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

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

ISBN 978-80-87437-97-1

Effects of chemicals present in sewage treatment plants´ effluents on fish

Kateřina Grabicová

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

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

Effects of chemicals present in sewage treatment plants´ effluents on fish

Vliv chemikálií přítomných ve výtocích z čistíren odpadních vod na ryby

Kateřina Grabicová

Czech Republic, Vodňany, 2014

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

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

Effects of chemicals present in sewage treatment plants´ effluents on fish

Vliv chemikálií přítomných ve výtocích z čistíren odpadních vod na ryby

Kateřina Grabicová

Czech Republic, Vodňany, 2014

I, Kateřina Grabicová, thereby declare that I wrote the Ph.D. thesis myself using results of my own work or collaborative work of me and colleagues and with help of other publication resources which are properly cited.

I hereby declare that, in accordance with the § 47b Act No. 111/1998 Coll., as amended, I agree with publicizing of my Ph.D thesis in full version electronically in a publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice on its web sites, with keeping my copyright to the submitted text of this Ph.D. thesis. I also agree so that the same electronic way, in accordance with above mentioned provision of the Act No. 111/1998 Coll., was used for publicizing reviews of supervisor and reviewers of the thesis as well as record about the progress and result of the thesis defence. I also agree with compering the text of my Ph.D. thesis with a database of theses "Theses.cz" operated by National Register of university theses and system for detecting of plagiarisms.

In Vodňany 2nd, May, 2014

Supervisor
Assoc. Prof. Dipl.-Ing. Tomáš Randák, Ph.D.
University of South Bohemia in České Budějovice (USB)
Faculty of Fisheries and Protection of Waters (FFPW)
Research Institute of Fish Culture and Hydrobiology (RIFCH)
South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses (CENAKVA)
Zátiší 728/II
389 25 Vodňany
Czech Republic

Consultant
Dipl.-Ing. Hana Kocour Kroupová, Ph.D.
University of South Bohemia in České Budějovice (USB)
Faculty of Fisheries and Protection of Waters (FFPW)
Research Institute of Fish Culture and Hydrobiology (RIFCH)
South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses (CENAKVA)
Zátiší 728/II
389 25 Vodňany
Czech Republic

Head of Laboratory of Environmental Chemistry and Biochemistry, USB, FFPW, RIFCH
Assoc. Prof. Dipl.-Ing. Tomáš Randák, Ph.D.

Dean of Faculty of Fisheries and Protection of Waters
Prof. Dipl.- Ing. Otomar Linhart, D.Sc.

Board of doctorate study defence with referees
Prof. Dipl.-Ing. Petr Ráb, D.Sc. – head of the board
Assoc. Prof. M.Sc. Milan Gelnar, CSc. – board member
Assoc. Prof. M.Sc. Zdeněk Adámek, CSc. – board member
Assoc. Prof. M.Sc. Jana Pěknicová, CSc. – board member
Assoc. Prof. M.Sc. Josef Matěna, CSc. – board member
Prof. Stanislav Navrátil, DMV, CSc. – board member

Assoc. Prof. M.Sc. Josef Cvačka, Ph.D. – referee
Assoc. Prof. Ali Moazzami, Ph.D. – international referee

Date, hour and place of Ph.D. defence
17th September 2014 at 13:30 in USB, FFPW, RICHF, Vodňany

Name: Kateřina Grabicová
Title of thesis: Effects of chemicals present in sewage treatment plants´ effluents on fish

Ph.D. thesis, USB FFPW, RIFCH, Vodňany, 2014, 85 pages, with the summary in English and Czech.
Graphic design & technical realisation: JENA Šumperk, www.jenasumperk.cz
ISBN 978-80-87437-99-5

CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. ANTHROPOGENIC POLLUTANTS AND THEIR FATE IN THE ENVIRONMENT

Municipal waste waters contain many compounds that are used or produced by humans, e.g. pharmaceuticals and personal care products (PPCPs), nutritive components (sweeteners), persistent organic pollutants (POPs), heavy metals, which are used in large quantities around the world.

Most of sewage treatment plants (STPs) are not designed to treat these types of substance and many of these compounds and their metabolites can escape the elimination in STPs and enter the aquatic environment via sewage effluents (Pal et al., 2010; Petrovic et al., 2003).

The thesis is focused especially on the evaluation of presence and potential toxicological effects of PPCPs in an aquatic environment. PPCPs are a very broad group of compounds consisting of every human and veterinary medicine and consumer products such as housecleaning products, fragrances, sunscreens and others (Daughton and Ternes, 1999).

Pharmaceuticals are used for diagnosis, cure, mitigation, treatment, prevention of diseases, or health conditions in humans and animals (Daughton and Ternes, 1999). According to their therapeutic purpose, they are classified to groups, for example antibiotic, analgesic, antidepressant, antiepileptic etc. (Fent et al., 2006). Many of the pharmaceuticals are used to treat civilization diseases: for example maintaining cholesterol balance, combating mental illness, treating stress, ulcers, asthma, etc., and they include specific beta blockers, lipid regulators, antidiabetics, antianginal drugs, as well as analgesics and antibiotics (Jones et al., 2002). Pharmaceuticals are designed to attack a specific target and in the environment, they can affect the same pathways in animals (Fent et al., 2006). Because of lipophilic and persistent chemical properties of some of these compounds, they can accumulate or induce effects in aquatic terrestrial system (Halling-Sorensen et al., 1998).

Mompelat et al. (2009) made a list of human and veterinary pharmaceuticals and few by-products (include both, metabolites excreted via urine or feces, and transformation products which can be formed in the environment from pharmaceuticals) of which the main concern is about non-steroidal anti-inflammatory drugs, anticonvulsants and lipid regulators. The concern is focused on antibiotics too because they can increase the occurrence of resistant bacteria in the environment (Daughton and Ternes, 1999).

Another group that has lately had received much attention due to risks to aquatic wildlife is antidepressants (Brodin et al., 2013; Lajeunesse et al., 2011). This group includes monoamine reuptake inhibitors, e.g. selective serotonin reuptake inhibitors (SSRIs), such as citalopram, fluoxetine and sertraline, and serotonin-norepinephrine reuptake inhibitors (SNRIs), such as venlafaxine. These pharmaceuticals metabolize to metabolites with retaining SSRI / SNRI activity (Aryal et al., 2012; DeVane, 1999; Hiemke and Hartter, 2000). The mechanism of action includes blocking monoamine reuptake in the presynaptic cells, which leads to an increase in the concentration of serotonin and/or norepinephrine within the synapses (Fent et al., 2006).

The degradation of pharmaceutically active compounds by metabolism is a two-step process (Daughton and Ternes, 1999; Halling-Sorensen et al., 1998). Phase I is usually linked with oxidation, reduction or hydrolysis. The products are often more reactive and sometimes more toxic than the parent drug. Phase II reactions involve conjugation (with glucuronic acid or sulfate) to increase their excretion. Both phase I and phase II reactions change the physicochemical properties of the substance because metabolites are usually more soluble in water than parent compounds before metabolism. However, some of them can be still active and leave the body in this active form (Heberer, 2002; Roberts and Thomas, 2006).

Another part of PPCPs are personal care products (PCPs) which include sun screen agents (UV filters), antiseptics (e.g. triclosan), fragrances (e.g. synthetic "musk" compounds) and preservatives in cosmetics. PCPs are not used for treatment of diseases (Daughton and Ternes, 1999). Contrary to pharmaceuticals, PCPs are not metabolized and leave households unchanged (e.g. Balmer et al., 2005).

UV filters are often used to protect skin from chronic (skin cancer) or acute (sunburn, photo-ageing) exposure to ultraviolet radiation from sunlight (Gasparro, 2000). Two general types of sunscreen have been developed: organic and inorganic (Gasparro et al., 1998). Individual organic UV filters have a relatively narrow absorption spectrum. Therefore, they are usually combined to obtain protection against the entire UV-radiation spectrum. A list of UV filters that are allowed in cosmetic products is given in EU Regulation No. 1223/2009, Annex VI (Regulation, 2009). UV filters can be added in concentrations up to 10% to sunscreen products (Schlumpf et al., 2001).

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenylether) is an antibacterial reagent used in households, e.g. in toothpaste, detergents, household sponges, plastic cutting boards, socks and underwear. Triclosan is active against both gram-positive and gram-negative bacteria.

Musk is one of the most important fragrances which are used in perfumes, cosmetics and laundry agents. Natural musk is very expensive so today almost all of the musks are produced synthetically. The polycyclic musk fragrances represent 70% of all musk fragrances (Dietrich, 1999). The most often used and studied musk fragrances are galaxolide (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta(g)-2-benzopyran) and tonalide (7-acetyl-1,1,3,4,4,6-hexamethyl-tetrahydronaphthalene) (Balk and Ford, 1999a; Balk and Ford, 1999b).

After use, the active pharmaceutical ingredients and/or its metabolites and PCP reach municipal wastewaters and enter municipal sewage treatment plants (STPs) where they are degraded (Halling-Sorensen et al., 1998). Most of STPs are not able to remove these substances during the treatment processes, hence some these compounds end up in the environment and will there have the potential to affect non-target aquatic organisms (Heberer, 2002; Huerta et al., 2012; Hughes et al., 2013; Petrovic et al., 2003; Santos et al., 2010; Ternes et al., 2004; Verlicchi et al., 2012). The second pathway how PPCPs enter the environment is directly when STP is missing or when they are washed off humans by bathing or swimming (Balmer et al., 2005).

Two main primary treatment processes are involved in degradation of PPCPs – microbial degradation and sorption to filterable solids (Daughton and Ternes, 1999). During these processes, these compounds are removed completely or partially (Golovko et al., 2014; Rodil et al., 2012; Verlicchi et al., 2012). According to their ability to be removed during treatment processes, compounds can be divided into four classes: no elimination (0%), poor elimination (< 40%), mild elimination (40–80%) and effectual elimination (> 80%) (Vieno et al., 2007). Some compounds can be effectively removed during treatment in STP from the liquid phase but they are concentrated in the sludge (Clarke and Smith, 2011; Pasquini et al., 2013; Ternes et al., 2004).

There are many studies about removal efficiency of individual pharmaceuticals in STP (e.g. Golovko et al., 2014; Kosma et al., 2014; Pasquini et al., 2013; Verlicchi et al., 2013). Removal efficiency of pharmaceuticals is variable for individual pharmaceuticals from low removal ability to almost 100% degradation during the year (Golovko et al., 2014). Some pharmaceuticals show negative removal efficiency because of higher concentration in effluent than in influent in STP (Golovko et al., 2014; Kosma et al., 2014) probably due to the conversion of their conjugates to the parent compound (Verlicchi et al., 2012; Vieno et al., 2007). Table 1 shows removal efficiency of a few selected pharmaceuticals in STP.

Table 1. *Removal efficiency of pharmaceuticals in STP.*

Pharmaceutical	Removal efficiency [%]		
Analgesics / NSAID			
Codeine	40–75 [a]	38 [b]	
Diclofenac	* – 32 [a]	35 [b]	* – 65 [c]
Ibuprofen	92 [b]	63–97 [c]	90 [d]
Naproxen	79 [b]	25–98 [c]	
Paracetamol	64–100 [c]		
Salicylic acid	76 [b]	75–100 [c]	
Tramadol	* [a]		
Antibiotics			
Azithromycin	* [b]		
Ciprofloxacin	71 [b]		
Erythromycin	66 [b]	75–90 [d]	
Norfloxacin	25 [b]		
Ofloxacin	61 [b]	50–75 [d]	
Roxithromycin	54 [b]		
Sulfamethoxazole	52 [b]	58–99 [c]	
Trimethoprim	31 [b]	23–91 [c]	
Cardio-vascular drug			
Beta-blockers			
Atenolol	15–38 [a]	65 [b]	
Bisoprolol	32–52 [a]		
Metoprolol	10–16 [a]	29 [b]	
Sotalol	* – 20 [a]	40 [b]	
Calcium channel blockers			
Diltiazem	48–79 [a]		
Isradipine	58–75 [a]		
Verapamil	25–55 [a]		
Angiotensin receptor blocker			
Valsartan	70–90 [a]		
Diuretics			
Furosemide	80–91 [a]	35 [b]	
Lipid regulators			
Bezafibrate	60 [b]	26–90 [c]	
Fenofibrate	50 [b]	55–72 [c]	
Psycho-active drugs			
Carbamazepine	36 [b]	* – 20 [c]	
Diazepam	100 [b]		
Fluoxetine	59 [b]		
Paroxetine	67 [b]		

[a] Golovko et al., 2014; [b] Verlicchi et al., 2013; [c] Kosma et al., 2014; [d] Pasquini et al., 2013
* = negative removal efficiency.

The removal efficiency of UV filters as phenylbenzimidazole sulfonic acid, benzophenone 1, benzophenone 3 and benzophenone 4 is seasonal variable with mean removal in summer – 10, 98, 60 and 27 percent, respectively (Golovko et al., 2014). UV filters were found in surface waters up to low μg L^{-1} (Wick et al., 2010) with the highest observed concentration in summer season (up to 2.7 μg L^{-1}) (Balmer et al., 2005; Poiger et al., 2004).

The removal efficiency of triclosan is 65–75% (Kosma et al., 2014) or even higher (80–90%) (Pasquini et al., 2013), but the removal from the liquid phase is partly due to the adsorption to the sludge (Pasquini et al., 2013). Triclosan is bioavailable to fish in the environment (Adolfsson-Erici et al., 2002). Methyl triclosan is a biotransformation product of triclosan formed during treatment processes and it can be used as a chemical marker for lipophilic STP-derived contaminants (Buser et al., 2006).

Synthetic musk compounds were determined in effluents from STPs, sludge and in fish from sewage ponds in ranges of low μg L^{-1} and mg kg^{-1}, respectively (Bester, 2004; Rimkus, 1999). Approximately 35% of tonalide and galaxolide was not changed during the treatment processes in STP (Bester, 2004).

PPCPs are present in ranges of nanograms to low micrograms per liter in surface water and ground waters, sometimes in drinking waters (e.g. Corcoran et al., 2010; Fent et al., 2006; Grabic et al., 2012; Halling-Sorensen et al., 1998; Kolpin et al., 2002; Roberts and Thomas, 2006; Schultzt and Furlong, 2008; Ternes, 1998). The final effect of contamination on aquatic biota is dependent on the concentration of pollutants in the environment. From this point of view, the dilution factor is very important, i.e. dilution of treated water in its recipient (stream, river). It means that organisms living in a small stream with low dilution of treated water are more affected than organisms in bigger recipients with high dilution of coming treated water (Li et al., 2011).

Treated STPs´ effluents contain a mixture of chemicals. It is very difficult to evaluate risks connected with a presence of these chemicals in an aquatic environment. A better way of risk assessment (but too complicated) is the using of the whole mixture for toxicity evaluation (Pomati et al., 2008) because the risk quotient for individual pharmaceuticals is much lower than the risk quotient of the mixture (Backhaus and Karlsson, 2014) and therefore the effects of mixture to exposed organisms could be stronger than the effect of a single compounds (Cleuvers, 2003; Cleuvers, 2004; Corcoran et al., 2010).

1.2. APPROACHES USED FOR RISK ASSESSMENT IN AQUATIC ENVIRONMENT

Three points are responsible for the distribution and fate of pollutants in the environment – physicochemical properties of the compound, environmental conditions and physicochemical properties of abiotic components, and the composition, mass, physiological, anatomical and behavioral characteristics of the species inhabiting exposed ecosystem (Huckins et al., 2006).

Th ere are several models how to study the infl uence of aquatic environment contamination to exposed organism. A laboratory approach is conventional and the most often used. Fish or other indicator organisms are exposed to selected compounds or to simply mixture under controlled conditions. Tested substances are usually dissolved in water fi lled experimental tanks (e.g. Holmberg et al., 2011; Nallani et al., 2011; Paterson and Metcalfe, 2008). It means that bioconcentration is prevailing. Th e intake of pollutants via contaminated natural food is eliminated. Th e experiments with caged fi sh are used for studying the eff ect of real contamination as well. Fish are placed into cages or tanks and exposed directly to effl uent or riverine water (e.g. Lajeunesse et al., 2011; Togunde et al., 2012). Depending on the exposure period, fi sh are usually not fed or artifi cial food is used. Although real water is used, fi sh are stressed and these types of experiments do not truly mimic the natural conditions.

Another approach with wild fish is also used. Wild fish are caught in polluted streams and the bioaccumulation and biomarkers are measured (e.g. Brooks et al., 2005; Brozinski et al., 2013; Hajslova et al., 2007; Li et al., 2011; Sanchez et al., 2011). The limitation of this approach is that the time of exposure is unknown and undefined due to fish migration. Their diet is also unknown.

Aquatic organisms are not exposed only via contaminated water but also via their diet. Benthic fauna is a very important part of the food chain in the aquatic environment (e.g. Greenberg and Dahl, 1998; Hellmann et al., 2013; Hildrew, 1992; Laine, 2001). Benthic organisms are often used as bioindicators for assessment of environmental pollution due to their limited movability and relatively easy sampling (e.g. Kolarikova et al., 2012; Pan et al., 2012; Smith et al., 1999).

The third approach is passive sampling (PS), a relatively new method for the determination of chemicals in the environment. PS is based on the free flow of analyte from the sampled medium to a collecting medium as a result of a difference in chemical potentials (Gorecki and Namiesnik, 2002). Therefore, only water dissolved (biologically available) fraction of pollutants is sampled. Passive samplers are applicable for screening of contaminants in the (aquatic) environment, for assessment of contaminant fate and distribution between environmental compartments, estimation of organism exposure or for measurement of time-weighted average aqueous concentrations (Gorecki and Namiesnik, 2002; Vrana et al., 2005).

There are two most often used samplers for sampling organic compounds in water. The first are semipermeable membrane devices (SPMDs) for non-polar compounds as pesticides, volatile organic compounds or persistent organic pollutants (Gorecki and Namiesnik, 2002). These SPMDs were invented by Huckins et al. (1990). The second type of samplers are polar organic chemical integrative samplers (POCIS) used for the determination of hydrophilic chemicals as pharmaceuticals, pesticides, hormones, illicit drugs and some PCPs (Alvarez et al., 2004; Bartelt-Hunt et al., 2011; Harman et al., 2011; Jalova et al., 2013). Both types are useful for sampling surface water, groundwater and sediments (Huckins et al., 2006).

1.3. EFFECTS OF CHEMICALS PRESENT IN TREATED SEWAGE WATER ON AQUATIC ORGANISMS

Fish are chronically exposed to many contaminants due to continual input into the stream their whole life, so compounds present in water can enter the organisms, accumulate and cause some effects (Corcoran et al., 2010). The continual uptake of pollutants to aquatic organisms is risky because effects could accumulate so slowly without detected changes until the level of these effects when changes are irreversible (Daughton and Ternes, 1999). The different sensitivity to pollution was observed in different fish species (Corcoran et al., 2010).

Some synthetic and natural compounds could mimic natural hormones in the endocrine systems of animals (Snyder et al., 2003). These substances are called endocrine disrupting compounds (EDCs) and are defined as "exogenous agents that interfere with the production, release, transport, metabolism, binding, action, or elimination of the natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" (EPA, 2011). There are three major types of EDCs, estrogenic compounds which mimic or block natural estrogen, androgenic compounds which mimic or block natural testosterone and thyroidal compounds with direct or indirect impacts to the thyroid (Snyder et al., 2003). EDCs have the ability to cause hormone-like effects and are able to disrupt the functions of endocrine system thereby to disturbance of regulation of many vital responses in the organism (Douxfils et al., 2007; Jobling et al., 1998). Vitellogenesis, the process of yolk formation during maturing of oocytes, is controlled hormonally by estrogens (Sumpter and

Jobling, 1995). Steroid or synthetic estrogens released to the environment could activate vitellogenin (female egg yolk precursor protein) production even in males or immature individuals (Tyler et al., 1999) which can lead to degenerative alterations of male gonads, reproductive defects and intersex (Allner et al., 2010; Jobling et al., 2002; Jobling et al., 1998; Lange et al., 2011; Rodgers-Gray et al., 2001; Sumpter and Jobling, 1995; Tyler and Jobling, 2008). The level of changes is dependent on the duration of exposure, timing of exposure, maturity of fish (Nash et al., 2004) as well as on species of fish because some species are more sensitive than other (Douxfils et al., 2007). Vitellogenin detected in plasma is used as biomarker for environmental estrogens (Heppell et al., 1995).

Many alterations in the male reproductive system are due to androgen receptors – exposure to anti-androgens (e.g. bisphenol A, vinclozin, butylbenzylphtalate) cause blocking of androgen action and leads to the same effects as exposure to estrogens (Sohoni and Sumpter, 1998).

Thyroidal alterations as hyperplasia (Schmidt et al., 2012) or changes on thyroidal hormone levels in plasma (Cyr and Eales, 1990; Cyr et al., 1988) were observed in fish exposed to contaminants. These results are worrying because the thyroidal system is vital for growth, development and reproduction (for review see Brown et al., 2004).

Some UV filters also interfere with the sex hormone system and can affect the reproduction of fish (Bluthgen et al., 2012; Gago-Ferrero et al., 2012; Zucchi et al., 2011).

There is not only endocrine disruption of fish which are living in streams with STPs´ effluents. Very low concentrations of antidepressants in the aquatic environment can affect biological processes, nervous and hormonal systems, reproduction and/or the behavior of fish and other aquatic organisms (Brodin et al., 2013; Corcoran et al., 2010; De Lange et al., 2006; Henry et al., 2004; Valenti et al., 2012). SSRIs can also decrease the Na/K-ATPase activity in the brain which has been proposed to be used as a biomarker for SSRIs (Lajeunesse et al., 2011). Histo-pathological changes of livers, kidneys and gills were observed in fish exposed to pollutants (Bernet et al., 2004; Liney et al., 2006; Norris et al., 2000; Schramm et al., 1998).

The effect of xenobiotics on aquatic organisms is possible to study by measurement of some biochemical responses (biochemical markers) or concentrations in different fish tissues and plasma. Biomarkers used in the Czech national monitoring program are vitellogenin and 11-ketotestosterone measured in male fish blood plasma and ethoxyresorufin-O-deethylase (EROD) measured in liver tissue (Randak et al., 2009).

Cytochrome P450 plays an important role in the degradation of xenobiotics (oxygenations in phase I) and in the metabolism of a lot of structurally diverse chemicals, both exogenous and endogenous (Lewis, 2001). Altered cytochrome P450 1A activity (measured as the catalytic activity of EROD) has been used as a marker for the impact of xenobiotics on fish for more than 15 years (Bucheli and Fent, 1995; Burkina et al., 2012; Fent, 2003).

Metabolic transformation of xenobiotics may lead to increased risk of oxidative stress (Lushchak, 2011). Therefore the activity of antioxidant enzymes such as superoxide dismutase, catalase and glutathione reductase studied in aquatic organisms exposed to pollutants could be changed due to exposure to xenobiotics (Brandao et al., 2013; Li et al., 2010; Oliveira et al., 2013). Many compounds present in STPs´ effluents are toxic to organisms and cause oxidative stress (Sturve et al., 2008). The increase or decrease of enzymatic activity after exposure of the pollutant is depended on the concentration, time, type of enzyme and species of fish (Oruc and Usta, 2007).

Moreover, some PPCPs have been detected in SPMDs which indicate that these compounds are potentially bioaccumulated (Huckins et al., 1990; Jalova et al., 2013; Poiger et al., 2004). Uptake and bioaccumulation/bioconcentration of PPCPs such as UV filters (Buser et al., 2006; Fent et al., 2008; Gago-Ferrero et al., 2012; Nagtegaal et al., 1997; Poiger et al., 2004),

non-steroidal anti-inflammatory drug ibuprofen (Nallani et al., 2011), antidepressants (Brooks et al., 2005; Du et al., 2012; Gelsleichter and Szabo, 2013; Ramirez et al., 2009; Ramirez et al., 2007; Schultz et al., 2010), antihistaminic diphenhydramine (Wang and Gardinali, 2013), PCP triclosan and stimulant drug caffeine (Jakimska et al., 2013), musk compounds tonalide and galaxolide (Balk and Ford, 1999a) and synthetic estrogen 17-α-ethinylestradiol (Al-Ansari et al., 2013) was found in aquatic organisms.

1.4. AIMS OF THE THESIS

The main aims of this study were to:

1. Investigate the presence of selected PPCPs in surface water and to study the effects of long-term exposure of UV filter 2-phenylbenzimidazole-5-sulfonic acid and cardio-vascular drug verapamil on rainbow trout (*Oncorhynchus mykiss*).
2. Investigate the bioconcentration of antidepressants in fish exposed to effluent from sewage treatment plants.
3. Assess the bioavailable concentrations of selected analgesics, psycholeptics, antidepressants and illicit drugs in riverine surface waters using passive samplers (POCIS).
4. Evaluate the pharmaceuticals presence in benthos living in "treated" sewage water recipient as potential exposure pathway of fish.

REFERENCES

Adolfsson-Erici, M., Pettersson, M., Parkkonen, J., Sturve, J., 2002. Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden. Chemosphere 46, 1485–1489.

Al-Ansari, A.M., Atkinson, S.K., Doyle, J.R., Trudeau, V.L., Blais, J.M., 2013. Dynamics of uptake and elimination of 17 alpha-ethinylestradiol in male goldfish (*Carassius auratus*). Aquat. Toxicol. 132, 134–140.

Allner, B., von der Gonna, S., Griebeler, E.M., Nikutowski, N., Weltin, A., Stahlschmidt-Allner, P., 2010. Reproductive functions of wild fish as bioindicators of reproductive toxicants in the aquatic environment. Environ. Sci. Pollut. Res. 17, 505–518.

Alvarez, D.A., Petty, J.D., Huckins, J.N., Jones-Lepp, T.L., Getting, D.T., Goddard, J.P., Manahan, S.E., 2004. Development of a passive, *in situ*, integrative sampler for hydrophilic organic contaminants in aquatic environments. Environ. Toxicol. Chem. 23, 1640–1648.

Aryal, B., Aryal, D., Kim, E.J., Kim, H.G., 2012. Pharmacokinetics of venlafaxine and its major metabolite o-desmethylvenlafaxine in freely moving mice using automated dosing/sampling system. Indian J. Pharmacol. 44, 20–25.

Backhaus, T., Karlsson, M., 2014. Screening level mixture risk assessment of pharmaceuticals in STP effluents. Water Res. 49, 157–165.

Balk, F., Ford, R.A., 1999a. Environmental risk assessment for the polycyclic musks AHTN and HHCB in the EU – I. Fate and exposure assessment. Toxicol. Lett. 111, 57–79.

Balk, F., Ford, R.A., 1999b. Environmental risk assessment for the polycyclic musks, AHTN and HHCB – II. Effect assessment and risk characterisation. Toxicol. Lett. 111, 81–94.

Balmer, M.E., Buser, H.R., Muller, M.D., Poiger, T., 2005. Occurrence of some organic UV filters in wastewater, in surface waters, and in fish from Swiss lakes. Environ. Sci. Technol. 39, 953–962.

Bartelt-Hunt, S.L., Snow, D.D., Damon-Powell, T., Brown, D.L., Prasai, G., Schwarz, M., Kolok, A.S., 2011. Quantitative evaluation of laboratory uptake rates for pesticides, pharmaceuticals, and steroid hormones using POCIS. Environ. Toxicol. Chem. 30, 1412–1420.

Bernet, D., Schmidt-Posthaus, H., Wahli, T., Burkhardt-Holm, P., 2004. Evaluation of two monitoring approaches to assess effects of waste water disposal on histological alterations in fish. Hydrobiologia 524, 53–66.

Bester, K., 2004. Retention characteristics and balance assessment for two polycyclic musk fragrances (HHCB and AHTN) in a typical German sewage treatment plant. Chemosphere 57, 863–870.

Bluthgen, N., Zucchi, S., Fent, K., 2012. Effects of the UV filter benzophenone-3 (oxybenzone) at low concentrations in zebrafish (*Danio rerio*). Toxicol. Appl. Pharmacol. 263, 184–194.

Brandao, F.P., Rodrigues, S., Castro, B.B., Goncalves, F., Antunes, S.C., Nunes, B., 2013. Short-term effects of neuroactive pharmaceutical drugs on a fish species: Biochemical and behavioural effects. Aquat. Toxicol. 144, 218–229.

Brodin, T., Fick, J., Jonsson, M., Klaminder, J., 2013. Dilute Concentrations of a Psychiaatric Drug Alter Behavior of Fish from Natural Populations. Science 339, 814–815.

Brooks, B.W., Chambliss, C.K., Stanley, J.K., Ramirez, A., Banks, K.E., Johnson, R.D., Lewis, R.J., 2005. Determination of select antidepressants in fish from an effluent-dominated stream. Environ. Toxicol. Chem. 24, 464–469.

Brown, S.B., Adams, B.A., Cyr, D.G., Eales, J.G., 2004. Contaminant effects on the teleost fish thyroid. Environ. Toxicol. Chem. 23, 1680–1701.

Brozinski, J.M., Lahti, M., Meierjohann, A., Oikari, A., Kronberg, L., 2013. The Anti-Inflammatory Drugs Diclofenac, Naproxen and Ibuprofen are found in the Bile of Wild Fish Caught Downstream of a Wastewater Treatment Plant. Environ. Sci. Technol. 47, 342–348.

Bucheli, T.D., Fent, K., 1995. Induction of Cytochrome-P450 as a Biomarker for Environmental Contamination in Aquatic Ecosystems. Crit. Rev. Environ. Sci. Technol. 25, 201–268.

Burkina, V., Zamaratskaia, G., Randak, T., Li, Z. H., Fedorova, G., Pickova, J., Zlabek, V., 2012. Verapamil does not modify catalytic activity of CYP450 in rainbow trout after long-term exposure. Ecotox. Environ. Safe. 79, 148–152.

Buser, H.R., Balmer, M.E., Schmid, P., Kohler, M., 2006. Occurrence of UV filters 4-methylbenzylidene camphor and octocrylene in fish from various swiss rivers with inputs from wastewater treatment plants. Environ. Sci. Technol. 40, 1427–1431.

Clarke, B.O., Smith, S.R., 2011. Review of 'emerging' organic contaminants in biosolids and assessment of international research priorities for the agricultural use of biosolids. Environ. Int. 37, 226–247.

Cleuvers, M., 2003. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicol. Lett. 142, 185–194.

Cleuvers, M., 2004. Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. Ecotox. Environ. Safe. 59, 309–315.

Corcoran, J., Winter, M.J., Tyler, C.R., 2010. Pharmaceuticals in the aquatic environment: A critical review of the evidence for health effects in fish. Crit. Rev. Toxicol. 40, 287–304.

Cyr, D.G., Eales, J.G., 1990. Influence of short-term estradiol treatment on plasma thyroid hormone kinetics in rainbow trout, *Salmo gairdneri*. J. Fish Biol. 36, 391–400.

Cyr, D.G., Maclatchy, D.L., Eales, J.G., 1988. The influence of short-term 17-beta-estradiol treatment on plasma-t3 levels and *in vitro* hepatic t45'-monodeiodinase activity in immature rainbow trout, *Salmo gairdneri*. Gen. Comp. Endocrinol. 69, 431–438.

Daughton, C.G., Ternes, T.A., 1999. Pharmaceuticals and personal care products in the environment: Agents of subtle change? Environ. Health Perspect. 107, 907–938.

De Lange, H.J., Noordoven, W., Murk, A.J., Lurling, M., Peeters, E.T.H.M., 2006. Behavioural responses of Gammarus pulex (Crustacea, Amphipoda) to low concentrations of pharmaceuticals. Aquat. Toxicol. 78, 209–216.

DeVane, C.L., 1999. Metabolism and pharmacokinetics of selective serotonin reuptake inhibitors. Cell. Mol. Neurobiol. 19, 443–466.

Dietrich, D.R., 1999. The toxicology of musk fragrances. Toxicol. Lett. 111, 1–4.

Douxfils, J., Mandiki, R., Silvestre, F., Bertrand, A., Leroy, D., Thome, J.-P., Kestemont, P., 2007. Do sewage treatment plant discharges substantially impair fish reproduction in polluted rivers? Sci. Total Environ. 372, 497–514.

Du, B., Perez-Hurtado, P., Brooks, B.W., Chambliss, C.K., 2012. Evaluation of an isotope dilution liquid chromatography tandem mass spectrometry method for pharmaceuticals in fish. J. Chromatogr. A. 1253, 177–183.

EPA, 2011. Endocrine Disruptors Research. on-line: http.//www.epa.gov/ncer/science/endocrine/index.html.

Fent, K., 2003. Ecotoxicological problems associated with contaminated sites. Toxicol. Lett. 140, 353–365.

Fent, K., Weston, A.A., Caminada, D., 2006. Ecotoxicology of human pharmaceuticals (vol 76, pg 122, 2006). Aquat. Toxicol. 78, 207–207.

Fent, K., Kunz, P.Y., Gomez, E., 2008. UV filters in the aquatic environment induce hormonal effects and affect fertility and reproduction in fish. Chimia 62, 368–375.

Gago-Ferrero, P., Diaz-Cruz, M., Barcelo, D., 2012. An overview of UV-absorbing compounds (organic UV filters) in aquatic biota. Anal. Bioanal. Chem. 404, 2597–2610.

Gasparro, F.P., 2000. Sunscreens, skin photobiology, and skin cancer: The need for UVA protection and evaluation of efficacy. Environ. Health Perspect. 108, 71–78.

Gasparro, F.P., Mitchnick, M., Nash, J.F., 1998. A review of sunscreen safety and efficacy. Photochem. Photobiol. 68, 243–256.

Gelsleichter, J., Szabo, N.J., 2013. Uptake of human pharmaceuticals in bull sharks (*Carcharhinus leucas*) inhabiting a wastewater-impacted river. Sci. Total Environ. 456, 196–201.

Golovko, O., Kumar, V., Fedorova, G., Randak, T., Grabic, R., 2014. Removal and seasonal variability of selected analgesics/anti-inflammatory, anti-hypertensive/cardiovascular pharmaceuticals and UV filters in wasterwater treatment plant. Environ. Sci. Pollut. Res. 21, 7578–7585.

Gorecki, T., Namiesnik, J., 2002. Passive sampling. Trac-Trends Anal. Chem. 21, 276–291.

Grabic, R., Fick, J., Lindberg, R.H., Fedorova, G., Tysklind, M., 2012. Multi-residue method for trace level determination of pharmaceuticals in environmental samples using liquid chromatography coupled to triple quadrupole mass spectrometry. Talanta 100, 183–195.

Greenberg, L.A., Dahl, J., 1998. Effect of habitat type on growth and diet of brown trout, Salmo trutta L., in stream enclosures. Fisheries Manag. Ecol. 5, 331–348.

Hajslova, J., Pulkrabova, J., Poustka, J., Cajka, T., Randak, T., 2007. Brominated flame retardants and related chlorinated persistent organic pollutants in fish from river Elbe and its main tributary Vltava. Chemosphere. 69, 1195–1203.

Halling-Sorensen, B., Nors Nielsen, S., Lanzky, P.F., Ingerslev, F., Holten Lutzheft, H.C., Jorgensen, S.E., 1998. Occurrence, Fate and Effects of Pharmaceutical Substances in the Environment – A Review. Chemosphere 36, 357–393.

Harman, C., Reid, M., Thomas, K.V., 2011. In Situ Calibration of a Passive Sampling Device for Selected Illicit Drugs and Their Metabolites in Wastewater, And Subsequent Year-Long Assessment of Community Drug Usage. Environ. Sci. Technol. 45, 5676–5682.

Heberer, T., 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol. Lett. 131, 5–17.

Hellmann, C., Wissel, B., Winkelmann, C., 2013. Omnivores as seasonally important predators in a stream food web. Freshw. Sci. 32, 548–562.

Henry, T.B., Kwon, J.W., Armbrust, K.L., Black, M.C., 2004. Acute and chronic toxicity of five selective serotonin reuptake inhibitors in Ceriodaphnia dubia. Environ. Toxicol. Chem. 23, 2229–2233.

Heppell, S.A., Denslow, N.D., Folmar, L.C., Sullivan, C.V., 1995. Universal assay of vitellogenin as a biomarker for environmental estrogens. Environ. Health Perspect. 103, 9–15.

Hiemke, C., Hartter, S., 2000. Pharmacokinetics of selective serotonin reuptake inhibitors. Pharmacol. Ther. 85, 11–28.

Hildrew, A., 1992. Food webs and species interactions. In: Petts, C., Petts, G.E. (eds), The rivers handbook: hydrological and ecological principles. Volume 1. Blackwell Scientific, Oxford, England, pp. 309–330.

Holmberg, A., Fogel, J., Albertsson, E., Fick, J., Brown, J.N., Paxeus, N., Forlin, L., Johnsson, J.I., Larsson, D.G.J., 2011. Does waterborne citalopram affect the aggressive and sexual behaviour of rainbow trout and guppy? J. Hazard. Mater. 187, 596–599.

Huckins, J.N., Tubergen, M.W., Manuweera, G.K., 1990. Semipermeable-membrane devices containing model lipid – a new approach to monitoring the bioavailability of lipophilic contaminants and estimating their bioconcentration potencial. Chemosphere 20, 533–552.

Huckins, J.N., Petty, J.D., Booij, K., 2006. Monitors of Organic Chemicals in the Environment: Semipermeable Membrane Devices. Springer Science+Business Media, LCC.

Huerta, B., Rodriguez-Mozaz, S., Barcelo, D., 2012. Pharmaceuticals in biota in the aquatic environment: analytical methods and environmental implications. Anal. Bioanal. Chem. 404, 2611–2624.

Hughes, S.R., Kay, P., Brown, L.E., 2013. Global Synthesis and Critical Evaluation of Pharmaceutical Data Sets Collected from River Systems. Environ. Sci. Technol. 47, 661–677.

Jakimska, A., Huerta, B., Barganska, Z., Kot-Wasik, A., Rodriguez-Mozaz, S., Barcelo, D., 2013. Development of a liquid chromatography-tandem mass spectrometry procedure for determination of endocrine disrupting compounds in fish from Mediterranean rivers. J. Chromatogr. A 1306, 44–58.

Jalova, V., Jarosova, B., Blaha, L., Giesy, J. P., Ocelka, T., Grabic, R., Jurcikova, J., Vrana, B., Hilscherova, K., 2013. Estrogen-, androgen- and aryl hydrocarbon receptor mediated activities in passive and composite samples from municipal waste and surface waters. Environ. Int. 59, 372–383.

Jobling, S., Coey, S., Whitmore, J.G., Kime, D.E., Van Look, K.J.W., McAllister, B.G., Beresford, N., Henshaw, A.C., Brighty, G., Tyler, C.R., Sumpter, J.P., 2002. Wild intersex roach (*Rutilus rutilus*) have reduced fertility. Biol. Reprod. 67, 515–524.

Jobling, S., Nolan, M., Tyler, C.R., Brighty, G., Sumpter, J.P., 1998. Widespread sexual disruption in wild fish. Environ. Sci. Technol. 32, 2498–2506.

Jones, O.A.H., Voulvoulis, N., Lester, J.N., 2002. Aquatic environmental assessment of the top 25 English prescription pharmaceuticals. Water Res. 36, 5013–5022.

Kolarikova, K., Stuchlik, E., Liska, M., Horecky, J., Tatosova, J., Hardekopf, D., Lapsanska, N., Horicka, Z., Hovorka, J., Mihaljevic, M., Fuksa, J. K., von Tumpling, W., 2012. Long-Term Changes in the Bioaccumulation of As, Cd, Pb, and Hg in Macroinvertebrates from the Elbe River (Czech Republic). Water Air Soil Pollut. 223, 3511–3526.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999–2000: A national reconnaissance. Environ. Sci. Technol. 36, 1202–1211.

Kosma, C.I., Lambropoulou, D.A., Albanis, T.A., 2014. Investigation of PPCPs in wastewater treatment plants in Greece: Occurrence, removal and environmental risk assessment. Sci. Total Environ. 466, 421–438.

Laine, A., 2001. Effects of peatland drainage on the size and diet of yearling salmon in a humic northern river. Arch. Hydrobiol. 151, 83–99.

Lajeunesse, A., Gagnon, C., Gagne, F., Louis, S., Cejka, P., Sauve, S., 2011. Distribution of antidepressants and their metabolites in brook trout exposed to municipal wastewaters before and after ozone treatment – Evidence of biological effects. Chemosphere 83, 564–571.

Lange, A., Paull, G.C., Hamilton, P.B., Iguchi, T., Tyler, C.R., 2011. Implications of persistent exposure to treated wastewater effluent for breeding in wild roach (*Rutilus rutilus*) populations. Environ. Sci. Technol. 45, 1673–1679.

Lewis, D. F. V., 2001. COMPACT: a structural approach to the modelling of cytochromes P450 and their interactions with xenobiotics. J. Chem. Technol. Biotechnol. 76, 237–244.

Li, Z. H., Zlabek, V., Turek, J., Velisek, J., Pulkrabova, J., Kolarova, J., Sudova, E., Berankova, P., Hradkova, P., Hajslova, J., Randak, T., 2011. Evaluating environmental impact of STPs situated on streams in the Czech Republic: An integrated approach to biomonitoring the aquatic environment. Water Res. 45, 1403–1413.

Li, Z.H., Zlabek, V., Velisek, J., Grabic, R., Machova, J., Randak, T., 2010. Physiological condition status and muscle-based biomarkers in rainbow trout (*Oncorhynchus mykiss*), after long-term exposure to carbamazepine. J. Appl. Toxicol. 30, 197–203.

Liney, K.E., Hagger, J.A., Tyler, C.R., Depledge, M.H., Galloway, T.S., Jobling, S., 2006. Health effects in fish of long-term exposure to effluents from wastewater treatment works. Environ. Health Perspect. 114, 81–89.

Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13–30.

Mompelat, S., Le Bot, B., Thomas, O., 2009. Occurrence and fate of pharmaceutical products and by-products, from resource to drinking water. Environ. Int. 35, 803–814.

Nagtegaal, M., Ternes, T. A., Baumann, W., Nagel, R., 1997. UV-Filtersubstanzen in Wasser und Fischen. Umweltwissenschaften und Schadstoff-Forschung. 9, 79–86.

Nallani, G.C., Paulos, P.M., Constantine, L.A., Venables, B.J., Huggett, D.B., 2011. Bioconcentration of ibuprofen in fathead minnow (*Pimephales promelas*) and channel catfish (*Ictalurus punctatus*). Chemosphere 84, 1371–1377.

Nash, J.P., Kime, D.E., Van der Ven, L.T.M., Wester, P.W., Brion, F., Maack, G., Stahlschmidt-Allner, P., Tyler, C.R., 2004. Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. Environ. Health Perspect. 112, 1725–1733.

Norris, D.O., Camp, J.M., Maldonado, T.A., Woodling, J.D., 2000. Some aspects of hepatic function in feral brown trout, Salmo trutta, living in metal contaminated water. Comp. Biochem. Physiol. C-Toxicol. Pharmacol. 127, 71–78.

Oliveira, R., McDonough, S., Ladewig, J.C.L., Soares, A., Nogueira, A.J.A., Domingues, I., 2013. Effects of oxytetracycline and amoxicillin on development and biomarkers activities of zebrafish (*Danio rerio*). Environ. Toxicol. Pharmacol. 36, 903–912.

Oruc, E.O., Usta, D., 2007. Evaluation of oxidative stress responses and neurotoxicity potential of diazinon in different tissues of *Cyprinus carpio*. Environ. Toxicol. Pharmacol. 23, 48–55.

Pal, A., Gin, K.Y.H., Lin, A.Y.C., Reinhard, M., 2010. Impacts of emerging organic contaminants on freshwater resources: Review of recent occurrences, sources, fate and effects. Sci. Total Environ. 408, 6062–6069.

Pan, B.Z., Wang, Z.Y., Xu, M.Z., 2012. Macroinvertebrates in abandoned channels: assemblage characteristics and their indications for channel management. River Res. Appl. 28, 1149–1160.

Pasquini, L., Munoz, J.F., Rimlinger, N., Dauchy, X., France, X., Pons, M.N., Gorner, T., 2013. Assessment of the fate of some household micropollutants in urban wastewater treatment plant. Chemical Papers. 67, 601–612.

Paterson, G., Metcalfe, C.D., 2008. Uptake and depuration of the anti-depressant fluoxetine by the Japanese medaka (*Oryzias latipes*). Chemosphere 74, 125–130.

Petrovic, M., Gonzalez, S., Barcelo, D., 2003. Analysis and removal of emerging contaminants in wastewater and drinking water. Trac-Trends Anal. Chem. 22, 685–696.

Poiger, T., Buser, H.R., Balmer, M.E., Bergqvist, P.A., Muller, M.D., 2004. Occurrence of UV filter compounds from sunscreens in surface waters: regional mass balance in two Swiss lakes. Chemosphere 55, 951–963.

Pomati, F., Orlandi, C., Clerici, M., Luciani, F., Zuccato, E., 2008. Effects and interactions in an environmentally relevant mixture of pharmaceuticals. Toxicol. Sci. 102, 129–137.

Ramirez, A.J., Mottaleb, M.A., Brooks, B.W., Chambliss, C.K., 2007. Analysis of pharmaceuticals in fish using liquid chromatography-tandem mass spectrometry. Analytical Chemistry 79, 3155–3163.

Ramirez, A.J., Brain, R.A., Usenko, S., Mottaleb, M.A., O'Donnell, J.G., Stahl, L.L., Wathen, J.B., Snyder, B.D., Pitt, J.L., Perez-Hurtado, P., Dobbins, L.L., Brooks, B.W., Chambliss, C.K., 2009. Occurrence of Pharmaceuticals and Personal Care Products in Fish: Results of a National Pilot Study in the United States. Environ. Toxicol. Chem. 28, 2587–2597.

Randak, T., Zlabek, V., Pulkrabova, J., Kolarova, J., Kroupova, H., Siroka, Z., Velisek, J., Svobodova, Z., Hajslova, J., 2009. Effects of pollution on chub in the River Elbe, Czech Republic. Ecotox. Environ. Safe. 72, 737–746.

Regulation, 2009. Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. Official Journal of the European Union. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:342:0059:0209:en:PDF.

Rimkus, G.G., 1999. Polycyclic musk fragrances in the aquatic environment. Toxicol. Lett. 111, 37–56.

Roberts, P.H., Thomas, K.V., 2006. The occurrence of selected pharmaceuticals in wastewater effluent and surface waters of the lower Tyne catchment. Sci. Total Environ. 356, 143–153.

Rodgers-Gray, T.P., Jobling, S., Kelly, C., Morris, S., Brighty, G., Waldock, M.J., Sumpter, J.P., Tyler, C.R., 2001. Exposure of juvenile roach (*Rutilus rutilus*) to treated sewage effluent induces dose-dependent and persistent disruption in gonadal duct development. Environ. Sci. Technol. 35, 462–470.

Rodil, R., Quintana, J.B., Concha-Grana, E., Lopez-Mahia, P., Muniategui-Lorenzo, S., Prada-Rodriguez, D., 2012. Emerging pollutants in sewage, surface and drinking water in Galicia (NW Spain). Chemosphere 86, 1040–1049.

Sanchez, W., Sremski, W., Piccini, B., Palluel, O., Maillot-Marechal, E., Betoulle, S., Jaffal, A., Ait-Aissa, S., Brion, F., Thybaud, E., Hinfray, N., Porcher, J.M., 2011. Adverse effects in wild fish living downstream from pharmaceutical manufacture discharges. Environ. Int. 37, 1342–1348.

Santos, L., Araujo, A.N., Fachini, A., Pena, A., Delerue-Matos, C., Montenegro, M., 2010. Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. J. Hazard. Mater. 175, 45–95.

Schlumpf, M., Cotton, B., Conscience, M., Haller, V., Steinmann, B., Lichtensteiger, W., 2001. *In vitro* and *in vivo* estrogenicity of UV screens. Environ. Health Perspect. 109, 239–244.

Schmidt, F., Schnurr, S., Wolf, R., Braunbeck, T., 2012. Effects of the anti-thyroidal compound potassium-perchlorate on the thyroid system of the zebrafish. Aquat. Toxicol. 109, 47–58.

Schramm, M., Muller, E., Triebskorn, R., 1998. Brown trout (*Salmo trutta* f. *Fario*) liver ultrastructure as a biomarker for assessment of small stream pollution. Biomarkers 3, 93–108.

Schultzt, M.M., Furlong, E.T., 2008. Trace analysis of antidepressant pharmaceuticals and their select degradates in aquatic matrixes by LC/ESI/MS/MS. Anal. Chem. 80, 1756–1762.

Schultz, M.M., Furlong, E.T., Kolpin, D.W., Werner, S.L., Schoenfuss, H.L., Barber, L.B., Blazer, V.S., Norris, D.O., Vajda, A.M., 2010. Antidepressant pharmaceuticals in two us effluent-impacted streams: occurrence and fate in water and sediment, and selective uptake in fish neural tissue. Environ. Sci. Technol. 44, 1918–1925.

Smith, M.J., Kay, W.R., Edward, D.H.D., Papas, P.J., Richardson, K.S., Simpson, J.C., Pinder, A.M., Cale, D.J., Horwitz, P.H.J., Davis, J.A., Yung, F.H., Norris, R.H., Halse, S.A., 1999. AusRivAS: using macroinvertebrates to assess ecological condition of rivers in Western Australia. Freshw. Biol. 41, 269–282.

Snyder, S.A., Westerhoff, P., Yoon, Y., Sedlak, D.L., 2003. Pharmaceuticals, personal care products, and endocrine disruptors in water: Implications for the water industry. Environ. Eng. Sci. 20, 449–469.

Sohoni, P., Sumpter, J.P., 1998. Several environmental oestrogens are also anti-androgens. J. Endocrinol. 158, 327–339.

Sturve, J., Almroth, B.C., Forlin, L., 2008. Oxidative stress in rainbow trout (*Oncorhynchus mykiss*) exposed to sewage treatment plant effluent. Ecotox. Environ. Safe. 70, 446–452.

Sumpter, J.P., Jobling, S., 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ. Health Perspect. 103, 173–178.

Ternes, T.A., 1998. Occurrence of drugs in German sewage treatment plants and rivers. Water Res. 32, 3245–3260.

Ternes, T.A., Joss, A., Siegrist, H., 2004. Scrutinizing pharmaceuticals and personal care products in wastewater treatment. Environ. Sci. Technol. 38, 392A–399A.

Togunde, O.P., Oakes, K.D., Servos, M.R., Pawliszyn, J., 2012. Optimization of solid phase microextraction for non-lethal *in vivo* determination of selected pharmaceuticals in fish muscle using liquid chromatography-mass spectrometry. J. Chromatogr. A 1261, 99–106.

Tyler, C.R., Jobling, S., 2008. Roach, sex, and gender-bending chemicals: the feminization of wild fish in English rivers. Bioscience 58, 1051–1059.

Tyler, C.R., van Aerle, R., Hutchinson, T.H., Maddix, S., Trip, H., 1999. An *in vivo* testing system for endocrine disruptors in fish early life stages using induction of vitellogenin. Environ. Toxicol. Chem. 18, 337–347.

Valenti, T.W., Gould, G.G., Berninger, J.P., Connors, K.A., Keele, N.B., Prosser, K.N., Brooks, B.W., 2012. Human therapeutic plasma levels of the selective serotonin reuptake inhibitor (SSRIs) sertraline decrease serotonin reuptake transporter binding and shelter-seeking behavior in adult male fathead minnows. Environ. Sci. Technol. 46, 2427–2435.

Verlicchi, P., Al Aukidy, M., Zambello, E., 2012. Occurrence of pharmaceutical compounds in urban wastewater: Removal, mass load and environmental risk after a secondary treatment-A review. Sci. Total Environ. 429, 123–155.

Verlicchi, P., Galletti, A., Petrovic, M., Barcelo, D., Al Aukidy, M., Zambello, E., 2013. Removal of selected pharmaceuticals from domestic wastewater in an activated sludge system followed by a horizontal subsurface flow bed – Analysis of their respective contributions. Sci. Total Environ. 454**,** 411–425.

Vieno, N., Tuhkanen, T., Kronberg, L., 2007. Elimination of pharmaceuticals in sewage treatment plants in Finland. Water Res. 41**,** 1001–1012.

Vrana, B., Mills, G.A., Allan, I.J., Dominiak, E., Svensson, K., Knutsson, J., Morrison, G., Greenwood, R., 2005. Passive sampling techniques for monitoring pollutants in water. Trac-Trends Anal. Chem. 24**,** 845–868.

Wang, J., Gardinali, P.R., 2013. Uptake and depuration of pharmaceuticals in reclaimed water by mosquito fish (*Gambusia holbrooki*): A worst-case, multiple-exposure scenario. Environ. Toxicol. Chem. 32**,** 1752–1758.

Wick, A., Fink, G., Ternes, T.A., 2010. Comparison of electrospray ionization and atmospheric pressure chemical ionization for multi-residue analysis of biocides, UV-filters and benzothiazoles in aqueous matrices and activated sludge by liquid chromatography-tandem mass spectrometry. J. Chromatogr. A. 1217**,** 2088–2103.

Zucchi, S., Bluthgen, N., Ieronimo, A., Fent, K., 2011. The UV-absorber benzophenone-4 alters transcripts of genes involved in hormonal pathways in zebrafish (*Danio rerio*) eleuthero-embryos and adult males. Toxicol. Appl. Pharmacol. 250**,** 137–146.

CHAPTER 2

LONG-TERM AQUARIA TESTS

2.1. Steinbach, C., Fedorova, G., Prokes, M., Grabicova, K., Machova, J., Grabic, R., Valentova, O., Kocour Kroupova, H., 2013. Toxic effects, bioconcentration and depuration of verapamil in the early life stages of common carp (*Cyprinus carpio* L.). Science of the Total Environment 461–462, 198–206.

2.2. Grabicova, K., Fedorova, G., Burkina, V., Steinbach, C., Schmidt-Posthaus, H., Zlabek, V., Kocour Kroupova, H., Grabic, R., Randak, T., 2013. Presence of UV filters in surface water and the effects of phenylbenzimidazole sulfonic acid on rainbow trout (*Oncorhynchus mykiss*) following a chronic toxicity test. Ecotoxicology and Environmental Safety 96, 41–47.

It was allowed by publisher on 25th February / 19th February, 2014 to include the papers in this Ph.D. thesis.

Science of the Total Environment 461–462 (2013) 198–206

Contents lists available at SciVerse ScienceDirect

Science of the Total Environment

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

Toxic effects, bioconcentration and depuration of verapamil in the early life stages of common carp (*Cyprinus carpio* L.)

Christoph Steinbach [a,*], Ganna Fedorova [a], Miroslav Prokes [b], Katerina Grabicova [a], Jana Machova [a], Roman Grabic [a], Olga Valentova [a], Hana Kocour Kroupova [a]

[a] Research Institute of Fish Culture and Hydrobiology, South Bohemian Research Centre of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, CZ-38925 Vodnany, Czech Republic
[b] Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, v.v.i., Kvetna 8, 603 65 Brno, Czech Republic

HIGHLIGHTS

- Study of the acute and sub-chronic toxicity of verapamil on early-life stages of common carp.
- Acute exposure to verapamil reduced the heart rate in early-life stages of common carp.
- The bioconcentration factor of verapamil ranged from 6.6 to 16.6.
- The half-life of verapamil in fish was estimated to be 10.2±1.6days.

ARTICLE INFO

Article history:
Received 17 January 2013
Received in revised form 11 April 2013
Accepted 2 May 2013
Available online xxxx

Editor: Mark Hanson

Keywords:
Bioconcentration
Embryo-larval test
Half-life time
Heart rate
Verapamil

ABSTRACT

Verapamil is a pharmaceutical that belongs to a group of calcium channel blockers and is mainly used as a treatment of angina pectoris and arterial hypertension. Verapamil has been detected in aquatic environments in concentrations ranging from ng L^{-1} to μg L^{-1}. In the present study, a series of acute toxicity tests of verapamil on various developmental stages of common carp (*Cyprinus carpio*) were conducted. As a result, 96hLC_{50} values of verapamil were estimated at 16.4 ± 9.2, 7.3 ± 1.5 and 4.8 ± 0.2 mg L^{-1} for embryos (E5–E9) and common carp larvae L2 and L5, respectively. Lethal concentrations of verapamil decreased with an increase in the age of the fish. Acute exposure to verapamil significantly reduced the heart rate in the embryos and larvae. In an embryo-larval toxicity test (sub-chronic exposure), the bioconcentration, depuration, and toxic effects of verapamil were assessed in common carp. The fish were exposed to verapamil in a concentration of 0.463 (environmentally relevant), 4.63, 46.3 and 463 μg L^{-1}. Verapamil had no effect on the accumulated mortality, hatching, condition factor, growth or ontogeny of the fish in any of the tested concentrations. In carp exposed to 463 and 46.3 μg L^{-1} of verapamil, significantly higher occurrences of malformations and edemas were observed compared to the control. The bioconcentration factor of verapamil in whole fish homogenates ranged between 6.6 and 16.6 and was therefore below the critical value for hazard substances (BCF > 500). The half-life and the 95% depuration time for the tested compound were estimated to be 10.2 ± 1.6 days and 44.2 ± 8.6 days, respectively. No effects of verapamil on the studied endpoints were observed at environmentally relevant concentrations.

1. Introduction

Typically, human pharmaceuticals are excreted after application and enter aquatic ecosystems via wastewater because they are not completely removed in sewage treatment plants (Khetan and Collins, 2007). Even if the concentrations of most pharmaceuticals in aquatic environments are relatively low (from ng L^{-1} up to low μg L^{-1}), the pharmaceuticals might pose a substantial threat to aquatic organisms (Corcoran et al., 2010; Gunnarsson et al., 2008) because they are designed to be effective in low doses. In general, effects on non-target species are supposed to occur as a result of specific drug target interactions rather than via unspecific modes of action, whereas pharmaceuticals are designed to interact with evolutionarily well-conserved drug targets (Corcoran et al., 2010; Gunnarsson et al., 2008). However, there is only limited knowledge available on the long-term influence of pharmaceuticals on aquatic organisms (Fent et al., 2006).

Verapamil, a phenylalkylamine, is a pharmaceutical belonging to a group of calcium channel blockers. This highly prescribed drug is an active antianginal, antiarrhythmic and antihypertensive that is used in the treatment of cardiovascular diseases (Johnston et al., 1981; Lüllmann et al., 2003). The reported concentrations of verapamil in

* Corresponding author. Tel.: +420 387774622.
E-mail address: steinbach@frov.jcu.cz (C. Steinbach).

0048-9697/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.scitotenv.2013.05.002

treated waste waters and surface waters lie in the range of μg L^{-1} and ng L^{-1}, respectively (Batt et al., 2008; Hummel et al., 2006; Khan and Ongerth, 2004).

Acute toxicity of verapamil is relatively low, as demonstrated by an acute toxicity test on rainbow trout (*Oncorhynchus mykiss*) by Li et al. (2010a). In their study, the 96hLC_{50} value for verapamil was estimated at 2.7 mg L^{-1}. The acute exposure of zebrafish larvae (*Danio rerio*) to verapamil at a concentration of 455 mg L^{-1} affected their heart rate and gut activity (Berghmans et al., 2008). However, verapamil has also caused effects in fish at lower concentrations. A short-term exposure (96 h) of rainbow trout (*O. mykiss*) to verapamil at a concentration of 270 μg L^{-1} caused oxidative stress (Li et al., 2010a) and an increase in the stress index (integrated biomarker response; Li et al., 2010b) after long-term exposure (42 days).

Fish and amphibians are vertebrates that undergo their metamorphosis, a very sensitive life period, in surface waters. Consequently, their embryo-larval stages are supposed to be highly sensitive to chemicals polluting the aquatic environment in which they live (Richard et al., 2008). In toxicological risk assessments, the fish embryo-larval test (Machova et al., 2009; OECD, 1992, 1996) and amphibian metamorphosis assay (OECD, 2009) are established test procedures. To date, few studies have focused on the effects of verapamil on early-life stages of fish and amphibians (Burgess and Vere, 1989; Overturf et al., 2011). A study by Burgess and Vere (1989) revealed that verapamil at a concentration of 72 μg L^{-1} caused abnormalities in the development of the African clawed frog (*Xenopus laevis*). In a recent study by Overturf et al. (2011), long-term exposure (28 days) of verapamil to the fathead minnow (*Pimephales promelas*) larvae at a concentration of 600 μg L^{-1} caused a significant decrease in growth rate. Verapamil is a cationic amphiphile substance that has a bioconcentration potential because it is slightly lipophilic (log P = 4.8 and 3.4 for its uncharged and charged form, resp.; Fick et al., 2010a,b; Lüllmann et al., 2003; OECD, 1996). However, only limited data on the bioconcentration of verapamil in fish are available (Fick et al., 2010a,b). Bioconcentration properties of a substance can be described by the bioconcentration factor (BCF), which is defined as the ratio of the concentration of the substance in the fish to the concentration in the water (Mackay and Fraser, 2000; OECD, 1996; Springer et al., 2008). The elimination rate, presented as the half-life, is another important pharmacokinetic parameter. The half-life is defined as the time it takes for the concentration of a substance in an organism to halve its steady-state (Lüllmann et al., 2003; OECD, 1996). It reflects the capacity for biotransformation and elimination of a substance in the exposed organisms. However, the bioconcentration factor and half-life are important endpoints for environmental hazard assessment (Franke, 1996; Mackay and Fraser, 2000; Nordberg and Rudén, 2007; OECD, 1996, 2001; Springer et al., 2008). In fact, only a limited number of studies have described them for waterborne human pharmaceuticals in fish (Berghmans et al., 2008; Alderton et al., 2010; Fick et al., 2010a, 2010b; Gomez et al., 2010; Nallani et al., 2011; Mehinto et al., 2010; Paterson and Metcalfe, 2008).

The aim of the present study was to determine the toxic effects of acute and long-term exposure of verapamil on the early-life stages of common carp, a well-established model species used to test the adverse effects of chemicals on the early development of fish (OECD, 1992, 1996). To our knowledge, this is the first study describing the effects of verapamil on the occurrence of malformations during early development of fish. Moreover, this study is among the first to investigate both the bioconcentration factor and half-life of verapamil in fish.

2. Material and methods

2.1. Experimental animals

Fertilized eggs and larvae of the common carp (*Cyprinus carpio* L.) were obtained from the breeding station of the Research Institute of Fish Culture and Hydrobiology in Vodnany (Czech Republic). Eggs were produced according to standard methods of artificial reproduction (Kocour et al., 2005). Twenty four hours post-fertilization, all eggs were treated with malachite green solution (0.25 mg L^{-1}) and formaldehyde (0.125 μg L^{-1}) for two hours to prevent possible fungal infection (Sudova et al., 2007). White unfertilized eggs were removed.

2.2. Experimental conditions

The fish were kept in dechlorinated tap water. Throughout the experiment, all basins were gently aerated. The water quality parameters were the following: dissolved oxygen >5.8 mg L^{-1}, temperature 20.1 ± 1.7 °C, pH 7.9 ± 0.2, $ANC_{4.5}$ (acid neutralization capacity) 1.1 mmol L^{-1}, COD_{Mn} (chemical oxygen demand) 1.10 mg L^{-1}, NH_4^+ 0.02 mg L^{-1}, NO_3^- 9.2 mg L^{-1}, PO_4^{3-} 0.02 mg L^{-1}, Cl^- 10.65 mg L^{-1}. The basins were placed in a laboratory (open-air conditions) with natural light exposure (16:8 h light: dark).

2.3. Chemicals

Verapamil hydrochloride (CAS number: 152-11-4) and Diltiazem hydrochloride (CAS number: 33286-22-5) were obtained from Sigma-Aldrich Corporation (USA). LC–MS grade acetonitrile (LiChrosolv Hypergrade) was purchased from Merck (Darmstadt, Germany). Verapamil concentrations are expressed as concentrations of the active substance verapamil throughout the study.

2.4. Acute toxicity tests

The 96 h acute toxicity tests were conducted using the OECD guideline No. 212 (OECD, 1998), with modifications as described below. Three early-life stages of common carp were used: embryos – stage E5 and larvae – stages L2 and L5. The larvae of the stages L2 and L5 were 6 and 20 days post hatched, respectively. At the conclusion of the tests, embryos reached stage E9 and larvae remained at stages L2 and L5, respectively. Developmental stages were classified according to Penaz et al. (1983). At each experimental replicate, 20 embryos or 15 larvae were randomly distributed to crystallization basins containing 190 mL of a solution corresponding to the following treatments: 1) eggs – control (verapamil-free water), 1, 2, 5, 10, and 20 mg L^{-1} verapamil and 2) larvae – control (verapamil-free water), 1, 2, 5, 10, 15, and 20 mg L^{-1} verapamil. Each treatment and the control were replicated three times. The test solutions were exchanged daily by light draining each basin and adding new solution. Mortalities were recorded and dead fish were removed on a daily basis. Once the larvae were able to take up food, they were fed daily *ad libitum* with freshly hatched brine shrimp (*Artemia salina*) nauplii after water was exchanged.

At the end of the exposure, the heart rate (beats per minute) of the fish (stages E9 and L2) was recorded from five fish per replicate in the following treatments: control, 1, 2, 5, and 10 mg L^{-1} verapamil. The embryos and larvae exposed to higher verapamil concentrations died before the end of the tests. The heart was not visible in the third stage (L5); therefore, the heart rate could not be measured in this case. The heart rate was determined by counting the number of visual beats under a stereomicroscope (Olympus SZXZ; magnification ×10) over a period of 30 s. At the end of the experiments, all surviving fish were fixed in a 4% formalin solution. Morphological anomalies were evaluated according to Jezierska et al. (2000).

2.5. Sub-chronic toxicity test

The embryo-larval toxicity test was conducted using the modified test design of the OECD guideline No. 210 (OECD, 1992). The test started one day after egg fertilization. At each experimental replicate, 100 eggs were randomly distributed to each of the crystallization basins containing 300 mL of the solution corresponding to the following

treatments: control (verapamil-free water); 0.463 μg L^{-1} verapamil (group 1); 4.63 μg L^{-1} verapamil (group 2); 46.3 μg L^{-1} verapamil (group 3); 463 μg L^{-1} verapamil (group 4). Each treatment and the control were replicated three times. The solutions from each treatment were renewed daily by light draining each basin and adding new solution. Hatching and mortality were recorded daily; dead eggs, embryos and larvae were removed. Once the larvae were able to take up food, they were fed daily *ad libitum* with freshly hatched brine shrimp (*A. salina*) nauplii after the water was exchanged. The experiment was terminated after 31 days.

To assess development, ten fish per replicate were sampled after 10, 15, 20, 24 and 31 days. The fish were stored in a 4% formalin solution. Determination of developmental periods and stages followed Penaz et al. (1983). Morphological anomalies were evaluated according to Jezierska et al. (2000). The total length, relative mass after fixation, developmental stages and occurrence of morphological anomalies were checked under a stereomicroscope (Olympus SZXZ). The total length was measured with an accuracy of 0.01 mm by a fine digital caliper. The relative weight after fixation, to a resolution of 0.1 mg, was measured by a Mettler Toledo AB204-S balance. The weight of fish was taken at the time when the weight was already stabilized, i.e. after 3 months of formalin preservation (Kristoffersen and Salvanes, 1998). The Fulton's condition factor (FCF; Nash et al., 2004) and the mean specific growth rate (SGR; Machova et al., 2009; Cook et al., 2000) for the fish in each of the experimental groups were calculated. After the termination of the embryo-larval test, the replicates of each treatment were put together and kept in five aquaria (40 L) in verapamil-free water. The fish were fed daily with dry food (Tetra). Fifty percent of the water in the tanks was exchanged at least every third day. These fish were kept in the aquaria five months post-termination of the exposure to verapamil to evaluate the half-life of verapamil in fish. The fish mortality was also recorded during this period.

2.6. Analysis of verapamil in water and fish

In the acute toxicity tests, water samples were taken from each basin immediately after the water was exchanged and 24 h post exchange during each test. In the sub-chronic toxicity test, water samples were taken from each basin after 0, 2, 6 and 24 h on days 10, 20 and 31. Additionally, tap water samples were taken. All water samples were taken using separate syringes. At the end of the exposure, seven fish from each replicate (105 in total) were sampled. Additionally, seven fish from each experimental group (35 in total in every sampling term) were sampled after two and six weeks post-exposure. All sampled fish were dried with filter paper and stored in 2 mL Eppendorf tubes at 20 °C until analysis. The concentration of verapamil was determined in whole fish homogenates. The homogenization and extraction of the samples followed modified Ramirez et al. (2009). The sample extraction was conducted with acetonitrile in the high-speed homogeniser with stainless-steel balls. Diltiazem was used as an internal standard.

Quality assurance/quality control (QA/QC) of the analytical method included blank samples (to be sure that target compound is not introduced from the sample handling), duplicates (every tenth sample was duplicated) and fortified samples (every tenth sample of fish from control group was spiked with the target compound at the concentration 100 ng g^{-1} to check the method accuracy). Matrix-matched standards ("clean" fish extracts spiked with both target compound and internal standard) were used to correct response factor of the calibration curve prepared in methanol.

The water samples were analyzed using line SPE liquid chromatography with tandem mass spectrometry (LC–MS/MS; Kvarnryd et al., 2011). Samples of two highest concentrations were diluted prior to analysis. Fish homogenate samples were analyzed using LC–MS/MS. These analyses were 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, San Jose, CA, USA) and a HTS-XT autosampler (CTC Analytics AG, Zwingen, Switzerland).

The bioconcentration factor (BCF) was calculated in accordance with OECD Guideline No. 305 (OECD, 1996) for each treatment group as follows: the mean concentration of verapamil in the fish sampled at the termination of the experiment was divided by the mean concentration of all analyzed water samples in the respective group.

First-order kinetics was assumed to determine the depuration rate. The half-life (50% depuration, t_{50}) and 95% loss in the depuration phase (t_{95}) of verapamil in fish were calculated using linear regression of the natural logarithm (ln) of the detected concentrations (group 2, group 3, group 4) and the value of the slope (k) of the graph: (t_{50}) = 0.693/k and (t_{95}) = 3.0/k, respectively (OECD, 1996). The first group was excluded from half-life calculations because the detected concentrations were too close or below the LOQ.

2.7. Statistical analysis

Statistical analysis was performed using Statistica version 9 (StatSoft, Czech Republic). The differences in the accumulated mortality, hatching time and occurrence of macroscopic morphological anomalies between the test groups were measured using the χ^2 test. Data for the total length, relative mass after fixation, and FCF were tested for normality using the Kolmogorov–Smirnov test and for homogeneity using Levene's test. If the statistical criteria for normality and homogeneity were fulfilled, a one-way analysis of variance (ANOVA) was implemented with subsequent Dunnett's multiple-range post hoc test. If these criteria were not satisfied, the nonparametric test (Kruskal–Wallis test) was applied. The results are presented as the mean ± standard deviation. Statistical differences between the experimental groups were considered significant when $p < 0.05$.

The 96hLC_{50} values were calculated by probit analysis using EKOTOX 5.1 software (Ingeo Liberec, Czech Republic).

3. Results

3.1. Determination of verapamil in water and fish

3.1.1. Analytical method performance

Performance of the analytical method was assessed including its linearity, repeatability, sensitivity and recovery. Six point calibration curves were prepared for water and fish analysis in the range 10–5000 ng L^{-1} and 1–500 ng g^{-1} respectively. Good linearity was observed with R^2 coefficient 0.998. Method repeatability was tested for ten replicates; relative standard deviation (RSD) of replicates was 10%. The recovery of verapamil from fish tissue was studied by spiking "clean" fish sample with the target compound before the extraction procedure. Average recovery of verapamil was 110% with the RSD of ten replicates 5%. The limits of quantification (LOQ) of verapamil in fish and water were 0.006 μg g^{-1} and 0.07 μg L^{-1}, respectively.

3.1.2. Concentration of verapamil in water

Concentrations of verapamil in all treatment baths measured immediately (0 h) and 24 h post-exchange in acute toxicity tests as well as sub-chronic test are shown in Table 1. A comparison of the determined verapamil water concentration with nominal values for all test groups of the sub-chronic test is listed in Table 2. Concentrations of verapamil in the controls and the tap water were below the limit of quantification.

3.1.3. Concentration of verapamil in fish

The concentrations of verapamil in fish homogenates at the conclusion of the sub-chronic test are summarized in Table 3. Verapamil concentrations in the fish were linearly correlated to the concentrations in

Table 1
Concentration of verapamil in water in the 96-h acute toxicity tests and in the 31-day embryo-larval toxicity test (concentrations measured immediately (0 h) and (24 h) after water exchange). Values are expressed as the mean ± SD. Acute toxicity tests: n = 9, concentrations in mg L^{-1}; 31-day embryo-larval toxicity test: n = 3, concentrations in μg L^{-1}.

Test	Treatment/sampling time	Concentration of verapamil	
		0 h	24 h
Acute tests	Control	<LOQ	<LOQ
	Nominal conc. 1 mg L^{-1}	0.7 ± 0.1	0.8 ± 0.1
	Nominal conc. 2 mg L^{-1}	1.2 ± 0.1	2.0 ± 0.1
	Nominal conc. 5 mg L^{-1}	4.2 ± 1.6	3.7 ± 1.8
	Nominal conc.10 mg L^{-1}	8.1 ± 1.8	8.5 ± 1.8
	Nominal conc. 15 mg L^{-1}	13.9 ± 4.4	16.6 ± 4.6
	Nominal conc. 20 mg L^{-1}	16.2 ± 3.3	21.6 ± 4.6
Sub-chronic test	Control	<LOQ	<LOQ
	Nominal conc. 0.463 μg L^{-1}	1.1 ± 0.5	0.7 ± 0.6
	Nominal conc. 4.63 μg L^{-1}	4.0 ± 0.9	5.5 ± 1.4
	Nominal conc. 46.30 μg L^{-1}	42.5 ± 13.8	26.1 ± 13.3
	Nominal conc. 463 μg L^{-1}	363 ± 129	222 ± 89

water (y = 3.9151x + 67.147, R^2 = 0.9947; x and y represent the concentration of verapamil in water and fish homogenates). The BCF calculated for groups 1 to 4 was as follows: 16.6, 14.0, 10.1, and 6.6. The half-life and 95% loss period in the depuration phase of verapamil in fish were estimated at 10.2 ± 1.6 and 44 ± 8.6 days, respectively. Hence, verapamil was still detectable in the fish six weeks after time of exposure.

3.2. Acute toxicity tests

3.2.1. Lethal concentrations

The 96hLC_{50} values of verapamil were estimated at 16.4 ± 9.2, 7.3 ± 1.5, and 4.8 ± 0.2 mg L^{-1} for embryos (E5–E9) and larvae L2 and L5, respectively. Lethal concentrations of verapamil decreased with increasing age of fish.

3.2.2. Heart rate

Verapamil exposure was found to significantly reduce heart rate. The exponential regression analysis of the heart rate (y) on verapamil concentration in the water (x) for embryos (E9) and larvae (L2) of common carp yielded values as follows: $y = 94.96e^{-0.1543x}$, $R^2 = 0.87$ and $y = 104.85e^{-0.1333x}$, $R^2 = 0.85$, respectively (Fig. 1). The heart rate could not be measured in stage L5 larvae, because their hearts were not visible.

3.2.3. Macroscopic morphological anomalies

Macroscopic morphological anomalies observed at the conclusion of the acute toxicity tests are given in Table 4. These included the axial and lateral curvature of the spine (kyphosis, lordosis, and scoliosis) and skull malformation, as well as yolk sac, visceral, and heart edemas (Fig. 2). A higher incidence of edemas compared to the controls was observed in most verapamil-treated groups of all stages (Table 4). Furthermore, at stage L2, all verapamil-treated groups had a significantly higher occurrence of malformations (Table 4) compared to the controls. Occurrence of malformations could not be assessed in groups treated with higher verapamil concentrations (higher than 10 or 5 mg L^{-1} in the case of E9 and L2, and L5, respectively), as all the fish in these treatments died before the termination of the test.

Table 2
Concentration of verapamil in water of all treatments and differences of determined concentrations to the nominal concentrations during the 31-day embryo-larval toxicity test.

	Control	Group 1	Group 2	Group 3	Group 4
		0.463 μg L^{-1}	4.63 μg L^{-1}	46.3 μg L^{-1}	463 μg L^{-1}
c (μg L^{-1})					
Mean ± SD	<LOQ	0.9 ± 0.6	4.0 ± 1.2	33.7 ± 15.5	298.8 ± 117.7
Min–max		0.1–2.3	1.8–6.5	9.3–64.2	88.3–612.8
Difference to nominal conc. (%)	–	196 ± 124	87 ± 27	72 ± 35	64 ± 25

Table 3
Concentration of verapamil in fish homogenates at the conclusion of the 31-day embryo-larval toxicity test (0 day) and after 14 and 42 days post-exposure. Values are expressed as the mean ± SD.

Group/sampling time	Concentration of verapamil (μg kg^{-1})		
	0 day	14 days	42 days
Control	<LOQ	<LOQ	<LOQ
Group 1: 0.463 μg L^{-1}	15.1 ± 6.6	<LOQ	<LOQ
Group 2: 4.63 μg L^{-1}	56.1 ± 22.0	17.3 ± 9.6	4.5 ± 7.0
Group 3: 46.3 μg L^{-1}	340 ± 91.2	66.2 ± 22.4	19.2 ± 5.9
Group 4: 463 μg L^{-1}	1870 ± 274	282 ± 57.1	42.8 ± 15.6

3.3. Sub-chronic toxicity test

3.3.1. Hatching

Hatching began 5 days after the initial time of exposure, and the majority of eggs in all treatment groups hatched by day 7. Verapamil had no significant effect on the hatching time (χ^2 test, p > 0.05).

3.3.2. Accumulated mortality

The accumulated mortality did not differ statistically among treatments (χ^2 test, p > 0.05). No mortality was observed five months after the termination of the experiment in all the groups, including in the control.

3.3.3. Length, weight, FCF and SGR

Exposure to verapamil had no significant effect on the length, relative mass after fixation or FCF of the tested groups (ANOVA, p > 0.05, Table 5). The specific growth rate in the control, group 1, group 2, group 3 and group 4 were 14.0, 13.4, 15.4, 13.1, and 15.7, respectively.

3.3.4. Macroscopic morphological anomalies

Verapamil caused the following macroscopic morphological anomalies in the exposed larvae: deformation of the intestine, axial and lateral curvature of the spine (kyphosis, lordosis, and scoliosis), skull malformation, fin malformations, underdeveloped eyes and visceral edema with hematocele (the number of checked fish in each treatment ranged between 24 and 31). The most predominant malformations were axial curvature of the spine and visceral edema with hematocele (Fig. 3, Table 6).

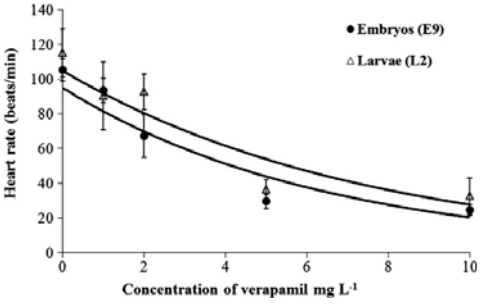

Fig. 1. Exponential regression of heart rate (y) on the verapamil concentration in water (x) for embryos (E9) and larvae (L2) of common carp, respectively; $y = 94.96e^{-0.1543x}$, $R^2 = 0.87$; $y = 104.85e^{-0.1333x}$, $R^2 = 0.85$.

Table 4
Occurrence of macroscopic morphological anomalies in embryos (E9) and larvae (L2, L5) of common carp at the conclusion of the 96-h acute toxicity tests with verapamil. MA — sum of all malformations, Sp — axial and lateral curvature of the spine, SM — skull malformation, E — sum of all edemas, VE — visceral edema, YE — yolk sac edema and CE — cardiac edema. Asterisks indicate a significant difference compared to the control: * $p < 0.05$, ** $p < 0.01$ (χ^2 test). N.E. — not evaluated.

Stage	c ($mg\ L^{-1}$)	MA (%)	Sp (%)	SM (%)	E (%)	YE (%)	VE (%)	CE (%)
E9	Control	4.7	4.7	0	2.3	2.3	0	0
	1	15.4	15.4	5.1	20.5**	17.9*	0	15.4*
	2	15.0	15.0	7.5	30.0**	12.5	0	22.5*
	5	17.0	17.0	10.6*	59.6**	36.2**	0	34.0**
	10	66.7**	59.0**	28.2**	82.1**	79.5**	0	69.2**
L2	Control	3.0	3.0	0	0	N.E.	0	0
	1	33.3**	25.9**	7.4	14.8*	N.E.	14.8*	3.7
	2	27.8**	27.8**	16.7*	11.1	N.E.	11.1*	5.6
	5	57.1**	57.1**	35.7**	75.0**	N.E.	53.6**	67.9**
	10	77.8**	66.6**	55.6**	55.6**	N.E.	44.4**	33.3**
L5	Control	0	0	0	2.4	N.E.	2.4	N.E.
	1	0	0	0	4.4	N.E.	4.4	N.E.
	2	4.5	4.5	0	15.9*	N.E.	15.9*	N.E.
	5	6.5	6.5	0	25.8**	N.E.	25.8**	N.E.

The highest malformation rates in verapamil-treated fish were observed on days 20 and 24 (Table 6). No malformations were observed at the last sampling time (day 31). In groups 3 and 4 (treated with the two highest concentrations), a significantly higher occurrence of malformations and edema with hematocele was observed compared to the control.

3.3.5. Early ontogeny

Verapamil did not cause a delay in the development of the exposed fish. The differences in the early ontogeny among the tested groups were not significant (Wilcoxon–Mann–Whitney-test, $p > 0.05$, Table 6).

4. Discussion

4.1. Acute toxicity tests

4.1.1. Lethal concentrations of verapamil

In the present study the $96hLC_{50}$ values of verapamil for common carp embryos and larvae ranged from 16.6 to 4.8 $mg\ L^{-1}$; therefore, verapamil can be classified as a harmful ($>10 < 100\ mg\ L^{-1}$) or toxic ($>1 < 10\ mg\ L^{-1}$; OECD, 2001) substance. This finding is in accordance with the studies by Li et al. (2010a) and Villegas-Navarro et al. (2003), who estimated the lethal concentration of verapamil for juvenile rainbow trout and *Daphnia magna* to be 2.7 $mg\ L^{-1}$ ($96hLC_{50}$) and 7.0 $mg\ L^{-1}$ ($48hLC_{50}$). Interestingly, acute lethal concentrations ($96hLC_{50}$) of verapamil determined in the present study were close to the comatose-lethal blood plasma concentration for humans (2.5 $mg\ L^{-1}$; Regenthal et al., 1999).

The toxicity of verapamil seemed to increase slightly with the age of the fish, with the oldest stage (L5) being the most sensitive. The tested developmental stages of common carp differed in food intake as well as in advancement of physiological development (Penaz et al., 1983). However, the differences in sensitivity might be explained by the advancement of gill development, as the fish were only exposed to waterborne verapamil in the study. Fish of the L5 stage had fully developed gills compared to the other tested stages (E5-9 and L2) (Penaz et al., 1983); therefore, verapamil could enter their body much easier. In general, larvae appear to be more sensitive to most toxic substances compared to embryos (Kroupova et al., 2010; Machova et al., 2009).

4.1.2. Effect of verapamil on heart rate

In the present study, verapamil reduced the heart rate of carp embryos and larvae in acute toxicity tests in a concentration-dependent manner, which is well in line with the finding of Berghmans et al. (2008), who exposed zebrafish larvae to verapamil for 3 h in the concentration range of 136–227 $mg\ L^{-1}$. Furthermore, Shin et al. (2010) found a reduced heart rate in zebrafish embryos exposed to verapamil at the concentration of 1 $mg\ L^{-1}$ for 12 h compared to the controls. The present study, together with the results of Berghmans et al. (2008) and Shin et al. (2010), reveal that verapamil has a similar pharmacological effect on fish as it does on humans, where it is used therapeutically to reduce the heart rate (Grossman and Messerli, 2004). The similarity in the mode of action in fish and humans is not surprising, whereas most drug targets are supposed to be highly conservative among vertebrate species (Gunnarsson et al., 2008). This finding is further supported by the fact that comparing the polypeptide sequences for the human and zebrafish orthologs of the α-subunit of the L-type calcium channel, which is one of the targets of the calcium channel blocker verapamil, revealed a 78% match (Lacinova et al., 1995; Rottbauer et al., 2001). Sequence of carp α-subunit of the L-type calcium channel is not available, but it is highly probable that carp and zebrafish polypeptide sequences are very similar since both fish species belong to the same family, family of cyprinid fishes.

4.2. Macroscopic morphological anomalies

Carp embryos and larvae exposed acutely to high verapamil concentrations (1–10 $mg\ L^{-1}$) had a higher occurrence of macroscopic morphological anomalies than fish exposed sub-chronically to lower concentrations (0.463–463 $\mu g\ L^{-1}$). However, in both cases, verapamil exposure was associated with significantly higher occurrence of spine malformations and edemas. Occurrence of edemas was also observed in frogs treated with verapamil during their early development (Burgess and Vere, 1989). Similarly, peripheral edema is a non-cardiovascular side effect of verapamil treatment in humans (Drenth et al., 1992). Burgess and Vere (1989) attributed this effect of verapamil to disturbances in the ionic equilibrium. Moreover, long-term exposure of carp larvae to verapamil resulted in a higher incidence of hematocele in the intestine compared to the control, whereas gastrointestinal hemorrhage is another side effect of verapamil treatment in humans (Pahor et al., 1996). Gastrointestinal hemorrhage is caused by inhibition of platelet aggregation as well as the inhibition of the vasoconstrictive response to bleeding (Pahor et al., 1996). These effects might also be responsible for hematocele in fish.

4.3. Sub-chronic toxicity test

4.3.1. Sub-chronic toxicity

Long-term exposure to verapamil at concentrations of 0.463–463 $\mu g\ L^{-1}$ had no effect on the survival and morphometric parameters (length, relative weight after fixation, and FCF) of the carp embryos and larvae in the present study. Similarly in a study by Overturf et al. (2011) that exposed fathead minnow embryos and larvae (*P. promelas*) to verapamil (600 $\mu g\ L^{-1}$) for 28 days, no effect on mortality was observed; however, verapamil exposure caused a significant reduction in dry weight. In the present study, the occurrence of macroscopic morphological anomalies appeared to be the most sensitive endpoint that was analyzed. Verapamil exposure resulted in a significantly higher occurrence of malformations, and edema with hematocele, in fish exposed to the two highest verapamil concentrations (46.3 and 463 $\mu g\ L^{-1}$) compared to the control. However, neither of the studied endpoints showed any effect of verapamil at environmentally relevant concentrations on carp larvae and embryos.

4.3.2. Bioconcentration factor

In the present study, the bioconcentration factor (BCF) of verapamil in fish homogenates ranged between 6.6 and 16.6. To the best of our knowledge, there are no other experimental data available on BCF in fish tissues or fish homogenates. Predicted BCF for whole fish is 3.2

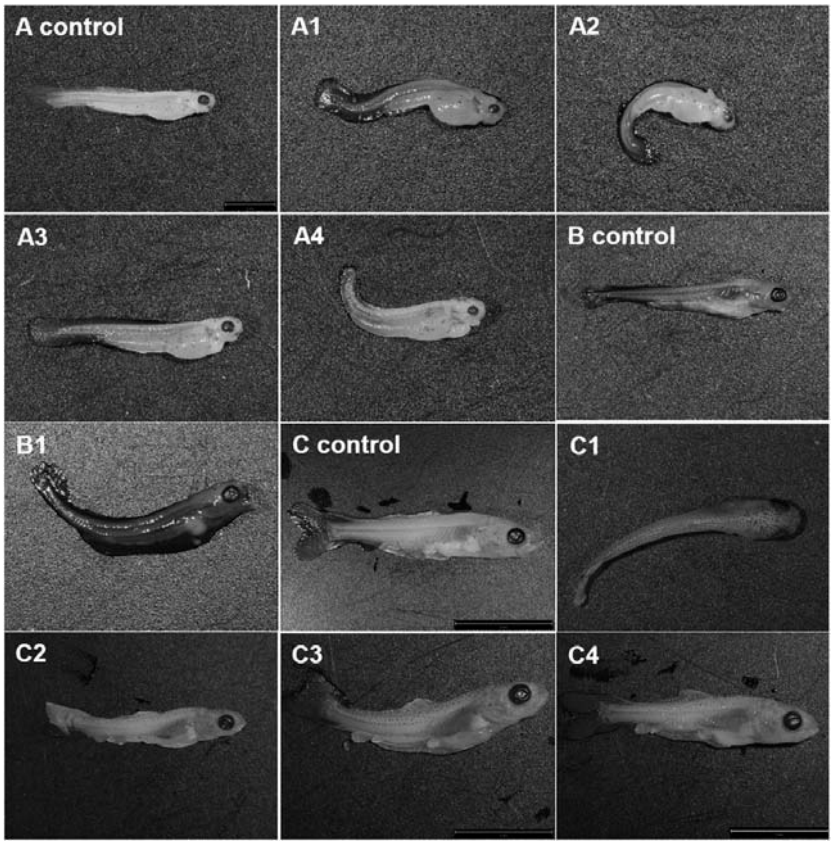

Fig. 2. Types of deformed embryos and larvae of common carp at the conclusion of the 96-h acute toxicity tests with verapamil; A — normal embryo (control, E9), A1 — axial curvature, kyphosis (5 mg L^{-1}, E9), A2 — deformed skull, axial curvature; kyphosis lateral spinal curvature; scoliosis, yolk sac edema (10 mg/l, E9), A3 — yolk sac edema, cardiac edema (1 mg L^{-1}, E9), A4 — cardiac edema, yolk sac edema, spinal curvature; c – shape (10 mg L^{-1}, E9), B — normal larva (control, L2), B1 — cranial malformation, cardiac edema, visceral edema, spinal curvature (5 mg L^{-1}, L2), C — normal larva (control, L5), C1 — lateral spinal curvature, scoliosis (2 mg L^{-1}, L5), C2 — visceral edema, spinal curvature; lordosis (5 mg L^{-1}, L5), C3 — visceral edema, axial spinal curvature; c – shape (5 mg L^{-1}, L5), C4 — visceral edema (5 mg L^{-1}, L5).

(based on log P = 3.4, calculated by EPI Suite™ v4.11 – US EPA, 2012) which is in agreement with our experimental values. Moreover, we can compare our values only with those found in fish blood plasma. Namely, Fick et al. (2010b) reported that the plasma of rainbow trout exposed to waste water containing verapamil had a BCF ranging from <33 to 175, whereas the predicted BCF value of the plasma of fish was 40 (based on log P = 3.4; Fick et al., 2010b). The BCF determined in the present study is lower, but follows the same order of magnitude. A BCF of <500 in fish is considered to be indicative of a low level of bioconcentration in the given substance (OECD, 2001).

Although the plasma BCF seems to be low, verapamil could reach dangerous levels in fish plasma. In accordance with "fish plasma model" proposed by Huggett et al. (2003), Fick et al. (2010a,b) calculated critical environmental concentrations (CECs) of verapamil causing fish plasma concentration equal to the therapeutic human plasma level (10 ng mL^{-1}; Schulz and Schmoldt, 2003). Based on predicted and measured blood BCFs, they estimated CECs at 230 and 53 ng L^{-1}, respectively (Fick et al., 2010a,b). Therefore, both similar pharmacological effect of verapamil in man and fish and relatively low critical environmental concentrations indicate a high risk of verapamil to fish.

4.3.3. Depuration of verapamil

The half-life of verapamil in carp larvae was estimated to be 10.2 days, which is much longer than the half-life in humans (4.5–12 h; Wood et al., 1999). Similar results were obtained for fluoxetine (Paterson and Metcalfe, 2008), which had a half-life three times greater when exposed in medaka compared to mammalian species. The longer half-life of verapamil in fish compared to humans implies the existence of differences in the biotransformation and/or excretion of the substance between these species. Verapamil undergoes extensive first-pass elimination in the liver of humans; therefore, only 20–30% of orally administrated verapamil is bioavailable (Eichelbaum et al., 1984). Similarly, Luurtsema et al. (2005)) who systemically administrated (by injection) verapamil to rats observed an intensive metabolism of this substance in liver (88.9% 60 h post-injection). Verapamil undergoes extensive oxidative metabolism in both humans and rats mediated by

Table 5
Total length (TL), relative mass after fixation (w), and Fulton's condition factor (FCF) of the common carp larvae during the 31-day embryo-larval toxicity test with verapamil. Values are expressed as the mean ± SD.

Sampling time		Control	Group 1	Group 2	Group 3	Group 4
			0.463 μg L^{-1}	4.63 μg L^{-1}	46.3 μg L^{-1}	463 μg L^{-1}
Day 10	TL (mm)	7.7 ± 0.3	7.5 ± 0.5	7.3 ± 0.4	7.5 ± 0.5	7.2 ± 0.3
	w (mg)	3.0 ± 0.4	2.7 ± 0.4	2.1 ± 0.5	2.8 ± 0.5	2.0 ± 0.4
	FCF	0.7 ± 0.1	0.7 ± 0.2	0.5 ± 0.1	0.9 ± 0.1	0.5 ± 0.1
Day 15	TL (mm)	8.6 ± 0.4	8.8 ± 0.5	8.8 ± 0.6	8.8 ± 0.5	8.8 ± 0.6
	w (mg)	4.2 ± 1.1	4.7 ± 0.8	4.7 ± 1.1	4.67 ± 1.2	5.0 ± 1.1
	FCF	0.7 ± 0.2	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
Day 20	TL (mm)	10.1 ± 0.7	10.3 ± 0.6	10.0 ± 0.6	10.3 ± 1.0	10.2 ± 0.9
	w (mg)	8.6 ± 2.5	9.3 ± 2.2	8.7 ± 1.8	9.5 ± 2.7	8.9 ± 2.2
	FCF	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.2
Day 24	TL (mm)	11.8 ± 0.7	12.0 ± 0.8	12.4 ± 0.9	12.3 ± 0.8	12.35 ± 0.9
	w (mg)	20.0 ± 5.5	16.8 ± 4.9	20.7 ± 5.2	16.7 ± 4.4	19.1 ± 5.4
	FCF	1.2 ± 0.2	1.0 ± 0.3	1.1 ± 0.11	0.9 ± 0.10	1.0 ± 0.2
Day 29	TL (mm)	14.8 ± 1.47	14.5 ± 0.91	14.8 ± 1.0	14.5 ± 1.5	14.7 ± 1.5
	w (mg)	35.0 ± 12.0	39.4 ± 2.9	38.5 ± 9.3	38.7 ± 12.4	41.7 ± 12.5
	FCF	1.5 ± 0.1	1.4 ± 1.6	1.2 ± 0.2	1.2 ± 0.1	1.3 ± 0.2
Day 31	TL (mm)	16.7 ± 1.0	15.9 ± 1.1	16.2 ± 1.1	16.3 ± 1.7	15.9 ± 1.3
	w (mg)	56.7 ± 12.4	45.5 ± 10.6	53.4 ± 11.9	56.1 ± 21.5	54.0 ± 15.7
	FCF	1.2 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1

several cytochrome P450 isoenzymes whereas the main pathways are N-dealkylation, N-demethylation and O-demethylation (Reder-Hilz et al., 2004). However, the metabolism of verapamil in fish is not yet fully understood. On the one hand, in a study by Alderton et al. (2010), verapamil produced similar metabolic products in zebrafish larvae and humans, indicating similarities in their metabolism. On the other hand, Burkina et al. (2012) reported that in contrast to humans, long-term exposure of verapamil to rainbow trout had no significant effect on the activity of the selected CYP450 enzymes that are involved in the metabolism of verapamil and other pharmaceuticals in humans. It is possible that other biotransformation enzymes might be involved in fish; however, the biotransformation and/or elimination of verapamil were far slower in fish than in humans.

5. Conclusion

In summary, the effects caused by verapamil in fish can be considered similar to the therapeutic effects and side effects that are found in humans (reduction of heart rate, bradycardia, peripheral edema and gastrointestinal hemorrhage). These similarities in the mode of action can be explained by the high structural similarities of L-type calcium channels in fish and humans (Rottbauer et al., 2001).

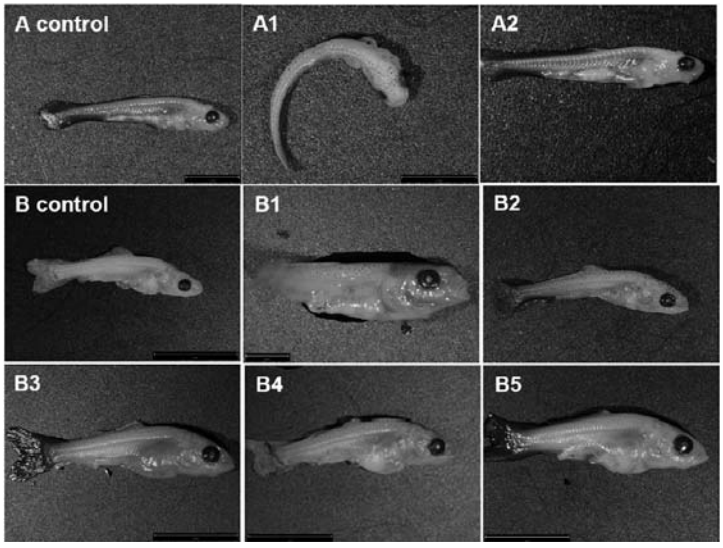

Fig. 3. Types of deformed larvae of common carp during the 31-day embryo-larval toxicity test with verapamil; A — normal larva (control, L4), A1 — axial curvature; kyphosis, underdeveloped eye (46.3 μg L^{-1}, L4), A2 — intestine edema (46.3 μg L^{-1}, L4), B — normal larva (control, L5), B1 — deformed skull (4.36 μg L^{-1}, L5), B2 — axial curvature; kyphosis (46.3 μg L^{-1}, L5), B3 — visceral edema with haematocele (46.3 μg L^{-1}, L5), B4 — coelom edema (463 μg L^{-1}, L5), B5 — intestine deformation; strangulation and dilatation (463 μg L^{-1}, L5).

Table 6
Developmental stages (steps, DS) of the larval period and occurrence of macroscopic morphological anomalies during the 31-day embryo-larval toxicity test on common carp. MA — sum of all malformations, EH — edema and hematocele. Asterisks indicate a significant difference compared to the control: * $p < 0.05$, ** $p < 0.01$ (χ^2 test).

Groups/Sampling Time		Day	Day	Day	Day	Day	Day	Σ
		10	15	20	24	29	31	
Control	DS	L3-L4	L4	L5	L5	L6	L6	–
	MA (%)	0	3.6	0	0	0	0	0.6
	EH (%)	0	0	0	7.7	0	0	1.3
Group 1	DS	L3-L4	L4	L5	L5	L6	L6	–
0.463 μg L^{-1}	MA (%)	7.1	0	3.4	3.2	0	0	2.4
	EH (%)	0	0	0	6.5	0	0	1.2
Group 2	DS	L3-L4	L4	L5	L5-L6	L6	L6	–
4.63 μg L^{-1}	MA (%)	0	0	0	0	3.5	0	0.6
	EH (%)	0	0	0	30.8*	0	0	4.7
Group 3	DS	L3-L4	L4	L2-L5	L5	L6	L6-J1	–
46.3 μg L^{-1}	MA (%)	7.4	11.1	3.7	3.7	0	0	4.2*
	EH (%)	3.7	0	0	48.1**	3.6	0	9.0*
Group 4	DS	L3-L4	L4	L4-L5	L4-L6	L5-L6	L6	–
463 μg L^{-1}	MA (%)	8	3.4	16.0*	4.2	3.3	0	5.6*
	EH (%)	0	3.4	0	37.5*	0	0	6.2*

Fish exposed sub-chronically to verapamil at a concentration of 4.63–463 μg L^{-1} appeared to bioconcentrate the pharmaceutical at a low level (BCF ranging between 6.6 and 16.6), but the half-life was relatively long (10.2 days) compared to that in humans, indicating the slow rate of biotransformation and/or elimination of verapamil in fish. Although we did not observe any effects of verapamil on studied endpoints at environmentally relevant concentration in the present study, we cannot exclude the possibility that it might affect fish after a longer exposure.

Acknowledgments

This study was funded by the CENAKVA project CZ.1.05/2.1.00/01.0024; project USB (GAJU) no. 047/2010/Z, project USB (GAJU) no. 087/2013/Z, Czech Science Foundation no. P503/11/1130 project and the project of the Ministry of Agriculture of the Czech Republic no. QH82117.

References

Alderton W, Berghmans S, Butler P, Chassaing H, Fleming A, Golder Z, et al. Accumulation and metabolism of drugs and CYP probe substrates in zebrafish larvae. Xenobiotica 2010;40:547–57.
Batt AL, Kostich MS, Lazorchak JM. Analysis of ecologically relevant pharmaceuticals in wastewater and surface water using selective solid-phase extraction and UPLC–MS/MS. Anal Chem 2008;80:5021–30.
Berghmans S, Butler P, Goldsmith P, Waldron G, Gardner I, Golder Z, et al. Zebrafish based assays for the assessment of cardiac, visual and gut function — potential safety screens for early drug discovery. J Pharmacol Toxicol Methods 2008;58:59–68.
Burgess A, Vere DW. Teratogenic effects of some calcium channel blocking agents in *Xenopus* embryos. Pharmacol Toxicol 1989;64:78–82.
Burkina V, Zamaratskaia G, Randak T, Li ZH, Fedorova G, Pickova J, et al. Verapamil does not modify catalytic activity of CYP450 in rainbow trout after long-term exposure. Ecotoxicol Environ Saf 2012;79:148–52.
Cook JT, McNiven MA, Richardson GF, Sutterlin AM. Growth rate, body composition and feed digestibility/conversion of growth-enhanced transgenic Atlantic salmon (*Salmo salar*). Aquaculture 2000;188:15–32.
Corcoran J, Winter MJ, Tyler CR. Pharmaceuticals in the aquatic environment: a critical review of the evidence for health effects in fish. Crit Rev Toxicol 2010;40:287–304.
Drenth JF, Michiels JJ, Van Joost T, Vuzevski VD. Verapamil-induced secondary erythermalgia. Br J Dermatol 1992;127:292–4.
Eichelbaum M, Mirkus G, Vogelgesang B. Pharmacokinetics of (+)-, (-)- and (+-)-verapamil after intravenous administration. Br. J. clin. Pharmac. 1984;17:453–8.
Fent K, Weston AA, Caminada D. Ecotoxicology of human pharmaceuticals. Aquat Toxicol 2006;76:122–59.
Fick J, Lindberg RH, Tysklind M, Larsson DG. Predicted critical environmental concentrations for 500 pharmaceuticals. Regul Toxicol Pharmacol 2010a;58:516–23.
Fick J, Lindberg RH, Parkkonen J, Arvidsson B, Tysklind M, Larsson DGJ. Therapeutic levels of levonorgestrel detected in blood plasma of fish: results from screening rainbow trout exposed to treated sewage effluents. Environ Sci Technol 2010b;44:2661–6.
Franke C. How meaningful is the bioconcentration factor for risk assessment? Chemosphere 1996;32:1897–905.
Gomez CF, Constantine L, Huggett DB. The influence of gill and liver metabolism on the predicted bioconcentration of three pharmaceuticals in fish. Chemosphere 2010;81:1189–95.
Grossman E, Messerli FH. Calcium antagonists. Prog Cardiovasc Dis 2004;47:34–57.
Gunnarsson L, Jauhiainen A, Kristiansson E, Nerman O, Larsson DGJ. Evolutionary conservation of human drug targets in organisms used for environmental risk assessments. Environ Sci Technol 2008;42:5807–13.
Huggett DB, Cook JC, Ericson JF, Williams RT. A theoretical model forutilizing mammalian pharmacology and safety data to prioritize potentialimpacts of human pharmaceuticals to fish. Hum. Ecol. Risk Assess. 2003;9:1789–99.
Hummel D, Löffler D, Fink G, Ternes TA. Simultaneous determination of psychoactive drugs and their metabolites in aqueous matrices by liquid chromatography mass spectrometry. Environ Sci Technol 2006;40:7321–8.
Jezierska B, Lugowska K, Witeska M, Sarnowski P. Malformations of newly hatched common carp larvae. Electron J Pol Agric Univ 2000;3.
Johnston A, Burgess CD, Hamer J. Systemic availability of oral verapamil and effect on PR interval in man. Br J Clin Pharmacol 1981;12:397–400.
Khan SJ, Ongerth JE. Modelling of pharmaceutical residues in Australian sewage by quantities of use and fugacity calculations. Chemosphere 2004;54:355–67.
Khetan SK, Collins TJ. Human pharmaceuticals in the aquatic environment: a challenge to green chemistry. Chem Rev 2007;107:2319–64.
Kocour M, Gela D, Rodina M, Linhart O. Testing of performance in common carp *Cyprinus carpio* L. under pond husbandry conditions I: top-crossing with Northern mirror carp. Aquacult Res 2005;36:1207–15.
Kristoffersen JB, Salvanes AGV. Effects of formaldehyde and ethanol preservation on body and otoliths of *Maurolicus muelleri* and *Benthosema glaciale*. Sarsia 1998;83:95-102.
Kroupova H, Prokes M, Macova S, Penaz M, Barus V, Novotny L, et al. Effect of nitrite on early-life stages of common carp (*Cyprinus carpio* L.). Environ Toxicol Chem 2010;29:535–40.
Kvarnryd M, Grabic R, Brandt I, Berg C. Early life progestin exposure causes arrested oocyte development, oviductal agenesis and sterility in adult *Xenopus tropicalis* frogs. Aquat Toxicol (Amst Neth) 2011;103:18–24.
Lacinova L, Welling A, Bosse E, Ruth P, Flockerzi V, Hofmann F. Interaction of Ro 40-5967 and verapamil with the stably expressed alpha 1-subunit of the cardiac L-type calcium channel. J Pharmacol Exp Ther 1995;274:54–63.
Li ZH, Li P, Randak T. Ecotoxocological effects of short-term exposure to a human pharmaceutical verapamil in juvenile rainbow trout (*Oncorhynchus mykiss*). Comp Biochem Physiol C: Toxicol Pharmacol 2010a;152:385–91.
Li ZH, Velisek J, Zlabek V, Grabic R, Machova J, Kolarova J, et al. Chronic toxicity of Verapamil on juvenile rainbow trout (*Oncorhynchus mykiss*): effects on morphological indices; hematological parameters and antioxidant responses. J Hazard Mater 2010b;185:870–80.
Lüllmann H, Mohr K, Wehling M. Pharmakologie und Toxikologie: Thieme, Stuttgart,15. komplett überarbeitete Auflage; 2003. [32, 115].
Luurtsema G, Molthoff CFM, Schuit RC, Windhorst AD, Lammertsma AA, Franssen EJF. Evaluation of (R)-[11C]verapamil as PET tracer of P-glycoprotein function in the blood–brain barrier: kinetics and metabolism in the rat. Nucl Med Biol 2005;32:87–93.
Machova J, Prokeš M, Kroupová H, Svobodová Z, Máčová S, Doleželová P, et al. Early ontogeny, growth and mortality of common carp (*Cyprinus carpio*) at low concentrations of dimethyl sulfoxide. Acta Vet Brno 2009;78:505–12.
Mackay D, Fraser A. Bioaccumulation of persistent organic chemicals: mechanisms and models. Environ Pollut 2000;110:375–91.
Mehinto AC, Hill EM, Tyler CR. Uptake and biological effects of environmentally relevant concentrations of the nonsteroidal anti-inflammatory pharmaceutical diclofenac in rainbow trout (*Oncorhynchus mykiss*). Environ Sci Technol 2010;44:2176–82.
Nallani GC, Paulos PM, Constantine LA, Venables BJ, Huggett DB. Bioconcentration of ibuprofen in fathead minnow (*Pimephales promelas*) and channel catfish (*Ictalurus punctatus*). Chemosphere 2011;84:1371–7.
Nash JP, Kime DE, Van der Ven LTM, Wester PW, Brion F, Maack G, et al. Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. Environ Health Perspect 2004;112:1725–33.
Nordberg A, Rudén C. The usefulness of the bioconcentration factor as a tool for priority setting in chemicals control. Toxicol Lett 2007;168:113–20.
OECD, Organization for Economic Cooperation and Development. Guidelines for the testing of chemicals. Section 2: Effects on Biotic Systems TG- No. 210: Fish, Early-Life Stage Toxicity Test. Paris, France: Organization for Economic Co-operation and Development; 1992.
OECD, Organization for Economic Cooperation and Development. Guidelines for the testing of chemicals. Section 2: Effects on Biotic Systems TG- No. 305: Bioconcentration: Flow-through Fish Test. Paris, France: Organization for Economic Co-operation and Development; 1996.
OECD, Organization for Economic Cooperation and Development. Guidelines for the testing of chemicals. TG No. 212. Fish, short-term toxicity test on embryo and sac-fry stages. Paris, France: Organization for Economic Co-operation and Development; 1998.
OECD, Organization for Economic Cooperation and Development. Harmonized integrated hazard classification system for human health and environmental hazards of chemical substances and mixtures. [8]Annex 2. Guidance Document 27, ENV/JM/MONO (2001). Paris, France: Organization for Economic Co-operation and Development; 2001.

OECD, Organization for Economic Cooperation and Development. Guidelines for the testing of chemicals. [TG No. 231]The amphibian metamorphosis assay. Paris, France: Organization for Economic Co-operation and Development; 2009.
Overturf MD, Overturf CL, Baxter D, Hala DN, Constantine L, Venables B, et al. Early life-stage toxicity of eight pharmaceuticals to the Fathead Minnow, *Pimephales promelas*. Arch Environ Contam Toxicol 2011;62:455–64.
Pahor M, Guralnik JM, Furberg CD, Carbonin P, Havlik RJ. Risk of gastrointestinal haemorrhage with calcium antagonists in hypertensive persons over 67 years old. Lancet 1996;347:1061–5.
Paterson G, Metcalfe CD. Uptake and depuration of the anti-depressant fluoxetine by the Japanese medaka (*Oryzias latipes*). Chemosphere 2008;74:125–30.
Penaz M, Prokes M, Kouril J, Hamackova J. Early development of the carp *Cyprinus carpio*. Acta Sci Nat Brno 1983;17:1-39.
Ramirez AJ, Brain RA, Usenko S, Mottaleb MA, O'Donnell JG, Stahl LL, et al. Occurrence of pharmaceuticals and personal care products in fish: results of a national pilot study in the United States. Environ Toxicol Chem 2009;28:2587–97.
Regenthal R, Krueger M, Koeppel C, Preiss R. Drug levels: Therapeutic and toxic serum/plasma concentrations of common drugs. J ClinMonit 1999;15:529–44.
Reder-Hilz B, Ullrich M, Ringel M, Hewitt N, Utesch D, Oesch F, et al. Metabolism of propafenone and verapamil by cryopreserved human, rat. mouse and dog hepatocytes: comparison with metabolism in vivo. Naunyn Schmiedebergs Arch Pharmacol 2004;369:408–17.
The toxicology of fishes. In: Richard T, Giulio D, Hinton DE, editors. New York: CRC Press; 2008. p. 4.
Rottbauer W, Baker K, Wo ZG, Mohideen M-APK, Cantiello HF, Fishman MC. Growth and function of the embryonic heart depend upon the cardiac-specific L-type calcium channel [alpha]1 subunit. Dev Cell 2001;1:265–75.
Schulz M, Schmoldt A. Therapeutic and toxic blood concentrations of more than 800 drugs and other xenobiotics. Pharmazie 2003;58:447–74.
Shin JT, Pomerantsev EV, Mably JD, MacRae CA. High-resolution cardiovascular function confirms functional orthology of myocardial contractility pathways in zebrafish. Physiol Genomics 2010;42:300–9.
Springer TA, Guiney PD, Krueger HO, Jaber MJ. Assessment of an approach to estimate aquatic bioconcentration factor using reduced sampling. Environ Toxicol Chem 2008;27:2271–80.
Sudova E, Machova J, Svobodova Z, Vesely T. Negative effects of malachite green and possibilities of its replacement in the treatment of fish eggs and fish: a review. Vet Med Praha 2007;52:527-539527.
US EPA. Estimation Programs Interface Suite™ for Microsoft® Windows, v 4.11. United States Environmental Protection Agency, Washington, DC, USA. http://www.epa.gov/opptintr/exposure/pubs/episuite.htm2012.
Villegas-Navarro A, Rosas-L E, Reyes JL. The heart of *Daphnia magna*: effects of four cardioactive drugs. Comp Biochem Physiol C: Toxicol Pharmacol 2003;136:127–34.
Wood AJJ, Abernethy DR, Schwartz JB. Calcium-antagonist drugs. N Eng J Med 1999;341:1447–57.

Ecotoxicology and Environmental Safety 96 (2013) 41–47

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

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

Katerina Grabicova [a,*], Ganna Fedorova [a], Viktoriia Burkina [a], Christoph Steinbach [a], Heike Schmidt-Posthaus [b], Vladimir Zlabek [a], Hana Kocour Kroupova [a], Roman Grabic [a], Tomas Randak [a]

[a] University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zatisi 728/II, 389 25 Vodnany, Czech Republic
[b] Centre for Fish and Wildlife Health, Institute of Animal Pathology, University of Berne, Laenggassstrasse 122, P.O. Box 8466, 3001 Berne, Switzerland

ARTICLE INFO

Article history:
Received 17 October 2012
Received in revised form
6 June 2013
Accepted 15 June 2013
Available online 29 July 2013

Keywords:
Rainbow trout
Chronic toxicity test
UV filters
2-Phenylbenzimidazole-5-sulfonic acid
Surface water

ABSTRACT

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

1. Introduction

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

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

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

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

* Corresponding author.
E-mail address: grabicova@frov.jcu.cz (K. Grabicova).

0147-6513/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ecoenv.2013.06.022

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

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

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

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

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

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

2. Materials and methods

2.1. Chemicals

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

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

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

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

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

2.2. Analytical system

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

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

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

2.4. Fish exposure experiment

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

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

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

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

2.5. Fish and water sampling

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

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

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

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

2.7. Liver microsome preparation and microsomal enzyme activity analysis

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

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

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

2.8. Antioxidant parameters

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

2.9. Biochemical and hematological parameters

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

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

2.10. Histology

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

2.11. Statistical analyses

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

3. Results and discussion

3.1. The presence of UV filters in surface waters

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

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

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

3.2. Accumulation of PBSA in fish

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

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

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

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

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

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

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

3.3. Cytochrome activity in fish liver

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

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

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

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

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

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

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

3.4. Oxidative stress in fish liver after exposure to PBSA

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

3.5. Biochemical and hematological parameters

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

Table 3
Activity of CYP450 enzymes in rainbow trout liver.

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

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

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

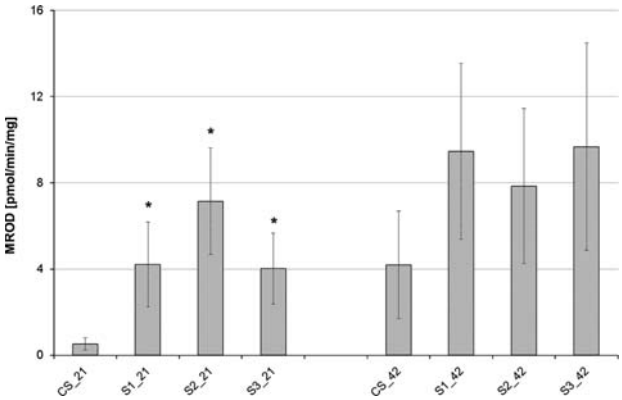

Fig. 1. Activity of 7-methoxyresorufin-*O*-deethylase (MROD) [pmol/min/mg]. CS – control group; S1 – concentration of PBSA 1 μg L^{-1}; S2 – concentration of PBSA 10 μg L^{-1}; S3 – concentration of PBSA 1000 μg L^{-1}; 21 – sampling after 21 days; 42 – sampling after 42 days. Significant differences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

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

Enzymes	Units	Tested groups	
		Control	S3
CAT	μmol H_2O_2min^{-1}mg^{-1}	0.035 ± 0.0055	0.041 ± 0.0077
SOD	nmol NBTmin^{-1}mg^{-1}	0.0074 ± 0.0021	0.0069 ± 0.0012
GR	nmol NADPHmin^{-1}mg^{-1}	0.040 ± 0.010	0.027 ± 0.0082*

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

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

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

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

3.6. Histology

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

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

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

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

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

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

4. Conclusions

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

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

Acknowledgments

This study was supported by the CENAKVA CZ.1.05/2.1.00/01.0024 project and the Grant Agency of Czech Republic P503/11/1130. We thank Elsevier Language Editing for their editing services.

Appendix A. Supporting information

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

References

Balmer, M.E., Buser, H.R., Muller, M.D., Poiger, T., 2005. Occurrence of some organic UV filters in wastewater, in surface waters, and in fish from Swiss lakes. Environ. Sci. Technol. 39, 953–962.

Bluthgen, N., Zucchi, S., Fent, K., 2012. Effects of the UV filter benzophenone-3 (oxybenzone) at low concentrations in zebrafish (Danio rerio). Toxicol. Appl. Pharmacol. 263, 184–194.

Bucheli, T.D., Fent, K., 1995. Induction of cytochrome-P450 as a biomarker for environmental contamination in aquatic ecosystems. Crit. Rev. Environ. Sci. Technol. 25, 201–268.

Burkina, V., Zamaratskaia, G., Randak, T., Li, Z.H., Fedorova, G., Pickova, J., Zlabek, V., 2012. Verapamil does not modify catalytic activity of CYP450 in rainbow trout after long-term exposure. Ecotox. Environ. Safe 79, 148–152.

Buser, H.R., Balmer, M.E., Schmid, P., Kohler, M., 2006. Occurrence of UV filters 4-methylbenzylidene camphor and octocrylene in fish from various swiss rivers with inputs from wastewater treatment plants. Environ. Sci. Technol. 40, 1427–1431.

Carlberg, I., Mannervik, B., 1975. Purification and characterization of flavoenzyme glutathione reductase from rat-liver. J. Biol. Chem. 250, 5475–5480.

Daughton, C.G., Ternes, T.A., 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change? Environ. Health Perspect. 107, 907–938.

El-Sayed, Y.S., Saad, T.T., El-Bahr, S.M., 2007. Acute intoxication of deltamethrin in monosex Nile tilapia, Oreochromis niloticus with special reference to the clinical, biochemical and haematological effects. Environ. Toxicol. Pharmacol. 24, 212–217.

Ewing, J.F., Janero, D.R., 1995. Microplate superoxide dismutase assay employing a nonenzymatic superoxide generator. Anal. Biochem. 232, 243–248.

Fedorova, G., Nebesky, V., Randak, T., Grabic, R. Simultaneous determination of 32 antibiotics in aquaculture products using LC–MS/MS. Chem. Papers, http://dx.doi.org/10.2478/s11696-013-0428-3, in press.

Fent, K., 2003. Ecotoxicological problems associated with contaminated sites. Toxicol. Lett. 140, 353–365.

Fent, K., Kunz, P.Y., Gomez, E., 2008. UV filters in the aquatic environment induce hormonal effects and affect fertility and reproduction in fish. Chimia 62, 368–375.

Fent, K., Zenker, A., Rapp, M., 2010. Widespread occurrence of estrogenic UV-filters in aquatic ecosystems in Switzerland. Environ. Pollut. 158, 1817–1824.

Gago-Ferrero, P., Diaz-Cruz, M.S., Barcelo, D., 2011. Occurrence of multiclass UV filters in treated sewage sludge from wastewater treatment plants. Chemosphere 84, 1158–1165.

Gago-Ferrero, P., Diaz-Cruz, M.S., 2012. An overview of UV-absorbing compounds (organic UV filters) in aquatic biota. Anal. Bioanal. Chem. 404, 2597–2610.

Gasparro, F.P., 2000. Sunscreens, skin photobiology, and skin cancer: The need for UVA protection and evaluation of efficacy. Environ. Health Perspect. 108, 71–78.

Gasparro, F.P., Mitchnick, M., Nash, J.F., 1998. A review of sunscreen safety and efficacy. Photochem. Photobiol. 68, 243–256.

Hasler, J.A., Estabrook, R., Murray, M., Pikuleva, I., Waterman, M., Capdevila, J., Holla, V., Helvig, C., Falck, J.R., Farrell, G., Kaminsky, L.S., Spivack, S.D., Bioitier, E., Beaune, P., 1999. Human cytochromes P450. Mol. Aspects Med. 20, 1–137.

Jonsson, E.M., Abrahamson, A., Brunstrom, B., Brandt, I., 2006. Cytochrome P4501A induction in rainbow trout gills and liver following exposure to waterborne indigo, benzo a pyrene and 3,3′,4,4′,5-pentachlorobiphenyl. Aquat. Toxicol. 79, 226–232.

Kennedy, S.W., Jones, S.P., 1994. Simultaneous measurement of cytochrome P4501A catalytic activity and total protein-concentration with a fluorescence plate reader. Anal. Biochem. 222, 217–223.

Leal, L.H., Vieno, N., Temmink, H., Zeeman, G., Buisman, C.J.N., 2010. Occurrence of xenobiotics in gray water and removal in three biological treatment systems. Environ. Sci. Technol. 44, 6835–6842.

Li, Z.H., Zlabek, V., Grabic, R., Li, P., Machova, J., Velisek, J., Randak, T., 2010a. Effects of exposure to sublethal propiconazole on intestine-related biochemical responses in rainbow trout, *Oncorhynchus mykiss*. Chem. Biol. Interact. 185, 241–246.

Li, Z.H., Velisek, J., Zlabek, V., Grabic, R., Machova, J., Kolarova, J., Randak, T., 2010b. Hepatic antioxidant status and hematological parameters in rainbow trout, *Oncorhynchus mykiss*, after chronic exposure to carbamazepine. Chem. Biol. Interact. 183, 98–104.

Li, Z.H., Zlabek, V., Velisek, J., Grabic, R., Machova, J., Kolarova, J., Li, P., Randak, T., 2011. Acute toxicity of carbamazepine to juvenile rainbow trout (*Oncorhynchus mykiss*): effects on antioxidant responses, hematological parameters and hepatic EROD. Ecotox. Environ. Safe 74, 319–327.

Meunier, B., de Visser, S.P., Shaik, S., 2004. Mechanism of oxidation reactions catalyzed by cytochrome P450 enzymes. Chem. Rev. 104, 3947–3980.

Nagtegaal, M., Ternes, T.A., Baumann, W., Nagel, R., 1997. UV-Filtersubstanzen in Wasser und Fischen. Umweltwiss. Schadstoff-Forschung. 9, 79–86.

Nebert, D.W., Dalton, T.P., Okey, A.B., Gonzalez, F.J., 2004. Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. J. Biol. Chem. 279, 23847–23850.

Nilsen, B.M., Berg, K., Goksoyr, A., 1998. Induction of cytochrome P450 1A (CYP1A) in fish a biomarker for environmental pollution. In: Phillips, I.R., Shephard, E.A. (Eds.), Cytochrome P450 Protocols, Methods in Molecular Biology, volume 107. Humana Press Inc., New Jersey, pp. 423–438.

Ochi, T., 1990. Effects of an organic hydroperoxide on the activity of antioxidant enzymes in cultured mammalian cells. Toxicology 61, 229–239.

Omura, T., Sato, R., 1964. Carbon Monoxide-Binding Pigment of Liver Microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem. 239, 2370–2378.

Paskova, V., Adamovsky, O., Pikula, J., Skocovska, B., Bandouchova, H., Horakova, J., Babica, P., Marsalek, B., Hilscherova, K., 2008. Detoxification and oxidative stress responses along with microcystins accumulation in Japanese quail exposed to cyanobacterial biomass. Sci. Total Environ. 398, 34–47.

Paskova, V., Paskerova, H., Pikula, J., Bandouchova, H., Sedlackova, J., Hilscherova, K., 2011. Combined exposure of Japanese quails to cyanotoxins, Newcastle virus and lead: oxidative stress responses. Ecotox. Environ. Safe 74, 2082–2090.

Petrovic, M., Gonzalez, S., Barcelo, D., 2003. Analysis and removal of emerging contaminants in wastewater and drinking water. Trac-Trends Anal. Chem. 22, 685–696.

Poiger, T., Buser, H.R., Balmer, M.E., Bergqvist, P.A., Muller, M.D., 2004. Occurrence of UV filter compounds from sunscreens in surface waters: regional mass balance in two Swiss lakes. Chemosphere 55, 951–963.

Portz, D.E., Woodley, C.M., Cech, J.J., 2006. Stress-associated impacts of short-term holding on fishes. Rev. Fish Biol. Fish. 16, 125–170.

Regulation, 2009. Regulation (EC) No 1223/2009of the European Parliament and of the Council of 30 November 2009 on cosmetic products. Official Journal of the European Union ⟨http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:342:0059:0209:en:PDF⟩.

Rodil, R., Quintana, J.B., Lopez-Mahia, P., Muniategui-Lorenzo, S., Prada-Rodriguez, D., 2008. Multiclass determination of sunscreen chemicals in water samples by liquid chromatography – Tandem mass spectrometry. Anal. Chem. 80, 1307–1315.

Rodil, R., Quintana, J.B., Concha-Grana, E., Lopez-Mahia, P., Muniategui-Lorenzo, S., Prada-Rodriguez, D., 2012. Emerging pollutants in sewage, surface and drinking water in Galicia (NW Spain). Chemosphere 86, 1040–1049.

Rousar, T., Parik, P., Kucera, O., Bartos, M., Cervinkova, Z., 2010. Glutathione reductase is inhibited by acetaminophen-glutathione conjugate in vitro. Physiol. Res. 59, 225–232.

Schlumpf, M., Cotton, B., Conscience, M., Haller, V., Steinmann, B., Lichtensteiger, W., 2001. In vitro and in vivo estrogenicity of UV screens. Environ. Health Perspect. 109, 239–244.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M. D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85.

Stara, A., Machova, J., Velisek, J., 2012. Effect of chronic exposure to simazine on oxidative stress and antioxidant response in common carp (*Cyprinus carpio L.*). Environ. Toxicol. Pharmacol. 33, 334–343.

Svobodova, Z., Pravda, D., Palackova, J., 1991. Unified methods of haematological examination of fish. Research Institute of Fish Culture and Hydrobiology, Vodnany p. 31, Methods No. 20.

Uno, T., Ishizuka, M., Itakura, T., 2012. Cytochrome P450 (CYP) in fish. Environ. Toxicol. Pharmacol. 34, 1–13.

Velisek, J., Svobodova, Z., 2004. Anaesthesia of rainbow trout (*Oncorhynchus mykiss*) with 2-phenoxyethanol: acute toxicity and biochemical blood profile. Acta Vet. BRNO 73, 379–384.

Verlicchi, P., Al Aukidy, M., Zambello, E., 2012. Occurrence of pharmaceutical compounds in urban wastewater: removal, mass load and environmental risk after a secondary treatment – a review. Sci. Total Environ. 429, 123–155.

Vessey, D.A., Lee, K.H., 1993. Inactivation of enzymes of the glutathione antioxidant system by treatment of cultured human keratinocytes with peroxides. J. Invest. Dermatol. 100, 829–833.

Wick, A., Fink, G., Ternes, T.A., 2010. Comparison of electrospray ionization and athmospheric pressure chemical ionization for multi-residues analysis of biocides, UV-filters and benzothiazoles in aqueous metrices and activated sludge by liquid chromatography–tandem mass spectrometry. J. Chromatogr. A 1217, 2088–2103.

Zucchi, S., Bluthgen, N., Ieronimo, A., Fent, K., 2011. The UV-absorber benzophenone-4 alters transcripts of genes involved in hormonal pathways in zebrafish (Danio rerio) eleuthero-embryos and adult males. Toxicol. Appl. Pharmacol. 250, 137–146.

CHAPTER 3

IMPACT OF EFFLUENTS FROM MUNICIPAL SEWAGE TREATMENT PLANTS TO AQUATIC ORGANISMS

3.1. Grabicova, K., Lindberg, R.H., Östman, M., Grabic, R., Randak, T., Larsson, D.G.J., Fick, J., 2014. Tissue-specific bioconcentration of antidepressants in fish exposed to effluent from a municipal sewage treatment plant. Science of the Total Environment 488–489, 46–50.

3.2. Grabicova, K., Grabic, R., Blaha M., Kumar, V., Cerveny, D., Fedorova, G., Randak, T., Presence of pharmaceuticals in benthic fauna living in a small stream affected by effluent from a municipal wastewater treatment plant. Water Research (submitted).

3.3. Fedorova, G., Randak, T., Golovko, O., Kodes, V., Grabicova, K., Grabic, R., 2014. A passive sampling method for detecting analgesics, psycholeptics, antidepressants and illicit drugs in aquatic environments in the Czech Republic. Science of the Total Environment 487, 681–687.

It was allowed by publisher on 9th May / 24th February 2014 to include the papers in this Ph.D. thesis.

According to the rules of the publisher it is allowed to include submitted manuscript in the author's and/or co-authors' Ph.D. theses. (http://www.elsevier.com/wps/find/journaldescription.cws_home/309?generatepdf=true)

Science of the Total Environment 488–489 (2014) 46–50

Contents lists available at ScienceDirect

Science of the Total Environment

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

Tissue-specific bioconcentration of antidepressants in fish exposed to effluent from a municipal sewage treatment plant

Katerina Grabicova [a,*], Richard H. Lindberg [b], Marcus Östman [b], Roman Grabic [a], Tomas Randak [a], D.G. Joakim Larsson [c], Jerker Fick [b]

[a] University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zatisi 728/II, CZ-389 25 Vodnany, Czech Republic
[b] Department of Chemistry, Umea University, SE-901 87 Umea, Sweden
[c] Department of Infectious Diseases, Institute of Biomedicine, University of Gothenburg, SE-413 46 Göteborg, Sweden

HIGHLIGHTS

- Antidepressants bioconcentrated in tissues of fish exposed to sewage effluent.
- Higher levels were found in brain and liver compared with muscle and blood plasma.
- Concentrations in brain were not far from mammalian therapeutic concentrations.
- Target-related effects on fish in effluent-dominated streams cannot be excluded.
- For some antidepressants, brain concentrations are valuable for assessing risk.

ARTICLE INFO

Article history:
Received 27 February 2014
Received in revised form 14 April 2014
Accepted 14 April 2014
Available online xxxx

Editor: D. Barcelo

Keywords:
Selective serotonin reuptake inhibitors
Caged fish
Rainbow trout
Liver
Brain
Venlafaxine

ABSTRACT

Tissue-specific bioconcentration of selected antidepressants was studied in rainbow trout (*Oncorhynchus mykiss*) exposed to undiluted effluent from a Swedish municipal sewage treatment plant for 13 days. Citalopram, sertraline and venlafaxine were found in the brains and livers of most fish, but not in blood plasma or muscle. Venlafaxine was the only drug found in plasma (3/20 fish). Fluoxetine was not detected in any fish tissue, in accordance with a low concentration in the effluent and a comparably high limit of quantification in tissues. Concentrations of citalopram, sertraline and venlafaxine in fish brain were up to 1/12, 1/8 and 1/26, respectively, of the lowest concentrations found in the brains of mammals treated with therapeutic doses. Thus, given co-exposure to several antidepressants and an assumed similar potency in fish, the margin of safety for target-related effects in fish residing in effluent-dominated streams is relatively low. Furthermore, the non-detectable levels of these drugs in blood plasma suggest that analyses of concentrations in target tissues (brain) would be more informative in field studies and other studies with environmentally realistic exposure concentrations.

1. Introduction

Pharmaceuticals form a diverse group of chemicals used in large quantities around the world. In contrast to the majority of chemicals used in society, pharmaceuticals are designed or selected to cause a biological effect in humans. After use, the active pharmaceutical ingredients (APIs) and/or their metabolites are excreted, reach municipal wastewaters and enter municipal sewage treatment plants (STPs) (Du et al., 2014; Halling-Sorensen et al., 1998; Verlicchi et al., 2012). STPs are not specifically designed to remove these substances during the treatment processes; hence, some pharmaceuticals end up in the environment and there will have the potential to affect non-target aquatic organisms (Heberer, 2002; Huerta et al., 2012; Hughes et al., 2013; Kidd et al., 2007; Petrovic et al., 2003; Santos et al., 2010; Verlicchi et al., 2012).

Pharmaceuticals can be divided into many groups according to their intended effect. A group that has lately received much attention due to risks for aquatic wildlife is antidepressants (Brodin et al., 2013; Lajeunesse et al., 2011). This group includes monoamine reuptake

Abbreviations: APIs, active pharmaceutical ingredients; CIT, citalopram; CR, concentration ratio; FLU, fluoxetine; SSRIs, selective serotonin reuptake inhibitors; SNRIs, serotonin–norepinephrine reuptake inhibitors; SER, sertraline; VEN, venlafaxine; STP, municipal sewage treatment plant.

* Corresponding author. Faculty of Fisheries and Protection of Waters, Zatisi 728/II, 389 25 Vodnany, Czech Republic. Tel.: +420 387774734.
E-mail address: grabicova@frov.jcu.cz (K. Grabicova).

http://dx.doi.org/10.1016/j.scitotenv.2014.04.052
0048-9697/

inhibitors, e.g. selective serotonin reuptake inhibitors (SSRIs), such as citalopram (CIT), fluoxetine (FLU) and sertraline (SER), and serotonin–norepinephrine reuptake inhibitors (SNRIs), such as venlafaxine (VEN).

Antidepressants are designed to treat depression, obsessive–compulsive disorder, panic disorder, etc., and the metabolic and pharmacokinetic properties of antidepressants have been presented in several publications (Aryal et al., 2012; DeVane, 1999; Hiemke and Hartter, 2000). According to these publications, CIT metabolizes to N-demethylcitalopram and N,N-didemethylcitalopram, which are much less active SSRIs than the parent compound. FLU metabolizes to norfluoxetin, which retains the SSRI activity. SER metabolizes mainly to desmethylsertraline, which has insignificant SSRI activity, and VEN metabolizes to O-desmethylvenlafaxine with the same SNRI potency as the parent compound. The mechanism of action includes blocking monoamine reuptake in the presynaptic cells, which leads to an increase in the concentration of serotonin and/or norepinephrine within the synapses (Fent et al., 2006). The chemical properties and occurrence of selected antidepressants in the environment are given in Table 1. The "critical environmental concentration" (CEC) for each drug is also indicated. This is the concentration in water that, based on the lipophilicity of the drug, is expected to result in a concentration in the fish blood similar to the human therapeutic blood plasma concentration (Fick et al, 2010).

Antidepressants in wastewater often degrade during the treatment processes in STPs (Vasskog et al., 2006), but residues are still detected in the STP effluent and in the aquatic environment at levels ranging from nanograms to lower micrograms per liter (Grabic et al., 2012; Schultzt and Furlong, 2008). There are reports suggesting that very low concentrations of SSRIs/SNRIs can affect biological processes, nervous and hormonal systems, reproduction and/or the behavior of fish and other aquatic organisms (Corcoran et al., 2010; de Lange et al., 2006; Henry et al., 2004; Valenti et al., 2012). Studies are however still scattered and more work is needed to define which water concentrations are required to cause biological effects in aquatic organisms. SSRIs can also decrease the Na/K-ATPase activity in the brain which has been proposed to be used as a biomarker for SSRIs (Lajeunesse et al., 2011).

There are many streams contaminated by municipal effluents where aquatic organisms are exposed to various xenobiotics, including antidepressants. Some antidepressants have been shown to accumulate in channel catfish (*Ictalurus punctatus*), black cappie (*Pomoxis nigromaculatus*), bluegill (*Lepomis macrochirus*), largemouth bass (*Micropterus salmoides*), common carp (*Cyprinus carpio*), white sucker (*Catostomus commersoni*), brown trout (*Salmo trutta*) or bull sharks (*Carcharhinus leucas*) (Brooks et al., 2005; Du et al., 2012; Gelsleichter and Szabo, 2013; Ramirez et al., 2007, 2009; Schultz et al., 2010) that live in effluent-dominated streams or estuaries, or in long-term tests where brook trout (*Salvelinus fontinalis*) were exposed to STP effluent (Lajeunesse et al., 2011). In some contrast to the results of the above-mentioned experiments, no bioconcentration or effects on fish behavior were observed during short-term aquaria tests with CIT (Holmberg et al., 2011). Similarly, no bioconcentration of SSRIs was found in fish from ponds receiving reclaimed wastewater (Wang and Gardinali, 2012) and in fathead minnow caged 10 m downstream the STP effluent (Metcalfe et al., 2010). The aim of this study was to describe the tissue-specific bioconcentration of SSRIs and venlafaxine in fish (rainbow trout) exposed to treated sewage effluent from a modern STP, thus containing concentrations of antidepressants and other unknown chemicals that would be realistic for an effluent-dominated stream. A comparison between tissue levels in fish and corresponding levels in humans taking the corresponding drug would allow a preliminary assessment of risk.

2. Material and methods

2.1. Chemicals

Liquid chromatography mass spectrometry (LC/MS) grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Formic acid (LC/MS grade) was obtained from Sigma-Aldrich (Steinheim, Germany). Internal standard stock solutions were prepared in methanol from mass-labeled compounds amitriptyline (D_6, CDN Isotopes, Canada) and trimethoprim ($^{13}C_3$, Cambridge Isotope Laboratories, USA). Stock solutions of citalopram hydrobromide (Sigma-Aldrich; no purity was stated), fluoxetine hydrochloride (Sigma-Aldrich; purity > 98%), sertraline hydrochloride (Sigma-Aldrich; purity > 98%) and venlafaxine hydrochloride (Sigma-Aldrich; purity > 98%) were prepared by dissolving the analytes in methanol.

2.2. Analytical system

A triple-stage quadrupole MS/MS TSQ Quantum Ultra EMR (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Accela LC pump (Thermo Fisher Scientific, San Jose, CA, USA) and a PAL HTC autosampler (CTC Analytics AG, Zwingen, Switzerland) operated using Xcalibur software (Thermo Fisher Scientific, San Jose, CA, USA) was used for the analysis of the fish tissue extracts and water. A detailed description of the method is given in Grabic et al. (2012).

2.3. Fish exposure experiment and sampling

Juvenile rainbow trout (*Oncorhynchus mykiss*) were purchased from Antens fiskodling AB, Alingsås, Sweden and acclimated to laboratory condition in aquaria with tap water at the Zoology Department, University of Gothenburg. The size of the fish ranged from 213 to 266 mm (total length) and 100 to 170 g (body weight). On the 19th of November 2009, the fish were transferred to the municipal STP of Gothenburg (Ryaverket), the second largest STP in Sweden. Here, the fish were exposed to final, treated effluent in an aerated 500-L tank with a flow rate of 7–10 L min^{-1} and a temperature of 11.7–13.3 °C. Oxygen saturation in the aquaria was always above 80% (8.2 mg L^{-1} and above) and the pH was approximately 7.5. Other, routine water quality parameters

Table 1
Characteristics of the investigated antidepressants, including lipophilicity (log K_{ow}), elimination half-life in humans, critical effect concentrations (CEC) in surface water and previously measured concentrations in the treated effluent from a range of Swedish municipal sewage treatment works.

	Citalopram	Sertraline	Venlafaxine	Fluoxetine
Log K_{OW}	3.74 [a]	5.29 [a], 2.9 [b]	3.28 [a], 0.5 [b]	4.05 [a]
Elimination half-life [h]	33 (23–45) [c]	26 (22–36) [c]	2 [d]	45 (24–144) [c]
CEC [ng L^{-1}] [e]	141	51	6112	489
Concentration in effluent from small sewage treatment plants in Sweden [ng L^{-1}] [f]	38–116	16–18	155–326	<LOQ–6.6

[a] Data from http://chem.sis.nlm.nih.gov/chemidplus/chemidheavy.jsp (accessed on 21st January 2014).
[b] Data from www.fass.se (accessed 21st January 2014).
[c] Data from DeVane (1999).
[d] Data from Aryal et al. (2012).
[e] Data from Fick et al. (2010).
[f] Data from Grabic et al. (2012).

were measured in the effluent by the treatment plant company (GRYAAB; Supplementary material). The fish were not fed during the experiment as rainbow trout of this size cope very well with food deprivation for several weeks at these temperatures (Kullgren et al., 2010, 2013). Food deprivation is expected to reduce differences between experimental individuals due to differences in food intake, which otherwise are common in captive rainbow trout.

The fish at Ryaverket were sampled after 13 day exposure on the 2nd of December. In total 20 fish were killed by a blow to the head, and blood samples were collected and centrifuged (Eppendorf centrifuge, 837 ×g, 10 min, 4 °C) to obtain plasma. Part of the liver, muscle from the dorsal part of the fillet in the middle of the fish and the entire brain of each fish were collected and stored at −80 °C until the analysis. A pre-exposure control group was sampled in a similar manner at the Zoology Department just before the experiment ($n = 10$). All fish were handled in accordance with the national and institutional guidelines for the protection of animal welfare with ethical pre-approval given to DGJ Larsson.

2.4. Tissue extraction and LC–MS/MS determination of SSRIs

Extracts from the fish plasma and tissues were prepared as detailed elsewhere (Fedorova et al., 2014). Briefly, the liver, muscle and brain samples were cut into small pieces, half gram of tissue was weighted, and 1 mL of acetonitrile with 0.1% formic acid and 50 ng of the internal standards were added into an Eppendorf-tube containing the tissue. The tissue was homogenized using a mini-beadbeater twice for 5 min with the speed set to 46 (mini-beadbeaterTM, biospec products, Techtum Lab, AB, SE) and, then centrifuged at 16,000 ×g for 10 min (Eppendorf centrifuge 5415C). The supernatant solution was frozen at −20 °C for 24 h. Later, the thawed supernatant was evaporated under nitrogen stream at room temperature to a volume of approximately 200 μL. The plasma samples were prepared in a similar way but without the homogenization in the mini-beadbeater. The extracts were diluted in water/methanol (50/50—*v*/*v*) at a 1:1 ratio prior to analysis. The extracts were analyzed using liquid chromatography with tandem mass spectrometry (LC–MS/MS) according to Grabic et al. (2012) and quantified with the matrix matching standard approach. The method was validated on control fish samples fortified with native compounds. The QA/QC samples, including duplicates, process blanks and fortified samples, were analyzed with each batch of processed samples. The limits of quantification (LOQs) for the fish samples are given in Table 2. Due to different sample amounts taken for extraction and differences in final volume, the LOQs are reported as a range—minimal to maximal value. The average recoveries for the fish liver fortified with CIT, FLU, SER and VEN to a concentration of 50 ng g^{-1} were 76% (with 5% RSD), 115% (with 40% RSD), 73% (with 1% RSD) and 78% (with 8% RSD), respectively. The average recoveries for fish muscle fortified with CIT, FLU, SER and VEN to a concentration of 50 ng g^{-1} were 166% (with 6% RSD), 50% (with 2% RSD), 136% (with 11% RSD) and 153% (with 5% RSD), respectively. The average recoveries for fish plasma fortified with CIT, FLU, SER and VEN to a concentration of 25 ng mL^{-1} were 107% (with 7% RSD), 120% (with 16% RSD), 112% (with 4% RSD) and 116% (with 5% RSD), respectively. Due to an insufficient amount of brain tissue, the recovery could not be measured in sampled fish. The trueness (recovery) of the method for brain was calculated from fortified brain samples of farmed rainbow trout. The average recoveries for fish brain fortified with CIT, SER and VEN to a concentration of 75 ng g^{-1} were 92% (with 14% RSD), 81% (with 21% RSD) and 97% (with 15% RSD), respectively.

Table 2
Concentrations [ng g^{-1}] of selected antidepressants in liver, brain, plasma and muscle of rainbow trout exposed to treated effluent from a Swedish sewage treatment works for 13 days, and in tissues from pre-exposure control fish.

	Antidepressant	Control fish	Exposed fish	LOQ [ng g^{-1}]
Liver	Citalopram	* (0/10)	12 ± 5 (18/20)	0.9–2.8
	Fluoxetine	* (0/10)	* (0/20)	1.0–6.4
	Sertraline	* (0/10)	4.5 ± 3.2 (8/20)	1.0–5.2
	Venlafaxine	* (0/10)	21 ± 11 (20/20)	1.0–3.4
Brain	Citalopram	* (0/10)	2.2 ± 1.3 (11/20)	1.0–2.0
	Fluoxetine	* (0/10)	* (0/20)	0.5–1.4
	Sertraline	* (0/10)	9.4 ± 3.8 (13/20)	1.0–8.0
	Venlafaxine	* (0/10)	3.1 ± 1.4 (17/20)	0.9–1.8
Plasma	Citalopram	* (0/10)	* (0/20)	1.4–2.4
	Fluoxetine	* (0/10)	* (0/20)	0.3–3.4
	Sertraline	* (0/10)	* (0/20)	1.3–5.6
	Venlafaxine	* (0/10)	2.6 ± 0.3 (3/20)	1.3–2.9
Muscle	Citalopram	* (0/10)	* (0/20)	1.4–2.4
	Fluoxetine	* (0/10)	* (0/20)	0.3–3.4
	Sertraline	* (0/10)	* (0/20)	1.3–5.6
	Venlafaxine	* (0/10)	* (0/20)	1.3–2.9

Data are mean ± S.D. Values in brackets are number of positive samples (values > LOQ) / number of analyzed samples. LOQ—limit of quantification. *—all values were below the LOQ.

2.5. Water sampling and analysis

Composite 24 hour effluent flow-proportional samples were collected during the entire experiment period. The concentrations of the target pharmaceuticals in the samples were determined using the LC–MS/MS method after offline SPE extraction (Grabic et al., 2012). Briefly, 100 mL of water samples was filtered through a 0.45 μm membrane filter (MF, Millipore, Sundbyberg, Sweden) and acidified to pH 3 using sulfuric acid. The internal standards were added to each sample and the solid phase extraction of samples was carried out with Oasis HLB (200 mg) cartridges. Methanol eluate was evaporated under a nitrogen stream to a volume of ca 20 μL and finally reconstituted in 1 mL of 5% acetonitrile in water.

2.6. Statistical analysis

Statistical analysis of the data of hepatosomatic index and Fulton's condition factor between pre-exposure controls and exposed fish was conducted with the STATISTICA v.10 software for Windows (StatSoft, Czech Republic). Normally distributed and homoscedastic data (as assessed by Cochran, Hartley and Barlett tests) were analyzed for differences in means using a *t*-test for independent samples. Differences were considered statistically significant at a $p < 0.05$.

3. Results and discussion

The calculated hepatosomatic index and Fulton's condition factor showed no statistically significant differences between the pre-exposure controls and the exposed fish. This suggests that the relatively short period of 13 days without feeding had no marked effect on the general physiology of the fish, as expected.

No antidepressants were detected in any of the pre-exposure control fish (Table 2). As no food was given to the experimental fish, this indicates that any antidepressants found in exposed fish have been taken up directly from the treated effluent.

The concentrations of antidepressants in the liver, brain, plasma and muscle of the fish exposed to the STP effluent are given in Table 2. The concentrations of FLU in all fish tissue samples were below the LOQ. This result agrees with the study by Lajeunesse et al. (2011), given their observed bioconcentration factor (BCF; i.e. the ration between body or tissue concentration and the concentration in the surrounding water), the low effluent concentration of FLU in the present study (18 ± 4 ng L^{-1}) and our LOQs for FLU in different tissues. The concentration of CIT and VEN observed in the exposed fish tissue were higher in liver than in brain, whereas the situation was opposite for SER. A similar pattern has previously been observed for SER in several fish species (bluegill—*L. macrochirus*, channel catfish—*I. punctatus* and black crappie—*P. nigromaculatus*) residing in municipal effluent-dominated

stream (Brooks et al., 2005). The previously published concentrations of SSRIs in brook trout exposed to 20% effluent for 3 months (Lajeunesse et al., 2011) agree with these results for CIT and VEN, but for SER, liver concentrations in brook trout were slightly higher than those found in the brain. The concentration of antidepressants in rainbow trout plasma and muscles was below LOQ in all samples (for CIT, FLU and SER) or in more than 80% of individuals (for VEN in plasma).

Based on the water concentration and concentrations in the tissues, the tissue-specific BCFs for the different tissues were estimated (Table 3). The BCFs in the brain (Table 3), the most lipid-rich tissue investigated, correspond rather well to the difference in log K_{ow} of CIT, VEN and SER, respectively (Table 1); the antidepressant with the highest BCF (SER) also has a rather high log K_{ow}, while CIT and VEN that have lower but similar log K_{ow} also have lower and similar brain BCFs. This could imply that a simple physic–chemical partitioning is a one driving force of the BCFs of the investigated antidepressants in the brain. The observed BCFs in the brain agree surprisingly well with the values estimated by Lajeunesse et al. (2011) for CIT, VEN and SER, despite several differences in experimental setup, including that different species were studied. In the liver, BCF data for CIT also agree well whereas the BCF for VEN is higher and lower for SER, in our study. Indeed, these relatively minor differences could be due to differences in experimental design; for example we exposed fish for a shorter time while, in the above-cited work, the fish were exposed to diluted effluent for 3 months (fish feeding is assumed as it is not given in the article). The liver enzyme activity might be different for the fed and unfed fish, which could lead to different drug concentrations in the liver samples. It should be stressed that we did not analyze desmethylsertraline, the common primary degradation product of SER.

Huggett et al. (2003) presented a hypothesis that because pharmaceuticals are designed to act on specific mammalian targets, they may have effects in non-target organisms at similar plasma concentrations. This "Read-Across Hypothesis", which was recently evaluated (Christen et al., 2010; Rand-Weaver et al., 2013), is based on the assumption that drug targets are conserved, which has been shown to be the case for the majority of targets in fish, including those of SSRIs and venlafaxine (Gunnarsson et al., 2008). This hypothesis makes it possible to estimate the probability of a pharmacological effect in exposed biota, primarily teleost fish, using a direct comparison of blood plasma levels between species.

In the present study, VEN was found in three out of the 20 exposed fish with an average plasma level of 2.6 ± 0.3 ng mL^{-1}. To our best knowledge, this is the first analyses of blood plasma concentrations of VEN in fish. The human therapeutic plasma concentration is 200 ng mL^{-1} (Schultz and Schmoldt, 2003), which gives a concentration ratio (CR; human therapeutic plasma concentration divided by the plasma concentration in the fish) of 77, only including the three samples where VEN was detectable. This means that these three exposed fish had plasma concentrations that were 77 times lower than the level that has a pharmacological effect in humans. For the other 17 fish the difference was even larger (154–77 times), based on measured water concentration and the LOQ of VEN in plasma.

Table 3
Concentration of selected antidepressants in the treated effluent from a Swedish municipal sewage treatment works, and estimated bioconcentration factors (BCFs) from water to different tissues of rainbow trout exposed to the same effluent for 13 days.

	Concentration in effluent		BCF			
	[ng L^{-1}]	LOQ [ng L^{-1}]	Liver	Brain	Plasma	Muscle
Citalopram	260 ± 60	5.0	47	9	<7	<7
Fluoxetine	18 ± 4	5.0	<337	<51	<84	<84
Sertraline	53 ± 16	10	85	180	<38	<38
Venlafaxine	330 ± 20	0.50	63	9	8	<6

Data of concentration in effluent are mean ± S.D., n = 14.
BCF = concentration in tissue [ng g^{1-}]/concentration in effluent [ng L^{-1}] × 1000.
The average BCFs were calculated only from the samples where the antidepressant was detectable (see Table 2).
For tissues where the analyte was not detected in any fish, the BCFs are indicated as smaller than a number based on the concentration found in the effluent and the average LOQ for that specific tissue.

CIT and SER could not be detected in plasma at exposure concentrations expected in an effluent-dominated stream. Hence, especially for field studies, but also for lab-based studies with environmentally realistic exposure concentrations, it can be difficult to assess risk based on plasma levels unless analytical detection methods are more sensitive. In contrast to the difficulties of detecting the antidepressants in plasma, CIT, SER and also VEN were easily detected in the target organ, the brain, of the fish. Given that therapeutic brain concentrations are known in mammals, we propose that it would be possible to assess risks for pharmacological interactions in fish a similar manner as with blood plasma. In fact, one can argue that comparing brain concentrations rather than plasma concentrations would be involving less assumptions as concentrations closer to the targets are compared.

Comparing levels of CIT and SER in fish brain tissue (2.2 ± 1.3 and 9.4 ± 3.8 ng g^{-1}, respectively) with the corresponding human concentrations that are in the range of 27 to 251 ng g^{-1} for CIT (Wille et al., 2009) and 80 to 5150 ng g^{-1} for SER (Lewis et al., 2013) gives the worst case CR (calculated on the lowest human concentration) of 12 for CIT and 8.5 for SER. The estimated CRs show that CIT and SER can reach levels in the target organs (brain) of fish not far from those known to cause effects in humans, while concentrations remain undetectable or only occasionally detectable in fish blood and muscle tissue. We have not found any human studies that include measurement of brain tissue concentrations of VEN, but there are corresponding studies in rats given doses leading to measurable effects (Unceta et al., 2010; Wikell et al., 2001). Comparing levels of VEN in fish brain tissue (3.1 ± 1.4 ng g^{-1}) with the corresponding rat concentrations that are in the range of 82 to 180 ng g^{-1} (Unceta et al., 2010; Wikell et al., 2001) gives a worst case CR (calculated on the lowest concentration in rat brain) of 26 for VEN.

For SER and CIT, the relations between brain and blood concentrations have been measured in humans. The distribution coefficients of SER, expressed as the brain/blood ratio, have been measured in the range of 17 to 22 (Lewis et al., 2013; Wille et al., 2009) and 7 for CIT (Wille et al., 2009). As we were not able to detect SER and CIT in plasma and as the study by Lajeunesse et al. (2011) did not include plasma analyses, we cannot confirm if there is a similar distribution coefficient in fish. Relations between brain and blood concentrations of VEN have been made in rat based studies and the brain/blood ratio of VEN was in the range 2.1–2.4 (Unceta et al., 2010; Wikell et al., 2001), which is quite similar to the observed brain/blood ratio of 1.2 of VEN, in this study, based on the three fish that had detectable VEN in plasma, i.e. it might be a slight underestimate of the true average ratio.

The concentrations of antidepressants in pooled muscle sample or whole body homogenates of wild fish are indeed often found below the LOQ (Brooks et al., 2005; Wang and Gardinali, 2012), even though the concentration in the target organ (brain) can be considerably higher. Similarly, we show that plasma levels may also be below the LOQ. We therefore propose that analyses of the brain levels of the investigated antidepressants, rather than blood plasma or whole body homogenates, would allow a more straightforward comparison to levels that have been shown to have pharmacological effects in mammals, and hence to assess risk or to identify compound of concern for future research. Having said this, we acknowledge the uncertainty that regardless what tissue levels that constitute the foundation for read-across, availability of the drug on the micro-scale at the target can be influenced by several unknown factors, such as binding to lipids or proteins.

4. Conclusions

This study confirms bioconcentration of three commonly used antidepressants in fish. Through a comparison of concentrations in different tissues, we propose that analyses of concentrations in the target organ

(brain) are most informative. The margins of safety compared to brain levels found in humans taking medication were relatively low. In the wild, fish are simultaneously co-exposed to several antidepressants acting through similar mechanisms. The investigated drugs tend to accumulate in the brain of the fish. As one would assume they would act additively, we therefore believe that there is concern for potentially disturbed monoaminergic signaling in the brain of fish residing in effluent-dominated streams. More research on the potential mixture effects of antidepressants at environmentally relevant concentrations is thus warranted.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2014.04.052.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This study was supported by the Foundation for Strategic Environmental Research (MISTRA), CENAKVA CZ.1.05/2.1.00/01.0024 project, the LO1205 project with financial support from the MEYS of the CR under the NPU I program, the Grant agency of the Czech Republic P503/11/1130 and the Swedish Research Council VR. We thank Jari Parkkonen for assistance with fish exposure, Professor Lars Förlin for valuable comments and Elsevier Language Editing for their editing services.

References

Aryal B, Aryal D, Kim EJ, Kim HG. Pharmacokinetics of venlafaxine and its major metabolite o-desmethylvenlafaxine in freely moving mice using automated dosing/sampling system. Indian J Pharmacol 2012;44:20–5.
Brodin T, Fick J, Jonsson M, Klaminder J. Dilute concentrations of a psychiatric drug alter behavior of fish from natural populations. Science 2013;339:814–5.
Brooks BW, Chambliss CK, Stanley JK, Ramirez A, Banks KE, Johnson RD, et al. Determination of select antidepressants in fish from an effluent-dominated stream. Environ Toxicol Chem 2005;24:464–9.
Christen V, Hickmann S, Rechenberg B, Fent K. Highly active human pharmaceuticals in aquatic systems: a concept for their identification based on their active mode of action. Aquat Toxicol 2010;96:167–81.
Corcoran J, Winter MJ, Tyler CR. Pharmaceuticals in the aquatic environment: a critical review of the evidence for health effects in fish. Crit Rev Toxicol 2010;40:287–304.
De Lange HJ, Noordoven W, Murk AJ, Lurling M, Peeters ETHM. Behavioural responses of *Gammarus pulex* (Crustacea, Amphipoda) to low concentrations of pharmaceuticals. Aquat Toxicol 2006;78:209–16.
DeVane CL. Metabolism and pharmacokinetics of selective serotonin reuptake inhibitors. Cell Mol Neurobiol 1999;19:443–66.
Du B, Perez-Hurtado P, Brooks BW, Chambliss CK. Evaluation of an isotope dilution liquid chromatography tandem mass spectrometry method for pharmaceuticals in fish. J Chromatogr A 2012;1253:177–83.
Du B, Price AE, Scott WC, Kristofco LA, Ramirez AJ, Chambliss CK, et al. Comparison of contaminants of emerging concern removal, discharge, and water quality hazards among centralized and on-site wastewater treatment system effluents receiving common wastewater influent. Sci Total Environ 2014;466–467:976–84.
Fedorova G, Nebesky V, Randak T, Grabic R. Simultaneous determination of 32 antibiotics in aquaculture products using LC-MS/MS. Chem Pap 2014;68:29–36.
Fent K, Weston AA, Caminada D. Ecotoxicology of human pharmaceuticals. Aquat Toxicol 2006;76:122–59.
Fick J, Lindberg RH, Tysklind M, Larsson DGJ. Predicted critical environmental concentrations for 500 pharmaceuticals. Regul Toxicol Pharmacol 2010;58:516–23.
Gelsleichter J, Szabo NJ. Uptake of human pharmaceuticals in bull sharks (*Carcharhinus leucas*) inhabiting a wastewater-impacted river. Sci Total Environ 2013;456:196–201.
Grabic R, Fick J, Lindberg RH, Fedorova G, Tysklind M. Multi-residue method for trace level determination of pharmaceuticals in environmental samples using liquid chromatography coupled to triple quadrupole mass spectrometry. Talanta 2012;100:183–95.
Gunnarsson L, Jauhiainen A, Kristiansson E, Nerman O, Larsson DGJ. Evolutionary conservation of human drug targets in organisms used for environmental risk assessments. Environ Sci Technol 2008;42:5807–13.
Halling-Sorensen B, Nors Nielsen S, Lanzky PF, Ingerslev F, Holten Lutzheft HC, Jorgensen SE. Occurrence, fate and effects of pharmaceutical substances in the environment—a review. Chemosphere 1998;36:357–93.
Heberer T. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol Lett 2002;131:5–17.
Henry TB, Kwon JW, Armbrust KL, Black MC. Acute and chronic toxicity of five selective serotonine reuptake inhibitors in *Ceriodaphnia dubia*. Environ Toxicol Chem 2004;23:2229–33.
Hiemke C, Hartter S. Pharmacokinetics of selective serotonin reuptake inhibitors. Pharmacol Ther 2000;85:11–28.
Holmberg A, Fogel J, Albertsson E, Fick J, Brown JN, Paxeus N, et al. Does waterborne citalopram affect the aggressive and sexual behaviour of rainbow trout and guppy? J Hazard Mater 2011;187:596–9.
Huerta B, Rodriguez-Mozaz S, Barcelo D. Pharmaceuticals in biota in the aquatic environment: analytical methods and environmental implications. Anal Bioanal Chem 2012;404:2611–24.
Huggett DB, Cook JC, Ericson JF, Williams RT. A theoretical model for utilizing mammalian pharmacology and safety data to prioritize potential impacts of human pharmaceuticals to fish. Hum Ecol Risk Assess 2003;9:1789–99.
Hughes SR, Kay P, Brown LE. Global synthesis and critical evaluation of pharmaceutical data sets collected from river systems. Environ Sci Technol 2013;47:661–77.
Kidd KA, Blanchfield PJ, Mills KH, Palace VP, Evans RE, Lazorchak JM, et al. Collapse of a fish population after exposure to a synthetic estrogen. Proc Natl Acad Sci U S A 2007;104:8897–901.
Kullgren A, Samuelsson L, Larsson DGJ, Björnsson Th, Jönsson Bergman E. A metabolomics approach to elucidate effects of food deprivation in juvenile rainbow trout (*Oncorhynchus mykiss*). Am J Physiol Regul Integr Comp Physiol 2010;299:R1440–8.
Kullgren A, Jutfelt F, Fontanillas R, Sundell K, Samuelsson LM, Wiklander K, et al. The impact of temperature on the metabolome and endocrine metabolic signaling in Atlantic salmon (*Salmo salar*). Comp Biochem Physiol A Mol Integr Physiol 2013;164:44–53.
Lajeunesse A, Gagnon C, Gagne F, Louis S, Cejka P, Sauve S. Distribution of antidepressants and their metabolites in brook trout exposed to municipal wastewaters before and after ozone treatment—evidence of biological effects. Chemosphere 2011;83:564–71.
Lewis RJ, Angier MK, Williamson KS, Johnson RD. Analysis of sertraline in postmortem fluids and tissues in 11 aviation accident victims. J Anal Toxicol 2013;37:208–16.
Metcalfe CD, Chu S, Judt C, Li H, Oakes KD, Servos MR, et al. Antidepressants and their metabolites in municipal wastewater, and downstream exposure in an urban watershed. Environ Toxicol Chem 2010;29:79–89.
Petrovic M, Gonzalez S, Barcelo D. Analysis and removal of emerging contaminants in wastewater and drinking water. Trac Trends Anal Chem 2003;22:685–96.
Ramirez AJ, Mottaleb MA, Brooks BW, Chambliss CK. Analysis of pharmaceuticals in fish using liquid chromatography–tandem mass spectrometry. Anal Chem 2007;79:3155–63.
Ramirez AJ, Brain RA, Usenko S, Mottaleb MA, O'Donnell JG, Stahl LL, et al. Occurrence of pharmaceuticals and personal care products in fish: results of a national pilot study in the United States. Environ Toxicol Chem 2009;28:2587–97.
Rand-Weaver M, Margiotta-Casaluci L, Patel A, Panter GH, Owen SF, Sumpter JP. The read-across hypothesis and environmental risk assessment of pharmaceuticals. Environ Sci Technol 2013;47:11384–95.
Santos L, Araujo AN, Fachini A, Pena A, Delerue-Matos C, Montenegro M. Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. J Hazard Mater 2010;175:45–95.
Schultz M, Schmoldt A. Therapeutic and toxic blood concentrations of more than 800 drugs and other xenobiotics. Pharmazie 2003;58:447–74.
Schultz MM, Furlong ET, Kolpin DW, Werner SL, Schoenfuss HL, Barber LB, et al. Antidepressant pharmaceuticals in two US effluent-impacted streams: occurrence and fate in water and sediment, and selective uptake in fish neural tissue. Environ Sci Technol 2010;44:1918–25.
Schultzt MM, Furlong ET. Trace analysis of antidepressant pharmaceuticals and their select degradates in aquatic matrixes by LC/ESI/MS/MS. Anal Chem 2008;80:1756–62.
Unceta N, Ugarte A, Sanchez A, Gomez-Caballero A, Aranzazu Goicolea M, Barrio RJ. Development of a stir bar sorptive extraction based HPLC-FLD method for the quantification of serotonin reuptake inhibitors in plasma, urine and brain tissue samples. J Pharm Biomed Anal 2010;51:178–85.
Valenti TW, Gould GG, Berninger JP, Connors KA, Keele NB, Prosser KN, et al. Human therapeutic plasma levels of the selective serotonin reuptake inhibitor (SSRI) sertraline decrease serotonin reuptake transporter binding and shelter-seeking behavior in adult male fathead minnows. Environ Sci Technol 2012;46:2427–35.
Vasskog T, Berger U, Samuelsen PJ, Kallenborn R, Jensen E. Selective serotonin reuptake inhibitors in sewage influents and effluents from Tromso, Norway. J Chromatogr A 2006;1115:187–95.
Verlicchi P, Al Aukidy M, Zambello E. Occurrence of pharmaceutical compounds in urban wastewater: removal, mass load and environmental risk after a secondary treatment —a review. Sci Total Environ 2012;429:123–55.
Wang J, Gardinali PR. Analysis of selected pharmaceuticals in fish and the fresh water bodies directly affected by reclaimed water using liquid chromatography–tandem mass spectrometry. Anal Bioanal Chem 2012;404:2711–20.
Wikell C, Hjorth S, Apelqvist G, Kullimgsjo J, Lundmark J, Bergqvist PBF, et al. Suistained administration of the antidepressant venlafaxine in rats: pharmacokinetic and pharmacodynamic findings. Naunyn Schmiedebergs Arch Pharmacol 2001;363:448–55.
Wille SMR, De Letter EA, Piette MHA, Van Overschelde LK, Van Peteghem CH, Lambert WE. Determination of antidepressants in human postmortem blood, brain tissue, and hair using gas chromatography–mass spectrometry. Int J Legal Med 2009;123:451–8.

PRESENCE OF PHARMACEUTICALS IN BENTHIC FAUNA LIVING IN A SMALL STREAM AFFECTED BY EFFLUENT FROM A MUNICIPAL SEWAGE TREATMENT PLANT

Katerina Grabicova[a]*, Roman Grabic[a], Martin Blaha[a], Vimal Kumar[a], Daniel Cerveny[a], Ganna Fedorova[a], Tomas Randak[a]

[a] University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zatisi 728/II, CZ-389 25 Vodnany, Czech Republic

**Corresponding author. Tel.: +420-387774734; E-mail address: grabicova@frov.jcu.cz; Faculty of Fisheries and Protection of Waters, Zatisi 728/II, 389 25 Vodnany, Czech Republic*

ABSTRACT

Aquatic organisms can be affected not only via polluted water but also via their food. In the present study, we examined bioaccumulation of seventy pharmaceuticals in two benthic organisms, *Hydropsyche* sp. and *Erpobdella octoculata* in a small stream affected by a sewage treatment plant (STP) in Prachatice (South Bohemia region, Czech Republic).

Furthermore, water samples from similar locations were analyzed for all seventy pharmaceuticals. In water samples from a control locality situated upstream of the STP, ten of the seventy pharmaceuticals were found with average total concentrations of 200 ng L^{-1}. In water samples collected at STP-affected sites (downstream the STP´s effluent), twenty-nine, twenty-seven and twenty-nine pharmaceuticals were determined at average total concentrations of 2000, 2100 and 1700 ng L^{-1}, respectively.

Six of the seventy pharmaceuticals (azithromycin, citalopram, clarithromycin, clotrimazole, sertraline, and verapamil) were found in *Hydropsyche*. Four pharmaceuticals (clotrimazole, diclofenac, sertraline, and valsartan) were detected in *Erpobdella*. Even pharmaceuticals present at low levels in water were found in benthic organisms at relatively high concentrations (up to 85 ng g^{-1} w.w. for azithromycin). Consequently, the uptake of pharmaceuticals via the food web could be an important exposure pathway for the wild fish population.

Keywords: *bioaccumulation, Hydropsyche, Erpobdella, natural fish diet, contamination*

1. INTRODUCTION

Effluent of municipal sewage treatment plants (STPs) contains numerous organic and inorganic pollutants due to insufficient removal efficiency during the treatment processes (Golovko et al., 2014a; Halling-Sorensen et al., 1998; Heberer, 2002; Petrovic et al., 2003). This incomplete removal broadly reflects the pharmaceuticals mixture to which fish and other aquatic organisms are typically exposed (Verlicchi et al., 2012).

Most of the research examining the possible bioconcentration of environmental contaminants in fish has focused on either controlled laboratory conditions associated with known individual pharmaceuticals or mixture exposure (e.g. Brozinski et al., 2011; Cuklev et al., 2012; Lahti et al., 2011; Nallani et al., 2011; Steinbach et al., 2013) or on fish placed into cages and exposed directly to the effluent (e.g. Lajeunesse et al., 2011; Togunde et al., 2012). It means that bioconcentration is the prevailing or only exposure mechanism. However, intake of pollutants via contaminated natural food is completely ignored in these experimental

setups. Depending on the exposure period, fish are either not fed or artificial food is used. In the case of real conditions, fish in cages can be stressed and outcomes of these experiments do not truly mimic the natural conditions.

Aquatic organisms are exposed not only via the discharge of sewage waters but also via their food. Benthic fauna is a very important part of the food web in aquatic environments (Hellmann et al., 2013; Hildrew, 1992). Benthic organisms are often used as bioindicators for assessing environmental pollution due to their limited movability and relatively easy sampling (Clews et al., 2014; Cortes et al., 2013; Pan et al., 2012; Smith et al., 1999). Larvae of caddisflies (*Hydropsyche* sp.) and leeches (*Erpobdella* sp.) are often used as indicators of water quality or pollution in streams (Azrina et al., 2006; Koperski, 2005; Koperski, 2010; Sola and Prat, 2006; Stuijfzand et al., 1999; Tessier et al., 2000). These species have been successfully used for monitoring of riverine pollution in the Czech Republic during the last decade (Kolarikova et al., 2012; Macova et al., 2009). These organisms significantly contribute to a fish diet as well (Elliott, 1967; Greenberg and Dahl, 1998; Laine, 2001; Reiriz et al., 1998).

The aim of this study was to investigate pharmaceutical levels in benthic organisms and consequently to reveal their importance in the exposure pathways of the fish.

2. MATERIAL AND METHODS

2.1. Sampling location

Benthic organisms and water samples were collected in the Zivny stream (a tributary of Blanice River) situated in the south part of the Czech Republic, during May 2013. The stream is 13 km long with an average depth of 30 cm and an average width of 3 m. Flow varied from 0.150 to 0.600 m^3 s^{-1}. The stream is highly impacted by effluent from the Prachatice STP. Prachatice (12000 inhabitants) is a district town. There is only light industry (food, machinery and electronics) and a hospital. The STP´s effluent can make up approximately 25% of the water flow in the Zivny stream. Except for stretches in Prachatice, the stream habitat is natural or semi-natural. The fish community is dominated by brown trout (*Salmo trutta* m. *fario* L.) with occurrences of other riverine species, including stone loach (*Barbatula barbatula* L.) and bullhead (*Cottus gobio*). Benthic invertebrate organisms, specifically *Hydropsyche* sp. and *Erpobdella octoculata,* were chosen for this study. Both of organisms are present in all (or in a majority) of sampled sites situated along the stream and make up an important part of the fish diet. Larvae of *Hydropsyche* are net-spinning omnivores (Benke and Wallace, 1997; Hellmann et al., 2013), using resources from multiple trophic levels. Leeches, *E. octoculata* (Hirudinea) are predators feeding especially on oligochates and chironomid larvae (Schenkova et al., 2007).

2.2. Sample collection

Water samples were collected seven times during two weeks in April-May 2013 in three locations: the area close to the place where the STP´s effluent enters the stream (site E), 1.5 km downstream (site B), and 3 km downstream (site R). Water samples from a non-contaminated area – 4.5 km upstream from the source of pollution (site C) – were used as a control (Fig. 1). Samples were filtered (0.45 μm regenerated cellulose filters, Labicom, Olomouc, Czech Republic) and frozen at -20 °C until the analysis.

The benthic organisms were collected from the same localities as the water samples. Individuals were picked up with tweezers, immediately frozen and stored at -20 °C until the analysis. *Hydropsyche* sp. was found in all of the sampled sites in sufficient amount, *E. octoculata* was found only in the sites situated downstream of the STP at Prachatice (i.e. sites E, B, R).

2.3. Chemicals

Liquid chromatography mass spectrometry- (LC/MS-) grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Formic acid (LC/MS grade) was obtained from Sigma-Aldrich (Steinheim, Germany).

Internal standard stocks were prepared from mass-labeled crystalline compounds of amitriptyline, atenolol, carbamazepine, clarithromycin, sulfamethoxazole, and trimethoprim. Sulfamethoxazole ($^{13}C_6$) and trimethoprim ($^{13}C_3$) were purchased from Cambridge Isotope Laboratories; carbamazepine (D_{10}) and amitriptyline (D_6) from CDN Isotopes; atenolol (D_6) from Alsa Chim; and clarithromycin (D_3) from Toronto Research Chemicals, Inc.

Native stocks were prepared from the crystalline compounds (Table 1). Stock solutions of all pharmaceuticals were prepared in methanol at the concentration of 1 mg mL^{-1}, and stored at -20 °C.

2.4. Water analysis

A triple-stage quadrupole MS/MS TSQ Quantum Ultra EMR (Thermo Fisher Scientific), equipped with Accela 1250 and 600 LC pumps (Thermo Fisher Scientific) and a PAL HTC autosampler (CTC Analytics AG) operated using Xcalibur software (Thermo Fisher Scientific), was connected with in-line solid phase extraction (SPE LC-MS/MS) and used for analysis of water.

Water samples were thawed and filtered through a regenerated cellulose syringe filter (0.45 μm pores). The samples were analyzed by the in-line SPE/LC-MS/MS method after internal standards addition. The description of the method can be found elsewhere (Fedorova et al., 2014a; Lindberg et al., 2014). Briefly, two analytical columns in two runs were used for separation of target analytes – and Cogent Bidentate column (50 mm × 2.1. mm ID × 4 μm particles; Microsolv Technology Corporation). In-line extraction was performed at Hypersil Gold column (20 mm × 2.1 mm ID × 12 μm particles; Thermo Fisher Scientific), in accordance with the method described by Fedorova et al. (2014a). A detailed description of the analytical method is given in the supplementary material (S1). The limits of quantification (LOQs) for the water samples are given in Table 2. The trueness of the analytical method has been reported previously (Fedorova et al., 2014a).

2.5. Benthos analysis

Extracts from benthos were prepared according to (Fedorova et al., 2014b). Collected amounts of both *Hydropsyche* sp. and *E. octoculata* from each locality were sufficient for five to six parallel samples. Briefly, three individuals of one species from the same locality were cut into small pieces when thawed. Around one half gram of this sample was weighted and internal standards were added to each sample. The mixture was then homogenized (TissueLyser II, Quiagen, Germany; 1800 min^{-1} for 10 min) with acetonitrile (acidified 0.1% formic acid) using a stainless steel ball. The samples were then centrifuged (Micro 200R centrifuge, Hettich Zentrifugen, Germany; 9500 × g for 10 min) and filtered (0.45 μm regenerated cellulose filters, Labicom, Olomouc, Czech Republic). The supernatant was frozen at -20 °C for 24 hours to precipitate and remove proteins and other solid particles from the samples. A Q-Exactive mass spectrometer (Thermo Fisher Scientific) coupled with an Accela 1250 LC pump (Thermo Fisher Scientific) and HTS XT-CTC autosampler (CTC Analytics AG), was used for the analysis of the extracts from benthic organisms. Extracts were analyzed by liquid chromatography with tandem mass spectrometry in high resolution product scan mode using the same two chromatography columns mentioned above. The analytical method is described in supplementary material (S2, S3). The trueness of the method (as a recovery of fortified samples) together with the LOQs is given in Table 2.

2.6. Statistical analysis

The data are presented as mean ± SD. Statistical analysis of the data was conducted with the STATISTICA v.12 software for Windows (StatSoft, Czech Republic). Normally distributed and homoscedastic data (as assessed by Cochran, Hartley and Barlett tests) were appraised by a one-way ANOVA (Tukey test). When data were not normally distributed and homoscedastic, the non-parametric method of data analysis was used (Kruskal-Wallis test). Differences were considered statistically significant at $p < 0.05$. One half of the LOQ value was used in the data sets when some of the results were found below the LOQ.

3. RESULTS AND DISCUSSION

3.1. Pharmaceuticals in water

Mean water concentrations of seventy pharmaceuticals measured in water samples are given in Table 2. Fifteen of the seventy analyzed pharmaceuticals with the highest observed concentrations belong to a group of antibiotics (clarithromycin, trimethoprim and sulfamethoxazole), cardiovascular drugs (irbesartan, valsartan, eprosartan, sotalol, atenolol, metoprolol and bisoprolol), psychoactive drugs (venlafaxine, citalopram and carbamazepine), and analgesics (diclofenac and tramadol). The highest concentrations were 130 (clarithromycin), 210 (metoprolol and valsartan), 110 (carbamazepine), and 270 (tramadol) ng L^{-1} for antibiotics, cardiovascular drugs, psychoactive drugs, and analgesics, respectively. This pattern corresponds to consumption within the Czech population and the resistance of pharmaceuticals to the treatment processes (Golovko et al., 2014a; Golovko et al., 2014b).

Figure 2 shows total concentration of pharmaceuticals in a longitudinal profile of the stream. Total concentration in site C (upstream), as well as the number of pharmaceuticals above LOQ, is lower than those in sites downstream of the effluent released from the STP. Statistical testing of the data was performed for all pharmaceuticals to determine whether or not the differences among the localities are significant. This observation suggests that this STP potentially contributed to the pollution by pharmaceuticals in the downstream sites. However, a few exceptions to this pattern were identified. For example, antifungal and psychoactive drugs, namely clotrimazole (4–7 ng L^{-1}), mirtazapine (2–3 ng L^{-1}), sertraline (2–5 ng L^{-1}) and risperidone (2–4 ng L^{-1}), were found in almost the same (very low) levels in all localities. Some other pharmaceuticals, such as memantine (4 ng L^{-1}) and diltiazem (2 ng L^{-1}), were determined at low levels but only at sites E and R. Statistically significant differences between upstream and all polluted downstream sites were found in the concentrations of all other pharmaceuticals. However, for most of pharmaceuticals, there were few differences in concentration among the downstream sites (R, E and B), particularly between E and R. These observations could be explained by the short distance between site B and site E (approx. 1.5 km), hence less time for degradation, sorption or transformation processes. However, we observed a decrease in atorvastatin, azithromycin, citalopram, clindamycin, diclofenac, dipyridamole, eprosartan, erythromycin, sotalol, sulfamethoxazole, trimethoprim, and verapamil concentrations at site R.

3.2. Pharmaceuticals in benthic organisms

The same range of pharmaceuticals as in the water samples was analyzed for in benthic organisms living in the recipient stream.

Six of the pharmaceuticals analyzed for were found above LOQ in *Hydropsyche* sp. – the antibiotics azithromycin and clarithromycin, the cardiovascular drug verapamil, the antifungal drug clotrimazole, and the antidepressants citalopram and sertraline (Table 3).

Four pharmaceuticals were detected in *E. octoculata* – the cardiovascular drug valsartan, the antidepressant sertraline, the anti-inflammatory drug diclofenac, and the antifungal drug clotrimazole (Table 4). A comparison of the pharmaceuticals concentrations of pharmaceuticals in extracts from *Erpobdella* between polluted sites (E, B and R) and non-polluted site is unfortunately not possible, because *Erpobdella* were not found in the non-polluted site. The areas where *Erpobdella* were collected in the Zivny stream correspond to areas in the Malaysian Langat river, where this subclass of benthic organisms was also found only in eutrophic areas (Azrina et al., 2006).

The concentration of pharmaceuticals in the benthic biota ranged from 1.2 to 85 ng g^{-1} wet weight (w.w.) in samples from polluted sites, whilst below LOQ in the upstream control site except for detected levels of clotrimazole (3.6 ng g^{-1}). However, this finding corresponds to a trend observed in concentrations of this compound in water. There was a significant decrease of concentration in sites downstream of the STP compared to the control site; on the other hand, only one half of the clotrimazole samples from the control site were found above LOQ. Unfortunately, the other sampled species was not present in the control site, so we could not confirm the presence of clotrimazole in biota there. *Erpobdella* samples from sites E, B and R contained clotrimazole at about the same level as in *Hydropsyche*. Sertraline and clotrimazole were detected in both species. Sertraline was not present in samples from the control site; concentration levels were about the same in *Hydropsyche* (4.3–5.5 ng g^{-1}) and *Erpobdella* (4.2–5.6 ng g^{-1}), and concentrations did not shown significant differences among polluted localities.

Azithromycin (up to 85 ng g^{-1} w.w.) and diclofenac (up to 33 ng g^{-1} w.w.) were the highest measured pharmaceuticals in *Hydropsyche* and *Erpobdella*, respectively. While bioaccumulation in fish was previously reported for diclofenac (Lahti et al., 2011; Mehinto et al., 2010), azithromycin has not yet been detected in aquatic organisms. A significant increase of azithromycin at site B compared to site E could be explained by possible veterinary usage of this antibiotic in farms situated close to site B and its excretion near the stream. There was no relationship between water and benthos azithromycin concentration. Based on previously reported strong sorption of azithromycin to bottom sediment (Gibs et al., 2013) and low concentration in sampled water, we can conclude that particles associated with this compound will be main source of this antibiotic in *Hydropsyche*. Diclofenac concentrations in *Erpobdella* followed a decrease of water concentration with longer distances from the STP. This trend was also obvious for the valsartan found in *Erpobdella*. Another three compounds found in *Hydropsyche* (citalopram, clarithromycin and verapamil) were not present in the control group, and there were no differences in these pharmaceuticals among all three polluted sites. However, clarithromycin was very close to LOQ and, contrary to all other compounds found in benthos, it was not present in all samples from sites B and R.

Clarithromycin, diclofenac, and valsartan represent the major water pollutants. All other compounds were found in water at very low concentrations (maximally low tens of ng L^{-1}). As benthic organisms are major components of the brown trout diet (Fochetti et al., 2003), pharmaceutical uptake via the food web can play an important role, especially for such compounds.

Both sampled species belong to predator benthic fauna, but they have different body composition, metabolism, behavior, and different feeding strategy. While *Hydropsyche* has a soft body with a high content of fat [up to 8% of w.w. (Meier et al., 2000)], *Erpobdella* has hard muscle body created by protein fibers. All above mentioned facts could be reasons for differences in the pharmaceutical patterns observed in whole body homogenates. The presence

of citalopram and sertraline in *Hydropsyche* can be associated with high accumulation of this antidepressant in fatty fish tissues, as reported in several papers (Brooks et al., 2005; Gelsleichter and Szabo, 2013; Grabicova et al., 2014).

There are only a limited number of articles reporting the fate and effect of STP-originated pharmaceutical mixtures on benthic organisms. The studies mainly focus on biochemical responses (Damasio et al., 2011), biodiversity (Azrina et al., 2006; Koperski, 2010; Pan et al., 2012), and survival (Stuijfzand et al., 1999). To our knowledge, this is the first report on pharmaceuticals in benthic fauna living in a stream affected by an STP´s effluent.

4. CONCLUSIONS

The results clearly indicate that STP effluent was the major source of pharmaceuticals in the Zivny stream (a tributary of Blanice River). Extended measurement of these pharmaceuticals in benthic organisms further showed their bioaccumulation potential. Eight of the seventy analyzed pharmaceuticals were found to have accumulated in benthic organisms *Hydropsyche* sp. or *E. octoculata* (azithromycin, citalopram, clarithromycin, clotrimazole, diclofenac, sertraline, valsartan, and verapamil), supporting the environmental stability of these pharmaceuticals.

Even pharmaceuticals present in low concentrations in water were bioaccumulated in benthic organisms. As these species of benthic organisms are an important part of the fish diet, the uptake of pharmaceuticals via the food web could be an important exposure pathway in the wild fish population. This study illustrates how the pharmaceuticals can enter into the aquatic food web, which is not necessarily limited to water-phase concentrations.

Acknowledgments

This project was supported by the CENAKVA CZ.1.05/2.1.00/01.0024 project, the LO1205 project with financial support from the MEYS of the CR under the NPU I program, the Grant Agency of USB GAJU 087/2013/Z and the Grant Agency of Czech Republic P503/11/1130. We thank to American Manuscript Editors for their editing services.

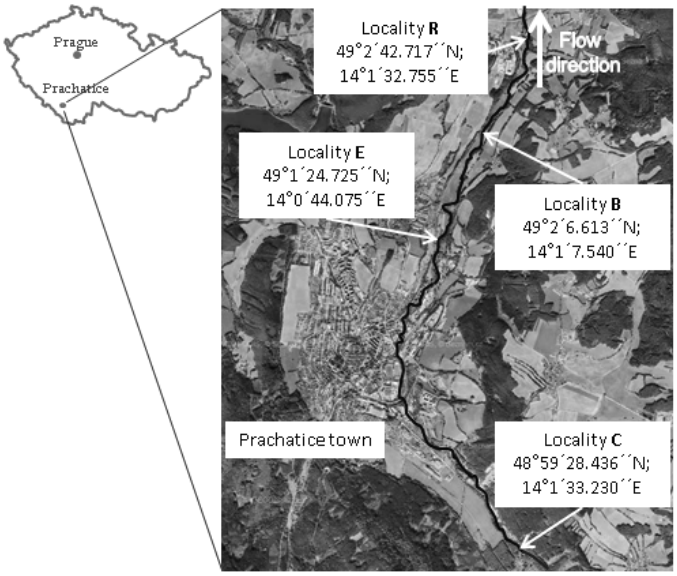

Figure 1. *Sampling localities of the Zivny stream.*
Legend: Site E – area where the STP´s effluent enters the Zivny stream; Site B – approx. 1.5 km downstream of the STP; Site R – approx. 3 km downstream of the STP; Site C – non-polluted area (approx. 4.5 km upstream of the STP).

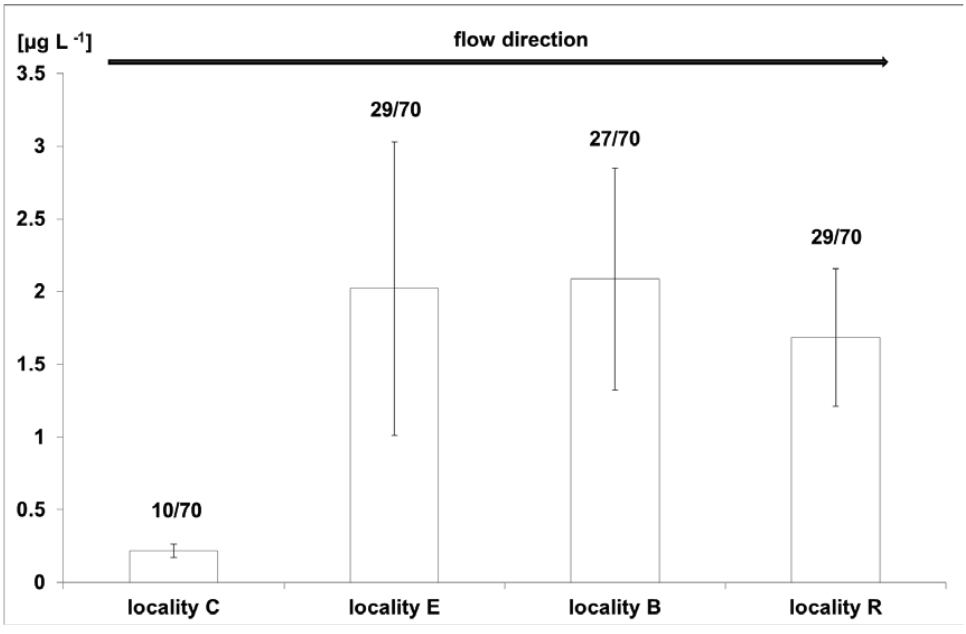

Figure 2. *Total concentrations of pharmaceuticals in water samples in a longitudinal profile of the Zivny stream. Numbers given in the graph refer to positive/analyzed pharmaceuticals.*
For legend see Figure 1.

Table 1. *Compounds used for preparation of native stocks. All purchased chemicals had analytical grade certificate or purity > 98%.*

Pharmaceutical	Producer	Pharmaceutical	Producer
Alfuzosin	Sigma Aldrich	Fulvestrant	Sigma Aldrich
Amitriptyline hydrochloride	Sigma Aldrich	Glibenclamide	Sigma Aldrich
Atenolol	Sigma Aldrich	Glimepiride	Sigma Aldrich
Atorvastatin calcium	Chemos GmbH	Haloperidol	Sigma Aldrich
Atracurium besylate	AK Scientific	Hydroxyzine dihydrochloride	Sigma Aldrich
Azithromycin	Sigma Aldrich	Irbesartan	Chemos GmbH
Bezafibrate	Sigma Aldrich	Levomepromazine hydrochloride	EPL
Biperiden hydrochloride	Sigma Aldrich	Loperamide hydrochloride	Sigma Aldrich
Bisoprolol hemifumarate	Sigma Aldrich	Maprotiline hydrochloride	Sigma Aldrich
Bupropion hydrochloride	Sigma Aldrich	Memantine hydrochloride	Sigma Aldrich
Carbamazepine	Sigma Aldrich	Metoprolol tartare salt	Sigma Aldrich
Chlorpromazine hydrochloride	Sigma Aldrich	Mianserin hydrochloride	Sigma Aldrich
Chlorprothixene hydrochloride	Sigma Aldrich	Miconazole nitrate salt	Sigma Aldrich
Cilazapril	Sigma Aldrich	Mirtazapine	Sigma Aldrich
Citalopram	AK Scientific	Nefazodone hydrochloride	Sigma Aldrich
Clarithromycin	Sigma Aldrich	Oxazepam	Sigma Aldrich
Clemastine fumarate	EPC	Perphenazine	Sigma Aldrich
Clindamycin hydrochloride	Sigma Aldrich	Promethazine hydrochloride	Sigma Aldrich
Clomipramine hydrochloride	Sigma Aldrich	Risperidone	AK Scientific
Clonazepam	Sigma Aldrich	Sertraline hydrochloride	AK Scientific
Clotrimazole	Sigma Aldrich	Sotalol hydrochloride	Sigma Aldrich
Cyproheptadine hydrochloride	Sigma Aldrich	Sulfamerazine	Sigma Aldrich
Desloratadine	Sigma Aldrich	Sulfamethazine	Sigma Aldrich
Diclofenac sodium salt	Sigma Aldrich	Sulfamethizole	Sigma Aldrich
Dicycloverine hydrochloride	EPD	Sulfamethoxazole	Riedel-de Haen
Dihydroergotamine tartare	Sigma Aldrich	Sulfamethoxypyridazine	Sigma Aldrich
Diltiazem hydrochloride	Sigma Aldrich	Sulfathiazole	Sigma Aldrich
Diphenhydramine hydrochloride	Sigma Aldrich	Terbinafine hydrochloride	Sigma Aldrich
Dipyridamole	Sigma Aldrich	Terbutaline hemisulfate	Sigma Aldrich
Econazole nitrate salt	Sigma Aldrich	Tramadol hydrochloride	Sigma Aldrich
Eprosartan	Chemos GmbH	Trimethoprim	Sigma Aldrich
Erythromycin	Sigma Aldrich	Valsartan	Sigma Aldrich
Fenofibrate	Sigma Aldrich	Venlafaxine hydrochloride	AK Scientific
Fexofenadine hydrochloride	Sigma Aldrich	Verapamil hydrochloride	Sigma Aldrich
Flecainide acetate	Sigma Aldrich	Zuclopenthixol	BPCL

Table 2. *Average concentration [ng L^{-1}] of pharmaceuticals in the water samples collected from the Zivny stream from 24th April to 10th May 2013.*

Pharmaceutical	Concentration in water [ng L^{-1}]				LOQ range
	Locality C	Locality E	Locality B	Locality R	[ng L^{-1}]
Analgesics					
Diclofenac	< LOQ	94 ± 52	91 ± 24	65 ± 23	2–3
Tramadol	7 ± 3	230 ± 150	270 ± 100	200 ± 80	1–2
Anesthetics					
Atracurium	< LOQ	< LOQ	< LOQ	< LOQ	3–5
Antihistaminics					
Cyproheptadine	< LOQ	< LOQ	< LOQ	< LOQ	1
Desloratadine	< LOQ	< LOQ	< LOQ	< LOQ	1
Diphenhydramine	< LOQ	< LOQ	< LOQ	< LOQ	2–4
Fexofenadine	< LOQ	15 ± 11	15 ± 8	9 ± 6	1
Hydroxyzine	< LOQ	< LOQ	< LOQ	< LOQ	3–5
Antibiotics					
Azithromycin	< LOQ	8 ± 7	6 ± 5	2 ± 1	1–2
Clarithromycin	< LOQ	120 ± 70	130 ± 60	88 ± 45	1
Clindamycin	< LOQ	7 ± 4	8 ± 4	5 ± 3	1
Erythromycin	< LOQ	23 ± 19	22 ± 13	15 ± 9	1– 2
Sulfamerazine	< LOQ	< LOQ	< LOQ	< LOQ	5–17
Sulfamethazine	< LOQ	< LOQ	< LOQ	< LOQ	1–2
Sulfamethizole	< LOQ	< LOQ	< LOQ	3 ± 1	1–4
Sulfamethoxazole	< LOQ	32 ± 20	28 ± 12	20 ± 11	3–4
Sulfamethoxypyridazine	< LOQ	< LOQ	< LOQ	< LOQ	1–2
Sulfathiazole	< LOQ	< LOQ	< LOQ	< LOQ	7–25
Trimethoprim	2 ± 1	75 ± 48	84 ± 28	55 ± 20	1–3
Anticholinergic					
Biperiden	< LOQ	< LOQ	< LOQ	< LOQ	1
Clemastine	< LOQ	< LOQ	< LOQ	< LOQ	1
Dicycloverine	< LOQ	< LOQ	< LOQ	< LOQ	1
Antidiabetics					
Glibenclamide	< LOQ	< LOQ	< LOQ	< LOQ	1–3
Glimepiride	< LOQ	< LOQ	< LOQ	< LOQ	2–3
Anti-diarrhea					
Loperamide	< LOQ	< LOQ	< LOQ	< LOQ	1
Antifungal drugs					
Clotrimazole	4± 1	5 ± 3	7 ± 4	6 ± 2	2–3
Econazole	< LOQ	< LOQ	< LOQ	< LOQ	2–3
Miconazole	< LOQ	< LOQ	< LOQ	< LOQ	1
Terbinafine	< LOQ	< LOQ	< LOQ	< LOQ	1
Anticancer, antihyperplasia					
Alfuzosin	< LOQ	< LOQ	< LOQ	< LOQ	3–4
Fulvestrant	< LOQ	< LOQ	< LOQ	< LOQ	1

Bronchodilator					
Terbutaline	< LOQ	< LOQ	< LOQ	< LOQ	4–13
Cardiovascular drugs					
Atenolol	< LOQ	95 ± 61	100 ± 40	69 ± 29	6–21
Bisoprolol	< LOQ	40 ± 24	38 ± 13	29 ± 9	3–5
Cilazapril	< LOQ	< LOQ	< LOQ	< LOQ	2–3
Diltiazem	< LOQ	2 ± 1	< LOQ	< LOQ	1
Dipyridamole	< LOQ	13 ± 10	11 ± 7	5 ± 1	1
Eprosartan	6 ± 2	67 ± 32	37 ± 17	22 ± 15	1
Flecainide	< LOQ	< LOQ	< LOQ	< LOQ	2–3
Irbesartan	5 ± 4	79 ± 43	88 ± 24	74 ± 25	1–2
Metoprolol	7 ± 4	210 ± 140	190 ± 70	130 ± 60	1–4
Sotalol	< LOQ	22 ± 3	24 ± 3	17 ± 2	7–23
Valsartan	8 ± 3	200 ± 110	210 ± 80	150 ± 50	1
Verapamil	< LOQ	9 ± 6	8 ± 3	4 ± 1	1
Lipid regulators					
Atorvastatin	< LOQ	25 ± 31	7 ± 5	8 ± 7	2–4
Bezafibrate	< LOQ	< LOQ	< LOQ	< LOQ	2–3
Fenofibrate	< LOQ	< LOQ	< LOQ	< LOQ	1–2
Psychoactive drugs					
Amitriptyline	< LOQ	< LOQ	< LOQ	< LOQ	1
Bupropion	< LOQ	< LOQ	< LOQ	< LOQ	3–4
Carbamazepine	< LOQ	110 ± 60	100 ± 40	76 ± 27	1–2
Chlorpromazine	< LOQ	< LOQ	< LOQ	< LOQ	1
Chlorprothixene	< LOQ	< LOQ	< LOQ	< LOQ	1
Citalopram	< LOQ	29 ± 19	27 ± 10	19 ± 3	3–5
Clomipramine	< LOQ	< LOQ	< LOQ	< LOQ	1
Dihydroergotamine	< LOQ	< LOQ	< LOQ	< LOQ	1– 2
Haloperidol	< LOQ	< LOQ	< LOQ	< LOQ	3–5
Levomepromazine	< LOQ	< LOQ	< LOQ	< LOQ	1
Maprotiline	< LOQ	< LOQ	< LOQ	< LOQ	2–4
Memantine	< LOQ	4 ± 2	< LOQ	4 ± 1	2–3
Mianserin	< LOQ	< LOQ	< LOQ	< LOQ	1
Mirtazapine	3 ± 2	3 ± 1	3 ± 1	2 ± 1	1–2
Nefazodone	< LOQ	< LOQ	< LOQ	< LOQ	1–3
Perphenazine	< LOQ	< LOQ	< LOQ	< LOQ	1–2
Promethazine	< LOQ	< LOQ	< LOQ	< LOQ	1
Risperidone	4 ± 2	2 ± 1	2 ± 1	4 ± 1	1
Sertraline	2 ± 1	5 ± 3	3 ± 1	2 ± 1	1–2
Venlafaxine	< LOQ	74 ± 42	76 ± 28	55 ± 21	4–5
Zuclopenthixol	< LOQ	< LOQ	< LOQ	< LOQ	1–3
Sedatives					
Clonazepam	< LOQ	< LOQ	< LOQ	< LOQ	2–3
Oxazepam	< LOQ	12 ± 6	11 ± 4	10 ± 3	1

For legend, see Figure 1. LOQ = limit of quantification; n = 7, ^{a,b,c} = levels of statistical significance ($p < 0.05$).

Table 3. *Concentration of positive pharmaceuticals [ng g^{-1} w.w.] in Hydropsyche sp.*

Pharmaceutical	Concentration [ng g^{-1}]								LOQ range	Recovery
	Locality C	F	Locality E	F	Locality B	F	Locality R	F	[ng g^{-1}]	[%]
Azithromycin	< LOQ ^{a}	0/6	40 ± 7 ^{b}	6/6	85 ± 8 ^{d}	5/5	68 ± 9 ^{c}	5/5	3.0–4.2	139
Clarithromycin	< LOQ ^{a}	0/6	3.1 ± 1.6 ^{a}	6/6	2.2 ± 0.8 ^{a}	3/5	2.9 ± 0.1 ^{a}	2/5	1.4–1.9	85
Verapamil	< LOQ ^{a}	0/6	3.5 ± 0.9 ^{b}	6/6	3.1 ± 0.9 ^{b}	5/5	2.7 ± 0.4 ^{b}	5/5	1.0–1.4	85
Citalopram	< LOQ ^{a}	0/6	4.2 ± 1.1 ^{b}	6/6	3.8 ± 0.8 ^{b}	5/5	3.6 ± 0.9 ^{b}	5/5	1.4–2.8	150
Sertraline	< LOQ ^{a}	0/6	4.6 ± 0.8 ^{b}	6/6	5.5 ± 1.6 ^{b}	5/5	4.3 ± 0.8 ^{b}	5/5	1.1–2.0	143
Clotrimazole	3.6 ± 0.3 ^{a}	3/6	2.3 ± 1.2 ^{a}	5/6	1.4 ± 0.1 ^{a}	4/5	1.4 ± 0.2 ^{a}	4/5	1.1–4.4	91

For legend see Figure 1; $^{a, b, c, d}$ – statistically significant differences ($p < 0.05$);
F – Frequency of detection, number of positive sample / number of all samples.

Table 4. *Concentration of positive pharmaceuticals [ng g^{-1} w.w.] in Erpodella octoculata.*

Pharmaceutical	Concentration [ng g^{-1}]						LOQ	Recovery
	Locality E	F	Locality B	F	Locality R	F	[ng g^{-1}]	[%]
Valsartan	2.3 ± 1.0 ^{a}	6/6	1.3 ± 0.5 ^{b}	6/6	< LOQ ^{b}	0/5	0.49–0.73	101
Sertraline	5.6 ± 0.8 ^{a}	6/6	4.9 ± 0.6 ^{a}	6/6	4.2 ± 2.6 ^{a}	6/6	0.82–1.1	125
Diclofenac	33 ± 13 ^{a}	6/6	14 ± 3 ^{b}	6/6	12 ± 4 ^{b}	5/5	1.3–1.9	120
Clotrimazole	2.5 ± 0.3 ^{a}	6/6	1.4 ± 0.2 ^{b}	6/6	1.2 ± 0.1 ^{b}	5/5	0.72–0.94	68

For legend see Figure 1; $^{a, b}$ – statistically significant differences ($p < 0.05$);
F – Frequency of detection, number of positive sample / number of all samples.

REFERENCES

Azrina, M.Z., Yap, C.K., Ismail, A.R., Ismail, A., Tan, S.G., 2006. Anthropogenic impacts on the distribution and biodiversity of benthic macroinvertebrates and water quality of the Langat River, Peninsular Malaysia. Ecotox. Environ. Safe. 64**,** 337–347.

Benke, A.C., Wallace, J.B., 1997. Trophic basis of production among riverine caddisflies: Implications for food web analysis Ecology 78**,** 1132–1145.

Brooks, B.W., Chambliss, C.K., Stanley, J.K., Ramirez, A., Banks, K.E., Johnson, R.D., Lewis, R.J., 2005. Determination of select antidepressants in fish from an effluent-dominated stream. Environ. Toxicol. Chem. 24**,** 464–469.

Brozinski, J.M., Lahti, M., Oikari, A., Kronberg, L., 2011. Detection of naproxen and its metabolites in fish bile following intraperitoneal and aqueous exposure. Environ. Sci. Pollut. Res. 18**,** 811–818.

Clews, E., Low, E.W., Belle, C.C., Todd, P.A., Eikaas, H.S., Ng, P.K.L., 2014. A pilot macroinvertebrate index of the water quality of Singapore's reservoirs. Ecol. Indic. 38**,** 90–103.

Cortes, R.M.V., Hughes, S.J., Pereira, V.R., Varandas, S.D.P., 2013. Tools for bioindicator assessment in rivers: The importance of spatial scale, land use patterns and biotic integration. Ecol. Indic. 34**,** 460–477.

Cuklev, F., Fick, J., Cvijovic, M., Kristiansson, E., Forlin, L., Larsson, D.G.J., 2012. Does ketoprofen or diclofenac pose the lowest risk to fish? J. Hazard. Mater. 229**,** 100–106.

Damasio, J., Fernandez-Sanjuan, M., Sanchez-Avila, J., Lacorte, S., Prat, N., Rieradevall, M., Soares, A., Barata, C., 2011. Multi-biochemical responses of benthic macroinvertebrate species as a complementary tool to diagnose the cause of community impairment in polluted rivers. Water Res. 45, 3599–3613.

Elliott, J.M., 1967. The food of trout (*Salmo trutta*) in a Dartmoor stream. J. Appl. Ecol. 4, 59–71.

Fedorova, G., Golovko, O., Randak, T., Grabic, R., 2014a. Storage effect on the analysis of pharmaceuticals and personal care products in wastewater. Chemosphere 111, 55–60.

Fedorova, G., Nebesky, V., Randak, T., Grabic, R., 2014b. Simultaneous determination of 32 antibiotics in aquaculture products using LC-MS/MS. Chemic. Papers 68, 29–36.

Fochetti, R., Amici, I., Argano, R., 2003. Seasonal changes and selectivity in the diet of brown trout in the River Nera (Central Italy). J. Freshw. Ecol. 18, 437-444.

Gelsleichter, J., Szabo, N.J., 2013. Uptake of human pharmaceuticals in bull sharks (*Carcharhinus leucas*) inhabiting a wastewater-impacted river. Sci. Total Environt. 456, 196–201.

Gibs, J., Heckathorn, H.A., Meyer, M.T., Klapinski, F.R., Alebus, M., Lippincott, R.L., 2013. Occurrence and partitioning of antibiotic compounds found in the water column and bottom sediments from a stream receiving two wastewater treatment plant effluents in Northern New Jersey, 2008. Sci. Total Environ. 458, 107-116.

Golovko, O., Kumar, V., Fedorova, G., Randak, T., Grabic, R., 2014a. Removal and seasonal variability of selected analgesics/anti-inflammatory, anti-hypertensive/cardiovascular pharmaceuticals and UV filters in wasterwater treatment plant. Environ. Sci. Pollut. Res. 21, 7578–7585.

Golovko, O., Kumar, V., Fedorova, G., Randak, T., Grabic, R., 2014b. Seasonal changes in antibiotics, antidepressants/ psychiatric drugs, antihistamines and lipid regulators in a wastewater treatment plant. Chemosphere111, 418–426.

Grabicova, K., Lindberg, R.H., Östman, M., Grabic, R., Randak, T., Larsson, D.G.J., Fick, J., 2014. Tissue-specific bioconcentration of antidepressants in fish exposed to effluent from a municipal sewage treatment plant Sci. Total Environ. 488–489, 46–50.

Greenberg, L.A., Dahl, J., 1998. Effect of habitat type on growth and diet of brown trout, Salmo trutta L., in stream enclosures. Fisher.Manag. Ecol. 5, 331–348.

Halling-Sorensen, B., Nors Nielsen, S., Lanzky, P.F., Ingerslev, F., Holten Lutzheft, H.C., Jorgensen, S.E., 1998. Occurrence, Fate and Effects of Pharmaceutical Substances in the Environment – A Review. Chemosphere 36, 357–393.

Heberer, T., 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol. Lett. 131, 5–17.

Hellmann, C., Wissel, B., Winkelmann, C., 2013. Omnivores as seasonally important predators in a stream food web. Freshw. Sci. 32, 548–562.

Hildrew, A., 1992. Food webs and species interactions. In: The rivers handbook: hydrological and ecological principles. Blackwell Scientific, Oxford, England, pp. 309–330.

Kolarikova, K., Stuchlik, E., Liska, M., Horecky, J., Tatosova, J., Hardekopf, D., Lapsanska, N., Horicka, Z., Hovorka, J., Mihaljevic, M., Fuksa, J.K., von Tumpling, W., 2012. Long-Term Changes in the Bioaccumulation of As, Cd, Pb, and Hg in Macroinvertebrates from the Elbe River (Czech Republic). Water Air Soil Pollut. 223, 3511–3526.

Koperski, P., 2005. Testing the suitability of leeches (Hirudinea, Clitellata) for biological assessment of lowland streams. Pol. J. Ecol. 53, 65–80.

Koperski, P., 2010. Diversity of macrobenthos in lowland streams: ecological determinants and taxonomic specificity. J. Limnol. 69, 88–101.

Lahti, M., Brozinski, J.M., Jylha, A., Kronberg, L., Oikari, A., 2011. Uptake from water, biotransformation, and biliary excration of pharmaceuticals by rainbow trout. Environ. Toxicol. Chem. 30, 1403–1411.

Laine, A., 2001. Effects of peatland drainage on the size and diet of yearling salmon in a humic northern river. Arch. Hydrobiol. 151, 83–99.

Lajeunesse, A., Gagnon, C., Gagne, F., Louis, S., Cejka, P., Sauve, S., 2011. Distribution of antidepressants and their metabolites in brook trout exposed to municipal wastewaters before and after ozone treatment – Evidence of biological effects. Chemosphere 83, 564-571.

Lindberg, R.H., Östman, M., Olofsson, U., Grabic, R., Fick, J., 2014. Occurrence and behaviour of 105 active pharmaceutical ingredients in sewage waters of a municipal sewer collection system. Water Res. 58, 221–229.

Macova, S., Harustiakova, D., Kolarova, J., Machova, J., Zlabek, V., Vykusova, B., Randak, T., Velisek, J., Poleszczuk, G., Hajslova, J., Pulkrabova, J., Svobodova, Z., 2009. Leeches as sensor-bioindicators of river contamination by PCBs. Sensors 9, 1807–1820.

Mehinto, A.C., Hill, E.M., Tyler, C.R., 2010. Uptake and Biological Effects of environmentally relevant concentrations of the nonsteroidal anti-inflammatory pharmaceutical diclofenac in rainbow trout (*Oncorhynchus mykiss*). Environ. Sci. Technol. 44, 2176–2182.

Meier, G.M., Meyer, E.I., Meyns, S., 2000. Lipid content of stream macroinvertebrates. Arch. Hydrobiol. 147, 447–463.

Nallani, G.C., Paulos, P.M., Constantine, L.A., Venables, B.J., Huggett, D.B., 2011. Bioconcentration of ibuprofen in fathead minnow (*Pimephales promelas*) and channel catfish (*Ictalurus punctatus*). Chemosphere 84, 1371–1377.

Pan, B.Z., Wang, Z.Y., Xu, M.Z., 2012. Macroinvertebrates in abandoned channels: assemblage characteristics and their indications for channel management. River Res. Appl. 28, 1149–1160.

Petrovic, M., Gonzalez, S., Barcelo, D., 2003. Analysis and removal of emerging contaminants in wastewater and drinking water. Trac-Trends Anal. Chem. 22, 685–696.

Reiriz, L., Nicieza, A.G., Brana, F., 1998. Prey selection by experienced and naive juvenile Atlantic salmon. J. Fish Biol. 53, 100–114.

Schenkova, J., Jarkovsky, J., Helesic, J., 2007. Strategies of coexistence of two species: *Erpobdella octoculata* and E-vilnensis (Hirudinea : Erpobdellidae). Inter. Rev. Hydrobiol. 92, 527–538.

Smith, M.J., Kay, W.R., Edward, D.H.D., Papas, P.J., Richardson, K.S., Simpson, J.C., Pinder, A.M., Cale, D.J., Horwitz, P.H.J., Davis, J.A., Yung, F.H., Norris, R.H., Halse, S.A., 1999. AusRivAS: using macroinvertebrates to assess ecological condition of rivers in Western Australia. Freshw. Biol. 41, 269–282.

Sola, C., Prat, N., 2006. Monitoring metal and metalloid bioaccumulation in hydropsyche (Trichoptera, Hydropsychidae) to evaluate metal pollution in a mining river. Whole body versus tissue content. Sci. Total Environ. 359, 221–231.

Steinbach, C., Fedorova, G., Prokes, M., Grabicova, K., Machova, J., Grabic, R., Valentova, O., Kocour Kroupova, H., 2013. Toxic effects, bioconcentration and depuration of verapamil in the early life stages of common carp (*Cyprinus carpio* L.). Sci. Total Environ. 461–462, 198–206.

Stuijfzand, S.C., Engels, S., van Ammelrooy, E., Jonker, M., 1999. Caddisflies (Trichoptera : Hydropsychidae) used for evaluating water quality of large European rivers. Arch. Environ. Contam. Toxicol. 36, 186–192.

Tessier, L., Boisvert, J.L., Vought, L.B.M., Lacoursiere, J.O., 2000. Effects of 2,4-dichlorophenol on the net-spinning behavior of Hydropsyche slossonae larvae (Trichoptera; Hydropsychidae), an early warning signal of chronic toxicity. Ecotox. Environ. Safe. 46, 207–217.

Togunde, O.P., Oakes, K.D., Servos, M.R., Pawliszyn, J., 2012. Optimization of solid phase microextraction for non-lethal in vivo determination of selected pharmaceuticals in fish muscle using liquid chromatography-mass spectrometry. J. Chromatogr. A. 1261, 99–106.

Verlicchi, P., Al Aukidy, M., Zambello, E., 2012. Occurrence of pharmaceutical compounds in urban wastewater: Removal, mass load and environmental risk after a secondary treatment-A review. Sci. Total Environ. 429, 123–155.

Science of the Total Environment 487 (2014) 681–687

Contents lists available at ScienceDirect

Science of the Total Environment

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

A passive sampling method for detecting analgesics, psycholeptics, antidepressants and illicit drugs in aquatic environments in the Czech Republic

G. Fedorova [a,*], T. Randak [a], O. Golovko [a], V. Kodes [b], K. Grabicova [a], R. Grabic [a]

[a] University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zatisi 728/II, 389 25 Vodnany, Czech Republic
[b] Czech Hydrometeorological Institute, Na Sabatce 17, 143 06 Prague 4-Komorany, Czech Republic

HIGHLIGHTS

- Field calibration of POCIS was carried out at a wastewater treatment plant.
- Bioavailable concentrations of psychoactive drugs were monitored in surface waters.
- Total concentrations of compounds ranged from 463 to 6447 ng POCIS^{-1}.
- Different pattern was found for water and POCIS concentration.

ARTICLE INFO

Article history:
Received 2 August 2013
Received in revised form 22 November 2013
Accepted 18 December 2013
Available online 30 January 2014

Keywords:
POCIS
Sampling rate
Field calibration
Surface water
Psychoactive compounds

ABSTRACT

The goal of this study was to assess the bioavailable concentrations of analgesics, psycholeptics, antidepressants and illicit drugs in the surface waters of the Czech Republic. All of the sampling sites are located within the most important water quality monitoring profiles at the Czech Hydrometeorological Institute. The total concentrations of the compounds ranged from 463 to 6447 ng POCIS^{-1} (Polar Organic Chemical Integrative Sampler). Carbamazepine (196–2690 ng POCIS^{-1}) and tramadol (160–2250 ng POCIS^{-1}) were the most abundant compounds at every site. The most polluted sites were those that received communal wastewater effluent and had a low dilution factor (ratio of wastewater effluent and river flow). The aqueous concentrations of the target compounds were estimated using sampling rate values obtained during a field calibration experiment. Patterns in the aqueous concentrations of the compounds (after back calculation from POCIS extracts) and the POCIS concentrations are different, possibly leading to discrepancies between the toxicity assessments conducted using POCIS extracts and those conducted using grab samples of water from the same location.

1. Introduction

Studies from around the world have reported the occurrence of drugs in waste- and surface water (Berset et al., 2010; Castiglioni et al., 2008; Gheorghe et al., 2008; Irvine et al., 2011). The primary sources of illicit drugs, as well as pharmaceuticals, in the aquatic environment are wastewater treatment plants (WWTPs). Compounds usually enter waterways via urban wastewater after being excreted (Buchberger, 2007). Analysing drug residues in influent wastewater can provide reliable information on drug usage patterns at the local (Zuccato et al., 2008), and international levels (Thomas et al., 2012); recent environmental drug studies have focused on wastewater analysis (Bijlsma et al., 2012; Karolak et al., 2010).

The presence of drugs in aquatic ecosystems is garnering increasing amounts of attention because the compounds might adversely affect aquatic life. It has been shown that anxiolytic drugs in surface waters alter animal behaviour and thus can have ecological and evolutionary consequences. Juvenile European perch were exposed to oxazepam at environmentally relevant concentrations, and the effects on their behaviour and feeding rates were observed (Brodin et al., 2013). Additionally, the bioaccumulation of oxazepam in fish muscle tissue was reported. Feito and co-authors determined evidence of acute lethal and chronic sub-lethal toxicity in vascular plants exposed to environmentally relevant concentrations of the antidepressant venlafaxine (Feito et al., 2013). Therefore, drug monitoring in aquatic environments is important because it forms a basis for risk assessment for aquatic organisms.

The goal of this study was to assess the bioavailable concentrations of analgesics, psycholeptics, antidepressants, and illicit drugs in surface waters from rivers in the Czech Republic. Water quality is regularly

* Corresponding author at: Zatisi 728/II, 389 25 Vodnany, Czech Republic. Tel.: +420 77 5360674.
E-mail address: gfedorova@frov.jcu.cz (G. Fedorova).

0048-9697/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.scitotenv.2013.12.091

monitored by the Czech Hydrometeorological Institute (CHMI) using a passive sampling (PS) approach. Thirty-seven compounds were analysed: three analgesics (buprenorphine, codeine, tramadol), one anaesthetic (ketamine), six antidepressants (amitriptyline, citalopram, mianserin, paroxetine, sertraline, venlafaxine), two anti-epileptics (carbamazepine, clonazepam), six drug metabolites (2-oxy-3-hydroxy-lysergic acid diethylamide (2-oxy-3-hydroxy-LSD), THC-COOH (Tetrahydrocannabinol) 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), benzoylecgonine, norbuprenorphine glucuronide, norketamine), 10 illicit drugs (amphetamine, cathinone, cocaine, lysergic acid diethylamide (LSD), N-methyl-1,3-benzodioxolylbutanamine (MBDB), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxy-N-ethylamphetamine (MDEA), 3,4-methylenedioxy-N-methylamphetamine (MDMA), mephedrone, methamphetamine), seven psycholeptics (alprazolam, haloperidol, methylphenidate, midazolam, oxazepam, risperidone, zolpidem), and two synthetic opioids (methadone, oxycodone). The list of selected drugs includes commonly consumed drugs (or their metabolites), such as methamphetamine, MDMA, cocaine, and THC-COOH (Thomas et al., 2012), as well as medicinal compounds for which nonmedical use is prohibited, such as ketamine and tramadol. Certain compounds, such as oxazepam, are known to have adverse effects on non-target species, which underscores the importance of including them in environmental risk assessments (Brodin et al., 2013).

Polar Organic Chemical Integrative Samplers (POCISs) were deployed for 21 days in the spring of 2012. Passive sampling can overcome the limitations of conventional sampling and provide time-integrated data (Bueno et al., 2009). A further advantage of passive sampling is the ability to mimic biological uptake precluding the use aquatic organisms for biomonitoring (Alvarez et al., 2005; Kot et al., 2000). Unfortunately, the limitation of this approach is a lack of calibration data. The available sampling rate values are limited to a small number of compounds (Bartelt-Hunt et al., 2011; Harman et al., 2011). Therefore, the data obtained from POCIS monitoring were used for comparative purposes.

2. Materials and methods

2.1. Chemicals and solvents

A standardised pesticide configuration of POCIS (Pest-POCIS) was purchased from Exposmeter AB (Tavelsjö, Sweden). The sequestration medium of Pest-POCIS consists of a triphasic admixture of a hydroxylated polystyrene-divinylbenzene resin (Isolute ENV+) and a carbonaceous adsorbent (Ambersorb 1500) dispersed on a styrene divinylbenzene copolymer (S-X3 Bio Beads).

Details regarding the preparation of analytical standards have been described previously (Fedorova et al., 2013b; Grabic et al., 2012). Mixtures of the standard solutions for all analytes and surrogate standards were prepared in methanol at 1 μg mL^{-1} and stored at 4 °C.

Methanol (LiChrosolv Hypergrade), acetonitrile (LiChrosolv Hypergrade), toluene (Suprasolv) and dichloromethane (Suprasolv) were purchased from Merck (Darmstadt, Germany). Formic acid (FA) was used to acidify the mobile phases (Labicom, Olomouc, Czech Republic). Ultrapure water was obtained from an aqua-MAX-Ultra system (Younglin, Kyounggi-do, Korea).

2.2. Sampling sites and field deployment of POCIS

A POCIS field calibration experiment was carried out at the WWTP in České Budějovice, Czech Republic from January 27 to February 16, 2011. The major source of input is domestic wastewater, as well as wastewater from two breweries and a dairy that account for less than 5%. Triplicates of POCIS were placed in protective cages and deployed in the effluent water for 1, 2, and 3 weeks. The mean water velocity at the outlet was 530 L s^{-1}, and the average integrated flow was 45 620 m^3 day^{-1}.

Wastewater samples for the POCIS calibration experiment were collected using an automated sampler (time proportional sampling, ASP-STATION 2000 sampler, Endress + Hauser). The samples were collected at 15-min intervals and were mixed after 24 h to obtain the daily mean. Each pooled sample was divided into three subsamples and frozen. The water temperature in the effluent channel was continuously monitored during the experiment and was stable throughout the calibration period. The concentrations of the target compounds ranged from low ng L^{-1} to several μg L^{-1}. The details of the POCIS calibration experiment can be found in a report by Fedorova et al. (2013a).

Passive sampling of river water was carried out at 21 sites located within the riverine profiles in the Czech Republic that are regularly monitored by the CHMI (Fig. 1). The locations of the sampling sites are listed with a short description in Table 1. All sampling sites are located within the most important water quality monitoring profiles in the CHMI. Most sites were situated at river outlets. The POCISs were placed in protective cages and deployed in the river water for 21 days during the spring of 2012. The water temperature was continuously monitored during the sampling period. After the exposure period, the samplers were cleaned with ultrapure water and transported on ice to the laboratory to be stored at −18 °C.

2.3. Extraction of target analytes

The chemical residues of interest were extracted from the passive samplers according to standardised procedures (Alvarez et al., 2005). The POCISs were carefully disassembled, and the sorbent was transferred into glass gravity-flow chromatography columns (1 cm i.d.) plugged with glass wool. A dichloromethane/methanol/toluene (8:1:1, v:v:v) mixture (50 mL) was used for elution during the recovery of sequestered analytes from the sorbent. Internal standards were added (20 ng per sample). The extracts were concentrated to approximately 1 mL by rotary evaporation. The residual water and traces of nonpolar solvents could cause the extract to separate into two phases. Because the presence of an eluent that is stronger than methanol is undesirable, we added methanol (10 ml) to the concentrated samples. Repeated evaporation removed any traces of toluene and DCM as an azeotropic mixture with methanol. Afterward, the samples were transferred to autosampler vials, and evaporated to 0.5 mL under a gentle stream of nitrogen.

The blank samples were processed the same way as the POCIS extracts to ensure that the target analytes were not introduced from laboratory procedures or sample handling.

2.4. In-line SPE/LC/MS/MS and LC/MS/MS analysis

A Q-Exactive mass spectrometer and a triple stage quadrupole MS/MS TSQ Quantum Ultra Mass Spectrometer (both from Thermo Fisher Scientific, San Jose, CA, USA) were each coupled with an Accela 1250 LC pump and Accela 600 LC pump (Thermo Fisher Scientific), and a HTS XT-CTC autosamplers (CTC Analytics AG, Zwingen, Switzerland) and used to analyse the water and POCIS samples, respectively. A Cogent Bidentate column (50 mm × 2.1 mm ID × 4 μm particles; Microsolv Technology Corporation, Eatontown, NJ, USA) was used to separate the target analytes. The POCIS extracts were diluted twice with ultrapure water and analysed using conventional LC injection (5 μL of sample per injection).

The filtered and spiked water samples were analysed using an in-line-SPE-LC fitted with a C18 column (Hypersil Gold, 20 mm × 2.1 mm i.d, 12 μm particles; Thermo Fisher Scientific) (Fedorova et al., 2013b). The in-line SPE enabled the pre-concentration and analysis of samples in a single run, making large volumes and time-consuming SPE extraction unnecessary. The extraction and analytical process required only 15 min and used only 1 mL of sample.

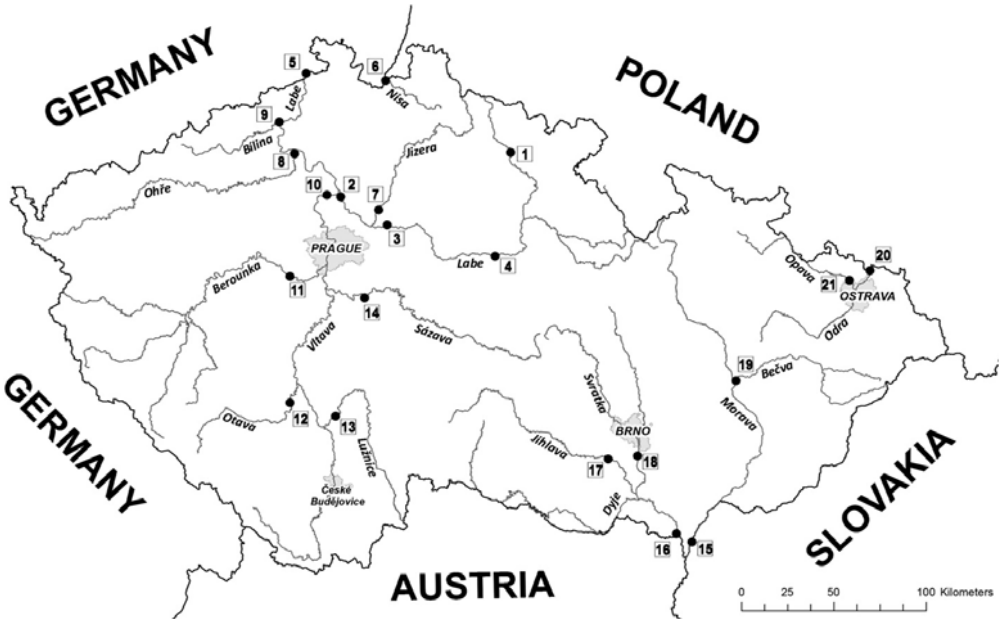

Fig. 1. Map of the sampling area.

Heated electrospray (HESI-II) in positive ion mode was used to ionise the target compounds. The key parameters were as follows: 3.5 kV ionisation voltage; 35 arbitrary units of sheath gas, 15 arbitrary units of auxiliary gas, 200 °C vaporiser, 325 °C capillary and 1.5 mTorr min^{-1} collision gas (argon). The details regarding the analytical methods are presented in two references (Fedorova et al., 2013b; Grabic et al., 2012).

3. Results and discussion

3.1. Wastewater sampling

PS to monitor drugs in aquatic environments is becoming more popular than grab sampling. PS can successfully combine sampling and pre-concentration of target analytes into a single step, thereby reducing the time and cost of further sample preparation. A limiting factor in the use of POCIS as a sampling tool for analysing drugs in water is the lack of calibration data. Sampling rates are available for a small number of compounds, but most of those were obtained under laboratory conditions and may vary significantly from field conditions. We carried out a calibration experiment under field conditions using wastewater effluent. The POCIS extracts were analysed with pooled water samples. For compounds occurring in amounts above the limit of quantification (LOQ) in water samples and in POCIS, determining the linear range of sampling and calculating the sampling rate value in this range were possible. The sampling rates of the selected drugs were calculated using the concentrations of the compounds adsorbed in POCIS and in water samples:

$$Rs = M_{POCIS} C_W^{-1} t^{-1}.$$

Rs is the sampling rate (L day^{-1}), M_{POCIS} is the amount of target compound in the POCIS (ng), Cw = average water concentration (ng L^{-1}), and t = exposure period (days). This equation is valid for the kinetic regime of uptake (POCIS is an integrative sampler) (Mazzella et al., 2007). Therefore, the period of linear uptake was determined for each compound. The field-calibrated sampling rates are reported in Table 2.

The uptake kinetics of selected compounds and their concentrations in water during the exposure period are shown in Fig. 2. For example, THC-COOH showed typical accumulation pattern with a linear uptake over 21 days of exposure. The uptake pattern of some compounds, such as oxazepam, was curvilinear (Fig. 2). Therefore, the sampling rates were calculated for a 14-day period.

Many target compounds were detected in effluent samples. Concentrations of some compounds (e.g., tramadol, carbamazepine, venlafaxine, citalopram, and methamphetamine) were quite high (up to 1 μg L^{-1}.) Some compounds (benzoylecgonine, ketamine, methadone, and midazolam) were found in the POCIS extract but not in wastewater samples, revealing the advantage of using POCIS as a sampling tool: the samples are pre-concentrated, enabling a lower LOQ (approximately 0.20 ng $POCIS^{-1}$).

3.2. Occurrence of selected compounds in river waters in the Czech Republic

The concentrations of target compounds recovered from POCIS at each sampling location are presented in Table 1. The total concentrations of the compounds ranged from 463 to 6447 ng $POCIS^{-1}$. Carbamazepine (196–2690 ng $POCIS^{-1}$) and tramadol (160–2250 ng $POCIS^{-1}$) were the most abundant compounds at every site. Three pharmaceuticals were found in relatively high concentrations: citalopram (18–429 ng $POCIS^{-1}$), venlafaxine (34–605 ng $POCIS^{-1}$), and oxazepam (9.1–276 ng $POCIS^{-1}$); these compounds were detected at all sites. The other compounds were detected sporadically. As expected, the lowest concentrations were observed at the control site (Labe Vestřev). This site has minimal inputs from municipalities and industry

Table 1
Description of sampling sites and amount of target compounds in POCIS extracts from each site (ng POCIS^{-1}).

No of sampling site	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
River	Labe	Labe	Labe	Labe	Labe	Nisa	Jizera	Ohře	Bílina	Vltava	Berounka	Otava	Lužnice	Sázava	Morava	Dyje	Jihlava	Svratka	Bečva	Odra	Opava
The name	Vestřev	Obříství	Lysá n.L.	Valy	Schmilka	Hrádek n. N	Předměřice	Terezín	Ústí nad Labem	Zelčín	Srbsko	Topělec	Bechyně	Nespeky	Lanžhot	Pohansko	Ivančice	Židlochovice/ Rajhrad	Troubky	Bohumín	Děhylov
What is monitored at the site	"Background" site at Labe river	Neratovice chloroalkali plant, POPs hot spot, ongoing chemical production	Oil refinery and chemicals production	Synthezia Pardubice, explosives and dyes production	City Ústí nad Labem (7th largest in CR), POP hot spot from chemicals production, Labe river outlet to Germany	City Liberec (5th largest in CR), Nisa river outlet to Germany	No special object	No special object	Highly polluted river from mining and chemical production	City Prague (the biggest communal population in CR)	No special object	No special object	No special object	No special object	No special object, Morava river outlet to Slovakia/ Austria	No special object, Dyje river outlet to Austria	No special object	City Brno (2nd largest in CR)	No special object	City Ostrava (3rd largest in CR), heavy and chemical industry, Odra river outlet to Poland	No special object
The basin outlet profile of River:	–	–	–	–	Labe	Nisa	Jizera	Ohře	Bílina	Vltava	Berounka	Otava	Lužnice	Sázava	Morava	Dyje	Jihlava	Svratka	Bečva	Odra	Opava
Amount of target compounds (ng POCIS^{-1})																					
2-Oxy-3-hydroxy-LSD	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Alprazolam	ND	ND	ND	ND	8.9	ND	ND	ND	5.7	6.6	3.3	ND	ND	ND	ND	13	ND	12	3.2	8.7	ND
Amitryptiline	ND	ND	ND	ND	ND	3.3	ND	ND	ND	ND	ND	ND	ND	ND	1.7	2.2	ND	2.8	ND	ND	ND
Amphetamine	ND	0.50	ND	0.90	0.90	5.6	0.70	ND	1.1	3.0	ND	ND	ND	1.3	0.70	ND	0.50	ND	ND	3.0	ND
Benzoylecgonine	ND	ND	ND	0.8	2.8	8.2	0.90	ND	2.4	6.4	0.50	1.0	ND	ND	ND	1.6	ND	8.4	ND	2.1	0.70
Buprenorphin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Carbamazepine	49	222	241	287	570	821	224	140	505	368	238	146	262	337	1012	764	290	937	476	771	317
Cathinone	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Citalopram	8	10	14	30	24	95	25	5	21	55	10	16	11	10	24	11	12	138	19	61	19
Clonazepam	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Cocaine	ND	ND	ND	ND	ND	3.1	ND	ND	1.6	2.7	ND	ND	ND	ND	ND	ND	ND	1.9	ND	0.80	ND
Codeine	ND	ND	ND	ND	13	ND	ND	ND	11	13	ND	ND	ND	ND	ND	ND	14	42	ND	12	ND
EDDP	ND	ND	ND	ND	1.2	ND	ND	ND	1.2	4.4	ND	ND	ND	ND	ND	0.60	ND	7.4	ND	ND	ND
Haloperidol	ND	ND	1.2	2.0	ND	ND	ND	1.1	1.1	ND	ND	ND	ND	ND	1.4	ND	ND	ND	0.80	3.0	ND
Ketamine	ND	ND	ND	ND	1.1	0.80	ND	ND	0.40	0.90	ND	ND	ND	0.40	1.4	ND	0.40	1.3	0.50	0.90	ND
LSD	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MBDB	ND	0.50	0.60	ND	ND	ND	ND	ND	0.60	2.1	0.40	ND	0.40	ND	1.2	ND	0.60	ND	0.70	ND	ND
MDA	5.7	ND	ND	ND	ND	5.9	ND	8.6	6.6	ND	ND	ND	ND	ND	6.5	8.8	ND	ND	ND	ND	ND
MDEA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MDMA	ND	ND	0.90	2.3	1.0	6.0	ND	ND	0.80	4.5	ND	ND	ND	ND	1.3	ND	ND	11	ND	1.1	ND
Mephedrone	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Metamphetamine	10	2.1	5.3	12	26	227	13	6.6	111	55	4.1	5.9	1.9	4.9	21	23	12	151	21	247	18
Methadone	ND	ND	ND	ND	0.60	ND	ND	ND	1.4	2.9	ND	ND	ND	ND	ND	0.50	ND	5.6	ND	ND	ND
Methylphenidate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Mianserin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Midazolam	ND	ND	0.80	ND	1.4	1.0	ND	ND	1.1	ND	ND	ND	ND	ND	1.1	1.0	0.70	2.5	ND	1.8	ND
Norbuprenorphine glucoronid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Norketamine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.2	ND	ND	ND
Oxazepam	1.9	24	37	65	107	169	25	30	135	62	38	20	53	50	123	85	62	198	49	88	25
Oxycodone	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Paroxetin	ND	ND	ND	4.6	ND	ND	ND	ND	ND	ND	3.7	ND	ND	ND	ND	ND	ND	4.7	ND	ND	ND
Risperidone	0.62	0.72	0.49	ND	ND	0.42	ND	ND	0.68	0.59	0.46	0.37	0.95	0.39	0.59	0.71	0.69	0.80	ND	1.3	ND
Sertraline	ND	ND	5.1	5.7	5.7	34	ND	ND	18	14	ND	5.2	ND	ND	ND	4.8	ND	42	ND	12	ND
THC-COOH	1.5	ND	ND	2.7	5.9	ND	2.9	ND	4.3	ND	4.2	ND	ND	ND	ND	4.8	3.4	13	ND	ND	3.1
Tramadol	38	123	133	194	198	441	124	66	354	231	130	92	148	113	435	313	150	663	310	404	115
Venlavafaxin	9.0	27	30	54	47	59	31	15	69	74	42	27	30	25	58	54	75	198	56	117	18
Zoldipem	ND	8.8	42	400	3.4	1.4	0.59	ND	1.8	3.2	ND	0.42	0.40	0.47	22	0.75	0.53	4.9	1.7	21	41

ND not detected.

Table 2
Calculated field sampling rates.

Compound	Rs, L day^{-1}	RSD[a], %	Linearity over 14 days[b]	Linearity over 21 days[b]
Benzoylecgonine	0.047[c]	5	0.9	0.77
Carbamazepine	0.26[d]	7	0.89	0.92
Citalopram	0.37[c]	9	0.96	0.84
Methamphetamine	0.11[d]	8	0.89	0.93
Oxazepam	0.17[c]	9	0.9	0.77
THC-COOH	0.065[d]	15	1	0.99
Tramadol	0.15[c]	8	0.92	0.84
Venlavafaxin	0.23[d]	4	0.96	0.94

[a] Relative standard deviation of the triplicates.
[b] Correlation coefficients of linear regression.
[c] Calculated for 14-day exposure period.
[d] Calculated for 21-day exposure period.

and was chosen as a background area for the Labe River. Low levels of the target compounds were also detected at sites 7, 8, 11–14, 17 and 21. As shown in Table 1, no special objects were monitored in this area. Sites 15, 16 and 19 are not exposed to municipal or industrial effluents; however, significantly higher concentrations of drugs were detected relative to the previous sites because sites 15, 16 and 19 are the basin outlets of rivers (Morava, Dyje and Bečva, respectively) that pass through highly inhabited areas and have relatively low dilution factors (ratio of wastewater effluent and river flow). Municipal wastewater input from five large cities contributed significantly to the contamination of various sites (10, 18, 20, 6, and 5) by target compounds, proving that WWTPs are the main source of pharmaceuticals in these aquatic environments. These findings agree with previous studies (Bartelt-Hunt et al., 2009; Kim et al., 2007). The highest concentrations were found at sites 18, 20 and 6. Those sites are located in highly inhabited municipalities. Their relatively low dilution factors contribute to the high concentrations observed. However, high dilutions of the WWTP effluent in rivers Labe and Vltava led to low concentrations of compounds at sites 5 and 10. Illicit drugs were detected mainly in the sites located in large municipalities that receive WWTP effluents.

3.3. Effects of environmental conditions on Rs and limitations for POCIS application

Passive samplers were designed to mimic the chemical exposure of aquatic organisms. Therefore, only water dissolved (biologically available) fraction of pollutants is sampled. This factor separates passive sampling from the more common sampling techniques and enables the use of the obtained information for risk assessment. The drawbacks of passive sampling include the need to determine the sampling rate values and to perform adjustments for site-specific environmental effects. The performance reference compound (PRC) approach was successfully used by Huckins and co-authors for SPMD samplers (Huckins et al., 2002). Unlike SPMDs, the POCIS uptake kinetics is more complicated with sorption being more important than the partition process; therefore, the release of PRCs and the uptake of target compounds are heterotopic. Although there were some attempts to find proper PRCs (Mazzella et al., 2007), this approach still is not applicable for POCIS. Currently, calibrating POCIS is the only way to obtain sampling rate values for compounds of interest. On the one hand, field calibration is limited by the number of compounds that naturally occur in water. On the other hand, these calibration studies are more helpful when carried out in situ rather than in the laboratory because reproducing real exposure conditions in the laboratory remains challenging. Currently, the lack of calibration data is the primary limitation hindering the application of POCIS for monitoring of polar organic pollutants.

The sampling rate values can be affected by environmental conditions during the exposure period. Factors such as temperature, biofouling and water flow might be involved. The temperature was stable during the entire calibration experiment (11.0 ± 0.4 °C). Temperature is an important factor because it may exert a significant compound-specific effect on the sampling rate (Kingston et al., 2000; Togola and Budzinski, 2007).

The uptake of the target compounds by the receiving phase can be decreased due to algal or microbial growth on the sampler membrane (Soderstrom et al., 2009). No visible differences in the levels of biofouling were observed during the sampling periods. Moreover, Alvarez and co-authors determined that the POCIS membrane is resistant to biofouling (Alvarez et al., 2004).

The changes in flow had little effect on the sampling rate according to Li and co-authors (Li et al., 2010). Another study by Li indicated slight variations in the Rs values under the conditions (pH and dissolved organic matter) observed in natural waters (Li et al., 2011).

The overall effect of combining different environmental factors on sampling rates remains unknown. Therefore, in the absence of a PRC-based approach, increasing the number of available sampling rate values for different polar compounds is critical.

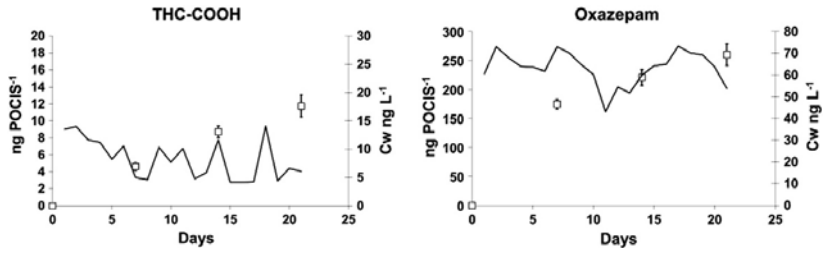

Fig. 2. Uptake in POCIS (left axis, n = 3) and water concentrations (right axis) of target compounds.

Table 3
Estimated surface water concentrations for selected compounds (ng L^{-1}).

Compound	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Benzoylecgonine	ND	ND	ND	0.81	2.8	8.3	0.91	ND	2.4	6.8	0.51	1.1	ND	ND	ND	1.6	ND	8.5	ND	2.1	0.71
Carbamazepine	8.9	41	44	53	104	150	41	26	92	71	44	27	48	62	185	140	53	172	87	141	58
Citalopram	1.1	1.3	1.8	3.9	3.1	12	3.2	0.64	2.7	7.4	1.3	2.1	1.4	1.3	3.1	1.4	1.5	18	2.4	7.9	2.4
Metamphetamine	4.3	0.91	2.3	5.2	11	98	5.6	2.9	48	25	1.8	2.6	0.82	2.1	9.1	9.9	5.2	65	9.1	107	7.8
Oxazepam	0.53	6.7	10	18	30	47	7.1	8.4	38	18	11	5.6	15	14	34	24	17	55	14	25	7.1
THC-COOH	1.1	ND	ND	1.9	4.3	ND	2.1	ND	3.1	ND	3.1	ND	ND	ND	ND	3.5	2.5	9.5	ND	ND	2.3
Tramadol	12	39	42	62	63	140	39	21	112	77	41	29	47	36	138	99	47	210	98	128	37
Venlavafaxin	1.9	5.6	6.2	11	9.7	12	6.4	3.1	14	16	8.7	5.6	6.2	5.2	12	11	16	41	12	24	3.7

ND not detected.

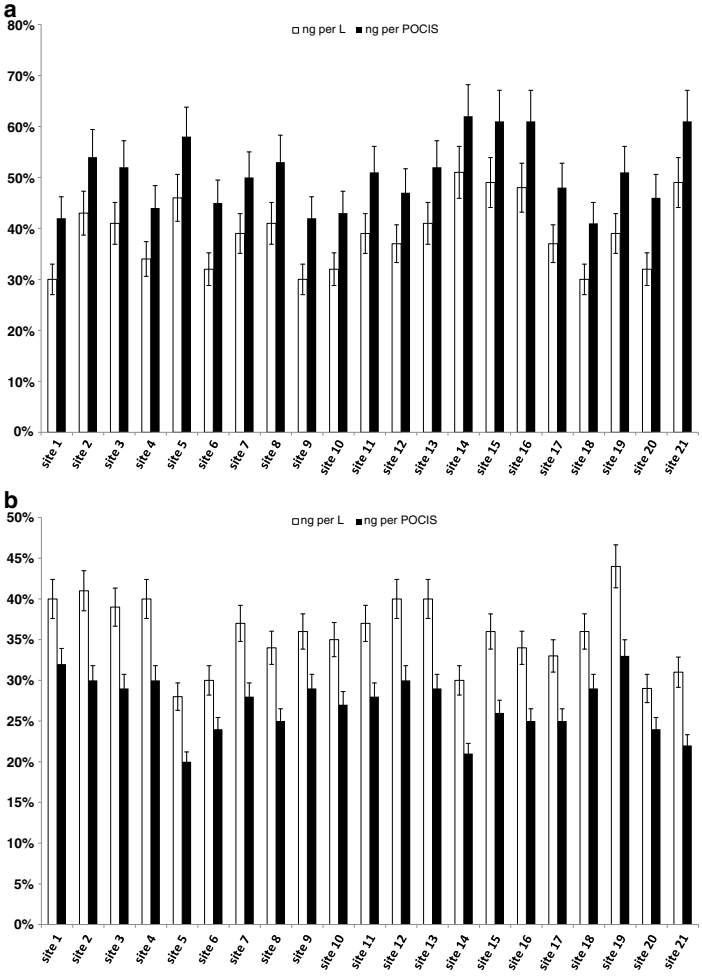

Fig. 3. Percentage of selected drug from total amount of drugs based on POCIS adsorbed amount and concentrations back calculated to water units (a. carbamazepine, b. tramadol).

3.4. Estimated aqueous concentrations of target compounds

The estimated concentrations of the target compounds in rivers are presented in Table 3. The sampling rate values obtained from the calibration experiment (Section 3.1) were used to back calculate the concentrations of the target compound in water based on the concentrations measured in the POCIS extract. High concentrations were estimated for methamphetamine, carbamazepine and tramadol at some of the sampling sites (Table 3). Interestingly, the proportional representation changed when the concentrations were recalculated from units of ng $POCIS^{-1}$ to ng L^{-1}. For example, the proportion of carbamazepine was higher when based on the POCIS concentration than the relative amount based on the water concentration (Fig. 3a); for tramadol, the pattern was the opposite (Fig. 3b). This effect is caused by the differences in sampling rates: the uptake by POCIS is faster for carbamazepine than for tramadol (Table 2). This fact should be accounted for when carrying out toxicity assays with POCIS extracts because the results might differ from those obtained during toxicity tests with water samples obtained as grab samples from the same location.

This type of estimate is only approximate because sampling rates calibrated under wastewater effluent conditions cannot be directly transformed into precise concentrations of water samples under ambient environmental conditions. The magnitude of the uptake rate can be influenced by the environmental conditions and the water matrix. However, POCIS is a robust tool for qualitative and semi-quantitative testing.

4. Conclusions

Passive sampling by POCIS is useful for monitoring pharmaceutical and illicit drug contents in aquatic environments. Drug monitoring in the Czech Republic has demonstrated that many target compounds enter river waters, and a number of these compounds reach high concentrations. A field calibration for POCIS was performed for selected compounds to calculate the sampling rates for a number of the compounds, enabling the estimation of the aqueous concentrations of target compounds at the monitored sites. This back-calculation changed the observed relative proportion of compounds in the sample. This trend might generate different results during toxicity assessments for POCIS extracts and grab samples of water from the same location. This finding stresses the importance of knowing the uptake rates during the back-calculation of concentrations of target compounds in waters using POCIS. Further research is needed to provide sampling rate values to calculate the aqueous compound concentrations, making POCIS a more attractive and precise tool for monitoring pharmaceutical and drug contents in aquatic environments.

Acknowledgements

This study was supported by the Ministry of Education, Youth and Sports of the Czech Republic CENAKVA No. CZ.1.05/2.1.00/01.0024, the Ministry of Education, Youth and Sports of the Czech Republic- the project LO1205 under the NPU I program, the project. Kontakt II LH12179 and the Grant Agency of the University of South Bohemia No. 087/2013/Z, CZ.1.07/2.3.00/20.0024 Strengthening of Excellence scientific teams in USB FFPW. We thank Elsevier editing service for the language correction of the manuscript.

References

Alvarez DA, Petty JD, Huckins JN, Jones-Lepp TL, Getting DT, Goddard JP, et al. Development of a passive, in situ, integrative sampler for hydrophilic organic contaminants in aquatic environments. Environ Toxicol Chem 2004;23:1640–8.

Alvarez DA, Stackelberg PE, Petty JD, Huckins JN, Furlong ET, Zaugg SD, et al. Comparison of a novel passive sampler to standard water-column sampling for organic contaminants associated with wastewater effluents entering a New Jersey stream. Chemosphere 2005;61:610–22.

Bartelt-Hunt SL, Snow DD, Damon T, Shockley J, Hoagland K. The occurrence of illicit and therapeutic pharmaceuticals in wastewater effluent and surface waters in Nebraska. Environ Pollut 2009;157:786–91.

Bartelt-Hunt SL, Snow DD, Damon-Powell T, Brown DL, Prasai G, Schwarz M, et al. Quantitative evaluation of laboratory uptake rates for pesticides, pharmaceuticals, and steroid hormones using POCIS. Environ Toxicol Chem 2011;30:1412–20.

Berset JD, Brenneisen R, Mathieu C. Analysis of llicit and illicit drugs in waste, surface and lake water samples using large volume direct injection high performance liquid chromatography–Electrospray tandem mass spectrometry (HPLC–MS/MS). Chemosphere 2010;81:859–66.

Bijlsma L, Emke E, Hernandez F, de Voogt P. Investigation of drugs of abuse and relevant metabolites in Dutch sewage water by liquid chromatography coupled to high resolution mass spectrometry. Chemosphere 2012;89:1399–406.

Brodin T, Fick J, Jonsson M, Klaminder J. Dilute concentrations of a psychiatric drug alter behaviour of fish from natural populations. Science 2013;339:814–5.

Buchberger WW. Novel analytical procedures for screening of drug residues in water, waste water, sediment and sludge. Anal Chim Acta 2007;593:129–39.

Bueno MJM, Hernando MD, Aguera A, Fernandez-Alba AR. Application of passive sampling devices for screening of micro-pollutants in marine aquaculture using LC–MS/MS. Talanta 2009;77:1518–27.

Castiglioni S, Zuccato E, Chiabrando C, Fanelli R, Bagnati R. Mass spectrometric analysis of illicit drugs in wastewater and surface water. Mass Spectrom Rev 2008;27:378–94.

Fedorova G, Golovko O, Randak T, Grabic R. Passive sampling of perfluorinated acids and sulfonates using polar organic chemical integrative samplers. Environ Sci Pollut Res 2013a;20:1344–51.

Fedorova G, Randak T, Lindberg RH, Grabic R. Comparison of the quantitative performance of Q Exactive high resolution mass spectrometer with that of triple quadrupole MS/MS for the analysis of illicit drugs in wastewater. Rapid Commun Mass Spectrom 2013b;27:1751–62.

Feito R, Valcarcel Y, Catala M. Preliminary data suggest that venlafaxine environmental concentrations could be toxic to plants. Chemosphere 2013;90:2065–9.

Gheorghe A, van Nuijs A, Pecceu B, Bervoets L, Jorens PG, Blust R, et al. Analysis of cocaine and its principal metabolites in waste and surface water using solid-phase extraction and liquid chromatography–ion trap tandem mass spectrometry. Anal Bioanal Chem 2008;391:1309–19.

Grabic R, Fick J, Lindberg RH, Fedorova G, Tysklind M. Multi-residue method for trace level determination of pharmaceuticals in environmental samples using liquid chromatography coupled to triple quadrupole mass spectrometry. Talanta 2012;100:183–95.

Harman C, Reid M, Thomas KV. In situ calibration of a passive sampling device for selected illicit drugs and their metabolites in wastewater, and subsequent year-long assessment of community drug usage. Environ Sci Technol 2011;45:5676–82.

Huckins JN, Petty JD, Lebo JA, Almeida FV, Booij K, Alvarez DA, et al. Development of the permeability/performance reference compound approach for in situ calibration of semipermeable membrane devices. Environ Sci Technol 2002;36:85–91.

Irvine RJ, Kostakis C, Felgate PD, Jaehne EJ, Chen C, White JM. Population drug use in Australia: a wastewater analysis. Forensic Sci Int 2011;210:69–73.

Karolak S, Nefau T, Bailly E, Solgadi A, Levi Y. Estimation of illicit drugs consumption by wastewater analysis in Paris area (France). Forensic Sci Int 2010;200:153–60.

Kim SD, Cho J, Kim IS, Vanderford BJ, Snyder SA. Occurrence and removal of pharmaceuticals and endocrine disruptors in South Korean surface, drinking, and waste waters. Water Res 2007;41:1013–21.

Kingston JK, Greenwood R, Mills GA, Morrison GM, Persson LB. Development of a novel passive sampling system for the time-averaged measurement of a range of organic pollutants in aquatic environments. J Environ Monit 2000;2:487–95.

Kot A, Zabiegala B, Namiesnik J. Passive sampling for long-term monitoring of organic pollutants in water. TrAC Trends Anal Chem 2000;19:446–59.

Li HX, Helm PA, Paterson G, Metcalfe CD. The effects of dissolved organic matter and pH on sampling rates for polar organic chemical integrative samplers (POCIS). Chemosphere 2011;83:271–80.

Li HX, Vermeirssen ELM, Helm PA, Metcalfe CD. Controlled field evaluation of water flow rate effects on sampling polar organic compounds using polar organic chemical integrative samplers. Environmental Toxicology and Chemistry 2010;29:2461–9.

Mazzella N, Dubernet JF, Delmas F. Determination of kinetic and equilibrium regimes in the operation of polar organic chemical integrative samplers. Application to the passive sampling of the polar herbicides in aquatic environments. J Chromatogr A 2007;1154:42–51.

Soderstrom H, Lindberg RH, Fick J. Strategies for monitoring the emerging polar organic contaminants in water with emphasis on integrative passive sampling. J Chromatogr A 2009;1216:623–30.

Thomas KV, Bijlsma L, Castiglioni S, Covaci A, Emke E, Grabic R, et al. Comparing illicit drug use in 19 European cities through sewage analysis. Sci Total Environ 2012;432:432–9.

Togola A, Budzinski H. Development of polar organic integrative samplers for analysis of pharmaceuticals in aquatic systems. Anal Chem 2007;79:6734–41.

Zuccato E, Chiabrando C, Castiglioni S, Bagnati R, Fanelli R. Estimating community drug abuse by wastewater analysis. Environ Health Perspect 2008;116:1027–32.

CHAPTER 4

GENERAL DISCUSSION

It is generally known that waste waters from households and hospitals contain many human used compounds, mainly PPCPs. STP are not able to remove these chemicals from water, so they enter the aquatic environment. Pharmaceuticals could be still in active form and can cause similar therapeutic effects in non-target aquatic organism as in humans. PCPs present in surface waters could affect the organism as well. This thesis is focused on the effects, mainly on bioconcentration / bioaccumulation of individual compounds or mixtures of them on aquatic organisms, fish and benthos.

The effect of individual compounds was studied on fish exposed to water stocks under laboratory condition (Steinbach et al., 2013, chapter 2.1.; Grabicova et al., 2013, chapter 2.2.).

The first tested compound was verapamil. This cardio-vascular drug acting as blocker of the calcium channel is frequently prescribed for treatment of high blood pressure and angina pectoris. Two types of tests were carried out: a 96h acute toxicity test and a sub-chronic test. The acute toxicity test was carried out on concentration levels 1, 2, 5, 10, 15 and 20 mg L^{-1}, respectively. The value of verapamil 96hLC_{50} for common carp (*Cyprinus carpio*) embryos and larvae ranged from 4.8 to 16.4 mg L^{-1}. This finding is in accordance with previous studies (Li et al., 2010b; Villegas-Navarro et al., 2003) where the lethal concentration was studied on juvenile rainbow trout (*Oncorhynchus mykiss*) and OECD guideline (OECD, 2001), verapamil could be classified as a harmful or toxic substance. Verapamil exposure caused the reduction of heart rate in embryo and larvae stage of common carp, this finding was observed in zebrafish (*Danio rerio*) embryo and larvae as well (Berghmans et al., 2008; Shin et al., 2010). Also spine malformation and edema were observed in fish exposed to verapamil. Edema was found in juvenile frogs treated by verapamil too (Burgess and Vere, 1989). All of the above described effects were observed at concentration levels far from those which can be expected in STP recipients.

In the consequent experiment, the common carp embryos and larvae were exposed to concentrations 0.463 (environmentally relevant concentration), 4.63, 46.3 and 463 μg L^{-1}, respectively for a sub-chronic test. Hatching, mortality, growth and anomalies were studied. No effects were observed for any of the studied endpoints on fish exposed to environmentally relevant concentration compare to control fish. However, results of the study were used for verapamil bioconcentration factor (BCF) and verapamil half-life estimations. The estimated BCF values were in good agreement with those predicted from K_{ow} (US EPA, 2012) while verapamil half-life was much longer than verapamil half-life in humans.

The second studied compound was 2-phenylbenzimidazole-5-sulfonic acid (PBSA), a UV filter commonly present in surface waters in the Czech Republic (Grabicova et al., 2013, chapter 2.2.), in Germany (Wick et al., 2010) or in Spain (Rodil et al., 2012). Rainbow trout were exposed to different concentration levels, 1 (environmentally relevant concentration), 10 and 1000 μg L^{-1}, respectively for 21 and 42 days. Cytochromes P450 (CYP, mainly the first three families CYP1, CYP2 and CYP3) are involved in metabolism of xenobiotics (Daughton and Ternes, 1999; Hasler et al., 1999; Meunier et al., 2004), therefore the activities of CYP1A (cytochrome P450, family 1, subfamily A) measured as the catalytic activity of EROD and 7-methoxyresorufin-O-deethylase (MROD), and CYP2B measured as activity of 7-penthoxyresorufin-O-deethylase (PROD) were determined. Activities of EROD, MROD and PROD were significantly elevated in fish exposed to PBSA in all tested groups compared to control fish, even after 21 days of exposure. These results indicate that even environmentally relevant concentration could affect CYP activity.

Anti-oxidative stress enzyme (superoxide dismutase, catalase and glutathione reductase) activities were measured in liver of fish exposed to the highest concentration of PBSA. Only the activity of glutathione reductase was changed. This decreased activity of glutathione reductase might be caused by the impairment of the enzyme by reactive oxygen species (Ochi, 1990; Rousar et al., 2010) or by the diminished availability of NADPH (Li et al., 2010d). Biochemical parameters (lactate, glucose, alanine aminotransferase, creatine kinase, triglycerides and total proteins) were measured in blood plasma. The concentration of glucose in exposed fish was higher compared to control fish. Elevated concentrations of glucose in blood indicate metabolic stress (Portz et al., 2006). Creatine kinase and alanine aminotransferase are markers of environmental stress (El-Sayed et al., 2007; Li et al., 2010c). While creatine kinase was elevated, alanine aminotransferase was not. There were no changes in measures of hematological parameters. The results of concentration measured in plasma and different tissues indicate that PBSA does not accumulate in fish but can enter the fish and can therefore induce a response in the liver (impacted CYP activity).

From these laboratory results is obvious, that even environmentally relevant concentrations represent risk for fish. And in this case, fish were exposed only to one pollutant. In the real conditions, in effluents from municipal STP, there are many compounds which enter surface waters. Some of them are found in higher concentrations, e.g. atenolol, carbamazepine, cetirizine, ciprofloxacin, citalopram, diclofenac, hydrochlorothiazide, metoprolol, tramadol and venlafaxine (Fedorova et al., 2014; Fick et al., 2009; Golovko et al., 2014; Verlicchi et al., 2013); some of them in trace-level, e.g. benzophenone 3, estrone, isradipine, loratadine, phenazone and timolol (Gabet-Giraud et al., 2014; Golovko et al., 2014; Kosma et al., 2014; Verlicchi et al., 2013); some of them even under the limit of quantification (LOQ), e.g. diazepam, fenofibrate, ketamine, methadone, midazolam, simvastatin (Fedorova et al., 2014; Kosma et al., 2014; Verlicchi et al., 2013); and some of them were not analyzed. Nobody can predict how this mixture could affect aquatic organisms living in this cocktail of pollutants for their whole life. Therefore, the effects on fish should be studied in real conditions, it means using real effluent or real surface water for the experiment.

In the next study (Grabicova et al., 2014, chapter 3.1.) the effluent from the second biggest STP in Sweden was used for determining the concentration of antidepressants in fish tissues. Rainbow trout were placed into tanks with this effluent for exposure time almost 2 weeks. The bioconcentration was determined. Even this quite short period of exposure led to the presence of citalopram, sertraline and venlafaxine in fish brain and liver. Fluoxetine was found below LOQ. Brain is the target organ for action of antidepressants and liver is the organ where the main detoxification activity is going on. The concentrations of venlafaxine and citalopram were higher in liver than in brain, which correspond with a previous study carried on brook trout (*Salvelinus fontinalis*) (Lajeunesse et al., 2011). The concentration of sertraline was higher in brain than in liver. The same findings were observed in fish living in an effluent-dominated stream (Brooks et al., 2005). These antidepressants are used for treatment anxiety disorders and major depression. Because they were found in fish brain, it suggests that the behavior of fish could be affected. Unfortunately, the changes in behavior of fish were not studied due to the unnatural surroundings for exposed fish. The concentration of antidepressants was measured in composite 24 hours effluent flow-proportional samples to calculate tissue-specific BCFs. Higher BCF was found in liver than in brain for citalopram and venlafaxine. For sertraline, BCF was higher in brain than in liver. The values of BCFs are similar to values estimated in brook trout (Lajeunesse et al., 2011). Because of the target organ, the concentration measured in brain is the most informative for evaluation of risk assessment.

But as it was mentioned, only bioconcentration was observed. Again, this type of experiment does not mimic real conditions where fish are living in. One of the factors are that fish are stressed so measured biomarkers could be misrepresented. The second factor, which is not included to "caged fish" or "aquarium fish" approach, is diet. The uptake of chemicals via the food chain is an important part for risk assessment.

An important part of salmonid fish diet consists of benthic organisms. Therefore concentration of different pharmaceuticals in extracts from benthic organisms was studied (Grabicova et al., chapter 2.2). Zivny stream, a small stream highly impacted by effluent from Prachatice STP was chosen as a model site. Two benthic species, *Hydropsyche* sp. and *Erpobdella octoculata* were picked up in different areas of the stream, area E (situated directly downstream the effluent from STP), area B and R (1.5 and 3 km downstream the effluent, respectively). For comparison, benthic organisms were picked up at non-contaminated area (area C, approx. 4.5 km upstream the effluent).

There are many studies where benthic organisms and aquatic pollution is discussed. However, these studies are focused mainly on biochemical responses (Damasio et al., 2011a; Damasio et al., 2011b), biodiversity (Azrina et al., 2006; Koperski, 2010; Pan et al., 2013; Pan et al., 2012a; Pan et al., 2012b) and survival (Stuijfzand et al., 1999). Our study was focused on bioaccumulation of pharmaceuticals. A multi-residue analytical method on LC/HRMS/MS was used for analysis of seventy compounds. Pharmaceuticals detected in benthos extracts belong to groups of antibiotics (azithromycin and clarithromycin), cardiovascular drugs (valsartan and verapamil), non-steroidal anti-inflammatory drugs (diclofenac), antifungal drugs (clotrimazole) and psychoactive drugs (sertraline and citalopram). The highest bioaccumulated pharmaceuticals were azithromycin and diclofenac in *Hydropsyche* and *Erpobdella*, respectively. The uptake of pharmaceuticals via the food web could be an important exposure pathway of wild fish populations.

As it was mentioned in the introduction, another sampling method without live organisms could be used for monitoring or checking the situation in the streams – passive sampling (Fedorova et al., chapter 3.3.). Because of lack of calibration data, the sampling rate for individual compounds had to be calculated. This calculation was done from effluent of STP in Ceske Budejovice. The sampling rate is calculated from the amount of compound in POCIS, average water concentration and time of exposure. This equation is valid for kinetic regime of uptake (Mazzella et al., 2007) therefore a linear period of uptake is needed. Sampling rate could be affected by several factors such as temperature (Kingston et al., 2000; Togola and Budzinski, 2007), biofouling (algae and microbial growth (Soderstrom et al., 2009)) and water flow (Li et al., 2010a). Concentration of some compounds (e.g. benzoylecgonine, ketamine, methadone and midazolam) in water samples was below LOQ, but these compounds were found in POCIS extracts. The advantage of POCIS as a sampling tool is that samples are preconcentrated and the LOQ could be lower.

The passive sampling of river water was carried out within water quality monitoring of the Czech Hydrometeorological Institute at twenty-one key profile localities in the Czech Republic. Psycho-active drugs like carbamazepine, venlafaxine, citalopram and opioid analgesic tramadol together with the illicit drug metamphetamine were found at a relatively high concentration in POCIS from all sampled sites. The most polluted sites were those that received effluent from STP with a low dilution factor (ratio of wastewater effluent and stream flow) and vice versa.

The sampling rates were used for back calculation of water concentrations from data measured in POCIS extracts. The proportions among individual compounds in POCIS were

different from the proportions in back calculated data. These differences could lead to discrepancy between toxicity assessment based on passive sampling and toxicity assessment based on conventional grab water samples.

Conclusions and future perspectives

It is obvious from laboratory carried out experiments that even environmentally relevant concentrations of one of PPCPs can affect aquatic organisms. The real waters contain a mixture of different pollutant so the effects could be multiplied.

An important part of natural fish diet is benthic organisms. These organisms are affected via pollutants as well as fish; the bioaccumulation of some pharmaceuticals was demonstrated (Grabicova et al., chapter 3.2.). The hypothesis was verified: Benthic organisms as a part of fish diet established bioaccumulation of pharmaceuticals. So the fish consuming benthic organisms are affected not only via pharmaceuticals dissolved in water but via the food chain too. The consequent experiment with fish settled to polluted sites was done. The fish were in their native environment with natural food and the time of exposure was known. Concentration in fish plasma and tissues will be measured.

PS is useful sampling tool for the monitoring of pollution in aquatic environment but further research is needed to make PS by POCIS more attractive and precise method for sampling aquatic environment.

REFERENCES

Azrina, M.Z., Yap, C.K., Ismail, A.R., Ismail, A., Tan, S.G., 2006. Anthropogenic impacts on the distribution and biodiversity of benthic macroinvertebrates and water quality of the Langat River, Peninsular Malaysia. Ecotox. Environ. Safe. 64, 337–347.

Berghmans, S., Butler, P., Goldsmith, P., Waldron, G., Gardner, I., Golder, Z., Richards, F. M., Kimber, G., Roach, A., Alderton, W., Fleming, A., 2008. Zebrafish based assays for the assessment of cardiac, visual and gut function — potential safety screens for early drug discovery. J. Pharmacol. Toxicol. Methods 58, 59–68.

Brooks, B.W., Chambliss, C.K., Stanley, J.K., Ramirez, A., Banks, K.E., Johnson, R.D., Lewis, R.J., 2005. Determination of select antidepressants in fish from an effluent-dominated stream. Environ. Toxicol. Chem. 24, 464–469.

Burgess, A.M.C., Vere, D.W., 1989. Teratogenic effects of some calcium-channel blocking agents in *Xenpulus embryos*. Pharmacol. Toxicol. 64, 78–82.

Damasio, J., Barcelo, D., Brix, R., Postigo, C., Gros, M., Petrovic, M., Sabater, S., Guasch, H., de Alda, M.L., Barata, C., 2011a. Are pharmaceuticals more harmful than other pollutants to aquatic invertebrate species: A hypothesis tested using multi-biomarker and multi-species responses in field collected and transplanted organisms. Chemosphere 85, 1548–1554.

Damasio, J., Fernandez-Sanjuan, M., Sanchez-Avila, J., Lacorte, S., Prat, N., Rieradevall, M., Soares, A., Barata, C., 2011b. Multi-biochemical responses of benthic macroinvertebrate species as a complementary tool to diagnose the cause of community impairment in polluted rivers. Water Res. 45, 3599–3613.

Daughton, C.G., Ternes, T.A., 1999. Pharmaceuticals and personal care products in the environment: Agents of subtle change? Environ. Health Perspect. 107, 907–938.

El-Sayed, Y.S., Saad, T.T., El-Bahr, S.M., 2007. Acute intoxication of deltamethrin in monosex Nile tilapia, Oreochromis niloticus with special reference to the clinical, biochemical and haematological effects. Environ. Toxicol. Pharmacol. 24, 212–217.

Fedorova, G., Nebesky, V., Randak, T., Grabic, R., 2014. Simultaneous determination of 32 antibiotics in aquaculture products using LC-MS/MS. Chemical Papers. 68, 29–36.

Fick, J., Soderstrom, H., Lindberg, R.H., Phan, C., Tysklind, M., Larsson, D.G.J., 2009. Contamination of Surface, Ground, and Drinking Water from Pharmaceutical Production. Environ. Toxicol. Chem. 28, 2522–2527.

Gabet-Giraud, V., Miege, C., Jacquet, R., Coquery, M., 2014. Impact of wastewater treatment plants on receiving surface waters and a tentative risk evaluation: the case of estrogens and beta blockers. Environ. Sci. Pollut. Res. 21, 1708–1722.

Golovko, O., Kumar, V., Fedorova, G., Randak, T., Grabic, R., 2014. Removal and seasonal variability of selected analgesics/anti-inflammatory, anti-hypertensive/cardiovascular pharmaceuticals and UV filters in wasterwater treatment plant. Environ. Sci. Pollut. Res. 21, 7578–7585

Hasler, J.A., Estabrook, R., Murray, M., Pikuleva, I., Waterman, M., Capdevila, J., Holla, V., Helvig, C., Falck, J.R., Farrell, G., Kaminsky, L.S., Spivack, S.D., Bioitier, E., Beaune, P., 1999. Human cytochromes P450. Mol. Aspects Med. 20, 1–137.

Kingston, J.K., Greenwood, R., Mills, G.A., Morrison, G.M., Persson, L.B., 2000. Development of a novel passive sampling system for the time-averaged measurement of a range of organic pollutants in aquatic environments. J. Environ. Monit. 2, 487–495.

Koperski, P., 2010. Diversity of macrobenthos in lowland streams: ecological determinants and taxonomic specificity. J. Limnol. 69, 88–101.

Kosma, C.I., Lambropoulou, D.A., Albanis, T.A., 2014. Investigation of PPCPs in wastewater treatment plants in Greece: Occurrence, removal and environmental risk assessment. Sci. Total Environ. 466, 421–438.

Lajeunesse, A., Gagnon, C., Gagne, F., Louis, S., Cejka, P., Sauve, S., 2011. Distribution of antidepressants and their metabolites in brook trout exposed to municipal wastewaters before and after ozone treatment – Evidence of biological effects. Chemosphere 83, 564–571.

Li, H.X., Vermeirssen, E.L. M., Helm, P.A., Metcalfe, C.D., 2010a. Controlled field evaluation of water flow rate effects on sampling polar organic compounds using polar organic chemical integrative samplers. Environ. Toxicol. Chem. 29, 2461–2469.

Li, Z.H., Li, P., Randak, T., 2010b. Ecotoxocological effects of short-term exposure to a human pharmaceutical Verapamil in juvenile rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. C-Toxicol. Pharmacol. 152, 385–391.

Li, Z.H., Velisek, J., Zlabek, V., Grabic, R., Machova, J., Kolarova, J., Randak, T., 2010c. Hepatic antioxidant status and hematological parameters in rainbow trout, *Oncorhynchus mykiss*, after chronic exposure to carbamazepine. Chem.-Biol. Interact. 183, 98–104.

Li, Z.H., Zlabek, V., Grabic, R., Li, P., Machova, J., Velisek, J., Randak, T., 2010d. Effects of exposure to sublethal propiconazole on intestine-related biochemical responses in rainbow trout, *Oncorhynchus mykiss*. Chem.-Biol. Interact. 185, 241–246.

Mazzella, N., Dubernet, J. F., Delmas, F., 2007. Determination of kinetic and equilibrium regimes in the operation of polar organic chemical integrative samplers – Application to the passive sampling of the polar herbicides in aquatic environments. J. Chromatogr. A. 1154, 42–51.

Meunier, B., de Visser, S. P., Shaik, S., 2004. Mechanism of oxidation reactions catalyzed by cytochrome P450 enzymes. Chem. Reviews. 104, 3947–3980.

OECD, 2001. Harmonized integrated hazard classification system for human health and environmental hazards of chemical substances and mixtures. [8]Annex 2. Guidance Document 27, ENV/JM/MONO (2001). Paris, France: Organization for Economic Co-operation and Development.

Ochi, T., 1990. Effects of an organic hydroperoxide on the activity of antioxidant enzymes in cultured-mammalian-cells. Toxicology 61, 229–239.

Pan, B.Z., Wang, Z.Y., Xu, M.Z., 2012a. Macroinvertebrates in abandoned channels: assemblage characteristics and their indications for channel management. River Res. Appl. 28, 1149–1160.

Pan, B.Z., Wang, Z.Y., Xu, M.Z., Xing, L.H., 2012b. Relation between stream habitat conditions and macroinvertebrate assemblages in three Chinese rivers. Quat. Int. 282, 178–183.

Pan, B.Z., Wang, Z.Y., Li, Z.W., Yu, G.A., Xu, M.Z., Zhao, N., Brierley, G., 2013. An exploratory analysis of benthic macroinvertebrates as indicators of the ecological status of the Upper Yellow and Yangtze Rivers. J. Geograph. Sci. 23, 871–882.

Portz, D.E., Woodley, C.M., Cech, J.J., 2006. Stress-associated impacts of short-term holding on fishes. Rev. Fish. Biol. Fish. 16, 125–170.

Rodil, R., Quintana, J.B., Concha-Grana, E., Lopez-Mahia, P., Muniategui-Lorenzo, S., Prada-Rodriguez, D., 2012. Emerging pollutants in sewage, surface and drinking water in Galicia (NW Spain). Chemosphere 86, 1040-1049.

Rousar, T., Parik, P., Kucera, O., Bartos, M., Cervinkova, Z., 2010. Glutathione reductase is inhibited by acetaminophen-glutathione conjugate *in vitro*. Physiol. Res. 59, 225–232.

Shin, J.T., Pomerantsev, E.V., Mably, J.D., MacRae, C.A., 2010. High-resolution cardiovascular function confirms functional orthology of myocardial contractility pathways in zebrafish. Physiol. Genomics. 42, 300–309.

Soderstrom, H., Lindberg, R.H., Fick, J., 2009. Strategies for monitoring the emerging polar organic contaminants in water with emphasis on integrative passive sampling. J. Chromatogr. A. 1216, 623–630.

Stuijfzand, S.C., Engels, S., van Ammelrooy, E., Jonker, M., 1999. Caddisflies (Trichoptera : Hydropsychidae) used for evaluating water quality of large European rivers. Arch. Environ. Contam. Toxicol. 36, 186–192.

Togola, A., Budzinski, H., 2007. Development of polar organic integrative samplers for analysis of pharmaceuticals in aquatic systems. Analytical Chemistry. 79, 6734–6741.

US EPA, 2012. Estimation Programs Interface Suite™ for Microsoft® Windows, v 4.11. United States Environmental Protection Agency, Washington, DC, USA. http://www.epa.gov/opptintr/exposure/pubs/episuite.htm, accessed 14th April 2014.

Verlicchi, P., Galletti, A., Petrovic, M., Barcelo, D., Al Aukidy, M., Zambello, E., 2013. Removal of selected pharmaceuticals from domestic wastewater in an activated sludge system followed by a horizontal subsurface flow bed – Analysis of their respective contributions. Sci. Total Environ. 454, 411–425.

Villegas-Navarro, A., Rosas-L, E., Reyes, J. L., 2003. The heart of Daphnia magna: effects of four cardioactive drugs. Comp. Biochem. Physiol. C-Toxicol. Pharmacol. 136, 127–134.

Wick, A., Fink, G., Ternes, T.A., 2010. Comparison of electrospray ionization and atmospheric pressure chemical ionization for multi-residue analysis of biocides, UV-filters and benzothiazoles in aqueous matrices and activated sludge by liquid chromatography-tandem mass spectrometry. J. Chromatogr. A. 1217, 2088–2103.

ENGLISH SUMMARY

Effects of chemicals present in sewage treatment plants´ effluents on fish

Kateřina Grabicová

There are many compounds that are used or produced by humans, e.g. pharmaceuticals and personal care products (PPCPs), nutritive components (sweeteners), persistent organic pollutants, heavy metals, which are used in large quantities around the world. These compounds end up in waste waters and enter sewage treatment plants (STPs). But not all of these compounds are removed during the treatment processes in STP and they enter the aquatic environment via sewage effluents. The thesis is focused especially on evaluation of presence and potential toxicological effects of PPCPs in aquatic environment.

The risk assessment for a single compound is usually based on laboratory experiments. The first tested compound was verapamil, a pharmaceutical for lowering blood pressure and treatment of cardiac diseases. Two types of tests were carried out, an acute one with high concentration levels and a sub-chronic one with levels that included environmentally relevant concentration. After the exposure, common carp larvae and embryos showed malformation and edemas and the heart rate was decreased. According to 96hLC_{50}, the verapamil could be classified as a harmful or toxic substance.

The second tested substance was UV filter 2-phenylbenzimidazole-5-sulfonic acid (PBSA) which was found at relatively high concentrations in south Bohemian surface waters. In spite of very low bioconcentration of PBSA in fish, activities of 7-ethoxyresorufin-O-deethylase, 7-methoxyresorufin-O-deethylase and 7-penthoxyresorufin-O-deethylase in exposed rainbow trout were increased which indicate that activity of cytochromes P450 were affected even in environmentally relevant concentration.

In a real aquatic system, fish are exposed to waters which contain not only a single pollutant but a mixture of them. A consequent experiment was carried out on rainbow trout caged in effluent for 13 days from STP in Gothenburg, the second biggest STP in Sweden. The concentration of selected antidepressants was measured in different fish tissues and plasma. Citalopram, sertraline and venlafaxine were found in fish brain and liver. As these antidepressants are used for the treatment of psychical disorders, behavior of fish could be affected.

The limitations of these experiments, even with real water with a mixture of chemicals, are that fish are stressed in aquaria or cages and the exposure via the food chain is not included.

Benthic organisms are an important part of fish diet. The impact of pollution on benthic organisms is often studied. However, there is limited knowledge about the bioaccumulation of pharmaceuticals in the benthos and consequently in fish. Therefore bioaccumulation in *Hydropsyche* and *Erpobdella* was studied. These organisms were collected in non-polluted and in effluent affected localities. Antibiotics azithromycin and clarithromycin, cardio-vascular drug verapamil, antifungal drug clotrimazole and antidepressants citalopram and sertraline were found in *Hydropsyche*. Cardio-vascular drug valsartan, antidepressant sertraline, anti-inflammatory drug diclofenac and antifungal drug clotrimazole were found in *Erpobdella*.

Passive sampling is an integrative sampling method which allows the obtaining of time average concentration over period of few weeks. POCIS (Polar Organic Chemical Integrative Samplers) are used for sampling of polar compounds. It also can mimic bioconcentration. Based on the field experiments, the sampling rate for individual pharmaceuticals and illicit drugs was calculated. Twenty-one localities in the Czech Republic were sampled by POCIS and the water concentration of psychoactive drugs was estimated. Further research is necessary to make POCIS passive sampling a more precise tool for monitoring pharmaceutical and drug contents in aquatic environments.

CZECH SUMMARY

Vliv chemikálií přítomných ve výtocích z čistíren odpadních vod na ryby

Kateřina Grabicová

Farmaka a látky každodenní potřeby (tzv. personal care products, PPCP), doplňky stravy, perzistentní organické polutanty a těžké kovy, to jsou chemikálie, které jsou používané či vyráběné po celém světě ve velkém množství. Tyto látky jsou vyloučeny či vyhozeny do odpadu a společně s odpadní vodou vstupují do čistíren odpadních vod (ČOV). Ne všechny látky jsou v několika stupňovém čisticím procesu odstraněny a prostřednictvím výtoků z ČOV se dostávají do životního prostředí. Tato dizertační práce je zaměřena na PPCP – na jejich přítomnost ve vodním prostředí a na zjišťování jejich možného vlivu na vodní organizmy.

Hodnocení rizik plynoucích z přítomnosti cizorodých látek ve vodním prostředí je obvykle založeno na laboratorních studiích. V rámci této práce bylo první testovanou látkou léčivo verapamil, který je hojně předepisován k léčbě srdečních nemocí a na snížení krevního tlaku. K testování tohoto léčiva byly použity dva typy testů – akutní s vysokými koncentracemi a subchronický, kde byla testována i koncentrace odpovídající reálným koncentracím této látky ve vodním prostředí. Embrya a larvy kapra obecného (*Cyprinus carpio*) vystavené působení verapamilu vykazovaly deformace a otoky těl. Bylo pozorováno i snížení tepové frekvence. Podle stanovené hodnoty 96hLC50 může být verapamil zařazen mezi škodlivé či jedovaté látky.

Další testovanou látkou byla 2-fenylbenzimidazole-5-sulfonová kyselina (PBSA). Tato kyselina patří do skupiny UV filtrů používaných např. v opalovacích krémech a jak bylo v rámci naší terénní studie zjištěno, vyskytuje se v povrchových vodách jižních Čech v relativně vysokých koncentracích. I přesto, že biokoncentrace PBSA v tkáních a plazmě pstruha duhového (*Oncorhynchus mykiss*) byla nízká, aktivita 7-ethoxyresorufin-O-deethylasy, 7-methoxyresorufin-O-deethylasy a 7-penthoxyresorufin-O-deethylasy u exponovaných ryb byla zvýšená, z čehož vyplývá, že byla ovlivněna aktivita cytochromů P450, a to i koncentrací PBSA běžně stanovovanou v povrchových vodách.

Ryby žijící ve volné přírodě jsou však vystaveny působení různorodých směsí chemických látek. V následné studii byl proto použit přístup, kdy byl pstruh duhový vystaven přímo působení výtoku z ČOV v Göteborgu, druhé největší ČOV ve Švédsku. Pozornost byla zaměřena na výskyt antidepresiv v krevní plazmě a tkáních ryb po jejich 13denní expozici. Citalopram, sertraline a venlafaxine byly nalezeny v mozku a játrech těchto ryb. Protože se tato léčiva používají k léčbě depresí a jiných psychických poruch, je možno předpokládat, že může být také ovlivněno i chování ryb vystavených působení těchto látek.

Akvarijní a klecové experimenty jsou však pro ryby stresující, a navíc nezohledňují příjem cizorodých látek potravou. Důležitou součástí potravy ryb jsou bentické organizmy. Je mnoho studií, které se zabývají vlivem znečištění na bentické organizmy, ale tyto studie jsou obvykle zaměřeny na biodiverzitu či ovlivnění biomasy. Dalším cílem práce bylo zjistit, zda se léčiva kumulují v tělech bentických organizmů. Byly studovány dva druhy bentických druhů, larvy chrostíků (*Hydropsyche* sp.) a pijavky (*Erpobdella octoculata*). V larvách chrostíků došlo ke kumulaci antibiotik azithromycinu a clarithromycinu, kardiovaskulárního léčiva verapamilu, protiplísňového léčiva klotrimazolu a antidepresiv sertralinu a citalopramu. V pijavkách byly nalezeny valsartan (kardiovaskulární léčivo), diklofenak (protizánětlivé léčivo) a také, stejně jako v larvách chrostíků, sertraline a klotrimazol.

Velmi perspektivní metodou používanou ke vzorkování životního prostředí je pasivní vzorkování. V reálných podmínkách byla provedena a ověřena kalibrace pasivních vzorkovačů. Pasivní vzorkovače POCIS (*Polar Organic Chemical Integrative Samplers*) byly použity ke vzorkování 21 lokalit České republiky pro stanovení psychoaktivních farmak. Pro využití pasivních vzorkovačů jako exaktní metody pro sledování farmak a drog ve vodním prostředí je zapotřebí dalšího výzkumu.

ACKNOWLEDGEMENTS

At last, space for acknowledgements. I would like to express my thanks to my supervisor Tomáš Randák for his guidance, advices and support during four years of my study, and for his friendship at work and after-work time too.

Then, I would like to thank to my colleagues from our Laboratory of Environmental Chemistry and Biochemistry: Roman, Vláďa, Jitka, Anja, Honza, Oksana, Vika, Dan, Olga, Sidika and Pavla as well as from symbiotic Laboratory of Aquatic Toxicology and Ichthyology. The working atmosphere in this team was (and still is) friendly, pleasant and joyful. Thank you for your help, inspiring suggestions and constructive discussions. I will always remember the time spent in laboratory, communication room and in Loucky mlyn.

I cannot forget to send the thanks to Sweden – to Department of Chemistry at Umeå University, especially to Jerker Fick, Richard Lindberg, Marcus Östman and Mats Tysklind. It was a great time in Sweden during my 3 months internship stay there. I would like to thank to Prof. Joakim Larsson for his help with our collective article.

I would like to thank to Willem for his language editing.

Finally, my warmly thanks belong to my dear family for their patience, support and love. THANK YOU.

This Ph.D. thesis could have arisen through the financial support of:

- The Ministry of Education, Youth and Sports of the Czech Republic, projects CENAKVA (CZ.1.05/2.1.00/01.0024), and CENAKVA II (project LO1205 under the NPU I program).
- The Grant Agency of the University of South Bohemia in České Budějovice, project no. 087/2010/Z.
- The Czech Science Foundation, project no. P503/11/1130

LIST OF PUBLICATIONS

PEER-REVIEWED JOURNALS WITH IF

Fedorova, G., Randak, T., Golovko, O., Kodes, V., **Grabicova, K.**, Grabic, R., 2014. A passive sampling method for detecting analgesics, psycholeptics, antidepressants and illicit drugs in aquatic environment in the Czech Republic. Science of the Total Environment 487: 681–687.

Grabicova, K., Lindberg, R.H., Östman, M., Grabic, R., Randak, T., Larsson, D.G.J., Fick, J., 2014. Tissue-specific bioconcentration of antidepressants in fish exposed to effluent from a municipal sewage treatment plant. Science of the Total Environment 488–489: 46–50.

Grabicova, K., Fedorova, G., Burkina, V., Steinbach, C., Schmidt-Posthaus, H., Zlabek, V., Kocour Kroupova, H., Grabic, R., Randak, T., 2013. Presence of UV filters in surface water and the effects of phenylbenzimidazole sulfonic acid on rainbow trout (*Oncorhynchus mykiss*) following a chronic toxicity test. Ecotoxicology and Environmental Safety 96: 41-47.

Steinbach, C., Fedorova, G., Prokes, M., **Grabicova, K.**, Machova, J., Grabic, R., Valentova, O., Kocour Kroupova, H., 2013. Toxic effects, bioconcentration and depuration of verapamil in the early life stages of common carp (*Cyprinus carpio* L.). Science of the Total Environment 461–462: 198–206.

ABSTRACTS AND CONFERENCE PROCEEDINGS

Fedorova, G., Randak, T., Golovko, O., Kodes, V., **Grabicova, K.**, Grabic, R., 2013. Analgesics, psycholeptic, antidepressants and illicit drugs in aquatic environment of Czech Republic. Book of abstracts, Diversification in Inland Finfish Aquaculture II (DIFA II), Vodnany, Czech Republic. p. 41.

Grabicova, K., Lindberg, R.H.., Ostman, M., Grabic, R., Randak, T., Fick, J., 2013. Bioconcentration of antidepressant in fish exposed to wastewater treatment plant´s effluent. Book of abstracts, Diversification in Inland Finfish Aquaculture II (DIFA II), Vodnany, Czech Republic. p. 45.

Kolarova, J., Zlabek, V., Grabic, R., Golovko, O., **Grabicova, K**., Burkina, V., Randak, T., 2013. The effect of environmentally relevant concentration of selected PPCPs on fish cell lines. 49th Congress of the European-Societies-of-Toxicology (EUROTOX), Interlaken, Switzerland, Toxicology letters, volume 221, p. S161.

Randak, T., Grabic, R., Fedorova, G., **Grabicova, K.**, Burkina, V., Golovko, O., Steinbach, Ch., Zlabek, V., 2013. Complex approach to monitoring of aquatic environment contamination in Czech Republic. Book of abstracts, Diversification in Inland Finfish Aquaculture II (DIFA II), Vodnany, Czech Republic. p. 38.

Steinbach, C., Burkina, V., Fedorova, G., **Grabicova, K.,** Velisek, J., Zlabek, V., Randak, T., Schmidt-Posthaus, H., Grabic, R:, Kroupova, HK, 2013. 49th Congress of the European-Societies-of-Toxicology (EUROTOX), Interlaken, Switzerland, Toxicology letters, volume 221, p. S60.

Grabicova, K., Fedorova, G., Burkina, V., Kroupova, H., Grabic, R., Randdak, T., 2012. UV-filters in aquatic environment and their effects on fish. Abstract book AQUA 2012, Prague, Czech Republic, p. 432.

Steinbach, C., **Grabicova, K.**, Fedorova, G., Machova, J., Grabic, R., Kroupova, H., 2012. Assessment of toxic effects, bioconcentration and depuration of verapamil in early-life stages of common carp (*Cyprinus carpio*). Abstract book AQUA 2012, Prague, Czech Republic, p. 1056.

TRAINING AND SUPERVISION PLAN DURING STUDY

Name	Kateřina Grabicová
Research department	2010–2014 – Laboratory of Environmental Chemistry and Biochemistry
Daily supervisor	
Supervisor	Dipl.-Ing. Tomáš Randák, Ph.D.
Period	1st October 2010 until 30th September 2014

Ph.D. courses	**Year**
Ichthyology and Fish Systematics	2010
Basics of Scientific Communication	2011
Pond Aquaculture	2011
Applied Hydrobiology	2012
English Language	2012

Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	2011 2012 2013 2014

International conferences	**Year**
Grabicova, K., Fedorova, G., Burkina, V., Zlabek, V., Kroupova, H., Grabic, R., Randak, T., 2012. UV filters in aquatic environment and its effects on fish. AQUA 2012, Prague, Czech Republic, September 1–5, 2012. (poster presentation)	2012
Grabicova, K., Lindgerg, R.H., Östman, M., Grabic, R., Randak, T., Fick, J., 2013. Bioconcentration of antidepressants in fish exposed to wastewater treatment plant´s effluent. Diversification in Inland Finfish Aquaculture II, Vodnany, Czech Republic, September 24–26, 2013. (oral presentation)	2013

Foreign stays during Ph.D. study at RIFCH and FFPW	**Year**
Assoc. Prof. Jerker Fick, Chemistry Department, Umea University, Umea, Sweden. (3 months, SPMDs extraction, sample preparation, determination of pharmaceuticals distribution in fish tissues)	2012

Teaching	**Year from**
Biochemistry – lectures and seminars	2011
Basics of biology – lectures and seminars	2011
Basics of analytical chemistry – seminars	2013

Summer school student supervision		**Year**
Despoina Sousoni	Bioaccumulation of pharmaceuticals in benthic organisms in a small stream affected by sewage treatment plant	2013
Stoyana Pecheva	Bioaccumulation of pharmaceuticals in benthic organisms in a small stream affected by sewage treatment plant	2013

CURRICULUM VITAE

Surname:	**Grabicová**
First name:	**Kateřina**
Title:	Dipl.-Ing.
Born:	1st August, 1978
Nationality:	Czech
Marital status:	Married

EDUCATION

2010 – present	Ph.D. student, University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, Laboratory of Environmental Chemistry and Biochemistry
1996 – 2002	Dipl.-Ing., Institute of Chemical Technology, Faculty of Food and Biochemical Technology, Czech Republic (Biochemistry and biotechnology)
2003 – 2005	B.Sc., Institute of Chemical Technology, Czech Republic (Specialization in pedagogy)

COURSES

Metabolomics course (Umea University), Poster course (Umea University), Histological seminar (FFPW)

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

Assoc. Prof. Jerker Fick, Chemistry Department, Umea University, Umea, Sweden. (3 months, SPMDs extraction, sample preparation, determination of pharmaceuticals distribution in fish tissues)