomes, which contain CYP450, are commonly used to identify and characterize xenobiotic metabolites, examine the potential for drug–drug and food–drug interactions, and suggest further *in vivo* testing (Chauret et al., 1998). The microsomes are also used to study CYP450 as a biomarker and indicator of environmental contamination. The activities of CYP1A1-dependent 7-ethoxyresorufin *O*-deethylase (EROD) and CYP3A-dependent 7-benzyloxy-4-trifluoromethylcoumarin-O-debenzylase (BFCOD) are the most common assays used in fish to study the effects of environmental pollutants on CYP450 (Uno et al., 2012; Wagner et al., 2013; Zamaratskaia & Zlabek, 2011). CYP2E1 (nitrophenol-hydroxylase), a member of the CYP450 superfamily, mediates activity in the metabolism of ethanol and other low-molecular-weight toxicants and volatile organic pollutants in mammals, and it has been studied in fish (Burkina et al., 2013; Geter et al., 2003; Zamaratskaia & Zlabek, 2011). As an *in vitro* model, microsomes have advantages over hepatocytes: they are less expensive, more readily available and kinetic analysis does not interact with Phase II metabolism. Common substrates and potential inhibitors of microsomal incubations are dissolved inorganic solvents such as methanol, ethanol, acetonitrile and dimethyl sulfoxide (DMSO). However, solvents used to solubilize substrates and inhibitors may themselves affect the rate of CYP450 probe reactions. It is well known that some organic solvents alter the activities of individual CYP450 enzymes in mammals (Kawalek & Andrews, 1980). The effect of organic solvents on the activity of CYP450 has been studied primarily in humans (Busby et al., 1999; Chauret et al., 1998; Easterbrook et al., 2001; Hickman et al., 1998; Vuppugalla et al., 2007) and rats (Wang et al., 1996). To the best of our knowledge, there are no published reports on the effects of organic solvents on CYP450 activities in fish hepatic microsomes. Such studies are, however, of great importance because fish microsomes are increasingly used to investigate

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the effects of environmental pollutants on fish detoxification systems (Burkina et al., 2013; Smith et al., 2012) and to identify their metabolic pathways (Shen et al., 2012). Inhibition of individual enzyme activities in fish hepatic microsomes are also used to identify the enzyme(s) responsible for metabolizing studied compounds (Anzenbacher & Anzenbacherová, 2008; Vestergren et al., 2012).

A majority of investigations on the effects of organic solvents on CYP450 activities were performed using simultaneous applications of a substrate and organic solvent in incubation media. However, investigations of time-dependent inhibitions of CYP450 require pre-incubation of the inhibitor with enzyme-containing media before the addition of substrate. To the best of our knowledge, effects of organic solvents on fish CYP450 activity after pre-incubation have not been studied. We hypothesize that solvents used for solubilization of time-dependent inhibitors might affect the activity of a given enzyme during pre-incubation.

The aim of this study was to determine the effects of organic solvents on 7-ethoxyresorufin-O-deethylase (EROD), 7-benzyloxy-4-trifluoromethylcoumarin-O-debenzylase (BFCOD) and *p*-nitrophenol hydroxylase (PNPH) in rainbow trout microsomes. For this purpose, five organic solvents (acetonitrile, acetone, methanol, ethanol and DMSO) were used at concentrations ranging from 0.01% to 3% in fish microsomal incubations with and without the pre-incubation step.

Material and methods

Chemicals

Acetonitrile (Ind. No. 608-001-003; purity 99.9%), ethanol (Ind. No. 603-002-005; purity 96%) and DMSO (CAS 67-68-5; purity 99.8%; Lichrosolv, Hypergrade) were obtained from Merck (Darmstadt, Germany). Methanol (CAS 67-56-1; purity 99.9%) and acetone (CAS 67-64-1; purity 99.9%) were purchased from Fluka and Scharlau, respectively.

Resorufin, 7-ethoxyresorufin, 7-hydroxy-4-trifluoromethylcoumarin (HFC), 7-benzyloxy-4-trifluoromethylcoumarin (BFC), *p*-nitrophenol (PNP), 4-nitrocatechol, reduced b-nicotinamide adenine dinucleotide phosphate (NADPH) and trichloroacetic acid (TCA) were obtained from Sigma–Aldrich (Czech Republic).

Based on the purity of each solvent, stock solutions of 86.67% (v/v) acetonitrile, acetone, methanol and DMSO and 90% (v/v) ethanol were prepared by dissolving pure solvent in distilled water. Further dilutions were carried out to achieve final concentrations of 0.01, 0.1, 0.5, 0.8, 1, 2 and 3%.

Fish and hepatic microsome preparation

Adult rainbow trout of both sexes of mean ± SD body length 318.1 ± 20.69 mm and weight 368.9 ± 35.78 g were obtained from a local commercial hatchery (Czech Republic). Fish were transferred to aquaria containing 250 L fresh water (temperature, 14 ± 1°C; pH, 7.7 ± 0.1; dissolved oxygen, 8.5–9.0 mg/L). Fish were not fed 24 h prior to sampling to avoid prandial effects during assay. Experimental animals were handled according to national and institutional guidelines for the protection of human subjects and animal welfare.

Liver samples collected from nine fish were immediately frozen and stored at −80 °C until used for microsomal preparation. The hepatic microsomal fraction was prepared from approximately 1 g liver sample using differential centrifugation as described by Burkina et al. (2013).

Protein levels were estimated spectrophotometrically using the method described by Smith et al. (1985), which used bovine serum albumin as a standard. The microsomes were diluted to a protein content of 10 mg/ml. To determine enzyme activity, three pools of nine fish were used.

Microsomal enzyme activity

The EROD activity was measured using the method described by Kennedy & Jones (1994) with modification. Incubation mixtures consisting of 20 µL (0.2 mg) microsomal protein, incubation medium (50 mM potassium phosphate buffer, pH 7.4), 0.5 µL 7-ethoxyresorufin (2 µM) and 15 µL solvent (ranging from 0.01% to 3%) were added to a black 96-well plate. Reaction was initiated by adding 10 µL NADPH (1 mM) to make a total volume of 260 µl in each well.

The BFCOD activity was estimated by finding the rate at which BFC was transformed to HFC using the method of Renwick et al. (2001) with modification. Incubation mixtures consisting of 20 µL (0.2 mg) microsomal protein mixture, incubation medium (50 mM potassium phosphate buffer, pH 7.4), 2.6 µL BFC (150 µM) and 15 µL solvent (ranging from 0.01% to 3%) were added to a 96-well plate. Reaction was initiated by adding 5 µL NADPH (0.5 mM) to make a total volume of 260 µL in each well.

The PNPH activity was estimated by finding the rate at which PNP was transformed to 4-nitrocatechol (Zamaratskaia & Zlabek, 2011). Incubation mixtures consisting of 50 µL (0.5 mg) microsomal protein, incubation medium (50 mM potassium buffer, pH 7.4), 5 µL *p*-nitrophenol (200 µM) and 15 µL solvent (ranging from 0.01% to 3%) were added to an Eppendorf. Reaction was initiated by adding 10 µL NADPH (1 mM) to make a total volume of 250 µL. Reaction was allowed to proceed for 30 min at 21°C. It was stopped by the addition of 10 µL ice-cold 40% TCA.

In the first set of the experiments, the substrate and organic solvents were added to the microsomes simultaneously. In the second set, solvents were pre-incubated with microsomes before the addition of substrates. Plates then were sealed to avoid solvent evaporation. The choice of pre-incubation time, 10 min for EROD and BFCOD and 30 min for PNPH, was based on the results from previous experiments. Incubation time was 15 min for all assays.

A fluorescence detector (Infinite M200; TECAN, Mannedorf, Switzerland) was used for detection of resorufin (excitation/emission 544/590 nm) and HFC (excitation/emission 410/538 nm). Detection limits were 2 and 1 pmol, respectively. The concentrations of 4-nitrocatechol were measured using high-performance liquid chromatography (HPLC; Zamaratskaia & Zlabek, 2011). The HPLC equipment consisted of a pumping system (L-6200A), autosampler (AS 2000), UV detector (L-4250) set at 345 nm and D-6000 HPLC Manager software (Merck, Hitachi, Tokyo, Japan). *p*-Nitrophenol and 4-nitrocatechol were separated on a Hypersil ODS (5 µm, 60 × 4.6 mm) HPLC column under

DOI: 10.3109/15376516.2015.1074326

isocratic conditions using a mobile phase composed of 0.1% TFA in water (75%) and acetonitrile (25%) at a flow rate of 1.0 mL/min. The detection limit of 4-nitrocatechol was 0.125 nmol.

Analyses were performed in duplicate, and enzyme activity was expressed as percentage of control activity. Activity changes of more than 25% were considered moderately affected, but those of more than 50% were considered severely affected.

Results and discussion

The EROD activity was not affected by organic solvents at concentrations up to 0.5% (Figure 1A). Addition of 1% acetonitrile or acetone or 0.8–1% ethanol moderately decreased EROD activity. In contrast, EROD activity was inhibited only at the highest tested concentration of DMSO or methanol, while it was severely inhibited by acetonitrile, acetone or ethanol at 2% and above. This is an important finding because in *in vitro* inhibition studies, CYP1A1 inhibitors are often dissolved in organic solvents, potentially confounding results. Thus, it is essential that controls in such experiments contain the same amount of organic solvent as treatment incubations. To the best of our knowledge, this is the first study to show a strong *in vitro* effect of organic solvents on EROD activity in fish hepatic microsomes. *In vitro* CYP1A activity in rat hepatic microsomes, measured as phenacetin O-deethylation, was reduced by approximately 20% in the presence of 1% methanol, ethanol, acetone or DMSO, while at least 2.5% acetonitrile was necessary for an inhibitory effect (Li et al., 2010). In contrast, a 5% concentration was needed to achieve human phenacetin *O*-deethylation, resulting in a 12% reduction in methanol and a 27% reduction in acetonitrile (Chauret et al., 1998). The effect of organic solvent on fish CYP1A, as demonstrated by our results, is much stronger. The activity was reduced by 50% in the presence of 2% to 3% organic solvent, suggesting that fish CYP1A is more sensitive to organic solvent than mammalian CYP1A. In the presence of 3% acetonitrile or ethanol, formation of resorufin was not detectable. A similar inhibition pattern was observed when a pre-incubation step was included (Figure 1B). The highest inhibitions were observed in the presence of 3% organic solvent, the highest concentration tested. Acetonitrile, acetone and ethanol had the greatest ability of the five solvents to inhibit EROD.

The effect of organic solvent on CYP3A activity was not only inhibited but also induced (Figure 2A). Only 3% acetonitrile inhibited more than 75% of BFCOD activity. Moreover, 3% ethanol resulted in reduction of activity by more than 25%. On the other hand, we found that neither

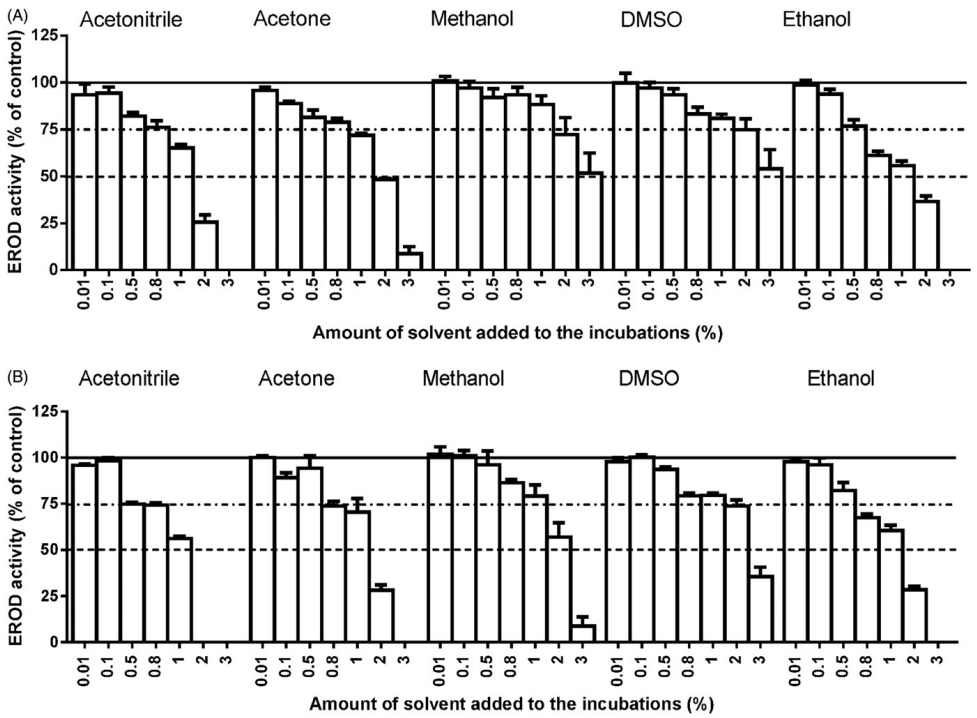

Figure 1. Effect of organic solvents at varying concentrations (0.01–3%) on the activity of 7-ethoxyresorufin *O*-deethylase (EROD) in fish hepatic microsomes as % of control (A) without pre-incubation and (B) with pre-incubation. The error bars indicate standard deviations. Activity changes more than 25% considered as moderately affected and more than 50% considered severely affected.

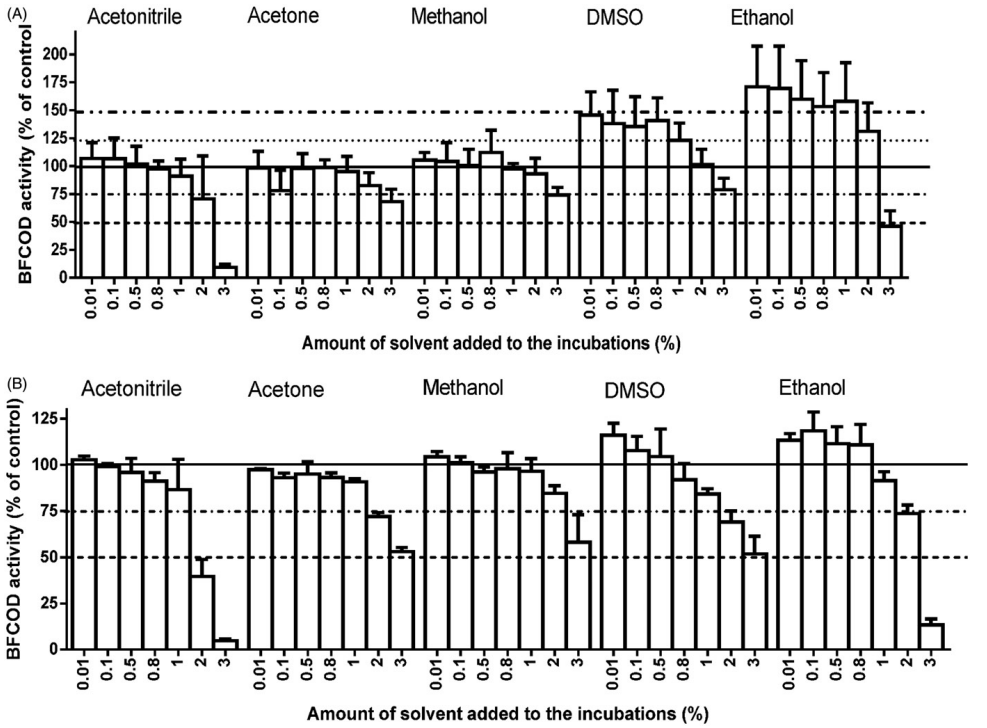

Figure 2. Effect of organic solvents at varying concentrations (0.01–3%) on the activity of 7-benzyloxy-4-trifluoromethylcoumarin O-debenzylase (BFCOD) in fish hepatic microsomes as % of control (A) without pre-incubation and (B) with pre-incubation. The error bars indicate standard deviations. Activity changes more than 25% considered as moderately affected and more than 50% considered severely affected.

methanol nor acetone had an apparent effect on CYP3A activity at any concentration. *In vitro* CYP3A activity in rat liver microsomes was reduced more than 50% in 10% acetonitrile, acetone, methanol, ethanol or DMSO (Li et al., 2010). In human cytochromes, 1% organic solvent inhibited CYP3A4/5 activity 20% in the presence of methanol or acetone (Hickman et al., 1998). Easterbrook et al. (2001) observed inhibition of CYP3A4 in human hepatocytes using 1–2% DMSO. Similarly, 5% acetonitrile or methanol was needed to reduce activity of CYP3A4 in humans, and 1.25% or more DMSO was needed to inhibit more than 50% activity (Li, 2009). Interestingly, in this study, at the lowest concentrations of ethanol (2% and lower) or DMSO (1% and lower), BFCOD activity was increased, a result not seen when a pre-incubation step was added (Figure 2B). A similar result was found by Iwase et al. (2006), who found that ethanol or 1-propanol (0.11%) or DMSO (0.1%) stimulated CYP3A activity in human liver microsomes. The nature of the induction is well understood. It was previously shown that some organic solvents might increase CYP450 activity, probably resulting from protein stabilization. For example, in human cDNA-expressed CYP450, Busby et al. (1999) observed an increase in CYP2C9-dependent diclofenac metabolism in the presence of 1 or 3% acetonitrile. Chauret et al. (1998) and Hickman et al. (1998) observed induction of CYP1A2 activity in the presence of acetonitrile (1%), and Moldeus & Gergely (1980) observed induction of acetaminophen oxidation by acetone.

The PNPH activity was not affected by up to 0.8% ethanol or up to 1% acetonitrile (Figure 3A). Moreover, 1–3% DMSO inhibited the activity more than 25%, in agreement with previous findings that DMSO inhibited CYP2E1 activity (Kim et al., 2007; Yoo et al., 1987). The PNPH activity was strongly inhibited by at least 1% ethanol or 3% acetnitrile. Acetone had no effect at any tested concentration. Hickman et al. (1998) observed no apparent effects at 1% acetonitrile, but over 50% inhibition at 1% acetone on CYP2E1 activity in human hepatocytes. Unlike with human hepatocytes, acetone may be a useful solvent for fish microsomes. With a pre-incubation step, PNPH activity was not affected by up to 1% ethanol, methanol or DMSO or up to 0.8% acetonitrile (Figure 3B). Acetone had no effect on the activity of PNPH at any tested concentration.

It is obvious that the inhibitory potency of organic solvents is substrate-dependent. Thus, the use of alternative substrate(s) might be advantageous when metabolisms of commonly used substrates are strongly inhibited by a given organic solvent. This, however, requires further investigation.

DOI: 10.3109/15376516.2015.1074326

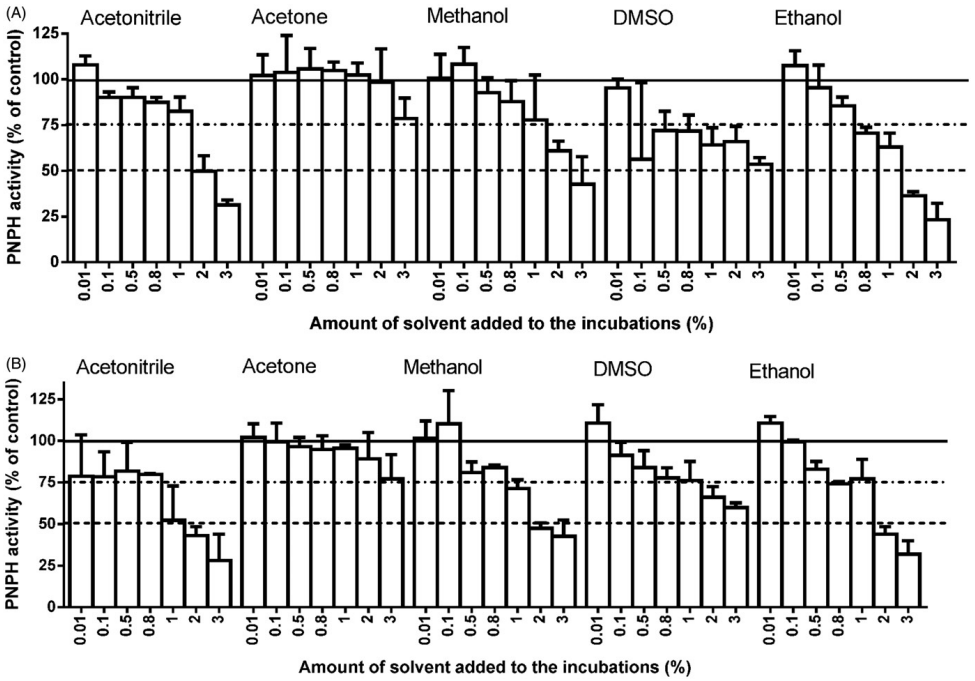

Figure 3. Effect of organic solvents at varying concentrations (0.01–3%) on the activity of *p*-nitrophenol hydroxylase (PNPH) in fish hepatic microsomes as % of control (A) without pre-incubation and (B) with pre-incubation. The error bars indicate standard deviations. Activity changes more than 25% considered as moderately affected and more than 50% considered severely affected.

Besides the use of fish microsomes, the novelty of this study is the inclusion of a pre-incubation step. Time-dependent inhibition of CYP450 caused by irreversible or quasi-irreversible interactions is commonly studied by pre-incubating the inhibitor with enzyme-containing media before the addition of the substrate. We found no indication that pre-incubation with organic solvent prior to substrate addition would result in severe inhibition. In general, the degree of inhibition was similar whether or not the pre-incubation step was included. However, it should be noted that we used only a single pre-incubation time for each reaction investigated, which was 10 min for EROD and BFCOD activities, and 30 min for PNPH activity. Longer pre-incubation times might have stronger effects on CYP450 activities.

Conclusion

The presence of organic solvents can greatly affect *in vitro* metabolism of commonly used CYP450 substrates in fish hepatic microsomes. The degree of this effect is substrate-, solvent- and concentration-dependent. Organic solvents at concentrations below 0.5% did not alter CYP450 activities, indicating that the amount used to solubilize substrates and inhibitors in *in vitro* microsomal studies should be kept minimal. Our results indicated that up to 1% methanol can be used in the studies based on rainbow trout hepatic CYP1A and CYP3A activities, and up to 2% acetone can be used in studies of CYP2E1 activity.

Acknowledgements

We would like to thank American Manuscript Editors, an affiliate of Grammar Labs, LLC, for English language editing.

Declaration of interest

The study was financially supported by the Ministry of Education, Youth and Sports of the Czech Republic – projects ''CENAKVA'' (No. CZ.1.05/2.1.00/01.0024) and ''CENAKVA II'' (No. LO1205 under the NPU I program), by the Grant Agency of the University of South Bohemia in Ceske Budejovice (No. 087/2013/Z) and by the Czech Science Foundation (No. GACR P503/11/1130).

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Chemosphere 192 (2018) 105–112

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

In vitro effects of diosmin, naringenin, quercetin and indole-3-carbinol on fish hepatic CYP1A1 in the presence of clotrimazole and dexamethasone

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HIGHLIGHTS

- Naringenin, diosmin and clotrimazole inhibited the EROD activity alone.
- Diosmin with high concentration of clotrimazole showed antagonistic behaviour.
- Quercetin, I3C and dexamethasone did not inhibit the EROD activity alone.
- Combination of dexamethasone and quercetin or I3C synergistically inhibited the EROD activity.

ARTICLE INFO

Article history:
Received 24 May 2017
Received in revised form
13 October 2017
Accepted 18 October 2017
Available online 23 October 2017

Handling Editor: Frederic Leusch

keywords:
Phytochemical
Pharmaceuticals
Combination index
EROD
Inhibition
Interaction

ABSTRACT

Phytochemicals are widely present in fruits, vegetables and other plants and have great health benefits owing to their antioxidant properties. They are naturally found in the aquatic environment as well as discharged from sewage treatment plants after their large consumption. Little is known about their impact on fish; particularly in light of their interactions with pharmaceuticals. Therefore, this study was designed to determine the effects of diosmin, naringenin, quercetin and idole-3-carbinol on CYP1A-dependent 7-ethoxyresorufin-O-deethylase (EROD) activity on rainbow trout hepatic microsomes in the presence of two pharmaceuticals: clotrimazole and dexamethasone. The interactions between the phytochemicals and pharmaceuticals used in this study were determined using a combination index. Hepatic microsomes were exposed to two concentrations (1-or 50 μM) of phytochemicals and pharmaceuticals separately and in combinations. Singly, clotrimazole inhibited EROD activity 40% and 90% of control, while dexamethasone did not. Naringenin and diosmin inhibited EROD activity alone up to 90% and 55% respectively, but activities were further inhibited in the presence of either pharmaceutical. The preliminary study of combinations of clotrimazole with phytochemicals primarily showed synergistic effects. While EROD activity was not inhibited in the presence of quercetin or indole-3-carbinol, significant and synergistic inhibition was detected when either of these was combined with clotrimazole or dexamethasone.

1. Introduction

Bioactive substances present as natural constituents in food are intensively studied to evaluate their potential beneficial effects on human health, namely anti-cancer, antioxidant, free radical scavenging, anti-viral or anti-inflammatory properties. Phytochemicals are secondary metabolites present in common foods, such as fruits and vegetables, and polyphenols and glucosinolates are major groups of phytochemicals. Flavonoids are the most common polyphenolic compounds present in plant-originated food (Chahar et al., 2011), diosmin, naringenin and quercetin which were used in this study belong to this group. The average daily intake of flavonoids among adults in Europe ranges between 851 and

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https://doi.org/10.1016/j.chemosphere.2017.10.106
0045-6535/

Abbreviations

CI	Combination index
CYP	Cytochrome P450
DMSO	Dimethyl sulfoxide
EROD	7-ethoxyresorufin-O-deethylase
I3C	Indole-3-carbinol
MeOH	Methanol

225 mg, but this varies greatly between countries. For example, flavonoid consumption in Sweden is 310 mg daily, whereas in Czech Republic, it is 225 mg daily (Vogiatzoglou et al., 2015). Diosmin and naringenin are present in citrus fruits such as grapefruit (Gattuso et al., 2007), and quercetin is in capers (Inocencio et al., 2000) and onions (Ewald et al., 1999). Glucosinolates are natural organic compounds found mainly in cruciferous vegetables and they are responsible for plant defence mechanism against damage, insects and diseases (Higdon et al., 2007). Indole-3-carbinol (I3C) is derived when the plant enzyme myrosinase hydrolyzes glucosinolates during the chopping, chewing or digestion of plant tissues (del Carmen Martínez-Ballesta et al., 2013). This metabolite is highly unstable and is transformed into many other metabolites, in acidic environment like stomach I3C transforms in to 3,3′-diindolylmethane (DIM; Fujioka et al., 2016; Thomson et al., 2016). In our previous *in vitro* study, rainbow trout hepatic microsomes treated with 3-methylindole were shown to produce I3C as a metabolite (Zlabek et al., 2016). The average daily intake of glucosinolates reportedly varies between 6.5 and 14.5 mg per person (Agudo et al., 2008; Steinbrecher and Linseisen, 2009). Indole-3-carbinol is often taken as a supplement in doses of 200–400 mg daily in order to boost general health or prevent of several cancer types, hormonal imbalance, cervical dysplasia. Found in broccoli, cauliflower and cabbage, I3C was suggested to exhibit inhibitory effects on skin and prostate tumours (Nachshon-Kedmi et al., 2003).

Because they have biologically beneficial effects, flavonoids and I3C are commercialised as high-dose supplements and are usually available in health food stores. However, use of high-dose flavonoids can make them pro-oxidants, causing certain adverse effects. Naringenin at 10 mg/L was reportedly teratogenic on amphibians (Pérez-Coll and Herkovits, 2004). Another phenolic compound, tannic acid, showed hepatotoxic effects on carp at 10 mg/kg (Varanka et al., 2001). Excessive use of these phytochemicals in supplement form, effluents from pulp mills, algae or naturally occurring in plant material in surface waters are some of the sources of these phytochemicals in aquatic ecosystem (Jarošová et al., 2015). Even though their occurrence has not yet received sufficient attention, Rocha et al. (2013) detected flavonoids biochanin A, daidzein and genistein at 19 μg/L, 137 ng/L and 24 ng/L respectively in surface waters in Portugal. In another study, genistein and daidzein were found in surface water in USA at 1.4 and 1.6 ng/L respectively (Rearick et al., 2014).

According to "The Global Use of Medicines," total spending on medicines will increase to $1.3 trillion by 2018 (IMS-Health, 2013). Many of those medicines are included on the list of compounds that are prioritized for environmental risk assessment (Roos et al., 2012). Clotrimazole and dexamethasone are drugs that were included on that list under several ranking methods. These pharmaceutically active compounds belong to the imidazole and glucocorticoid classes of drugs, respectively. Each has a wide spectrum of use in medicine. Their interactions with fish CYP systems have previously been studied *in vivo* (Baudiffier et al., 2012; Burkina et al., 2015a; Corcoran et al., 2014; Hinfray et al., 2011; Levine and Oris, 1999) and/or *in vitro* (Burkina et al., 2013; Levine and Oris, 1999). It has been reported that clotrimazole inhibits the CYP1A activity 50% at 0.1 μM and more than 80% at 10 μM concentration where dexamethasone was not able to inhibit or induce the EROD activity in fish (Burkina et al., 2013; Levine and Oris, 1999).

Pharmaceuticals and dietary bioactive compounds are continually discharged post-excretion into environmental bodies of water by municipal and hospital effluents, either unchanged or as metabolites, where they can affect non-target aquatic organisms. Because, removal of such compounds at wastewater treatment plants is inefficient, clotrimazole and dexamethasone can appear in surface water. Thus, clotrimazole has been detected in surface water at the nanogram-per-litre level (Loos et al., 2013; Peschka et al., 2007), and dexamethasone has been detected at the nanogram-to microgram-per-litre level in surface water (Chang et al., 2007; Liu et al., 2011, 2012).

The effect of dexamethasone has been intensively studied in fish (Bhattacharyya and Butler, 1980; Burkina et al., 2015a; DellaGreca et al., 2004; LaLone et al., 2012; Wassmur et al., 2010). It can interfere with fish reproduction systems and with normal growth, thus negatively affecting embryonic development, increasing deformities in fathead minnows (LaLone et al., 2012). Dexamethasone has low plasma concentrations in mammals and the highest plasma concentration of 0.01 μM was found in pigs (PubChem, 2017). No reports of blood plasma concentrations can be found for fish.

A number of researchers argue that clotrimazole is toxic to aquatic organisms (OSPAR, 2013). Recent investigations into these adverse effects of clotrimazole on aquatic organisms show that it induces cyp17a1 mRNA levels associated with steroidogenesis in adult zebrafish (*Danio rerio*; Baudiffier et al., 2012; Hinfray et al., 2011). It is a potent modulator of several CYP-mediated reactions in fish (Burkina et al., 2013), and it can bioconcentrate as well (Burkina et al., 2016a; Corcoran et al., 2014).

Recently, CYP-mediated metabolisms in fish aroused the attention of researchers, and fish were repeatedly evaluated as potential models in drug discovery and toxicology studies (Bailey et al., 1996; Goldstone et al., 2010). Moreover, hepatic CYP in rainbow trout (*Oncorhynchus mykiss*) was affected by the same flavonoids that affected mammalian CYP, suggesting that rainbow trout can be used for selecting compounds for further research on flavonoid-drug interactions (Burkina et al., 2016b). CYP1A1 is the most prominent xenobiotic-metabolizing enzyme in fish and its activity is important in the detoxification processes. CYP1A1 is known to be induced by various environmental contaminants including pharmaceuticals in the aquatic environment (Beijer et al., 2013; Burkina et al., 2015b; Wassmur et al., 2013). Our *in vitro* study, clotrimazole identified as a potent inhibitor of CYP1A1-dependent EROD activity with Ki value of 0.5 μM (Burkina et al., 2013). In contrast, the same study showed that dexamethasone did not affect EROD activity (Burkina et al., 2013). Thus, clotrimazole and dexamethasone were selected in the present study due to their different potency to modulate CYP1A1-dependent EROD activity. However, it is not known how these effects on EROD activity might be changed in the presence of phytochemicals. 50 μM quercetin and naringenin showed competitive and non-competitive inhibition on fish hepatic EROD activity respectively. When individual effects of flavonoids were studied, quercetin and naringenin inhibited EROD in fish with Ki values of 0.12 μM and 2.63 μM, respectively (Arinc et al., 2015). In mice, quercetin inhibited EROD activity with Ki values from 1.6 to 2.7 μM, while naringenin had no effect (Pilipenko et al., 2017). Thus, in the current study we used phytochemicals and pharmaceutical concentrations close to Ki value (1 μM). The concentration of 50 μM was also included as at this high concentration EROD activity

reduced to 25% of control activity maximum (Burkina et al., 2013).

Generally, combination behaviours of chemical compounds are classified as antagonistic, additive or synergistic. Antagonistic effects occur when the mixture of chemicals results in an effect that is less than the sum of the effects of each chemical lead alone. Synergistic effects occur when the mixture results in an effect that is greater than the sum of the individual effects. Additive effects occur when the mixture results in an effect that is the same as the sum of the individual effects.

The effects of certain individual flavonoids on CYP are well-described. However, knowledge about the effects of combinations of phytochemicals and drugs on fish CYP is limited. The combined effects can be weaker (antagonistic), stronger (synergistic) or additive. The primary aim of this study was to evaluate the effects of diosmin, naringenin, quercetin and I3C separately on hepatic EROD activity and in combination with clotrimazole or dexamethasone. Another aim was to determine combination indices (CIs) to quantify the combined effects of phytochemicals and pharmaceuticals.

2. Materials and methods

2.1. Chemicals

Diosmin (analytical standard, PubChem CID: 5353588), naringenin (purity 95%, PubChem CID: 932), quercetin (purity 95%, PubChem CID: 5280343), I3C (purity 95%, PubChem CID: 3712), 7-ethoxyresorufin, resorufin, reduced β-nicotinamide adenine dinucleotide phosphate, dimethyl sulfoxide (DMSO) and ethanol were obtained from Sigma Aldrich (Steinheim, Germany). Clotrimazole (CAS Number 23593-75-1, $C_{22}H_{17}ClN_2$, PubChem CID: 2812) and dexamethasone sodium phosphate (CAS Number 2392-39-4, $C_{22}H_{28}FNa_2O_8P$, PubChem CID: 16961) were obtained from Voight (USA). Acetonitrile and methanol (MeOH) of HPLC grade were purchased from Merck (Darmstadt, Germany).

Stock solutions of clotrimazole and dexamethasone were prepared in DMSO and MeOH, respectively. The flavonoids diosmin and naringenin were dissolved in DMSO, quercetin was dissolved in MeOH and I3C was dissolved in ethanol. Stock solutions of clotrimazole, dexamethasone, diosmin, naringenin, quercetin and I3C were diluted further to obtain the two concentrations used during incubations (1 and 50 μM).

2.2. Fish and hepatic microsome preparation

Rainbow trout ($n = 12$; mean ± standard deviation, body length, 268 ± 13 mm; mean body weight, 254 ± 9.0 g) were obtained from a local commercial hatchery (Czech Republic). Fish were transferred into aquaria containing 250 L fresh water (mean temperature, 14 ± 1 °C; mean pH 7.7 ± 0.1; dissolved oxygen 8.5–9.0 mg/L). During the two-week' acclimation period, the fish were fed commercial fish food (Bio Mar, Denmark) at 1% body weight daily. The fish were not fed 24 h prior to sampling to avoid prandial effects during assay. Fish were handled according to national and institutional guidelines for the protection of human subjects and animal welfare, and the facility is licensed (No. 53100/2013-MZE-17214) according to the Czech National Directive (the Law against Animal Cruelty, No. 246/1992). Before sampling, fish were anaesthetized in an ice bath, and the spinal cord was cut immediately. Liver samples were collected from 12 fish, immediately frozen and stored at −80 °C until the preparation of microsomal fractions.

The hepatic microsomal fraction was prepared out of approximately 1 g liver samples using differential centrifugation as previously described (Burkina et al., 2013). Protein levels were measured spectrophotometrically using bovine serum albumin as a standard (Smith et al., 1985). The microsomes were diluted to a protein content of 10 mg/mL.

To determine enzyme activity, 12 rainbow trout were used to prepare 4 pools, each providing microsomal fractions from 3 individuals (2 female and 1 male). Pooled microsomal fractions were stored at −80 °C until analyzed.

2.3. Determination of EROD activity in hepatic microsomes

The catalytic activity of EROD using fish microsomes was measured with a slight modification as previously described (Zamaratskaia and Zlabek, 2009). Briefly, incubation mixtures (500 μL) were prepared using 0.2 mg microsomal protein, 50 mM potassium phosphate buffer (pH 7.4) and 2 μM 7-ethoxyresorufin. The reaction was started by the addition of 0.5 mM reduced β-nicotinamide adenine dinucleotide phosphate followed by incubation for 10 min at 21 °C. Reactions were terminated by the addition of 500 μL 100% ice-cold MeOH. Reaction mixtures were centrifuged at 7500 *g* for 3 min. The amount of product formation was analyzed by HPLC. Enzyme activity was calculated by determining the amount (pmol) of resorufin formed per minute per milligram of microsomal protein.

Resorufin was detected using a fluorescence detector (L-7480, Hitachi, Japan) at 560 nm and 586 nm (excitation and emission, respectively). The quantification limit of resorufin was 0.5 pmol/mL. The HPLC system comprised a pump (L-7100), autosampler (L-7200), fluorescence detector (L-7485), D-7000 HPLC Manager software (Merck, Hitachi, Tokyo, Japan) and a reverse-phase LiChrospher RP-18 column (5 μm) equipped with a guard column.

2.4. Inhibition assays

The individual abilities of clotrimazole, dexamethasone, diosmin, naringenin, quercetin and I3C to inhibit EROD activity were determined using 1 and 50 μM concentrations of each in incubation mixtures treated as described above. Then, the process was repeated using mixtures of two compounds, one drug and one phytochemical, at the same concentrations. All incubations were performed in duplicate with variability below 15% and mean values were used for statistical analysis. The degree of inhibition was assessed by comparing EROD activities in presence of the tested compound(s) with control incubations using the same amount of DMSO, MeOH or ethanol in the absence of any compound. The final solvent content was 0.2% in all incubations. The control data were presented in Supplementary Material 1.

2.5. Data analysis

Statistical analysis was performed using Statistical Analysis System, version 9.1 (SAS Institute, Cary, NC, USA). The comparison between treatments were performed by ANOVA using the Mixed procedure. The mixed model (PROC MIXED) included the pharmaceutical (clotrimazole or dexamethasone), pharmaceutical concentration (0,1 μM or 50 μM), the phytochemical concentration (0,1 μM or 50 μM), and the interactions between them as fixed factors. Individual microsomal preparations were regarded as the random factor. Analyses were performed separately for diosmin, naringenin, quercetin and I3C. Differences between incubations at the same phytochemical concentration and at various drug concentrations were considered significant when $p \leq 0.05$. Data are presented as least squares means and standard errors.

Synergistic, additive, or antagonistic effects of pharmaceuticals and phytochemicals were derived using the median-effect principle of the mass-action law according to the method of Chou and Talalay (1984) and were calculated using the CI equation: $CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2$, where $(D_x)_1$ and $(D_x)_2$ are the

individual doses of Drug $_1$ and Drug $_2$ that inhibit *x*%, and $(D)_1$ and $(D)_2$ are the portions of Drug $_1$ and Drug $_2$ in combination that also inhibit *x*%. CompuSyn software Version 1.0 (CompuSyn, Inc. Paramus, NJ, USA) was used to determine the quantitative relationships between non-constant combinations of pharmaceuticals and phytochemicals. When CI values were 1, <1, or >1, the effects were additive, synergistic, or antagonistic, respectively.

3. Results

3.1. Exposure to a single compound

Effects of pharmaceuticals or phytochemicals on CYP1A enzyme activity alone are presented in Fig. 1. Clotrimazole, but not dexamethasone, inhibited EROD activity in both tested concentrations (Fig. 1A). In the presence of 1 μM naringenin alone EROD activity was reduced 70% compared with control, while at 50 μM the observed inhibition was stronger (91%). Diosmin at both concentrations similarly inhibited EROD activity (55%–45% of control) while I3C and quercetin did not inhibit EROD activity in the fish hepatic microsomes (Fig. 1B).

3.2. Exposure to combination of pharmaceutical and phytochemical

Naringenin at 1 μM combined with clotrimazole at 1 or 50 μM further inhibited enzyme activity compared with the observed individual inhibitions, but combined with dexamethasone at either concentration had no further effect (Fig. 2A). Neither clotrimazole nor dexamethasone at either concentration further altered the inhibition of 50 μM naringenin. Based on CI values, the combination of naringenin and clotrimazole was found to be primarily synergistic except in one combination, which had an additive effect (Table 1).

Diosmin at 1 μM in combination with 1 μM clotrimazole further inhibited EROD activity (76% of control, $p > 0.05$); however, in combination with 50 μM clotrimazole, inhibition was slightly weaker than with clotrimazole alone (Fig. 2B). This pattern was evidenced by CI values. The combination of diosmin and 1 μM clotrimazole showed synergism while the combination of diosmin and 50 μM clotrimazole showed antagonism. The combination of diosmin and either concentration of dexamethasone did not inhibit EROD activity.

Indole-3-carbinol, when combined with clotrimazole at either concentration or with dexamethasone at 50 μM, reduced EROD activity significantly (69%–91% of control, $p < 0.001$; Fig. 2C), and CI values indicate synergism. When 1 μM I3C was combined with 1 μM dexamethasone, EROD activity was slightly inhibited, but less than 50%.

The combination of quercetin with either pharmaceutical inhibited EROD activity to a similar degree regardless of concentration (60%–86% of control, $p < 0.001$; Fig. 2D). Interactions between the tested pharmaceuticals and quercetin showed synergism based on CI values except when the pharmaceutical was 50 μM clotrimazole, which showed antagonism.

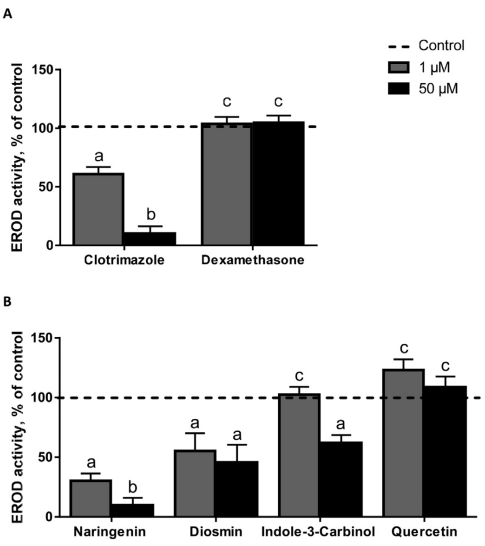

Fig. 1. *In vitro* EROD activity inhibition in hepatic microsomes of rainbow trout by pharmaceuticals (A) and phytochemicals (B): a single concentration of substrate (2 μM 7-ER) and two concentrations (1 μM, light grey bars) or (50 μM, black bars) show inhibition in percent of that found in controls (mean ± standard error) using four pools of fish. The dashed line shows control and letters represent the significant differences (significance at $p < 0.05$).

4. Discussion

To the best of our knowledge, this study is the first to investigate the combined effects of pharmaceuticals and dietary phytochemicals on piscine hepatic EROD activity. A great deal of current research addresses the effects of a cocktail of environmental pollutants on various species (Celander, 2011; Vasquez et al., 2014). Less is known about the combined effects of human pharmaceuticals and natural bioactive compounds on fish.

The major finding of this study was that some phytochemicals, such as I3C and quercetin, which by themselves do not affect EROD activity, might have greater impacts on the presence of the pharmaceuticals clotrimazole and dexamethasone. Thus, the effects of clotrimazole and dexamethasone on fish CYP1A cannot be accurately predicted from controlled studies because unknown chemicals could potentially be present in the environmental media, possibly enhancing any effect. The potential for 'cocktail' effects has been repeatedly demonstrated in various laboratory studies, especially those focusing on hormone disrupters (Carvalho et al., 2014). These studies were performed primarily on mammals. For example, You et al. (2002) used rats to study the effects of the phytoestrogen genistein on the toxicity of the pesticide methoxychlor. Kumi-Diaka et al. (1999) tested the combined cytotoxicity effect of genistein and dexamethasone on mice. Co-treatment induced apoptosis in mouse testicular cells. The effects of three phenolic acids, rosmic, caffeic and ferulic acid, as well as their combinations, were determined in the *in vitro* CYP1A activities of human and rat hepatocyte cells. Their combinations were primarily synergistic in human cells but antagonistic in rat hepatocytes (Liu et al., 2013). These results suggest that phytochemicals can act either way, thus bringing benefits to or posing hazards on the health of various species.

Cocktail effects of compounds on fish have been reviewed (Celander, 2011). Rainbow trout injected simultaneously with 17α-ethynylestradiol and ketoconazole showed significant increases in EROD activities compared to those injected with only one of the compounds (Hasselberg et al., 2008). Atlantic cod treated with a mixture of ketoconazole and a nonylphenolic compound showed induced EROD activities, increased CYP1A protein levels when compared with those injected with ketoconazole, nonylphenol or

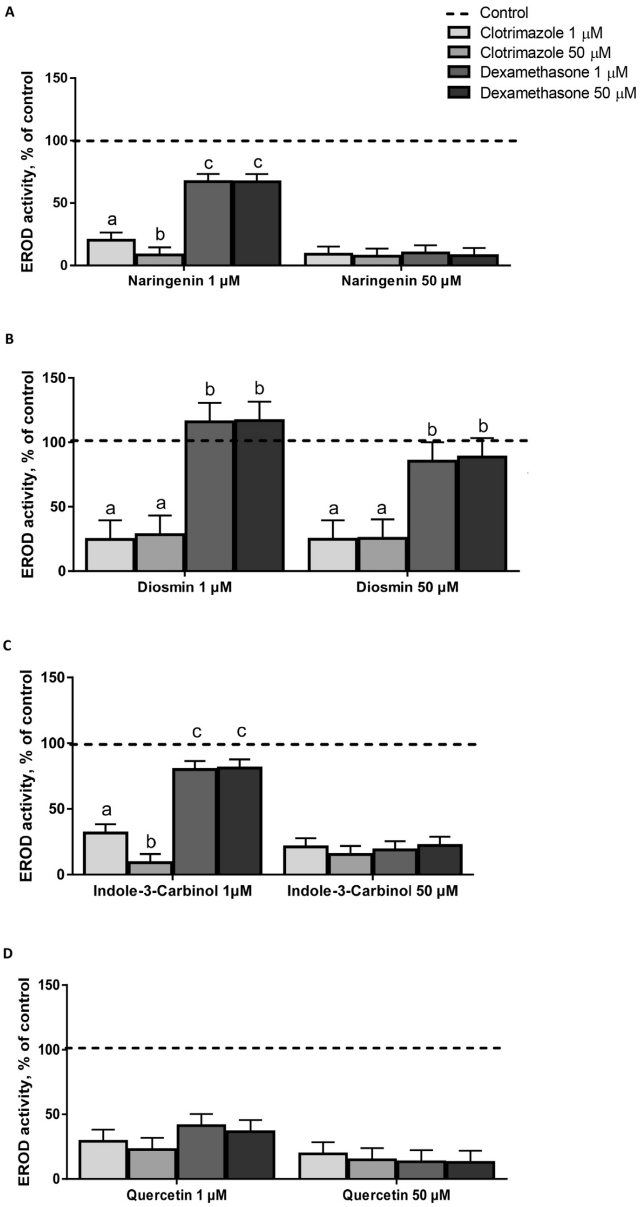

Fig. 2. *In vitro* EROD activity in rainbow trout hepatic microsomes with combinations of 1 or 50 μM clotrimazole (lighter bars) or dexamethasone (darker bars) and 1 or 50 μM naringenin (A), diosmin (B), indole-3-carbinol (C) or quercetin (D) in incubations conducted in the presence of a single substrate concentration (2 μM 7-ER) and represented as the percent of that found in controls (mean ± standard error) using four pools of fish. The dashed line shows control and letters represent the significant differences (significance at $p < 0.05$).

Table 1
Interaction behaviours between phytochemicals and pharmaceuticals, represented by the combination index (CI; a refinement of that described by Chou (2006)), where CI < 1 indicates synergism, CI = 1 indicates additive effects and CI > 1 indicates antagonism.

Phytochemical	Phyto-chemical concentration (μM)	Inhibitor	Inhibitor concentration (μM)	Combination index	Drug combination
Naringenin	1	Clotrimazole	1	0.31	Synergism
	1		50	0.71	Moderate Synergism
	50		1	1.02	Nearly Additive
	50		50	0.19	Strong Synergism
	1	Dexamethasone	1	NA	
	1		50	NA	
	50		1	1.36	Moderate Antagonism
	50		50	0.48	Synergism
Diosmin	1	Clotrimazole	1	0.10	Very Strong Synergism
	1		50	6.57	Strong Antagonism
	50		1	0.10	Very Strong Synergism
	50		50	5.21	Strong Antagonism
	1	Dexamethasone	1	NA	
	1		50	NA	
	50		1	NA	
	50		50	NA	
I3C	1	Clotrimazole	1	0.18	Strong Synergism
	1		50	0.84	Moderate Synergism
	50		1	0.24	Strong Synergism
	50		50	2.12	Antagonism
	1	Dexamethasone	1	NA	
	1		50	NA	
	50		1	0.14	Strong Synergism
	50		50	0.18	Strong Synergism
Quercetin	1	Clotrimazole	1	0.14	Strong Synergism
	1		50	4.06	Strong Antagonism
	50		1	0.06	Very Strong Synergism
	50		50	1.78	Antagonism
	1	Dexamethasone	1	0.00	Very Strong Synergism
	1		50	0.00	Very Strong Synergism
	50		1	0.00	Very Strong Synergism
	50		50	0.00	Very Strong Synergism

NA: data not available; I3C: indole-3-carbinol.

ethynylestradiol alone (Hasselberg et al., 2005).

Numerous studies have characterised a variety of naturally-occurring dietary compounds that can activate and/or inhibit mammalian CYP1A (Moon et al., 2006). The pharmaceuticals and dietary compounds selected for our study were previously shown to interact with mammalian CYP systems and to modify the activities of some isoforms (Chahar et al., 2011; Foti and Wahlstrom, 2008). These compounds have repeatedly been detected in the aquatic environment around the world (Carlsson et al., 2006). In recent years, a piscine CYP system was extensively studied to gain a better understanding of the role of each CYP in drug metabolism. The effects of active pharmaceuticals in the aquatic environment on piscine CYP systems have been reviewed (Burkina et al., 2015b; Uno et al., 2012). Recently, the effects of dietary phenolic compounds on fish CYP systems have also received attention (Arinc et al., 2015; Burkina et al., 2016b; Davila et al., 2010). However, no information on the combined effects of pharmaceuticals and common human dietary components on fish detoxification mechanisms is available. Dietary compounds such as flavonoids, flavones, isoflavones, I3C have been previously reported as aryl hydrocarbon receptor (AhR) activators either as ligand or agonist (Ciolino et al., 1998, 1999; Mohammadi-Bardbori et al., 2012; Nguyen and Bradfield, 2008). Therefore, we speculate that mechanism of action in food-drug interactions could be related to their direct effects on the CYP system. For example, CYP1A1 expression is mediated via an AhR, and because of that receptor's high affinity to these compounds, enzyme activity is induced.

In mammals, dexamethasone does not affect CYP1A activity (Kishida et al., 2008) but in fish, conflicting results have been found. Some studies suggest dexamethasone has no effect on CYP activity (Burkina et al., 2013, 2015a; Guiloski et al., 2015; Smith and Wilson, 2010), but others show that exposure may cause a reduction (Dasmahapatra and Lee, 1993) or induction (Celander et al., 1997) of CYP1A1 protein and total CYP450 content in rainbow trout (Burkina et al., 2015a). Dexamethasone did not affect EROD activity in fish hepatic microsomes. This agrees with our previous results on hepatic microsomes from rainbow trout (Burkina et al., 2013, 2015a). Similarly, neither I3C nor quercetin alone were found to affect EROD activity. This is surprising, because previous studies showed that I3C inhibited CYP1A activity in humans, rats and fish through the formation of DIM (Stresser et al., 1995). The absence of inhibition found in the current study might be the result of DIM being scarcely produced. The lack of a preincubation step in the experimental design could explain this. Nevertheless, when dexamethasone was combined with I3C or quercetin, EROD activity was significantly reduced, suggesting that such combinations might have harmful effects on CYP and detoxification mechanism(s).

Combination behaviour of the phytochemicals and pharmaceuticals presented as synergism, antagonism or additive in the table according to the user's guideline of the CompuSyn software. However, the table that represent the degree of synergism or antagonism by the developers have some limitations. For example, some observed results that showed as synergism can be named as potentiation. Potentiation is a sort of synergism which one drug is not effective by itself but increases the effect of another drug. In this study, dexamethasone with naringenin or 1 μM of I3C with clotrimazole or quercetin with clotrimazole show potentiation effect where dexamethasone, I3C and quercetin did not affect the EROD enzyme activity alone but increased the inhibition of others when combined.

We found that a high concentration of clotrimazole combined with diosmin or I3C showed antagonistic effects. Although

inhibition remained high, the effect was smaller than that of clotrimazole alone. Similarly, *in vivo* study on gold fish showed antagonistic effect of co-exposure of mixture of caffeine and sulfamethoxazole on hepatic EROD activity (Li et al., 2012). Generally, to the best of our knowledge, only limited information is available on the antagonistic behaviour of combined chemicals.

In this study, dexamethasone is strongly synergistic with I3C and quercetin, and clotrimazole is strongly synergistic with naringenin and I3C. Synergistic effects were also observed by Fernández et al. (2012), who evaluated the effect of mixture of pharmaceuticals and fragrance on EROD activity in rainbow trout gonadal cell line.

An additive effect is usually observed when the compounds are similar in structure. For example, estrogenic compounds (i.e. phytoestrogens and xenoestrogens) act additively (Brian et al., 2007). In this study, we found that only 50 μM naringenin combined with 1 μM clotrimazole showed a nearly additive effect. Hasselberg et al. (2005) were reported that ketoconazole, nonylphenol or their mixtures inhibited EROD activity in Atlantic cod liver microsomes and ketoconazole acted as non-competitive inhibitor of the EROD activity *in vitro* while, *in vivo,* the mixture induced EROD activity. In another study, 1 μM of carbofuran and β-naphthoflavone combination showed additive effect on EROD activity using primary cell culture of catfish hepatocytes (Ghosh et al., 2000).

This study was limited by our focus on combinations of only two compounds at a time. Studies of multi-component mixtures would provide a pattern more similar to that seen in the environment. However, exposure to more than two substances or to a large mixture cannot provide data for interactions between individual components or their combined actions. Therefore, using large mixtures can provide only the effect of the mixture but not how the various compounds affect each other. It should be noted that determination of combined behaviour of compounds that was calculated in this study only gives preliminary result. To fully understand the behaviour of tested compounds with each other, further research should be conducted using more concentration and dose-responsive curves.

It is worthy to note that a majority of studies on CYP regulation are based on single-component testing. A single component, when assessed alone, might exert different effects than the component combined with others. Therefore, the effect of a mixture cannot be predicted from the effects of individual components. While recognizing the limitations of our analysis, we believe that our results provide valuable information for potential interactions between selected phytochemicals and pharmaceuticals at multiple concentrations on fish hepatic microsomes.

Acknowledgements

This study was financially supported by the Swedish University of Agricultural Sciences, NJ faculty, Uppsala, Sweden and by the Ministry of Education, Youth and Sports of the Czech Republic – projects "CENAKVA" (No. CZ.1.05/2.1.00/01.0024) and "CENAKVA II" (No. LO1205 under the NPU I program) by the Grant Agency of the University of South Bohemia in Ceske Budejovice (No. 012/2016/Z), by the Czech Science Foundation (No.15-04258S). We also thank American Manuscript Editors for editing the English manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2017.10.106.

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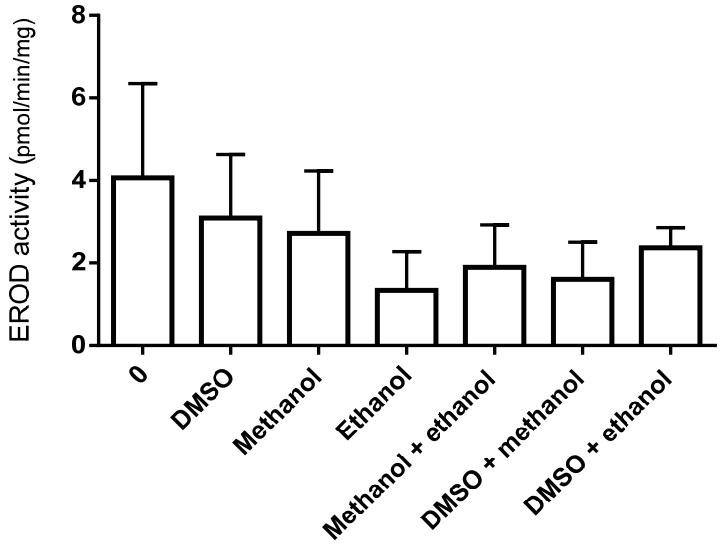
Appendix A.

Supplementary Material 1.



This graph presents tested controls without, with or combined solvents. No significant differences were observed between control groups (n=4 pools of microsome, One-way ANOVA, *P*- value= 0.1364)

CHAPTER 3

IN VIVO EXPERIMENTS: EFFECTS OF SELECTED PHARMACEUTICALS ON FISH UNDER LABORATORY CONDITIONS

Burkina, V., Sakalli, S., Rasmussen, M.K., Zamaratskaia, G., Koba, O., Thai, G.P., Grabic, R., Randak, T., Zlabek, V., 2015. Does dexamethasone affect hepatic CYP450 system of fish? Semi-static *in vivo* experiment on juvenile rainbow trout. Chemosphere 139, 155–162.

Burkina, V., Zamaratskaia, G., Oliveira, R., Fedorova, G., Grabicova, K., Schmidt-Posthaus, H., Steinbach, C., Domingues, I., Golovko, O., Sakalli, S., Grabic, R., Randak, T., Zlabek, V., 2016. Sub-lethal effects and bioconcentration of the human pharmaceutical clotrimazole in rainbow trout (*Oncorhynchus mykiss*). Chemosphere 159, 10–22.

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My contributions to these works were 24% and 5% respectively.

Chemosphere 139 (2015) 155–162

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Does dexamethasone affect hepatic CYP450 system of fish? Semi-static *in-vivo* experiment on juvenile rainbow trout

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HIGHLIGHTS

- Juvenile rainbow trout were sub-chronically exposed to DEX.
- DEX increased CYP3A-like protein levels in fish hepatic microsomes.
- DEX has no effect at the highest tested concentration.
- DEX has no effect on studied CYP450 mediated reaction.
- DEX might have a negative impact on non-target species such as fish.

ARTICLE INFO

Article history:
Received 9 April 2015
Received in revised form 1 June 2015
Accepted 16 June 2015

Keywords:
Dexamethasone
Rainbow trout
CYP1A
CYP3A
CYP2E1-like protein

ABSTRACT

Effects of aquatic pollutants on fish are of increasing concern. Pharmaceutical-based contaminants are prioritized for further study in environmental risk assessment using several approaches. Dexamethasone (DEX) was one such contaminant recognised for its effect on fish health status. Thus, we carried out an *in vivo* experiment to identify potential effects of DEX on rainbow trout. Fish were exposed to 3, 30, 300 and 3000 ng L^{-1} DEX in a semi-static system over a period of 42 d. The concentrations of DEX that fish were exposed to was confirmed by LC–LC–MS/MS. Using hepatic microsomes, we determined cytochrome P450 content, activities of ethoxyresorufin O-deethylase (EROD), p-nitrophenol hydroxylase (PNPH), 7-benzyloxy-4-trifluoromethylcoumarin O-debenzylase (BFCOD) and benzyloxyquinoline O-debenzylase (BQOD), as well as protein expression. Our results showed that fish do not change the catalytic activity of CYP450-mediated reactions after high DEX concentration exposure. These results disagree with mammalian studies, where DEX is a well-known inducer of CYP450. We showed a significant effect of DEX exposure on CYP450-mediated reactions (EROD, BCFOD, BQOD and PNPH) when expressed as amount of product formed per min per nmol total CYP450 at 3, 30 and 300 ng L^{-1} after 21 d exposure. Moreover, BFCOD and BQ activities showed matching trends in all groups. Western blot analysis showed induction of CYP3A-like protein in the presence of the lowest environmentally relevant concentration of DEX. Based on these findings, continued investigation of the effect of DEX on fish using a battery of complementary biomarkers of exposure and effect is highly relevant.

1. Introduction

Corticosteroids are a class of chemicals that includes natural steroid hormones (glucocorticoids and mineralocorticoids), produced from cholesterol in the adrenal cortex of vertebrates, and their synthetic analogues. Fish interrenal glands are capable of secreting adrenocortical steroids. Cortisol has been found in the blood and tissues of several fish species, including rainbow trout

Abbreviations: AhR, aryl hydrocarbon receptor; BFCOD, 7-benzyloxy-4-trifluor omethylcoumarin O-debenzylase; BQ, 7-benzyloxyquinoline; BQOD, 7-benzyloxyquinoline O-debenzylase; DEX, dexamethasone; EROD, ethoxyresorufin O-deethylase; CYP450, cytochrome P450; PNPH, p-nitrophenol hydroxylase.

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http://dx.doi.org/10.1016/j.chemosphere.2015.06.031
0045-6535/

(*Oncorhynchus mykiss*), brown trout (*Salmo trutta*) (Sloman et al., 2001), and Mozambique tilapia (*Oreochromis mossambicus*); (Johnstone et al., 2013). Fish kidneys and associated adrenal glands are structured differently than other vertebrates (Perry and Capaldo, 2011). Natural corticosteroids are involved in a broad range of physiological processes (e.g., inflammation) by reducing the response to stress as well as glucose, lipid and protein metabolism. Synthetic equivalents of corticosteroids act in similar ways and can have higher corticosteroid potencird than natural variants (Bentz, 2014).

Dexamethasone (DEX), a potent synthetic glucocorticoid drug, is effective for treatment of a range of inflammatory and autoimmune conditions as well as the reduction of side effects associated with chemotherapy. On the Czech Republic pharmaceutical market, DEX is incorporated into eighteen drugs used for treatment of systemic hormonal and sensory organ problems. The Czech Republic was the eighteenth largest EU pharmaceutical market in 2012 (EFPIA, 2013), and its DEX use was 46.13 kg in 2013 (SUKL, 2015).

In the last few decades, a vast range of synthetic steroid drugs has been produced and released into the aquatic environment where, as a group, they are potential contaminants that disrupt non-target organisms in aquatic environments (Kumar et al., 2015). The concentration of DEX was reportedly 38 ng L^{-1} downstream from a swine farm (Liu et al., 2012). It was reportedly between 1.2 and 23 ng L^{-1} in wastewater influent (Chang et al., 2007; Liu et al., 2011). In river water downstream from a pharmaceutical manufacturing plant discharge, it was 23 μg L^{-1} (Creusot et al., 2014).

Because of growing concerns about the potential adverse impacts of pharmaceuticals on non-target aquatic organisms, DEX was prioritized by ranking schemes for environmental risk assessment (Roos et al., 2012); it is prescribed for its potent glucocorticoid effect. Several recent studies investigated the effects of glucocorticoids at environmentally relevant levels on fish secondary sexual characteristics (Kugathas and Sumpter, 2011; Kugathas et al., 2012, 2013). Dexamethasone was found to affect reproduction, growth and development in fathead minnows after chronic exposure at 500 μg L^{-1} (LaLone et al., 2012). It also significantly reduced resting plasma cortisol levels and induced interrenal cell atrophy following treatment with 50 mg g^{-1} DEX for 7 d in Chinook salmon (McQuillan et al., 2011) and was found to potentially cause oxidative stress in liver tissue at 0.3 and 3.0 μg kg^{-1} doses, disturbing the antioxidant system in the gonads of male *Hoplias malabaricus* after trophic DEX exposure at 0.03–3.0 μg kg^{-1} doses (Guiloski et al., 2015). Currently, there is limited information about its toxicity in the aquatic environment and the eventual effects on physiological processes in fish.

The hepatic cytochrome P450 (CYP450) superfamily is essential for metabolising foreign chemicals, fatty acids, vitamins, hormones and other compounds and consists of 18 subfamilies (Uno et al., 2012). The number of discovered CYP genes is increasing as a result of intense work with CYP450 gene structures (Kubota et al., 2013). The first three subfamilies (CYP1, CYP2 and CYP3) are mainly responsible for metabolising xenobiotics, and variations in their expression and activity can be used as indicators of exposure to environmental contaminants. CYP1A is a catalyst of environmental pollutants, including human pharmaceuticals (Laville et al., 2004; Navas et al., 2004; Smith et al., 2012); therefore it is critical in finding the pathways leading to detoxification. This enzyme is highly conservative among vertebrates. Mammals, birds and some fish species (eel and rainbow trout) possess genes for two CYP1A isoforms (Berndtson and Chen, 1994; Rifkind et al., 1994; Gorman et al., 1998; Mahata et al., 2003). A widely used assay for CYP1A is the measurement of EROD activity, which is routinely used as a biomarker to determine the presence of organic pollutants (Mandal, 2005). The majority of CYP450s are substrate-inducible via mechanisms often including ligand activation of transcription factors such as the aryl hydrocarbon receptor (AhR), pregnane X receptor, constitutive androstane receptor and others. The mechanism of CYP1A induction in fish is well known; numerous bioactive compounds induce CYP1A via binding to AhR and subsequent initiation of transcription. The less-studied mechanism of CYP450 regulation involves stabilization of mRNA and changes in protein turnover, leading to an increase in CYP450 activity, which largely depends on biotransformation of environmental pollutants in aquatic organisms. Factors that alter this activity might also alter the toxicity of CYP450 substrates.

Natural and synthetic glucocorticoids perform their biological action in the organism through receptor-dependent mechanisms (e.g., by binding to the glucocorticoid receptors). It has been determined that glucocorticoid receptors cross-talk with other nuclear receptors, including AhR (Celander et al., 1996b; Dvorak et al., 2008; Vrzal et al., 2009). Dexamethasone is a typical inducer of mammalian CYP3A (Donato et al., 1995; McCune et al., 2000; Pascussi et al., 2000a,b) and CYP2E1 activity (Tamasi et al., 2001). In fish, DEX increased protein expression and CYP1A catalytic activity in hepatocellular carcinoma cells from *Poeciliopsis lucida* (Celander et al., 1996a).

In this study, we investigated the *in vivo* effect of DEX on the catalytic activity of selected hepatic CYP450s in rainbow trout. Because DEX concentrations vary greatly in environmental waters, two environmentally relevant DEX concentrations (3 and 30 ng L^{-1}) and two higher concentrations (300 and 3000 ng L^{-1}) were included. Activities of the following CYP450s were measured: CYP1A (7-ethoxyresorufin O-deethylase [EROD]), CYP2E1-like (p-nitrophenol hydroxylase [PNPH]) and CYP3A-like (7-benzyloxy-4-trifluoromethylcoumarin O-debenzylase [BFCOD] and 7-benzyloxyquinoline O-debenzylase [BQOD]). Moreover, total CYP450 content as well as CYP1A and CYP3A-like proteins were determined.

2. Materials and methods

2.1. Chemicals

Dexamethasone sodium phosphate (CAS Number 2392-39-4, $C_{22}H_{28}FNa_2O_8P$) and diltiazem, (2S,3S)-5-(2-dimethylaminoethyl)-2-(4-methoxyphenyl)-4-oxo-2,3-dihydro-1,5-benzothiazepin-3-yl acetate (CAS Number 42399-41-7, purity 98%), were obtained from Voight (USA) and dissolved in water to make a stock 45 mg L^{-1} DEX solution. Resorufin, 7-ethoxyresorufin, p-nitrophenol, 7-benzyloxy-4-trifluoromethylcoumarin (BFC), 7-hydroxy-7-benzyloxy-triflouromethylcoumarin, 7-benzyloxyquinoline (BQ), and nicotinamide adenine dinucleotide phosphate were obtained from Sigma–Aldrich (Steinheim, Germany). Hydroxyquinoline was purchased from Acros (Geel, Belgium), and HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Immunoblotting gels (Novex® 10% Tris–Glycine) were obtained from Life Technologies. Anti-fish CP-226 antibody from Biosense Laboratories AS (Bergen, Norway) was used to detect CYP1A, and anti-human CYP3A antibody (SC-25845, Santa Cruz, Inc.) was used to detect CPY3A-like protein. We verified that the latter recognised a band corresponding to approximately 50 kDa in both human and fish microsomes in a preliminary trial (Supplement 1).

2.2. Fish and treatment

Juvenile rainbow trout ($n = 240$) weighing 175 ± 30 g (mean ± standard deviation) were purchased from a local

commercial hatchery (Husinec, Czech Republic). Fish were transferred to twelve aquaria, each containing 250 L fresh water, and parameters such as temperature, dissolved oxygen content and pH were measured daily. Similar water conditions (14.2 ± 1 °C, pH 7.7 ± 0.1, dissolved oxygen 8–9 mg L^{-1} and natural light photoperiod) were maintained during acclimation (two weeks) and the experimental period. The fish were fed commercial fish food (BioMar, Denmark) at 1% total body weight daily. Feeding was stopped 24 h before sampling events to avoid prandial effects. Fish were handled according to national and institutional guidelines for the protection of human subjects and animal welfare, the Law against Animal Cruelty (082/2002-V2), and approval was received according to Czech National Directive No. 419/2012 on the protection of experimental animals.

At the start of the experimental period, twenty fish were randomly distributed into each of twelve aquaria containing 250 L water in a semi-static system and exposed to sub-lethal concentrations of DEX for 21 and 42 d. A control group was unexposed. Dexamethasone was formed from hydrolysis of dexamethasone sodium phosphate. A working dexamethasone sodium phosphate solution of 1 mg L^{-1} was prepared daily from the stock solution and stored overnight at 4 °C before use. Nominal concentrations of DEX used were 0 ng L^{-1} (control), 3 ng L^{-1} (lowest environmentally relevant concentration), 30 ng L^{-1} (highest environmentally relevant concentration), 300 ng L^{-1} and 3000 ng L^{-1}. Each experimental condition was duplicated. At days 0, 21 and 42, four fish from each duplicate aquarium were sacrificed and combined into a single group of 8. The livers were collected and stored at −80 °C until microsomes were prepared.

2.3. Determination of DEX content in water using LC–LC–MS/MS

An analytical LC–LC–MS/MS method based on dual column liquid chromatography connected to an electrospray ionisation hybrid high resolution mass spectrometer was developed for detection and quantification of DEX in water. A hybrid quadrupole/orbital trap mass spectrometer, QExactive (Thermo Fisher Scientific, San Jose, CA, USA), was used to detect the target compound.

An Accela 1250 LC pump (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Accela 600 LC pump (Thermo Fisher Scientific) and an HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland) was used for LC analysis.

Two high-performance liquid chromatography columns were used, the first (Hypersil Gold [20 mm × 2.1 mm, 12 μm particle size]; Thermo Fisher Scientific) for extracting the analyte from the water and the second (Hypersil Gold Phenyl [50 mm × 2.1 mm, 3 μm particle size]; Thermo Fisher Scientific) for analytical separation.

The LC–LC conditions for water sample analysis are described in Supplement 2. Samples were filtered through a regenerated cellulose syringe filter (0.45 μm pore size) and stored at 4 °C in 10-mL vials in a cooled autosampler tray prior to analysis. A 5-mL sample was injected. A heated electrospray ionisation (HESI-II) source in positive ion mode was used for ionisation of the target compound. The operating parameters were set: spray voltage, 3.5 kV; sheath gas, 40 arbitrary units; auxiliary gas, 10 arbitrary units; capillary temperature, 325 °C and vaporizer temperature, 250 °C. The hybrid quadrupole/orbital trap mass spectrometer QExactive was operated in high resolution product scan (HRPS) mode at 17,500 full width at half maximum resolution. The AGC target was set to 3,000,000, and its maximum injection time was 100 ms. Optimised high resolution parameters for the target analyte and internal standard are given in Supplement 3. Quality assurance/quality control (QA/QC) of the analysis included blank water samples to reveal cross contamination during exposure and sample handling. Native diltiazem was used as an internal standard for quantification. Matrix-matched standard responses (water from control aquaria spiked with target compound and internal standard) were used as factors for correcting the response derived from the calibration curve.

This method was validated in the range of tested concentrations and exhibited good linearity in the concentration range between 5 and 5000 ng L^{-1} DEX; $R^2 = 0.998$. Recovery of DEX from aquaria water was evaluated by spiking unexposed water samples with the target compound at the four exposure concentration levels (3, 30, 300, 3000 ng L^{-1}). Average relative recovery of DEX was 95% (range, 77–107%); relative standard deviation across eight replicates was 11%. Average limit of quantification was 1.3 ng L^{-1}.

The treatment solution was renewed each day to maintain water quality and the appropriate DEX concentration. The proportion of "new" treatment solution (time 0 h) to "old" treatment solution (time 24 h) was 2.5:1. Water samples were analysed within one hour after being taken from aquaria. Using LC–MS/MS, DEX concentration in the water was measured weekly (six times) during the experimental period immediately after solution renewal (0 h) and after 24 h.

2.4. Microsome preparation

The microsome fraction was prepared using differential centrifugation as described by Burkina et al. (2013). Protein levels were estimated spectrophotometrically using the method described by Smith et al. (1985) using bovine serum albumin as a standard. The microsomes were diluted to a protein content of 5 mg mL^{-1}.

2.5. Determination of total CYP450 content and microsomal enzyme activity

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

The catalytic activities of EROD, BFCOD, PNPH and BQOD were determined. The EROD activity was determined as the transformation rate of 7-ethoxyresorufin to resorufin, the BFCOD activity as the transformation rate of BFC to 7-hydroxy-4-trifluoromethylcoumarin and the PNPH activity as the transformation rate of p-nitrophenol to 4-nitrocatechol using incubation procedures previously described (Burkina et al., 2013). The BQOD activity was determined as the transformation rate of BQ to hydroxyquinone (Tomankova et al., 2012). Briefly, incubation mixtures (500 μL) containing 0.1 mg microsomal protein in 50 mM potassium buffer (pH 7.8) and BQ (10 μM) were prepared. The reaction was started by the addition of 0.5 mM nicotinamide adenine dinucleotide phosphate, and it was allowed to proceed for 10 min at 21 °C. The reaction was stopped by the addition of 500 μL 100% MeOH. Enzyme activity was either normalised to the total protein amount in mg or to the total CYP450 content in nmol.

2.6. Immunoblotting

Microsomal samples of control groups (on days 0 and 21) and all exposed groups after 21 d exposure to DEX were analysed. Western blotting was performed using a protocol modified from Rasmussen et al. (2011a,b). In brief, equal amounts of protein were separated on a 10% gel and electro-blotted onto a PVDF membrane. Following treatment with primary antibodies, proteins were visualised using HRP-conjugated secondary antibodies, an ECL substrate and a CDD camera. To verify equal protein loading and electro-transfer, total protein was stained using Ponceau S staining followed by visual inspection.

All samples to be compared were quantified relative to a standard sample. The control group average was normalised to 100, and the exposed groups were measured relative to that standard.

2.7. Statistical analyses

Statistical analysis was performed using Statistical Analysis System, version 9.1 (SAS Institute, Cary, NC, USA). Fish were divided into eleven groups according to exposure time and applied DEX concentration. A mixed procedure was used to evaluate the results. The model included the effect of treatment as a fixed factor, and the tank as a random factor. If the overall effect of treatment resulted in $p > 0.05$, no further analyses were performed. Otherwise, the differences between the least squares mean values of the eleven groups were determined by including the PDIFF option in the LSMEANS statement. A logarithmic transformation was applied to normalise data on enzymatic activity. Results are presented as least squares means and 95% confidence intervals after back-transformation per group of fish. The differences between two tanks did not exceed 12% in any assay. Results were considered significant when $p < 0.05$.

3. Results

3.1. Water chemistry

The concentration of DEX in the water was checked six times during the experimental period. Samples from each aquaria were taken immediately after water renewal and again after 24 h. The average concentration of DEX was always within 30% of the intended concentration. The time average concentrations over the experimental period were 3.5 ± 1.0, 26 ± 3.9, 235 ± 29 and 2121 ± 39 ng L^{-1} corresponding to the nominal concentrations of 3, 30, 300 and 3000 ng L^{-1}. In the controls, it was below the limit of quantification (<1.2–2.6 ng L^{-1}).

3.2. Total CYP450 content

Total CYP450 content in liver tissue was compared between control and DEX-exposed groups. There was a significant overall effect of treatment on total CYP450 content ($p = 0.015$; Fig. 1). After 21 d exposure, CYP450 content was lower in the groups exposed to 3, 30 and 300 ng L^{-1} DEX, compared with the control. At day 42, this difference was not present.

3.3. Specific CYP450 activity

Neither DEX concentration nor period of exposure altered the rates of measured CYP450-mediated reactions, as calculated per mg microsomal protein (Fig. 2).

Though a significant overall treatment effect on EROD activity was observed, variations between groups were random and unrelated to either DEX concentration or period of exposure.

The EROD activity was significantly induced by DEX at 3, 30 and 300 ng L^{-1} after 21 d exposure when activities were normalised to nmol total CYP450 content (Fig. 3). At a concentration of 3000 ng L^{-1}, DEX did not affect EROD activity. No effect of DEX was observed after 42 d exposure. Similar effects were observed for other measured reactions (Fig. 3).

3.4. CYP1A1 and CYP3A-like protein expression

Using specific polyclonal antibodies, immunoblotting was performed in hepatic microsomes after 21 d exposure to assess the effect of DEX on CYP1A, CYP3A-like and CYP2E1-like proteins

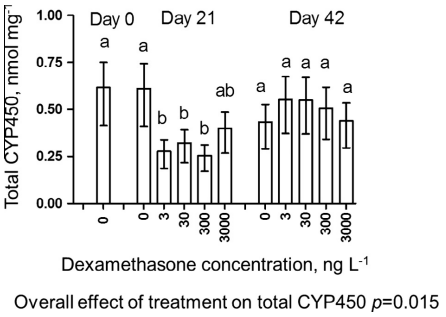


Fig. 1. Total CYP450 content in microsomal samples prepared from rainbow trout liver after chronic exposure to DEX. Differences between least squares means were determined with the probability difference test of mixed model with treatment as a fixed factor, and the tank as a random factor. Results are presented as least squares means and 95% confidence intervals after back-transformation per group of fish. Means with different superscripts are significantly different ($p < 0.05$) compared to control group; $N = 8$.

occurrence. Western blot analysis showed that fish exposed at the lowest environmentally relevant concentration of DEX had elevated CYP3A-like protein compared to the control group, but CYP1A protein levels remained unchanged (Fig. 4A and B). The CYP2E1-like protein was not detected.

4. Discussion

The induction of CYP450 in fish has been evaluated as a sensitive early warning signal of organic xenobiotics in the aquatic environment (van der Oost et al., 2003). In this sub-chronic *in vivo* experiment, we observed that DEX at concentrations ranging from 3 to 300 ng L^{-1} can modify CYP450 activity when expressed as amount of product formed per min per nmol total CYP450, and it can affect total CYP450 content. Several recent studies focused on the modulation of CYP450 activity by numerous pharmaceuticals including DEX (Smith and Wilson, 2010; Wassmur et al., 2010). In general, no consistent response of fish CYP450 to DEX was found across published studies. In one study, increased metabolism of a number of CYP450 substrates was found after treating rainbow trout with DEX at a dose of 2 mg/kg body weight (Haasch et al., 1994). Rainbow trout injected with DEX at a dose of 100 mg/kg body weight showed significant induction in 3-cyano-7-ethoxycoumarin metabolism (mainly metabolised by CYP1A enzymes), while EROD and 7-methoxyresorufin O-demethylation activities were not altered (Smith and Wilson, 2010). Similarly, injection of DEX at 0.03–3 μg/kg into male *H. malabaricus* did not alter EROD activity (Guiloski et al., 2015). CYP3A activity was induced in grass carp after DEX treatment (Li et al., 2008). This dissimilarity in responses can be associated with many factors, such as the age of the fish, type of exposure and differences in experimental concepts.

There is evidence that the response of CYP450 activity to DEX is species-specific. CYP1A and CYP3A expressions were strongly induced by DEX in porcine hepatocytes (Rasmussen et al., 2014) and in rat and human, but not minipig or beagle dog hepatocytes (Lu and Li, 2001). Within fish, there are also species-specific differences. Thus, in the same study, the effects of DEX were observed in rainbow trout but not in killifish (Smith and Wilson, 2010). Additionally, DEX induced CYP3A activity (induction of aminopyrine N-demethylase and erythromycin N-demethylase) in two grass carp cell lines (Li et al., 2008). Given that DEX is a

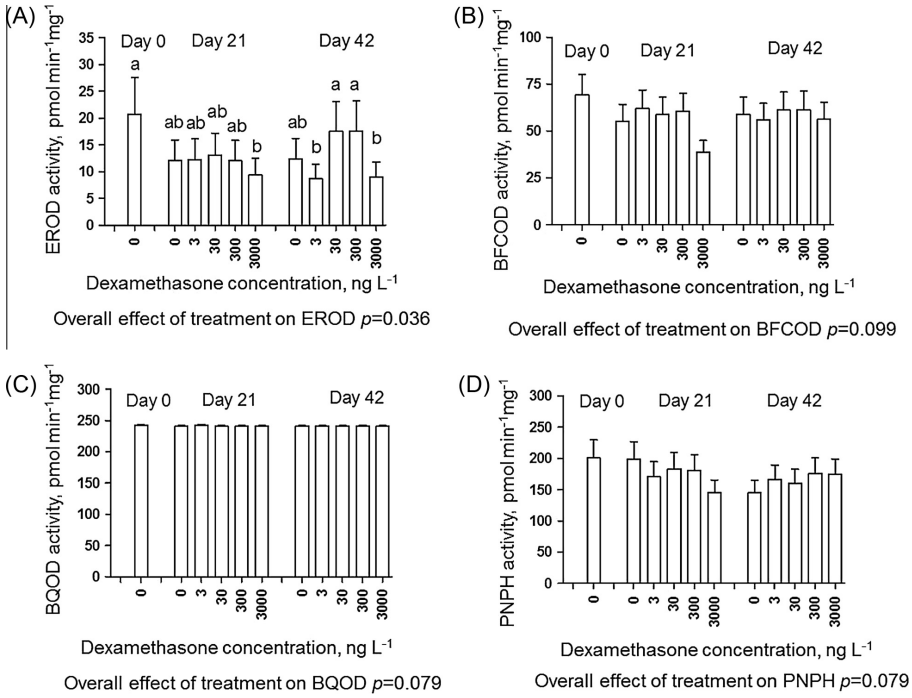


Fig. 2. The effects of DEX on (A) EROD, (B) BFCOD, (C) BQOD and (D) PNPH activity in hepatic microsomes prepared from rainbow trout liver ($n = 8$ in each group). Values are specific activities given as pmol/min normalised to the total amount of protein. Differences between least squares means were determined with the probability difference test of mixed model with treatment as a fixed factor, and the tank as a random factor. Results are presented as least squares means and 95% confidence intervals after back-transformation per group of fish. Means with different superscripts are significantly different ($p < 0.05$) compared to control group; $N = 8$. (A) EROD, 7-ethoxyresorufin O-deethylase; (B) BFCOD, 7-benzyloxy-4-trifluoromethylcoumarin O-debenzylase activity; (C). BQOD, 7-benzyloxyquinoline O-debenzylase activity; and (D) PNPH, p-nitrophenol hydroxylase activity.

well-known inducer of mammalian CYP3A, we included two catalyzed reactions (BFCOD and BQOD) to evaluate CYP3A activity. Both affected rainbow trout in similar ways, and induction followed 21 d exposure.

In mammals, DEX can initiate transcription of *CYP2E1* (Sampol et al., 1997). The CYP2E1-like protein activity has also been identified in fish (Kaplan et al., 1999; Wall and Crivello, 1999; Zamaratskaia and Zlabek, 2011). To date, there are no reports on the *in vivo* effect of DEX on PNPH activity. *In vitro*, DEX did not modify PNPH activity in rainbow trout (Burkina et al., 2013); however, we found that PNPH activity was induced by DEX after 21 d exposure. Compared to mammals, the regulation of CYP2E1 in fish is less investigated and requires further study. The molecular mechanisms by which DEX regulates PNPH activity appear to be similar to those of other studied CYP450-mediated reactions in fish liver.

At 3000 ng L^{-1}, DEX did not affect our investigated parameters, suggesting that DEX is regulated by CYP450 in a biphasic way, a response to xenobiotics that is not uncommon (Heinrichs et al., 1994). These data might also suggest that fish adapted to DEX exposure. It was previously shown that, depending on concentration, DEX might act as either a permissive or suppressive factor in enzyme induction (Ringel et al., 2002). We found that CYP1A1, CYP2E1-like and CYP3A-like enzyme activities increased in the presence of environmentally relevant concentrations of DEX, but not in the presence of the highest tested concentration (3000 ng L^{-1}) when activities were calculated per nmol total CYP450 content. No effect was observed when the activities were calculated relative to total protein content. Differences in the CYP450 responses to DEX depended on the calculation, either relative to total protein or to total CYP450 content. This is an important observation. Activities of CYP450-mediated reactions expressed relative to total protein content more closely reflected an *in vivo* situation, suggesting that overall DEX did not alter measured CYP450 activities. Nevertheless, an increase in the activities of CYP1A, CYP3A and CYP2E1-like proteins, expressed relative to total CYP450 content, suggest an eventual increase in the protein content of individual CYP450 isoforms. In studies using mammals, significant differences in enzyme catalytic activities were also found only after calculation of enzyme catalytic activity per total CYP450 (Wandel et al., 1998; Anzenbacherova et al., 2008). This means that the estimation of enzyme activity, expressed relative to total CYP450, is another way to illustrate a complete picture of the effects of various pharmaceutical drugs other than by calculation per milligram of protein.

To further test this concept, protein expressions of CYP1A and CYP3A were measured in the same sample. Samples from fish exposed to DEX for 21 d were used because no effect was observed

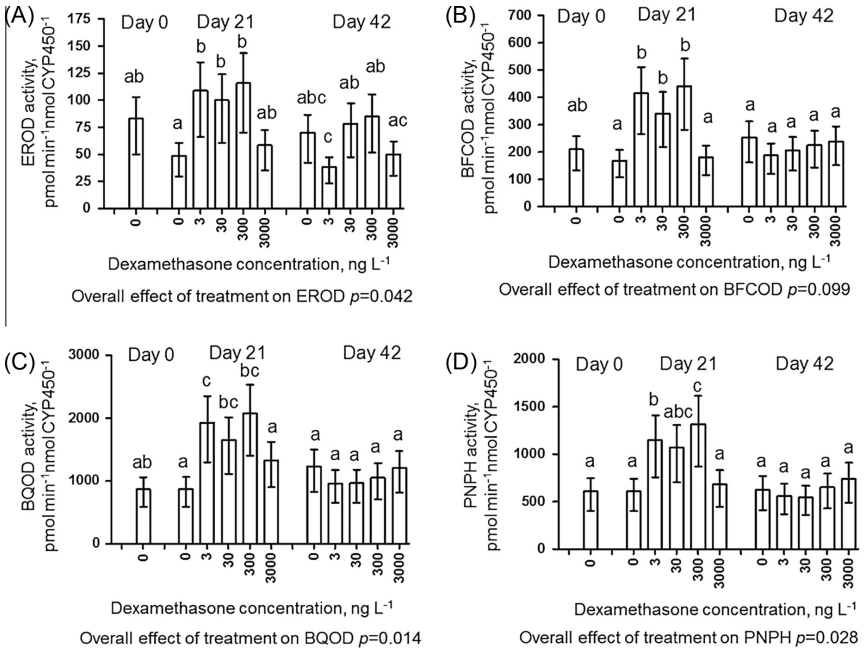


Fig. 3. The effects of DEX on (A) EROD, (B) BFCOD, (C) BQOD and (D) PNPH activity in hepatic microsomes prepared from rainbow trout liver (n = 8 in each group). Values are specific activities given as pmol/min normalised to the total amount of CYP450. Differences between least squares means were determined with the probability difference test of mixed model with treatment as a fixed factor, and the tank as a random factor. Results are presented as least squares means and 95% confidence intervals after back-transformation per group of fish. Means with different superscripts are significantly different ($p < 0.05$) compared to control group; N = 8. (A) EROD, 7-ethoxyresorufin O-deethylase activity; (B) BFCOD, 7-benzyloxy-4-trifluoromethylcoumarin O-debenzylase activity; (C) BQOD, 7-benzyloxyquinoline O-debenzylase activity; and (D) PNPH, p-nitrophenol hydroxylase activity.

after 42 d. Previous studies showed diverse evidence in the regulation of CYP1A and CYP3A proteins in fish and fish cell cultures. For example, DEX down regulated CYP1A1 in trout hepatocytes (Dasmahapatra and Lee, 1993), but did not affect CYP3A in rainbow trout (Lee et al., 1993). Moreover, in combination with beta-naphthoflavone (a known AhR activator), DEX increased CYP1A1 protein levels in the *P. lucida* hepatocellular carcinoma cell line (Celander et al., 1997).

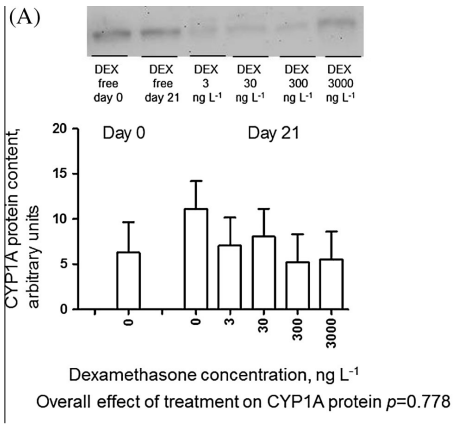
Following DEX exposure, the levels of CYP1A1 protein and activity remained unchanged, as seen in our previous *in vitro* study (Burkina et al., 2013). Dasmahapatra and Lee (1993) reported that 3.9 μg L^{-1} DEX, similar to our highest tested concentration, did not change CYP1A1 protein content. An increase in CYP3A protein content can be related to an adaptive mechanism of fish to DEX exposure. Immunoblotting data did not support the CYP450 activity data. CYP3A-like protein level was increased only at 3 ng L^{-1} DEX, but not for other doses. This study showed that treatment with the same dose of DEX did not cause a change in the activity of BFCOD or BQOD (Fig. 2).

Determining the protein content in higher concentrations might be helpful in understanding the exact mechanism of CYP450 activity induction and potential interactions of glucocorticoids with receptors such as AhR and pregnane X receptor.

In this study, the CYP2E1-like protein was not detected using several antibodies against human and rat epitopes; however the CYP2E1 protein level was successfully determined and responded to carbon tetrachloride treatment in common carp liver (Jia et al., 2014). In future studies, it could be desirable to develop CYP2E1 antibodies against fish CYP2E1.

A number of *in vitro* data have been reported for the effects of DEX on mammalian and fish CYP450 systems, but data from *in vivo* studies remains limited. It is important to investigate the effects of DEX at concentrations that commonly occur in the aquatic environment to mimic its possible interaction with fish-specific protein targets. Here, we tested the effects of low levels of residual DEX on specific pharmacological targets in fish and found that DEX can modify CYP450 activity in rainbow trout. We also clearly showed that DEX induces hepatic CYP3A-like proteins. This observation is important because there may be further interactions of environmentally relevant DEX concentrations with cellular recovery mechanisms and non-investigated xenobiotic-metabolizing proteins in fish. However, treatment of juvenile rainbow trout with DEX failed to demonstrate a clear and significant effect at high concentrations. In actual conditions, combinations of DEX with other glucocorticoids can raise synergistic actions of this key xenobiotic detoxification pathway.

Our results, together with the results of previous studies, highlight the need to further investigate the effects of glucocorticoids in aquatic environments on fish CYP450 and their impacts on biochemical processes.



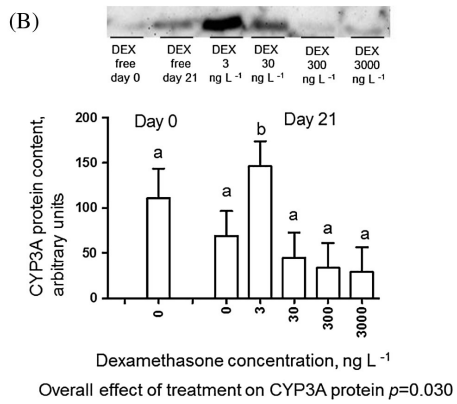


Fig. 4. (A) The effects of DEX on CYP1A protein in hepatic microsomes prepared from rainbow trout ($n = 8$ in each group). Band with a molecular weight of approx. 50 kDa. (B) The effects of DEX on CYP3A-like protein in hepatic microsomes prepared from rainbow trout ($n = 8$ in each group). Means with different superscripts within bar differ ($p < 0.05$).Band with a molecular weight of approx. 50 kDa.

Conflict of interest statement

The authors declare no conflicts of interest.

Acknowledgement

The study was financially supported by the Ministry of Education, Youth and Sports of the Czech Republic – projects "CENAKVA" (No. CZ.1.05/2.1.00/01.0024) and "CENAKVA II" (No. LO1205 under the NPU I program), by the Grant Agency of the University of South Bohemia in Ceske Budejovice (No. 087/2013/Z) and by the Czech Science Foundation (No. P503/11/1130). Individual CYP450 activities were analysed at Swedish University of Agricultural sciences, NJ Faculty. We also thank American Manuscript Editors for editing the English manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2015.06.031.

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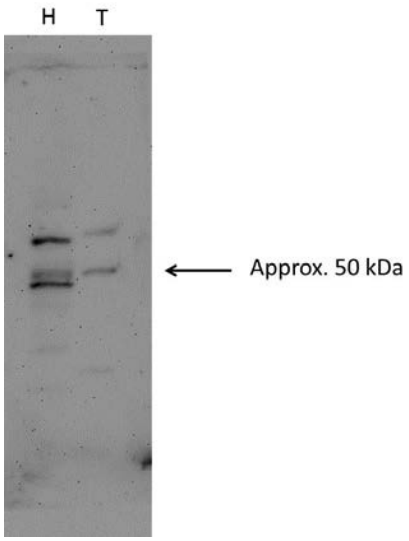
Appendix B.

Supplement 1. *Western blot of microsomes from human (H) and rainbow trout (T) liver tissue. Experimental conditions are given in section 2.6 in the manuscript. Only the band with a molecular weight of approx. 50 kDa were included in the analysis.*



Supplement 2*. LC-MS/MS gradient for the elution of DEX.*

Analytical pump 1			
Time, min	A, %	B, %	Flow, µL/min
0.00	85	15	300
2.70	85	15	300
10.00	0	100	400
12.00	0	100	400
12.01	85	15	300
13.00	85	15	300

Analytical pump 2			
Time, min	A, %	B, %	Flow, µL/min
0.00	100	0	2000
2.60	100	0	2000
2.71	0	100	300
8.00	0	100	500
10.00	0	100	2000
11.00	100	0	2000
13.00	100	0	2000

A – ultrapure water (Milli-Q; with 0.1% formic acid); B – acetonitrile (with 0.1% FA). It took 1 min to load the sample; 6.55 min to elute the target compound.

Supplement 3. *Optimized MS/MS parameters for SRM analysis.*

Compound	Precursor ion (m/z)	Quantification ion (m/z)	Confirmation ion (m/z)	NCE	RT
Dexamethasone	393.2070	291.1752	279.1743	30	6.55
Diltiazem	415.1689	178.0320	*	35	6.53

*No confirmation ion.

NCE, normalized collision energy add (%); SRM, selected-reaction monitoring; RT, retention time.

Chemosphere 159 (2016) 10–22

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Sub-lethal effects and bioconcentration of the human pharmaceutical clotrimazole in rainbow trout (*Oncorhynchus mykiss*)

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HIGHLIGHTS

- Juvenile rainbow trout were sub-chronically exposed to CLO.
- A six-week exposure is sufficient to initiate histopathological changes in fish organs.
- CLO was found to have a high concentration level in fish liver and kidney.
- The depuration half-life of CLO in fish was estimated to be 28 days in fish kidneys.
- CLO affect the activities of the studied CYP450 isoforms in the liver.

ARTICLE INFO

Article history:
Received 12 January 2016
Received in revised form
3 May 2016
Accepted 13 May 2016

Handling Editor: Jim Lazorchak

Keywords:
Biomarkers
Depuration half time
Hematology
Histopathology

ABSTRACT

The aim of this study was to characterize biomarker responses, haematological profiles, structural changes and uptake in juvenile rainbow trout exposed to clotrimazole (CLO) at three concentrations (0.01 – [lowest environmentally relevant concentration], 1.0 [highest environmentally relevant concentration] and 10 μg L^{-1}) in a semi-static system over a period of 42 days. Antioxidant defence enzymes, which responded to CLO exposure, changed the oxidative stress status of cells, but no differences were observed in lipid peroxidation. Clotrimazole triggered a biphasic response of CYP3A-like activity in liver microsomes, which may indicate a detoxification process in the liver. Histopathological alterations were most pronounced in kidneys and testes in the group exposed to 10 μg 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 testes were changed: The number of spermatozoa was reduced, and the spermatogonia and spermatocytes were increased. The highest CLO concentration was detected in fish liver (3710 ng per gram wet tissue) and kidney (4280 ng per gram wet tissue). Depuration half-life was estimated to be 72, 159, and 682 h in liver, muscle, and kidney, respectively.

Taken together, these results provide valuable toxicological data on the effects of CLO on aquatic non-target organisms, which could be useful for further understanding of the potential risks in the real aquatic environment.

Abbreviations: BFCOD, 7-benzyloxy-4-trifluoromethylcoumarin O-debenzylase; CAT, Catalase; CF, Condition factor; CLO, clotrimazole; CYP450, cytochrome P450; EROD, ethoxyresorufin O-deethylase; GPx, Glutathione peridoxase; GR, Glutathione reductase; GST, Glutathione S-transferase; PCV, Haematocrit; Hb, Haemoglobin concentration; HIS, Hepatosomatic index; LDH, Lactate dehydrogenase; Leuko, Leukocyte count; LPO, Lipid peroxidation; MCV, Mean corpuscular erythrocyte volume; MCHC, Mean corpuscular haemoglobin concentration; MCH, Mean corpuscular haemoglobin; ROS, Reactive oxygen species; RBC, Red blood cell count.

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http://dx.doi.org/10.1016/j.chemosphere.2016.05.042
0045-6535/

1. Introduction

Residues and metabolites of pharmaceuticals that reach aquatic systems can exert adverse effects on non-target organisms (Kasprzyk-Hordern et al., 2008; Brausch and Rand, 2011; Verlicchi et al., 2012; Baumann et al., 2013). Clotrimazole (CLO), bisphenyl-2-chlorophenyl-1-imidazolyl methane, is an antifungal agent widely used in human and veterinary medicine for treating fungal, dermatophyte and yeast infections. In the Czech Republic, CLO prescriptions for the treatment of dermatological and genitourinary problems amounted to 3.94 tons in 2013 (SUKL).

Clotrimazole is presumably introduced into the aquatic environment mainly through domestic (Thomas and Hilton, 2004; Peschka et al., 2007) and hospital wastewater discharge (Escher et al., 2011; Frédéric and Yves, 2014). It is expected in numerous aquatic environments because of its relative resistance to hydrolysis (at pH 7, its half-life at 4 °C and 25 °C is 242 days and 20 days, respectively) and photolysis (half-life is 3–310 days, depending on sunlight conditions) (OSPAR, 2013). Biotransformation and adsorption to particles are the main processes for its elimination in wastewater treatment plants (WWTPs') (Peng et al., 2012), and it has been detected in river water at 6–71 ng L^{-1} (Thomas and Hilton, 2004; Roberts and Thomas, 2006; Huang et al., 2010) and in wastewater at 0.6–111 ng L^{-1} (Huang et al., 2010; Loos et al., 2013). The Guangzhou (China) WWTP influent, containing between 256 and 1834 ng L^{-1}, had its highest mass load inflow in the winter and its lowest in the fall, ranging between 0.0028 and 0.28 mg person^{-1} d^{-1} (Peng et al., 2012). Clotrimazole has recently attracted the interest of the research community as a contaminant of concern (Gyllenhammar et al., 2009; Shi et al., 2012) for its diverse toxicity to aquatic organisms [PNEC = 1 μg L^{-1} (Peng et al., 2012) to 0.05 pg L^{-1} (Huang et al., 2010)].

Recently, experimentally-exposed common carp took up CLO from water and maintained a consistent concentration in the liver and blood plasma (Corcoran et al., 2014). Clotrimazole may influence other aquatic organisms. Survival and development of marine shrimp larvae (*Palaemon serratus*) were affected at 2.78 μg L^{-1} (González-Ortegón et al., 2015), and morphological changes were observed at 3.16 μg L^{-1} (González-Ortegón et al., 2013).

Because CLO belongs to the antifungal group of medications containing an imidazole ring, it can affect the activity of cytochrome P450 (CYP450) (Verras and Ortiz de Montellano, 2006). Antifungals act through the inhibition of CYP450-dependent 14α-demethylase, which is critical to ergosterol biosynthesis (Rupp et al., 2005). Despite anatomical differences, many physiological and biochemical processes in fish are remarkably similar to those in humans. For example, the fish CYP450 enzyme system shares 50% identity with the corresponding mammalian system (Huggett et al., 2003). The enzymes form an extremely important metabolic system because of their involvement in regulating the titres of endogenous compounds such as hormones, fatty acids and steroids. Thus, the physiological processes in fish may be affected by the presence of CLO. 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 (Ronis et al., 1998; Zhang et al., 2002; Hinfray et al., 2006; Wassmur et al., 2013). In addition, it modulates cellular Ca^{2+} homeostasis in mammals (Snajdrova et al., 1998; Klokouzas et al., 2002) and steroidogenesis in aquatic organisms (Hinfray et al., 2011; Baudiffier et al., 2012, 2013).

In general, fungicides may induce cellular damage in aquatic organisms e.g. [(Li et al., 2010a,b,c; Toni et al., 2011)]. Among cellular toxicity mechanisms, oxidative stress, defined as an imbalance between pro-oxidant and antioxidant cellular forces, causes oxidative damage to tissues (Ashtiani et al., 2011). This occurs mainly in the endoplasmic reticulum of exposed cells where CYP450 activities may generate reactive oxygen species (ROSs) as by-products of detoxification processes. Liver and gill tissues are considered to be the most susceptible in fish, and both enzymatic (superoxide dismutase, catalase and peroxidases) and non-enzymatic (e.g., vitamins and glutathione) antioxidants are used to manage this. The alterations of these enzymes during antioxidant defence are used as biomarkers in response to oxidative stress (van der Oost et al., 2003). So far, little information has been available on the oxidative effects of CLO in fish. Sub-chronic toxicity tests can provide useful information for understanding possible ecotoxicological effects of human pharmaceuticals on aquatic organisms.

The aim of this study was to assess the sub-chronic effects of CLO at two environmentally relevant CLO concentrations and one sub-lethal concentration on juvenile rainbow trout (*Oncorhynchus mykiss*). Specifically, it aimed to 1) investigate the impact of CLO exposure on the haematological and biochemical profiles of blood plasma and the histology of the kidney, liver, gill and gonads; 2) measure the enzymatic activities involved in detoxification and levels of lipid peroxidation in the liver, gills and brain; 3) examine tissue-specific CLO concentrations followed by a depuration period of 7 or 13 days; and 4) determine CLO concentrations in faeces.

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, obtained from Sigma Aldrich as the nitrate salt (purity 98%), was used as an internal standard (due to similarity with structure and retention time of econazole to CLO). 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). All other chemicals were obtained from Sigma Aldrich Europe.

2.2. Fish

Juvenile rainbow trout (n = 240) weighing 220 ± 40 g (mean ± standard deviation, S.D.), were obtained from a local commercial hatchery (Husinec, Czech Republic). Fish were transferred to aquaria containing 250 L of freshwater with continuous aeration under a 12:12 L:D photoperiod. The temperature, dissolved oxygen concentration and pH were 15.6 ± 0.9 °C, 7.5–8.5 mg L^{-1} and 7.2 ± 0.15, respectively. Details on water hydrochemical analysis are given in Supplementary Material 1. 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. They were handled according to national and institutional guidelines for the protection of human subjects and animal welfare. The unit is licensed (No. 53100/2013-MZE-17214) according to the Czech National Directive (the Law against Animal Cruelty, No. 246/1992).

2.3. Exposure to clotrimazole and chemical analyses

Fish were randomly divided into groups of 20 placed in each of twelve aquaria containing 200 L of water or testing solution in a semi-static system. In semi-static system (without flow though) each aquaria had their own sink drain and water tap. During the test, 75% of water was daily changed and replaced by new water

containing the corresponding concentration of CLO. Fish were exposed to concentrations of CLO at 0.01 (lowest environmentally relevant concentration), 1.0 (highest environmentally relevant concentration), and 10 μg L^{-1} (sub lethal concentration) for 21 and 42 days. A control group (C) was maintained in clean freshwater. Solvent control (SC) group was exposed to the volume of DMSO (v/v, 0.01%) used with CLO exposed fish. All concentrations were tested in duplicate.

A stock solution of CLO was prepared in DMSO (2 mg mL^{-1}) and used to make three working solutions in water (2, 200, and 2000 μg). Test solutions were prepared from working solutions daily. 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 and working solutions of CLO were protected from light and stored at +2 °C and +10 °C, respectively.

Testing solutions were renewed each day to maintain water quality and the appropriate CLO concentration. The concentration of CLO in the water was measured six 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 μg L^{-1} CLO treatment were used to assess the adsorption level of CLO in conditions without fish. The water sampling regime was the same as described above for the experimental period. Water samples from each exposure aquarium were analysed 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 analysed 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 were as described by Grabic et al. (2012). Methods details are in Supplement 2.

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. Tissue samples

Fish were not fed for 24 h prior to sampling to avoid prandial effects. At days 21 and 42, four fish from each duplicate aquarium were sacrificed and combined into a single group of 8. Blood, brain, gills, gonads, liver, kidney, and white muscle were quickly removed.

Blood was taken by caudal vein puncture using a heparinized syringe. An aqueous solution of heparin sodium salt (5000 IU mL^{-1}) at 0.01 mL mL^{-1} blood was used to stabilize the samples. A small volume of blood was immediately used for determination of haematological variables and differential leukocyte counts (Svobodova et al., 1991). Blood plasma for chemical analysis was obtained from cooled centrifuged blood (10 min, 837*g*, 4 °C). Blood was stored at −80 °C for determination of biochemical catalase and glutathione reductase activity and at −20 °C for chemical analyses. Gills from both sides and whole brain were removed and stored at −80 °C for biochemical analysis. Two liver samples for biochemical analyses, one for preparation of the microsomal fraction (~0.4 g) and one for the post-mitochondrial supernatant (~0.1 g) were stored at −80 °C until use. A third liver sample (~0.4–0.5 g) along with kidney and muscle samples were stored at −20 °C for chemical analyses.

Fork length (FL), body weight (BW), and liver weight (LW) of fish were recorded. Condition factor (CF) and hepatosomatic index (HSI) were calculated according to White and Fletcher (1985): CF = BW (g)/FL^3 (cm) × 100; HSI = LW (g)/BW (g) × 100.

After exposure period, fish exposed to CLO at 1.0 μg L^{-1} treatment were placed into CLO-free water to determine the depuration half-life of CLO in the fish. Depuration was determined after 7 days (day 49; 6 fish) and 13 days (day 55; 8 fish). First-order depuration kinetics was assumed. 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

On day 21 of exposure, fish faeces were collected over a 24 h period from the bottom of aquaria in all experimental aquaria, and CLO concentrations were measured.

A modified extraction procedure (Fedorova et al., 2014) was used to extract CLO from fish tissue and faeces. Briefly, samples of liver, kidney, muscle, and faeces (approximately 0.5 g), internal standard (econazole, 50 ng per sample), and 1 mL of extraction solvent (acetonitrile:isopropanol, 1:1) were homogenized with stainless-steel balls for 10 min, 30 s^{-1} (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. The extract was diluted with water (1:3) for liquid chromatography tandem high resolution mass spectrometry (LC-MS/HRMS) analysis. Faeces samples were assayed immediately after extraction.

Clotrimazole was quantified using a 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 V. The method details are documented in Supplementary Material 3.

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

2.6. Validation of clotrimazole quantification 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 1–500 ng g^{-1} (R^2 = 0.994). Method repeatability was tested for ten replicates with 0.1 μg L^{-1} concentration levels; relative standard deviation (RSD) for each concentration in replicates was 8%. Recovery of CLO from fish tissue was evaluated from fortified samples. Average recovery of CLO was 98% with an RSD of ten replicates of 9% for fish

muscle, 101% with an RSD 7% for plasma, and 100% with an RSD 8% for kidney. The limit of quantification (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. Biochemical assays

2.7.1. Preparation of post-mitochondrial supernatant and microsomal fraction

The post-mitochondrial supernatant (PMS) was obtained as described by Howcroft et al. (2009). Samples were homogenized in 1.5 mL 0.1 M K-phosphate 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 10,000g for 20 min at 4 °C to isolate the PMS.

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.

Protein levels were estimated spectrophotometrically as 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}.

2.7.2. 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} cm^{-1} (Omura and Sato, 1964a,b).

Two CYP450-mediated reactions were used to estimate catalytic activities of CYP450 isoforms: 7-ethoxyresorufin O-deethylation (EROD, CYP1A) and 7-benzyloxy-4-trifluoromethylcoumarin O-debenzylation (BFCOD, CYP3A).

The catalytic activity of EROD was determined as the transformation rate of resorufin to resorufin (Kennedy and Jones, 1994). The 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). Reaction incubations were described by Grabicova et al. (2013).

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 (limits of detection for resorufin was 2 pmol and for HFC 1 pmol).

2.7.3. Glutathione S-transferase

Glutathione S-transferase (GST) activity was determined using 1-chloro-2,4-dinitrobenzene as a substrate, using the methods of Habig et al. (1974) adapted for the microplate reader by Frasco and Guilhermino (2002). 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} cm^{-1}.

2.7.4. Catalase

Catalase (CAT) activity was determined as a decrease in hydrogen peroxide in a 96-well flat-bottomed UV-transparent microtitre plate. The decomposition of the substrate (H_2O_2) was recorded at 240 nm. Calculations were made using a molar extinction coefficient of 40 $M^{-1}cm^{-1}$.

2.7.5. Glutathione reductase

Glutathione reductase (GR) activity was determined by the method of Cribb et al. (1989). 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}.

2.7.6. Glutathione peroxidase

Glutathione peridoxase (GPx) activity was measured by the method of Mohandas et al. (1984). Oxidation of NADPH was recorded at 340 nm. A molar extinction coefficient of 6220 M^{-1} cm^{-1} was applied.

2.7.7. Lipid peroxidation

Oxidative damage was assessed by determining the level of LPO following the methodology of Ohkawa et al. (1979). Briefly, 100 μL homogenate was mixed with 333 μL cold 12% trichloroacetic acid, 200 μL 60 mM Tris-HCl with 0.1 mM DTPA, and 333 μL 0.73% 2-thiobarbituric acid. Tubes were held 1 h at 100 °C. After centrifugation at 3000g for 5 min and 25 °C, absorbance was determined at 535 nm. A molar extinction coefficient of 156 000 M^{-1} cm^{-1} was applied.

2.7.8. Lactate dehydrogenase

Lactate deydrogenase (LDH) activity was determined by the method of Vassault (1983) adapted for microplate, as described by Diamantino et al. (2001). A molar extinction coefficient of 6300 M^{-1} cm^{-1} was applied.

Details on concentration of substrate, protein and preparation of reaction mixtures are given in Supplementary Material 4.

All assays were performed spectrophotometrically in quadruplicate using a 96-well microplate reader (Labsystem Multiscan EX). Samples were held on ice; measurements were made at 25 °C. Variation in absorbance at each reaction well was linear over time ($R^2 > 0.8$). Enzyme activity was expressed as units of substrate hydrolysed per minute per mg protein. Oxidative damage was expressed as the mmol of thiobarbituric acid-reactive substances (TBARS) per gram wet tissue.

2.8. Haematological variables

Transformation solution (0.1 g potassium ferricyanide, 0.025 g potassium cyanide, 0.07 g potassium dihydrogen phosphate made up to 0.5 L with distilled water) was used to determine haemoglobin concentration.

Eight haematological indices were measured: haemoglobin concentration (Hb), red blood cell count (RBC), haematocrit (PCV), mean corpuscular erythrocyte volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), leukocyte count (Leuko), and granulocyte count. Haemoglobin level was determined using a spectrophotometer (Helios Epsilon, UNICAM). The conventional biomarkers MCV and MCHC were quantified from blood count analysis. Conventional biomarkers were used for prediction of the average volume of a red blood corpuscle (MCV), average haemoglobin concentration in individual erythrocytes (MCH) and concentration of haemoglobin in unit volume of erythrocytes (MCHC). Procedures were based on unified methods for haematological examination of fish (Svobodova et al., 1991).

2.9. Histology

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

Fixed samples of kidney, liver, gill and gonads of fish from SC and 10 μg L^{-1} aquaria were paraffin-embedded and cut into 5 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).

2.10. Statistical analysis

All statistical analyses were conducted using STATISTICA (v. 10.0 for Windows, StatSoft). One-way analysis of variance (ANOVA) was employed to find significant differences in measured variables between experimental groups (n = 8) after normality and variance homoscedasticity tests were conducted. The *t*-test was used to compare SC to the control group. The two were combined into a single control to assess biomarker responses at days 21 and 42. 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 recognized when $p < 0.05$. Data are presented as least-squares means after back-transformation with 95% confidence intervals.

3. Results

Controls and exposed groups showed normal growth and feeding behaviours. No mortalities occurred during the 42 days in any group. No significant differences in biochemical variables were found between control and solvent control groups.

3.1. Clotrimazole concentrations in aquarium water, fish tissues and faeces

Table 1 shows CLO concentrations as measured in aquarium water. Water samples from all controls were below the LOQ (<0.009 μg L^{-1}). No significant fluctuations in CLO at the environmentally relevant concentration were observed, likely because the concentration was near the LOQ (uncertainty in the assessment method was 30–40% at LOQ). Among aquaria at 10 μg L^{-1}, levels decreased to 49% over 24 h without fish and to 3%–30% with fish.

Table 2 shows the concentrations of CLO in fish tissue and blood plasma for the exposure periods. No CLO was detected in any tissue from the control or solvent-only control groups. More than 89% of the analysed samples in the experimental groups were positive at both sampling times.

We found no CLO (<6.2 ng g^{-1}) in the blood plasma of fish from the 0.01 and 1.0 μg L^{-1} treatment groups, but at 10 μg L^{-1}, it was 350 ng g^{-1} and 450 ng g^{-1} at days 21 and 42, respectively. Muscle from this group contained 490 ng g^{-1} at day 21, and this increased 2.27-fold at day 42. The greatest CLO concentrations were found in kidney and liver tissues. At the highest CLO exposure, liver contained 2960 ng g^{-1} at day 21 and 1.25 times more at day 42. Kidney contained 1260 ng g^{-1} at day 21 day and 3.4 more at day 42.

Supplement 5 shows CLO levels in faeces. They ranged from 3% to 7% of the total amount in each aquarium (2200 and 2000 μg). Actual concentrations in faeces reflect the CLO exposure level (0.037, 2.3 and 29 μg g^{-1} w/w for 0.1, 1.0, and 10 μg L^{-1}, respectively).

3.2. Depuration of CLO from fish tissue

Depuration rates of CLO were measured only in fish exposed to 1.0 μg L^{-1} (Table 3). After 7 and 13 days' depuration, plasma levels were below the LOQ (<6.2 ng g^{-1}). Levels remained detectable in organs (liver < muscle < kidney). The estimated half-lives were 72 h in liver, 159 h in muscle and 682 h in kidney.

3.3. Biometric indices

Body weight and fork length did not significantly differ between treated groups ($p > 0.05$) on day 21 (Table 4). At day 42, liver weight was significantly greater among fish exposed to 1.0 and 10 μg L^{-1}. Significantly higher HSIs were observed in the 1.0 and 10 μg L^{-1} treatment groups (Table 5).

3.4. Biochemical effects

3.4.1. Total CYP450 content, EROD and BFCOD activity

Treatment did not affect CYP450 content (see Supplement 6). At 0.01 and 10 μg L^{-1} treatment, EROD activity was higher at day 42 than in corresponding controls (Fig. 1). In controls, it was lower at day 42 than at day 21, indicating a decrease in EROD activity among controls rather than an increase among exposed fish. Variations in EROD activity in exposed fish (3.6- and 2.4-fold differences in 0.01 and 10 μg L^{-1} groups, respectively, over controls at day 42) were significant, but not concentration-dependent.

At day 21, BFCOD activity was significantly different only at the greatest CLO concentration (Fig. 1). Longer exposure times were associated with inhibition of BFCOD activity in the 0.01 μg L^{-1} treatment group, but activity induction was seen at 10 μg L^{-1}.

3.4.2. Oxidative stress, antioxidant and GSH-antioxidant responses

Lipid peroxidation levels (expressed by TBARS levels) were not significantly different between controls and exposed fish in any tissue or at any treatment time (see Supplement 7). Levels of antioxidant enzyme activities in tissues after sub-chronic exposure to CLO are presented in Fig. 2 (a–o).

In controls, CAT activity in gills was significantly lower on day 21 than on day 42. Higher CAT levels were found in gills at day 21 and brains at day 42 in 1.0 and 10 μg L^{-1} treatment groups than in

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.

Added concentration of clotrimazole (nominal concentration), μg L^{-1}	Measured concentrations of clotrimazole in experimental water (with fish), μg L^{-1}						Measured concentrations of clotrimazole in water (without fish), μg L^{-1}	
	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	0.050	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%

Limit of quantification (LOQ) was 0.009 μg L^{-1}.

Table 2
Concentration of clotrimazole (ng g^{-1}) in rainbow trout. Groups exposed to clotrimazole 0.01, 1.0, and 10 μg L^{-1}. Data are means ± S.D., n = 8.

Tissue	Experimental groups					
	21 d			42 d		
	0.01 μg L^{-1}	1.0 μg L^{-1}	10 μg L^{-1}	0.01 μg L^{-1}	1.0 μg L^{-1}	10 μg L^{-1}
Plasma	≤ LOQ	≤ LOQ	350 ± 40 (8/8)	≤ LOQ	≤ LOQ	450 ± 50 (8/8)
Muscle	2.6 ± 0.56 (8/8)	15 ± 8.9 (7/8)	490 ± 200 (8/8)	5.5 ± 2.6 (8/8)	28 ± 4.3 (8/8)	1110 ± 260 (8/8)
Liver	51 ± 26 (8/8)	1140 ± 700 (8/8)	2960 ± 1780 (8/8)	251 ± 130 (8/8)	1300 ± 350 (8/8)	3710 ± 1470 (8/8)
Kidney	16 ± 12 (8/8)	110 ± 40 (8/8)	1260 ± 310 (8/8)	180 ± 70 (8/8)	240 ± 120 (8/8)	4280 ± 1980 (8/8)

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 analysed samples.

controls.

Glutathione reductase activity was significantly induced in livers of fish exposed at the highest concentration of CLO at day 42, but was significantly decreased in the brains of those exposed to 1.0 μg L^{-1} at day 21, compared to controls. At the end of exposure, activity of GR in fish from all treatment groups was not significantly different from that of control in gill and brain.

In livers, GPx activities in controls were significantly lower on day 42 compared to day 21, and on day 42 the activities were also lower in fish exposed to 10 μg L^{-1} compared to control. In gills, on day 21 the activities were lower in fish exposed to 1.0 and 10 μg L^{-1} compared to control, yet higher on day 42 after exposure to 1.0 μg L^{-1} at day 42.

At day 21, significantly higher LDH activities were seen in livers after exposure to 0.01 and 1.0 μg L^{-1} than no exposure, whereas these values in the highest concentration did not differ from the control. The LDH activities in brains exposed to 1 μg L^{-1} for 21 days were significantly lower compared to control and other treatment groups. During the follow-up exposure, significant decrease in LDH activity were observed at concentrations of 1.0 and 10 μg L^{-1} compared to control. The values in the concentration of 0.01 μg L^{-1} did not differ from control.

Compared to controls, sub-chronic exposure to CLO resulted in significantly higher levels of GST in livers after exposure to 0.01 and 1.0 μg L^{-1} at day 42 (Fig. 2).

3.5. Haematological profiles

Table 6 shows erythrocyte and leukocyte profiles. At day 42, RBC was significantly higher among those exposed to 0.01 μg L^{-1} than all other groups. Granulocytes in those exposed to 0.01 and 10 μg L^{-1} at day 42 were 2.7 and 2.4 times higher, respectively, than seen in the controls.

3.6. Histopathological changes

3.6.1. Gills

The SC and 10 μg L^{-1} treatment groups had similar results in gills at day 42. Pathologies were characterized by mild to moderate epithelial cell hyperplasia and hypertrophy, multifocal epithelial cell detachment and capillary aneurisms (see Supplement 8).

3.6.2. Kidney

Among the SC group, 4 of 8 fish showed mild cytoplasmic vacuolation or granulation of tubular epithelial cells. At day 42, among those exposed to 10 μg L^{-1}, 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 homogenous eosinophilic material in the cytoplasm of tubular epithelial cells (hyaline droplet degeneration). In two fish, greater tubuloneogenesis (newly formed tubules) was observed. Three fish showed dilation of the Bowman's capsule, and in one, this included deposition of granular eosinophilic material (Fig. 3).

3.6.3. Liver

Similar structural changes were observed among those in the SC and 10 μg L^{-1} treatment groups, including mild to moderate lipid vacuolation of hepatocytes and granulation of cytoplasm (see Supplement 8).

3.6.4. Gonads

In the ovaries, no differences were observed between the SC and 10 μg L^{-1} treatment groups. In each, two males were found. Composition of testicular germ cells differed between treatment and control animals at day 42. Compared to controls, treated fish had fewer spermatozoa, whereas the numbers of spermatogonia and spermatocytes were greater (Fig. 4).

4. Discussion

The results of this work demonstrate the effects of a widely-used fungicide, CLO, on the activity of enzymes related to biotransformation and antioxidant enzyme activity. Effects on haematological indices and mild histopathological alterations of several tissues were observed.

Verifying actual exposure under laboratory conditions is crucial for compounds with high partition coefficients (K_{ow}) such as CLO (Peschka et al., 2007; González-Ortegón et al., 2013; Corcoran et al., 2014). The significantly different adsorptions of CLO between aquaria containing fish and those without fish suggests that under experimental conditions, CLO may have been partly adsorbed by aquaria surfaces (insignificant portions), and it was probably first

Table 3
Depuration half-life of clotrimazole in fish tissue. Group exposed to CLO 1 μg L^{-1}, 7 (n = 6) and 13 (n = 8) days after termination of exposure experiment. Data are means ± S.D. Units ng g^{-1}.

Groups	Post exposure period (days)	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)		N.D.	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 analysed samples. Half-life (mean) calculated as $t_{50} = 0.693/k$. N.D. – not detectable.

Table 4
Morphological parameters in rainbow trout sub-chronically exposed to CLO. Significant differences compared with solvent control group, $P < 0.05$ (means with different superscripts are significantly different, ANOVA). Data are means ± S.D., n = 8.

Indices	Exposure (days)	C	SC	0.01 μg L^{-1}	1.0 μg L^{-1}	10 μg L^{-1}
FL	21	25 ± 1.1^{bcde}	24 ± 1.3^{abc}	23 ± 0.68^{cde}	24 ± 1.8^{cde}	25 ± 2.3^{bcde}
(cm)	42	26 ± 0.63^{abc}	26 ± 0.85^{abc}	26 ± 1.0^{abcd}	27 ± 0.88^{ab}	28 ± 1.1^{cde}
BW	21	218 ± 31^{abc}	183 ± 25^{bc}	175 ± 17^{c}	188 ± 36^{bc}	203 ± 45^{bc}
(g)	42	225 ± 18^{ab}	230 ± 32^{ab}	218 ± 26^{abc}	256 ± 25^{a}	257 ± 18^{a}
LW	21	2.0 ± 0.43^{ab}	1.6 ± 0.68^{ab}	1.6 ± 0.29^{ab}	1.7 ± 0.35^{ab}	1.8 ± 0.35^{ab}
(g)	42	1.7 ± 0.20^{b}	1.7 ± 0.35^{ab}	1.7 ± 0.19^{ab}	2.2 ± 0.45^{a}	2.2 ± 0.20^{a}

FL, fork length; BW, body weight; LW, liver weight.

Table 5
Effect of CLO on biometric characteristics of rainbow trout. Significant differences compared with solvent control group, $P < 0.05$ (means with different superscripts are significantly different, ANOVA). Data are means ± S.D., n = 8.

Indices	Unit	Exposure (days)	C	SC	0.01 μg L^{-1}	1.0 μg L^{-1}	10 μg L^{-1}
CF	g/cm^3	21	1.3 ± 0.13^{a}	1.3 ± 0.17^{a}	1.4 ± 0.13^{a}	1.4 ± 0.27^{a}	1.4 ± 0.21^{a}
		42	1.3 ± 0.09^{a}	1.3 ± 0.14^{a}	1.2 ± 0.05^{a}	1.2 ± 0.05^{a}	1.2 ± 0.10^{a}
HSI	%	21	0.92 ± 0.11^{ab}	0.87 ± 0.34^{ab}	0.91 ± 0.15^{ab}	0.92 ± 0.13^{ab}	0.92 ± 0.16^{ab}
		42	0.78 ± 0.08^{b}	0.75 ± 0.12^{b}	0.78 ± 0.05^{b}	0.87 ± 0.11^{a}	0.87 ± 0.06^{a}

CF, condition factor; HSI, hepatosomatic index.

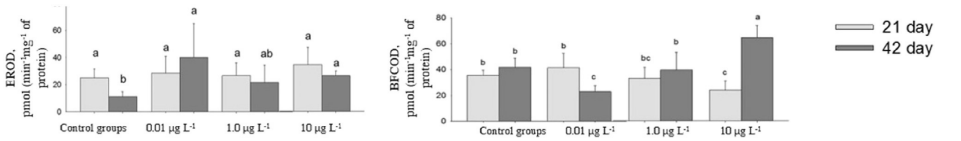

Fig. 1. Effect of CLO on hepatic ethoxyresorufin-O-deethylase (EROD) and 7-benzyloxy-4-trifluoromethylcoumarin O-debenzylase (BFCOD) activity in rainbow trout. Significant differences compared with control groups, P < 0.05 (means with different superscripts are significantly different, ANOVA). Data are back-transformed means with 95% confidence interval, n = 8.

taken up by fish and then absorbed by organic matter.

4.1. Clotrimazole in fish tissue and faeces

Clotrimazole belongs to a group of substances that have lipophilic properties. They are accumulated in body tissues according to fat content (liver > kidney > muscle). Our results comply except in fish exposed to the greatest CLO concentration at day 42. Our finding that the highest CLO concentrations were in kidneys may be explained by kidney damage, which was demonstrated at histopathological examination. This damage could have caused insufficient excretion of CLO and its persistence in this organ.

The human therapeutic plasma concentration of CLO is 5 ng mL^{-1} (http://www.drugs.com). Plasma concentrations in exposed fish were 90 times higher than that which would cause pharmacological effects in humans.

In clinical observations by Holt and Newman (1972), children treated with oral doses of CLO (100 mg kg^{-1} day^{-1} for 14, 21, 28 and 63 days) presented low levels in body fluids (urine, faeces and blood plasma). The authors suggested that CLO is a drug with long-term action in the body. To determine pathways of excretion, we evaluated the presence of CLO in fish faeces and found that excretion levels could not be confirmed when faeces were collected from the bottoms of aquaria because sorption from the water might contribute to faecal CLO. Concentrations in faeces determined in this study are tentative.

Clotrimazole had longer elimination half-lives in all analysed tissues (72–682 h) than has been reported for human blood (3.5–5.5 h after a 1.5-g oral dose) (Reynolds and Prasad, 1982). The relatively short half-life in livers (72 h) suggests that the liver actively eliminates CLO. Relatively long half-lives in kidneys, almost 28.4 days, implies a greater potential for adverse effects in non-target species. This finding is significant from the view of structural changes in the fish kidney after CLO exposure.

4.2. Morphological parameters

The HSI is associated with liver energy reserves and metabolic activity, while CF is used to assess the general condition of fish. The effects of human pharmaceuticals on HSI and CF have been described in various fish species (Li et al., 2011; Xu and Jing, 2012). We found significantly higher HSIs among CLO-treated groups only at the two highest concentrations. This increase in liver/body ratios might be associated with increased hepatic detoxification, showing that the liver responds to the presence of an exogenous compound.

4.3. Biochemical effects

Enzyme activity can decrease as a result of either excess substrate or damage induced by oxidative modification (Lushchak, 2011). However, the role of CLO in biotransformation and oxidative stress biomarkers is unclear because no concentration-response relationship was found in most of the enzyme activities.

Numerous *in vitro* and *in vivo* studies have demonstrated that CLO inhibits CYP450 activity in fish. Clotrimazole has also reportedly interacted with the steroidogenic enzymes CYP17 and CYP19 (Monod et al., 1993; Hinfray et al., 2011; Baudiffier et al., 2012, 2013). The antifungals CLO and ketoconazole have been shown to act as potent inhibitors of CYP1A activity in fish liver microsomes (Hegelund et al., 2004; Hasselberg et al., 2008; Wassmur et al.,

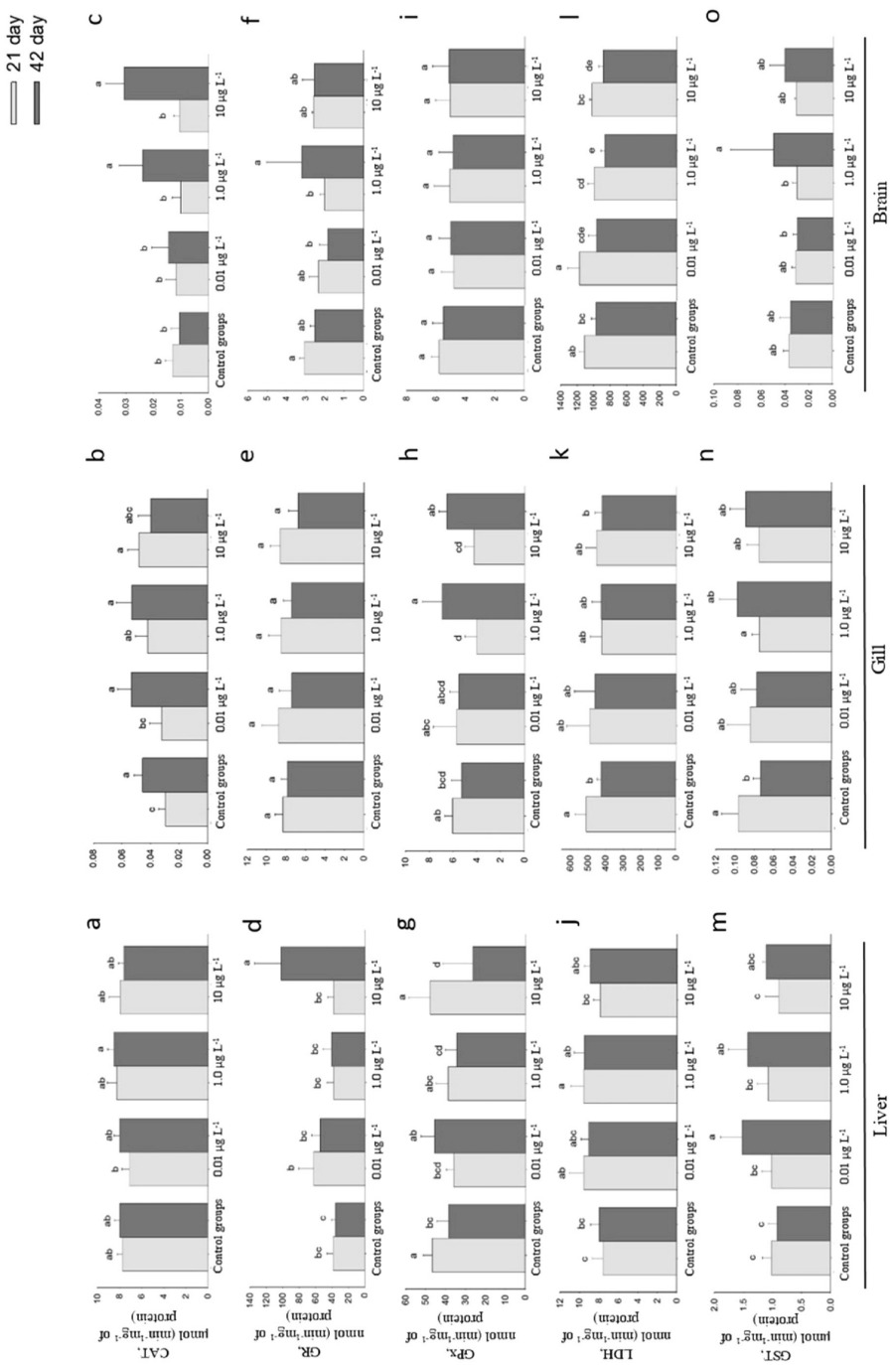

Fig. 2. Effect of CLO on catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), lactate dehydrogenase (LDH) and glutathione S-transferase (GST) activity in rainbow trout tissues [liver [a, d, g, j, m], gill [b, e, h, k, n], brain [c, f, i, l, o]). Significant differences compared with control groups. $P < 0.05$ (means with different superscripts are significantly different, ANOVA). Data are means ± S.D., n = 8.

Table 6
Haematological parameters and leukocyte differential count in rainbow trout chronically exposed to clotrimazole. Significant differences compared with solvent control (SC), $P < 0.05$ (means with different superscripts are significantly different, 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}
RBC	T/l	21	1.3 ± 0.12^{b}	1.1 ± 0.07^{b}	1.2 ± 0.05^{b}	1.2 ± 0.06^{b}	1.1 ± 0.06^{b}
		42	1.4 ± 0.07^{b}	1.4 ± 0.13^{b}	1.7 ± 0.09^{a}	1.3 ± 0.08^{b}	1.2 ± 0.11^{b}
Hb	g/l	21	78 ± 5.0^{a}	75.01 ± 3.2^{a}	71.61 ± 3.4^{a}	77.69 ± 1.9^{a}	86 ± 12^{a}
		42	81 ± 1.9^{a}	77 ± 3.8^{a}	83 ± 2.3^{a}	76 ± 4.2^{a}	75 ± 3.9^{a}
PCV	l/l	21	0.38 ± 0.03^{a}	0.39 ± 0.02^{a}	0.39 ± 0.02^{a}	0.41 ± 0.02^{a}	0.37 ± 0.03^{a}
		42	0.43 ± 0.01^{a}	0.42 ± 0.02^{a}	0.49 ± 0.02^{a}	0.45 ± 0.03^{a}	0.41 ± 0.02^{a}
MCV	fl	21	294 ± 27^{a}	358 ± 10^{a}	329 ± 12^{a}	335 ± 17^{a}	329 ± 24^{a}
		42	327 ± 17^{a}	319 ± 16^{a}	305 ± 20^{a}	361 ± 16^{a}	360 ± 16^{a}
MCH	pg	21	60 ± 2.3^{bc}	69 ± 2.2^{ab}	61 ± 2.2^{c}	63 ± 1.7^{abc}	76 ± 7.9^{a}
		42	62 ± 3.5^{abc}	59 ± 3.0^{bc}	50.86 ± 3.0^{c}	62 ± 3.20^{abc}	67 ± 3.3^{ab}
MCHC	g/l	21	214 ± 21^{ab}	194 ± 4.5^{b}	185 ± 4.8^{b}	192.07 ± 9.3^{b}	236 ± 21^{a}
		42	188 ± 2.9^{b}	184 ± 3.8^{b}	168 ± 5.4^{b}	171 ± 2.4^{b}	186 ± 3.1^{b}
Leuko	G/l	21	99 ± 14^{ab}	62 ± 10^{b}	91 ± 19^{ab}	83 ± 15^{ab}	73 ± 11^{ab}
		42	106 ± 12^{ab}	131 ± 26^{a}	128 ± 11^{ab}	124 ± 15^{ab}	93 ± 7.0^{ab}
Lymphocytes small	%	21	94 ± 2.1^{a}	92 ± 3.3^{a}	96 ± 1.8^{a}	87 ± 5.7^{a}	96 ± 1.7^{a}
		42	97 ± 0.6^{a}	97 ± 0.6^{a}	94 ± 2.6^{a}	98 ± 0.6^{a}	95 ± 1.1^{a}
Monocytes	%	21	2.6 ± 1.0^{ab}	3.3 ± 1.2^{ab}	3.3 ± 1.7^{ab}	7.9 ± 3.8^{a}	3.0 ± 1.4^{ab}
		42	1.9 ± 0.4^{b}	1.4 ± 0.2^{b}	2.1 ± 0.6^{b}	0.7 ± 0.2^{b}	1.3 ± 0.4^{b}
Granulocytes	%	21	2.1 ± 0.7^{a}	3.9 ± 1.8^{a}	0.61 ± 0.3^{ab}	3.62 ± 1.5^{ab}	0.75 ± 0.4^{ab}
		42	1.3 ± 0.5^{b}	1.1 ± 0.3^{b}	3.5 ± 1.8^{a}	1.1 ± 0.5^{ab}	3.13 ± 0.5^{a}

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.

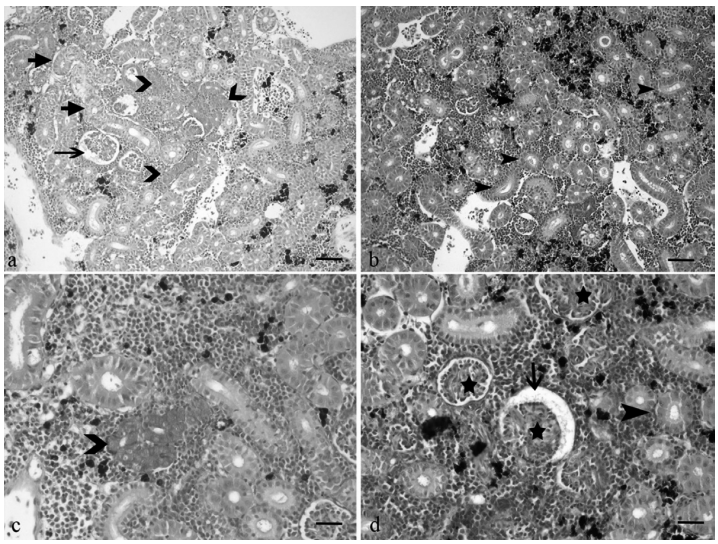

Fig. 3. Histopathological changes in the kidney. a. Animals exposed to 10 μg L^{-1} of CLO for 42 days showed vacuolation of tubular epithelial cells together with kyryopycnosis (tubulonephrosis) (closed arrows), increased amount of tubuloneogenesis (open arrowheads) and dilation of Bowman's space (open 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.

2010; Burkina et al., 2013). 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 CLO is a non-competitive inhibitor in hepatic microsomes of juvenile rainbow trout (Burkina et al., 2013). Finding significant differences between controls at days 21 and 42 might indicate that aging changes normal physiological variation in EROD activity. Similar results were found by Zlabek et al. (2016). A two-fold difference in EROD activity was found between juvenile and adult rainbow trout. However, our results show that the EROD activity is induced by CLO in the water, suggesting induction of CYP1A activity. The difference in our results from those previously published may have resulted from the CLO concentrations used and/or different *in vitro* and

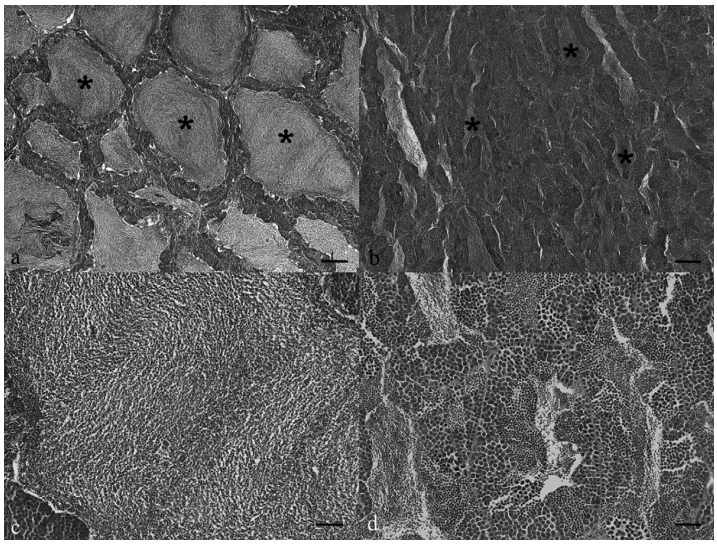

Fig. 4. Histopathology of testis. a, c: testis of control group, exposed to DMSO. b, d: testis of fish exposed to 10 μg L^{-1} of CLO for 42 days, showing decreased amount of spermatozoa and increased amount of spermatogonia and spermatocytes. Stars = tubular lumen filled with spermatozoa; a, b: bar = 100 μm, c, d: bar = 25 μm. HE stain.

in vivo models.

At day 21, BFCOD activities in exposed fish differed from that in controls only at the highest CLO concentration. Longer duration of exposure was associated with its inhibition in fish exposed to 0.01 μg L^{-1} and with its induction in fish exposed 10 μg L^{-1}. Observed changes in BFCOD activity suggest response of CYP3A-like enzyme activities to CLO exposure.

An *in-vitro* study by Burkina et al. (2013) showed that EROD and BFCOD were both inhibited by CLO in a concentration-dependent manner, but this *in-vivo* study does not support those results, possibly because different types of toxicological tests were used. In *in-vivo* tests, cells hold various types of regulatory mechanisms, and their responses are triggered by a multitude of intracellular signalling events that can influence the relevance of the cellular toxicological response.

This biphasic variation in CYP450 activity might be a result of a differential release of CLO and/or its metabolites from fish. The metabolite profile of CLO (Brugnara et al., 1995; Rittenhouse et al., 1997; Shah et al., 2001; Fazlul, 2007) includes at least 3 major metabolites (2-chlorophenyl-4-hydroxyphenyl-phenylmethane, 2-chlorophenyl-bis-phenylmethane and 2-chlorophenyl-bis-phenylmethanol). The nature of these variations and their relevance to fish physiology require further investigation.

At day 42, after exposure to 0.01 and 1.0 μg L^{-1}, CLO levels were higher in livers than in kidneys, confirming the elimination process (involving CYP3A-like enzymes). However, after exposure to 10 μg L^{-1} levels were higher in kidneys, suggesting that fish cannot eliminate CLO over time, and adverse effects can occur in kidneys (see Section 4.5).

We examined the status of the protective antioxidant system in rainbow trout under sub-chronic CLO exposure. Many studies have demonstrated the adverse effects of pharmaceuticals on fish including triggering the production of ROSs, which may be scavenged by the antioxidant defence system (Li et al., 2010a,d, 2011a; Sanchez-Muros et al., 2013).

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., 2010a,b,c) and in three-spined stickleback *Gasterosteus aculeatus* (Sanchez et al., 2008) depending on the intensity and duration of the stress. In our study as well as in Toni et al. (2011), no effect on liver CAT activity was observed, but we observed that CAT activity was induced in gills and brains. Because changes were observed in gills, CAT activities in controls at days 21 and 42 cannot be necessarily attributed to CLO exposure. High brain CAT activities imply the presence of a large amount of H_2O_2.

The antioxidant enzymes expressed by GR and GPx activities can be induced by an adaptive mechanism reacting to low levels of oxidative stress. The detoxification of ROSs and hydroperoxides implies the oxidation of GSH to GSSG by GPx (Ahmad, 1995). At the expense of NADPH, GSSG is then reduced to GSH by GR, and it is recycled by the pentose phosphate pathway. Decreased GR activity may lead to GSH depletion and increased GSSG if its loss cannot be compensated by the synthesis of new glutathione molecules (Srikanth et al., 2013). We found no changes in gill GR activity, but significant differences in brain GR activity might be a sufficient to their normal physiological state. However, GR was significantly increased in livers in concentration of 10 μg L^{-1} after 42 days exposure, probably resulting from differences in the availability of NADPH; this trend was also observed by Li et al. (2010a,b).

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 alterations in brain GPx activities were observed. Despite the significant differences of hepatic GPx between control at the two sampling times, generation of

ROSs effected GPx catalytic activity on day 42 after exposure to 10 μg L^{-1} CLO. In gills, this effect occurred after exposure to 1 and 10 μg L^{-1} on day 21, however after longer exposure, GPx activity was relatively balanced across exposure groups. These changes might indicate an adaptive response to ROSs production.

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

The metabolites formed by phase I biotransformation are conjugated via phase II enzymes (e.g., GST) before excretion. Bastos et al. (2013) showed that GST is an effective protective mechanism against an array of hydrophobic and electrophilic compounds, including peroxidized lipids and secondary metabolites of LPO, through the −SH group. GST is an effective protective mechanism against an array of hydrophobic and electrophilic compounds, including peroxidized lipids and secondary metabolites of LPO, through the −SH group. The GST response varies with species, organ and the type of toxicant (Song et al., 2006). Corcoran et al. (2014) reported up-regulation of hepatic gsta and gstp genes in common carp after 10 days' exposure to 14.63 μg L^{-1} CLO. We found a clear inverted U-shaped GST response in gill tissue, indicating that GST was inhibited in the presence of 10 μg L^{-1} CLO, resulting in decreased capacity of phase II glutathione conjugation to xenobiotic substrates. Molecular modelling of the metabolism of CLO suggests that it can react with cellular nucleophiles such as glutathione, causing its depletion (Klokouzas et al., 2002; Fazlul, 2007).

Goncalves et al. (2012) and Motawi et al. (2015) demonstrated that CLO can affect oxidative phosphorylation, glycolytic enzymes, and, therefore, the energy pathways in cells. Lactate dehydrogenase is a cytoplasmic enzyme, and commonly it reflects the energy metabolic capacity of tissues after acute (Rao, 2006) or long-term (Osman et al., 2010) exposure to a contaminated environment because of enzymatic involvement in the supply of energy demand under oxygen-limiting conditions. The LDH is involved in the final step of anaerobic glycolysis to regulate the pyruvate-lactate conversion under stress conditions. We found that CLO affected hepatic and brain LDH activities. Hepatic LDH activity was induced only after initial exposure in the two lowest CLO concentrations. This could be explained by depletion of LDH and a failure to metabolize pyruvate in the liver. As a result of these changes, CLO can interfere with the aerobic metabolic pathway through depletion of LDH and a failure to metabolize pyruvate in fish tissues. Brain LDH activity was inhibited by higher CLO concentrations at the last sampling point. Differences in tissue LDH response according to CLO concentration might be linked to how much those tissues are involved in energy metabolism. There were no significant differences in the highest concentration after 42 days of exposure in all tested concentrations in liver and gill. It might be connected with adaptive response to CLO exposure.

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 tissues in 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 μg L^{-1} tebuconazol and, in laboratory exposure, in livers at 33.47 and 36.23 μg L^{-1} and muscle at 33.47 μg L^{-1}. Li et al. (2010a,d) exposed rainbow trout to 0.2–500 μg L^{-1} propiconazol over 30 days and found a significant increase of TBARS levels in brains and intestines. Sanchez et al. (2008) reported unchanged TBARS levels in three-spined stickleback after 7 days' exposure to 10–500 μg L^{-1} prochloraz. Our results showed that sub-chronic exposure to CLO did not lead to oxidative damage in the investigated tissues as indicated by TBARS levels, compared to controls. In further studies, we recommend choosing different biomarkers to identify oxidative stress, such as GSH and GSSG/GSH ratio.

In present study, among all investigated fish tissues, only hepatic tissue showed response to CLO environmentally relevant concentration exposure. Also, in some cases, fish enzymatic systems showed similar effects in catalytic enzyme activities at zero and the highest CLO concentration. This might be explained by activation of alternative detoxification mechanisms in fish, such as activities of uninvestigated CYP450 isoforms that may be similar to that seen in controls. Another explanation could be that fish exhausted detoxification mechanisms of CLO in livers at the highest concentration.

4.4. Haematological profiles

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 physiological changes following various stress conditions. In this study, the main haematological response to the chronic effects of CLO was significantly lower RBC counts at the environmentally relevant concentration. Fish likely attempt to improve their O_2^-carrying capacity to maintain gas transfer by producing additional red cells.

Of the leukocytes, only the granulocytes increased in number. 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 counts are recognized as sensitive indicators of environmental stress response (Li et al., 2011). In this study, such changes may relate to a stress response of the immune system.

4.5. Histopathological changes

Histological changes were most obvious in kidneys and testes, while gills, liver and ovaries were not significantly different than those of controls. Degenerative changes in the kidney, including tubulonephrosis and hyaline droplet degeneration were most prominent in the tubular epithelial cells. An increase in tubuloneogenesis was observed in some exposed fish and can be interpreted as attempts to regenerate. Regeneration in the kidney has been reported in several fish species (Reimschuessel et al., 1990, 1996; Reimschuessel, 2001; Salice et al., 2001; Watanabe et al., 2009; Diep et al., 2011). Tubular degeneration and regeneration is probably a result of CLO exposure. At present, information of histological alteration in fish kidney after CLO exposure is limited.

Changes in the gonads were visible only in the two males, which complicates making definite conclusions. However, in animals exposed to CLO, a clear decrease in the number of spermatozoa and increases in spermatogonia and spermatocytes were observed. These findings agree with the increase in spermatogonia reported by Baudiffier et al. (2013) in zebrafish exposed to 197 μg L^{-1} CLO for 21 days. These effects may be a result of disruption to steroidogenesis by CLO (a known EDC and priority hazardous substance), capable of inhibiting P450-steroidogenic enzymes (Ankley et al., 2007; Brown et al., 2011). Studies with a greater number of males are needed.

5. Conclusions

Our data provide an indication of sub-lethal impacts of CLO in the aquatic environment. We determined that CLO is concentrated

in fish organs (liver and kidney). Fish excretory organs were not able to properly eliminate the highest concentration from the body and histopathological damage occurred in kidneys. Interference with CYP450-mediated reactions, antioxidant enzyme activities and histopathological alterations in gonads and kidneys may be of concern. Presence of CLO in the environment might have consequences for fish health as a result of prolonged presence in the body (the maximal depuration half-life is up to 28 days).

Acknowledgements

The study was financially supported by the Ministry of Education, Youth and Sports of the Czech Republic – projects "CENAKVA" (No. CZ.1.05/2.1.00/01.0024), "CENAKVA Center Development" (No. CZ.1.05/2.1.00/19.0380) and "CENAKVA II" (No. LO1205 under the NPU I program), by the Grant Agency of the University of South Bohemia in Ceske Budejovice (No. 012/2016/Z) and the Grant Agency of the Czech Republic P503/11/1130. We thank American Manuscript Editors for English language editing.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.05.042.

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Appendix C.

Supplementary Material 1. *Hydro-chemical analysis of after 42days of exposure. All units in mg L^{-1}*

Sample	NH_4^+-N	NO_3^--N	NO_2^--N	PO_4^{3-}-P	CHSKMn	KNK4,5
Control group	1,32	1,74	0,063	0,013	3,8	1,15
10 μg L^{-1}	1,27	1,80	0,060	0,010	3,5	1,10

Supplementary Material 2. *Parameters of LC-LC method for the analysis of CLO in water samples.*

Analytical pump				*High-flow pump for in-line extraction*				
Time, min	A, %	C, %	Flow, μL/min	Time, min	A, %	B, %	C, %	Flow, μL/min
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% formic acid, 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.

Supplementary Material 3. *Parameters of LC method for the analysis of fish tissue and faeces.*

Time, min	A, %	B, %	Flow, μL 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).

Supplementary Material 4. *Experimental conditions for enzyme activity assays in rainbow trout post-mitochondrial supernatant.*

Reaction	PMS, µl, [Protein concentration for each reaction, mg/ml]	Reaction solution	Total volume of reaction	molar extinction coefficient, $M^{-1}cm^{-1}$
CAT	15, [~ 0.02]	285 µl (a mixture of 100 µL 0.03 M H2O2 and 185 µL 0.05 M K-phosphate (pH 7.0)	300	40
GR	50, [~ 0.2]	150 µl (0.4 mM NADPH, 2 mM GSSG, and 1 mM DTPA in 0.05 M K-phosphate buffer, pH 7.0)	200	6220
GPx	15, [~ 0.2]	285 µl (1 mM EDTA, 1 mM sodium azide and glutathione reductase from baker's yeast (7.5 mL from stock containing 1 U mL^{-1}). Added to the substrate was 30 µL 4 mM GSH and 0.8 mM NADPH. The reaction was started by the addition of 0.5 mM of H2O2)	300	6220
LDH	15, [~ 0.02]	285 µl (a mixture of 35 µL Tris-NaCl buffer (0.1 M, pH 7.2), 250 µL 0.24 mM Tris-NaCl-NADH, and 40 µL 10 mM Tris-NaCl-pyruvate)	300	6300
GST	100, [~ 0.04]	200 µl (a mixture of 2.55 mL 10 mM CDNB, 15.30 mL 10 mM GSH, and 84.5 mL 0.1 M K-phosphate buffer, pH 6.5)	300	9600

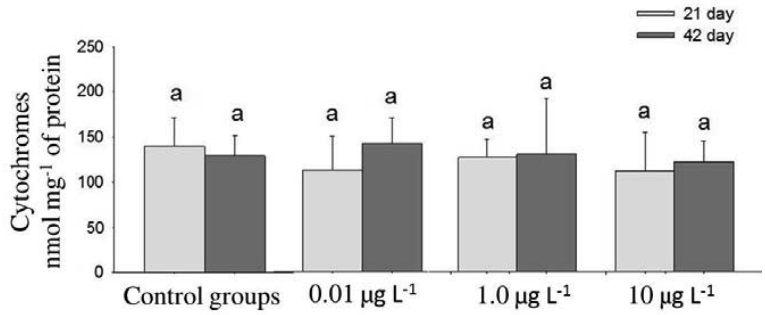
CAT – catalase; CDNB-1-chloro-2,4-dinitrobenzene (Sigma D6518, Lot # SLBB4940V); DTPA - diethylenetriaminepentaacetic acid (Sigma 2373329, Lot # BCBH7402V); GPx- glutathione peroxidase; GR - glutathione reductase; GSH - reduced glutathione (Sigma G4251 Lot # SLBD3561V); GSSG - L-glutathione oxidized (Sigma G4501, Lot # 100K7276V); GST - glutathione S-transferase; LDH - lactate dehydrogenase; PMS- post-mitochondrial supernatant.

Supplementary Material 5. *Concentration of clotrimazole in faeces collected after 24 h of exposure in experimental aquaria (n = 2).*

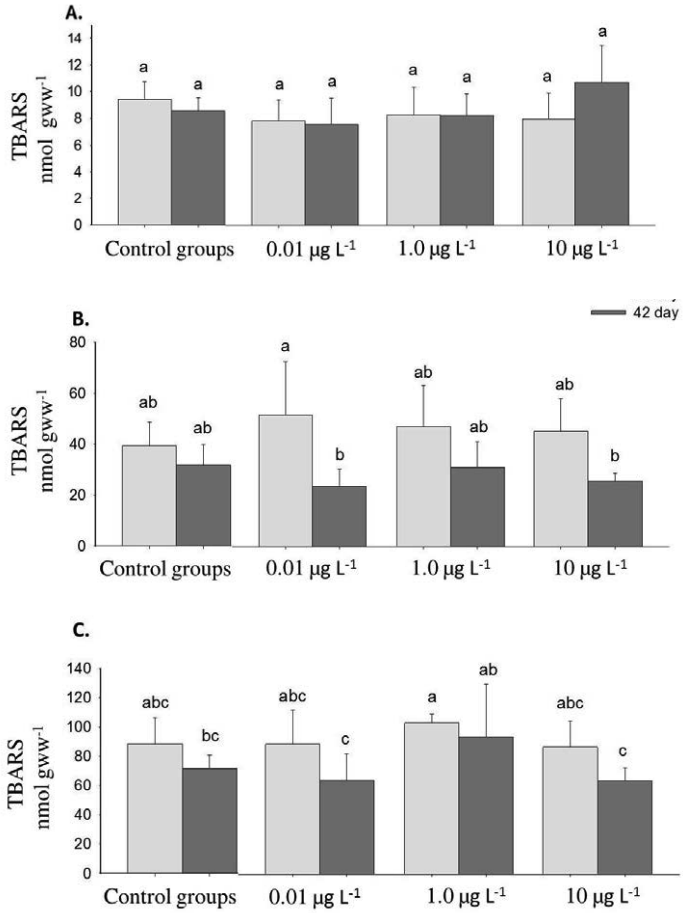
Clotrimazole	Total amount of CLO in 200 L	Faeces analysis	Amount of CLO in faeces collected after 24 h	Faeces/water
(nominal concentration), µg L^{-1}	[µg $aquaria^{-1}$]	[µg g^{-1}w.w.], (RSD, %)	[µg $aquaria^{-1}$]	[CLO in faeces/ nominal CLO aq.] ×100, [%]
0.01	2	0.037 (15)	0.14	7
1	200	2.3 (29)	6.3	3
10	2000	29 (9)	83	4

LOQ for faeces = 5.6 ng g^{-1}. RSD- relative standard deviation

Supplementary Material 6. *Effect of clotrimazole on CYP450 content in the hepatic microsomes from rainbow trout. No significant differences compared with control group, P < 0.05 (ANOVA). Data are back-transformed means with 95% confidence interval, n = 8.*



Supplementary Material 7. *Effect of CLO on level of thiobarbituric acid reactive substances (TBARS) in rainbow trout tissues. A: liver, B: gill, C: brain. No significant differences compared with controls group, P < 0.05 (ANOVA). Data are means ± S.E., n = 8.*



Supplementary Material 8. *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 sufficient developed

CHAPTER 4

IN SITU EXPERIMENT: COCKTAIL EFFECTS OF PHARMACEUTICALS AND PERSONAL CARE PRODUCTS ON COMMON CARP (*CYPRINUS CARPIO* L.)

Sakalli, S., Giang, P.T., Burkina, Zamaratskaia G., Rasmussen, M.K., Khalili, T.S., Bakal, T., Sampels, S., Kolarova, J., Grabic, R., Turek, J., Randak, T., Zlabek, V., 2018. The effects of sewage treatment plant effluents on hepatic and intestinal biomarkers in common carp (*Cyprinus carpio*). Science of the Total Environment 635, 1160–1169.

My contributions to this work was 50%.

Science of the Total Environment 635 (2018) 1160–1169

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

The effects of sewage treatment plant effluents on hepatic and intestinal biomarkers in common carp (*Cyprinus carpio*)

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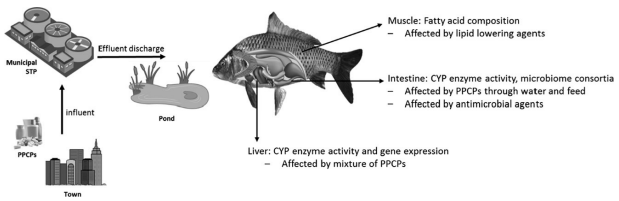
HIGHLIGHTS

- Common carp were exposed to treated sewage water for a year.
- EROD and BFCOD activity both in liver and intestine were affected.
- Treated sewage water had a great impact on the intestinal microbiome taxa.
- The observed effects might be different in fish from running surface waters due to dilution factor.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 9 February 2018
Received in revised form 9 April 2018
Accepted 14 April 2018
Available online xxxx

Editor: Henner Hollert

Keywords:
CYP1A
CYP3A
Gene expression
Lipids
Fatty acids
Microbiome

ABSTRACT

Sewage treatment plants (STPs) are one of the major source of pharmaceuticals and personal care products in the aquatic environment. Generally, the effects of individual chemicals on fish are studied under laboratory conditions, which leads to results that are potentially not realistic regarding the effects of these chemicals under environmental conditions. Therefore, in this study, common carps were held in exposed pond that receive water from STP effluents for 360 days under natural conditions. Elimination of xenobiotics starts in the fish intestine, in which the microbial community strongly influences its function. Moreover, the fish intestine functions as crucial organ for absorbing lipids and fatty acids (FA), with consequent transport to the liver where their metabolism occurs. The liver is the primary organ performing xenobiotic metabolism in fish, and therefore, the presence of pollutants may interact with the metabolism of FA. The catalytic activity of CYP1A and CYP3A-like enzymes, their gene expression, FA composition and intestinal microbiome consortia were measured. The catalytic activity of enzymes and their gene and protein expression, were induced in hepatic and intestinal tissues of fish from the exposed pond. Also, fish from the exposed pond had different compositions of FA than those from the control pond: concentration of 18:1 n-9 and 18:2 n-6 were significantly elevated and the longer chain n-3 FA 20:5 n-3, 22:5 n-3 and 22:6 n-3 were significantly lowered. There were clear differences among microbiome consortia in fish intestines across control and exposed groups. Microbiome taxa measured in exposed fish were also associated with those found in STP activated sludge. This study reveals that treated STP water, which is assumed to be clean, affected measured biomarkers in common carp.

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https://doi.org/10.1016/j.scitotenv.2018.04.188
0048-9697/

1. Introduction

Pharmaceuticals and personal care products (PPCPs) represent one of the major groups of compounds that are used to improve life quality. In the European Union, there are nearly 3000 chemicals used in medicines such as antibiotics, anti-inflammatories, beta-blockers, steroidal hormones and lipid regulators (Scholz, 2015). Their extensive use, direct discharge into waters, improper disposal, and insufficient treatment of wastewaters results in the release of some of these compounds (or their metabolites) into the aquatic environment. Sewage treatment plants (STPs) are designed to remove the main nutrients, such as organic carbon, nitrates, ammonia and phosphates using physical, chemical, and biological processes. However, STPs are inefficient to degrade some PPCPs, which make them one of the major sources of these compounds in the aquatic environment. PPCPs have been found in sewage, treated wastewater, surface waters, groundwater and drinking water (Fedorova et al., 2014; Golovko et al., 2014; Grabicova et al., 2017; Koba et al., 2018; Kozisek et al., 2013). Their concentrations are generally as low as ng L^{-1} or μg L^{-1} (Ebele et al., 2017; Grabicova et al., 2017; Rozman et al., 2017; Yin et al., 2017). 64 personal care products recognized as 'emerging contaminants' were found in STP effluent, 43 of which in surface water and 23 in groundwater within 30 countries (Montes-Grajales et al., 2017). Consequently, these compounds are accepted as emerging contaminants due to their persistency in the aquatic environment. Therefore, their fate and exposure to non-target organisms have been extensively studied. However, information regarding their ecotoxicological effects in aquatic organisms and wildlife is limited (Carlsson et al., 2006; Fent et al., 2006).

Intestine, beside gills and skin, is part of the first barrier of pollutant entry in fish, either by direct exposure through water or indirect exposure through feed, and it is responsible for digestion, absorption and transportation of nutrients and the prior elimination of xenobiotics (Martin et al., 2016). Functions of the intestine are strongly influenced by the associated microbial community, which in turn is affected by antimicrobial agents (antibiotics, antifungal, etc.) present in STP effluents (Nayak, 2010). Lipids and fatty acids (FA) are absorbed by the intestine and transported by the lymphatic system, circulatory system or directly via portal system to the liver where they are metabolized (Tocher, 2003). However, lipid lowering agents such as statins, fibrates, etc., might affect their metabolism in the liver (Du et al., 2008; Tocher, 2003). After chemical uptake, the liver is the main metabolizing organ in which major biotransformation and elimination of compounds of STP effluents occur (van der Oost et al., 2003). The biotransformation of xenobiotics generally includes Phase I and Phase II metabolic reactions, Phase I enzymes increase the polarity where Phase II enzymes increase the hydrophilicity of the compounds for further elimination. The most important group of enzymes in Phase I metabolism is cytochrome P450 (CYP). In fish, CYP1A1 and CYP3A are widely used as biomarkers, and their enzymatic activity are generally measured as 7-ethoxyresorufin-O-deethylase (EROD) and 7-benzyloxy-4-trifluoromethylcoumarin O-debenzylase (BFCOD), respectively (Burkina et al., 2015b).

The chemical composition of the ponds included in the experiment has been evaluated previously using Polar Organic Chemical Integrated Samplers (POCISs). According to POCIS analysis, 62 pharmaceuticals out of 171 polar compounds such as antibiotics, cardiovascular drugs, psychoactive drugs etc. were detected in the water from the exposed pond (Giang et al., 2017). In addition, pharmaceuticals and their metabolites were previously assessed in carp liver from the exposed pond, and eight pharmaceuticals and three metabolites were found (Koba et al., 2018). The highest measured concentrations of pharmaceuticals in carp liver were sertraline, ranging from 0.6 to 61 ng g^{-1}, followed by metoprolol acid, which ranged from 0.1 to 26 ng g^{-1}. *N*-desmethylcitalopram ranged from 0.5 to 24 ng g^{-1} and citalopram at 22 ng g^{-1} (Koba et al., 2018).

It is likely that different groups of compounds affect different biomarker responses in fish. In the present study, hepatic and intestinal CYP activity, gene and protein expression of *CYP1A* and *CYP3A*, FA composition and intestinal microbiome consortia were tested as biomarkers under exposure to a mixture of PPCPs from STP effluent discharge water.

2. Materials and methods

2.1. Chemicals

PCR reagents were purchased from Bio-Rad (Canada). Acetonitrile and methanol of HPLC grade were purchased from Merck (Darmstadt, Germany). Immunoblotting gels (Novex 10% Tris-Glycine Mini Protein) were obtained from Life Technologies (Czech Republic). Anti-fish CP-226 antibody from Biosense Laboratories AS (Bergen, Norway) was used to detect CYP1A. Anti-rabbit CYP3A antibody (H300, Santa Cruz, Inc.) was used to detect CPY3A-like protein, and anti-Goat I-19 (Santa Cruz, Inc) was used to detect β-actin. In preliminary trail, we verified that the anti-CYP3A and anti-β-actin antibody recognized a corresponding protein band in both fish and human liver in a preliminary trial (data not shown). All other chemicals were purchased through Sigma Aldrich (EU).

2.2. Experimental setup and fish sampling

Experimental sites have been described previously (Giang et al., 2017). Briefly, common carp were held in the exposed pond, receiving water only from STP effluents from the town of Vodňany, Czech Republic. The control pond received water from upstream of Blanice River (South Bohemia region, Czech Republic) for 360 days under natural conditions. Fish were sampled at the beginning of the experiment at time 0 and then at 30, 90, 180 and 360 day time points to cover all the seasons, detail information can be found in Supplementary Material 1, Section 1. The water temperatures measured at 30, 90, 180 and 360 day at the control pond were 14.2 °C, 22.0 °C, 12.1 °C, 10.1 °C respectively while at the exposed pond they were 11.1 °C, 19.6 °C, 15.8 °C, 12.8 °C respectively. More details about water and rearing conditions of both exposed and control pond can be found elsewhere (Giang et al., 2017).

Common carp (*Cyprinus carpio*) weighed 65.8 ± 3.4 g (mean weight ± standard deviation) and were 17 ± 0.3 cm (mean total length ± standard deviation) in length. These carp were obtained from Faculty of Fisheries and Protection of Water (Czech Republic) and were randomly stocked in control and exposed ponds to a density of 0.14 fish per m^2. Fish relied on natural feed in each pond. Twelve fish were collected from each pond in each of the five sampling events. Fish were handled according to the national and institutional guidelines for the Protection of Human Subjects and Animal Welfare. The unit is licensed (No. 53100/2013-MZE –17214; CZ01676, 21.01.2015) according to the Czech National Directive (Act No. 246/1992 Coll., on the Protection of Animals Against Cruelty). Fish were sacrificed by cutting the spinal cord. The liver samples, for gene and protein expression, were sampled quickly under sterile conditions and were immediately stored in liquid nitrogen. The liver and intestinal tissues, for measuring the two CYP-mediated reactions, and muscle tissues, for measuring FA composition, were collected and immediately frozen and stored at −80 °C until further use.

2.3. Microsome preparation

The hepatic and intestine microsomal fractions were prepared from approximately 1 g of tissue by differential centrifugation (Burkina et al., 2013). Prior to fraction preparation, intestine samples were cleaned from connective tissue and fat; then, intestinal lumen was washed with an isotonic solution to remove digested food residues. The intestine was then cut into small pieces to allow easy homogenization.

Protein content was measured spectrophotometrically using bovine serum albumin as a standard (Smith et al., 1985). The microsomes were diluted with microsomal buffer (0.25 M sucrose, 0.01 M Tris, EDTA

0.1 mM, pH 7.4) to a protein content of 10 mg ml^{-1}. Microsomal fractions were stored at −80 °C until analysis.

2.4. Determination of EROD and BFCOD activity in fish hepatic and intestinal microsomes

EROD activity was measured as previously described, with a slight modification for fish microsomes (Zamaratskaia and Zlabek, 2009). Briefly, incubations with a total volume of 500 μL contained 0.2 mg of microsomal protein in 50 mM potassium phosphate buffer (pH 7.4) and 7-ethoxyresorufin (2 μM). The reaction was started by the addition of 0.5 mM reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) followed by incubation for 7 min at 25 °C. The reactions were terminated with 500 μL 100% ice-cold methanol. Reaction mixtures were centrifuged at 7500 g for 3 min. Resorufin was detected using a fluorescence detector (L-7480; Merck, Hitachi, Tokyo, Japan) at 560 nm and 586 nm (excitation and emission, respectively). The limit of quantitation of resorufin was 0.5 pmol ml^{-1}.

BFCOD activity was measured as previously described with slight modification for fish microsomes (Zlabek and Zamaratskaia, 2012). The BFCOD activity was measured as the transformation rate of 7-benzoyloxy-4-trifluoromethyl coumarin (BFC) to 7-hydroxy-4-trifluoromethylcoumarin (HFC). Briefly, incubations with total volume of 500 μL contained 0.2 mg microsomal protein in an incubation medium of 50 mM potassium phosphate buffer (pH 7.4) and BFC (12.5 μM). The reaction was started by adding 0.5 mM NADPH and incubated for 10 min in a water bath at 25 °C. The reaction was stopped by adding 500 μL ice-cold 100% methanol. The reaction mixtures were then centrifuged for 5 min at 7500 g. The HFC was detected using a fluorescence detector (L-7480) at 410 nm and 538 nm (excitation and emission, respectively). The limit of quantitation of HFC was 0.5 pmol ml^{-1}.

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 Li-Chrospher® RP-18 column (250 mm length, 4 mm inner diameter and 5 μm particle size; Merck, Hitachi, Tokyo, Japan) equipped with a guard column was used. Enzyme activity was calculated as pmol of the product (resorufin or HFC) formed per min per mg of microsomal protein.

2.5. Specific mRNA expression and immunoblotting

2.5.1. Gene expression

Total RNA isolation was carried out using the Trizol reagent according to the manufacture. In brief, a total of 20 mg of frozen tissue was homogenized in Trizol. RNA was isolated by adding chloroform and incubating the solution at room temperature for 10 min. After centrifugation, the aqua phase was collected, and the RNA was precipitated using 2-propranol and subsequently washed in 70% ethanol and resuspended in RNAase-free water. The RNA concentration was estimated spectrophotometrically (Picodrop). The isolated RNA was converted to cDNA by reverse transcription using the iScript cDNA synthesis kit, according to the manufacturer's instruction. cDNA was diluted in RNAase free water and stored at −20 °C until further use.

Semi-quantitative PCR was conducted as described in Rasmussen et al. (2011b) using TaqMan probes. Primers and TaqMan probes were designed with Primer Express 3.0.1 using common carp specific sequences of genomic DNA. Sequence specificity was verified by nucleotide BLAST search (https://blast.ncbi.nlm.nih.gov). The primers and probes are listed in the Supplementary Material 1, Section 2. Control samples with no mRNA were analyzed to confirm that no unspecific amplification occurred. All samples were analyzed as duplicates. The relative mRNA expression was calculated by relating the obtained values for threshold cycles to a standard curve obtained by running a serial dilution of one cDNA sample. The relative gene expression was normalized to the expression of β-actin. The obtained Ct-values for β-actin did not differed between treatments and time-points to be compared. The average of the control groups according to the time of exposure was normalized to 1, and the experimental groups were expressed relative to the corresponding control group.

2.5.2. Immunoblotting

Liver samples of fish caught in both ponds on days 30 and 360 were analyzed. Liver tissues were homogenized in RIPA buffer (50 nM Tris, 150 nM NaCl, 2 nM EDTA, 1% triton, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, pH = 7.0) supplemented with 2 mM phenylmethanesulfonylfluoride. Afterwards, samples were centrifuged at 20,000 g for 20 min at 40 °C. Western blotting was performed using a protocol modified according to Rasmussen et al. (2011a, 2011c). In brief, equal amounts of protein were separated on a 12% polyacrylamide gel and electro-blotted onto a polyvinylidene difluoride membrane. Following treatment with primary antibodies (anti-CYP1A; anti-CYP3A; anti-β-actin diluted in TBS-buffer with 0.1% Tween 20) overnight at 4 °C, proteins were visualized using HRP-conjugated secondary antibodies (rabit anti-goat and goat- anti rabit) and enhanced chemiluminescence substrate. The relative protein concentration was calculated by scanning the blot using the ChemiDoc-XRS^{+} (Bio-Rad) and quantified with Image Lab software (Bio-Rad). The obtained values for protein expression were normalized to the relative expression of β-actin (loading control).

2.6. Fatty acid composition

Muscle taken from skinless fillets of the fish on days 0, 180 and 360 ($n = 8$ in each group) were homogenized, and 1.5 g sample from each fillet was taken for analysis. The lipid extraction was performed on sample duplicates using the methods of Hara and Radin (1978). The lipid content was quantified gravimetrically. For FA analyses, methylation of total lipids was performed according to Appelqvist (1968) using boron trifluoride. FA composition was analyzed by gas chromatography (Trace Ultra FID; Thermo Scientific, Milan, Italy) using a BPX-70 50 m fused silica capillary column (id. 0.22 mm, 0.25 μm film thickness, SGE, USA). The individual FAs peak were identified by comparing their retention times to the retention times of the standard mixture GLC-68-D (Nu-Chek Prep, Elysian, USA) and other single FA standards (Nu-Chek Prep, Elysian, USA). Peak areas were integrated using Xcalibur 3.0 ISQ integration software (Thermo Scientific, USA).

2.7. Intestinal microbiome: DNA extraction and analysis

The sixteen carp intestinal microbiome samples, eight from each pond, were analyzed. For taxonomic characterization of the intestinal microbiome, the intestinal content (minimum 0.25 g) was collected on day 180. For each sample, the total DNA was extracted from 0.25 g of the intestine content using PowerFecal® DNA Isolation Kit (MO BIO laboratories, USA) according to the manufacturer's instructions and used as a template for primary PCR in 16S rDNA amplicon library preparation.

The procedure of 16S rDNA amplicon library preparation for Illumina MiSeq was adjusted according to Baldrian et al. (2012). The universal primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) (Turner et al., 1999) and 907R (5′-CCGTCAATTCCTTTRAGTTT-3′) (Muyzer et al., 1995) were used to amplify the V4–V5 region of bacterial 16S rDNA in primary PCR. The composite primers for multiplex assay using MiSeq Illumina sequencing contained additional 5–7 nucleotide long sample tags that were separated from primers by 2 nucleotide long spacers (Parameswaran et al., 2007). PCR amplifications were performed in two steps (Baldrian et al., 2012), additional details can be found in Supplementary Material 1, Section 3. The purified barcoded amplicons from different samples were mixed in equimolar concentrations, and the amplicon library was constructed using TruSeq DNA

Library Preparation Kit v2 (Illumina, USA) and sequenced by Illumina MiSeq platform (paired-end reads, 2 × 250 =bp).

The sequencing data were processed according to the pipeline SEED 2.0.2 (Vetrovsky and Baldrian, 2013a) including the pair-end reads merged by fastq-join (Aronesty, 2013) and chimeric sequences removal using USEARCH 8.1.1861 (Edgar, 2010). Sequences were clustered using the UPARSE-OTU algorithm (Edgar, 2013) at a level of 97% identity. Consensus sequences representing each cluster were assigned at the genus level using blast algorithm (Altschul et al., 1990) against the SILVA database (Update Release 123.1) (Quast et al., 2013; Yilmaz et al., 2014). The clusters that represented identical genera were merged. The closest higher taxon was used when the genus assignment was not possible. Sequences identified as nonbacterial and sequences with coverage lower than 90% of the reference were discarded. The bacterial genome count estimates were calculated based on the 16S rDNA copy numbers in the closest available sequenced genome, as described previously (Vetrovsky and Baldrian, 2013b). Number of reads for each taxon was converted to the relative abundance for further analysis. Non-metric multidimensional scaling (nMDS), based on Bray-Curtis distance matrix was used for the visualization of the results in the program PAST (PAleontological STatistics version 3.14).

2.8. Statistical analyses

Statistical analyses were performed using Statistical Analysis System, version 9.1 (SAS Institute, Cary, NC, USA). For FA, hepatic and intestinal CYP activity, ANOVA Mixed procedure were performed. The Mixed model included fixed factors of group (control and exposed groups), time of exposure and interactions between group and time. Data were calculated as a percentage of identified FA and are presented as mean values, and standard deviation while EROD and BFCOD activity analysis were performed on $\log_{10}$-transformed data to improve the distribution of values and data are presented as least squares means and standard errors after back-transformation. For gene and protein expression, non-parametric Mann-Whitney *t*-test was applied to investigate the differences between control and exposed group at the same time point. A Pearson product-moment correlation coefficient was computed to assess the relationship between the pharmaceuticals measured in fish liver and the biomarkers measured in fish. Statistical differences were considered significant when $p \leq 0.05$.

3. Results

3.1. EROD and BFCOD activity in fish hepatic and intestinal microsomes

Significant induction of EROD activity was observed in the liver at 180 days and in the intestine at 30 and 180 days of fish exposed to STP effluent (Fig. 1). BFCOD activity in the liver was significantly lower at only 30 days of exposure compared to control, while BFCOD activity in the intestine was induced at 90 and 180 days of exposure (Fig. 2). At the end of the exposure period, hepatic and intestinal EROD and BFCOD activity did not show any significant differences from those in the control group.

3.2. mRNA and protein expression of CYP1A and CYP3A

3.2.1. Genes expression

Expression of *CYP1A* and *CYP3A* are reported in Fig. 3. *CYP1A* expression was significantly higher in the exposed group after 30 and 90 days exposure (9.3 and 4.2-fold, respectively). At 180 and 360 days exposure, there was no difference observed between exposed and control groups in *CYP1A* expression (Fig. 3A). *CYP3A* expression is reported in Fig. 3B. No significant differences were found between control and exposed groups in all investigated events.

3.2.2. Protein expression

Protein expression of CYP1A and CYP3A were investigated at the 30 and 360 day time points to confirm the results in gene expression. These results are shown in Fig. 4. Protein expression of CYP1A in exposed fish was significantly higher than in control fish at 30 days exposure (94-folds). Although, no significant difference between these two groups was seen at 360 days exposure. No significant difference between control and exposed groups was measured in protein expression of CYP3A, in both 30 and 360 day time points.

3.3. Fatty acid composition

The fat content and FA composition of the muscle tissue of common carp sampled at the 0, 180 and 360 day time points in both control and exposed groups are presented in Table 1. Total fat content was significantly higher in the exposed group. In addition, exposure changed several FA compared to the values of the control group. 18:1 n-9 and 18:2 n-6 were significantly higher, while the longer chain n-3 FA eicosapentaenoic acid (EPA; 20:5 n-3), 22:5 n-3 and docosahexaenoic acid (DHA, 22:6 n-3) were significantly lower in the exposed group, compared to the control which can be due to the decrease in the synthesis. It is also interesting to note that in the exposed group 18:2 n-6 increased at 180 days and then decreased at 360 days.

Total saturated fatty acids (SFA) and poly unsaturated fatty acids (PUFA) were significantly lower and monounsaturated fatty acids (MUFA) were higher in the exposed group, compared to the control group. Total n-3 content was significantly higher after 360 days in the control group, whereas in the group exposed with STP effluents, a decrease in the n-3 content occurred, which subsequently resulted in the combination with a higher proportion of n-6 in a lower n-3/n-6 ratio, indicating a lower nutritional value for human consumption.

3.4. Correlations

The correlation data between pharmaceuticals identified in fish liver and biomarkers result of EROD, BFCOD, CYP1A and CYP3A gene expression can be found in the Supplementary Material 1, Section 4. The strong negative correlation was found between EROD activity measured in liver and clindamycin ($r = -0.59$, $p \leq 0.001$) while, weak correlation was found with *O*-desmethylvenlafaxine ($r = 0.31$, $p \leq 0.05$). The BFCOD activity measured in liver were found significantly correlated with clindamycin ($r = 0.89$, $p \leq 0.001$) and *O*-desmethylvenlafaxine ($r = 0.31$, $p \leq 0.05$). The EROD activity measured in intestine showed significant correlation with carbamazepine ($r = 0.50$, $p \leq 0.01$).

3.5. Intestinal microbiome

The sequencing data were deposited in the MG-RAST public database at http://metagenomics.anl.gov/ under accession numbers mgs629743 and mgs629746. The average number of reads per sample obtained after the initial processing (length, quality check and removal of non-bacterial sequences) was 2819 (min. 543, max. 5036). Only the taxa that had a relative abundance ≥0.5% in at least one sample were considered significant and were included in further evaluation. In total, 118 such significant taxa were identified, the sampled environments being distinguished primarily by the relative abundance in 7 of them: planctomycetes *Pirellula* and *Rhodopirellula*, unclassified Oscillatoriales (*Phormidium*), unclassified Rhizobiales (*Nordella*), *Desulfocapsa*, *Hyphomicrobium*, unclassified *Actinobacteria* (*Nakamurella*) and unclassified Bacillaceae (*Bacillus*). The only exception was if the sum of their relative abundance in the samples from the exposed pond exceeded 30% (min. 29.8%-max. 76.1%) and the total relative abundance of these bacteria in the control pond samples were lower than 6.5% (except two samples that had a relative abundance of 14.2% and 23.5%, min. 0.5%-max. 23.5%), see Supplementary Material 2. The Bray-Curtis

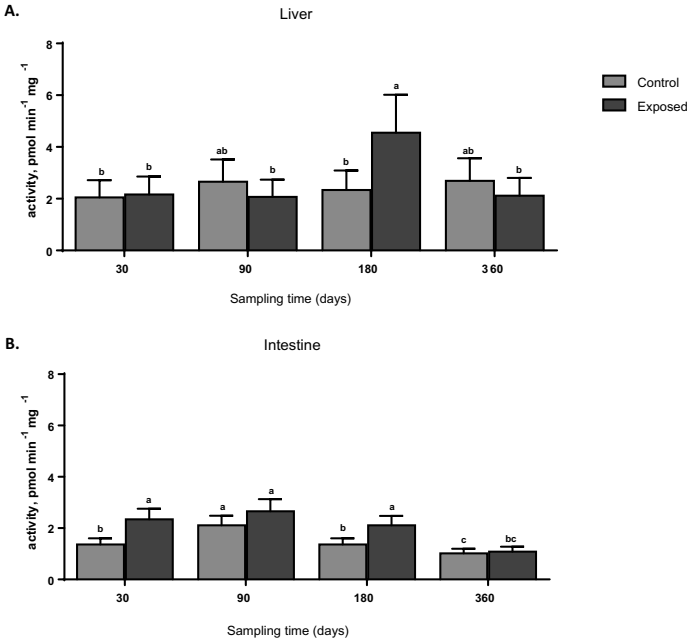

Fig. 1. EROD activity in hepatic (A) and intestinal (B) microsomes of common carp, in control pond (light grey bars) and in exposed pond (dark grey bars) receiving discharged water from STP. Enzyme activity is shown as pmol per min per mg of microsomal protein (mean ± standard error). The letters indicate statistical differences (ANOVA Mixed procedure, $p < 0.05$).

similarity indices reveal the difference among the carp intestinal microbiome samples from the control and exposed ponds, see Supplementary Material 2. The difference among the intestinal microbiome composition of carps are visualized on nMDS based on the Bray-Curtis distance matrix (Fig. 5).

4. Discussion

This study is one of the few reports that demonstrates the effects of chemical mixtures present in the aquatic environment under natural conditions. The unique design of these experiments allowed us to perform a real case study in which fish were held in a pond receiving water from STP discharge.

The presence of PPCPs in the POCIS was analyzed in both control and STP effluent discharge exposed ponds (Giang et al., 2017) while, in the water, sediment, and fish tissue were analyzed only in STP effluent exposed pond (Koba et al., 2018). Antibiotics, psychoactive drugs, anti-inflammatory agents, cardiovascular/hypertension drugs, and their metabolites were mainly detected groups of PPCPs in both control and exposed ponds (see Supplementary Material 1, Section 5). The seasonal variation in the total concentration of PPCPs, both in water and POCIS samples, showed similar trends. During autumn period (180 days) the concentration of PPCPs was lower, while in spring and summer periods (30, 90 and 360 days) PPCPs concentration was the highest. This is probably due to seasonal consumption and STP removal efficiency (Golovko et al., 2014). Bioaccumulation factor of the PPCPs in carp liver from the exposed pond showed that citalopram, its metabolite *N*-desmethylcitalopram and sertraline were the highest; however, only sertraline exceeded the 2000 bioaccumulation factor threshold (Koba et al., 2018). Which means that metabolism of the compound is altered and the compound may pose adverse or harmful effects to the organism (OECD, 2001). On the other hand, observed chemical results from exposed pond which receives water from STP effluents discharge are much higher than the chemical results observed in running surface water (Giang et al., 2018) which suggest that high dilution factor may observed in running surface waters such as streams.

Intestine, as a first barrier of pollutant entry in fish, showed multiple biomarker responses that included microbiome consortia and both CYP enzyme activities. Our results support that CYP1A gene and enzyme activity are responsive biomarkers for xenobiotics metabolism in piscine liver. In addition, FA metabolism might be affected by the presence of certain groups of pharmaceuticals such as statins and fibrates.

The CYP enzymes are involved in Phase I metabolism of xenobiotic chemicals and drugs. CYP1A dependent activity measured as an EROD is a well-established biomarker and an "early warning" sign of exposure to environmental pollutants. Moreover, AhR-regulated pathways activated by xenobiotics regulates the transcription of *CYP1A*, which results in the translation of CYP1A protein. Induction of CYP1A activity may cause toxic responses due to the activation of protoxins, or decrease toxicity because of an adaptive response (Pelkonen et al., 1998). In addition to AhR agonists, other compounds can effect CYP1A activity through other mechanisms without activating AhR (Hu et al., 2007). Several drugs received attention because of their effects on fish CYPs (Blunt et al., 2017; Creusot et al., 2015; Hamilton et al., 2017; Li et al., 2016). For example, fluoxetine, an antidepressant, inhibits the activity of EROD (Laville et al., 2004; Ribalta and Solé, 2014; Smith et al., 2012) and BFCOD (Ribalta and Solé, 2014). In the fish hepatic microsome, clotrimazole inhibits both EROD and BFCOD activity (Burkina et al., 2013), while fluoxetine, bezafibrate and roxithromycin induced the EROD activity *in vitro* (Thibaut and Porte, 2008) and *in vivo* (Liu et al., 2014).

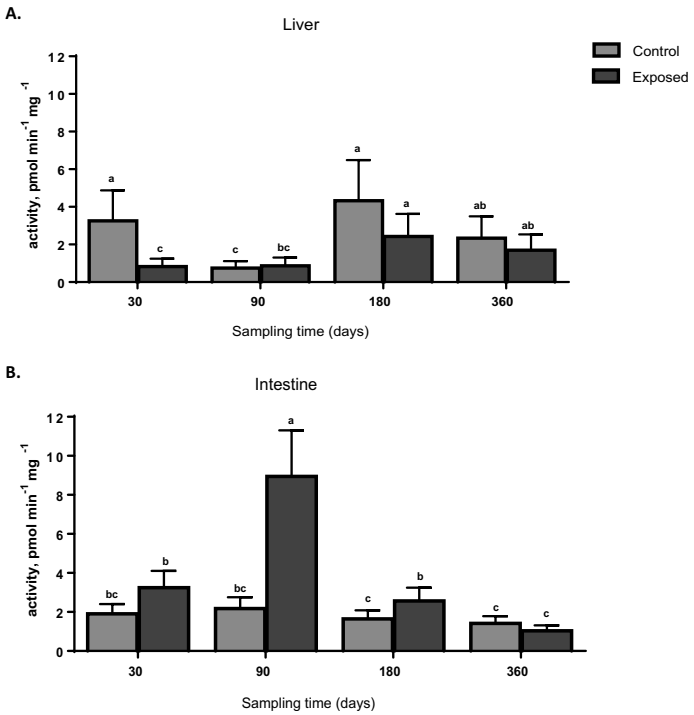

Fig. 2. BFCOD activity in hepatic (A) and intestinal (B) microsomes of common carp, in control pond (light grey bars) and in exposed pond (dark grey bars) receiving discharged water from STP. Enzyme activity is shown as pmol per min per mg of microsomal protein (mean ± standard error). The letters indicate statistical differences (ANOVA Mixed procedure, $p < 0.05$).

We observed that *O*-desmethylvenlafaxine, metabolite of antidepressant venlafaxine, or antibiotic clindamycin showed significant correlation with hepatic EROD and BFCOD activity in common carp.

Mixture or cocktails of pollutants might have different effects than individual compounds. For example, zebrafish (*Danio rerio*) exposed to a mixture of pharmaceuticals (caffeine, ibuprofen, carbamazepine and tamoxifen) increased the transcription of *CYP1A* and accordingly, up-regulated the activity of EROD while single drug exposure cause decreased *CYP1A* expression (Aguirre-Martínez et al., 2017). In another study, three-spined sticklebacks (*Gasterosteus aculeatus*) that were exposed to different percentages of effluents from a drug manufacturing plant, showed induction in gill EROD activity and in *CYP1A* expression (Beijer et al., 2013). Similarly, our data showed increased hepatic *CYP1A* mRNA levels at 30 and 90 day time points and up-regulated hepatic EROD activity at the 180 day time point. According to the results of this study, we can speculate that the adaptive responses of fish hepatic metabolism occurred after a half year. It is well known that induction of mRNA does not always result in increased catalytic activity of the corresponding enzyme (Hamilton et al., 2017). This was repeatedly reported in fish (Arinç et al., 2001; George et al., 2004; Kammann et al., 2008; Tom et al., 2003). Therefore, the differences between *CYP1A* mRNA and EROD activity in the present study are not surprising. Different transcriptional and post-transcriptional CYP1A regulation can be a reason for this.

In both mammals and fish, the CYP3A enzyme is considered a drug metabolizing enzyme that is primarily present in the liver and small intestine (Li et al., 2008). CYP3A-like activity and/or *CYP3A* expression have been measured in fish or other aquatic organisms exposed to single contaminants (Burkina et al., 2015a; Burkina et al., 2016; Cubero-Leon et al., 2012; Zanette et al., 2013), mixtures of contaminants (Ding et al., 2016) or STP effluents (Blunt et al., 2017). Observed hepatic CYP3A-like activity in this study were lower, especially after 30 days there was significantly lower activity compared with control, which could be a result of low CYP3A protein. Although there are limited studies on the effects of STP effluents on fish hepatic CYP3A-like activity, in wild gudgeons (*Gobio gobio*) living in the downstream water that receive pharmaceutical factory discharge, CYP3A activity did not significantly differ from the control group (Sanchez et al., 2011). Also in goldfish (*Carassius auratus*) exposed to reuse water from municipal waste water treatment plant, *CYP3A* expression was unchanged (Blunt et al., 2017). Similarly, in this study *CYP3A* expression was also unchanged at sampling time points, because this enzyme is associated with the metabolism of pharmaceuticals, it was expected to observe significant changes in *CYP3A* expression from the exposed pond.

It is known that the transcription of CYP enzymes in extrahepatic tissue, such as fish intestine, may occur after exposure to dietary xenobiotics or orally administrated drugs (Yuen and Au, 2006). Moreover, antibiotics that alter the intestinal microbiome can indirectly affect intestinal CYP activity (Martin et al., 2016; Zhang et al., 2014). Information on intestinal CYPs response to contaminants in fish is very limited. Intestinal CYP1A activity (Doering et al., 2012; Yuen and Au, 2006) or CYP3A (Hegelund and Celander, 2003; James et al., 2005) in several fish species were investigated in previous studies. To the best of our knowledge, this is the first study that measures intestinal EROD and

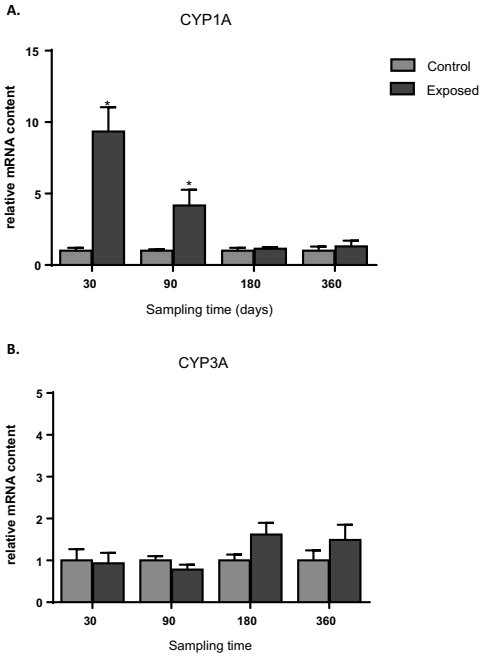

Fig. 3. mRNA expression of CYP1A (A) and CYP3A (B) in common carp in control pond (light grey bars) and in exposed pond (dark grey bars) receiving discharged water from STP. Data are reported as mean ± SD fold changes compared to control (normalized to 1 within each fish). Asterisks indicate statistical differences between control and exposed ponds within the same time point (Mann-Whitney t-test, $p \leq 0.05$).

BFCOD activity in fish chronically exposed to STP effluents. Fish exposed to dietary benzo[*a*]pyrene induced significant intestinal EROD activity across 28 days of exposure (Yuen and Au, 2006). In another study, white sturgeon (*Acipenser transmontanus*) and rainbow trout (*Oncorhynchus mykiss*) injected with 50 and 500 mg kg^{-1} β-naphthoflavone induced intestinal EROD activity only in white sturgeon and not rainbow trout (Doering et al., 2012). In the present study, differently from EROD activity in the liver, intestine induced at day 30, where the highest concentration of PPCPs observed. In addition, we observed that intestinal CYP3A activity in common carp was more sensitive than hepatic activity. Fish can be exposed to contaminants via water and/or via the food web. It was reported that benthic organisms from polluted areas can bio-concentrate the pharmaceuticals and this can subsequently cause bioaccumulation in fish (Grabicova et al., 2015; Grabicova et al., 2017). Exposure via both water and the food web could be the reason that intestinal CYP3A enzyme activity was found to be higher than the liver enzyme activity.

The naturally available diet differs from pond to pond. This is caused by changes in water quality and pond environment. However, conditions and general water parameters were similar in both ponds and therefore, the diet composition in both ponds was relatively similar. In general, feed FA composition reflects the FA composition in the fish flesh (Pettersson et al., 2009; Zajic et al., 2016) and therefore, feed is one of the main factors that affect the FA composition in fish. This explains the changes in FA composition in both the control group from time 0 and the major changes in the exposed group. In line with this, it is also clear that some of the FA are affected similarly in both the control and the exposed groups; for example, the similar increase found in 14:0. However, other factors, such as the presence of lipid regulators (statins or fibrates), can affect lipid storage and FA metabolism (Cheng et al., 2016; Rochman et al., 2013). We assumed that the large differences in some FA, such as 18:3 n-3, 22:5 n-3, EPA, DHA, SFA, PUFA in between control and exposed groups are related to altered FA metabolism due to the presence of the above mentioned chemicals.

Decreased n-3 and SFA percentages in the group exposed to the effluents, compared to the control group, is in agreement with the results of the study performed by Cheng et al. (2016), in which, lower percentages of n-3 and SFA were measured in fish fed on contaminated feed, compared to the non-contaminated feed group. Our results are also in

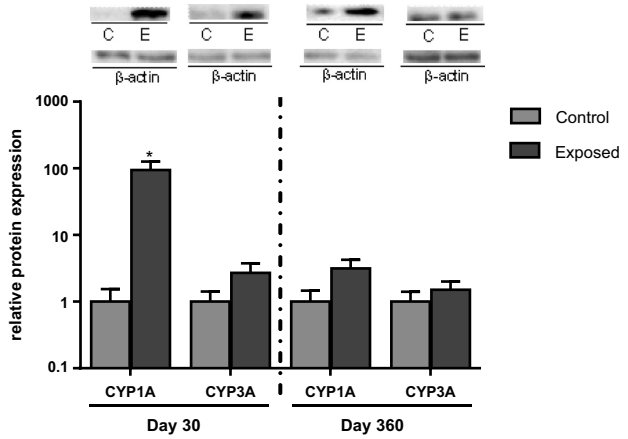

Fig. 4. Protein expression of CYP1A and CYP3A in common carp in control pond (light grey bars) and in exposed pond (dark grey bars) receiving discharged water from STP. Data are reported as mean ± SD fold changes compared to control (normalized to 1 within each fish). Asterisk indicates statistical differences between control and exposed ponds within the same time point (Mann-Whitney t-test, $p \leq 0.05$). The relative protein concentration was visualized by scanning on a Molecular Imager FX (Bio-Rad). Actin, reference gene; C, Control group; E, Exposed group.

Table 1
Total lipid fatty acid composition of carp muscle sampled at three different time points (0, 180, 360 day) and two different sites: Control, receives water from Blanice River; Exposed, receiving water from STP effluents.

		Control		Exposed	
	0	180	360	180	360
Fat content	$0.92^{c} \pm 0.15$	$2.32^{a} \pm 1.08$	$1.20^{a} \pm 0.36$	$3.06^{b} \pm 1.19$	$3.65^{b} \pm 1.43$
14:00	$0.53^{b} \pm 0.13$	$1.94^{a} \pm 0.39$	$1.52^{a} \pm 0.4$	$1.53^{a} \pm 0.2$	$1.68^{a} \pm 0.28$
16:00	$18.6^{a} \pm 1.24$	$19.1^{a} \pm 0.65$	$18.2^{a} \pm 0.86$	$17.0^{b} \pm 1.18$	$17.1^{b} \pm 0.74$
16:1 n-9	$3.13^{c} \pm 0.62$	$8.74^{a} \pm 1.46$	$6.98^{b} \pm 1.95$	$7.47^{ab} \pm 1.39$	$9.20^{a} \pm 1.60$
16:1 n-7	$0.63^{c} \pm 0.20$	$0.95^{bc} \pm 0.23$	$1.62^{b} \pm 0.31$	$1.55^{ab} \pm 0.65$	$1.79^{a} \pm 0.74$
17:00	$0.37^{c} \pm 0.10$	$0.95^{a} \pm 0.23$	$1.02^{a} \pm 0.15$	$0.66^{b} \pm 0.08$	$0.61^{b} \pm 0.10$
18:00	$5.97^{a} \pm 0.37$	$5.14^{a} \pm 0.84$	$5.64^{a} \pm 0.50$	$3.66^{b} \pm 0.74$	$3.54^{b} \pm 0.35$
18:1 n-9	$14.1^{b} \pm 3.41$	$14.7^{b} \pm 4.92$	$11.2^{b} \pm 1.67$	$21.5^{a} \pm 4.41$	$21.3^{a} \pm 3.57$
18:1 n-7	$3.37^{d} \pm 0.29$	$5.79^{a} \pm 1.11$	$5.95^{ab} \pm 0.49$	$4.40^{cd} \pm 0.42$	$4.93^{bc} \pm 0.74$
18:2 n-6	$9.11^{bc} \pm 1.47$	$7.04^{cd} \pm 4.56$	$4.57^{d} \pm 0.38$	$17.1^{a} \pm 2.27$	$12.2^{b} \pm 3.44$
18:3 n-3	$0.94^{d} \pm 0.27$	$8.15^{a} \pm 1.12$	$5.31^{bc} \pm 1.47$	$6.24^{b} \pm 0.87$	$4.72^{c} \pm 0.75$
20:1 n-9	$2.01^{a} \pm 0.19$	$0.65^{c} \pm 0.27$	$0.73^{c} \pm 0.18$	$1.07^{b} \pm 0.13$	$1.31^{b} \pm 0.19$
20:2 n-6	$1.12^{a} \pm 0.19$	$0.24^{c} \pm 0.12$	$0.36^{c} \pm 0.15$	$0.73^{b} \pm 0.11$	$0.78^{b} \pm 0.18$
20:3 n-6	$2.25^{a} \pm 0.30$	$0.49^{b} \pm 0.21$	$0.69^{b} \pm 0.08$	$0.68^{b} \pm 0.29$	$0.77^{b} \pm 0.16$
20:4 n-6	$11.0^{a} \pm 1.61$	$3.98^{c} \pm 1.26$	$5.87^{b} \pm 1.13$	$2.99^{c} \pm 1.13$	$3.29^{c} \pm 1.12$
20:3 n-3	$0.38^{b} \pm 0.11$	$0.47^{ab} \pm 0.07$	$0.54^{a} \pm 0.06$	$0.42^{b} \pm 0.06$	$0.45^{ab} \pm 0.07$
20:5 n-3	$3.98^{c} \pm 1.00$	$10.1^{b} \pm 2.53$	$14.0^{a} \pm 1.67$	$4.60^{c} \pm 1.03$	$6.19^{c} \pm 1.31$
22:3 n-3	$0.92^{ab} \pm 0.55$	$0.32^{c} \pm 0.12$	$0.58^{bc} \pm 0.13$	$0.36^{c} \pm 0.17$	$0.42^{c} \pm 0.14$
22:4 n-6	$2.84^{a} \pm 0.50$	$0.29^{b} \pm 0.11$	$0.40^{b} \pm 0.07$	$0.23^{b} \pm 0.08$	$0.27^{b} \pm 0.11$
22:5 n-3	$2.93^{b} \pm 0.60$	$2.60^{bc} \pm 0.91$	$4.00^{a} \pm 0.68$	$1.50^{d} \pm 0.63$	$1.76^{cd} \pm 0.46$
22:6 n-3	$13.0^{a} \pm 3.05$	$5.50^{bc} \pm 1.65$	$7.89^{b} \pm 1.43$	$3.72^{c} \pm 1.99$	$4.68^{c} \pm 1.97$
SFA	$25.8^{a} \pm 1.10$	$27.3^{a} \pm 1.84$	$26.6^{a} \pm 0.69$	$22.9^{b} \pm 1.02$	$23.1^{b} \pm 1.18$
MUFA	$23.6^{d} \pm 3.79$	$31.3^{bc} \pm 4.09$	$26.7^{cd} \pm 3.51$	$36.2^{ab} \pm 5.26$	$38.9^{a} \pm 3.89$
PUFA	$48.4^{a} \pm 4.72$	$39.2^{bc} \pm 2.70$	$44.2^{ab} \pm 3.50$	$38.6^{c} \pm 5.49$	$35.5^{c} \pm 2.98$
n-3	$22.1^{bc} \pm 3.85$	$27.1^{ab} \pm 5.16$	$32.3^{a} \pm 2.48$	$16.8^{c} \pm 3.83$	$18.2^{c} \pm 3.37$
n-6	$26.3^{a} \pm 1.69$	$12.0^{d} \pm 3.50$	$11.9^{d} \pm 1.20$	$21.7^{b} \pm 2.80$	$17.3^{c} \pm 3.04$
$\sum$n-3/$\sum$n-6	$0.84^{b} \pm 0.14$	$2.46^{a} \pm 0.71$	$2.73^{a} \pm 0.16$	$0.78^{b} \pm 0.17$	$1.10^{b} \pm 0.34$

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, poly unsaturated fatty acids.
Data are expressed as a percentage of identified fatty acids (mean ± S.D.), ($n = 8$).
The letters indicate significant differences between all experimental groups within the same acid.

line with earlier data on brown trout (*Salmo trutta* m. fario L.), in which, fish exposed to effluent water discharge showed an increase in fat content, increase 18:1 n-9, decrease n-3 and total PUFA (Giang et al., 2018). However, the 18:2 n-6 percentage did not change in that study. It seems the cocktail of chemicals from the sewage effluent affected lipid metabolism and caused an increase storage of lipids and thereby increase in MUFA deposition. Also, the higher fat content in the exposed group could be partly caused by lower n-3 PUFA percentage, as fat is stored as triacylglycerols that are usually higher in SFA and MUFA (Henderson and Tocher, 1987). Furthermore, there was possibly a decreased

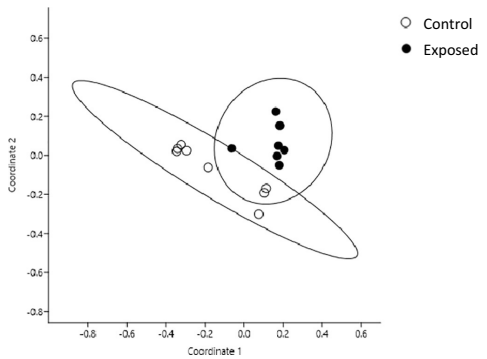

Fig. 5. The nMDS plot of Bray-Curtis distance matrix for individual intestinal microbiome samples from carp; taxon relative abundance cut-off 0.5% in at least one sample (stress value = 0.12). The values for samples of the carp are marked as follows: circle, control pond; dot, exposed pond. Ellipses estimate a region where 95% of population points are expected to fall.

synthesis/storage or increased oxidation of 20:5 n-3, as well as the longer metabolites 22:5 n-3 and 22:6 n-3, most probably due to the statins. The lower proportions of 20:5 n-3, 22:5 n-3 and 22:6 n-3 could in addition be partly due to decreased synthesis, from 18:3 n-3 towards the longer chain n-3 FA as also, 18:3 n-3 is lower in the exposed groups and hence there is a lower proportion of the substrate. This hypothesis is supported by the fact that the proportion between 20:5 n-3 and 22:5 n-3, as well as 22:5 n-3 and 22:6 n-3 is relatively similar. However, the increased proportion of 18:2 n-6 should then lead to an increased proportion of 20:4 n-6 as the metabolic pathways use the same desaturases and elongases (De Henauw et al., 2007; Palmquist, 2009). Since this is not the case, we suggest that the necessary enzymes for elongation and desaturation could have been negatively affected by the pollutants. To confirm this, gene and protein expression of the related elongases and desaturases should be evaluated.

The second possible factor for a decreased proportion of unsaturated FA, namely increased oxidation due to oxidative stress caused by the chemicals present in the water (Giang et al., 2017), is further strengthened by the fact that 18:2 n-6 first increased in the exposed group and then decreased; likely a result of the increased oxidative stress with longer exposure. Considering that the fish are produced for human consumption, the changes in the exposed group also seen by the lower n-3/n-6 ratio can be considered negatively, as a high content and proportion of n-3 FA is desired in fish and has shown positive effects on human health (Simopoulos, 2003).

From the highly abundant intestinal microbiome taxa identified in samples from the exposed pond, four (*Hyphomicrobium*, unclassified Actinobacteria (*Nakamurella*), unclassified Oscillatoriales (*Phormidium*) and *Pirellula*) were previously associated with the activated sludge from wastewater treatment plants (Martins et al., 2011; McIlroy et al., 2016; Tian et al., 2015). The increased abundance of these genera could thus be considered a biomarker of anthropogenic pollution. The acceptance of these taxa with the intake of sludge contaminated food cannot be excluded, but additional studies are necessary to confirm

this assumption. These, or related genera, were already identified in the intestinal microbiome of cyprinids (Li et al., 2015; Li et al., 2013; van Kessel et al., 2011) however, in our study, the medians of the relative abundances of these four genera were at least one order higher in the samples from the exposed pond, in comparison to controls. The analysis of the intestinal microbiome composition, confirmed the effect of the STP on the ecology of the exposed pond.

There were several limitations in this study. First, biomarker responses present in another study (Giang et al., 2017) indicated adaptive responses to contaminants developed during the first 30 days of the experiment. Thus, weekly sampling at the beginning of the experiment would allow more insight in to the early response mechanism(s) of fish exposed to STP discharge. Second, lack of data regarding the feed sources (i.e. bioaccumulation, species variation) in both ponds made it difficult to explain intestinal CYP activity as well as FA composition.

In conclusion, this study reveals effects of PPCPs found in STP effluents under realistic conditions on common carp. Long term exposure to STP effluents in the exposed pond affected hepatic and intestinal CYP enzymes, FA metabolism and consequently, the intestinal microbiome in common carp. STP effluent discharge has clear impacts on the intestinal microbiome taxa as well as CYP metabolism. However, it should be noted that the observed effects might differ in fish living in running surface waters due to dilution factor of the STP effluent discharge. Further *in situ* studies focused on various STP discharge scenarios would bring more insights on the effects of PPCPs under real conditions.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgements

The study was financially supported by the Ministry of Education, Youth and Sports of the Czech Republic, projects CENAKVA (No. CZ.1.05/2.1.00/01.0024), CENAKVA II (No. LO1205 under the NPU I program), by the Grant Agency of the University of South Bohemia in Ceske Budejovice (No. 12/2016/Z) and by the Czech Science Foundation (No. 15-04258S). The authors would also like to thank the town of Vodňany and CEVAK a.s. for supporting this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.04.188.

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Appendix D.

Supplementary Material 1.

Material 1. *The sampling event of each pond.*

Time point	Date	Pond
0 Day	April 2015	
30 days	May 2015	Control
		Exposed
90 days	July 2015	Control
		Exposed
180 days	October 2015	Control
		Exposed
360 days	April 2016	Control
		Exposed

Material 2. *Primer and TaqMan probe used for gene expression. All primers and probes are given in the 5′-3′ direction.*

	CYP1A
Forward Primer	CAACGTGATCTGTGGGATATGC
Reverse Primer	AAACCCACCAGCTCGTCATC
Probe	TCGGCCGGCGCTACAGCC
	CYP3A
Forward Primer	GCAGCGAGGAACACACAGAA
Reverse Primer	GCCGAAGATGAAGATCATGGA
Probe	AAGGTCTGAGCGACCACGAGATCCTCTT
	Beta-actin
Forward Primer	GGTATGTGCAAAGCCGGATT
Reverse Primer	GGCGACCCACGATGGA
Probe	AGATGATGCTCCCCGTGCCGTC

Material 3. *Detailed description of PCR amplification method*

In the first step, three independent 50 µl reactions per sample were performed, each containing 5 µl of 10x Supreme NZYTaq polymerase buffer, 2 µl of 50 mM $MgCl_2$ (NZYTech, Portugal), 1 µl of 10mM dNTP (Thermo Fisher Scientific, USA), 2 µl of each primer (0.25 mM), 35 µl of dH_2O, 1 µl of 5U μl^{-1} Supreme NZYTaq polymerase (NZYTech, Portugal) containing 4% Phusion High-Fidelity DNA polymerase (NEB, USA) and 2 µl of template DNA. The cycling conditions were 94°C for 5min; 35 cycles of 94°C for 1 min, 57°C for 50 s and 72°C for 30 s; followed by final extension at 72°C for 10 min. PCR products of all three independent reactions for each sample were pooled and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA). The DNA concentration of each pooled purified primary amplicon solution was adjusted to 50 ng μl^{-1} and 2 µl of DNA solution was further used as a template in the secondary PCR performed under the same conditions except that the fusion primers were used, and the number of cycles was 10. The secondary PCR was performed in two independents 50 µl reactions per sample. The PCR products for each sample were pooled and gel purified using the Wizard SV

Gel and PCR Clean-Up System (Promega, USA) and finally purified and concentrated using the MinElute PCR Purification Kit (QIAGEN, Germany). The DNA concentration was measured by Qubit 2.0 Fluorometer (Life Technologies, USA) using dsDNA BR Assay Kit chemistry and standards.

Material 4. *The correlations between pharmaceuticals measured in fish liver and biomarkers*

	Correlation coefficient (r) values					
	CY1A	CYP3A	EROD Liver	EROD intestine	BFCOD liver	BFCOD intestine
Carbamazepine	-0.10	0.08	-0.16	0.50**	-0.05	-0.34
Cetirizine	0.23	-0.10	-0.21	-0.04	-0.17	-0.20
Citalopram	0.11	-0.20	-0.04	-0.04	-0.04	-0.07
Clindamycin	-0.21	0.08	-0.59***	-0.15	0.89 ***	-0.09
Metoprolol	0.38	-0.11	-0.02	-0.07	-0.07	-0.07
Metoprolol acid	-0.24	0.09	-0.01	-0.01	-0.09	-0.02
N-desmethylcitalopram	-0.10	0.13	0.22	-0.24	0.24	-0.12
O-desmethylvenlafaxine	-0.33	-0.02	0.31*	0.04	0.31*	-0.08
Sertraline	0.11	-0.23	-0.14	0.20	-0.16	0.04
Tramadol	0.20	-0.17	0.07	-0.24	-0.09	-0.04
Venlafaxine	0.31	-0.14	0.01	0.02	-0.05	-0.06

A Pearson product-moment correlation coefficient was computed to assess the relationship between the pharmaceuticals found in fish liver (data can be found in Koba et al., 2018) and biomarkers measured in fish. * *p*-value < 0.05; ** *p*-value < 0.01; ****p*-value < 0.001. EROD - 7-ethoxyresorufin-O-deethylase; BFCOF - 7-benzyloxy-4-trifluoromethylcoumarin O-debenzylase.

Material 5. *Concentrations of group of pharmaceuticals*

Group of PPCPs	30 days		90 days		180 days		360 days	
	Control	Exposed	Control	Exposed	Control	Exposed	Control	Exposed
NSAIDs	7	1023	8	739	13	266	41	486
Beta blockers	22	1210	36	391	36	308	77	467
Hypertension	47	2772	105	1874	88	644	124	1460
Antibiotic	48	4592	80	2313	109	1146	138	2181
Psychoactive	55	3580	165	2006	164	1059	180	1652
Others	127	776	86	395	81	70	320	329
Sum of PPCPs	305	13952	478	7719	490	3494	880	6575

All data are presented as ng/POCIS; data in exposed pond were calculated by the average result of POCIS from 3 sampling points (near the inlet, mid-way location and near the outlet). Data was taken from Giang et al., 2017.

Supplementary Material 2.

Table 1. *The list of identified taxon and their relative abundances (cut-off 0.5%)*

taxon	Control pond samples								Exposed pond samples							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
Acinetobacter	0.06	0.64	0.00	0.03	0.23	0.00	0.00	3.08	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.00
Ahrensia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.67	0.00	0.00	0.00
Alsobacter	0.00	0.00	0.00	0.00	0.12	0.00	0.16	0.00	0.00	0.07	0.62	0.11	0.00	1.31	0.39	0.00
Aminobacter	0.00	0.00	0.08	0.00	0.06	0.05	0.00	0.00	0.00	0.00	0.02	0.00	4.45	0.04	0.12	0.00
Brevibacterium	0.61	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Brevundimonas	0.61	1.15	0.00	0.00	0.23	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Burkholderia	2.19	1.03	0.04	1.80	1.05	0.02	0.12	2.56	1.23	0.03	0.00	0.00	0.00	0.00	0.04	0.06
candidatus *Cardinium*	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	41.48	0.87	0.00	0.80	3.64	0.24
candidatus *Odyssella*	0.00	0.00	0.03	0.00	0.04	0.00	0.00	0.68	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00
Clostridium	0.00	0.01	0.24	0.00	0.37	0.19	0.09	0.00	0.04	0.14	0.06	0.18	0.10	0.01	0.03	1.35
Corynebacterium	0.00	0.30	0.04	0.00	0.15	0.02	0.03	1.06	0.50	0.00	0.00	0.00	0.09	0.00	0.00	0.00
Cupriavidus	1.23	2.24	0.00	2.24	0.70	0.00	0.06	1.98	1.41	0.04	0.00	0.00	0.00	0.00	0.00	0.00
Defluviicoccus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.89	0.00	0.00	0.00
Dechloromonas	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.64	0.01	0.00	0.11	0.10	0.02	0.00
Desulfobulbus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.60	0.00	0.22	0.22	0.08	0.00	0.00
Desulfocapsa	0.06	0.00	0.05	0.05	0.16	0.00	0.01	0.03	0.00	19.72	0.69	1.77	1.78	1.19	0.70	7.67
Desulfomonile	0.00	0.00	0.16	0.00	0.47	0.09	0.16	0.00	0.00	0.36	0.25	2.51	0.00	0.40	0.08	1.19
Desulforhabdus	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.04	0.02	0.60	0.00	0.08	0.12	0.12
Desulfovibrio	0.00	0.02	0.00	0.00	0.02	0.00	0.00	0.00	0.00	1.99	0.01	0.10	0.15	0.08	0.05	0.24
Eubacterium	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.58	0.00	0.00	0.00	0.00	0.02	0.00	0.00
Gallionella	1.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00
Halomonas	32.79	25.60	0.66	53.73	25.43	0.14	1.67	43.28	0.00	0.00	0.00	0.05	0.22	0.02	0.12	0.00
Hyphomicrobium	0.18	0.00	0.33	0.16	0.23	0.00	0.36	0.00	0.31	0.22	1.21	1.09	3.11	1.95	0.77	1.42

taxon	Control pond samples								Exposed pond samples							
Chryseobacterium	3.16	0.75	0.00	0.00	0.00	0.00	0.00	0.63	0.16	0.00	0.00	0.00	0.00	0.08	0.54	0.12
Labrys	0.00	0.00	0.00	0.00	0.00	0.54	0.00	0.00	0.00	0.00	0.47	0.00	2.22	2.48	3.87	0.24
Lactococcus	0.00	0.02	0.03	0.24	0.00	0.35	0.00	0.03	5.54	3.78	0.01	0.06	0.98	0.00	0.00	0.00
Leifsonia	0.00	0.88	0.00	4.00	1.16	0.00	0.24	0.19	1.57	0.02	0.00	0.00	0.00	0.00	0.00	0.00
Leptotrichia	0.00	0.00	0.00	0.00	0.42	0.00	0.00	0.00	0.00	0.58	0.00	0.00	0.00	0.00	0.00	0.00
Methylocaldum	0.00	0.00	0.16	0.00	0.41	0.36	0.26	0.00	0.16	0.04	0.17	0.00	0.22	0.48	1.08	0.36
Methylocystis	0.00	0.27	3.78	0.00	0.58	1.72	7.85	0.00	0.31	0.07	0.89	0.33	4.00	1.44	1.01	0.24
Moraxella	0.57	0.20	0.00	0.00	0.00	0.00	0.01	0.70	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mycobacterium	0.00	0.00	7.15	0.00	0.99	1.45	13.75	0.00	0.31	0.00	0.40	0.22	0.00	0.64	1.51	0.00
Pelomonas	0.26	1.05	0.00	0.08	0.35	0.00	0.06	1.82	0.00	0.00	0.00	0.00	0.00	0.08	0.00	0.00
Phenylobacterium	1.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pirellula	0.18	0.34	6.08	0.00	1.51	1.18	5.67	0.00	9.10	1.38	9.76	1.91	3.11	16.00	18.70	5.22
Planktothrix	0.00	0.10	1.31	0.00	2.89	3.65	4.76	0.00	0.00	0.00	0.06	0.05	2.00	0.04	0.08	0.00
Polaromonas	0.00	0.00	0.00	0.00	2.91	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Polynucleobacter	0.00	0.61	0.00	0.00	0.58	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Propionibacterium	1.21	0.16	0.05	0.11	0.04	0.00	0.00	0.75	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00
Propionivibrio	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.51	0.00	0.00	0.00	0.00	0.00	0.00
Pseudomonas	0.00	0.00	0.05	0.40	0.02	0.02	0.01	0.68	0.00	0.03	0.01	0.00	0.00	0.01	0.01	0.00
Ralstonia	1.70	1.42	0.00	0.48	0.43	0.00	0.01	0.61	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00
Rhizobium	0.00	0.02	0.00	0.00	0.00	0.05	0.00	0.00	0.16	0.27	0.42	0.44	0.41	0.47	2.50	0.47
Rhodococcus	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.52	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Rhodopirellula	0.00	0.20	3.29	0.00	0.47	0.36	6.42	0.00	0.94	3.11	2.62	4.17	3.11	3.84	5.65	6.17
Rothia	0.99	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Shewanella	5.68	4.23	0.05	4.71	2.35	0.03	0.12	5.51	0.00	0.00	0.01	0.00	0.00	0.01	0.01	0.00
Schlegelella	0.00	0.00	0.16	0.00	0.00	0.00	0.00	0.00	0.94	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sphingomonas	22.09	11.75	0.41	12.32	4.83	0.05	0.67	17.46	22.13	0.04	0.04	0.00	0.00	0.08	0.23	0.24

taxon	Control pond samples								Exposed pond samples							
Staphylococcus	0.07	0.26	0.00	0.42	0.16	0.02	0.00	1.39	0.06	0.00	0.00	0.00	0.00	0.00	0.02	0.00
Stenotrophomonas	0.88	1.79	0.00	0.04	0.84	0.00	0.02	0.94	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Streptococcus	1.01	0.52	0.03	0.00	0.04	0.02	0.00	0.36	0.24	0.01	0.23	0.04	0.09	0.02	0.00	0.05
unclassified Acidimicrobiales (*Iamia*)	0.00	0.00	0.16	0.00	0.12	0.00	0.04	0.00	0.63	0.04	0.12	0.05	0.00	0.36	0.15	0.00
unclassified Acidobacteria (*Blastocatella*)	0.00	0.00	0.00	0.32	0.00	0.00	0.00	0.77	0.94	0.00	0.00	0.00	0.00	0.00	0.00	0.00
unclassified Actinobacteria	0.00	0.00	0.00	0.00	0.12	0.00	0.00	0.00	1.26	0.00	0.12	0.00	0.00	1.28	0.85	0.24
unclassified Actinobacteria (*Atopobium*)	0.00	0.00	0.16	0.80	0.12	0.00	0.16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
unclassified Actinobacteria (*Modestobacter*)	0.00	0.00	0.16	0.00	0.00	0.00	0.00	0.00	0.31	0.00	0.21	0.33	2.67	0.08	0.62	0.00
unclassified Actinobacteria (*Nakamurella*)	0.00	0.07	0.38	0.00	0.39	0.09	0.22	0.10	2.82	0.07	3.17	1.20	6.67	9.00	3.49	0.24
unclassified Actinomycetales	0.00	0.00	0.16	0.00	0.08	0.06	0.07	0.00	0.42	0.10	0.38	0.22	0.30	0.92	0.76	0.24
unclassified Alphaproteobacteria (*Prosthecomicrobium*)	1.40	0.00	0.16	0.00	0.12	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
unclassified Anaerolineaceae (*Bellilinea*)	0.00	0.00	0.16	0.00	0.06	0.00	0.00	0.00	0.63	0.00	0.02	0.05	0.00	0.00	0.00	0.12
unclassified Anaerolineaceae (*Levilinea*)	0.00	0.00	0.00	0.00	0.06	0.00	0.12	0.00	0.00	0.07	0.25	1.95	0.00	0.08	0.08	0.12

taxon	Control pond samples								Exposed pond samples							
unclassified Anaerolineaceae (*Longilinea*)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.19	0.60	0.44	0.12	0.35	0.00
unclassified Anaerolineaceae (*Ornatilinea*)	0.00	0.00	0.16	0.00	0.06	0.00	0.36	0.05	0.00	0.22	0.04	1.04	0.00	0.08	0.00	0.24
unclassified Anaplasmataceae (candidatus *Xenohaliotis*)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.67	0.00	0.00	0.00	0.00	0.00
unclassified Anaplasmataceae (*Neorickettsia*)	6.84	8.81	0.00	4.16	4.19	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
unclassified Bacillaceae (*Bacillus*)	0.04	0.19	11.83	0.04	3.05	0.63	0.99	0.09	22.11	0.51	3.74	0.46	7.06	1.19	5.74	2.23
unclassified Caldilineaceae (*Caldilinea*)	0.00	0.00	0.08	0.00	0.06	0.00	0.16	0.00	0.16	0.11	0.15	0.98	2.45	0.28	0.46	0.24
unclassified Caldilineaceae (*Litorilinea*)	0.00	0.03	0.74	0.00	0.64	0.00	0.56	0.77	0.16	0.87	0.88	3.16	5.67	0.76	1.32	0.83
unclassified Clostridiales (*Tissierella*)	0.00	0.00	0.03	0.02	0.02	0.02	0.00	0.00	1.07	0.00	0.15	0.00	0.13	0.51	0.64	0.02
unclassified Coxiellaceae (*Aquicella*)	0.00	0.00	0.89	0.00	0.17	0.49	0.31	0.00	0.10	0.00	0.01	0.00	0.29	0.00	0.04	0.00
unclassified Coxiellaceae (*Coxiella*)	0.00	0.00	0.33	0.00	0.12	0.27	0.00	0.00	0.00	0.07	0.01	0.11	0.89	0.12	0.15	0.00
unclassified Deltaproteobacteria	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.02	0.00	0.67	0.16	0.00	0.00
unclassified Dependentiae	0.00	0.07	0.00	0.00	0.03	0.02	0.00	0.00	2.20	0.07	0.00	0.79	0.59	0.36	0.31	0.00

taxon	Control pond samples								Exposed pond samples							
unclassified Desulfohalobiaceae (*Desulfonatronospira*)	0.00	0.03	0.74	0.00	0.20	0.63	1.24	0.00	0.00	0.00	0.01	0.03	0.00	0.02	0.12	0.00
unclassified Desulfohalobiaceae (*Desulfonatronovibrio*)	0.00	0.00	0.96	0.00	0.17	0.43	0.77	0.00	0.00	0.00	0.03	0.00	0.00	0.02	0.06	0.00
unclassified Dietziaceae (*Dietzia*)	1.26	0.05	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
unclassified Enterobacteraceae (*Escherichia-Shigella*)	8.19	10.04	0.09	10.52	9.49	0.04	0.12	1.95	1.57	0.02	0.02	0.11	0.13	0.05	0.01	0.00
unclassified Firmicutes	0.00	0.00	0.05	0.00	0.00	0.00	0.05	0.00	3.71	0.00	0.04	0.00	0.07	0.19	0.04	0.00
unclassified Gammaproteobacteria	0.00	0.00	0.00	0.00	0.12	0.00	0.00	0.00	0.63	0.00	0.17	0.11	1.78	0.24	0.46	0.12
unclassified Hyphomicrobiaceae (*Pedomicrobium*)	2.45	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
unclassified Christensenellaceae	0.00	0.00	0.00	0.00	0.00	0.07	0.05	0.00	1.48	0.02	0.00	0.00	0.06	0.00	0.00	0.00
unclassified Chroococcales (*Cyanobacterium*)	0.00	0.34	0.82	0.00	0.21	0.79	1.43	0.00	0.21	0.00	0.06	0.04	0.15	0.13	0.36	0.00
unclassified Chroococcales (*Gleocapsa*)	0.00	0.00	0.49	0.00	0.17	0.14	0.38	0.02	0.00	0.04	0.75	0.00	0.44	0.84	2.39	0.12
unclassified Chroococcales (*Chroogloeocystis*)	0.00	1.49	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.03	0.08
unclassified Legionellaceae (*Legionella*)	0.00	0.04	0.71	0.00	0.29	0.33	0.29	0.00	0.42	0.00	0.93	0.13	0.44	1.41	2.00	0.00
unclassified Nostocales (*Brasilonema*)	0.00	0.42	19.24	0.00	2.58	13.48	22.06	0.10	0.10	0.02	0.26	0.20	0.15	0.41	0.88	0.00

taxon	Control pond samples								Exposed pond samples							
unclassified Nostocales (*Calothrix*)	0.09	0.00	0.70	0.04	0.17	0.32	0.55	0.07	2.75	0.20	0.60	0.38	1.11	2.59	1.12	0.65
unclassified Oscillatoriales (*Hydrocoleum*)	0.09	1.71	0.00	0.08	0.00	0.00	0.02	0.00	0.00	0.43	0.14	0.90	0.00	0.72	0.41	0.53
unclassified Oscillatoriales (*Phormidium*)	0.09	2.46	0.41	0.60	0.59	0.47	0.22	0.24	0.78	45.25	11.77	49.22	4.11	17.65	9.08	53.97
unclassified Oxalobacteraceae (*Undibacterium*)	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.77	0.00
unclassified Parachlamydiaceae (*candidatus Protochlamydia*)	0.00	0.00	0.05	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.01	0.29	0.00	0.03	0.77	0.00
unclassified Phyllobacteriaceae (*Mesorhizobium*)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.09	0.19	0.05	0.67	0.37	0.35	0.12
unclassified Planctomycetaceae	0.00	0.27	4.44	0.16	0.64	0.27	3.33	0.19	0.00	0.87	0.89	3.33	3.11	1.28	2.23	0.83
unclassified Planctomycetaceae (*Blastopirellula*)	0.00	0.00	0.00	0.00	0.00	0.09	0.52	0.00	0.00	0.43	0.19	0.44	1.33	0.65	0.27	0.95
unclassified Planctomycetaceae (*Planctomyces*)	0.00	0.07	0.49	0.00	0.17	0.05	0.65	0.05	0.31	0.04	0.48	0.16	0.22	0.84	1.20	0.95
unclassified Planctomycetaceae (*Singulisphaera*)	0.00	0.01	1.03	0.00	0.09	0.36	1.72	0.00	0.12	0.01	0.03	0.04	0.48	0.04	0.16	0.06
unclassified Planctomycetaceae (*Zavarzinella*)	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.10	0.00	0.01	0.05	0.59	0.05	0.13	0.00
unclassified Rhizobiales (*Ancylobacter*)	0.00	0.00	0.00	0.00	0.00	0.18	0.00	0.00	0.00	0.00	0.04	0.55	0.00	0.40	0.15	0.00

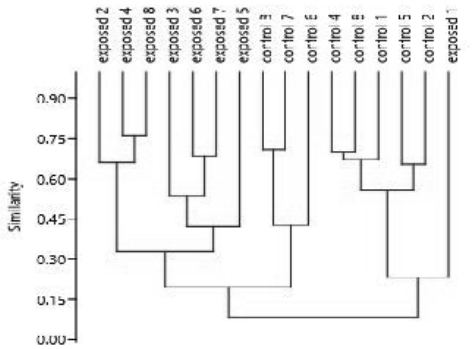
taxon	Control pond samples								Exposed pond samples							
unclassified Rhizobiales (*Aquamicrobium*)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00	1.52	0.08	0.00
unclassified Rhizobiales (*Nordella*)	0.00	0.07	1.15	0.16	0.12	0.45	0.36	0.00	0.63	0.65	2.12	1.69	0.89	3.86	3.52	1.42
unclassified Rhizobiales (*Phreatobacter*)	0.00	0.00	0.00	0.00	0.00	0.03	0.08	0.00	0.63	0.00	0.75	0.11	0.59	1.57	0.31	0.06
unclassified Rhodobacteraceae (*Albidovulum*)	0.00	0.00	0.66	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
unclassified Rickettsiales (candidatus *Defluviella*)	0.00	0.00	1.64	0.00	0.00	50.82	0.52	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
unclassified Ruminococcaceae (candidatus *Soleaferrea*)	0.00	0.20	6.57	0.16	8.72	6.70	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00
unclassified Saccharibacteria	0.00	0.00	0.00	0.00	0.12	0.00	0.00	0.00	0.00	0.01	0.46	0.11	4.56	0.81	2.03	0.00
unclassified Solirubrobacterales (*Conexibacter*)	0.00	0.00	0.00	0.00	0.23	0.27	0.36	0.00	0.00	0.00	0.21	0.46	1.78	0.56	0.31	0.00
unclassified Spirochaetales (*Brevinema*)	0.35	13.28	0.00	0.00	6.63	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
unclassified Synechococcales (*Arthronema*)	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.78	0.00	0.03	0.00
unclassified Synechococcales (*Leptolyngbya*)	0.06	0.07	4.85	0.00	0.62	3.79	5.00	0.03	1.10	0.16	2.62	1.07	1.96	1.99	4.45	1.50

taxon	Control pond samples								Exposed pond samples							
unclassified Synechococcales (*Neosynechococcus*)	0.00	0.00	0.74	0.00	0.08	0.32	0.52	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.12
unclassified Synechococcales (*Synechococcus*)	0.00	0.03	5.59	0.00	1.05	4.88	7.92	0.05	0.00	0.09	0.21	0.05	0.00	0.45	0.27	0.12
unclassified Syntrophaceae (*Desulfobacca*)	0.00	0.00	0.33	0.00	0.93	0.18	0.59	0.00	0.00	0.29	0.62	4.04	0.00	0.56	1.03	1.19
unclassified Thermomicrobia	0.00	0.00	0.08	0.00	0.00	0.05	0.04	0.87	0.00	0.04	0.19	0.16	0.89	0.12	0.19	0.00
unclassified Vibrionaceae (*Photobacterium*)	0.00	0.88	0.00	0.09	0.37	0.00	0.00	8.83	0.00	0.34	0.00	0.00	0.06	0.23	0.00	0.00
unclassified Xanthomonadaceae (*Ignatzschineria*)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.26	0.07	0.00	0.00	0.00	0.60	0.00	0.00
unclassified Xanthomonadaceae (*Wohlfahrtiimonas*)	0.00	0.07	0.00	0.00	0.00	0.09	0.00	0.10	0.00	8.69	0.00	0.00	0.00	0.00	0.00	0.00
unclassified Xanthomonadales (candidatus *Competibacter*)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.31	0.14	0.00	2.00	1.23	0.14	0.00

Table 2. *Bray-Curtis distance matrix calculated for individual control pond samples and exposed pond samples (cut-off 0.5%).*

	control 1	control 2	control 4	control 5	control 8	control 7	control 6	control 3	exposed 1	exposed 3	exposed 5	exposed 6	exposed 7	exposed 2	exposed 4	exposed 8
control 1		0.66	0.68	0.52	0.67	0.04	0.01	0.02	0.28	0.01	0.01	0.01	0.02	0.01	0.01	0.01
control 2	0.66		0.64	0.66	0.55	0.06	0.04	0.05	0.21	0.05	0.06	0.07	0.07	0.05	0.06	0.06
control 4	0.68	0.64		0.54	0.70	0.04	0.02	0.03	0.21	0.01	0.02	0.02	0.02	0.02	0.02	0.02
control 5	0.52	0.66	0.54		0.43	0.22	0.23	0.28	0.20	0.14	0.16	0.14	0.17	0.08	0.11	0.12
control 8	0.67	0.55	0.70	0.43		0.05	0.01	0.04	0.25	0.02	0.03	0.03	0.03	0.02	0.02	0.02
control 7	0.04	0.06	0.04	0.22	0.05		0.39	0.71	0.15	0.22	0.27	0.26	0.32	0.10	0.19	0.22
control 6	0.01	0.04	0.02	0.23	0.01	0.39		0.46	0.07	0.11	0.15	0.13	0.16	0.06	0.08	0.08
control 3	0.02	0.05	0.03	0.28	0.04	0.71	0.46		0.28	0.27	0.35	0.27	0.37	0.11	0.18	0.21
exposed 1	0.28	0.21	0.21	0.20	0.25	0.15	0.07	0.28		0.26	0.27	0.29	0.31	0.10	0.12	0.16
exposed 3	0.01	0.05	0.01	0.14	0.02	0.22	0.11	0.27	0.26		0.34	0.53	0.54	0.24	0.33	0.36
exposed 5	0.01	0.06	0.02	0.16	0.03	0.27	0.15	0.35	0.27	0.34		0.46	0.46	0.19	0.33	0.27
exposed 6	0.01	0.07	0.02	0.14	0.03	0.26	0.13	0.27	0.29	0.53	0.46		0.68	0.34	0.45	0.47
exposed 7	0.02	0.07	0.02	0.17	0.03	0.32	0.16	0.37	0.31	0.54	0.46	0.68		0.22	0.36	0.38
exposed 2	0.01	0.05	0.02	0.08	0.02	0.10	0.06	0.11	0.10	0.24	0.19	0.34	0.22		0.64	0.69
exposed 4	0.01	0.06	0.02	0.11	0.02	0.19	0.08	0.18	0.12	0.33	0.33	0.45	0.36	0.64		0.76
exposed 8	0.01	0.06	0.02	0.12	0.02	0.22	0.08	0.21	0.16	0.36	0.27	0.47	0.38	0.69	0.76	

ANOSIM		**PERMANOVA**	
Permutation N:	9999	Permutation N:	9999
Mean rank within:	41.82	Total sum of squares:	4.84E+00
Mean rank between:	76.84	Within-group sum of squares:	3.37E+00
R:	0.5837	F:	6.1
p (same):	0.0005	p (same):	0.0006



CHAPTER 5

GENERAL DISCUSSION

CYPs are important biomarkers in aquatic toxicology because they metabolise a vast variety of endogenous and exogenous compounds. Therefore, the fish CYP system has been widely studied to understand their role in xenobiotic metabolism. Microsomal fractions are rich sources of CYP enzymes, therefore, they are commonly used for investigating the metabolic fate of xenobiotics and potential drug-drug interactions.

This thesis includes five studies in which bioassays using fish hepatic and/or intestinal microsomes exposed to environmental pollutants either *in vitro, in vivo* or *in situ* were performed. Chapter 2 includes two *in vitro* studies; the first one studied the effects of several organic solvents at different concentrations on fish hepatic CYP activities (CYP1A, CYP2E1-, and CYP3A-like). The second paper studied effects of selected phytochemicals (naringenin, diosmin, quercetin, and indole-3-carbinol) and pharmaceuticals (dexamethasone and clotrimazole) alone and in combination to identify drug-drug interactions in fish hepatic microsomes. Chapter 3 includes two papers in which dexamethasone- and clotrimazole-induced chronic effects were studied in rainbow trout using *in vivo* systems. Chapter 4 includes an *in situ* study, which presents the most realistic case study in which common carp were exposed to STP effluent discharge water under natural conditions.

In vitro experiments

Prior to the start of each pollutant experiment, organic solvents were tested for their capability to interfere with CYP activity (Chapter 2.1). Organic solvents, including acetonitrile, acetone, methanol, ethanol, and DMSO were tested at 0.01%, 0.1%, 0.5%, 0.8%, 1%, 2%, and 3% concentrations on CYP1A (EROD), CYP3A-like (BFCOD), and CYP2E1-like (PNPH) activities using rainbow trout microsomes. EROD activity was affected when organic solvents exceeded 0.5%. Acetonitrile at 1% and ethanol at 0.8% to 1% caused a moderate decrease in EROD activity. DMSO and low ethanol concentrations (0.01%–0.8%) induced BFCOD activity while PNPH activity was inhibited at high concentration (2%–3%) of acetonitrile, methanol, and ethanol. DMSO caused a slight reduction in CPY activity at 0.5% to 0.8% concentrations. Organic solvents affected different CYPs activities at different concentrations. Our results demonstrated that the inhibitory potency of organic solvents is substrate-dependent. Previously, it was reported that 1% acetone, DMSO, ethanol, and methanol reduced CYP1A activity by 20% in rat hepatic microsomes (Li et al., 2010), and 5% methanol and acetonitrile inhibited CYP1A activity in human hepatic microsomes by 12% and 27%, respectively (Chauret et al., 1998). CYP2E1 activity in human hepatocytes was inhibited >50% with 1% acetone (Hickman et al., 1998). Human CYP3A4 activity was inhibited in the presence of DMSO at 1-2% (Easterbrook et al., 2001) and 1.25% (Li, 2009). On the other hand, ethanol or DMSO at 0.1% inhibited CYP3A activity in human hepatic microsomes (Iwase et al., 2006). Similarly, BFCOD activity in fish hepatic microsomes was also induced in the presence of ethanol and DMSO (Sakalli et al., 2015). Generally, the use of 1% of organic solvents in *in vitro* bioassays is accepted as safe (Timm et al., 2013); however, our study revealed that fish CYP activities are more sensitive to organic solvents than mammalian activities are. The use of organic solvents in *in vitro* assays should always be kept at a minimum concentration, and controls containing the same amount of solvents should be included in the experiment. In addition, selection of alternative substrates during the experimental design can be advantageous.

Effects of the pharmaceuticals, clotrimazole and dexamethasone, and/or the phytochemicals naringenin, diosmin, quercetin, and indole-3-carbinol on piscine hepatic CYP1A activity was studied (Chapter 2.2). Excessive use of pharmaceuticals and dietary bioactive compounds,

effluents from pulp mills, and STPs or demolition of naturally occurring plants cause these compounds to enter surface waters either in an unchanged form or as metabolites (Dykstra et al., 2015; Jarošová et al., 2015; Rearick et al., 2014). The occurrence of these compounds in the environment can negatively affect non-target organisms such as fish (Jarošová et al., 2015; Lecomte et al., 2017). Previously, the phytochemicals glucosinolates, flavonoids, and isoflavones have been reported as AhR activators (Ciolino et al., 1999; Ciolino et al., 1998; Mohammadi-Bardbori et al., 2012; Nguyen and Bradfield, 2008). *In vitro* effects of clotrimazole and dexamethasone (Burkina et al., 2013; Celander et al., 1997; Dasmahapatra and Lee, 1993; Levine et al., 1997), and the effects of certain phytochemicals on fish CYPs have been previously studied (Arinc et al., 2015; Burkina et al., 2016b). However, such studies cannot provide information on the combined effects of phytochemicals and pharmaceuticals. The combined effects are generally classified as antagonistic (weaker), synergistic (stronger), or additive. Therefore, the effects on fish will depend on combinations of the compounds and their concentrations. In our study, dexamethasone had no effect on the EROD activity alone, but clotrimazole inhibited fish hepatic EROD activity, which agrees with a previous study (Burkina et al., 2013). The phytochemicals, naringenin and diosmin, inhibited EROD activity, while quercetin and indole-3-carbinol did not. An important finding from this experiment was the lack of either quercetin or indole-3-carbinol effects on EROD activity; however, when either one of these agents was combined with clotrimazole or dexamethasone, EROD activity was strongly inhibited. Thus, a single compound might exert different effects when combined with other compounds, and the effects of mixed compounds cannot be predicted from individual compounds' effects. Therefore, our results provide useful information for different interactions of selected pharmaceuticals and phytochemicals on fish hepatic microsomes.

***In vivo* experiments**

In vivo experiments were performed with dexamethasone at environmentally relevant concentrations of 0.003 and 0.03 μg L^{-1} and at higher concentrations of 0.3 and 3 μg L^{-1} (Burkina et al., 2015); clotrimazole was tested at an environmentally relevant concentration of 0.01 μg L^{-1}, and at higher concentrations of 1 and 10 μg L^{-1} (Burkina et al., 2016a) at 21 and 42 days. Dexamethasone and clotrimazole belong to the glucocorticoid and imidazole groups, respectively. They are found in the aquatic environments up to μg L^{-1} (Liu et al., 2011; Liu et al., 2012; Loos et al., 2013), and they are potential pollutants that can affect non-target organisms such as fish. Dexamethasone is included both in the list of pharmaceuticals which pose environmental risks (Roos et al., 2012) and in the list of pharmaceuticals with potential critical concentration for the environment (Fick et al., 2010) while, clotrimazole increases concerns of scientists due to its toxicity for aquatic organisms (Huang et al., 2010; Peschka et al., 2007).

The total of CYP (CYP1A, CYP2E1- and CYP3A-like) activities were measured using hepatic dexamethasone-exposed microsomes from rainbow trout (Chapter 3.1). The total CYP content was significantly inhibited at 0.003, 0.03 and 0.3 μg L^{-1} after 21 days of exposure. No effects were observed at 42 days after exposure, which might suggest that fish had adapted to the conditions. EROD, PNPH, and BFCOD activities were not affected by any of the dexamethasone concentrations; however, there was a significant biphasic inhibition of CYP activities at 21 days after exposure in which results were normalised to total CYP content. Several studies have reported dexamethasone effects on fish CYP activities (Li et al., 2008; McQuillan et al., 2011; Smith and Wilson, 2010; Wassmur et al., 2010). Results show that there is a species-specific response to dexamethasone with respect to CYP activity. For example, in rainbow trout treated with 2 mg kg^{-1} body weight of dexamethasone, several CYP substrates were altered

(Haasch et al., 1994). In another study, rainbow trout injected with 100 mg kg^{-1} body weight of dexamethasone did not present altered EROD activity, while in killifish, EROD alterations were observed (Smith and Wilson, 2010).

The total CYP and CYP1A and CYP3A-like activities were measured in rainbow trout hepatic microsomes that were exposed to clotrimazole (Chapter 3.2). Total CYP activity was not affected by any of the clotrimazole concentrations, while at 0.01 and 10 µg L^{-1} both EROD and BFCOD activity was affected. EROD activity was induced after longer exposure periods, while BFCOD activity was inhibited at 0.01 µg L^{-1} concentration rather than induced at the highest test concentration. Previous studies reported that clotrimazole has inhibitory capabilities on rainbow trout EROD activity *in vitro* (Burkina et al., 2013; Hegelund et al., 2004; Sakalli et al., 2018). In contrast, clotrimazole produced EROD activity induction when fish were exposed *in vivo*; moreover, concentration-dependant inhibition was not observed either with respect to EROD or BFCOD activities when fish underwent *in vivo* exposure. This finding might be due to additional factors that may contribute to metabolism such as first-pass metabolism or intracellular signalling responses.

The results suggest that clotrimazole could negatively affect fish CYPs at environmentally relevant concentrations. However, in the light of previous *in vitro* studies, the effects of clotrimazole and dexamethasone on fish CYPs system cannot be truly predicted from single exposures in *in vivo* studies because unknown chemicals could potentially be present in the environmental media, which could enhance any effects from these two compounds. Therefore, further investigations were done to identify the effects of mixture compounds using an *in situ* model.

***In situ* experiment**

Fish were exposed to a cocktail of environmentally found xenobiotics (Celander, 2011), and it was determined that the effects of xenobiotic mixtures might lead to unpredicted health effects (Li et al., 2012; Sakalli et al., 2018). Information on the ecotoxicological effects of cocktail compounds in aquatic organisms and wildlife is limited (Carlsson et al., 2006; Fent et al., 2006). Therefore, in this study, common carp (*Cyprinus carpio*) were held in a pond that receives water from STP effluent discharge water for one year (Chapter 4.1). Five sampling events (0, 30, 90, 180, and 360 days) were conducted during the experimental period, which allowed us to observe seasonal changes. The control pond received water from a clean river and had similar seasonal conditions as the exposed pond during the experiment. The details of the chemical analysis are presented elsewhere (Giang et al., 2017; Koba et al., 2018). Briefly, PPCP concentrations were the highest at day 30 (spring time), then slightly lower at day 90 (summer time), and the lowest at 180 days (autumn time). EROD and BFCOD activities were measured in hepatic and intestinal microsomes, while *CYP1A* and *CYP3A* expressions were measured in hepatic tissues.

The results demonstrated that hepatic EROD activity was induced only at 180 days during the sampling period, while in the intestine, significant induction was observed at days 30 and 180. Hepatic BFCOD activity was significantly lower at 30 days after exposure. Intestinal BFCOD activity was induced at 90 and 180 days of exposure. *CYP1A* expression was significantly induced at 30 and 90 days after exposure compared to that in control fish. *CYP3A* expression was not changed during the experiment. No effects were observed at experimental day 360, which might suggest that adaptive responses of the fish CYP system occurred after 180 days. Several studies have been conducted in which the effects of mixture of drugs on fish CYPs were observed (Aguirre-Martínez et al., 2017; Beijer et al., 2013; Blunt et al., 2017; Burkina et al., 2018; Creusot et al., 2015; Hamilton et al., 2017). Similarly, in our

data, the pharmaceuticals mixture caused an increase in *CYP1A* expression and EROD activity in zebrafish (*Danio rerio*) (Aguirre-Martínez et al., 2017). When three-spined sticklebacks (*Gasterosteus aculeatus*) were exposed to drug manufacturing effluents, EROD activity and *CYP1A* expression were also induced (Beijer et al., 2013). CYP3A-like activity was reported in wild gudgeons (*Gobio gobio*) exposed to pharmaceutical manufactory discharge water, but in contrast to our findings, hepatic CYP3A-like activity did not change (Sanchez et al., 2011). Similar to our findings, *CYP3A* expression was not affected in goldfish *(Carassius auratus)* when exposed to reused water from STPs (Blunt et al., 2017). These results were surprising since this enzyme is associated with the metabolism of pharmaceuticals, and significant changes in *CYP3A* expression from the exposed pond were expected.

Another finding from this study was that intestinal EROD and BFCOD activities were affected in exposed fish. Even so, intestinal CYP3A activity in common carp was more sensitive than its hepatic activity. This supports suggestions that fish could be exposed to the contaminants via both their water and the food web (Grabicova et al., 2015; Grabicova et al., 2017). Clearly, fish CYP activity is affected by pharmaceuticals. However, exposure pathways might add additional information on drug metabolism, and this cannot be determined from *in vivo* and *in vitro* studies performed under laboratory conditions.

Conclusions and future investigations

In vitro effects of xenobiotics that are studied in this thesis provide valuable information with respect to the mode of action of dexamethasone and clotrimazole on piscine CYPs. However, drug-drug or food-drug interactions are observed when two compounds are combined in *in vitro* experiments. Therefore, *in vitro* studies alone are not enough to use as monitoring tools for environmental contaminants. In addition, *in vivo* activity of the piscine CYP system yielded different observations than the *in vitro* system, suggesting additional factors may have contributed to piscine metabolism such as first-pass or extrahepatic metabolism. Moreover, several environmental stress factors are often eliminated in *in vivo* studies, which are performed under laboratory conditions, and these factors may additionally affect piscine CYPs. *In situ* studies represent the most realistic scenarios for the effects of environmental contaminants on fish CYP metabolism as a non-target species. The lifestyle, marketing, and consumption in this modern era has caused an increase in new drug or personal care product production, which might be more potent and more persistent in the human body and therefore in the environment. Although comprehensive research has been done on the effects of environmental pollutants, much research is still needed. Future aspects of the drug metabolism in fish should include phase II metabolism in addition to identification of hepatic and intestinal metabolite capacity *in vitro*, which could be evaluated using fish subcellular fractions.

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Sakalli, S., Burkina V., Zlabek V., Zamaratskaia G., 2015. Effects of acetone, acetonitrile, ethanol, methanol and DMSO on cytochrome P450 in rainbow trout *(Oncorhynchus mykiss)* hepatic microsomes. Toxicology Mechanisms and Methods 25, 501–506.

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ENGLISH SUMMARY

Bioactive compounds in the aquatic environment and their effects on fish – a special focus on piscine cytochrome P450

Sidika Sakalli

The term "biologically active compounds" covers a wide range of substances originating from natural or synthetic origins. These compounds can enter the aquatic environment through wastewater treatment plants, manufactural discharge or they are naturally present in the aquatic plants or microorganisms. Their adverse effects on fish has been widely studied and accepted. This thesis is focussed on the adverse effect of several bioactive compounds (i.e. pharmaceuticals, phytochemicals, or complex mixture of pollutants) on fish using different experimental design as *in vitro, in vivo* and *in situ*.

In the first part of this thesis, *in vitro* effects of pharmaceuticals and phytochemicals or their combinations on fish CYP system were investigated. Moreover, effects of standard carrier solvents used in enzyme activity assays were also investigated. An important finding in this study was the lack of effects of either dexamethasone, quercetin, or indole-3-carbinol on EROD activity; however, when these agents were combined, EROD activity was strongly inhibited. This demonstrates that combination of compounds might exert different effects than single compounds, and the effects of mixture compounds cannot be predicted from the effects of individual compounds.

In the second part of the thesis, the effects of chronic exposures of rainbow trout to dexamethasone and clotrimazole were investigated. The results regarding dexamethasone showed similarities with *in vitro* studies, and both *in vitro* and *in vivo* exposure of dexamethasone did not alter the CYP enzyme activities. On the other hand, *in vivo* exposure of clotrimazole yielded conflicting results with findings from the *in vitro* studies. Clotrimazole induced EROD activity in chronically exposed rainbow trout, and BFCOD activity showed biphasic pattern in which it was inhibited at environmentally relevant concentrations and induced at high concentrations. Thus, the observed effects suggest that clotrimazole could negatively affect fish CYPs at environmentally relevant concentrations. However, in the environment, the effects of clotrimazole and dexamethasone on fish CYPs system might be modified because of unknown compounds in these mixtures. Therefore, further investigations were done to identify the effects of mixture compounds using an *in situ* model.

The last part of the dissertation addresses the effects of cocktail PPCPs on common carp under natural conditions. *In situ* studies provide valuable information on both hepatic and intestinal CYP activities. Both EROD and BFCOD activities were affected by the PPCPs that are present in the exposed fish. Moreover, changes in intestinal CYP activities suggest that fish can ingest some of these contaminants through their feed. Therefore, the intestines might be responsible for elimination of some of these pollutants and act as the first barrier of pollutant entry in fish. Despite the extensive studies concerning aquatic pollution, further studies are necessary. Development of new pharmaceuticals, their occurrence in the aquatic environment, and their effects on non-target organisms should be continuously monitored.

CZECH SUMMARY

Bioaktivní látky ve vodním prostředí a jejich vliv na ryby – zaměření na rybí cytochromy P450

Sidika Sakalli

Pojem biologicky aktivní sloučeniny zahrnuje širokou škálu látek přírodního nebo syntetického původu. Tyto sloučeniny mohou vstupovat do vodního prostředí prostřednictvím čistíren odpadních vod, průmyslovými výrobními procesy nebo se přirozeně vyskytují ve vodních rostlinách nebo mikroorganizmech. Jejich nepříznivé účinky na ryby jsou intenzivně studovány a dokládány. Tato práce je zaměřena na studium nepříznivých účinků několika bioaktivních látek (tj. léčiv, fytochemikálií nebo komplexních směsí znečišťujících látek) na ryby s odlišným experimentálním provedením zahrnujícím *in vitro, in vivo* a *in situ* experimenty.

V první části práce byly *in vitro* zkoumány účinky léčiv a fytochemikálií nebo jejich kombinací na systém CYP u ryb. Dále byl zjišťován vliv organických rozpouštědel použitých v metodice esejí enzymové aktivity. Důležitým zjištěním v tomto experimentu bylo, že dexamethason, kvercetin a indol-3-karbinol samotně neovlivňují aktivitu EROD, nicméně v jejich kombinaci byla aktivita EROD silně inhibována. Tento výsledek ukazuje, že kombinace sloučenin může mít odlišné účinky oproti působení jednotlivých látek a účinky směsí nemohou být předpovězeny pouze na základě studia působení jednotlivých sloučenin.

Ve druhé části práce byly zkoumány účinky chronické expozice pstruha duhového dexamethasonu a clotrimazolu. Výsledky týkající se dexamethasonu vykazovaly podobnosti s *in vitro* studiemi, a to jak *in vitro*, tak i *in vivo* expozice dexamethasonu neměla vliv na aktivity enzymů CYPs. Na druhé straně výsledky *in vivo* expozice clotrimazolu vykazovaly v porovnání s *in vitro* experimentem nejednoznačné výsledky. Clotrimazol indukoval aktivitu EROD u chronicky exponovaného pstruha duhového a aktivita BFCOD vykazovala dvojfázový průběh, inhibici v environmentálně relevantních koncentracích, a naopak indukci vyvolanou vysokými koncentracemi. Pozorované účinky naznačují, že clotrimazol může negativně ovlivnit CYP ryb již při environmentálně relevantních koncentracích. Nicméně v životním prostředí mohou být účinky clotrimazolu a dexamethasonu na systém CYP u ryb modifikovány při působení neznámých směsí. Z tohoto důvodu byla provedena navazující studie zaměřená na identifikaci účinků směsí sloučenin za použití *in situ* experimetu.

Poslední část disertační práce se zabývá dopady směsí PPCP na kapra obecného v přirozených podmínkách. *In situ* experiment poskytuje cenné informace o aktivitě jaterních a střevních cytochrómů u exponovaných ryb. Obě sledované enzymové aktivity EROD a BFCOD byly ovlivněny PPCP identifikovanými u exponovaných ryb. Změny v aktivitách střevních CYP naznačují, že ryby mohou některé z těchto kontaminantů přijmout prostřednictvím potravy. Střeva plnící funkci první bariéry vstupu znečišťujících látek do organizmu mohou být odpovědná za eliminaci některých z těchto znečišťujících látek. I přes rozsáhlé studie o znečištění vodních zdrojů vyvstává potřeba provádět specifické experimenty. Neustálý vývoj léčiv, jejich nekontrolovaný výskyt ve vodním prostředí a nepříznivé působení na necílové organizmy přináší potřebu průběžného sledování.

ACKNOWLEDGMENTS

Firstly, I would like to express my sincere appreciation to my supervisor Assoc. Prof. Vladimir Žlábek for his wholehearted support, patience, inspiration, and profound knowledge throughout my Ph.D study. His guidance helped me all the time of my research and study. I could not have imagined having a better supervisor for my Ph.D study. I am also grateful to him for introducing me to my Swedish co-supervisor Assoc. Prof. Galia Zamaratskaia. It is a great privilege to know and to have worked with her. I would like to thank her for her cooperation, research ideas, feedback, and support. I would also like to thank my other co-supervisor and a friend Dr. Viktoriia Burkina for guiding and teaching me, for the inspiring discussions we had, for the long nights we worked together in the lab, and for all the fun we have had in the last five years.

I would like to thank my fellow doctoral student Pham Thai Giang for his endless help, cooperation, and friendship. In addition, I would like to express my gratitude to the staff of our Laboratory of Environmental Chemistry and Biochemistry for the last-minute favors.

My sincere thanks also go to Prof. Dr. Henner Hollert, Dr. Honxia Xiao and Dr. Jochen Kuckelkorn, who provided me with the opportunity to join their team as an intern, access to the laboratory and research facilities. With their precious support, I've gained valuable knowledge and new skills that helped me throughout my research.

I would also like to thank the Deans of Faculty of Fisheries and Protection of Waters Prof. Otomar Linhart (former) and Prof. Pavel Kozák (current) for giving me the opportunity to study at the faculty.

I would like to thank my family: my mother Mevhibe and my father Yusuf for their financial and emotional support during my studies. And, many thanks to my fiancé and his family. They made my stay in Czech Republic extra special!

And finally, last but by no means least, I would like to thank my uncle Hüseyin and especially my aunt Alev for her guidance, support, English editing and for always being there for me.

The study was financially supported by:

- The Ministry of Education, Youth and Sports of the Republic – projects ''CENAKVA'' (No. CZ.1.05/2.1.00/01.0024) and ''CENAKVA II'' (No. LO1205 under the NPU I program)
- The Grant Agency of the University of South Bohemia in Ceske Budejovice (No. 087/2013/Z, 012/2016/Z, 090/2016/Z)
- The Czech Science Foundation (No. GACR P503/11/1130, 15-04258S).

LIST OF PUBLICATIONS

Peer-reviewed journals with IF

Burkina, V., **Sakalli, S.,** Pilipenko, N., Zlabek, V., Zamaratskaia, G., 2018. Effect of human pharmaceuticals found in aquatic environment on hepatic CYP1A and CYP3A-like activity in rainbow trout (*Oncorhynchus mykiss*): An *in vitro* study. Chemosphere 205, 380–386. (IF 2017 = 4.427)

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Giang, P.T., **Sakalli, S**., Fedorova, G., Khalili, T.S., Bakal, T., Najmanova, L., Grabicova, K., Kolarova, J., Sampels, S., Zamaratskaia, G., Grabic, R., Randak, T., Zlabek, V., Burkina, V., 2018. Biomarker response, health indicators, and intestinal microbiome composition in wild brown trout (*Salmo trutta* m. *fario* L.) exposed to a sewage treatment plant effluent-dominated stream. Science of the Total Environment 625, 1494–1509. (IF 2017 = 4.61)

Sakalli, S., Burkina, V., Pilipenko, N., Zlabek, V., Zamaratskaia, G., 2018. *In vitro* effects of diosmin, naringenin, quercetin and indole-3-carbinol on fish hepatic CYP1A1 in the presence of clotrimazole and dexamethasone. Chemosphere 192, 105–112. (IF 2017 = 4.427)

Sakalli, S., Giang, P.T., Burkina, V., Zamaratskaia, G., Rasmussen, M.K., Khalili, T.S., Bakal, T., Sampels, S., Kolarova, J., Grabic, R., Turek, J., Randak, T., Zlabek, V., 2018. The effects of sewage treatment plant effluents on hepatic and intestinal biomarkers in common carp (*Cyprinus carpio*). *Science of the Total Environment* 635, 1160–1169. (IF 2017 = 4.61)

Giang, P.T., Burkina, V., **Sakalli, S**., Schmidt-Posthaus, H., Rasmussen, M.K., Randak, T., Grabic, R., Grabicova, K., Fedorova, G., Koba, O., Golovko, O., Turek, J., Cerveny, D., Kolarova, J., Zlabek, V., 2017. Effects of multi-component mixtures from sewage treatment plant effluent on common carp (*Cyprinus carpio*) under fully realistic condition. Environmental Management. (IF 2017 = 2.177)

Burkina, V., Zamaratskaia, G., Oliveira, R., Fedorova, G., Grabicova, K., Schmidt-Posthaus, H., Steinbach, C., Domingues, I., Golovko, O., **Sakalli, S**., Grabic, R., Randak, T., Zlabek, V., 2016. Sub-lethal effects and bioconcentration of the human pharmaceutical clotrimazole in rainbow trout (*Oncorhynchus mykiss*). *Chemosphere* 159, 10–22.

Zlabek, V., Burkina, V., Borrisser-Pairó, F., **Sakalli, S**., Zamaratskaia, G., 2016. Phase I metabolism of 3-methylindole, an environmental pollutant, by hepatic microsomes from carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*). Chemosphere 150, 304–310. (IF 2015 = 3.698)

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Dezfuli, B.S., Giari, L., Squerzanti, S., Lui, A., Lorenzoni, M., **Sakalli, S.**, Shinn, A.P., 2011. Histological damage and inflammatory response elicited by *Monobothrium wageneri* (Cestoda) in the intestine of *Tinca tinca* (Cyprinidae). Parasites and Vectors 4, 225. (IF 2010 = 2.130)

Manuscripts

Khalili, T.S., Turek, J., Červený, D., Lepič, P., Kozák, P., Burkina, V., **Sakalli, S**., Noguchi, S., Sampels, S. 2017. Insect meal as a partial replacement for fish meal in a formulated diet for perch (*Perca fluviatilis*). Submitted to Journal of Applied Ichthyology (IF 2017 = 0.774).

Sakalli, S., Burkina, V., Steinbach, C., Grabicova, K., Grabic, R., Kolarova, J., Randak, T., Vladimir, Z., 2018. Effects of chronically exposed to environmentally relevant concentration of dexamethasone, a synthetic glucocorticoid, on rainbow trout.

Abstracts and conference proceedings

Sakalli, S., Giang, P.T., Burkina, V., Randak, T., Golovko, O., Fedorova, G., Zamaratskaia G., Zlabek, V., 2017. The effects of multi contaminants on cytochrome P450 in common carp *(Cyprinus carpio*), exposed to sewage treatment plant effluents. XVIII. Toxikologické conference 2017, Vodňany, 23–25 August 2017

Pham, G.T., Burkina, V., **Sakalli, S**., Randak, T., Grabic, R., Fedorova, G., Grabicova, K., Koba O., Golovko O., Turek J., Cerveny D., Kolarova J., Rasmussen K.M., Zlabek V., 2017. Effects of multi-component mixtures from sewage treatment plant effluent on common carp under fully realistic condition – a real case study. The 3rd International Conference on Environmental Pollution, Restoration, and Management. Quy Nhon, Vietnam, 6–10 March 2017.

Sakalli, S., Burkina, V., Pilipenko, N., Zlabek, V., Zamaratskaia, G., 2016. Effects of quercetin and indole-3-carbinol on CYP1A in presence of a therapeutic drug, clotrimazole on fish hepatic microsomal fractions. 16th International Nutrition and Diagnostics Conference. Prague, 3–6 October 2016.

Burkina, V., Žlábek, V., Borrisser-Pairó, F., **Sakalli, S.,** Zamaratskaia, G., 2016. Metabolite formation from 3-methylindole by hepatic microsomes from carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) during phase I metabolism. SETAC Europe 26th Annual Meeting, Nantes, France, 22–26 May 2016.

Burkina, V., **Sakalli, S.,** Koba, O., Zamaratskaia, G., Pham G.T., Grabic, R., Randák, T., Žlábek, V, 2015. The effect of dexamethasone on the hepatic CYP450 system of rainbow trout (*Oncorhynchus mykiss*). SETAC Europe 25th Annual Meeting, Barcelona, Catalonia, Spain, 3–7 May 2015.

Sakalli, S., Burkina, V., Grabicová, K., Kolářová, J., Borik, A., Koba, O., Rasmussen, K.M., Zamaratskaia, G., Grabic, R., Randák, T., Žlábek, V., 2015. Effects of dexamathasone chronical exposure on rainbow trout. Toxikologické konference. Toxicita a biodegradabilita odpadů a látek významných ve vodním prostředí. Vodňany, Czech Republic, 26–28 August 2015.

Sakalli, S., Burkina, V., Žlábek, V., Zamaratskaia, G., 2015. Effects of organic solvents on cytochrome P450 in fish hepatic microsomes. SETAC Europe 25th Annual Meeting, Barcelona, Catalonia, Spain, 3–7 May 2015.

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Sakalli, S., Burkina, V., Grabicová, K., Kolářová, J., Borik, A., Koba, O., Rasmussen, K.M., Zamaratskaia, G., Grabic, R., Randák, T., Žlábek, V. Effects of dexamethasone chronical exposure on rainbow trout. Toxikologické konference. Toxicita a biodegradabilita odpadů a látek významných ve vodním prostředí. 26–28 August, Vodňany, Czech Republic (Oral presentation).	2015
Sakalli, S., Burkina, V., Žlábek, V., Zamaratskaia, G. Effects of organic solvents on cytochrome P450 in fish hepatic microsomes. SETAC Europe 25th Annual Meeting. 3–7 May, Barcelona, Spain (Poster presentation).	2015
Sakalli, S., Burkina, V., Pilipenko, N., Zlabek, V., Zamaratskaia, G. Effects of quercetin and indole-3-carbinol on CYP1A in presence of a therapeutic drug, clotrimazole on fish hepatic microsomal fractions. 16th International Nutrition and Diagnostics Conference. 3–6 October, Prague (Poster presentation).	2016
Sakalli, S., Giang, P.T., Burkina, V., Randak, T., Golovko, O., Fedorova, G., Zamaratskaia G., Zlabek, V. The effects of multi contaminants on cytochrome P450 in common carp (*Cyprinus carpio*), exposed to sewage treatment plant effluents. XVIII. Toxikologické conference, 23–25 August Vodňany (Oral presentation)	2017

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RESEARCH INTERESTS

Occurrence, accumulation, and biotransformation of environmental pollutants mostly pharmaceuticals and personal care products. Identification of pollutants effects *in vitro, in vivo* and *in situ* on aquatic organisms, their interactions with hepatic and intestinal metabolism in fish using Cytochrome P450 (CYP) biomarkers, moreover, identify the combination or cocktail mixtures of the pollutants effects on fish health.

LANGUAGE SKILLS

English (B2 level, FCE), Turkish (native), Czech (A2 Level)