JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE
239

Marja Lahti

The Fate Aspects of Pharmaceuticals in the Environment

Biotransformation, Sedimentation and Exposure of Fish

JYVÄSKYLÄN YLIOPISTO

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Esitetään Jyväskylän yliopiston matemaattis-luonnontieteellisen tiedekunnan suostumuksella
julkisesti tarkastettavaksi yliopiston Ambiotica-rakennuksen salissa YAA303
toukokuun 26. päivänä 2012 kello 12.

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UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2012

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Cover pictures by Marja Lahti, Aimo Oikari and Risto Retkin

URN:ISBN:978-951-39-4729-3
ISBN 978-951-39-4729-3 (PDF)

ISBN 978-951-39-4728-6 (nid.)
ISSN 1456-9701

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ABSTRACT

Lahti, Marja
The fate aspects of pharmaceuticals in the environment – Biotransformation, sedimentation and exposure of fish
Jyväskylä: University of Jyväskylä, 2012, 76 p.
(Jyväskylä Studies in Biological and Environmental Science
ISSN 1456-9701; 239)
ISBN 978-951-39-4728-6 (nid.)
ISBN 978-951-39-4729-3 (PDF)
Yhteenveto: Lääkeaineiden ympäristökohtalo – Biotransformaatio, sedimentaatio ja kalojen altistuminen
Diss.

Pharmaceuticals are bioactive chemicals that are mostly released to the environment via municipal wastewater treatment plants. Although they are widely detected in surface waters, the environmental fate and exposure of biota are still largely unknown. The results in this thesis show that microbial transformation was more efficient under aerobic than anaerobic conditions, however large differences were found between three pharmaceuticals studied experimentally. Diclofenac was recalcitrant, bisoprolol partially biotransformed and naproxen readily biodegradable. Many pharmaceuticals were present in settleable particulate material collected from the vicinity of four wastewater treatment plants in Finland. Cationic pharmaceuticals were also found in deeper sediments and citalopram was the most abundant one in particles and sediments. The sediment profile of citalopram correlated well with its increasing consumption by human population. Hence, it could be usable also for sediment dating. The waterborne exposure and efficient metabolism was found for the five pharmaceuticals studied in the laboratory experiments with rainbow trout. The concentrations of diclofenac, naproxen and ibuprofen (parent drug and its metabolites) were two to four orders of magnitude higher in the rainbow trout bile than in the blood plasma. Same compounds were also found in field-exposed rainbow trout, supporting the usability of bile in the exposure assessment. Cationic compounds were not found in the bile or plasma of field exposed fish. The measurements from particulate material, passive samplers and fish implied that of the four sites studied here, the wastewater treatment plant of Riihimäki in River Vantaa caused the highest local impacts of pharmaceuticals in the environment.

Keywords: Bioavailability; biotransformation; environmental fate; fish; pharmaceuticals; sediment.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-V. I am the first author in each article and have had significant role in planning, data collection, analyses, and writing of them.

I Lahti M. & Oikari A. 2011. Microbial transformation of pharmaceuticals naproxen, bisoprolol, and diclofenac in aerobic and anaerobic environments. *Archives of Environmental Contamination and Toxicology* 61: 202–210.

II Lahti M. & Oikari A. 2011. Pharmaceuticals in settleable particulate material in urban and non-urban waters. *Chemosphere* 85: 826–831.

III Lahti M. & Oikari A. 2012. Occurrence of pharmaceuticals in sediment cores – Citalopram as chemomarker of past consumption. Submitted manuscript.

IV Lahti M., Brozinski J.-M., Jylhä A., Kronberg L. & Oikari A. 2011. Uptake from water, biotransformation and biliary excretion of pharmaceuticals by rainbow trout. *Environmental Toxicology and Chemistry* 30: 1403–1411.

V Lahti M., Brozinski J.-M., Segner H., Kronberg L. & Oikari A. 2012. Bioavailability of pharmaceuticals in waters close to wastewater treatment plants – Use of fish bile for exposure assessment. Accepted to *Environmental Toxicology and Chemistry*.

ABBREVIATIONS

BCF	bioconcentration factor
BOD	biolocigal oxygen demand
CYP1A	cytochrome P450 1A
dw	dry weight
EC_x	effective concentration (effect in *x* % of individuals)
EMA	European Medicines Agency
ERA	environmental risk assessment
EROD	7-ethoxyresorufin *O*-deethylase
ESI	electrospray ionization
GC-FID	gas chromatography – flame ionization detector
GC-MS	gas chromatography – mass spectrometry
K_d	sorption coefficient
K_{oc}	sorption coefficient adjusted to organic carbon fraction
K_{ow}	octanol water partition coefficient
LC_x	lethal concentration (*x* % lethality)
LC	liquid chromatography
LOEC	lowest observed effect concentration
log D	logarithm of octanol water partition coefficient corrected with dissociation at ambient pH
log P	logarithm of octanol water partition coefficient of neutral species (same as log K_{ow})
LOI	loss on ignition
MoA	Mode of action
MS/MS	tandem mass spectrometry
NOEC	no observed effect concentration
PAH	polycyclic aromatic hydrocarbon
PEC	predicted environmental concentration
PNEC	predicted no effect concentration
POCIS	polar organic chemical integrative sampler
RSD	relative standard deviation
SE	standard error
SPM	settleable particulate material
ThOD	theoretical oxygen demand
TOC	total organic carbon
Vtg	vitellogenin
WWTP	wastewater treatment plant

1 INTRODUCTION

1.1 Pharmaceuticals in the environment

1.1.1 Pharmaceuticals as environmental pollutants

Pharmaceuticals as so-called emerging pollutants have recently gained increasing scientific interest due to several reasons, such as:

- Increasing consumption
- Constant release
- Non-traditional sources of pollution
- Physico-chemical properties
- Mixture exposure
- Acute sublethality
- Biological activity (known mode of actions in human)

The use of pharmaceuticals has increased enormously during the last few decades and for instance the wholesale has doubled in Finland within 10 years (from 1998 to 2008). Especially the consumptions of anti-inflammatory (i.e. pain killers), antidepressant and diabetes drugs have increased (FIMEA 2010). There are over 900 approved drugs on the Finnish market today and the use may vary from grams to tons per year (Appendix 1). Due to the constant use, pharmaceuticals are continuously released to the environment.

Traditional xenobiotics are classified according to their similar structure (e.g. dioxins, polychlorinated biphenyls and polycyclic aromatic hydrocarbons (PAH)). Due to the similarities in the structures, some generalizations of the environmental fate and toxicity can be made. The classification of compounds as a pharmaceutical is based on their demand. Even compounds within the same therapeutic class may have a totally different structure, properties and hence environmental fate (Fig. 1, Table 1; Cunningham 2008).

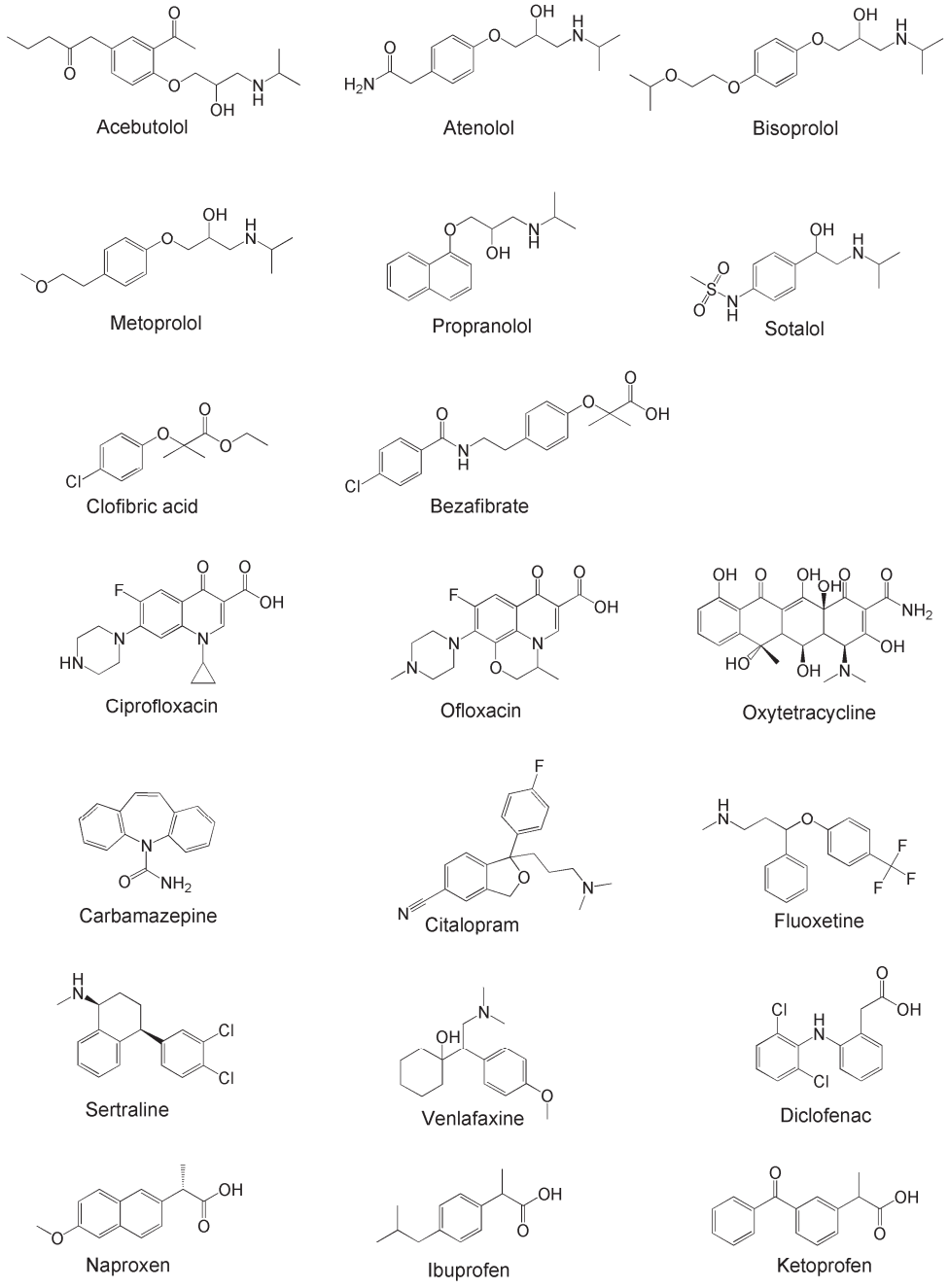

FIGURE 1 Molecular structures of the pharmaceuticals studied in this thesis.

TABLE 1 Selected physico-chemical properties of the pharmaceuticals studied in this thesis.

Compound	MW[a]	pKa[a]	log P (log K_{ow})[a]	log D pH 7[b] (est)	log Kd	log K_{oc}	Water solubility mg l^{-1} [a]	Henry's law constant atm m^3 mol^{-1} [a]
Acebutolol	336.43	9.2[c]	1.71 (exp)	–0.64	0.5–1.0[c]	2.35–2.47[c]	259	1.34×10^{-20}
Atenolol	266.34	9.6	0.16 (exp)	–2.09	0.05–0.9[c,d]	1.85–3.2[c,d]	13 300	1.37×10^{-18}
Bisoprolol	325.45	9.6[c]	1.87 (exp)	–0.54	0.3–0.8[c]	2.17–2.3[c]	2 240	2.89×10^{-15}
Metoprolol	267.36	9.7[c]	1.69 (est)	–0.81	0.2–0.9[c]	2.22–2.24[c]	4 780	1.40×10^{-13}
Propranolol	257.34	9.4	3.48 (exp)	0.45	0.7–2.2[c,d]	2.43–4.0[c,d]	61.7	7.98×10^{-13}
Sotalol	308.83	9.6[c]	0.24 (exp)	–2.01	0.1–0.6[c]	1.94–2.15[c]	137 000	2.66×10^{-14}
Bezafibrate	361.83	3.6[e]	4.25 (est)	–0.93	-	-	0.355	2.12×10^{-15}
Clofibric acid	214.65	2.8[f]	2.57 (exp)	–1.06	0.5[f]	0.4[f]	583	2.19×10^{-8}
Ciprofloxacin	331.35	5.9, 8.9[g]	0.28 (exp)	–0.33	2.6[g]	4.79[g]	30 000	5.09×10^{-19}
Ofloxacin	361.38	6.0, 8.3[g]	–0.39 (exp)	–0.20	2.5[g]	4.64[g]	28 300	4.98×10^{-20}
Oxytetracycline	460.44	3.3, 7.3, 9.1[g]	–0.90 (exp)	–2.25	2.6–3.0[g]	4.44–4.97[g]	313	1.70×10^{-25}
Carbamazepine	236.28	13.9[i]	2.45 (exp)	1.89	–1.1–5.3[d,h]	2.00–3.42[d,h]	17.7	1.08×10^{-10}
Citalopram	324.39	9.6[j]	3.74 (est)	1.02[b], 1.39[k] (exp)	3.9–4.6[k]	5.32–6.02[k]	31.1	2.69×10^{-11}
Fluoxetine	309.33	10.1[j]	4.05 (exp)	1.15[b], 1.22[k] (exp)	2.9–4.1[f,k]	4.09–5.49[f,k]	60.3	8.90×10^{-8}
Sertraline	306.24	9.5[j]	5.29 (est)	2.70[b], 1.37[k] (exp)	2.2–2.9[k]	3.80–4.85[k]	3.52	5.10×10^{-8}
Venlafaxine	277.41	9.3[l]	3.28 (est)	0.39	-	-	267	2.04×10^{-11}
Diclofenac	296.16	4.2	4.51 (exp)	1.77	4.7[h]	2.45–3.74[h]	2.37	4.73×10^{-12}
Ibuprofen	206.29	4.9	3.97 (exp)	0.19	–1.0–1.7[d,h]	1.3–2.21[d,h]	21	1.50×10^{-7}
Ketoprofen	254.28	4.5	3.12 (exp)	0.94	-	-	51	2.12×10^{-11}
Naproxen	230.27	4.2	3.18 (exp)	0.73	2.34[i]	-	15.9	3.39×10^{-10}
Paracetamol	151.17	9.4	0.46 (exp)	0.47	0.4–1.0[d]	2.4–4.1[d]	14 000	6.42×10^{-13}

[a]SRC (2011) [b]ACD/Labs V10.02 [c]Ramil et al.(2010) [d]Yamamoto et al. (2009) [e]Nikolaou et al. (2007) [f]Löffler et al. (2005) [g]Tolls (2001) [h]Scheytt et al. (2005) [i]Jones et al. (2002) [j]Vasskog et al. (2006) [k]Kwon et al. (2008) [l]Cherkaoui et al. (2001) (exp) experimental value (est) calculated value

Most of the pharmaceuticals are ionizable and water soluble. Under pH-range of surface waters (pH 6–8), they occur as cations, anions or zwitterions. The proportion of ionized and neutral species of naproxen (acid) and citalopram (base) are presented in Fig. 2. At the ambient pH 7, both are almost totally in ionized form (> 99.5 % ionized). Octanol/water distribution (log K_{ow} /log P) is usually low (Table 1). It is widely used to assess bioconcentration and sorption of hydrophobic chemicals (Mackay 1982, Spacie & Hamelink 1985, Mackay & Fraser 2000). For ionizable compounds, octanol/water distribution must be corrected to account for the neutral and ionized fractions at the environmentally relevant pH. This corrected parameter is log D (Kah & Brown 2008). Quite often pH 7 is used for the environmental risk assessments (Cunningham 2008).

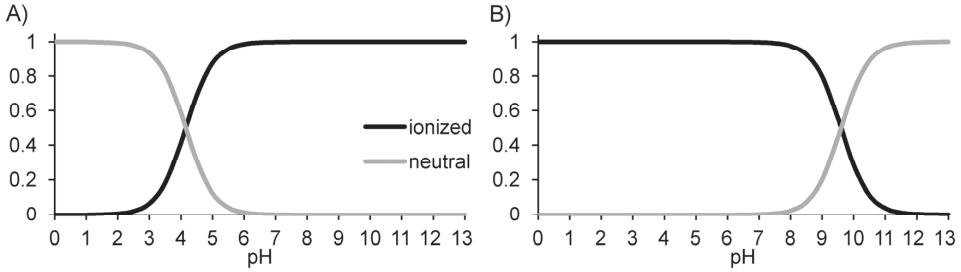

FIGURE 2 Proportion of ionized and neutral species of A) acid naproxen (pKa 4.15) and B) base citalopram (pKa 9.6) calculated according to Henderson-Hasselbalch equation.

1.1.2 Sources and occurrence of pharmaceuticals

The pathways and distribution of pharmaceuticals in the environment are depicted in Fig. 3, and those covered in this thesis are highlighted with grey. Unlike industrial chemicals, pharmaceuticals are mostly used in private households and hospitals, which are also considered as the main route of entrance to the wastewaters and environment (Daughton & Ternes 1999, Heberer 2002, Kümmerer 2008). After administration, drugs are excreted via urine and feces entering the sewage system as unaltered parent compounds or as their metabolites (Daughton & Ternes 1999, Heberer 2002, Celiz et al. 2009). Part of the purchased drugs are not consumed e.g. due to expiration. These unused drugs may be disposed of via a drain or in household waste ending up in wastewater and landfill.

Previously, manufacturing of pharmaceuticals was considered as a minor source of pollution due to controlled production and the high value of the product (Williams 2005). However, e.g. in some Asian countries, releases from factories can be very high (Larsson et al. 2007, Fick et al. 2009).

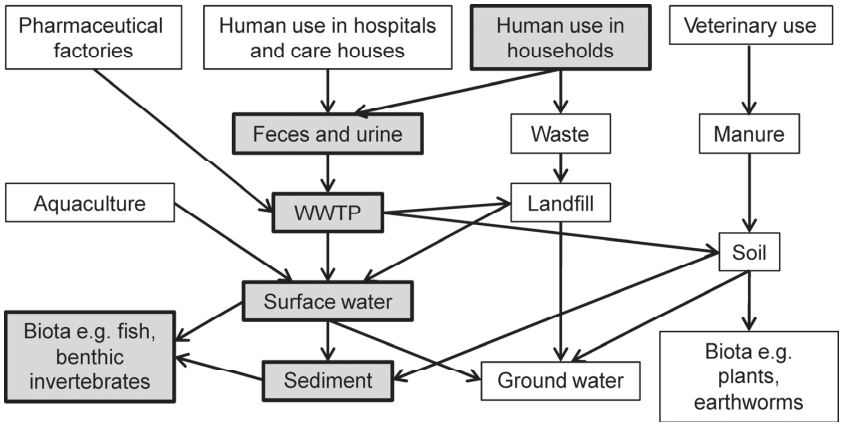

FIGURE 3 Sources and distribution of pharmaceuticals in the environment. Those aspects covered in this work are darkened.

Veterinary pharmaceuticals and their metabolites are excreted in urine and feces to manure, which is often applied to the field as a fertilizer or the manure can end up in soils when animals graze outside. In fish medication, drugs are released directly to the surrounding water and some settle into sediments (Daughton & Ternes 1999, Heberer 2002, Kümmerer 2008).

The removal efficiency of pharmaceuticals in wastewater treatment plants (WWTP) varies considerably, from negligible to total elimination. Biotransformation (section 1.2.1) and sorption (1.2.2) to sludge are considered the main removal processes in WWTPs (Fent et al. 2006). Although many pharmaceuticals are efficiently treated, they are continuously discharged from WWTP to surface waters. Thus they can be characterized as pseudopersistent.

The concentrations of pharmaceuticals (as original active ingredient) generally range from 500 to 5000 ng l^{-1} in influents (Carballa et al. 2004, Quintana & Reemtsma 2004, Petrovic et al. 2006, Vieno 2007, Gros et al. 2010, da Silva et al. 2011), from 50 to 3000 ng l^{-1} in effluents (Ternes 1998, Kolpin et al. 2002, Metcalfe et al. 2003b, Quintana & Reemtsma 2004, Petrovic et al. 2006, Nikolaou et al. 2007, Vieno 2007, Gros et al. 2010, da Silva et al. 2011) and from 1 to 500 ng l^{-1} in surface waters (Ternes 1998, Kolpin et al. 2002, Metcalfe et al. 2003a, Quintana & Reemtsma 2004, Vieno 2007, Gros et al. 2010, da Silva et al. 2011). However, much higher concentrations have been reported from areas with intense drug manufacturing. For instance, up to 31 000 µg l^{-1} of ciprofloxacin was measured from treated effluents, 25 000 µg l^{-1} from surface waters 150 m downstream from the WWTP and 14 µg l^{-1} from a nearby domestic well (Larsson et al. 2007, Fick et al. 2009).

1.1.3 Effects of pharmaceuticals

Toxic mechanisms can be roughly divided into three classes: non-specific interactions (e.g. narcosis), specific interactions and chemical reactions (Escher & Hermens 2002). Pharmaceuticals are biologically active compounds designed to have a specific mode of action (MoA), which may increase the likelihood of receptor-mediated interactions (Escher et al. 2005). As many genes are conserved across animal taxa (Seiler 2002, Fent et al. 2006, Gunnarson et al. 2008), same drug-biomolecule interactions may occur also in non-target organisms such as in fish. However, the same genes may express differently in different species (Escher et al. 2005). In addition to therapeutically targeted effects, drugs cause unwanted side effects in humans and even these side effects may be the reasons for adverse effects in wildlife. Moreover, drugs can induce totally different, unknown and unpredictable effects than the therapeutic ones (Länge & Dietrich 2002, Seiler 2002, Fent et al. 2006).

Low concentrations of pharmaceuticals are continuously released to the environment causing long-lasting exposure of biota. Hence, chronic effects from exposure to low concentrations are more likely than acute effects from high pulsed emissions (like accidents). Many of the standardized toxicity tests measure short-term toxicity and endpoints are rough e.g. acute fish toxicity, immobilization of *Daphnia magna* and algae growth tests. As these endpoints are often insensitive, the effects of pharmaceuticals are not discovered or appear to exceed the environmentally relevant concentrations (Fent et al. 2006). Therefore more specific endpoints are needed to reveal possible effects of pharmaceuticals in the biota and environment (Henchel et al. 1997, Länge & Dietrich 2002, Escher et al. 2005, Crane et al. 2006, Clubbs & Brooks 2007). For instance, diclofenac caused histological changes in the kidney and liver of rainbow trout (*Oncorhynchus mykiss*, 28 d experiment) and brown trout (*Salmo trutta*, 21 d experiment) at 5 µg l^{-1} (Schwaiger et al. 2004, Hoeger et al. 2005) and cytological effects in rainbow trout (28 d experiment) and medaka (*Oryzias latipes*, 4 d experiment) at 1 µg l^{-1} (Triebskorn et al. 2004, Hong et al. 2007, Triebskorn et al. 2007). In comparison, acute EC_{50} (immobilization of *Daphnia magna*, 2 d) for diclofenac is 22 mg l^{-1} (Ferrari et al. 2003), which is four to five orders of magnitude higher than the chronic lowest observed effect concentration (LOEC) in fish (Triebskorn et al. 2004, Hong et al. 2007, Triebskorn et al. 2007).

Biota is exposed to undefined and fluctuating combinations of pharmaceuticals in the environment, which should be taken into account when the effects of pharmaceuticals are evaluated. Effects caused by a mixture may deviate from expected impacts due to the concentrations of single compounds i.e. there can be joint effect (Backhaus et al. 2008). These impacts can be evaluated and described with two concepts: concentration addition and independent action. In the concentration addition, the effects of every similarly acting (same MoA) compounds are summing up. Hence, the effects are observed even if concentrations of single compounds in a mixture are below individual no observed effect concentrations (NOEC; Backhaus et al. 2008). In the independent action, compounds have different MoAs and the whole

toxicity is based on the toxicity of a single compound. In general, no effects are observed below individual NOECs (Backhaus et al. 2008). Joint effects have been found e.g. in a mixtures of anti-inflammatory drugs (Cleuvers 2003, 2004), beta-blockers (Cleuvers 2005), quinolone antibiotics (Backhaus et al. 2000) and antidepressants (Christensen et al. 2007).

So far there is only little information about the effects of pharmaceuticals in sediments as most of the studies have focused on water-column species and systems. Effects of fluoxetine (an antidepressant) on benthic invertebrates have been most widely studied. Fluoxetine decreases survival (LC_{50} 15.2 µg g^{-1} dw) and growth (LOEC 1.3 µg g^{-1} dw) of *Chironomus tentans* (Brooks et al. 2003). In addition, fluoxetine changes sex ratio (more females), decreases emergence frequency and increases clutch size of *Chironomus riparius* (Nentwig 2007, Sánchez-Argüello et al. 2009). Low concentrations of fluoxetine can stimulate reproduction (increased egg mass) of *Physa acuta* snails, whereas inhibition can occur at higher concentrations (Sánchez-Argüello et al. 2009). Carbamazepine is relatively toxic to benthic *C. riparius* with EC_{10} and EC_{50} in the range of 70–140 and 160–210 ng g^{-1} dw, respectively (Oetken et al. 2005). In laboratory experiments, diclofenac causes oxidative stress in *Hyaella azteca* at sediment concentration 47 ng g^{-1} dw (Oviedo-Gomez et al. 2010). The same study showed LC_{50} 467 ng g^{-1} dw. Unexpectedly, there is no data on tissue exposure of benthic biota to human pharmaceuticals.

In the 1990s, the populations of *Gyps* vultures collapsed, revealing over 90 % decline in less than 10 years in Pakistan and India (Prakash et al. 2003). The cause of the high mortality was the anti-inflammatory drug diclofenac (Oaks et al. 2004). The exposure of local populations of vultures was due to a common practice of leaving dead domestic livestock for scavengers. Diclofenac causes kidney malfunction and accumulation of uric acid in the blood, finally leading to death (Oaks et al. 2004, Meteyer et al. 2005, Naidoo & Swan 2009). The sensitivity of *Gyps* vultures may be due to genetic deficiency in the metabolic system that causes saturation of uric acid transport (Naidoo et al. 2010). In 2006, the production and use of diclofenac as veterinary pharmaceutical was banned in India, Nepal and Pakistan (Taggart et al. 2009). However, ketoprofen causes same toxicological effects (Naidoo et al. 2010).

1.1.4 Environmental risk assessment of pharmaceuticals

The European Medicines Agency (EMA) has released guidelines on the environmental risk assessment (ERA) of medicinal products for human (EMEA 2006) and veterinary (EMEA 1997) use. Guideline for veterinary drugs was later replaced by international guidelines (VICH 2000, 2004) and a supporting document (EMEA 2008). The ERA has to be performed during registration of a new drug or reregistration of an old veterinary drug.

Pharmaceuticals for human use are assessed in a two phased ERA procedure (EMEA 2006). In phase I, exposure is estimated and, in phase II, environmental fate and effects are analyzed (Fig. 4). Exposure (phase I) is estimated by calculating predicted environmental concentration (PEC) in

aquatic compartment based on consumption doses. Several simplifying assumptions are made such as no metabolism, microbial transformation or retention in sludge. The action limit is 0.01 µg l^{-1}. In the ERA of veterinary drugs, the terrestrial compartment is also evaluated (VICH 2000).

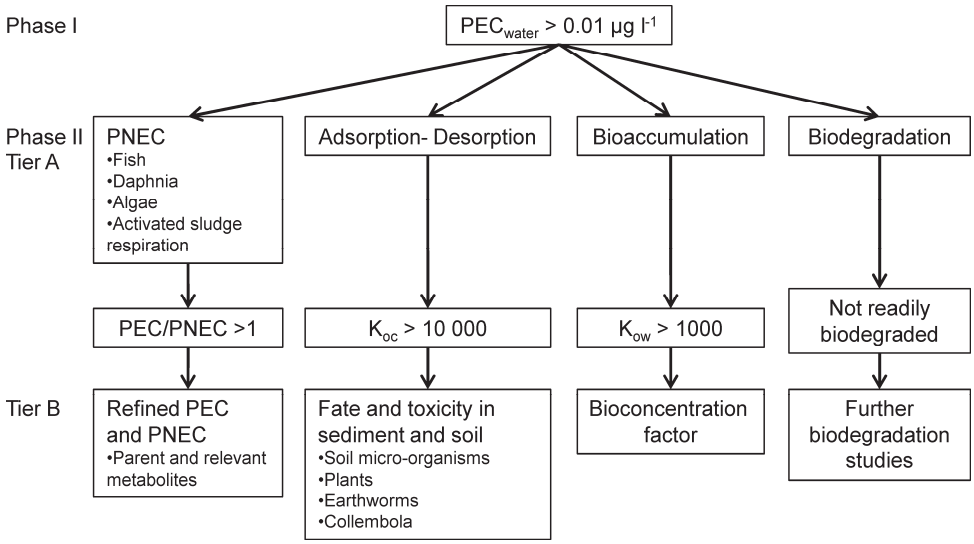

FIGURE 4 Tiered scheme of environmental risk assessment (ERA) of pharmaceuticals for human use (EMEA 2006).

Phase II is divided into two tiers (EMEA 2006). Tier A of human pharmaceuticals consists of basic toxicity and fate tests, such as adsorption-desorption, ready biodegradation, activated sludge respiration inhibition, algae growth inhibition, *Daphnia* reproduction and fish early life stage toxicity tests. Predicted no effect concentration (PNEC) is used for the initial prediction of risk by calculating PEC/PNEC-ratios. Further investigations in Tier B are needed, if PEC/PNEC is over 1, if partitioning to organisms or sludge is likely, or if a compound is not readily biodegradable. Otherwise, no further investigations are demanded.

In Tier B, risk assessment is refined with case by case tests e.g. to study the fate in sediments and effects on sediment dwelling organisms (EMEA 2006). In this tier, also the fate and effects of the relevant metabolites may be studied. PEC may be refined by using modeling. The fate and effects in the terrestrial environment is studied if notable sorption is likely. End-points in terrestrial tests can include biotransformation in soil, plant growth, earthworm acute toxicity and *Collembola* reproduction. For veterinary pharmaceuticals, there are multiple routes of entry to the environment (Fig. 3). For this reason, phase II for veterinary pharmaceuticals may include additional toxicological studies e.g. with dung or marine organisms.

If risk-benefit assessment reveals possible risks to the environment, appropriate risk mitigation measures must be used, e.g. appropriate labeling and disposal instructions or restrictions on application. The use of veterinary drugs may be banned on specified target animals or even totally due to environmental risks (EMEA 2008). However, the benefits of human drugs are considered so significant that the authorization of a drug cannot be interfered due to environmental reasons (EMEA 2006).

1.2 Fate of organic chemicals in the environment

1.2.1 Microbial transformation

After its release, several processes may define the fate of a chemical in the environment. The most important fate processes are biotransformation, sorption, chemical transformation, phototransformation and evaporation (Rogers 1996). The importance of each process depends on the compound and its spatial distribution (Fig. 5). For pharmaceuticals, biotransformation and sorption are considered the most important ones (Quintana et al. 2005).

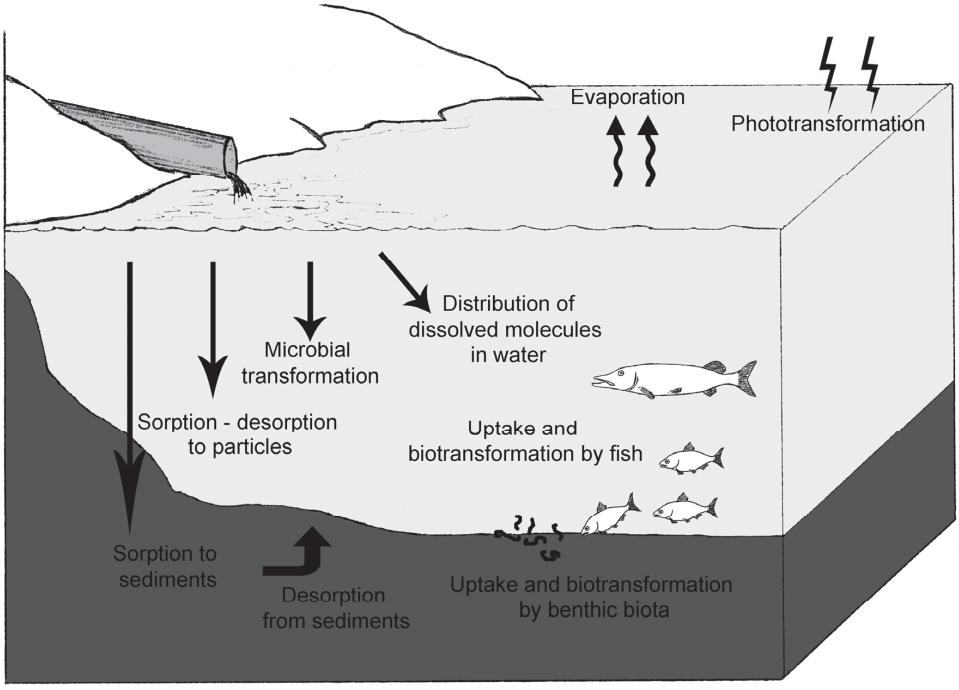

FIGURE 5 Fate processes of organic chemicals, such as pharmaceuticals, in the aquatic environment.

Structures of organic compounds change due to microbiologically mediated processes. The rate of transformation may vary considerably, being crucial in the biogeochemical cycles of e.g. carbon and nitrogen (Leadbetter 1997). Finally these pathways can lead to the mineralization of the compound through degradation into simple inorganic constituents. Terminologically, in the context of the present study, biodegradation is used to refer the mineralization of the compound, whereas biotransformation recognizes any biologically mediated change in the structure of the foreign molecule (Walter & Crawford 1997).

Microbes work as aerobes and anaerobes depending on whether they can use oxygen or not (Leadbetter 1997). The principal end products in aerobic (oxic) environments are water and carbon dioxide, and in anaerobic (anoxic) environments carbon dioxide and methane. Often the change from oxic to anoxic conditions is gradual and the term hypoxic is used for the intermediate phase.

Biotransformation can be direct or co-metabolic. In the direct transformation, microbes use a compound as a carbon and energy source, whereas in the co-metabolic transformation, certain microbes gain energy from more abundant and easily degradable organic compounds (Walter & Crawford 1997). Hence, the way the microbial community may work is dependent on presence of other substrates simultaneously in the same habitat, i.e. on trophic or saprobic set-ups.

Biotransformation is considered to be the main process for removing pharmaceuticals, both in WWTPs and in the aquatic environments (Quintana et al. 2005). Microbial transformation of pharmaceuticals varies from recalcitrant, like clofibric acid and carbamazepine, to readily transformed, like ibuprofen, ketoprofen and paracetamol (Zwiener & Frimmel 2003, Yu et al. 2006, Joss et al. 2006, Kunkel & Radke 2008, Jelic et al. 2011). The biotransformation is often slower in anaerobic conditions (Gröning et al. 2007, Kunkel & Radke 2008). Hence, it is important to predict the fate of pharmaceuticals both in aerobic and anaerobic environments.

1.2.2 Sorption and desorption

Sorption has an important role in determining the fate of chemicals in WWTPs as well as in the environment. In addition of determining where a compound will end up, sorption may hinder the biotransformation of pharmaceuticals due to reduced bioavailability (Alexander 2000).

The estimation of sorption of hydrophobic organic chemicals is traditional, since they tend to sorb to the organic fraction of the particles and sediment. This phenomenon can be described e.g. with equilibrium partition theory (Di Toro et al. 1991, Pignatello & Xing 1996). It assumes that with time the distribution of a hydrophobic organic chemical is in equilibrium between the pore water, organic carbon of the sediment and lipids of an animal or other organism. The equilibrium is directly dependent on the hydrophobicity (K_{ow}) of the chemical and the amount of organic carbon in the solid matrix (Karickhoff et al. 1979, Di Toro et al. 1991, Pignatello & Xing 1996). Sorption is often described with

sorption coefficient (K_d), which is the ratio between the sorbed and free compound. This can be further normalized to the organic carbon content to derive K_{oc}.

Due to the ionization and pH dependence of dissociation (Fig. 2), the distribution of ionizable compounds between water and solid matrices (such as water – sediment, water – sludge) is not solely based on hydrophobicity (K_{ow}). Rather, it is based on ionic interactions and is pH dependent (Tolls 2001, Schwarzenbach et al. 2003, Pan et al. 2009, Williams et al. 2009). Sorption mechanisms involve surface related adsorption, ion exchange, complexation and hydrogen bonding (Tolls 2001, Schwarzenbach et al. 2003, Williams et al. 2009). The normalization of sorption to organic carbon content does not necessarily decrease the variation in the sorption coefficients of pharmaceuticals (Tolls 2001). Especially the sorption of cations may be higher than expected from the K_{ow}, because of the negative surface charges of clay particles, organic materials and biofilms (Tolls 2001, Schwarzenbach et al. 2003, Pan et al. 2009, Williams et al. 2009, Yamamoto et al. 2009). Citalopram and fluoxetine sorb very strongly to sediment and soil ($\log K_d > 3$), although their $\log K_{ow}$-values are rather low (Table 1; Kwon et al. 2008, Yamamoto et al. 2009).

The variation in the degree of sorption between pharmaceuticals is large (Williams et al. 2009, Jelic et al. 2011). For instance, the sorption of hydrochlorthiazide, fenofibrate and diazepam is especially high, whereas the sorption of metoprolol, chrolamphenicol and salbutamol is negligible (Jelic et al. 2011).

1.2.3 Bioavailability and exposure assessment

Generally it is assumed that only truly dissolved fraction can be taken up by aquatic organisms (Arnot & Cobas 2006). Thus the measured total concentrations in water do not necessary correlate with the bioavailable part and hence are not adequate in exposure assessment. In sediment studies, the bioavailable fraction is presented by the free concentration in pore water (Di Toro et al. 1991), but many benthic organisms are exposed to sediments also via ingested particles (Kaag et al. 1997, Forbes et al. 1998, Leppänen & Kukkonen 1998, Sormunen et al. 2008). Bioavailability of chemical can be experimentally assessed by using organisms, such as fishes or mollusks, or with passive samplers.

Exposure of aquatic animals can be demonstrated and assessed by measuring internal concentration e.g. in blood, muscle or adipose tissue. Xenobiotics and their metabolites can be secreted into fish bile resulting in total concentrations several orders of magnitude higher than those found in the surrounding water (e.g. Statham et al. 1976, Oikari et al. 1984, Larsson et al. 1999). A prerequisite for this approach is the knowledge of formed metabolites. To assess exposure of fishes, bile is especially suitable for organic compounds which are readily metabolized and secreted hepatocellularly to the bile instead of accumulated in muscle or other organs (Statham et al. 1976).

Passive sampling is a technology that is based on free flow of analyte from target to collecting medium until equilibrium or its fraction is reached (Górecki & Namieśnik 2002, Stuer-Lauridsen 2005, Seethapathy et al. 2008). In the aquatic environment, passive samplers can be used instead of several water samples for the measurement of average water concentrations. Once calibrated with an organism, it also can be used for the exposure assessment, because commonly the bioavailable fraction is susceptible for this process (Seethapathy et al. 2008). Usually passive sampling refers to non-living devices, although living organisms (with negligible biotransformation) also fulfill the criterion (Górecki & Namieśnik 2002). Polar Organic Chemical Integrative Samplers (POCIS) have been used for the sampling of hydrophilic compounds like pharmaceuticals (Togola & Budzinski 2007, MacLeod et al. 2007), but also estrogens (Alvarez et al. 2004, Vermeirssen et al. 2005).

1.2.4 Uptake, metabolism and excretion of chemicals in fish

The bioconcentration of hydrophobic chemicals is due to the lipophilic partition into the lipids of the animal. At the stage of equilibrium of uptake and elimination, the bioconcentration factor (BCF) can be calculated by dividing the amount of chemical in organism with that in the surrounding water (Spacie & Hamelink 1985, Arnot & Cobas 2006). In addition to experimental measurements, the BCF of hydrophobic compounds can be estimated from the K_{ow} of the compound (Mackay 1982, Spacie & Hamelink 1985, Mackay & Fraser 2000, Arnot & Cobas 2006). Methods to calculate the BCF of ionizable compounds also have been proposed (Meylan et al. 1999, Fu et al. 2009). The BCF of ionizable compounds is highly dependent on K_{ow} and pKa of the compound, and hence on the ambient pH (Meylan et al. 1999, Fu et al. 2009). Pharmaceuticals are usually in ionized form under pH of the ambient water and fish blood. This generally decreases the uptake, bioconcentration and eventually the toxicity of these chemicals (Nakamura et al. 2008, Valenti et al. 2009).

The exposure of fish occurs only if compound is passed through biological membrane (i.e. is absorbed) for instance in the gills, intestine or skin. The uptake process of a foreign chemical in fish gills includes the transfer onto the gill membrane surface and diffusion through epithelial cells (Erickson et al. 2006a, 2006b). The uptake can occur also by binding to carrier molecules (Miller 2008). In addition to these, possible pH variations at the gill structures are important for ionizable compounds, because pH determines the proportions of neutral and ionized forms. In addition, the flux of neutral molecules across the epithelia sustains continuous dissociation from ionized to neutral forms and thus enhances the uptake (Erickson et al. 2006a, 2006b). Dissociation of neutral molecule to its ionized form inside the cell can prevent the flux out of the cell, a phenomenon called ion trap (Rendal et al. 2011) Overall, the uptake rate of ionized compounds is lower than that of neutral ones (Saarikoski & Viluksela 1981, Nakamura et al. 2008, Fu et al. 2009, Valenti et al. 2009, Rendal et al. 2011),

although ionized molecules may also be absorbed by ion carriers (Miller 2008). After uptake, a compound reaches the target site via blood circulation.

Xenobiotics can be efficiently metabolized in the vertebrate liver. The main function of biotransformation is to change the properties of the chemicals so that they become more water soluble and more excretable Biotransformation reactions are grouped into two phases. Phase I metabolites are formed by oxidation, reduction, hydrolysis and some other less frequent reactions. These reactions introduce a reactive functional group to the molecule. Phase II reactions are conjugations, the most common products being glucuronide, sulfate and glutathione conjugates. A conjugating molecule may be added directly to the parent compound or to the phase I metabolite (Di Guilio 1995, Parkinson 1996, Celiz 2009).

The predominant metabolites of pharmaceuticals in human are determined during the drug development, as many of them are mainly eliminated as metabolites. For instance, only 0 to 15 % of diclofenac, ibuprofen and naproxen are excreted as unmetabolized parent compound (Goodman Gilman et al. 1990, Rainsford 2009). In fish, the biotransformation of these three pharmaceuticals is efficient and several metabolites have been identified. Acyl glucuronides are predominating in bile, but also sulfate conjugates as well as hydroxylated metabolites are found (Kallio et al. 2010, Brozinski et al. 2011, 2012a).

After hepatic biotransformation, the formed metabolites are excreted to small intestine via the bile and perhaps some renally and branchially. However, enterohepatic cycling may prolong the half-lives of xenobiotics, as a compound or its metabolite may be reabsorbed in the intestine. Elimination of the ionic forms of compounds in fish gills is usually rather fast compared to the neutral forms (Erickson et al. 2006a, 2006b).

1.2.5 Other fate processes

Phototransformation is a process where solar radiation changes the structure of a chemical directly or indirectly. In direct phototransformation, a chemical absorbs the energy from the light. Phototransformation can take place in the atmosphere or in upper aquatic phase, if a compound is absorbing light at > 290 nm. The transformation is indirect when one molecule (called a photosensitizer) absorbs the energy from solar light and the energy is transferred to another chemical that undergoes transformation (Zepp & Cline 1977, Zepp et al. 1981, Mill 1999). Common photosensitizers are dissolved organic carbon (humus) and nitrate (Zepp et al. 1981, Mill 1999, Andreozzi et al. 2003). Several pharmaceuticals are known to undergo phototransformation (Boreen et al. 2003), such as diclofenac (Buser et al. 1998, Poiger et al. 2001, Andreozzi et al. 2003, Packer et al. 2003, Tixier et al. 2003, Eriksson et al. 2010), naproxen (Packer et al. 2003, Lin & Reinhard 2005, Lin et al. 2006) and clofibric acid (Andreozzi et al. 2003, Packer et al. 2003). Phototransformation may form products that are more toxic than the original ones (DellaGreca et al. 2004, Isidori et al. 2005, Schmitt-Jansen et al. 2007).

Chemical transformation processes are oxidation, reduction, hydrolysis, substitution, and elimination reactions, all of which may take place in the dark (Schwarzenbach et al. 2003). Due to preferred stability in human gut, these have minor importance in determining the fate of pharmaceuticals (Fent et al. 2006).

Evaporation of chemicals depends on vapor pressure and water solubility (Henry's law constant). Compounds with high Henry's law constant ($> 10^{-4}$) can evaporate to the atmosphere and be transported even long distances in the environment (Rogers 1996, Schwarzenbach et al. 2003). Pharmaceuticals have low Henry's law constants (Table 1), so evaporation is not considered a significant fate process (Fent et al. 2006).

2 OBJECTIVES

The objective of this thesis was to study the fate of pharmaceuticals in the fresh-water environment through discharges from WWTP. The measurements of effluent releases from WWTPs were defined out of the focus of this study. The research followed the presence of pharmaceuticals in solid compartments and considered the possibility of internal exposure of aquatic animals to pharmaceuticals. In more detail, the objectives were:

- To improve the knowledge of the microbial transformation of naproxen, bisoprolol and diclofenac in aerobic and anaerobic conditions (I)
- To determine occurrence of pharmaceuticals in settleable particulate material close to WWTPs as well as in rural and in reference sites (II)
- To measure the vertical distribution of pharmaceuticals in sediments at three locations affected by municipal effluents (III)
- To widen the knowledge of the uptake and metabolism of five pharmaceuticals in rainbow trout in a laboratory experiment (IV)
- To define the exposure of fish to pharmaceuticals close to wastewater treatment plant and to compare the applicability of passive sampling, fish blood plasma and bile analyses in exposure assessment (V)

3 MATERIALS AND METHODS

3.1 Chemicals

Pharmaceuticals from different therapeutic classes were chosen to experimental settings based on their consumption, properties and toxicity. The aim was to choose compounds expected to have different fates in the environment (such as anions and cations, readily transformed and persistent). Most of the studied compounds are also frequently detected in surface waters. Between fifteen to seventeen compounds were analyzed in the field studies (II, III, V), whereas in the laboratory experiments (I, IV) three to five compounds were analyzed. The structures of the pharmaceuticals are presented in Fig. 1 and physico-chemical properties in Table 1.

3.2 Study areas

Field studies were conducted in twelve locations in Finland. These sites were grouped into three classifications based on human influence: sites affected by municipal effluents from WWTPs (A–F; Fig. 6), reference sites (G–I) and rural sites with human settlement but without WWTP (J–L). Samples collected at each site are presented in Fig. 6. The characteristics of WWTPs are presented in Table 2 and surface waters in papers II, III and V.

3.3 Passive sampling (V)

In order to evaluate freely dissolved fraction in a water column, POCIS were kept in the water for 10 days at the time of the fish experiment in the field. Samplers were put to a stainless steel deployment devices attached to the fish cage. POCIS were stored in –20 °C until the extraction.

Site	Watercourse	Name of city or municipality	POCIS	SPM	Sediment	Fish
A	Lake Päijänne	Jyväskylä WWTP	x	x		x
A	Lake Päijänne	Jyväskylä WWTP (~1 km)			x	
B	River Vantaa	Riihimäki WWTP	x	x		x
C	River Aura	Aura WWTP	x	x		x
D	Lake Jämsänvesi	Petäjävesi WWTP		x		
E	Lake Haapajärvi	Lappeenranta WWTP			x	
F	Lake Lievestuoreenjärvi	Lievestuore WWTP			x	
G	Lake Palosjärvi	Reference Toivakka	x	x		x
H	Lake Konnevesi	Reference Konnevesi	x	x		x
I	Lake Valkea-Kotinen	Reference Hämeenlinna		x		
J	Lake Kakskerta	Rural settlement Turku		x		
K	Lake Jämsänvesi A	Rural settlement Petäjävesi		x		
L	Lake Jämsänvesi B	Rural settlement Petäjävesi		x		

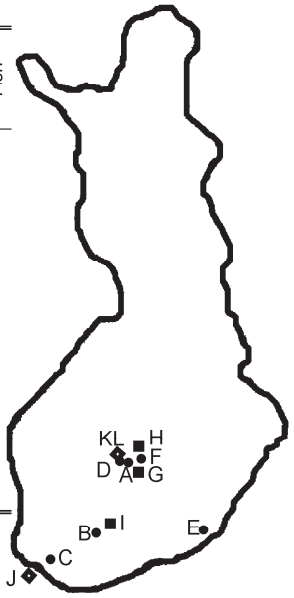

FIGURE 6 Locations of the study sites and specification of the samples taken at each site. Further details of the locations can be found in the publications (II, III, V).

TABLE 2 Characteristics of wastewater treatment plants on locations A–F. Data was collected from monitoring reports of respective units.

Site	Population serviced	Treatment process	Influent flow $m^3\ d^{-1}$	Effluent SS $mg\ l^{-1}$	BOD load $kg\ d^{-1}$
A	130 000	Biol + $FeSO_4$	44 700	11	481
B	27 000	Biol + $FeSO_4$	15 300		130
C	2390	Biol + $FeSO_4$	868	22	15
D	1600	Biol + $FeSO_4$	266	12	1
E	64 000	Biol + $FeSO_4$	15 000	12	116
F	2600	Biol + $FeSO_4$	443	12	13

SS = suspended solids, BOD = biological oxygen demand, Biol = biological treatment, $FeSO_4$ = chemical phosphorus precipitation

3.4 Sampling of SPM (II)

In this thesis, the floating material with high enough gravity is defined as settleable particulate material (SPM). SPM was collected for two months in summers 2008 and 2009. The funnel-like collectors (area 0.25 m^2, volume 90 l) were made from stainless steel. The upper edge of a collector was about 35 cm from the sediment surface. After two months, collectors were lifted up to the surface and gently moved to shoreline water. The first fraction (2.5 l) was pumped from the bottom of the collector (called strong sample) and the second

sample was taken after mixing (4 × 2.5 l, called mixed sample). The SPM fraction was decanted after two days settling period in 4 °C. Four mixed samples were pooled. SPM were frozen and dried with freeze-drier.

3.5 Sampling of sediments (III)

Sediment cores in Lakes Päijänne and Lievestuoreenjärvi were taken with Kajak-sampler and divided into 2.5 cm thick slices in the laboratory after one day settling time. In Lake Haapajärvi, sediment was sliced in the field with Limnos-corer (2 cm slices). Sediments were frozen and dried with freeze-drier.

3.6 Microbial transformation experiments (I)

The biotransformation of bisoprolol, diclofenac and naproxen were studied in two sets of experiments: aerobic and anaerobic. The extent of aerobic transformation was determined by measuring oxygen consumption and the disappearance of the parent compound. Experiments were conducted by adding individual pharmaceutical to aquatic solution containing microbial inoculum from the WWTP of Jyväskylä and mineral solution according to OECD 301F guideline (OECD 1992). For half of the bottles, sodium acetate was added as an additional carbon source. Subsamples for chemical analyses were taken in the beginning and after 7, 14, 21, 28, 43, 57 and 75 days of incubation in 20 °C. Oxygen consumption was measured with an OxiTop® device that automatically calculates the consumed oxygen from the pressure drop in a closed bottle, where formed CO_2 is bound to solid NaOH. The biological oxygen consumption (BOD) was compared to the theoretical oxygen demand (ThOD), which is the calculated amount of oxygen required to oxidize the compound completely.

Anaerobic transformation was evaluated by determining the methane production and the depletion of parent compound. Incubations were conducted in serum bottles. Digested sludge from the WWTP of Jyväskylä was used as an inoculum. Aqueous solution containing pharmaceutical, inoculum, sodium acetate and mineral water according to ISO11734 guideline (ISO 1995) was added to the serum bottle. The headspace was purged with nitrogen to remove oxygen. Two bottles for chemical analysis were sacrificed in the beginning and after 14, 35, 97 and 161 days of incubation in 15 °C. Gaseous methane was measured from the headspace once a week using GC-FID. Separate bottles were used for the methane measurements and chemical analyses.

3.7 Laboratory experiment with rainbow trout (IV)

One year-old juvenile rainbow trout (*Oncorhynchus mykiss*, in average 169 g and 24 cm) were purchased from a local hatchery (Savon Taimen Inc., Rautalampi, Finland) and acclimatized to the laboratory conditions for 11 days before the experiments.

Fish were exposed for 10 days in flow-through system to a mixture of five pharmaceuticals: diclofenac, ibuprofen, naproxen, carbamazepine and bisoprolol. Low and high mixture experiments (nominally equimolar, 1–2 μg l^{-1} and 25–50 μg l^{-1}) as well as control were made. Concentrations in the lower experiment were relatively close to those common in effluents. Four fish were studied per treatment. The actual water concentrations were monitored daily during the experiments.

3.8 Field experiment with rainbow trout (V)

One year-old juvenile rainbow trout (*Oncorhynchus mykiss*, in average 180 g and 26 cm) were purchased from a local hatchery (Savon Taimen Inc., Rautalampi, Finland) and acclimatized to the laboratory conditions from 16 to 23 days before their transport to field sites.

Rainbow trout were held in 240 l cages for 10 days in early June 2008. Experiment was done in three sites near WWTP (Lake Päijänne, River Vantaa and River Aura) and in three reference sites (Lake Palosjärvi, Lake Konnevesi and laboratory of the University of Jyväskylä). Three cages were used in WWTP sites and two in reference sites with four fish in each (i.e. total number of fish per site were 8–12). Sampling was done in the field (Oikari 2006).

3.9 Analytical methods

3.9.1 Dry weight, loss on ignition and total organic carbon (II, III)

Dry weight (dw) and loss on ignition (LOI) were determined from fresh SPM or sediment according to SFS 3008 (SFS 1990). The total organic carbon (TOC) was analyzed from freeze-dried samples with Flash EA1112 elemental analyzer (Carlo Erba) connected to a Finnigan DeltaPlus Advantage continuous flow mass spectrometer (ThermoFisher Scientific Corp., Waltham, USA). Inorganic carbon was removed with HCl.

3.9.2 Analysis of pharmaceuticals with LC-MS/MS

Water (I, IV) and blood plasma (IV, V) samples were acidified and extracted with HLB-cartridges (Oasis, Waters) using methanol as elution solvent. SPM and sediments (II, III) were first extracted three times with acetonitrile and phosphate buffer using an ultrasonic bath. The extract was further purified with a HLB-cartridge (Oasis, Waters). The sorbent in POCIS was extracted with methanol (V). The extracts were divided into two for the analysis of acidic and basic pharmaceuticals.

For bile, separate extraction was used for acidic and basic compounds. Acidics were extracted with HLB-cartridges using 2 % ammonium hydroxide in 80 % methanol as eluent. Basics were extracted with MCX-cartridges (Oasis, Waters) using methanol and 2 % ammonium hydroxide in methanol as eluents. Biles were either extracted directly (IV) or deconjugated first with β-glucuronidase/arylsulfatase from *Helix pomatia* (V). The deconjugation hydrolysed phase II metabolites into respective phase I metabolites, simplifying the chromatographic identification. The identification of diclofenac, naproxen and ibuprofen metabolites was based on previous works of the group (Kallio et al. 2010, Brozinski et al. 2011, 2012a).

The analysis was performed with a Waters Alliance® 2795 (I, II, III, IV) or Agilent 1100 (V) LC system both coupled with Quattro Micro™ electrospray-tandem mass spectrometer (LC-ESI-MS/MS). Compounds were separated with a reversed phase C18 column (Waters XBridge™). In the negative ionization mode (ESI−), the mobile phase consisted of 0.01 M ammonium acetate and 0.01 M ammonium acetate in 90 % acetonitrile (II, IV) or 0.01 M ammonium hydroxide and 0.01 M ammonium hydroxide in 90 % acetonitrile (III, V). In the positive ionization mode (ESI+), acetonitrile and 0.1 % (v/v) formic acid (I, II, IV) or acetonitrile and 0.5 % (v/v) acetic acid (III, V) were used as eluents.

Bioconcentration in blood plasma (BCF_{plasma}) and bile (BCF_{bile}; IV) were calculated by dividing the measured plasma or bile concentration with the measured average water concentration. The sum (as molar basis) of all quantified metabolites was used for the calculation of BCF_{bile}.

3.9.3 Analysis of fecal sterols with GC-MS (III)

Dry sediment was first Soxhlet-extracted with hexane:2-propanol solution (2:1 v/v; Lahdelma & Oikari 2006) and the extract purified with a silica cartridge (Sep-Pak 1 g). Sterols were eluted with 12 ml of chloroform. After silylation, cholesterol and coprostanol were analyzed with a HP 6890 gas chromatograph (Hewlett Packard, Germany) equipped with a HP 5973 mass spectrometer (Hewlett Packard, USA) using HP-5 column (30 m × 0.25 mm ID, 0.25 μm).

3.9.4 Biomarkers (V)

Liver activity of ethoxyresorufin-*O*-deethylase (EROD) was analyzed from the liver mitochondrial supernatant (S9) according to Hodson et al. (1996). EROD-activity was measured spectrofluorometrically by following the conversion of 7-ethoxyresorufin to resorufin after the addition of NADPH as energy. As a positive control, three rainbow trout were intraperitoneally injected with a known CYP1A-inducer, β-naphtoflavone (31 μg g^{-1} wet weight). The protein concentration was measured with a Bio-Rad DC Protein Assay kit (CA, USA) using bovine serum albumin as a standard.

For real-time PCR of vitellogenin (Vtg) mRNA, hepatic samples were extracted using TRIzol-chloroform method according to Wenger et al. (2011). After extraction, DNA was removed and cDNA synthetized. Primer and probe efficiencies were tested by generating a dilution curve representing 10-fold dilution steps. All measurements were performed on 7500 Real-Time PCR System (Applied Biosystems, Rotkreutz, Switzerland). Expression levels of Vtg-mRNA were normalized against the expression level of 18s mRNA. Male rainbow trout fed with 17β-estradiol (20 mg kg^{-1} diet) were used as positive control.

4 RESULTS

4.1 Characterization of field sites

4.1.1 Pollution from municipal wastewater treatment plants (III, V)

Relative contamination by pharmaceuticals at locations A–C, G and H (Fig. 7) was evaluated with POCIS in 2008. The sum of pharmaceuticals in passive samplers was highest at River Vantaa (B, 2884 ng), followed by Lake Päijänne (A, 867 ng) and River Aura (C, 544 ng). Diclofenac was also detected at the reference Lakes Palosjärvi (G, 4 ng) and Konnevesi (H, 3 ng). Almost all the 15 pharmaceuticals monitored could be detected and quantified in the passive samplers deployed in the vicinity of WWTPs. The only exceptions were the antidepressants fluoxetine, which could not be found at any of the sites, and sertraline, which could not be detected at River Aura (Fig. 7). At all locations in the vicinity of WWTP, the concentrations of venlafaxine and carbamazepine were the highest. The quantity of the other pharmaceuticals varied between sites.

The overall human population impact from municipal effluents at Lakes Päijänne, Haapajärvi and Lievestuoreenjärvi was evaluated by measuring the amount of coprostanol and cholesterol from the sediment cores. Based on the literature, a coprostanol concentration of > 0.5 μg g^{-1} in sediment as well as a coprostanol/cholesterol ratio of > 0.2 indicates wastewater input (Grimalt et al. 1990, Vane et al. 2010).

The concentrations of coprostanol and cholesterol in the surface sediment at Lake Päijänne (0–2.5 cm) were 9 and 13 μg g^{-1} dw, respectively (Fig. 8A). After a steep decrease towards the depth of 5 cm, concentrations decreased more gradually to below 0.1 and 2 μg g^{-1} dw. The coprostanol/cholesterol ratio was < 0.2 below the depth of 10 cm, which indicates fecal contamination until at least that depth.

At Lake Haapajärvi, the amount of coprostanol was high in the uppermost 12 cm (4–6 μg g^{-1} dw), decreasing then to about 0.7 μg g^{-1} dw (Fig. 8B). The

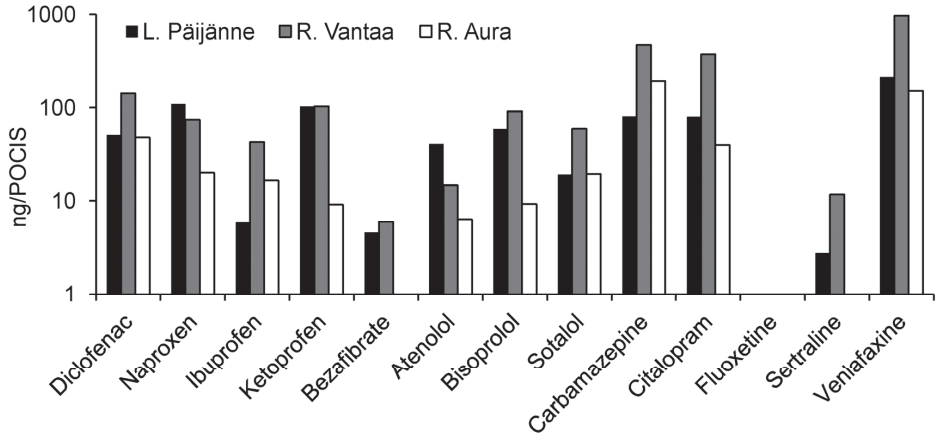

FIGURE 7 Concentrations of pharmaceuticals (ng/POCIS) in Polar Organic Chemical Integrative Samplers (POCIS) deployed at the sites next to wastewater treatment plants for 10 days in June 2008. Note the logarithmic scale on y-axis.

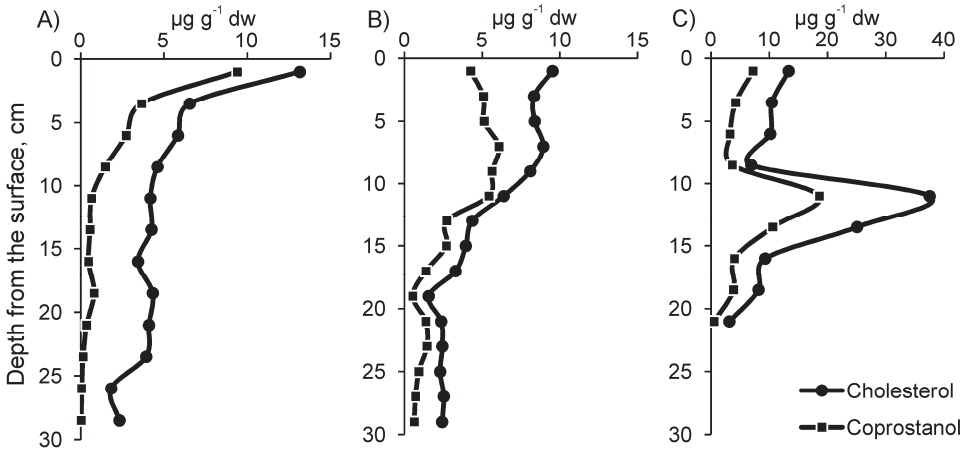

FIGURE 8 Concentrations of cholesterol and coprostanol (µg g^{-1} dw) in A) Lake Päijänne, B) Lake Haapajärvi and C) Lake Lievestuoreenjärvi sediment cores. Note different scales on x-axis.

cholesterol concentration was rather steady until 8 cm (8–10 µg g^{-1} dw) decreasing then gradually to 2 µg g^{-1} dw. The coprostanol/cholesterol ratio was over 0.2 in the whole sediment column. Also sterol concentrations indicated fecal pollution in the whole sediment column.

High concentrations of cholesterol and coprostanol were found at Lake Lievestuoreenjärvi ranging between 7–37 µg g^{-1} dw and 3–19 µg g^{-1} dw, respectively (Fig. 8C). Coprostanol/cholesterol ratio was 0.4–0.5 in the whole sediment column (0–22.5 cm). The highest concentrations were found at the depth of 10 to 15 cm, coinciding with the darkest sediment layers (Fig. 9).

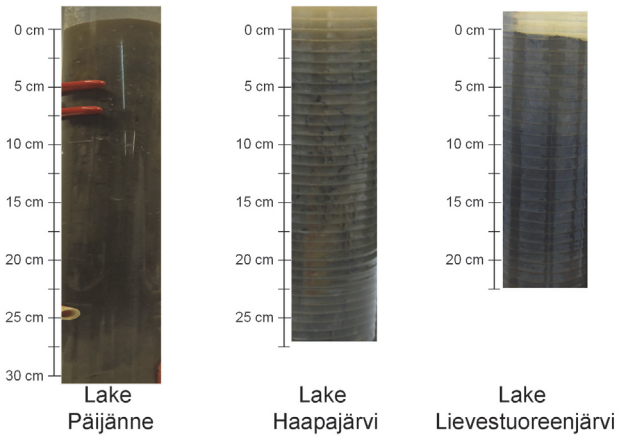

FIGURE 9 Photograph of sediment profiles.

4.1.2 Polyaromatic (CYP1A-inducing) and estrogenic chemicals (V)

The EROD-activity and Vtg-mRNA were measured from field exposed rainbow trout. There were no differences in the EROD-activity between sites and overall the activity was low (0.47–1.1 pmol resorufin min^{-1} mg^{-1} protein). The unaltered EROD-activity indicates that effluents did not contain AhR-positive agonists such as polychlorinated dioxins, polychlorinated biphenyls or certain polycyclic aromatic hydrocarbons to induce liver CYP1A.

The Vtg-mRNA expression in fish exposed at the reference sites and at River Vantaa was minor (< 1 Vtg copy numbers/1000 18s copies). At Lake Päijänne and River Aura, the induction was measurable (4 and 11 Vtg copy numbers/1000 18s copies). However, this response was very low compared to the positive control. The Vtg results indicate that the estrogenicity type of loading by the investigated wastewater sources was low.

4.1.3 General characteristics of SPM and sediment (II, III)

Characteristics of SPM varied considerably between ten locations (II). Generally, LOI and TOC were higher in reference sites than in rural or WWTP sites. Due to the lower amount of mineral material and hence lower settling, dry weight in reference sites was lower. The total amount of accumulated material was highest in WWTP sites, especially at Rivers Vantaa and Aura (Table 3), which were rivers containing high amounts of suspended solids. The deposition rate varied from < 1 kg dw m^{-2} $year^{-1}$ in reference sites to > 50 kg dw m^{-2} $year^{-1}$ in WWTP sites.

TABLE 3 Properties of the settleable particulate material (SPM) collected for two months in summers 2008 and 2009. For locations, see Fig. 6.

Location	Year	Deposition rate kg dw m^{-2} $year^{-1}$	strong or mixed[a]	dw %	LOI % dw	TOC % dw
Lake Päijänne (WWTP)	2008	5.0	strong	13.5	20.2	13.0
			mixed	15.4	19.7	13.8
Lake Päijänne (WWTP)	2009	53.2	strong	15.3	22.9	10.2
			mixed	7.1	35.4	14.6
River Vantaa (WWTP)	2008	103	strong	3.7	34.5	8.6
			mixed	2.4	40.7	7.3
River Aura (WWTP)	2008	70.9	strong	14.6	16.9	5.8
			mixed	16.8	15.8	5.4
Lake Jämsänvesi (WWTP)	2009	6.4	strong	7.9	20.0	8.9
			mixed	7.9	20.3	9.0
Lake Palosjärvi (Ref)	2008	0.4	strong	2.8	43.9	19.7
			mixed	0.6	45.0	-
Lake Palosjärvi (Ref)	2009	0.6	strong	1.9	39.7	16.2
			mixed	1.2	38.7	15.5
Lake Konnevesi (Ref)	2008	0.3	strong	3.2	36.5	-
			mixed	0.3	40.3	-
Lake Konnevesi (Ref)	2009	0.6	strong	4.5	31.7	12.7
			mixed	2.3	38.2	15.3
Lake Valkea-Kotinen (Ref)	2009	1.1	strong	2.0	84.2	36.6
			mixed	0.4	81.1	35.6
Lake Kakskerta (Rural)	2009	3.3	strong	8.6	15.3	5.7
			mixed	7.5	16.3	6.2
Lake Jämsänvesi A (Rural)	2009	4.0	strong	4.1	17.3	7.5
			mixed	5.7	18.8	8.0
Lake Jämsänvesi B (Rural)	2009	2.8	strong	7.4	19.0	8.0
			mixed	5.5	19.0	7.9

[a]Strong means the first bottom sample (2.5 l), mixed the homogenized pooled sample

Based on visual evaluation of the Lake Päijänne core, there was a clear change towards mineral material at the depth of 25 cm (Fig 9). At Lake Haapajärvi sediment, bioturbation was evident (visible burrowing channels). Black layers (10–20 cm) at the Lake Lievestuoreenjärvi sediment indicated strongly anaerobic conditions.

In the sediment at Lake Päijänne, LOI varied from 4 to 10 % and TOC from 0.9 to 3.3 %, both decreasing with depth (Fig. 10A). At Lake Haapajärvi, LOI and TOC varied from 7.3 to 10.9 % and from 2.9 to 4.3 %, respectively (Fig. 10B). A steep increase in LOI and TOC was observed at Lake Lievestuoreenjärvi, where they increased from 11 to 26 % and from 5 to 13 % below 5 cm. Below 12.5 cm, their proportions decreased reaching 13 and 5 % at 20 cm (Fig. 10C). This trend was similar to that of cholesterol and coprostanol concentrations.

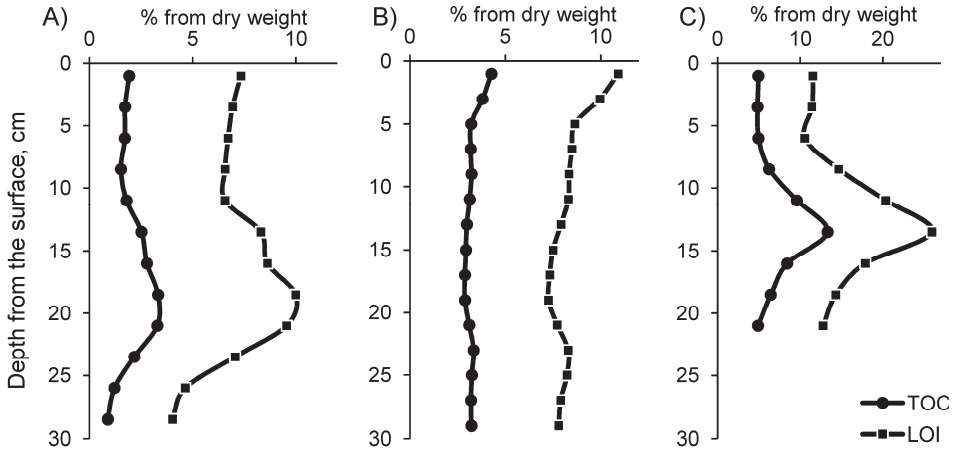

FIGURE 10 Loss on ignition (LOI, % of dry weight) and total organic carbon (TOC, % of dry weight) in A) Lake Päijänne, B) Lake Haapajärvi and C) Lake Lievestuoreenjärvi sediment cores. Note different scales on x-axis.

4.2 Microbial transformation of pharmaceuticals in water (I)

4.2.1 Aerobic conditions

The concentrations of diclofenac remained unchanged in treatments without an additional carbon source, sodium acetate (Fig. 11). With an additional carbon source, although the concentrations appeared to vary to some extent, the overall transformation of diclofenac was negligible over time. In addition, BOD was nearly equal to that of the corresponding control units (Table 4), supporting the conclusion of recalcitrance i.e. that diclofenac was not microbially transformed.

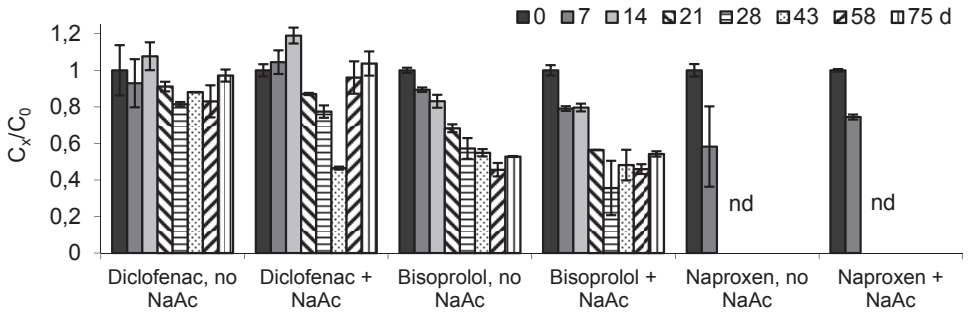

FIGURE 11 Changes in normalized concentrations of pharmaceuticals during the 75-day aerobic experiment. Pharmaceuticals were monitored with and without addition of sodium acetate (NaAc), an additional carbon source. Two parallel units were analyzed, thus error bars depict maximum and minimum values. nd = not detected.

TABLE 4 Oxygen consumption in the 75-day aerobic biotransformation experiments. Oxygen consumption of the respective control units has been substracted. BOD/ThOD describes the ratio (as %) of consumed oxygen compared to the theoretical amount of oxygen required to completely mineralize the compound.

	Oxygen consumption mg l^{-1}		BOD/ThOD %	
Added NaAc	no	yes	no	yes
Diclofenac	-2	1	-3	1
Naproxen	90	84	81	73
Bisoprolol	38	76	38	75

After 75 days, about 35 % of the bisoprolol had decayed (Fig. 11). The additional carbon source did not seem to have an influence on the transformation. The oxygen consumption of bisoprolol was 38 % and 75 % of the ThOD without and with additional carbon source, respectively (Table 4). Hence, BOD indicated a more efficient mineralization in the presence of additional carbon.

The transformation of naproxen was efficient. In fact it was not detected after 14 days of incubations (Fig. 11). A suggested intermediate metabolite 6-*O*-desmethyl naproxen was not found under aerobic conditions. However, it was most likely formed, but transformed further very efficiently. Decay in sterile treatments was < 10 %, so microbial transformation was the principal removal mechanism. Although there were differences in the kinetics of BOD with and without additional carbon, at the end (75 d) from 73 to 81 % of the ThOD was reached (Table 4).

4.2.2 Anaerobic conditions

The methane production in the reference without drugs (14.3 ml) and in the diclofenac, bisoprolol and naproxen treatments (14.7–15.7 ml) were about equal. No mineralization was evident.

About 26 % of diclofenac disappeared during 161 days (Fig. 12), but when compared to the sterile treatments, the removal turned out to be mainly abiotic (22 %). The total removal of bisoprolol averaged 28 %, from which approximately half was abiotic and the other half microbiological. In contrast to nil or low decay of diclofenac and bisoprolol, naproxen was efficiently transformed. Over 97 % of naproxen decayed during the incubation. A transformation product 6-*O*-desmethyl naproxen was detected after 14 days. At day 97, approximately 28 % (as molar basis) of the originally analyzed naproxen was detected as 6-*O*-desmethyl naproxen, which seemed to be relatively persistent under anaerobic conditions.

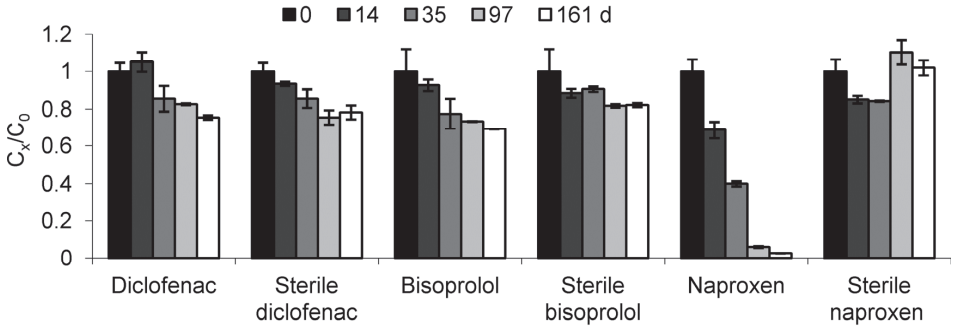

FIGURE 12 Changes in normalized concentrations of pharmaceuticals during 161-day anaerobic incubations. Results are means of two parallel experimental units sampled at each sampling point, thus error bars depict maximum and minimum values.

4.3 Contamination of benthic habitats by pharmaceuticals

4.3.1 Pharmaceuticals in SPM (II)

Of the 17 pharmaceuticals monitored, from 8 to 13 were found in samples collected downstream from WWTPs. High concentrations, generally over 200 ng g^{-1} dw, of citalopram, ciprofloxacin and bisoprolol were found (Fig. 13). Concentrations and detection frequency decreased with decreasing WWTP size. The highest concentrations were found in the collector at Lake Päijänne. However, due to the high amount of suspended solids, the largest load of sedimenting pharmaceuticals was found at River Vantaa. The annual deposition rates of individual pharmaceuticals ranged considerably, from 17 to 79400 µg m^{-2} y^{-1}. Ibuprofen and ofloxacin were detected in the rural Lake Kakskerta (some human settlement but no centralized WWTP). Pharmaceuticals were not detected in any of the reference sites or in the other two rural sites.

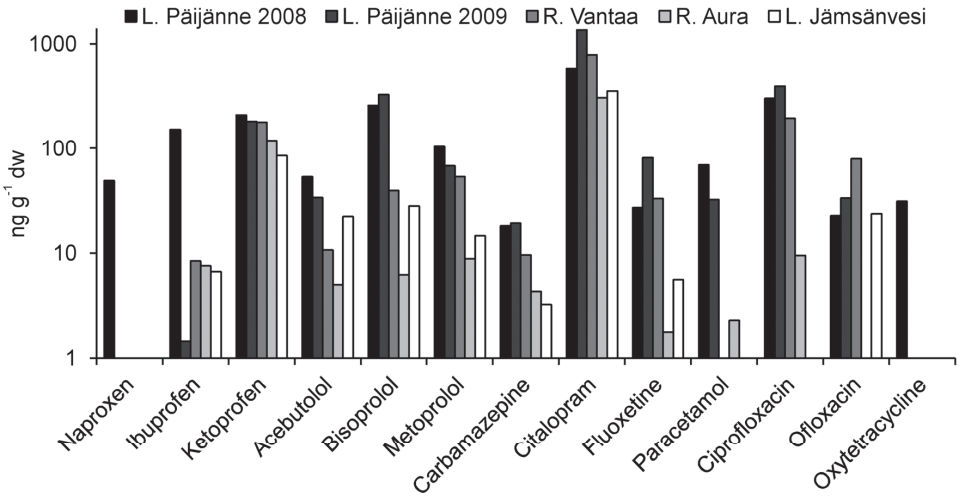

FIGURE 13 Concentrations of pharmaceuticals in settleable particulate material (ng g^{-1} dry weight) collected from sites adjacent to wastewater treatment plants for two months in summers 2008 and 2009. Atenolol, bezafibrate, diclofenac and sotalol are not presented, since they were not detected in any of the samples. Note the logarithmic scale on y-axis.

4.3.2 Pharmaceuticals in sediments (III)

Several pharmaceuticals were detected in sediments at Lakes Päijänne and Haapajärvi (Fig. 14). The most abundant pharmaceutical found in the sediments was citalopram (15–290 ng g^{-1} dw), but also bisoprolol (7–38 ng g^{-1} dw), acebutolol (4–13 ng g^{-1} dw), propranolol (9–43 ng g^{-1} dw) and sertraline (4–14 ng g^{-1} dw) were found. Pharmaceuticals were not found at Lake Lievestuoreenjärvi.

Pharmaceuticals were mainly found in the uppermost 0–12.5 cm at Lake Päijänne, but there were no common trend in the profiles of different pharmaceuticals (Fig. 14A). At Lake Haapajärvi, pharmaceuticals were found in the whole sediment column (0–30 cm) and the concentrations seemed to vary randomly (Fig. 14B). The variation of citalopram was especially high. In general, higher concentrations were found in Lake Haapajärvi than in Lake Päijänne.

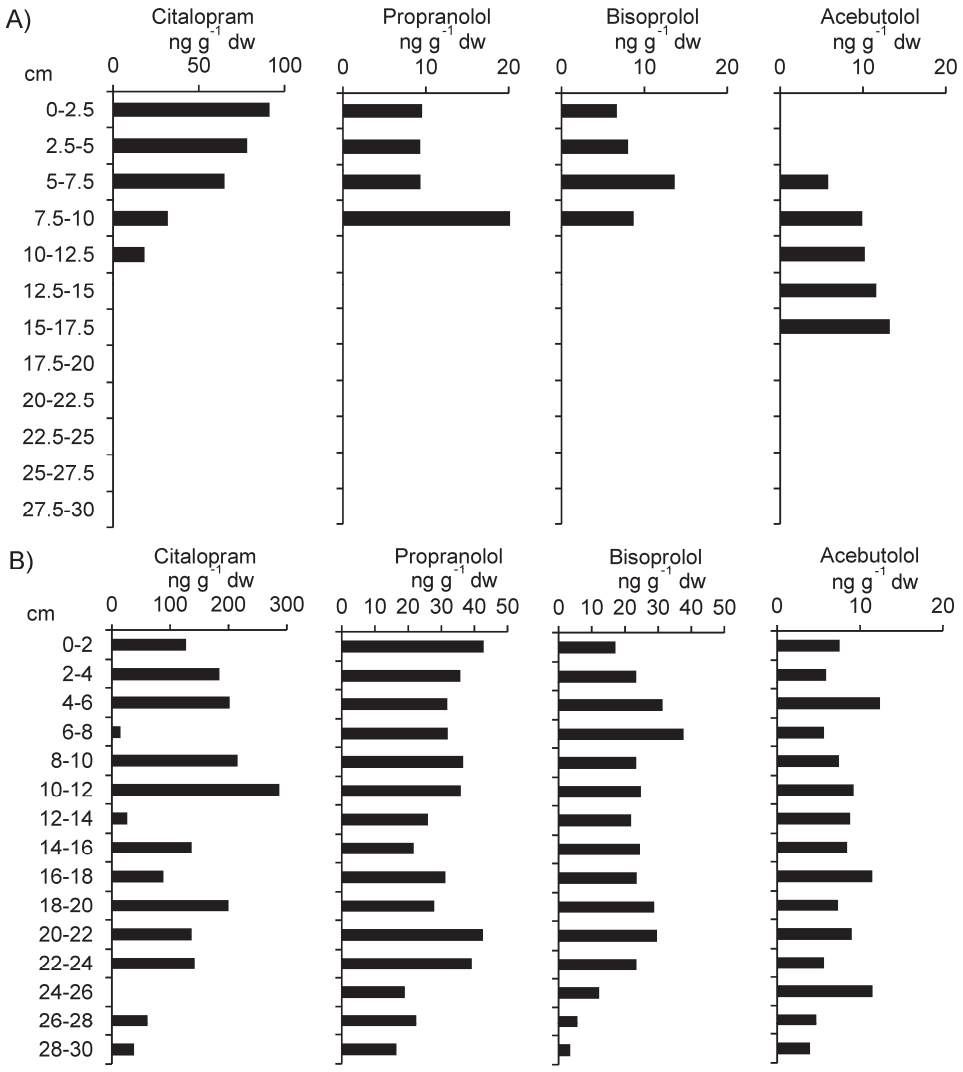

FIGURE 14 Concentrations (ng g^{-1} dw) of selected pharmaceuticals in A) Lake Päijänne and B) Lake Haapajärvi sediment cores. Note different scales on x-axis. When no value is given compound was not detected.

4.4 Exposure of rainbow trout to pharmaceuticals

4.4.1 Water exposure in the laboratory (IV)

After the 10-day flow-through experiment, the concentrations of the five pharmaceuticals in trout blood plasma varied from compound to compound (Fig. 15A). The bioconcentration factor in plasma (BCF_{plasma}) was highest for diclofenac (4.9–7.7) and lowest for bisoprolol (< 0.01–0.02). There were no statistical ($p > 0.05$) differences in the plasma bioconcentration between low and high water concentrations, although bisoprolol was not detected in the low concentration experiment. The variances in uptake between individuals were high (RSD up to 86 %).

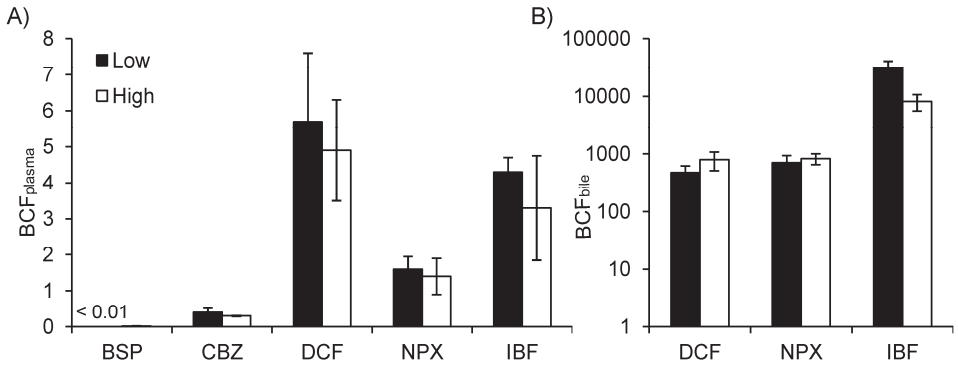

FIGURE 15 Mean (± SE) bioconcentration factors A) in plasma (BCF_{plasma}) and B) in bile (BCF_{bile}) of juvenile rainbow trout exposed in the laboratory for 10 days to mixture of pharmaceuticals at low and high water concentrations. BCF_{plasma} was calculated as ratio of plasma concentration to that in water. BCF_{bile} was calculated as ratio of bile (free parent compound + metabolites) concentration to that in water (free parent compound). Note logarithmic scale on BCF_{bile}. BSP = bisoprolol, CBZ = carbamazepine, DCF = diclofenac, NPX = naproxen, IBF = ibuprofen.

Several metabolites of diclofenac, naproxen and ibuprofen were detected in rainbow trout bile both in low and high water concentrations. Chromatogram and structures of the naproxen metabolites are presented in Fig. 16. The identity of the metabolites was based on Kallio et al. (2010) and Brozinski et al. (2011, 2012a). The most abundant metabolites were acyl glucuronides of either parent compound (naproxen) or hydroxylated metabolites (diclofenac and ibuprofen) (Fig. 17). The amount of unmetabolized pharmaceuticals in bile was low (0–14 % of the total). None of the metabolites were detected in the control fish. The average BCF_{bile} of ibuprofen was four times higher in the low than in the high concentration experiment ($p < 0.05$; Fig. 15B). Instead, there were no differences in BCF_{bile} of diclofenac and naproxen between experiments ($p > 0.05$). Compared to the plasma, concentrations of metabolites in bile were 10^2–10^4 fold higher.

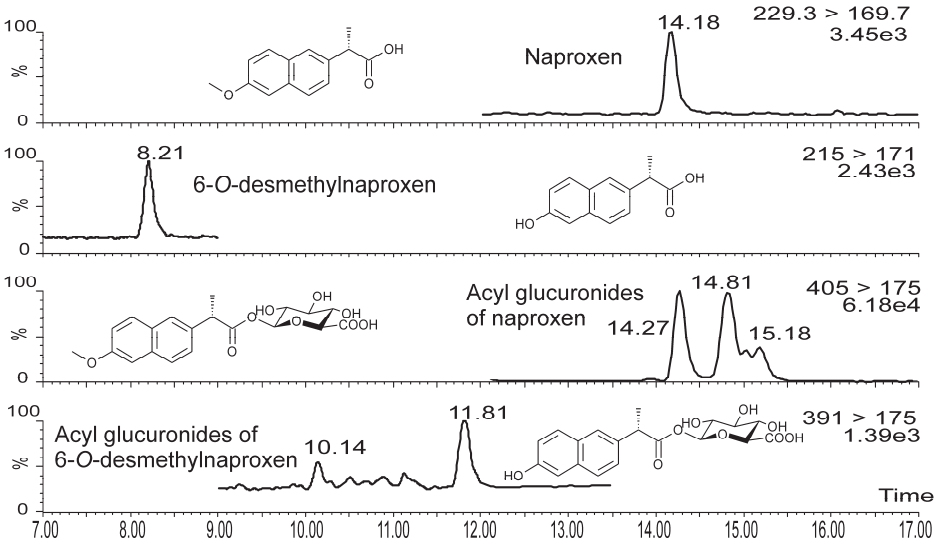

FIGURE 16 Chromatogram and structures of naproxen metabolites in a bile from rainbow trout exposed to a mixture of pharmaceuticals for 10 days at water concentration 40 µg l^{-1}.

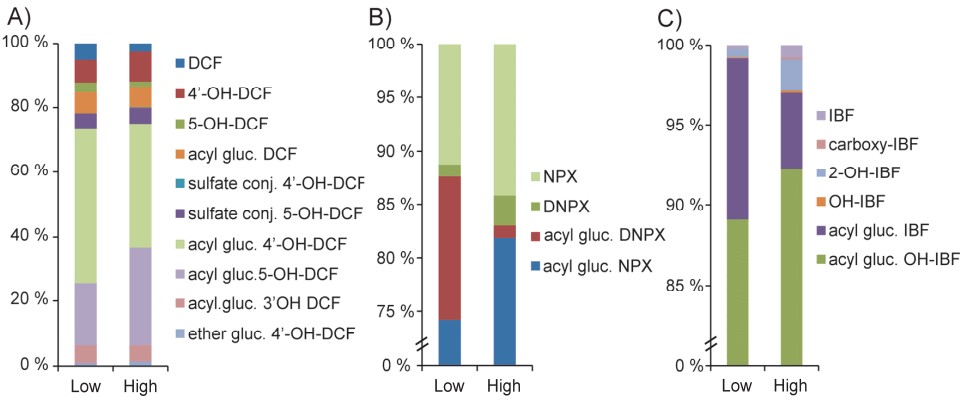

FIGURE 17 Proportions (%) of the metabolites of A) diclofenac, B) naproxen and C) ibuprofen identified from the rainbow trout bile after 10-day exposure to the mixture of five pharmaceuticals. Note differences in y-axis scale. DCF = diclofenac, OH-DCF = hydroxy-diclofenac, NPX = naproxen, DNPX = 6-*O*-desmethyl naproxen, IBF = ibuprofen, OH-IBF = hydroxy-ibuprofen.

4.4.2 Exposure in the field (V)

Of the 15 pharmaceuticals monitored, only three pharmaceuticals (diclofenac, naproxen and ibuprofen) were detected in the blood plasma (Table 5). The exposure of fish to these compounds was highest at River Vantaa, followed by Lake Päijänne and finally River Aura. Pharmaceuticals were not found in

plasma of fish held at the reference sites (Lake Palosjärvi, Lake Konnevesi and laboratory).

TABLE 5 Mean (± SE) concentrations of pharmaceuticals in the plasma of rainbow trout exposed in the field for 10 days in three sites next to wastewater treatment plants. The mean is based on only those samples where compound was quantified. N = number of samples analyzed, n = number of samples where compound was detected, nd = not detected.

	Lake Päijänne		River Vantaa		River Aura	
	ng ml^{-1}	n/N	ng ml^{-1}	n/N	ng ml^{-1}	n/N
Diclofenac	18 ± 5	4/11	21 ± 3	8/8	30 ± 15	2/11
Ibuprofen	nd	0/11	20 ± 6	4/8	nd	0/11
Naproxen	7.7 ± 1.5	4/11	13 ± 1	8/8	nd	0/11

For the analysis, the bile samples of field exposed rainbow trout were deconjugated in order to increase the concentrations of the parent pharmaceuticals. After deconjugation, parent drugs as well as previously identified phase I metabolites of diclofenac, naproxen and ibuprofen were analyzed from the bile. Only parent compounds of the other 12 pharmaceuticals were quantified, but not found.

Diclofenac (10–4000 ng ml^{-1}), naproxen (40–1900 ng ml^{-1}), ibuprofen (33–450 ng ml^{-1}) and metabolite hydroxy-diclofenac (210–4300 ng ml^{-1}) were found in the fish caged downstream the WWTPs (Table 6). None of the metabolites of naproxen and ibuprofen could be observed in the bile. Being in accordance with the plasma results, concentrations were highest in the bile of fish exposed at River Vantaa and lowest at River Aura. However, the concentration of naproxen was higher at Lake Päijänne than at River Vantaa. Pharmaceuticals were not found in the bile of fish held at the reference sites (Lake Palosjärvi, Lake Konnevesi and laboratory).

TABLE 6 Mean (± SE) concentrations of pharmaceuticals in the bile of rainbow trout exposed in the field for 10 days in three sites next to wastewater treatment plants. The mean is based on only those samples where compound was quantified. N = number of samples analyzed, n = number of samples where compound was detected.

	Lake Päijänne		River Vantaa		River Aura	
	ng ml^{-1}	n/N	ng ml^{-1}	n/N	ng ml^{-1}	n/N
Diclofenac	990 ± 200	5/5	4100 ± 500	3/3	100 ± 30	5/5
Hydroxy-diclofenac	1400 ± 360	5/5	4300 ± 420	3/3	300 ± 64	3/5
Ibuprofen	53 ± 11	2/5	450 ± 18	3/3	71	1/5
Naproxen	1900 ± 430	5/5	1200 ± 230	3/3	40 ± 15	5/5

5 DISCUSSION

5.1 Overall contamination of the studied locations by pharmaceuticals

A suitable passive sampling device, such as a POCIS, accumulates compounds that are dissolved in the water phase. Although nearly all the measured pharmaceuticals were present in the POCIS used in this study, distinct patterns were present in the SPM, sediment and fish. In general, cationic compounds were found in the SPM and sediments, and acidic compounds in fish plasma and bile.

Lake Päijänne was the most comprehensively studied of the six sites in the vicinity of WWTPs. Therefore it was possible to compare the patterns of pharmaceuticals in the POCIS, the SPM, sediment and field exposed fish. The most abundant pharmaceuticals in the POCIS were venlafaxine, naproxen, ketoprofen and citalopram. Venlafaxine was not found in the sediment or fish, although it was the most abundant pharmaceutical in the POCIS. Naproxen was detected in fish, but it was not found in the SPM or sediment. Finally, citalopram was abundant in the SPM and sediment, but it was not detected in the fish plasma or bile. These differences emphasize the diverse fates of pharmaceuticals.

No sediments were studied from Rivers Vantaa and Aura, because sediment is not accumulating in these river systems like in more slowly flowing lakes. Two lakes (Lake Haapajärvi and Lake Lievestuoreenjärvi) with different type of sedimentation were chosen for sediment studies in addition to Lake Päijänne (III). The SPM was collected to study the contamination by pharmaceuticals more widely and samples were collected from three types of areas: sites in the vicinity of WWTPs, rural locations without centralized wastewater treatment and reference sites (II).

Three approaches POCIS (V), SPM (II) and fish (V) revealed highest contamination at River Vantaa, followed by Lake Päijänne and River Aura. Thus, different evaluation methods seemed to complement each others. Earlier,

Vieno (2007) measured pharmaceuticals in effluent and river water at these locations and found the highest concentrations at River Vantaa (Lindqvist et al. 2005, Vieno 2007, Vieno et al. 2007). Although low concentrations of some compounds were detected in a POCIS and SPM in rural or reference areas, they seemed to be relatively free of pharmaceutical contamination in Finland.

5.2 Fate of pharmaceuticals in the environment

5.2.1 Can pharmaceuticals be persistent in the aquatic environment?

Some pharmaceuticals can be persistent in the aquatic environment. However, the microbial transformation begins already at the WWTP and continues in the receiving waters. Large variation in the microbial transformation of individual pharmaceuticals has been observed both in the WWTPs as well as in the environment (Yu et al. 2006, Kunkel & Radke 2008, Jelic et al. 2011). Also the results presented here (I) revealed markedly different persistency of naproxen, bisoprolol and diclofenac, the three drugs chosen for detailed examination. The added amount of readily degradable organic carbon did not contribute to the biotransformation efficiency of any of the studied drugs. Therefore, the rate of biotransformation seems to be more dependent on the properties of the compound than on the content of organic carbon in natural waters.

Biotransformation of naproxen was evident in both aerobic and anaerobic environmental conditions (I). The efficient biotransformation of naproxen was observed also in other studies (Urase & Kikuta 2005, Yu et al. 2006, Carballa et al. 2007, Tran et al. 2009) and it represented well those drugs that are not expected to persist in the environment.

Compared to naproxen, the transformation rate of bisoprolol was slow especially in anaerobic experiments (I). Partial removal was observed also in other studies (Wick et al. 2009, Ramil et al. 2010).

No decay of diclofenac was observed in the aerobic or anaerobic conditions (I). Diclofenac has been classified as persistent in some studies (Quintana et al. 2005, Joss et al. 2006), but able to biotransform by others (Yu et al. 2006, Carballa et al. 2007, Gröning et al. 2007, Kosjek et al. 2008). The incomplete biotransformation and elimination of diclofenac and bisoprolol suggests that these compounds are detected in the surface waters. Therefore, in the future, more awareness is needed to pick-out drugs persisting in the environment.

The transformation rate of naproxen and bisoprolol in the anaerobic conditions was slower than in the aerobic ones. Therefore the time naproxen and bisoprolol persists after release depends on the degree of saproby in the environment they end up. Bisoprolol is likely to remain in the anaerobic zone for a long time due to its slow decay rate. Studies have shown that biotransformation of many pharmaceuticals and endocrine-disrupting chemicals is notably lower in anaerobic than aerobic conditions, if

biotransformation takes place at all anaerobically (Ying & Kookana 2003, Gröning et al. 2007, Kunkel & Radke 2008, Jiang et al. 2010, Suarez et al. 2010).

5.2.2 Are pharmaceuticals susceptible for sedimentation?

Based on the results presented in this thesis (II), they are. Sorption and desorption have key roles in the distribution and bioavailability of pharmaceuticals in the environment. They determine for instance whether pharmaceuticals are sedimented close to the discharge point, transported with particulate material or remain fully dissolved. WWTPs are important sources of suspended solids and SPM originating from them can contain considerable amounts of xenobiotics (Byrns 2001). Sorption to particles may decrease the bioavailability by microbes and thus hinder the biotransformation of chemicals (Alexander 2000, Styrishave et al. 2011). The toxicity may decrease due to lower exposure, although benthic invertebrates as well as bottom feeding fish may be exposed also via ingested sediment particles (Kaag et al. 1997, Forbes et al. 1998, Leppänen & Kukkonen 1998, Sormunen et al. 2008).

The variation in the sorption between individual pharmaceuticals and solid matrices (soils and sediments) may be high (Williams et al. 2009, Jelic et al. 2011). Sorption of cations tends to be higher than that of anions due to ionic interactions with negatively charged minerals and biofilms (Tolls 2001, Pan et al. 2009, Williams et al. 2009), presumably also SPM. Both organic and inorganic fractions are important for the sorption of drugs (Pan et al. 2009). Although lipophilic partition into organic carbon is not considered the main sorption mechanism, an increasing TOC seems to enhance the sorption of some pharmaceuticals (Varga et al. 2010, Xu & Li 2010).

High concentrations of citalopram, ciprofloxacin and bisoprolol were abundant in SPM (II). Because of accumulation in the POCIS (VI), they were also present at the dissolved phase. The potential of a compound to sedimentate depends on sorption and biotransformation. Sediment burial is likely, if compound biotransform slowly enough and sorb to SPM. Citalopram and ciprofloxacin sorb strongly to the sediments and soils (Toll 2001, Kwon et al. 2008). Styrishave et al. (2011) suspected that strong sorption limits the biotransformation of citalopram, because of lesser bioavailability. In WWTPs, biotransformation of citalopram is moderate (40–60 %) being lower in anaerobic than aerobic conditions (Suarez et al. 2010). Carbamazepine and diclofenac were abundant in the POCIS, but not in SPM. Their sorption is low (Williams et al. 2009, Jelic et al. 2011) and they are recalcitrant in the environment (Zwiener & Frimmel 2003, Joss et al. 2006, Jelic et al. 2011, Suarez et al. 2010). Thus carbamazepine and diclofenac persist in the water phase and spread long distances with water flows. Low concentrations of ibuprofen were found in the SPM, but not in the sediments (II, III). Even the most recent layer, the uppermost 2 cm, was free of it. Sediment burial of ibuprofen is unlikely due to the ready biotransformation and low sorption (Zwiener & Frimmel 2003, Yu et al. 2006, Joss et al. 2006, Suarez et al. 2010).

5.2.3 Can discharge history of pharmaceuticals be demonstrated from sediments?

Yes. According to results presented here (III), the most widely detected compounds in sediments were citalopram, propranolol and bisoprolol, all positively charged at the ambient lake pH (6.5–7). None of the negatively charged pharmaceuticals, like rather persistent diclofenac (I), were detected in the sediment. The maximum concentration of the antidepressant citalopram was notably high (up to 290 ng g^{-1} dw; III). Surprisingly, this far, pharmaceuticals have been reported only from the surface layer of sediment. The reported concentrations of pharmaceuticals in surface sediments have been below 100 ng g^{-1} dw (Antonic & Heath 2007, Stein et al. 2008, Ramil et al. 2010, Schultz et al. 2010, Varga et al. 2010, da Silva et al. 2011). For instance, the concentrations of citalopram and bisoprolol were up to 15 and 86 ng g^{-1} dw, respectively (Ramil et al. 2010, Schultz et al. 2010). Also naproxen and diclofenac have been found in surface sediments (Rice & Mitra 2007, Varga et al. 2010), although they were not detected in the present study (III).

Benthic biota is mostly exposed to uppermost sediments. However, compounds in deeper sediments are not necessary permanently buried, but may become bioaccessible with time e.g. due to bioturbation by benthic biota (Gunnarson et al. 1999). Josefsson et al. (2010, 2011) detected mobilization of hydrophobic compounds from marine sediments due to bioturbation to a sediment depth of 10 cm or even lower. This demonstrates that remobilization may occur from the deeper sediment layers than the immediate subsurface. Xenobiotics in sediments can be released to the overlaying water during events that disturb sediment such as flooding or dredging (Eggleton & Thomas 2004). In addition, sediment dynamics and water flows have effect on the persistency of pharmaceuticals because of the substantial variation in the vertical profiles of oxygen (Kunkel & Radke 2008).

Occurrence of pharmaceuticals in deeper sediment layers (III) suggested that sediments can act as a sink for certain strongly sorbing compounds, such as citalopram. Increased desorption of pharmaceuticals was observed in the presence of sediment dwelling *Chironomus dilutus*. The influence of bioturbation on desorption was higher for less hydrophobic carbamazepine than for EE2 (Gilroy et al. 2012). Desorption of carbamazepine demonstrates that sediments can act as a long term source of pharmaceuticals.

Exposure via sediments can be important especially for benthic invertebrates but also for benthic fishes. The accumulation of EE2 in *Chironomus tentans* was greater in an experiment with sediment than water only (Dussault et al. 2009). The bioaccumulation of pharmaceuticals from sediments to benthic invertebrates is currently unknown. A strong bioconcentration of fluoxetine (BCF 185900) and lesser degree of carbamazepine (BCF 7.1) from water to aquatic invertebrate *Gammarus pulex* was recently reported (Meredith-Williams et al. 2012). Bioconcentration to *Notonecta glauca* was substantially lower (BCF fluoxetine 1.4, carbamazepine 0.2; Meredith-Williams et al. 2012). The evident

contamination of benthic habitats (III) highlights that there is need for invertebrate bioaccumulation and exposure studies.

Sediment profiles have been successfully used in tracking of historical contamination by industry e.g. that of pulp mill operations (Leppänen & Oikari 2001, Hynynen at al. 2004, Lahdelma & Oikari 2006). Although the total consumption of pharmaceuticals has been increasing, there are some drugs which have a decreased usage or have even ceased being used. With citalopram, a clear correlation (r^2 = 0.97) between annual sales (FIMEA 2011) and concentration in Lake Päijänne sediment was found (III). Furthermore, acebutolol and bisoprolol corresponded well with their introduction to the markets, even though the sediment varving was not ideal in Lake Päijänne (Meriläinen & Hamina 1993). Because consumption records of pharmaceuticals are well documented in many countries, some of them, like citalopram in the present study (III), could be used in dating of sediments with varving tendency. The age of sediment depth can be evaluated from the first occurrence of a pharmaceutical and the year it came to markets. Based on the present study, citalopram is one candidate for this kind of further study.

5.2.4 Are pharmaceuticals bioconcentrated and metabolized by fish?

The uptake and metabolism of pharmaceuticals was evident in fish (IV). Neutral compounds are taken up via lipophilic partition through epithelial membranes (Mackay & Fraser 2000). With ionizable compounds, the degree of ionization generally modifies the uptake, because ionized form is poorly absorbed (Erickson et al. 2006a, 2006b, Fu et al. 2009). The higher the proportion of the compound is in neutral form the higher is the uptake rate and hence toxicity (Saarikoski & Viluksela 1981, Nakamura et al. 2008, Fu et al. 2009, Valenti et al. 2009, Rendal et al. 2011). This means that increasing pH decreases the toxicity of acidic pharmaceuticals and increases that of basic pharmaceuticals (see example of ionization on Fig. 2). For ionic compounds, the uptake via active transporters is possible (Miller 2008, Fu et al. 2009), although better knowledge on their kinetics and regulation is warranted.

At the stage approaching equilibrium (10 d), the bioconcentration factors of pharmaceuticals in plasma were low (BCF_{plasma} 0.02–6; IV) compared to the hydrophobic chemicals such as PAHs (BCF_{plasma} 43–76; Kennedy & Law 1990). The bioconcentration of acidic pharmaceuticals (diclofenac, naproxen and ibuprofen) was slightly higher than that of bisoprolol and carbamazepine (IV). Basically no bioaccumulation in blood plasma was observed for bisoprolol, which was most likely readily eliminated due to fast cation transport.

The overall conclusion for slight accumulation of pharmaceuticals in blood is conclusive. The published bioconcentration factors of those pharmaceuticals studied during this thesis are presented in Table 7. The pH of the test water was not reported in all studies, although the accumulation of pharmaceuticals is highly dependent on water pH. According to laboratory studies, fluoxetine and its metabolite norfluoxetine have rather high potential for bioaccumulation (Nakamura et al. 2007, Paterson & Metcalfe 2008, Zhang et al. 2010). Actually

higher accumulation of the metabolite than the parent fluoxetine was detected (Nakamura et al. 2007, Paterson & Metcalfe 2008). Overall, it appears that pharmaceuticals are not accumulative in blood plasma, muscle or liver (Table 7), because of their high ionization and quick elimination (Erickson et al. 2006a, 2006b, Fu et al. 2009).

In fish bile, hydroxides as well as glucuronide and sulfate conjugates are common biotransformation products of PAHs, resin acids and pharmaceuticals (Oikari et al. 1984, Law et al. 1994, Kallio et al. 2010, Brozinski et al. 2011, 2012a). In the results reported here (IV) only trace amounts of the unmetabolized parent drugs were detected in bile. Diclofenac was mainly found as glucuronide and sulphate conjugates of hydroxy-diclofenac. Naproxen was mainly present as glucuronide. Microbial transformation product 6-*O*-desmethyl naproxen (I) was also formed by fish (IV). Ibuprofen was mainly metabolized into hydroxy-ibuprofen and glucuronides. Further studies by Brozinski et al. (2012a) revealed that ibuprofen is metabolized into several rather unusual products such as taurine-conjugates.

The metabolization of pharmaceuticals is obvious in fishes. To compare the bioconcentration in bile, all the quantified metabolites were summed up on a molar basis (IV). The bioconcentration of naproxen in the bile was approximately equal to diclofenac (~10^2), while the amount of ibuprofen metabolites in the bile was higher (~10^3). Mehinto et al. (2010) have reported similar bioconcentration of diclofenac (~10^2; Table 7).

Large differences in plasma and bile concentrations were found between individual rainbow trout (IV, V). Accordingly, large differences were found in organ distribution of diclofenac in brown trout (*Salmo trutta* f. *fario*; Hoeger et al. 2008). About 50 % of diclofenac was excreted within 36 h. Six hours after dosing 34–66, 2–4 and < 1 % of it was found in bile, muscle and blood, respectively. In addition, diclofenac undergoes enterohepatic recycling, i.e. reuptake from the intestine to the blood (Hoeger et al. 2008).

Higher lipid content of an organ can increase the accumulation of most hydrophobic pharmaceuticals. Zhang et al. (2010) measured higher concentrations of fluoxetine, carbamazepine and ibuprofen in adipose fin than in muscle (Table 7). In addition, the concentrations of fluoxetine and sertraline were ten times higher in the brain and liver than in the muscle tissue (Table 7; Brooks et al. 2005, Ramirez et al. 2009, Lajeunesse et al. 2011).

The plasma and the bile concentrations of rainbow trout had an inverse correlation suggesting individual differences in the efficiency of hepatic uptake, rate of biotransformation, or excretion to the bile (IV). The capacities of transporters may be limited, as secretion of ionic compounds is largely mediated by them (Miller 2008).

TABLE 7 Bioconcentration factors (BCF) of the studied pharmaceuticals in laboratory experiments.

Pharmaceutical	Exposure duration	Species [1]	Tissue	pH	BCF mean ± sd [2]	Reference
Fluoxetine	30 d	Japanese medaka	body	7	8.8 ± 5.2	Nakamura et al. (2007)
Fluoxetine	30 d	Japanese medaka	body	9	260 ± 150	Nakamura et al. (2007)
Fluoxetine	30 d	Japanese medaka	liver	7	330 ± 90	Nakamura et al. (2007)
Fluoxetine	30 d	Japanese medaka	liver	9	3100 ± 400	Nakamura et al. (2007)
Fluoxetine	7 d	Japanese medaka	whole fish	7.4	74 (mean)	Paterson & Metcalfe (2008)
Fluoxetine	8 d	Rainbow trout	muscle	7.8	58.98 ± 16.81	Zhang et al. (2010)
Fluoxetine	8 d	Rainbow trout	adipose fin	7.8	143.36 ± 21.50	Zhang et al. (2010)
Norfluoxetine [3]	30 d	Japanese medaka	body	7	84 ± 8	Nakamura et al. (2007)
Norfluoxetine [3]	30 d	Japanese medaka	body	9	650 ± 180	Nakamura et al. (2007)
Norfluoxetine [3]	30 d	Japanese medaka	liver	7	1500 ± 200	Nakamura et al. (2007)
Norfluoxetine [3]	30 d	Japanese medaka	liver	9	3700 ± 2300	Nakamura et al. (2007)
Norfluoxetine [3]	7 d	Japanese medaka	whole fish	7.4	117 (mean)	Paterson & Metcalfe (2008)
Citalopram	1 d	Rainbow trout	plasma	7.5	< 0.008	Holmberg et al. (2011)
Propranolol	21 d	Fathead minnow	plasma	nr [4]	0.4–15 (mean)	Giltrow et al. (2009)
Bisoprolol	10 d	Rainbow trout	plasma	7.7	< 0.01–0.02 (mean)	Publication IV
Carbamazepine	8 d	Rainbow trout	muscle	7.8	0.52 ± 0.11	Zhang et al. (2010)
Carbamazepine	8 d	Rainbow trout	adipose fin	7.8	4.16 ± 0.87	Zhang et al. (2010)
Carbamazepine	10 d	Rainbow trout	plasma	7.7	0.3–0.4 (mean)	Publication IV
Diclofenac	14 d	Rainbow trout	plasma	7.4	4.02 ± 0.75	Cuklev et al. (2011)
Diclofenac	14 d	Rainbow trout	liver	7.4	2.54 ± 0.36	Cuklev et al. (2011)
Diclofenac	21 d	Rainbow trout	bile	nr [4]	509–657 (min–max)	Mehinto et al. (2010)
Diclofenac	2 d	Rainbow trout	plasma	nr [4]	7 (mean)	Brown et al. (2007)
Diclofenac	10 d	Rainbow trout	plasma	7.7.	4.9–5.7 (mean)	Publication IV

Diclofenac	10 d	Rainbow trout	bile	7.7	476–797 (mean)	Publication IV
Naproxen	2 d	Rainbow trout	plasma	nr [4]	4 (mean)	Brown et al. (2007)
Naproxen	10 d	Rainbow trout	plasma	7.7	1.4–1.6 (mean)	Publication IV
Naproxen	10 d	Rainbow trout	bile	7.7	703–829 (mean)	Publication IV
Ketoprofen	2 d	Rainbow trout	plasma	nr [4]	0.1 (mean)	Brown et al. (2007)
Ibuprofen	2 d	Rainbow trout	plasma	nr [4]	9 (mean)	Brown et al. (2007)
Ibuprofen	8 d	Rainbow trout	muscle	7.8	1.50 ± 0.25	Zhang et al. (2010)
Ibuprofen	8 d	Rainbow trout	adipose fin	7.8	23.69 ± 2.23	Zhang et al. (2010)
Ibuprofen	28 d	Fathead minnow	muscle	7.8	0.69 (mean)	Nallani et al. (2011)
Ibuprofen	28 d	Fathead minnow	liver	nr [4]	0.69 (mean)	Nallani et al. (2011)
Ibuprofen	28 d	Fathead minnow	gill	nr [4]	1.09 (mean)	Nallani et al. (2011)
Ibuprofen	7 d	Channel catfish	muscle	nr [4]	0.08 (mean)	Nallani et al. (2011)
Ibuprofen	7 d	Channel catfish	liver	nr [4]	0.51 (mean)	Nallani et al. (2011)
Ibuprofen	7 d	Channel catfish	gill	nr [4]	0.44 (mean)	Nallani et al. (2011)
Ibuprofen	7 d	Channel catfish	kidney	nr [4]	0.63 (mean)	Nallani et al. (2011)
Ibuprofen	7 d	Channel catfish	plasma	nr [4]	1.4 (mean)	Nallani et al. (2011)
Ibuprofen	10 d	Rainbow trout	plasma	7.7	3.3–4.3 (mean)	Publication IV
Ibuprofen	10 d	Rainbow trout	bile	7.7	8170–31000 (mean)	Publication IV

[1] Japanese medaka (*Oryzias latipes*), Rainbow trout (*Oncorhynchus mykiss*), Fathead minnow (*Pimephales promelas*), Channel catfish (*Ictalurus punctatus*)

[2] mean ± standard deviation if not stated otherwise, mean = mean or range of means, min–max = minimum and maximum values

[3] Metabolite of fluoxetine, the given value is a pseudo-BCF (concentration of norfluoxetine in fish divided with fluoxetine in water)

[4] nr = not reported

5.2.5 Are fish exposed to pharmaceuticals discharged from wastewater treatment plants?

The exposure of fish to widely used acidic anti-inflammatory drugs diclofenac, naproxen and ibuprofen in the vicinity of WWTPs was evident (V). The published concentrations of pharmaceuticals in wild and field exposed fish are presented in Appendix 2. Atenolol, bezafibrate, bisoprolol, metoprolol, paracetamol and propranolol were not found in fish (Ramirez et al. 2007, Ramirez et al. 2009, Fick et al. 2011). Acebutolol and sotalol have not been measured from fish before this study (V).

None of the studied antidepressants were detected in the bile or plasma (V), although those drugs are among the most widely found pharmaceuticals in wild and field exposed fish (Brooks et al. 2005, Chu & Metcalfe 2007, Kwon et al. 2009, Ramirez et al. 2009, Zhang et al. 2010, Metcalfe et al. 2010, Schultz et al. 2010, Lajeunesse et al. 2011). The uptake and accumulation of sertraline was higher than that of the other antidepressants (Brooks et al. 2005, Metcalfe et al. 2010, Schultz et al. 2010). However, for better understanding of the mechanisms of internal exposure, laboratory experiments reporting the BCF of sertraline should be conducted.

Exposure itself is not an adverse effect. The effect is evoked in a target organ or receptor and will occur when certain internal concentration is exceeded. Huggett et al. (2003, 2004) have proposed that the Fish Plasma Model could be used to evaluate the possibility of effect. The model is based on assumption that the same internal concentration will cause the effect in humans and fish. Thus human data could be used in ERA of pharmaceuticals, but many uncertainties related to fish toxicokinetics exist.

5.3 Fish bile as evidence of exposure of fish to pseudopersistent xenobiotics

Our knowledge of functional time-courses of hepato-biliary excretion in fishes is rather sporadic. In vertebrates, the cycle of bile formation and release to the intestine is dependent on nutritional status (Talbot & Higgins 1982, Förlin & Wachtmeister 1989). Bile tends to be stored in the gall bladder during fasting, being periodically emptied post-fed to enhance digestion in the intestine. To a certain extent, the longer the fasting time is the larger and darker the bile inside gall bladder is (Talbot & Higgins 1982). It is also evident that water is reabsorbed from the bile during long fasting i.e. over several days. Reabsorption reduces bile volume in the gall bladder and concentrates the xenobiotics and their metabolites that have been secreted from the liver (Talbot & Higgins 1982, Förlin & Wachtmeister 1989, Brumley et al. 1998). It is not known whether there is some modifications of bile composition under its storage e.g. due to hydrolases from liver. Due to practical reasons to avoid small bile volumes, rainbow trout were not fed during the experiments (IV, V).

In nature, boreal fishes seem to have periods when they do not eat (Oikari 2006).

In 1976, Statham et al. suggested that fish bile could be used for the assessment of exposure to readily metabolized xenobiotics (Statham et al. 1976). Since then several compounds have been measured from the bile, such as estrogens, polyaromatic hydrocarbons, resin acids, and chlorophenolics (Statham et al. 1976, Oikari 1986, Förlin & Wachtmeister 1989, Wachtmeister et al. 1991, Law et al. 1994, Larsson et al. 1999, Oikari et al. 1999, Gibson et al. 2005, Vermeirssen et al. 2005, Meriläinen et al. 2007). Bile has been especially suitable for compounds which are readily metabolized and excreted via the bile instead of accumulation in muscle or other organs (Statham et al. 1976, Förlin & Wachtmeister 1989).

There are many advantages in using bile. First of all, xenobiotics that are taken up by fish and secreted from the liver can accumulate in bile. In this study, laboratory experiment with diclofenac, naproxen and ibuprofen showed 10^2–10^3 fold accumulation from water to bile (IV). Earlier, Larsson et al. (1999) measured a 10^4–10^6 fold accumulation of estrogenic compounds (e.g. EE2, nonylphenol and bisphenol-A) in the bile of field-exposed rainbow trout compared to the surrounding water. The metabolic accumulation of resin acids and chlorophenols in bile is about 10^5 and 10^6 fold, respectively (Oikari et al. 1984, Oikari & Kunnamo-Ojala 1987, Meriläinen et al. 2007). Emissions of resin acids and chlorophenolics from the pulp industry have been very successfully monitored by measuring their concentrations in the fish bile (Meriläinen & Oikari 2008).

In the laboratory experiment (IV), the total concentrations (parent and metabolites) of diclofenac, naproxen and ibuprofen were 80 to 7000 times higher in the bile than in the plasma. In the field experiment (V), the differences varied from 10 to 400 fold. These results suggest that the exposure to pharmaceuticals can be detected in the bile at lower ambient water concentrations than from the plasma. In humans, the secretion of diclofenac glucuronides into bile is via an active ATP-dependent pump, which enables a several fold higher concentration in the bile than in the blood (Boelsterli 2003).

Secondly, bile can be used for comparative analysis of biotransformation (Oikari et al. 1984, Meriläinen & Oikari 2008). The metabolism may vary among species, which leads either to different structures of the metabolites or to variation in the relative abundances of them. With modern chromatographic techniques, metabolites can be identified and metabolite profiles of species compared. In previous studies, the metabolites of three pharmaceuticals diclofenac, naproxen and ibuprofen were identified in the bile of rainbow trout (Kallio et al. 2010, Brozinski et al. 2011, 2012a). Most of them were known as human metabolites. Thus human data can be used for predicting the metabolites in other vertebrates. For instance, fluoxetine is efficiently desmethylated to norfluoxetine in humans (Hiemke & Härtter 2000) and medaka (*Oryzias latipes*; Nakamura et al. 2008, Paterson & Metcalfe 2008). Apparently, more comparative data is needed among fishes, but also from those invertebrates inhabiting benthic environments.

However, identification can be demanding and lack of numerous surrogate standards may prevent quantification of the analytes. These obstacles can be overcome by hydrolyzing conjugates into respective parent compounds or phase I metabolites. Due to the more simple structure, the identification of phase I metabolites is easier than that of phase II conjugates. Importantly, where ambient concentrations in waters are low, deconjugation increases sensitivity, because one compound (parent or phase I metabolite) can form several conjugates. Compounds that undergo efficient phase I metabolism are not detected as parent compounds even after deconjugation and identification of the primary metabolite(s) is needed. Deconjugation of metabolites can be done with specific hydrolases or with alkaline and acidic solutions (Oikari et al. 1984, Oikari & Ånäs 1985, Legler et al. 2002, Gibson et al. 2005). Enzymes β-glucuronidase and arylsulfatase hydrolyse glucuronide and sulfate conjugates, respectively. In the field experiments (V), the bile samples were enzymatically deconjugated with β-glucuronidase/arylsulfatase solution. As a method validation, the bile of an unexposed rainbow trout was spiked with the 1-β-*O*-acyl glucuronides of diclofenac, naproxen and ibuprofen and subsequently the liquid was treated with the enzyme. LC-MS/MS analyses of the hydrolysate revealed that peaks due to the acyl glucuronides were absent, whereas peaks due to the diclofenac, naproxen and ibuprofen had emerged. Thus the deconjugation was efficient and quantitative.

In conclusion, for regulatory purposes, bile analyses are a useful tool for monitoring the exposure of fish to pharmaceuticals, while tissue residues may not trace low levels of exposure. Due to the strong bioconcentration, xenobiotics can be detected in the bile although chemical concentrations in ambient waters are very low and fluctuating.

5.4 Future directions

This thesis expands the knowledge of the different fates of pharmaceuticals in the environment. However, it also highlights several important research topics that should be studied in the near future. Among others these include:

Firstly, understanding the chronic effects of single substance and mixtures of pharmaceuticals on fish should be improved. This includes comparative studies with different species and drugs. Secondly, bile metabolites of numerous drugs (5–10 more) should be identified. Pharmaceuticals could be chosen based on their chemical characteristics, persistency, toxicity and consumption. Metabolism should also be compared between different species. To further improve the interpretation of bile analyses, excretory and secretory system time-courses, e.g. gall bladder dynamics, needs to be explored. Thirdly, the results revealed clear contamination of benthic habitats by pharmaceuticals. However, there is basically no knowledge of the exposure of benthic biota via sediments. Furthermore, the possible long-term effects on benthic invertebrates are still largely unknown.

6 CONCLUSIONS

The aim of this thesis was to determine several aspects of the environmental fate of pharmaceuticals after their release from WWTPs. In addition to pharmaceuticals in true soluble form next to discharge, this thesis studied the sedimentation, exposure of fish, and biotransformation by microbes and fish. These approaches were chosen in order to study the alternative fate possibilities of pharmaceuticals in the aquatic environment.

The presence of pharmaceuticals in water was evident and nearly all target compounds were found in passive samplers in the vicinity of WWTPs. In addition, some of the pharmaceuticals accumulated in SPM indicating that sediment burial is possible. The presence in SPM also highlighted that the true loading from the WWTP can be revealed only by measuring pharmaceuticals from both dissolved and suspended fractions.

The microbial transformation of pharmaceuticals in aquatic systems is variable and only partially understood today. Some are readily biotransformed whereas others even recalcitrant. Importantly, pharmaceuticals that biotransform quickly are detected in the environment due to their constant emissions. Drugs that have a slow rate of biotransformation or that are persistent are prone to accumulation in the environment.

Analyses of the sediments revealed high concentrations of few pharmaceuticals even at the deeper layers (citalopram, bisoprolol, propranolol and acebutolol). This gave the novel possibility to date the sediment and especially citalopram was found applicable for dating. Although the sorption of ionizable molecules is dependent on water and sediment characteristics, the same drugs occurred at the sediment cores in Lake Päijänne and Lake Haapajärvi. Their presence in deeper sediments showed that pharmaceuticals can persist therein and can cause delayed exposure of the benthic invertebrates.

Due to the ionization, pharmaceuticals may not be readily taken up from water. However, the ingestion of sediment may increase the exposure through the absorption during digestion, because one important property of an effective pharmaceutical is its efficient absorption in the human gut.

Demonstrating the existence and identification of the compound in the environment is the first step in the environmental risk assessment. There were clearly some compounds that should be studied further. Hazard identification revealed that citalopram occurs in water phase, accumulates in sediments and is persistent. Thus, the exposure and effects should be determined. Finally, the bioavailability and toxicity to water organisms determines the possible risks of pharmaceuticals to the aquatic life. However, citalopram was not detected in the rainbow trout. Either it was not absorbed due to low bioavailability or it was metabolized and eliminated very quickly. The exposure and the toxicity of citalopram to benthic invertebrates is currently not known, but should be studied in the future.

Regarding to the fate from water to fish, diclofenac, naproxen and ibuprofen were studied by plasma and bile analysis. Diclofenac was taken up by fish and accumulated in blood plasma. Exposure was demonstrated both in the laboratory and field study. Its persistency in the environment prolongs the time when exposure and effects are possible. Diclofenac is known to cause severe toxic effects on terrestrial and aquatic biota at the environmentally relevant concentrations. Thus hazard identification, exposure and effect assessments indicate that diclofenac may pose environmental risks more widely than currently realized.

As an overall conclusion, the fate of each pharmaceutical can be unique and must be known individually. Due to the variable properties, some compounds may be readily biotransformed, others sedimented or taken up by biota. Fate processes are overlapping and the relative importance of biotransformation, sedimentation and uptake by biota vary considerably from one site to another.

Acknowledgements

This study was carried out at the University of Jyväskylä, Department of Biological and Environmental Science during the years 2007–2012. The work was financially supported by the Finnish Graduate School in Environmental Science and Technology, Maa- ja vesitekniikan tuki ry and Academy of Finland (Grant no 109823).

I am grateful to my supervisor professor Aimo Oikari for accepting me as a PhD student and thus enabling this work. Knowing that the door was always open, when questions or problems arose, was very important. I believe your devotion to science is special.

I also want to thank my co-authors Jenny-Maria Brozinski, Antti Jylhä, Professor Leif Kronberg and Professor Helmut Segner. Jenny-Maria, the days with bile analyzes were often long, but I really enjoyed sharing them with you. The help from Antti during the experimental work and in the field was crucial, what would I have done without you. I acknowledge all the other people helping me during the hard field work or sample processing Lasse Alanko, Aarno Karels, Viljami Kinnunen, Silja Kujala, Siiri Latvala, Heli Ratia, Risto Retkin, Heli Rämänen, Tarini Sahoo, Jesper Svanfelt and Erkki Vesterinen.

Nothing would work without our laboratory technicians Mervi Koistinen and Leena Siitonen. You were always there when extra eyes, arms or cheering up were needed. I would not probably be here now without Päivi Meriläinen, who introduced me to the world of science (and fish bile). Eeva and Tarini, you shared the office with me and without you many problems (related to work and life in general) would have remained unsolved. I want to thank all the people working at the environmental science division, especially those visiting regularly at the coffee room. Those breaks often saved the day.

Warmest gratitude to my friends, who have reminded me about life outside the science, and to my family, who have always encouraged me and never doubted my decisions. Finally, Kari I am fortunate to have you in my life and no words can describe my gratitude.

YHTEENVETO (RÉSUMÉ IN FINNISH)

Lääkeaineiden ympäristökohtalo – Biotransformaatio, sedimentaatio ja kalojen altistuminen

Huolimatta vuosikymmeniä jatkuneesta ja alati kasvavasta lääkkeiden kulutuksesta, niiden ympäristövaikutukset ovat saaneet huomiota vasta viime vuosina. Lääkeannoksen ottamisen jälkeen sen vaikuttavat aineet imeytyvät elimistöön ja aiheuttavat terapeuttisen vaikutuksen. Lääkemolekyyli ei kuitenkaan häviä vaan suurin osa siitä eritetään pois elimistöstä virtsan ja ulosteiden mukana. Siten lääkkeet päätyvät jätevedenpuhdistamoiden kautta ympäristöön, erityisesti pintavesiin. Tämän tutkimuksen tarkoituksena oli selvittää lääkkeiden ympäristökohtaloa ja kertymistä kaloihin. Tutkimuksessa määritettiin lääkkeiden mikrobiologista hajoamista ja mitattiin kertymistä vesien partikkeliainekseen sekä järvien sedimenttiin (eli vesistön pohjaan). Kirjolohien (*Oncorhynchus mykiss*) altistumista lääkkeille selvitettiin sekä laboratoriossa että jätevedenpuhdistamoiden läheisyydessä.

Lääkkeiden muuntuminen mikrobiologisesti osoittautui hyvin vaihtelevaksi. Särkylääke naprokseeni hajosi hyvin sekä hapellisissa että hapettomissa olosuhteissa, joskin hajoamisnopeus oli suurempi hapellisissa oloissa. Sitä vastoin toisella särkylääkkeellä, diklofenaakilla, ei muuntumista havaittu, ja beetasalpaaja bisoprololi hajosi vain osittain. Lääkkeen pysyvyys ympäristössä määräytyykin sekä sen rakenteen että ympäristöolosuhteiden mukaan.

Tässä tutkimuksessa kerättiin laskeutuvaa partikkeliainesta eli ainesta, josta myöhemmin muodostuu sedimenttiä, yhteensä kymmenestä eri vesistöstä. Jätevedenpuhdistamon läheisyydessä sijaitsevilla näytteenottopaikoilla partikkeliaines sisälsi useita eri lääkkeitä, erityisesti masennuslääke sitalopraamia, beetasalpaaja bisoprololia sekä antibiootti siprofloksasiinia. Eniten lääkkeitä mitattiin Vantaanjoesta Riihimäen puhdistamon alapuolelta sekä Päijänteeltä Jyväskylän jätevedenpuhdistamon läheisyydestä. Haja-asutusalueilta lääkkeitä löydettiin vain vähän ja eristäytyneiltä puhtailta alueilta ei lainkaan. Tutkimus osoitti, että jotkin lääkkeet voivat kertyä voimakkaasti partikkeliainekseen ja myöhemmin myös sedimenttiin.

Tutkimuksia jatkettiin mittaamalla lääkkeitä sedimenteistä. Sedimentin syvyysprofiilit kolmelta eri järveltä paljastuivat hyvin erilaisiksi. Lappeenrannan Haapajärvessä lääkkeitä löytyi koko sedimenttipatsaasta, jopa 30 cm syvyydeltä. Järvi on matala ja sedimentit eivät kerrostu vuosittaisesti vaan sekoittuvat pohjaeläinten, kalojen ja veden virtausten vuoksi. Päijänteellä Jyväskylän jätevedenpuhdistamon läheisyydessä lääkkeitä löytyi vain ylimmästä 12,5 cm:stä ja erityisesti sitalopraamin pitoisuudet kasvoivat sedimentin pintaa kohti. Sedimentti oli siis Päijänteellä verrattain sekoittumatonta. Lievestuoreenjärven sedimenttinäytteestä ei löytynyt lääkkeitä luultavasti pienen jätevesimäärän vuoksi. Pitoisuudet Haapajärvessä olivat suurempia kuin Päijänteellä.

Kirjolohen laboratorioaltistus viiden lääkkeen seokselle paljasti lääkkeiden erilaisen potentiaalin kertyä kaloihin. Kalojen veriplasmassa diklofenaakin pi-

toisuudet olivat suurimmat ja bisoprololin pienimmät. Kirjolohen aineenvaihdunta muunsi lääkkeitä tehokkaasti ja sapesta mitattiin useita metaboliitteja. Pitoisuudet sapessa olivat moninkertaisia vesi- ja veriplasmapitoisuuksiin nähden ja sappi osoittautuikin hyväksi näytteeksi lääkeainealtistumista mitattaessa.

Kun lääkeainealtistuminen oli osoitettu laboratoriossa, voitiin tutkimuksia jatkaa kenttäolosuhteissa. Kirjolohia altistettiin sumpuissa kolmen jätevedenpuhdistamon läheisyydessä. Yhteensä 15 mitatusta lääkkeestä kolmea pystyttiin havaitsemaan kirjolohen plasmassa ja sapessa (särkylääkkeet diklofenaakki, naprokseeni ja ibuprofeeni). Pitoisuudet olivat jälleen suurempia sapessa kuin plasmassa. Kalojen altistuminen lääkkeille oli suurempaa Vantaanjoessa Riihimäen jätevedenpuhdistamon alapuolella kuin Päijänteellä Jyväskylän jätevedenpuhdistamon läheisyydessä, vaikka Jyväskylän puhdistamolla käsitellään huomattavasti enemmän jätevettä. Puhdistettu jätevesi sekoittuu järvessä nopeasti suureen vesitilavuuteen alentaen siten lääkepitoisuuksia. Alhaisinta altistuminen oli Auran jätevedenpuhdistamon alapuolella Aurajoessa.

Mittaukset partikkeleista, sedimentistä ja kaloista osoittivat, että eri lääkkeet kertyvät ympäristössä eri paikkoihin. Positiivisesti varautuneet masennuslääke sitalopraami sekä beetasalpaaja bisoprololi kertyivät sedimenttiin ja negatiivisesti varautuneet särkylääkkeet diklofenaakki, naprokseeni ja ibuprofeeni kaloihin. Lääkkeiden ympäristökohtalo voi siis vaihdella huomattavasti lääkkeestä toiseen ja ympäristöriskit tulisi arvioida jokaiselle lääkkeelle tai lääkeryhmälle erikseen.

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APPENDICES

APPENDIX 1 Consumption of pharmaceuticals

Table 1 Consumption of pharmaceuticals in Finland in 2007–2010 (FIMEA 2011).

Compound	Therapeutic class	CAS number	Defined daily dose g	Consumption in 2007 kg year^{-1}	Consumption in 2008 kg year^{-1}	Consumption in 2009 kg year^{-1}	Consumption in 2010 kg year^{-1}
Acebutolol	Betablocker	37517-30-9	0.4	735	663	715	539
Atenolol	Betablocker	29122-68-7	0.075	663	613	557	519
Bisoprolol	Betablocker	66722-44-9	0.01	517	564	595	635
Metoprolol	Betablocker	56392-17-7	0.15	5 200	5 070	4 850	4 710
Propranolol	Betablocker	525-66-6	0.16	600	627	613	613
Sotalol	Betablocker	3930-20-9	0.16	359	318	287	253
Bezafibrate	Lipid regulator	41859-67-0	0.6	174	152	128	117
Clofibrate	Lipid regulator	637-07-0	2.0	-	-	-	-
Ciprofloxacin	Antibiotic	85721-33-1	0.75	900	892	919	952
Ofloxacin	Antibiotic	82419-36-1	0.4	54	47	39	31
Oxytetracycline	Antibiotic, veterinary	79-57-2					
Carbamazepine	Anti-epileptic	298-46-4	1.0	4 180	4 040	3 810	3 730
Citalopram	Anti-depressant	59729-33-8	0.02	827	859	889	913
Fluoxetine	Anti-depressant	54910-89-3	0.02	205	204	196	186
Sertraline	Anti-depressant	79617-96-2	0.05	537	563	587	653
Venlafaxine	Anti-depressant	93413-69-5	0.1	1 050	1 180	1 370	1 510
Diclofenac	Anti-inflammatory	15307-86-5	0.1	1 040	1 080	1 020	1 070
Ibuprofen	Anti-inflammatory	15687-27-1	1.2	93 200	113 000	113 000	114 000
Ketoprofen	Anti-inflammatory	22071-15-4	0.15	856	792	671	600
Naproxen	Anti-inflammatory	22204-53-1	0.5	6 330	6 400	6 350	6 200
Paracetamol	Anti-inflammatory	103-90-2	3.0	106 000	123 000	138 000	151 000

APPENDIX 2 Pharmaceuticals in fish.

Table 1 Concentrations of pharmaceuticals in tissues of feral or field exposed (caged) fish.

Pharmaceutical	Experiment	Species[a]	Tissue	Concentration mean ± SD[b]	Unit	Reference
Fluoxetine	Wild	Various[c]	brain	1.58 ± 0.74	ng g^{-1}	Brooks et al. (2005)
Fluoxetine	Wild	Various[c]	liver	1.34 ± 0.65	ng g^{-1}	Brooks et al. (2005)
Fluoxetine	Wild	Various[c]	muscle	0.11 ± 0.03	ng g^{-1}	Brooks et al. (2005)
Fluoxetine	Wild	Gizzard shad	whole fish	0.16–1.02 (min–max)	ng g^{-1}	Chu & Metcalfe (2007)
Fluoxetine	Wild	Brown bullhead	whole fish	0.20–0.31 (min–max)	ng g^{-1}	Chu & Metcalfe (2007)
Fluoxetine	Wild	Various[d]	liver	80 (max)	ng g^{-1}	Ramirez et al. (2009)
Fluoxetine	Wild	White sucker	brain	0.02–1.65 (min–max)	ng g^{-1}	Schultz et al. (2010)
Fluoxetine	Mesocosm	Brook trout	liver	0.20 ± 0.06	ng g^{-1}	Lajeunesse et al. (2011)
Fluoxetine	Mesocosm	Brook trout	brain	0.08 ± 0.0.2	ng g^{-1}	Lajeunesse et al. (2011)
Fluoxetine	Mesocosm	Brook trout	muscle	0.09 ± 0.01	ng g^{-1}	Lajeunesse et al. (2011)
Fluoxetine	Wild	Perch	muscle	6.7 (pool)	ng g^{-1}	Fick et al. (2011)
Sertraline	Wild	Various[c]	brain	4.27 ± 1.4	ng g^{-1}	Brooks et al. (2005)
Sertraline	Wild	Various[c]	liver	3.59 ± 1.67	ng g^{-1}	Brooks et al. (2005)
Sertraline	Wild	Various[c]	muscle	0.34 ± 0.09	ng g^{-1}	Brooks et al. (2005)
Sertraline	Wild	Various[d]	muscle	19 (max)	ng g^{-1}	Ramirez et al. (2009)
Sertraline	Wild	Various[d]	liver	545 (max)	ng g^{-1}	Ramirez et al. (2009)
Sertraline	Wild	White sucker	brain	0.17–4.24 (min–max)	ng g^{-1}	Schultz et al. (2010)
Sertraline	Caged	Fathead minnow	whole fish	3.83 ± 1.81	ng g^{-1}	Metcalfe et al. (2010)
Sertraline	Caged	Rainbow trout	plasma	1.1–1.2 (mean)	ng ml^{-1}	Fick et al. (2010)
Sertraline	Mesocosm	Brook trout	liver	0.29 ± 0.05	ng g^{-1}	Lajeunesse et al.(2011)
Sertraline	Mesocosm	Brook trout	brain	0.21 ± 0.08	ng g^{-1}	Lajeunesse et al. (2011)
Sertraline	Mesocosm	Brook trout	muscle	0.12 ± 0.03	ng g^{-1}	Lajeunesse et al. (2011)

Sertraline	Wild	Perch	muscle	14 (pool)	ng g^{-1}	Fick et al. (2011)
Citalopram	Wild	White sucker	brain	0.02–0.21 (min–max)	ng g^{-1}	Schultz et al. (2010)
Citalopram	Caged	Fathead minnow	whole fish	2.90 ± 1.31	ng g^{-1}	Metcalfe et al. (2010)
Citalopram	Mesocosm	Brook trout	liver	0.41 ± 0.19	ng g^{-1}	Lajeunesse et al. (2011)
Citalopram	Mesocosm	Brook trout	brain	0.18 ± 0.11	ng g^{-1}	Lajeunesse et al. (2011)
Citalopram	Mesocosm	Brook trout	muscle	nd	ng g^{-1}	Lajeunesse et al. (2011)
Venlafaxine	Wild	White sucker	brain	1.12 (max)	ng g^{-1}	Schultz et al. (2010)
Venlafaxine	Caged	Fathead minnow	whole fish	1.20 ± 0.36	ng g^{-1}	Metcalfe et al. (2010)
Venlafaxine	Mesocosm	Brook trout	liver	0.69 ± 0.14	ng g^{-1}	Lajeunesse et al. (2011)
Venlafaxine	Mesocosm	Brook trout	brain	0.43 ± 0.10	ng g^{-1}	Lajeunesse et al. (2011)
Venlafaxine	Mesocosm	Brook trout	muscle	0.08 ± 0.03	ng g^{-1}	Lajeunesse et al. (2011)
Carbamazepine	Wild	Sunfish	muscle	0.83–1.44 (min–max)	ng g^{-1}	Ramirez et al. (2007)
Carbamazepine	Wild	Various[d]	muscle	3.1 (max)	ng g^{-1}	Ramirez et al. (2009)
Carbamazepine	Wild	Various[d]	liver	8 (max)	ng g^{-1}	Ramirez et al. (2009)
Carbamazepine	Caged	Rainbow trout	plasma	0.3–1.0 (mean)	ng ml^{-1}	Fick et al. (2010)
Diclofenac	Caged	Rainbow trout	plasma	12 ± 14	ng ml^{-1}	Brown et al. (2007)
Diclofenac	Caged	Rainbow trout	plasma	2.2–20 (mean)	ng ml^{-1}	Fick et al. (2010)
Diclofenac	Caged	Rainbow trout	plasma	18–30 (mean)	ng ml^{-1}	Publication V
Diclofenac	Caged	Rainbow trout	bile	101–4081 (mean)	ng ml^{-1}	Publication V
Diclofenac	Wild	Roach, bream	bile	50–88 (mean)	ng ml^{-1}	Brozinski et al. (2012b)
Naproxen	Caged	Rainbow trout	plasma	14 ± 9	ng ml^{-1}	Brown et al. (2007)
Naproxen	Caged	Rainbow trout	plasma	33–46 (mean)	ng ml^{-1}	Fick et al. (2010)
Naproxen	Caged	Rainbow trout	plasma	8–15 (mean)	ng ml^{-1}	Publication V
Naproxen	Caged	Rainbow trout	bile	40–1882 (mean)	ng ml^{-1}	Publication V
Naproxen	Wild	Roach, Bream	bile	21–38 (mean)	ng ml^{-1}	Brozinski et al. (2012b)
Ibuprofen	Caged	Rainbow trout	plasma	84 ± 62	ng ml^{-1}	Brown et al. (2007)

Ibuprofen	Caged	Rainbow trout	plasma	5.5–102 (mean)	ng ml^{-1}	Fick et al. (2010)
Ibuprofen	Caged	Rainbow trout	plasma	20 (mean)	ng ml^{-1}	Publication V
Ibuprofen	Caged	Rainbow trout	bile	71–451 (mean)	ng ml^{-1}	Publication V
Ibuprofen	Wild	Roach, Bream	bile	21–25 (mean)	ng ml^{-1}	Brozinski et al. (2012b)
Ketoprofen	Caged	Rainbow trout	plasma	15–107 (mean)	ng ml^{-1}	Fick et al. (2010)

[a]Gizzard shad (*Dorosoma cepedianum*), Brown bullhead (*Ameiurus nebulosus*), White suckers (*Catostomus commersoni*), Perch (*Perca fluvialitis*), Brook trout (*Salvelinus fontinalis*), Fathead minnow (*Pimeaphales promelas*), Rainbow trout (*Oncorhynchus mykiss*), Sunfish (*Lepomis sp*), Common roach (*Rutilus rutilus*), Common bream (*Abramis brama*)

[b]mean ± standard deviation if not stated otherwise, min–max = minimum and maximum values, pool = value of pooled sample consisting of several individuals, mean = mean or range of means

[c]Several species, Bluegill (*Lepomix macrochirus*), Channel catfish (*Ictalurus punctatus*), Black crappie (*Pomoxis nigromaculatus*)

[d]Species depending on location, Sonora sucker (*Catostomus insignis*), Largemouth bass (*Micropterus salmoides*), Common carp (*Cyprinus carpio*), Bowfin (*Amia calva*), White sucker, Smallmouth buffalo (*Ictiobus bubalus*)

II

PHARMACEUTICALS IN SETTLEABLE PARTICULATE MATERIAL IN URBAN AND NON-URBAN WATERS

by

Marja Lahti & Aimo Oikari 2011

Chemosphere 85: 826-831.

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Chemosphere 85 (2011) 826–831

Contents lists available at ScienceDirect

Chemosphere

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

Pharmaceuticals in settleable particulate material in urban and non-urban waters

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ARTICLE INFO

Article history:
Received 24 March 2011
Received in revised form 21 June 2011
Accepted 22 June 2011
Available online 19 July 2011

Keywords:
Pharmaceuticals
Settleable particulate material
Wastewater treatment plant
Sediment

ABSTRACT

Wastewater treatment plants (WWTP) are important sources of settleable particulate material (SPM), heading to sediments with natural suspended solids. To date, there is little information about the fate of pharmaceuticals in sediment systems. In this study, the objective was to determine if pharmaceuticals are detected in SPM at locations near WWTPs or even in rural areas, thus being susceptible for sedimentation.

SPM samples were collected from 10 sites in Finland, grouped as reference, rural and wastewater effluent sites. SPM collectors were placed about 35 cm above bottom for about 2 months during summer. After extraction, a set of 17 pharmaceuticals was analyzed.

Several pharmaceuticals were detected in SPM accumulated at sites next to WWTPs. The concentration of citalopram was notably high (300–1350 ng g^{-1} dw). Also bisoprolol and ciprofloxacin were detected at high concentrations (6–325 and 9–390 ng g^{-1} dw, respectively). In contrast, none of the pharmaceuticals were detected from reference sites and only two were found from a single rural site.

There is no previous information about the presence of pharmaceuticals in SPM. The results showed that pharmaceuticals are sorbed to particles in WWTP and nearby, eventually ending up in sediments. These results also indicate that pharmaceuticals are not markedly contaminating sediments of rural areas in Finland.

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1. Introduction

Pharmaceuticals are a diverse group of chemicals, continuously being released into the environment, mainly via municipal effluents. They are frequently detected in surface waters in a range from ng L^{-1} to μg L^{-1} (Calisto and Esteves, 2009; Kümmerer, 2009a,b), so concern has risen about their environmental effects. All pharmaceuticals are bioactive chemicals with specific mode-of-actions in humans. Instead of structural similarities, they are classified according to their uses. Due to this, compounds within the same class may have remarkably different structures and properties from each other (Kümmerer, 2009a) and hence different environmental fates.

According to equilibrium partition theory, hydrophobic chemicals distribute between pore water, lipids of the organism and organic carbon of the sediment, pore water concentration being the key determinant of the bioavailable fraction (Di Toro et al., 1991). Due to the polar and often ionic nature of pharmaceuticals, sorption to solid materials such as soil and sediment is not solely based on this hydrophobic partition. Rather, it is based on ionic interactions and is pH dependent (Tolls, 2001; Schwarzenbach et al., 2003). Thus sorption cannot be evaluated from the single value of log K_{ow}. To avoid underestimation of the sorption of polar ionic compounds, surface related adsorption, ion exchange, complexation and hydrogen bonding must also be considered (Tolls, 2001; Schwarzenbach et al., 2003). Normalization to organic carbon content does not necessarily decrease the variation in the sorption coefficients of veterinary pharmaceuticals (Tolls, 2001). At present, the sorption and desorption of pharmaceuticals have been understudied; despite the fact that sorption has a key role in distribution of pharmaceuticals between the phases and compartments in the environment, e.g. being sedimented or transported with particulate material. Sediments are often considered as a sink for xenobiotics, but compounds may be released from sediments during events of sediment disturbance such as in human activities or bioturbation (Eggleton and Thomas, 2004; Josefsson et al., 2010). In addition to contact with pore water, many benthic organisms are exposed to sediment-sorbed compounds *via* ingested particles (Sormunen et al., 2008). Sorption may hinder the microbial transformation of pharmaceuticals due to reduced bioavailability (Alexander, 2000). To date, there is only little information about the occurrence, distribution, and effects of pharmaceuticals in sediments.

In surface waters, particulates originating from a catchment area due to natural or anthropogenic reasons will eventually form sediment. This still floating material is defined here as settleable particulate material (SPM). Wastewater treatment plants (WWTP) are

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0045-6535/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.chemosphere.2011.06.084

important sources of SPM and suspended solids originating from them may contain remarkable amounts of xenobiotics (Byrns, 2001).

The objective of this study was to determine to what extent pharmaceuticals are detected from SPM next to WWTPs, hence being susceptible for sedimentation. Possible ubiquitous distribution of pharmaceuticals in rural areas of Finland with wastewater treatment in private households was also monitored. The field study was conducted in 10 locations. These sites were further divided into three groups based on the magnitude of effluent load: municipal WWTP, rural and reference sites. Target pharmaceuticals, altogether 17, were selected based on their consumption, and occurrence or effects in the environment. Pharmaceuticals were chosen from different classes so that wider range of properties and use were covered.

2. Materials and methods

2.1. Chemicals

Table S1 (Supplementary material) provides therapeutic classes, consumptions in Finland, and physicochemical properties of pharmaceuticals studied in this research. Analytical standards of acebutolol, atenolol, bezafibrate, carbamazepine, citalopram, diclofenac, fluoxetine, ibuprofen, ketoprofen, metoprolol, naproxen, ofloxacin, oxytetracycline, paracetamol, sotalol and internal standards of alprenolol, demeclocycline, 10,11-dihydrocarbamazepine and D5-fluoxetine were purchased from Sigma Aldrich Inc. (St. Louis, USA). Bisoprolol hemifumarate was obtained from Heuman Pharma GmbH (Nürnberg, Germany). Ciprofloxacin and internal standards enrofloxacin and D3-ibuprofen were purchased from Fluka (Seelze, Germany). All the standards were of purity ⩾98%. Stock solutions (1 mg mL^{-1}) of the standards were prepared in methanol, except antibiotics in 1:1 (v/v) methanol:0.01 M HCl, and stored at −20 °C. Ultrapure water was obtained by Ultra Clear UV plus (SG, Barsbüttel, Germany). The solvents methanol (Rathburn Chemicals Ltd., Walkerburn, Scotland, UK) and acetonitrile (Merck, Darmstadt, Germany) were HPLC grade.

2.2. Sampling and sample pretreatment of settleable particulate material (SPM)

Campaigns were conducted during two consecutive summers (2008 and 2009). SPM collectors made of stainless steel (area: 50 × 50 cm, volume 90 L, funnel-like shape with 5 × 5 cm grid, Fig. 1) were placed in the lake or river bottom for about 2 months. The upper edge of a collector was about 35 cm from the sediment surface.

SPM was collected from 10 sites (Fig. 1). Sites were divided into three groups. In sites next to WWTP (A–D), the distance from the effluent pipe was about three meters and water depth 0.5–7 m. At site A, collectors were placed at the proper distance with the help of a diver. Rural sites (E–G) were lakes with permanent settlements but without municipal WWTP, and reference sites (H–J) were lakes having no or little human influence (no permanent settlements). At reference and rural settlement sites, water depth ranged from two to six meters. Some characteristics of the surface waters and WWTPs therein are presented in Tables S2 and S3 (Supplementary material). Two collectors were situated at sites A–C in summer 2008. As the deviation between two collectors was low, only one collector per site was used in summer 2009.

After 2 months, collectors were slowly lifted above the water surface and transferred to the shore. Samples were pumped into brown glass bottles with the aid of well-cleaned manual bilge pump. The first sample (2.5 L) was taken without mixing of the

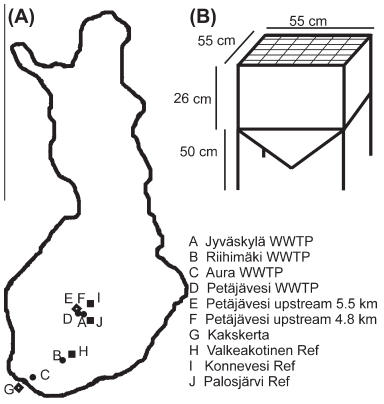

Fig. 1. (A) Sampling sites of settleable particulate material collected for 2 months in summers 2008 and 2009. ● next to wastewater treatment plant (WWTP) ■ reference site ♦ rural site (B) Scheme of the sample collector.

contents from the bottom of a collector. This was supposed to be the most densely settled and oldest sample (identified here as strong sample). After the strong sample, the steel-grid was brushed thoroughly and removed. The remaining sample was mixed well and four 2.5 L samples were taken (identified as mixed sample).

To prevent contamination of reference samples and thus false positives, assured qualities of the sampling and determinations were conducted throughout. Collectors were brushed with tap water and detergent (Deconex), soaked in water (5 d) and rinsed with water, ethanol and finally with water from the collection site. Separate devices (e.g. bilge pumps) were used for reference and WWTP sites during collection of the accumulated sample to avoid chemical contamination.

Samples were left to settle in the dark at 4 °C for 2 d, after which time the overlay water was decanted. The four mixed samples were pooled. Solid SPM samples were stored frozen at −20 °C until freeze drying.

The dry weight (dw, 105 °C/16 h) of the wet SPM was analyzed after decanting and the loss on ignition (LOI, 550 °C/2 h) after drying (SFS, 1990). LOI was calculated as percentage of mass lost during ignition and it describes the amount of organic material in the sample. Frozen samples were dried (30–60 h) in a freeze-dryer (Christ Alpha 2-4, Martin Christ, Osterode, Germany), each sampling location done separately. After drying, samples were homogenized by grounding in a mortar. The content of total organic carbon (TOC) was analyzed from the dried sample with Flash EA1112 elemental analyzer (Carlo Erba) connected to a Finnigan Delta^{Plus} Advantage continuous flow mass spectrometer (CF-IRMS) (ThermoFisher Scientific Corp., Waltham, USA). Inorganic carbon was removed from the sample by treating it with HCl vapor for 16 h.

The sedimentation rates of SPM and pharmaceuticals were calculated by dividing the amount of SPM in the collector with surface area (m^2) of the collector and collection time (in years).

2.3. Extraction and LC-MS/MS analysis of pharmaceuticals

Dried SPM sample was extracted with modified EPA method 1694 (EPA, 2007). Internal standards (D3-ibuprofen,

D5-fluoxetine, enrofloxacin, alprenolol, dihydrocarbamazepine and demeclocycline, 200 ng), phosphate buffer (pH 2, 15 mL) and acetonitrile (20 mL) were added to about 0.6 g of dry sample, mixed for 5 min, kept in ultrasonic bath for 30 min and centrifuged for 5 min (3000 rpm). The extraction cycle was repeated for total of three times. However, at the last cycle, only acetonitrile was used. Extract was evaporated by rotary evaporator to about 15 mL and 200 mL of water was added. EDTA (500 mg) was used to prevent chelation of tetracyclines. Before solid phase extraction, sample was filtered through prewashed filter (VWR glass microfiber 691). Cartridges (Oasis HLB, 6 cc, 200 mg) were conditioned with 8 mL methanol and 8 mL ultrapure water (pH 2 by HCl) and after sample loading washed with 10 mL water. APIs were eluted with 4 mL methanol. The well mixed sample was then divided into two, and both evaporated to dryness in a water bath (50 °C) with gaseous nitrogen. The first fraction was dissolved to 0.5 mL of 20% acetonitrile in 0.01 M ammonium acetate (ESI−) and the second one to 0.5 mL of 20% acetonitrile in 0.1% formic acid (ESI+).

Separation was performed with Waters Alliance 2795 (MA, USA) liquid chromatography (LC) consisting of tertiary pump, vacuum degasser, autosampler and column oven. A reversed phase C18 column (Waters XBridge 3.5 μm, 2.1 × 100 mm with 3.5 μm, 2.1 × 10 mm guard column) was used. The column temperature was set to 30 °C and that of autosampler to 20 °C. The injection volume was 30 μL.

In negative ionization mode (ESI−), the mobile phase consisted of 0.01 M ammonium acetate in 90% acetonitrile (A) and 0.01 M ammonium acetate (B) with a flow of 0.30 mL min^{-1}. The percentage of A was raised from 5% to 90% during 15.5 min, held in this percentage of A for 1.5 min (15.5–17 min) and lowered back to 5% of A during 1 min (17–18 min). At the end, the column was equilibrated for 8 min before the next injection (19–26 min).

In positive ionization mode (ESI+), acetonitrile (C) and 0.1% (v/v) formic acid (D) were used as eluents with a flow of 0.25 mL min^{-1}. The percentage of C was raised from 20% to 55% during 17 min and to 100% C during 3 min (17–20 min), held at this percentage of C for 1 min (21–22 min), and lowered back to 20% of C during 1 min (22–23 min). At the end, the column was equilibrated for 8 min before next injection (23–30 min).

A Quattro Micro triple-quadrupole mass spectrometer (MS/MS) (Waters, MA, USA) with electrospray interface was used as detector. Nitrogen was used as desolvation gas (ESI− 640 L h^{-1}, ESI+ 500 L h^{-1}) and as cone gas (50 L h^{-1}). Desolvation temperatures for ESI− and ESI+ were 250 °C and 200 °C and source temperatures 130 °C and 100 °C, respectively. Collision gas (argon) was used at collision cell pressure 2.8×10^{-4} mBar. Data acquisition was performed with multiple reaction monitoring (MRM) mode. Precursor and product ions, collision energies and cone voltages were optimized for each compound separately and are presented in Table S4 (Supplementary material).

Calibration of the compounds was done from 0.1 to 5000 ng mL^{-1}. Recovery of the extraction method was determined by spiking the surrogate standards into pure sediment, and extracting as described above. Recoveries varied from 50% to 105%. Limit of detection (LOD) and limit of quantification (LOQ) were set to signal to noise ratio 3 and 10, thus ranging from 0.1 to 0.5 ng g^{-1} and from 0.3 to 1.6 ng g^{-1}, respectively. Relative standard deviation of repeated standard injections was less than 6% for all the pharmaceuticals. Detailed method performance parameters are described in Table S5 (Supplementary material). To detect possible contamination of the samples during extraction, a blank sample was extracted at each batch. Pharmaceuticals were not detected from any of these extraction blanks.

3. Results

3.1. Characteristics of SPM

The annual deposition of SPM (kg dw m^{-2} y^{-1}), calculated from the amount of material in the collectors, was clearly higher in sites adjacent to WWTP (Table 1). However, it must be noted that sites B and C were rivers containing a high amount of suspended solids compared to the lake sites (Table S2 Supplementary material). There was also a 10-fold difference in site A (Jyväskylä WWTP) between sampling years. This was probably either due to the slightly different location of the collector or due to a difference in the suspended solids load from the WWTP. Based on monitoring information from WWTP, there was no deviation in operational measures in 2008 and 2009. Also rural sites had a higher amount of SPM compared to the reference sites with basically no permanent, long-lasting human influence. SPM in these rural sites originated mainly from agriculture and peat production in the catchment area or from a river outlet near the collection site.

In this study, the dry weight was measured after decanting the excess water from the top of well-settled material. Probably due to the lower amount of mineral matter, the solid material at limnic reference sites did not settle as well as at rural and WWTP sites. This was evident from the low dry weight after decanting (mainly <2%, Table 1).

The proportion of material from organic sources varied considerably between sites (Table 1). No correlation was found between TOC and LOI (r^2 = 0.365). Generally, TOC and LOI were higher at reference sites (>13% and >30%, respectively) than in rural or WWTP sites (<15% and <25%, respectively). However some exceptions were found as, for instance, in 2008, site B (Riihimäki WWTP in river) had high LOI amounts (35–40%) but low TOC (7.3–8.6%). The proportion of organic material at reference site H was remarkably high, TOC being over 80% and LOI over 35%. By visible evaluation, this sample was enriched by plant detritus. This site is a small headwater lake practically without any human influenced erosion (ICP IM, 2011). There was a small increase in the TOC and LOI at site D near WWTP compared to the sites E and F locating in the same lake upstream from the sewer outflow.

3.2. Concentrations of pharmaceuticals in SPM

Pharmaceuticals were not detected from any of the reference or rural sites, except ibuprofen and ofloxacin, which were detected from rural site G (Lake Kakskerta) (Table S6 Supplementary material). From eight to 13 pharmaceuticals out of the 17 monitored by LC–MS/MS were found from samples collected downstream from WWTPs (A–D) (Fig. 2). There was an apparent correlation between size of WWTP and the occurrence of pharmaceuticals. It was expected that the total load of pharmaceuticals depends on the population serviced as well as volume and distribution of the influent. The site adjacent to the largest WWTP (130 000 inhabitants; site A) had the highest frequency of detected pharmaceuticals with the highest concentrations. Concentrations and detection frequency decreased with decreasing WWTP size. However, the properties of the SPM may also have a large effect on the sorption of the compound.

The antidepressant citalopram was detected with highest concentrations (max 1350 ng g^{-1} dw) from each site. Also concentrations of ciprofloxacin and bisoprolol were generally over 200 ng g^{-1} dw. However, ciprofloxacin was not detected from site D (Petäjävesi WWTP), whereas oxytetracycline and naproxen were detected only from site A (Jyväskylä WWTP). Atenolol, bezafibrate, diclofenac, and sotalol were not detected from any of the samples. By comparing the sorption coefficients of the measured

Table 1
Properties of the settleable particulate material (SPM) collected for 2 months in summers 2008 and 2009. Sites A–D situated next to wastewater treatment discharge pipe, E–G in rural areas without municipal wastewater treatment, and H–J in reference areas without permanent settlement. For detailed location, please refer to map on Fig. 1. Strong sample was taken from the bottom of the collector, and mixed sample after stirring. LOI loss on ignition, TOC total organic carbon, dw dry weight, WWTP wastewater treatment plant, Ref reference area.

Site code	Location	Year	Sample	dw (%)	LOI (% dw)	TOC (% dw)	Deposition rate (kg dw m^{-2} y^{-1})
A	Jyväskylä WWTP	2008	Strong	13.5	20.2	13.0	5.0
			Mixed	15.4	19.7	13.8	
A	Jyväskylä WWTP	2009	Strong	15.3	22.9	10.2	53.2
			Mixed	7.1	35.4	14.6	
B	Riihimäki WWTP	2008	Strong	3.7	34.5	8.6	103
			Mixed	2.4	40.7	7.3	
C	Aura WWTP	2008	Strong	14.6	16.9	5.8	70.9
			Mixed	16.8	15.8	5.4	
D	Petäjävesi WWTP	2009	Strong	7.9	20.0	8.9	6.4
			Mixed	7.9	20.3	9.0	
E	Petäjävesi upstream 5.5 km	2009	Strong	4.1	17.3	7.5	4.0
			Mixed	5.7	18.8	8.0	
F	Petäjävesi upstream 4.8 km	2009	Strong	7.4	19.0	8.0	2.8
			Mixed	5.5	19.0	7.9	
G	Kakskerta	2009	Strong	8.6	15.3	5.7	3.3
			Mixed	7.5	16.3	6.2	
H	Valkea-Kotinen Ref.	2009	Strong	2.0	84.2	36.6	1.1
			Mixed	0.4	81.1	35.6	
I	Konnevesi Ref.	2008	Strong	3.2	36.5	–	0.3
			Mixed	0.3	40.3	–	
I	Konnevesi Ref.	2009	Strong	4.5	31.7	12.7	0.6
			Mixed	2.3	38.2	15.3	
J	Palosjärvi Ref.	2008	Strong	2.8	43.9	19.7	0.4
			Mixed	0.6	45.0	–	
J	Palosjärvi Ref.	2009	Strong	1.9	39.7	16.2	0.6
			Mixed	1.2	38.7	15.5	

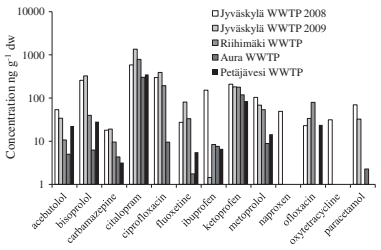

Fig. 2. Concentrations of pharmaceuticals in settleable particulate material (ng g^{-1} dw) collected from sites adjacent to wastewater treatment plants (WWTP) in Finland for 2 months in summers 2008 and 2009. Atenolol, bezafibrate, diclofenac, and sotalol are not presented, since they were not detected from any of the samples. Note logarithmic scale on *y*-axis.

pharmaceuticals, it can be generalized that those with higher sorption capacity were also detected at higher concentration from SPM (Fig. 2, Table S1 Supplementary material). The dry weight based concentrations in strong and mixed samples were rather equal and no general trend was observed (strong/mixed-ratio 1.02 ± 0.34 mean ± standard deviation, data not shown). Thus, both fractions seemed to be representative of the whole accumulated sample.

The detection profile in SPM did not follow the consumption pattern of pharmaceuticals in Finland (Fig. 2, Table S1 Supplementary material). In 2008, the most consumed pharmaceuticals were paracetamol, ibuprofen, naproxen, metoprolol and carbamazepine (122 000, 111 000, 6 400, 5 070 and 4 040 kg, respectively). However, relatively low concentrations of these compounds were detected from SPM (<200 ng g^{-1} dw). In contrast, although widely detected from SPM (generally >200 ng g^{-1} dw), only 660 kg of citalopram, 892 kg of ciprofloxacin and 564 kg of bisoprolol were consumed in 2008 in Finland.

The annual deposition rates of individual pharmaceuticals ranged considerably, from 17 to 79 400 μg m^{-2} y^{-1} (Table S7 Supplementary material). The largest load of sedimenting pharmaceuticals was found from site B (Riihimäki WWTP), although concentrations in SPM were higher in site A (Jyväskylä WWTP). This was due to the higher amount of SPM in site B than site A (Table 1).

4. Discussion

4.1. Pharmaceuticals in rural areas

The sources of pharmaceuticals within SPM in 10 water bodies in Finland were evaluated by grouping the sampling sites into three categories in relation to population density. Finland is a sparsely populated country with over 900 000 inhabitants (about 15% of the population) living in rural areas without centralized treatment of municipal wastewater. Based on the results presented here, pharmaceuticals today are not markedly contaminating the sediments in rural areas of Finland. Such areas are therefore similar to areas without permanent human habitation. The detection of pharmaceuticals in SPM samples collected from these areas would have indicated that these households are underestimated sources of pharmaceutical pollution that may need special attention.

4.2. Occurrence of pharmaceuticals adjacent to WWTPs

Compared to the previously measured surface water concentrations near sites B (WWTP Riihimäki) and C (WWTP Aura), the detection profiles in this study were rather different. While in surface waters near WWTPs, metoprolol and naproxen were detected with the highest concentrations of the 13 drugs monitored during 2003–2006 (data combined in Vieno, 2007), whereas in SPM, some metoprolol was detected but naproxen was not. Earlier, ciprofloxacin

was not detected in any of the surface water samples (Vieno, 2007) but, in SPM samples, the concentrations of ciprofloxacin were up to 392 ng g^{-1} dw. Anti-depressants citalopram and fluoxetine, and the betablocker bisoprolol have not been monitored in Finland's surface waters. In SPM, the concentrations of these compounds were up to 1 350, 81, and 325 ng g^{-1} dw, respectively.

There are large differences in the removal efficiencies in WWTPs between pharmaceuticals due to variation in biodegradability by a microbial community and sorption of the compounds in particles. Importantly, the removal in WWTPs has been mainly studied by comparing the disappearance of pharmaceuticals from the dissolved phase, without specifying the removal mechanism such as biotransformation or sorption (Miège et al., 2009). Some of the pharmaceuticals, like naproxen and ketoprofen, were found to be readily biodegradable but did not sorb to sludge, whereas hydrochlorthiazide (not monitored in this study) sorbed extremely efficiently (Jelic et al., 2011). For many pharmaceuticals, the removal efficiency and importance of sorption depends on the wastewater treatment plant as observed in the study of Jelic et al. (2011).

The sorption of pharmaceuticals in SPM and sediments depends on both the properties of the pharmaceuticals and SPM. In this study, citalopram was measured at highest concentrations in SPM. Supporting this finding, Kwon and Armbrust (2008) reported that citalopram and fluoxetine sorb very strongly to sediment and soil, although their log K_{ow}-values are rather low (citalopram 1.39, fluoxetine 1.22). These compounds are cationic in the environmentally relevant pH-range. In addition to hydrophobic interactions, the strong sorption includes several other mechanisms such as cation exchange, complexation and hydrogen bonding (Tolls, 2001; Schwarzenbach et al., 2003; Kwon and Armbrust, 2008). Although lipophilic sorption in organic carbon is not considered a main mechanism, the sample TOC seems to enhance the sorption of some pharmaceuticals (Varga et al., 2010; Xu and Li, 2010). Accordingly, Maoz and Chefetz (2010) concluded that the sorption of pharmaceuticals to (dissolved) organic material depends on the pH and hydrophilicity of organic material.

This study is the first one to report concentrations of pharmaceuticals in SPM, the material forming the sediment. Many pharmaceuticals were detected in several fold higher concentration from SPM than previously found in the receiving sediments (generally <100 ng g^{-1} dw) (Antonić and Heath, 2007; Stein et al., 2008; Ramil et al., 2010; Schultz et al., 2010; Varga et al., 2010). However, Rice and Mitra (2007) measured naproxen, ibuprofen and ketoprofen concentrations in sediment to vary from 50 to 10 000 ng g^{-1} dw. Ternes et al. (2005) studied the occurrence of several pharmaceuticals in sludge at WWTP and found only diclofenac, with concentration up to 450 ng g^{-1} dw. On the other hand, Jelic et al. (2011) also detected several other pharmaceuticals (<100 ng g^{-1} dw) from sludge. In all, it might be more predictable to compare the concentrations of pharmaceuticals in natural SPM to those measured from the sludge of WWTP, since these chemicals sorb in particles already at WWTP or nearby, thus entering the waterway in SPM.

As they were collected during the warm season, the SPM samples in this study were annual catches heading to surface sediments. Thus, biotransformation of the compounds has not proceeded as long time as it is apparent in surface sediments in nature. Furthermore, the bottom layers in the SPM collectors were probably more hypoxic, or even anaerobic, than the pooled subsample from the rest accumulated inside the collector. Anaerobic conditions usually tend to slow down the rate of biotransformation. For instance, biotransformation of selected pharmaceuticals and endocrine-disrupting chemicals were notably lower in anaerobic than aerobic conditions (Ying and Kookana, 2003; Jiang et al., 2010; Lahti and Oikari, in press; Lin and Gan, 2011). Hence, anaerobic conditions may enable sediment deposition of those pharmaceuticals that are readily biotransformed in aerobic conditions.

In the water bodies investigated here during warm season, the estimated annual deposition rates of pharmaceuticals are probably within their lower range. Regarding to the influx into waters, the consumption of pharmaceuticals by a given human population can be assumed to be relatively stable within a timeframe of 1 y. However, their transformation varies circannually, as besides thermal differences, there is less phototransformation in the boreal winter due to ice cover and darkness. In fact, Vieno et al. (2005) observed seasonal variation in the concentrations of pharmaceuticals in effluents and river waters on one of the current research areas, site C.

Due to unavailable knowledge on the long-term toxicity of pharmaceuticals to benthic invertebrates, risk assessment of sediment habitats contaminated by those chemicals is not possible. Some studies of effects of fluoxetine, carbamazepine and diclofenac on benthic invertebrates have been published (Brooks et al., 2003; Oetken et al., 2005; Nentwig, 2007; Sánchez-Argüello et al., 2009; Oviedo-Gómez et al., 2010). Based on the scant data available, the concentrations of fluoxetine and carbamazepine in SPM were in the range of NOEC and lethal concentrations in sediment (Brooks et al., 2003; Oetken et al., 2005; Nentwig, 2007; Sánchez-Argüello et al., 2009). So these compounds may pose long-term risks to the benthic biota and more studies are needed to assess their bioavailability from sediments.

5. Conclusions

This is the first study measuring pharmaceuticals in settleable particulate material (SPM). Wastewater treatment plants (WWTP) being sources of particle bound pharmaceuticals, thus supply the benthic environment with several compounds. The most widely detected pharmaceuticals were citalopram, ciprofloxacin and bisoprolol with concentrations exceeding 200 ng g^{-1} dw. Compared to the benthic habitats near WWTPs that presumably are contaminated by pharmaceuticals, rural and pristine reference areas were free of them in Finland. These results highlight the fact that measuring only the dissolved fraction of pharmaceuticals in the WWTP effluent may underestimate the loading and risks to the aquatic environment.

Acknowledgements

This study was supported by Grants from the Academy of Finland (No. 109823) and the Finnish Graduate School in Environmental Science and Technology. The authors wish to acknowledge the following individuals for their assistance in the field or with sample processing: Lasse Alanko, Jenny-Maria Brozinski, Antti Jylhä, Viljami Kinnunen, Silja Kujala, Siiri Latvala, Risto Retkin, Jesper Svanfelt and Erkki Vesterinen.

Appendix A. Supplementary materials

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2011.06.084.

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SUPPLEMENTARY MATERIAL

Pharmaceuticals in settleable particulate material in urban and non-urban waters

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Table S1. Selected physico-chemical properties of the studied pharmaceuticals.

Compound	Therapeutic class	MW	pKa	log K_{ow}	log K_{oc}	log Kd	Water solubility mg L^{-1}	Consumption in 2008 kg $year^{-1}$ [a]
Acebutolol [b,c]	Betablocker	336.43	9.2	1.71	2.35-2.47	0.5-1.0	259	663
Atenolol [b,c]	Betablocker	266.34	9.6	0.16	1.85-2.05	0.05-0.5	13 300	613
Bezafibrate [b,d]	Lipid regulator	361.83	3.6	4.25	1.41	-0.5	0.355	152
Bisoprolol [b,c]	Betablocker	325.45	9.5	1.87	2.17-2.3	0.3-0.8	2 240	564
Carbamazepine [b,e]	Anti-epileptic	236.28	13.9	2.45	2.00-3.42	0.2-2.3	17.7	4 040
Ciprofloxacin [b,f]	Antibiotic	331.35	5.9, 8.9	0.40	4.79	2.6	30 000	892
Citalopram [b,g]	Anti-depressant	324.39	9.6	1.39	5.32-6.02	3.9-4.6	31.1	661
Diclofenac [b,e]	Anti-inflammatory	296.16	4.2	4.51	2.45-3.74	4.7	2.37	1 080
Fluoxetine [b,g]	Anti-depressant	309.33	10.1	1.22	4.09-5.49	2.9-4.1	60.3	204
Ibuprofen [b,e]	Anti-inflammatory	206.29	4.9	3.97	2.14-2.21	1.7	21	111 000
Ketoprofen [b]	Anti-inflammatory	254.28	4.5	3.12			51	792
Metoprolol [b,c]	Betablocker	267.36	9.7	1.69	2.22-2.24	0.2-0.9	4 780	5 070
Naproxen [b]	Anti-inflammatory	230.27	4.2	3.18			15.9	6 400
Ofloxacin [b,f]	Antibiotic	361.38	6.0, 8.3	0.35	4.64	2.5	28 300	47
Oxytetracycline [b,f]	Antibiotic	460.44	3.3, 7.3, 9.1	-1.22	4.44-4.97	2.6-3.0	313	
Paracetamol [b]	Anti-inflammatory	151.17	9.4	0.46			14 000	122 000
Sotalol [b,c]	Betablocker	308.83	9.6	0.24	1.94-2.15	0.1-0.6	137 000	318

[a] Finnish Medicines Agency, 2009 [b] SRC PhyspProp Database, 2011 [c] Ramil, 2010 [d] Löffler et al., 2005 [e] Scheytt et al., 2005 [f] Tolls, 2001 [g] Kwon et al., 2008

Characteristics of waters and their catchments

Lake Päijänne is the second largest lake in Finland with area of 1 118 km^2 and total catchment area of 26 480 km^2. The sampling location (A), next to the Jyväskylä WWTP, was in the Poronselkä basin.

River Vantaa is a 101 km long river in Southern Finland. The catchment area is 1685 km^2 and it is most densely populated area in Finland. River water is turbid due to clay particles. Nutrients and suspended solids originate mainly from scattered loading. Collector was placed next to Riihimäki WWTP (B).

River Aura is a 70 km long river in the SW Finland. Water in the river is turbid due to clay particles and it is eutrophicated due to intense farming in the catchment area (874 km^2). Collector was placed next to small Aura WWTP (C).

Lake Jämsänvesi is a small lake in central Finland. The area of the northern part is about 3.8 km^2 and the southern part about 4.5 km^2. Water is strongly humic and the lake is classified as eutrophic. Wastewaters from Petäjävesi WWTP are discharged to southern part of the lake. Two collectors (E, F) were positioned to the northern part of the lake about 5 km upstream from the WWTP and one to the southern part of the lake next to the WWTP discharge pipe (D). Catchment area in the region of WWTP is about 460 km^2.

Lake Kakskerta (G) (area 1.6 km^2) is inside a small isle of Kakskerta. The island is located SW Finland and it belongs to Finnish Archipelago. It is classified as eutrophic and most of the nutrient load comes from agriculture in the catchment area (7.1 km^2). There is no municipal wastewater treatment in the area so effluent load originates from scattered private households treatment plants.

Lake Valkea-Kotinen (H) is a small (0.02 km^2) humic headwater lake in the Southern Finland belonging to International Cooperative Program on Integrated Monitoring on Air Pollution Effects on Ecosystems (ICP IM, www.environment.fi/syke/im). Lake is especially suitable as a reference area since there is no significant local pollution, such as settlement, agriculture or industry, in the catchment area (0.3 km^2).

Lake Konnevesi (I) is a clear oligotrophic lake in central Finland with area of 189 km^2. The collector was placed to the southern part of the lake. There is no loading industry in the water system.

Lake Palosjärvi (J) is a medium-size clear headwater lake in central Finland. Lake is rather pristine since there are only few inhabitants (mainly summer house settlement) and no industry in the catchment area.

Table S2. Characteristics of surface waters near the sampling locations. Data collected from annual monitoring reports. SS = suspended solids.

Code	Water system	pH	Turbidity FNU	Color Pt mg L^{-1}	SS mg L^{-1}	Conductivity mS m^{-1}	Total-P µg L^{-1}
A	Lake Päijänne Poronselkä basin	7.0	1.5	40-50		5.8	12
B	River Vantaa, City of Riihimäki	7.4	3.5-43	55-120[a]	25	31	117
C	River Aura, Town of Aura	7.0	26-64		15	13-26	130
D	Southern Lake Jämsänvesi	6.3		180-350		3.9	62
E & F	Northern Lake Jämsänvesi	6.4		170-240		3.3	28
G	Lake Kakskerta	7.2	4.2-14	20-80	3.5	12	30
H	Lake Valkea-Kotinen	5.3		100-160	11	3	18
I	Southern Lake Konnevesi	7.0	0.4	25-35	<1	4.6	7
J	Lake Palosjärvi	7.0	0.5	15-20		3.1	5

[a] Value upstream from the sampling location

Table S3. Characteristics of wastewater treatment plants on sampling locations A–D. Data collected from monitoring reports of respective units. SS = suspended solids, BOD = biological oxygen demand.

Code	Recipient water	Town	Population serviced	Influent flow m^3 d^{-1}	Effluent SS mg L^{-1}	BOD load kg d^{-1}
A	Lake Päijänne	Jyväskylä	130 000	44 700	11	481
B	River Vantaa	Riihimäki	27 000	15 300		130
C	River Aura	Aura	2390	868	22	15
D	Lake Jämsänvesi	Petäjävesi	1600	266	12	1

Table S4. Parameters used in the LC-MS/MS analysis of the monitored pharmaceuticals. ISTD = internal standard.

Compound	Ionization mode	ISTD	Retention time min	Precursor ion m/z	Product ion m/z	Cone V	Collision eV
Bezafibrate	ESI-	D3-IBF	8.0	360.1	274.0	21	17
Diclofenac	ESI-	D3-IBF	9.4	294.0	250.2	22	14
Ibuprofen	ESI-	D3-IBF	10.0	205.3	161.1	17	10
Ibuprofen-d3 (D3-IBF)	ESI-		10.0	208.0	164.0	17	8
Ketoprofen	ESI-	D3-IBF	7.7	253.1	209.3	12	8
Naproxen	ESI-	D3-IBF	7.7	229.0	170.2	11	17
Acebutolol	ESI+	APR	3.2	337.2	116.0	33	30
Alprenolol (APR)	ESI+		8.2	250.3	173.2	30	17
Atenolol	ESI+	APR	1.6	267.2	145.1	33	27
Bisoprolol	ESI+	APR	6.3	326.4	116.2	33	17
Carbamazepine	ESI+	HCBZ	8.9	237.2	194.0	28	19
Ciprofloxacin	ESI+	EFX	2.3	332.1	288.1, 314.0	35	20
Citalopram	ESI+	D5-FLX	9.6	325.1	109.1	33	35
Demeclocycline (DMC)	ESI+		3.8	465.0	448.0	29	20
Dihydrocarbamazepine (HCBZ)	ESI+		9.2	239.0	194.0	35	25
Enrofloxacin (EFX)	ESI+		2.7	359.9	316.0	30	23
Fluoxetine	ESI+	D5-FLX	13.7	310.2	148.1	22	9
Fluoxetine-d5 (D5-FLX)	ESI+		13.7	315.2	153.1	18	9
Metoprolol	ESI+	APR	3.7	268.4	116.1, 191.2	31	19
Ofloxacin	ESI+	EFX	2.2	362.1	318.0	31	21
Oxytetracycline	ESI+	DMC	2.3	461.1	426.2	25	20
Paracetamol	ESI+	APR	1.8	152.1	110.1	23	15
Sotalol	ESI+	APR	1.7	255.2	133.1	36	27

Table S5. Validation of the extraction method for the monitored pharmaceuticals. LOD = limit of detection, LOQ = limit of quantification.

Compound	Repeatability RSD %	Recovery %	LOD ng/g dw	LOQ ng/g dw
Bezafibrate	5.4	100	0.21	0.69
Diclofenac	5.9	95	0.14	0.47
Ibuprofen	3.0	102	0.14	0.47
Ketoprofen	4.5	105	0.17	0.56
Naproxen	3.7	104	0.18	0.60
Acebutolol	1.7	73	0.28	0.92
Atenolol	5.9	90	0.47	1.58
Bisoprolol	2.4	75	0.19	0.64
Carbamazepine	2.3	91	0.10	0.32
Ciprofloxacin	1.3	52	0.47	1.58
Citalopram	1.8	76	0.10	0.34
Fluoxetine	5.3	67	0.27	0.89
Metoprolol	1.4	72	0.45	1.49
Ofloxacin	2.3	50	0.14	0.47
Oxytetracycline	3.5	78	0.48	1.60
Paracetamol	4.1	93	0.33	1.10
Sotalol	3.4	82	0.22	0.74

Table S6. Concentrations of pharmaceuticals (ng g^{-1} dry weight) in settleable particulate material, dw = dry weight, nd = not detected.

Concentration ng g^{-1} dw	A Jyväskylä WWTP 2008	A Jyväskylä WWTP 2009	B Riihimäki WWTP	C Aura WWTP	D Petäjävesi WWTP	E Petäjävesi upstream 5.5 km	F Petäjävesi upstream 4.8 km	G Kakskerta	H Valkea-Kotinen Ref	I Konnevesi Ref	J Palosjärvi Ref
Acebutolol	53.8	34.1	10.7	5.0	22.5	nd	nd	nd	nd	nd	nd
Atenolol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Bezafibrate	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Bisoprolol	257	325	39.7	6.2	28.4	nd	nd	nd	nd	nd	nd
Carbamazepine	18.0	19.1	9.6	4.3	3.2	nd	nd	nd	nd	nd	nd
Citalopram	587	1350	787	302	353	nd	nd	nd	nd	nd	nd
Ciprofloxacin	299	392	194	9.5	nd	nd	nd	nd	nd	nd	nd
Diclofenac	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Fluoxetine	27.3	81.1	33.3	1.8	5.6	nd	nd	nd	nd	nd	nd
Ibuprofen	152	1.4	8.4	7.6	6.7	nd	nd	5.0	nd	nd	nd
Ketoprofen	209	181	178	119	85.3	nd	nd	nd	nd	nd	nd
Metoprolol	104	68.3	53.8	8.9	14.5	nd	nd	nd	nd	nd	nd
Naproxen	49.2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Ofloxacin	23.0	33.7	79.2	nd	23.9	nd	nd	13.4	nd	nd	nd
Oxytetracycline	31.4	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Paracetamol	69.6	32.5	nd	2.3	nd	nd	nd	nd	nd	nd	nd
Sotalol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Table S7. Sedimenting deposition rates of pharmaceuticals (µg m^{-2} y^{-1}) and solid material (kg dw m^{-2} y^{-1}), nc = not calculated.

Pharmaceutical deposition rate µg m^{-2} y^{-1}	A Jyväskylä WWTP 2008	A Jyväskylä WWTP 2009	B Riihimäki WWTP	C Aura WWTP	D Petäjävesi WWTP	E Petäjävesi upstream 5.5 km	F Petäjävesi upstream 4.8 km	G Kakskerta	H Valkea-Kotinen Ref	I Konnevesi Ref	J Palosjärvi Ref
Acebutolol	342	1820	1280	409	144	nc	nc	nc	nc	nc	nc
Atenolol	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc
Bezafibrate	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc
Bisoprolol	1630	17300	4600	444	182	nc	nc	nc	nc	nc	nc
arbamazepine	115	1020	1000	295	20.6	nc	nc	nc	nc	nc	nc
Citalopram	3730	71900	79400	21600	2270	nc	nc	nc	nc	nc	nc
Ciprofloxacin	1900	20900	19500	659	nc	nc	nc	nc	nc	nc	nc
Diclofenac	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc
Fluoxetine	174	4310	3190	95.3	35.9	nc	nc	nc	nc	nc	nc
Ibuprofen	966	76.7	837	523	42.8	nc	nc	16.6	nc	nc	nc
Ketoprofen	1330	9650	20800	8780	548	nc	nc	nc	nc	nc	nc
Metoprolol	659	3640	7080	612	93.4	nc	nc	nc	nc	nc	nc
Naproxen	313	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc
Ofloxacin	146	1790	9480	nc	153	nc	nc	44.4	nc	nc	nc
Oxytetracycline	199	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc
Paracetamol	443	1730	nc	239	nc	nc	nc	nc	nc	nc	nc
Sotalol	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc
Total sediment deposition rate kg dw m^{-2} y^{-1}	5.0	53.2	103	70.9	6.4	4.0	2.8	3.3	1.1	0.3-0.6	0.4-0.6

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IV

UPTAKE FROM WATER, BIOTRANSFORMATION AND BILIARY EXCRETION OF PHARMACEUTICALS BY RAINBOW TROUT

by

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2011

Environmental Toxicology and Chemistry 30: 1403-1411.

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Environmental Toxicology and Chemistry, Vol. 30, No. 6, pp. 1403–1411, 2011
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Printed in the USA
DOI: 10.1002/etc.501

UPTAKE FROM WATER, BIOTRANSFORMATION, AND BILIARY EXCRETION OF PHARMACEUTICALS BY RAINBOW TROUT

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(*Submitted* 3 *September* 2010; *Returned for Revision* 8 *December* 2010; *Accepted* 18 *January* 2011)

Abstract—An urgent need exists to assess the exposure of fish to pharmaceuticals. The aim of the present study was to assess the uptake and metabolism of waterborne pharmaceuticals in rainbow trout (*Oncorhynchus mykiss*). A further objective was to determine the possibility of monitoring exposure to low levels of pharmaceuticals by bile assays. Rainbow trout were exposed for 10 d under flow-through conditions to mixtures of five pharmaceuticals (diclofenac, naproxen, ibuprofen, bisoprolol, and carbamazepine) at high and low concentrations. The low concentration was used to mimic the conditions prevailing in the vicinity of the discharge points of wastewater treatment plants. The uptake and the bioconcentration were determined by blood plasma and bile analyses. The average bioconcentration factor in plasma ranged from below 0.1 for bisoprolol to 4.9 for diclofenac, the values being approximately similar at low and high ambient concentrations. The biotransformation of diclofenac, naproxen, and ibuprofen was considered efficient, because several metabolites could be detected in concentrations clearly exceeding those of the unmetabolized compounds. The glucuronides were the dominant metabolites for all three pharmaceuticals. The total bioconcentration in the bile was two to four orders of magnitude higher than in the plasma. The results of this work show that the exposure of fish to pharmaceuticals in environmentally relevant concentrations may be monitored by blood plasma and bile analyses, the latter allowing detection at markedly lower ambient concentration. Environ. Toxicol. Chem. 2011;30:1403–1411. © 2011 SETAC

Keywords—Pharmaceuticals Rainbow trout Metabolism Bioconcentration Bile

INTRODUCTION

The consumption of pharmaceuticals has increased substantially over the past few decades. In Finland, for example, the use of ibuprofen (IBF) has doubled within 10 years (National Agency for Medicines, 2010; Finnish drug consumption statistics: www.nam.fi). Pharmaceuticals used in human communities are eliminated into wastewaters intact or as metabolites. However, because of incomplete removal in wastewater treatment plants, the pharmaceuticals are found in recipient waters [1].

For environmental assessment of any chemical, it is necessary to have direct evidence of whether animals living in recipient waters are actually exposed to the chemical. Presently, perhaps the most urgent need in research of pharmaceuticals in the environment is the ability to assess exposure of local biota to these compounds. Assessment of uptake and the body burden of chemicals readily metabolized is as important as assessment of persistent ones. Thus far, research on pharmaceuticals in the aquatic environment has focused largely on their occurrence in water. This is due mainly to the fact that technical knowledge is lacking regarding the ways in which animals biotransform and excrete pharmaceuticals. Some studies on the bioaccumulation of pharmaceutical in tissues, mainly in the blood plasma, have been conducted [2–9]. Because of the continuous discharge of pharmaceuticals from wastewater treatment plants to surface waters, the compounds can be characterized as pseudopersistent, and, consequently, the aquatic organisms are chronically exposed.

The bioconcentration of nonpolar chemicals is due to the lipophilic partition process into the lipids of the animal [10], whereas the bioconcentration of ionic compounds depends on the water pH and dissociation constant of a chemical. Ionization usually decreases the uptake, bioconcentration, and toxicity of acidic and basic chemicals because of the loss of pure lipophilic characteristics [11–13].

Pharmaceuticals are a heterogeneous group of compounds [1], obeying no simple generalizations for their toxicokinetic properties related to their environmental fate. Despite being regularly ionic and water-soluble, they tend to sorb to environmental matrices [1], potentially leading to delayed uptake by aquatic animals. When fish are exposed to waterborne xenobiotics, they absorb them via their gills and skin [10,14].

The biotransformation reactions occur mainly in the liver, although other organs may also be important in certain reactions [15]. After biotransformation in the liver, the metabolites formed are excreted to the small intestine via bile. However, enterohepatic cycling may prolong the half-life of xenobiotics, because the compound or its metabolites may be reabsorbed in the intestine. Before excretion, xenobiotics either are directly conjugated (phase II) or are conjugated after phase I functionalization. Both phases (I and II) of metabolism are found in fish [14–16]. In humans, the main metabolites of pharmaceuticals have been determined during drug development [16]. The differences in biotransformation routes between humans and fish may lead to qualitative and quantitative differences in metabolite occurrence.

Xenobiotics and their metabolites can be secreted into fish bile, resulting in concentrations of several orders of magnitude higher than those found in the surrounding water [17]. Larsson

All Supplemental Data may be found in the online version of this article.
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Published online 19 February 2011 in Wiley Online Library (wileyonlinelibrary.com).

et al. [2] were the first to report pharmaceuticals (ethinylestradiol) in the bile of field-exposed fish. Resin acids, for example, are present at low concentrations in effluent discharges from the pulp and paper industry. Oikari [18] measured 100,000-fold total accumulation of resin acids, including metabolites, in fish bile. Hence, exposure to resin acids at less than 1 μg/L has been assessed with the aid of bile metabolites in feral and laboratory-exposed fish [18,19].

The aim of the present study was to determine the uptake and metabolism in rainbow trout (*Oncorhynchus mykiss*) of the anti-inflammatory drugs diclofenac (DCF), naproxen (NPX), and IBF; the β-blocker bisoprolol (BSP); and the antiepileptic carbamazepine (CBZ). Exposure to these pharmaceuticals was evaluated by measuring the parent compounds in blood plasma and their metabolites secreted into the bile.

MATERIALS AND METHODS

Chemicals

Hexane, acetone, methanol (Rathburn Chemicals), and acetonitrile (Merck) were all of high-performance liquid chromatography (HPLC) grade. Ultrapure water was obtained by Ultra Clear™ ultraviolet plus (SG). Analytical standards of DCF (purity > 99%), NPX (98%), IBF (> 98%), CBZ (> 99%), 10,11-dihydro-CBZ (99%), and alprenolol-HCl (> 99%) as well as ammonium hydroxide (≥ 25%) and ammonium acetate (99.99%) were purchased from Sigma Aldrich. Bisoprolol hemifumarate (> 99%) was obtained from Heuman Pharma. 4′-Hydroxy-DCF (98%), 5-hydroxy-DCF (98%), 1-β-*O*-acyl glucuronide of DCF (98%), 1-β-*O*-acyl glucuronide of NPX (98%), carboxy-IBF (98%), 2-hydroxy-IBF (98%), 1-hydroxy-IBF (98%), and 1-β-*O*-acyl glucuronide of IBF (98%) were purchased from Toronto Research Chemicals. Internal standard IBF-d3 (≥ 98%, 99 atom% D) was manufactured by Fluka and formic acid as well as internal standard fenoprop (99%) by Riedel-de Haën®. Phosphoric acid was purchased from Merck. 6-*O*-desmethylnaproxen (DNPX) was synthesized according to Brozinski et al. [20].

Fish maintenance and exposure setting

One-year-old juvenile rainbow trout (*Oncorhynchus mykiss*, weight 169 ± standard deviation [SD] 33 g and total length 24.3 ± SD 1.0 cm) were purchased from a local hatchery (Savon Taimen) and acclimatized to the laboratory conditions for 11 d before the experiments. During laboratory acclimatization, fish were kept in a steel tank (volume 2,160 L) with a water flow of approximately 1,000 ml/min, with photoperiod 16:8 h light: dark. The quality of unchlorinated artesian well water was monitored daily following the transfer of the fish. Water temperature was 13.6°C ± 0.4°C (SD) and pH 7.7 ± 0.2 (SD). Dissolved oxygen concentration remained above 9 mg/L before and during the experimental period. Fish were fed every other day (0.5% of fish biomass, Vital Plus 3.5 mm) ad libitum, but no food was given during the 3 d before the start of the experiments. Feces and uneaten food were removed from the aquarium daily. Maintenance and experiments were performed in accordance with the valid laws on animal testing and were licensed (ESLH-2007-06053/Ym-23) by the Finnish authority.

Fish were exposed to pharmaceuticals with three exposure regimes: control, low, and high. Concentrations in the low exposure were close to those found in effluents (nominally 6 nM, 1–2 μg/L) and in the high approximately 25 times those of the low exposure (nominally 150 nM, 25–50 μg/L). Two ambient concentrations at relatively wide range were used to study the possible concentration dependence of bioconcentration. This information is crucial when the exposure in the environment is evaluated. The mixture was prepared in an adjacent aquarium and pumped into the experimental aquarium (water volume 490 L) with an average flow of 152 ± SD 14 ml/min. The mixture was prepared fresh daily, and it was shielded from light. Four randomly chosen rainbow trout were transferred into each aquarium, yielding an average biomass load of 0.4 L/g/d (1.4 g fish/L). Fish were not fed during the 10-d exposure at 13.6°C to balance the volume of bile. Aquaria waters were collected every day during the experiment to confirm nominal exposure concentrations. The studied pharmaceuticals are presented in Table 1 and the measured exposure concentrations in Table 2.

At the end of exposure, fish were netted individually and immobilized with a blow to the head. Blood samples were taken by heart puncture using a prewashed and heparinized syringe and needle (2 ml and 22 G, respectively) and centrifuged immediately (3 min, 9,000 *g*, 10°C). Separated plasma was transferred into a new tube. After blood sampling, the fish were killed with a nurchal break. Bile was taken with a 25-G needle and 1-ml syringe (both prewashed with methanol). All the samples were snap frozen with liquid nitrogen and transferred to a −80°C freezer.

Extraction of water, blood plasma, and bile samples

After the addition of internal standards (fenoprop, alprenolol, and dihydro-CBZ), water samples (30 ml) were extracted according to Kallio et al. [21]. The centrifuged plasma sample (300 μl) was diluted to 1,000 μl with 50 μl of internal standard solution (containing fenoprop, alprenolol, and dihydro-CBZ 53, 56, and 49 ng, respectively), 630 μl ultrapure water, and 20 μl phosphoric acid (85%). Solid-phase cartridges (Oasis® HLB, 1 cc, 30 mg; Waters) were conditioned with 1 ml methanol and 1 ml water. After sample addition, the cartridges were washed with 1 ml 5% methanol in water, and the pharmaceuticals were eluted with 2 × 0.5 ml methanol.

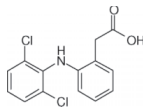
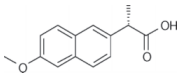
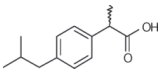
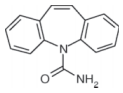
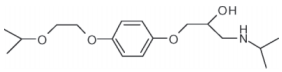
Water and plasma extracts were divided in two, both dried with a gentle stream of nitrogen. The first fraction, analyzed by liquid chromatography–mass spectrometry (LC-MS) with negative ionization mode, was reconstituted to 0.01 M ammonium acetate in 30% acetonitrile. The second fraction was reconstituted to 0.1% formic acid:acetonitrile (8:2 v/v) and analyzed by LC-MS in positive ionization mode. Water and plasma samples were dissolved into volumes of 1,000 μl and 200 μl, respectively.

The workup procedure for bile samples has been reported elsewhere [21]. Briefly, after internal standard addition (IBF-d3) and extraction, samples were reconstituted to 200 μl of 0.01 M ammonium acetate in 5% acetonitrile. The metabolism of DCF, NPX, and IBF is presented in Figure 1 and the structures of the metabolites in Figure 2 and Supplemental Data, Figure S1.

LC-MS/MS method

The chromatographic separation of the analytes was performed with a Waters Alliance® 2795 HPLC system consisting of tertiary pump, vacuum degasser, autosampler (set to 20°C), and column oven (set to 30°C). A reversed-phase C18 column (Waters XBridge™, 3.5 μm, 2.1 × 100 mm with 3.5 μm, 2.1 × 10 mm guard column) was used with a flow rate of 0.25 ml/min (water and plasma samples) or 0.30 ml/min (bile samples). Injection volume was 10 μl for water and plasma samples and 30 μl for bile samples.

Table 1. Structures and physicochemical properties of the pharmaceuticals studied

Compound	Molecular weight	Structure	pK_a	Log P[a]	Log D[a]	Water solubility (mg/L)
Diclofenac (DCF)	296.2		4.2	4.51	1.28	2.4
Naproxen (NPX)	230.3		4.2	3.18	0.85	15.9
Ibuprofen (IBF)	206.3		4.9	3.97	1.16	21.0
Carbamazepine (CBZ)	236.3		13.9	2.45	1.89	17.7
Bisoprolol (BSP)	325.5		9.5	1.87	0.03	2,240

[a] Log P octanol–water partition coefficient of neutral form, Log D octanol–water partition coefficient of neutral and ionized form at pH 7 (calculated with ACD/Labs V10.02, Advanced Chemistry Development).

In negative ionization mode (ESI−), the mobile phase consisted of 0.01 M ammonium acetate and 0.01 M ammonium acetate in 90% acetonitrile [21]. Plasma samples were analyzed with the same method as for water. In positive ionization mode (ESI+), 0.1% formic acid (A) and acetonitrile (B) were used as eluents. The percentage of B was raised from 20 to 55% during 17 min, held there for 1 min (minute 17–18), and lowered back to 20% of B during 1 min (minute 18–19). The column was equilibrated for 6 min before the next injection.

A Quattro Micro™ triple-quadrupole mass spectrometer (Waters) with electrospray interface was used as detector. Nitrogen was used as desolvation gas (ESI− 640 L/h, ESI+ 500 L/h) and as cone gas (50 L/h). Desolvation temperatures for ESI– and ESI+ were 325°C and 150°C and the source temperatures 130°C and 100°C, respectively. Collision gas (argon) was used at collision cell pressure 4.7×10^{-3} mBar. Data were acquired with multiple reaction monitoring (MRM) mode. Precursor and product ions, collision energies, and cone voltages were optimized for those compounds for which surrogate standards were available and are presented in Supplemental Data Tables S1 and S2. Cone voltages and collision energies of DCF, NPX, and IBF were used for the respective bile metabolites for which surrogate standards were not available.

Calibration of the compounds in water, plasma, and bile matrix was made from 0.1 to 5,000 μg/L, from 0.1 to 5,000 ng/ml, and from 10 to 5,000 ng/ml, respectively. Available surrogate standards were spiked into ultrapure water or diluted blood plasma or bile and extracted as described above. Relative recoveries in water and plasma ranged from 83 to 104% and from 92 to 105%, respectively. Relative standard deviation (RSD) of repeated standard injections was less than 5% for all the pharmaceuticals. The limit of quantification (LOQ) ranged from 0.01 to 0.15 μg/L, from 0.86 to 20 ng/ml, and from 20 to 100 ng/ml for water, plasma, and bile samples, respectively. Low sample volumes increased the LOQs of water and bile samples. The LOQ was determined only for those bile metabolites for which surrogate standard was available. Recoveries and repeatabilities were not determined for bile metabolites.

The bioconcentration of a drug into the blood plasma (BCF_{plasma}) was calculated as the ratio of concentrations (free parent compound) in blood plasma and water. The total

Table 2. Mean (±standard deviation) concentrations in water (at pH 7.7, $n = 11$ determinations) and plasma ($n = 4$ fish) with bioconcentration factors (BCF_{plasma}) in juvenile rainbow trout exposed for 10 d at low and high levels of pharmaceuticals as a mixture[a]

	Water (μg/L)		Plasma (ng/ml)		BCF_{plasma}	
	Low	High	Low	High	Low	High
Carbamazepine	1.6 ± 0.2	43 ± 5	0.62 ± 0.37	25 ± 2	0.40 ± 0.24	0.30 ± 0.03
Bisoprolol	1.9 ± 0.2	49 ± 7	< LOD	1.0 ± 0.4	< 0.01[b]	0.02 ± 0.01
Diclofenac	1.8 ± 0.2	43 ± 3	10 ± 7	210 ± 120	5.7 ± 3.8	4.9 ± 2.8
Naproxen	1.6 ± 0.1	40 ± 3	2.7 ± 1.1	55 ± 39	1.6 ± 0.7	1.4 ± 1.0
Ibuprofen	1.0 ± 0.1	25 ± 2	4.2 ± 0.8	82 ± 71	4.3 ± 0.8	3.3 ± 2.9

[a] BCF_{plasma} was calculated as ratio of plasma concentration to that in water. LOD = limit of detection.
[b] Maximum estimated BCF. Calculated by setting plasma concentration to the LOD.

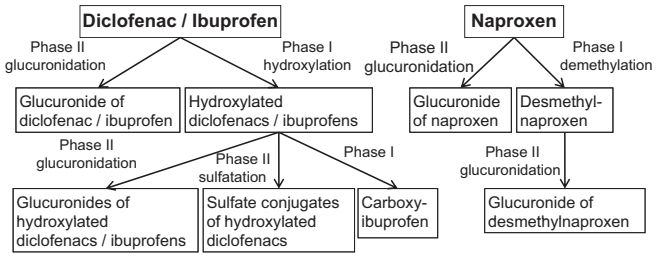


Fig. 1. The conceptual pathways for diclofenac, naproxen, and ibuprofen metabolism in rainbow trout [20–22].

bioconcentration of a pharmaceutical and its metabolites into the bile ($BCF_{total\ bile}$) was calculated as the ratio of total concentration (free parent compound + metabolites) in bile and concentration (free parent compound) in water.

Statistical analysis

Statistical analyses were made with SPSS 15.0 software. The significance of difference was set to $p < 0.05$. Normal distribution and equality of variances were tested with Shapiro–Wilk and Levene's tests, respectively. The comparisons between low and high exposures were made with the *t* test.

RESULTS

During the 10-d flow-through experiment, water samples were withdrawn daily for determination of the concentration of the pharmaceuticals. The results of the analysis showed almost no day-to-day variations in the concentrations (RSD 5.2–16.0%; Table 2). In the control exposures, CBZ was detected in mean concentrations of 0.13 μg/L (± SD 0.02). The source of the contamination originated from the extraction, but could not be traced. The other studied pharmaceuticals were not detected from the control exposure.

Bioconcentration in plasma

No statistical ($p > 0.05$) differences in bioconcentration (BCF_{plasma}) were noted between low and high exposures of the pharmaceuticals studied. Bisoprolol was not detected in the low-exposure experiments, so the maximum BCF_{plasma} of the compound was calculated from the limit of detection and was found to be 0.01. Because some CBZ was detected in plasma of control fish, the background contamination was taken into account (subtracted) when the BCF of the compound was calculated. Overall, bioconcentration in blood plasma was highest for DCF (4.9–7.7) and lowest for BSP (< 0.01–0.02, Table 2). The variances in uptake between individuals were high (RSD up to 86%).

Metabolism and bioconcentration in bile

Several metabolites of DCF, NPX, and IBF were detected at both low and high exposures (Table 3, Fig. 2, and Supplemental Data, Fig. S2). None of the metabolites was detected in the control fish. The identity of the metabolites was based on the previous studies of the research group [20–22]. The variation in the metabolite abundances was high between individuals (RSD 37–150%, $n = 4$ fish). Because of a large variation and several

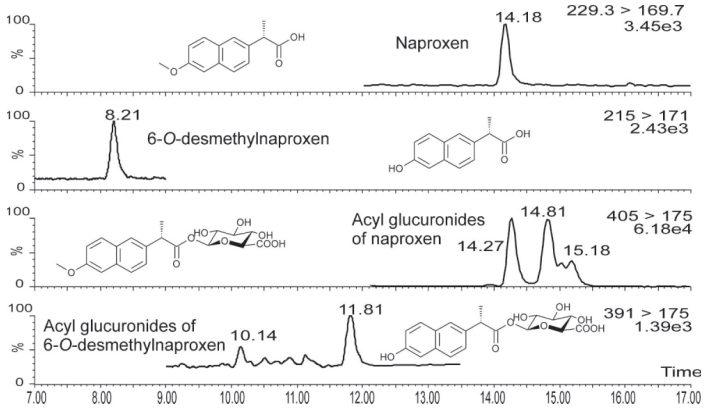


Fig. 2. Liquid chromatography–mass spectrometry chromatogram (multiple reaction monitoring) of naproxen metabolites in a bile sample from rainbow trout exposed to a mixture of pharmaceuticals for 10 d in aquaria with 40 μg/L of naproxen. Acyl migration caused several glucuronide peaks and peak broadening. However, only the structures of 1-β-*O*-acyl glucuronide isomers are shown (for further details see Kallio et al. [21]).

Table 3. Bile metabolites of diclofenac, naproxen, and ibuprofen (minimum and maximum, nM) of juvenile rainbow trout exposed for 10 d in mixtures of five pharmaceuticals (see Table 2)[a]

	Low exposure		High exposure	
	Min nM	Max nM	Min nM	Max nM
DCF	< LOD	552	< LOD	5,810
4′-OH-DCF	75	344	2,000	27,400
5-OH-DCF	< LOD	208	135	4,650
Acyl glucuronide of DCF	< LOD	671	1,560	17,200
Sulfate conjugate of 4′-OH-DCF[b]	< LOD	< LOD	47	691
Sulfate conjugate of 5-OH-DCF[b]	67	213	1900	12,000
Acyl glucuronide of 4′-OH-DCF[b]	845	1,810	19,300	70,300
Acyl glucuronide of 5-OH-DCF[b]	276	1,160	14,500	74,000
Acyl glucuronide of 3′-OH-DCF[b]	< LOD	362	1,290	24,800
Ether glucuronide of 4′-OH-DCF[b]	< LOD	124	590	2,000
Total nM (mean ± SD)	2,840 ± 1,750		116,000 ± 82,600	
Water nM (mean ± SD)	5.97 ± 0.70		145 ± 11	
$BCF_{total\ bile}$ (mean ± SD)	476 ± 294		797 ± 569	
NPX	< LOD	1,130	6,060	31,300
DNPX	< LOD	129	676	12,700
Acyl glucuronide of NPX	1,600	8,070	63,300	181,000
Acyl glucuronide of DNPX[b]	151	863	492	3,080
Total nM (mean ± SD)	4,870 ± 3,250		144,000 ± 61,700	
Water nM (mean ± SD)	6.93 ± 0.36		174 ± 11	
$BCF_{total\ bile}$ (mean ± SD)	703 ± 468		829 ± 355	
IBF	< LOD	667	1,650	9,790
Carboxy-IBF	< LOD	105	237	2,330
2-OH-IBF	283	1,090	7,120	36,100
OH-IBF[b]	24	144	216	3,750
Acyl glucuronide of IBF	5,920	26,300	10,900	53,200
Acyl glucuronides of OH-IBFs[b]	50,200	250,000	350,000	176,0000
Total nM (mean ± SD)	148,000 ± 93,500		974,000 ± 620,000	
Water nM (mean ± SD)	4.77 ± 0.39		119 ± 9	
$BCF_{total\ bile}$ (mean ± SD)	31,000 ± 19,600		8,170 ± 5,200	

[a] Mean ± standard deviation (SD) total bile ($n = 4$ fish) and water concentrations (nM; $n = 11$) and bioconcentration factors ($BCF_{total\ bile}$). $BCF_{total\ bile}$ was calculated as ratio of bile (free parent compound + metabolites) concentration to that in water (free parent compound). LOD = limit of detection (estimated nM), DCF = diclofenac, OH-DCF = hydroxylated diclofenac, NPX = naproxen, DNPX = 6-*O*-desmethylnaproxen, IBF = ibuprofen, OH-IBF = hydroxylated ibuprofen.

[b] No surrogate standard was available.

nondetectable metabolites, concentrations of individual metabolites are presented as minimum and maximum values (Table 3). No surrogate standards were available for acyl and ether glucuronides of hydroxy-DCFs (OH-DCF), sulfate conjugates of OH-DCFs, acyl glucuronide of DNPX, or acyl glucuronides of OH-IBFs, so concentrations of these metabolites must be considered as semiquantitative. Abbreviations of the metabolites can be found in Table 3 and the structures in Figure 2 and Supplemental Data, Figure S1.

Two phase I and seven phase II metabolites of DCF were detected from the bile of every trout in the high-exposure group (Table 3). At low exposure, all metabolites but sulfate conjugate of 4′-OH-DCF were detected in the bile of at least one trout. However, many of the metabolites were not detected in some trout, so variation existed between individuals. The most abundant metabolites were the acyl glucuronides of 4′-OH-DCF (30–62% of the total) and of 5-OH-DCF (16–32%). The acyl glucuronide of 4′-OH-DCF was more dominant with low exposure than with high. Only a small portion (0–11%) of unmetabolized DCF was found in the bile. No statistically significant difference in $BCF_{total\ bile}$ was observed between low- and high-exposure groups ($p > 0.05$; Fig. 3).

6-*O*-Desmethylnaproxen (phase I) and acyl glucuronides of NPX and DNPX were tentatively observed. The metabolites were found in the bile of trout from the high-exposure group (Fig. 2), but, in the low-exposure group, only some of them were detected (Table 3). Acyl glucuronide of NPX was the most dominant metabolite in low- and high-exposure groups, accounting for 63 to 90% of the total NPX metabolites. Acyl glucuronide of DNPX predominated more in the low- than in the high-exposure group. However, unmetabolized NPX was slightly more abundant in high- than in low-exposure groups (14 and 11%, respectively). As can be seen in Figure 3, similarly to DCF, $BCF_{total\ bile}$ was equal ($p > 0.05$) in trout exposed to low and high concentrations of NPX.

IBF was biotransformed into phase I metabolites (OH-IBFs and carboxy-IBF) and several acyl glucuronides representing phase II metabolites. Unmetabolized IBF and carboxy-IBF were not detected in the bile of some of the trout at low exposure, but could however be observed in trout subjected

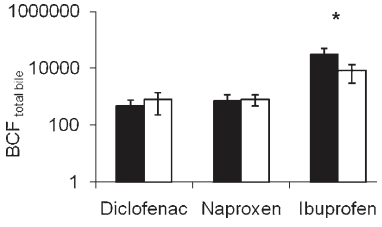


Fig. 3. Total bioconcentration of pharmaceuticals in the bile of rainbow trout exposed for 10 d at low (solid bars) and high (open bars) concentrations in water. Bioconcentration factor ($BCF_{total\ bile}$) was calculated as ratio between bile (free parent compound + metabolites) and water concentrations (free parent compound). Means with standard deviations of four individuals are depicted. Asterisk denotes statistically significant difference between low and high exposures ($p < 0.05$).

to high concentration of pharmaceuticals (IBF max 2%; Table 3). Acyl glucuronides of OH-IBF were the most dominant metabolites, forming from 83 to 96% of the total IBF metabolites. The acyl glucuronide of IBF was more abundant in the bile of trout in low- versus high-exposure groups. Variation in the relative proportions of the IBF metabolites was quite small, but the average $BCF_{total\ bile}$ was four times higher at low than at high exposures ($p < 0.05$; Fig. 3).

The concentrations of NPX and DCF in bile were two orders of magnitude and of IBF three to four orders of magnitude higher than in plasma. High variation between individuals in both bile and plasma concentrations gave rise to high variation also in bile-plasma ratio (RSD 77–106%). In general, the bile concentrations seemed to be higher in individuals with lower plasma concentration and vice versa (negative correlation, $r^2 = 0.37$–0.94), perhaps reflecting differences in the liver uptake or bile secretion capacity between individuals. However, these correlations were not statistically significant because of the limited number of animals ($p > 0.05$, $n = 4$).

DISCUSSION

Steady state of pharmaceuticals in fish

Our goal was to assess the degree of exposure of fish from long-term contact with pharmaceuticals in water using plasma and bile analyses. Because of this, the duration of the experiment in relation to buildup of the body burden should be assessed. This can be approximated from the elimination half-life; five half-lives considered enough for dynamically stable levels in tissues and organs [23]. We suggest that 10-d exposure in stable water concentration would be sufficient to reach steady-state concentrations in rainbow trout; i.e., the exposure was several times longer than the half-life of elimination. Unfortunately, we have no previous experimental knowledge regarding the pharmaceuticals studied in the present work. However, comparison with other chemicals, such as antibiotics and industrial pollutants, which are readily metabolized in fish, can be made. After repeated oral exposure of trout to sulfadimethoxine, oxolic acid, and oxytetracycline, the half-life in blood ranged from 36 to 134 h [24]. Hoeger et al. [25] determined that the body half-life of DCF in brown trout was 36 h after a single intraperitoneal injection. Resin acids and 4,5,6-trichloroguaiacol reached steady-state concentrations in fish bile within 24 and 144 h, respectively [19,26]. According to the available literature on warm-blooded mice and rats, the plasma half-lives of the five drugs studied in the present work ranged from 1 to 5 h. In humans, the half-life of elimination ranged from 2 to 20 h after a single oral administration, IBF being eliminated fastest and CBZ slowest [27]. In a toxicokinetic sense, the 10-d exposure of rainbow trout to pharmaceuticals at 13.6°C approached steady state of uptake in relation to elimination.

Uptake from water to blood

The uptake process of a foreign chemical in fish gills includes transfer onto the gill membrane surface, diffusion through epithelial cells, and binding to carrier molecules. In addition to these, possible pH variations at the gill structures are important for ionized compounds because pH affects the proportions of neutral and ionized forms. Also, the flux of neutral molecules across the epithelia sustains continuous dissociation from ionized to neutral forms and thus enhances uptake [28].

All the pharmaceuticals studied occurred mainly in ionized form in the pH range of water (pH 7.7, > 98.5% ionized) and in the blood of rainbow trout. The uptake rate of ionized compounds is lower than that of neutral compounds [11–13], although ionized molecules may also be absorbed by ion carriers [13]. In fish, as in humans, a mixed variety of anionic, cationic, and neutral transporters affects the active or passive uptake and elimination of xenobiotics [29]. Elimination of ionic compounds is usually rather fast compared with neutral ones [28,30].

Present results indicate that the exposure concentration at a relatively wide range did not affect to a significant degree the average bioconcentration of pharmaceuticals in the blood. Previous studies have shown that BCF_{plasma} for DCF in wastewater range from 2.5 to 29, whereas in the present study the factor was five to six [4,9]. However, relative bioconcentration of unmetabolized DCF into liver, gills, kidney, and muscle has been shown to increase with decreasing concentration of DCF [31]. With regard to the bioconcentration of NPX, previously reported BCF_{plasma} values in fish exposed to wastewater were somewhat higher than observed in the present study [4,9]. Although we found that IBF is only marginally bioconcentrated in blood, high variation in the bioconcentration factor in plasma has been reported, and, depending on site-specific factors, the bioconcentration may vary significantly [4,9].

No bioaccumulation in blood plasma was observed for BSP; the compound is most likely readily eliminated because of its high water solubility. Uptake from water, bioconcentration, and elimination has not been studied in fish, but in mammals the compound is known to be effectively absorbed from the diet and readily eliminated [32]. Carbamazepine has been detected in fish muscle and liver in a monitoring campaign near wastewater treatment plants [8], whereas in other studies CBZ was not detected [7,33]. Fick et al. [9] reported only slight bioconcentration of CBZ in plasma.

In comparison with neutral chemicals in the environment such as polyaromatic hydrocarbons (PAHs), bioconcentration in blood plasma ($BCF_{plasma} < 6$) was low for all studied pharmaceuticals, which is supported by the low octanol–water partitioning coefficients in pH 7 (log*D* 0.03–1.89) and the high water solubility of the pharmaceuticals studied (Table 1). Several-fold higher bioconcentrations in blood have been reported, e.g., for PAHs (BCF_{plasma} 43–76) [34]. The bioconcentration factors of the acidic pharmaceuticals studied (DCF, NPX, and IBF) were slightly higher than those of BSP and CBZ, which relates to the octanol–water partitioning of the neutral form (log*P*). Recently, it was shown that bioconcentration of some pharmaceuticals in the adipose fin were higher than in muscle, which was related to the higher lipid content of that organ. However, the most hydrophilic and ionic pharmaceuticals were not detected in either tissue [35].

Concentrations of bile metabolites

Concentrations of metabolites were quantified according to our previous work, which focused on the structural elucidation of the metabolites [20–22]. However, in the present study, kinetic and environmental dependences are emphasized. To compare the bioconcentration in bile, all the quantified metabolites were summed on a molar basis. These BCF ratios should be considered as semiquantitative, because the surrogate standards were not available for all the compounds. Furthermore, some unidentified metabolites may have been present as well.

With regard to the overall bioconcentration of DCF, a BCF_{bile} from 509 to 657 was recently reported [36], which coincides with the present results ($BCF_{total\ bile}$ 291–1,610). The glucuronic conjugates were the most abundant metabolites

(total 63–85%) in rainbow trout. Although detoxification is generally suggested to be due to glucuronidation, acyl glucuronides of DCF are potentially protein-reactive metabolites [37]. In humans, the excretion of DCF glucuronides into bile is via active adenosine triphosphate-dependent pump, which allows several-fold higher concentrations in bile than in blood [37]. With regard to NPX, the same metabolites have been identified in human urine [38] as from the rainbow trout bile in the present study. Acyl glucuronic conjugates of NPX were the most abundant in rainbow trout, as in humans [38]. Hydroxyibuprofen metabolites were identified from zebrafish fry [39] that are also the main metabolites in humans [40]. However, in rainbow trout, these metabolites accounted for only a trace amount of the total IBF (~1%), glucuronic conjugates being dominant (95–99%). Also, similar types of metabolites (hydroxides as well as glucuronide and sulfate conjugates) have been identified from the bile of rainbow trout exposed to, e.g., PAHs and resin acids [41,42].

Bile as monitoring tool for ambient concentrations

The cycle of bile formation and release to the intestine is dependent on nutritional status [43,44]. In the present study, fish were not fed during the experiment, in order to stabilize the volume of bile. Although bile tends to be stored in the gall bladder during fasting, this organ is periodically emptied post-feeding to enhance digestion in the intestine. To a certain extent, the longer the fasting time, the more abundant and darker the bile inside the gall bladder [43]. It is also evident that, during long fasting (several days), water is reabsorbed from the bile, reducing its volume in the gall bladder and concentrating the xenobiotics and their metabolites secreted from liver [43–45].

Previously, fish bile has been successfully used for the monitoring of chemicals in the aquatic environment [18,19,44]. It has been especially suitable for compounds that are readily metabolized and excreted via the bile instead of accumulating in muscle or other organs [17,44]. In addition, compounds that are metabolized in liver are secreted to the bile, allowing comparative analysis of biotransformation [41].

After release of the bile into the intestine, the metabolites and parent compounds that it contains will be eliminated mainly via feces. However, reabsorption in the intestine (enterohepatic cycling) may prevent the first-pass elimination and prolong the body half-life, as observed for DCF in brown trout [25]. The DCF concentration in blood of brown trout (*Salmo trutta f. fario*) decreased rapidly within a few hours after a single intraperitoneal injection, whereas, in bile, the concentration of DCF reached the maximum after 6 h and again after 36 h. Approximately half of the DCF was eliminated by 36 h [25].

In the present study, the bioconcentration of NPX in bile was approximately equal to that of DCF ($BCF_{total\ bile}$ 317–1,380 and 291–1,610, respectively), whereas higher amounts of IBF metabolites were bioconcentrated in bile ($BCF_{total\ bile}$ 3,520–57,900). Efficient metabolism and secretion of IBF into the bile is in accordance with findings of Ramirez et al. [8], who could not detect IBF from muscle or liver of fish caught near wastewater treatment plants. Thus, tissue residues are not able to trace exposure to low levels of pharmaceuticals. It is possible that DCF affected the metabolism of other pharmaceuticals, as suggested from up- or down-regulation of metabolizing enzymes [16], such as induction of cytochrome P4501A in rainbow trout liver [36].

Large differences in plasma and bile concentrations were found between individuals. The plasma and the bile concentrations had an inverse correlation, indicating some individual differences in the efficiency of hepatic uptake or excretion to bile. The capacity of active transporters may be limited, because secretion of ionic compounds is partially mediated by them [29]. The significantly larger $BCF_{total\ bile}$ of IBF in the low-exposure group also indicates that, with high exposure, the excretion capacity might have been saturated.

Although glucuronide conjugates of carboxylic acid containing pharmaceuticals would be the most convenient metabolites for monitoring purposes because of the highest concentrations in bile, their separation into several geometric isomers and the lack of hydroxylated glucuronide standards hinders the reliability of their quantification. However, the MRM transition ($[M\text{-}H\text{-anhydroglucuronic acid}]^-$) used for their quantification should not depend greatly on the structure of the parent compound. Only low amounts of unconjugated DCF, NPX, and IBF were detected from the bile (0–26%). However, it was impossible to distinguish between the originally unconjugated compound and those being deconjugated as a result of some hydrolyzing enzymes in the bile. Deconjugating enzymes and hydrolyzing alkaline solutions have been successfully used to determine $BCF_{total\ bile}$ in previous environmental studies with resin acids [19,41] and estrogens [46]. The gradual deconjugation of formed metabolites into phase I metabolites could be an alternative approach for the monitoring of pharmaceuticals. In gradual deconjugation, different conjugates are broken down in turn by using, e.g., β-glucuronidase, sulfatase, and finally alkaline solutions [41]. However, some information about differences in metabolite profiles would be lost. Also, acyl migration products (other isomers than β-acyl form) will not be deconjugated by β-glucuronidase, because the enzyme is conformation specific.

The IBF was most effectively traced from the bile among the three compounds (IBF, DCF, and NPX). Thus, this widely used pharmaceutical could be used as a chemomarker of drug exposure. In the present work, because of the lack of identification data in trout, metabolites of CBZ and BSP were not monitored in the bile. However, we expect that even at the low exposure it would have been possible to detect metabolites of these compounds in the bile.

The present results are significant, because bile analyses were proved to be an efficient tool for monitoring the exposure of fish to the pharmaceuticals studied. This approach most probably allows evaluation of exposure to even lower concentrations of pharmaceuticals than used in the present study.

CONCLUSIONS

The results indicate that exposure of rainbow trout to pharmaceuticals at concentrations close to those found in the environment can be measured from the blood plasma and, in particular, as metabolites in the bile. Metabolism of DCF, NPX, and IBF was efficient, and both phase I and phase II metabolites were detected from the bile. With the exception of IBF, exposure concentration did not have an effect on bioconcentration into plasma or bile. Bioconcentration in plasma was low, but total concentration of the parent pharmaceutical and its metabolites was two to four orders of magnitude higher in bile than in plasma. IBF bioconcentrated the most and could be used as a chemomarker of wide exposure of fishes to drugs. The present results imply that bile metabolites can be a useful tool for monitoring fish exposure to low concentrations of drugs in surface waters, even when concentrations in other tissues or body fluids are below the detection limit.

SUPPLEMENTAL DATA

Table S1. Retention times, precursor and product ions, and mass parameters of the pharmaceuticals analyzed from water and blood plasma samples.

Table S2. Retention times, precursor and product ions, and mass parameters of the metabolites and their surrogate standards analyzed from the bile samples.

Fig. S1. Structures of diclofenac, ibuprofen, and their metabolites detected from rainbow trout bile.

Fig. S2. Chromatogram of diclofenac and ibuprofen metabolites in bile sample from rainbow trout exposed to a mixture of pharmaceuticals for 10 d.

Acknowledgement—The study was supported by grants from the Academy of Finland (109823) and the Finnish Graduate School of Environmental Science and Technology.

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SUPPLEMETAL DATA

Uptake from water, biotransformation, and biliary excretion of pharmaceuticals by rainbow trout

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Table S1. Retention times, precursor and product ions and mass parameters of the pharmaceuticals analyzed from water and blood plasma samples. IS = internal standard

Compound	Ion mode	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (eV)
Diclofenac	ESI-	6.1	294	250	22	14
Naproxen	ESI-	2.7	229	170	11	17
Ibuprofen	ESI-	6.8	205	161	17	10
Fenoprop (IS)	ESI-	3.3	267	195	12	14
Bisoprolol	ESI+	5.9	326	116	33	17
Alprenolol (IS)	ESI+	7.7	250	173	30	17
Carbamazepine	ESI+	8.9	237	194	28	19
Dihydrocarbamazepine (IS)	ESI+	9.1	239	194	35	25

Table S2. Retention times, precursor and product ions, and mass parameters of the metabolites and their surrogate standards analyzed from the bile samples [1-3]. Negative ionization mode (ESI-) was used for all the compounds. DCF = diclofenac, OH-DCF = hydroxylated diclofenac, NPX = naproxen, DNPX = 6-*O*-desmethylnaproxen, IBF = ibuprofen, OH-IBF = hydroxylated ibuprofen, IS = internal standard.

Compound	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (eV)
DCF	17.4	294	250	22	14
4'-OH-DCF	15.1	310	266, 230	22	14
5-OH-DCF	15.5	310	266, 230	22	14
Acyl glucuronide of DCF	15.4, 15.6	470	175	22	10
Acyl glucuronides of OH-DCFs [a]	6.4, 8.1, 13.1-15.5	486	175, 193	22	10
Ether glucuronide of 4'-OH-DCF [a]	9.7	486	175, 193, 442	22	10
Sulfate conjugates of OH-DCFs [a]	11.7, 12.7	390	310, 266	22	14
NPX	14.2	229	170	11	17
DNPX	8.2	215	171	25	15
Acyl glucuronide of NPX	14.1-15.3	405	175, 229	11	10
Acyl glucuronide of DNPX [a]	10.1-11.8	391	175	11	17
IBF	18.6	205	161	17	11
OH-IBFs	8.5-10.0	221	177, 159	17	11
Carboxy-IBF	3.7	235	191	17	8
Acyl glucuronide of IBF	15.2, 15.5, 18.1	381	175	17	11
Acyl glucuronides of OH-IBFs [a]	8.2-14.2	397	175, 193	17	11
D3-ibuprofen (IS)	16.9	208	164	17	8

[a] no surrogate standard was available, cone voltages and collision energies were not optimized

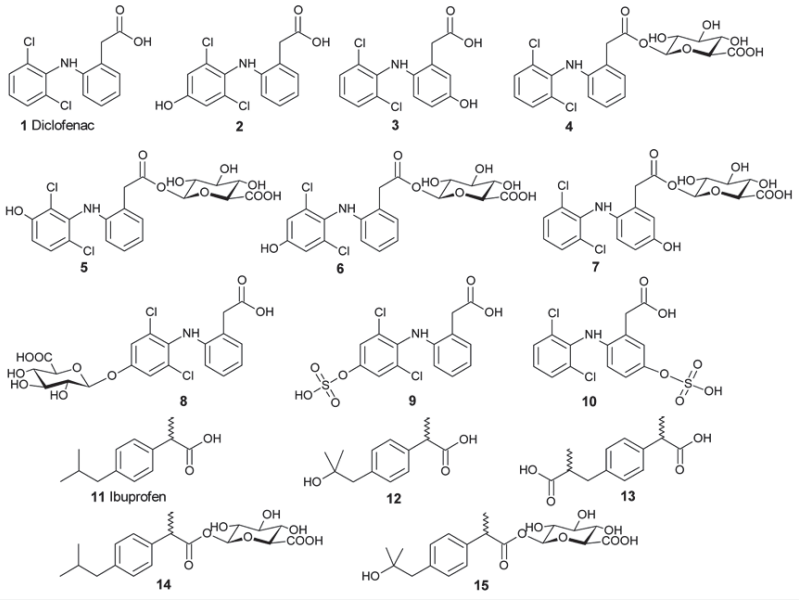

Fig. S1. Structures of diclofenac, ibuprofen, and their metabolites detected from the rainbow trout bile. Diclofenac (1), 4'-hydroxydiclofenac (2), 5-hydroxydiclofenac (3), acyl glucuronide of diclofenac (4), acyl glucuronide of 3'-hydroxydiclofenac (5), acyl glucuronide of 4'-hydroxydiclofenac (6), acyl glucuronide of 5-hydroxydiclofenac (7), ether glucuronide of 4'-hydroxydiclofenac (8), sulfate conjugate of 4'-hydroxydiclofenac (9), sulfate conjugate of 5-hydroxydiclofenac (10), ibuprofen (11), 2-hydroxyibuprofen (12), carboxyibuprofen (13), acyl glucuronide of ibuprofen (14) and acyl glucuronides of hydroxyibuprofen (15). Only the structures of 1-β-O-acyl glucuronide isomers are presented in the figure.

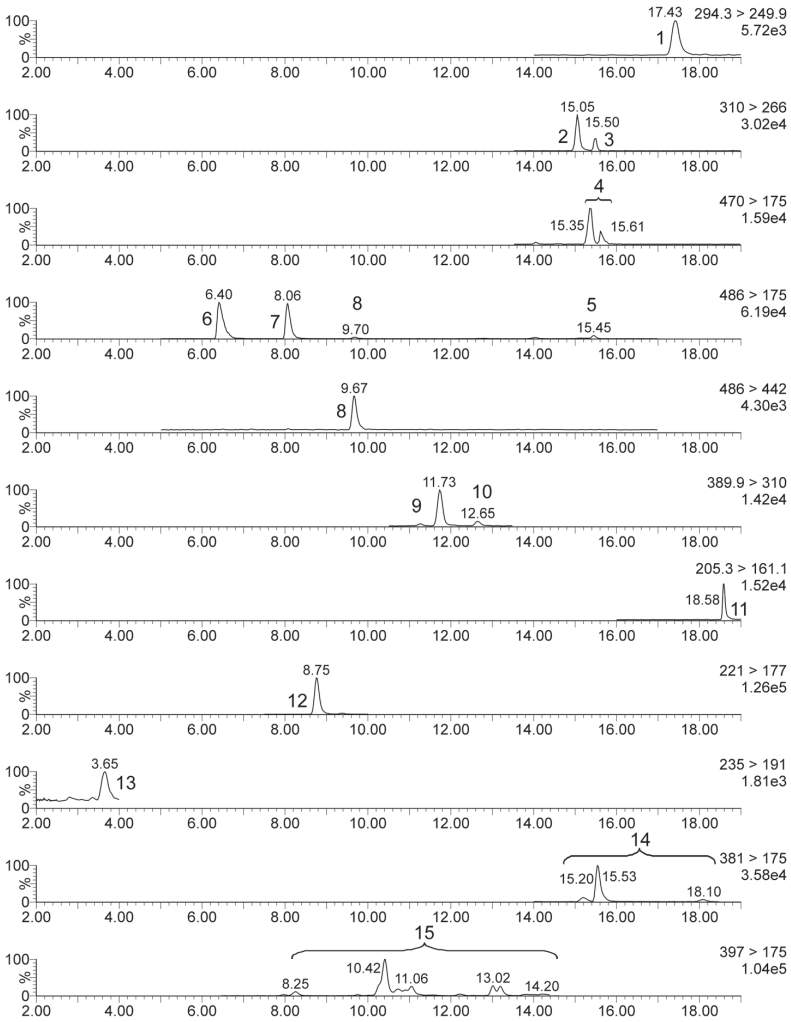

Fig. S2. LC-MS/MS chromatogram (MRM) of diclofenac and ibuprofen metabolites in bile sample from rainbow trout exposed to mixture of pharmaceuticals for ten days. The numbers next to peak corresponds to the structures in Fig. S1.

[1] Kallio J-M, Lahti M, Oikari A, Kronberg L. 2010. Metabolites of the environmental pollutant diclofenac in fish bile. *Environ Sci Technol* 44 :7213-7219.

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