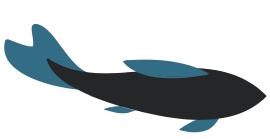
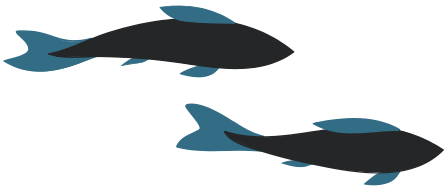
Jenny-Maria Brozinski

Identification and application of bile metabolites to assess the exposure of fish to pharmaceuticals in the environment



Åbo Akademi University



Jenny-Maria Brozinski (b. Kallio)

Master of Science, University of Turku 2007

Identification and application of bile metabolites to assess the exposure of fish to pharmaceuticals in the environment

Jenny-Maria Brozinski

Laboratory of Organic Chemistry

Department of Natural Sciences

Division of Natural Sciences and Technology

Åbo, Finland, 2013

SUPERVISORS Professor Leif Kronberg
Laboratory of Organic Chemistry
Åbo Akademi University
Åbo, Finland

Professor Aimo Oikari
Department of Biological and Environmental Sciences
University of Jyväskylä
University of Jyväskylä, Finland

REVIEWERS Assistant professor Jerker Fick
Department of Chemistry
Umeå University
Umeå, Sweden

Professor Juha-Pekka Salminen
Department of Chemistry
University of Turku
Turku/Åbo, Finland

OPPONENT Professor Margareta Törnqvist
Department of Material and Environmental Chemistry
Stockholm University
Stockholm, Sweden

Cover: Anna Stenman

ISBN 978-952-12-2875-9
Painosalama Oy – Turku, Finland 2013

Äidille ja isälle

ABSTRACT

Thousands of tons of pharmaceuticals are consumed yearly worldwide. Due to the continuous and increasing consumption and their incomplete elimination in wastewater treatment plants (WWTP), pharmaceuticals and their metabolites can be detected in receiving waters, although at low concentrations (ng to low µg L^{-1}). As bioactive molecules the presence of pharmaceuticals in the aquatic environment must be considered potentially hazardous for the aquatic organisms.

In this thesis, the biotransformation and excretion of pharmaceuticals in fish was studied. The main biotransformation pathways of three anti-inflammatory drugs, diclofenac, naproxen and ibuprofen, in rainbow trout were glucuronidation and taurine conjugation of the parent compounds and their phase I metabolites. The same metabolites were present in fish bile in aquatic exposures as in fish dosed with intraperitoneal injection. Higher bioconcentration factor in bile (BCF_{bile}) was found for ibuprofen when compared to diclofenac and naproxen.

Laboratory exposure studies were followed by a study of uptake of pharmaceuticals in a wild fish population living in lake contaminated with WWTP effluents. Of the analyzed 17 pharmaceuticals and six phase I metabolites, only diclofenac, naproxen and ibuprofen was present in bream and roach bile. It was shown, that diclofenac, naproxen and ibuprofen excreted by the liver can be found in rainbow trout and in two native fish species living in the receiving waters. In the bream and roach bile, the concentrations of diclofenac, naproxen and ibuprofen were roughly 1000 times higher than those found in the lake water, while in the laboratory exposures, the bioconcentration of the compounds and their metabolites in rainbow trout bile were at the same level as in wild fish or an order of magnitude higher. Thus, the parent compounds and their metabolites in fish bile can be used as a reliable biomarker to monitor the exposure of fish to environmental pharmaceuticals present in water receiving discharges from WWTPs.

SAMMANFATTNING

Läkemedel används i stora mängder runtom i hela världen. Kontinuerlig och växande användning av läkemedel kombinerad med deras ofullständig eliminering i reningsverk för avloppsvatten leder till att läkemedel och deras metaboliter påträffas i vattenmiljön. Läkemedel är bioaktiva molekyler och därmed bör deras förekomst i vattenmiljö betraktas som en potentiell risk för vattenorganismer.

I den här doktorsavhandlingen undersöktes upptag, bio-omvandling och utsöndring av läkemedel i fiskar i akvarieförhållanden och i vattendrag. I akvarieförsöken visades via undersökningar av fiskgalla att bio-omvandlingen av de anti-inflammatoriska läkemedelen diklofenak, naproxen och ibuprofen resulterade huvudsakligen i bildning av glukuronid- och taurinkonjugat. Både ursprungsförteningarna och deras fas I metaboliter bildade dessa konjugat. I fiskgalla var biokoncentrationsgrade hos ibuprofen större än hos diklofenak och naproxen.

Braxen och mört infångades från ett vattendrag som var kontaminerad med avloppsvatten. I fiskarnas gallvätska kunde diklofenak, naproxen och ibuprofen och deras metaboliter identifieras. Koncentrationerna av läkemedlen i gallan var ungefär 1000 gånger högre än de som påvisades i vattendraget. Detta visar att fiskgalla kan användas för att påvisa i vilken mån vilda fiskar är utsatta läkemedelsrester som härrör från vattenreningsverk.

PREFACE AND ACKNOWLEDGEMENTS

This work was carried out at the Laboratory of Organic Chemistry, Åbo Akademi University between 2008 and 2012. Financial support was received from Åbo Akademi University, Maj and Tor Nessling Foundation and the Finnish Doctoral Programme in Environmental Science and Technology (EnSTe).

I wish to thank my supervisors, Professor Leif Kronberg and Professor Aimo Oikari for providing me the opportunity to do research in a highly topical field of research. In addition to scientific work, I have had the opportunity to act as the secretary of the Finnish Society for Environmental Science (FSES) for two years. During my time as the secretary, I have acquired many new and valuable skills, such as realizing science management. Thank you Leif! Also, a big thanks to Aimo for prompt replies to my emails and all the encouraging comments. Your knowledge in fish physiology has been invaluable!

My roommates and LK group mates also deserve a big thank you. Jesper Svanfelt, Johan Eriksson and Axel Meierjohann, I couldn't have done this without your help! Jesper, thank you for helping me getting started my work. Axel, it has been a pleasure working with you in different projects. I hope to keep up the good work of importing illicit drugs (legally) to Finland!

Also, I am extremely thankful for Marja Lahti from University of Jyväskylä, for working side-by-side with me, providing me the fish samples and sharing the love and hate towards the fish bile analysis. Lastly, I want to acknowledge Tarini Sahoo (University of Jyväskylä) and Aarno Karels (South Karelian Fisheries Centre) for fish sampling, and Marja Kauppi, Jyrki Vuorinen and Jukka Höytämö (Centre for Economic Development, Transport and the Environment for Southeastern Finland) for their most valuable help with water sampling and with information about the characteristics of River Rakkolanjoki and Lake Haapajärvi.

My United Organic Tiger colleagues, thank you for good company in laboratory, coffee room, parties and trips to Sweden. Especially I want to thank Tiina Saloranta and Otto Långvik for your friendship. Päivi Pennanen is acknowledged for her big heart and ongoing support. I want to thank Peter Holmlund, Esa Nummelin, Roger Nordqvist, Janne Lönnqvist and Olli Martiskainen for maintaining the LC-MS instruments, helping me with sampling and other

important (or not so important) tasks. I also want to thank Jussi Meriluoto, Jaakko Mannio and Professor Bjarne Holmbom for successfully organizing FCES'11 and NECC2012 together with me and Leif.

I want to thank my friends for reminding me of life outside academia, and Volvo Garage for keeping the Brozinskis on the road.

Last but not least, I want to thank my mom and dad for giving me the opportunity to maintain the path I chose in high school and encouraging me when needed. My dear husband Ari, I could not have succeeded without you. You are my rock where I and our beautiful daughter Hildegard can always lean on. I love you.

Åbo, March 2013

Jenny-Maria Brozinski (b. Kallio)

LIST OF ORIGINAL PUBLICATIONS AND MANUSCRIPTS

This thesis is based on the following articles and manuscripts, which are referred to by their Roman numerals in the text.

I **Kallio, J.-M.**; Lahti, M.; Oikari, A.; Kronberg, L. Metabolites of the aquatic pollutant diclofenac in fish bile. *Environ. Sci. Technol.* **2010**, *44*, 7213–7219.

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

III **Brozinski, J.-M.**; Lahti, M.; Oikari, A.; Kronberg, L. Identification and dose dependency of ibuprofen biliary metabolites in rainbow trout. Submitted to *Chemosphere*.

IV **Brozinski, J.-M.**; Lahti, M.; Meierjohann, A.; Oikari, A.; Kronberg, L. The Anti-inflammatory drugs diclofenac, naproxen and ibuprofen are found in the bile of wild fish caught downstream of a wastewater treatment plant. *Environ. Sci. Technol.* **2013**, *47*, 342–348.

LIST OF RELATED PUBLICATIONS (not discussed in this thesis)

Svanfelt, J.; **Kallio, J.-M.**; Eriksson, J.; Kronberg, L. Environmental fate and hazards of the pharmaceutical diclofenac. In *Contaminants of Emerging Concern in the Environment: Ecological and Human Health Considerations*; Halden, R. ed.; American Chemical Society, Washington DC, USA, **2010**, pp 243–255.

Lahti, M.; **Brozinski, J.-M.**; Jylhä, A.; Kronberg, L.; Oikari, A. Uptake and biotransformation of pharmaceuticals by fish. *Environ. Toxicol. Chem.* **2011**, *30*, 1403–1411.

Carrasco Navarro, V.; **Brozinski, J.-M.**; Leppänen, M.T.; Honkanen, J.O.; Kronberg, L.; Kukkonen, J.V.K. Inhibition of pyrene biotransformation by piperonyl butoxide and identification of two pyrene derivatives in *Lumbriculus variegatus* (Oligochaeta). *Environ. Toxicol. Chem.* **2011**, *30*, 1069–1078.

Lahti, M.; **Brozinski, J.-M.**; Segner, H.; Kronberg, L.; Oikari, A. Bioavailability of pharmaceuticals in waters close to wastewater treatment plants - use of fish bile for exposure assessment. *Environ. Toxicol. Chem.* **2012**, *31,* 1831–1837.

Brozinski, J.-M.; Kronberg, L.; Mannio, J. Mihin lääkeaineet päätyvät ympäristössä? *Lääketieteellinen aikakauskirja Duodecim*, **2012**, 128, 1376–1380.

Thomas, K.; Bijlsma, L.; Castiglioni, S.; Covaci, A.; Emke, E.; Grabic, R.; Hernández, F.; Karolak, S.; Kasprzyk-Hordern, B.; Lindberg, R.H.; Lopez de Alda, M.; Meirjohann, A.; Ort, C.; Pico, Y.; Quintana, J.B.; Reid, M.; Rieckermann, J.; Terzic, S.; van Nuijs, A.L.N.; de Voogt, P. Comparing illicit drug use in 19 European cities through sewage analysis. *Sci. Tot. Environ.* **2012**, *432*, 432–439.

CONTRIBUTION OF THE AUTHOR

Jenny-Maria Brozinski has contributed to the papers in this thesis as stated below:

I Jenny-Maria Brozinski was involved in the method development, analysis and data interpretation. She was responsible for writing the paper.

II Jenny-Maria Brozinski was involved in the analysis and data interpretation, and she performed the synthetical work. Jenny-Maria Brozinski was responsible for writing the paper.

III Jenny-Maria Brozinski was involved in the analysis and data interpretation. She was responsible for writing the paper.

IV Jenny-Maria Brozinski was involved in the method development, analysis and data interpretation. She was responsible for writing the paper.

LIST OF ABBREVIATIONS

APCI	atmospheric pressure chemical ionization
BCF	bioconcentration factor for the parent compound e.g. in plasma, muscle, liver; it is calculated as the ratio of the concentration of the compound in tissue (mass kg^{-1} or mass L^{-1}) and the concentration in water (mass L^{-1})
BCF_{bile}	bioconcentration factor for the parent compound and its metabolites in bile; it is calculated as the ratio of the total concentration of the compound and its known metabolites in bile (mass L^{-1}) and the concentration of the parent compound in water (mass L^{-1})
CID	collision induced dissociation
CYP	cytochrome P450 isoforms
D	distribution coefficient; takes account of the neutral compound and its ionized form
EIC	extracted ion chromatogram
ESI	electrospray ionization; ESI+ (positive ESI) and ESI− (negative ESI)
IS	internal standard
K_{OW}	octanol-water partition coefficient; for neutral compound
LC	liquid chromatography
LOD	limit of detection
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MS^n	multiple tandem mass spectrometry
m/z	mass-to-charge ratio
MW	molecular weight
NMR	nuclear magnetic resonance
pK_a	acid dissociation constant
Q-ToF-MS	quadrupole-time-of-flight mass spectrometer
QqQ-MS	triple-quadrupole mass spectrometer
SD	standard deviation
SPE	solid phase extration
UV	ultraviolet
WWTP	wastewater treatment plant

TABLE OF CONTENTS

1 INTRODUCTION

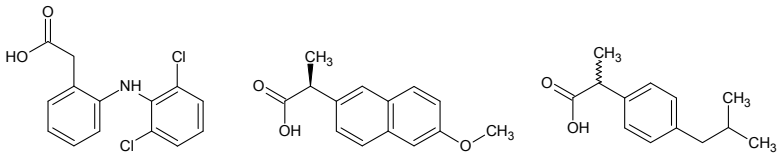
1.1 Presence of pharmaceuticals in the aquatic environment

Thousands of tons of pharmacologically active compounds are consumed every year worldwide for the treatment of human and animal illnesses. Due to this continuous and increasing consumption and their incomplete elimination in wastewater treatment plants (WWTP), pharmaceuticals and their metabolites can be detected in rivers, lakes, and coastal waters (1−7). The removal efficiency of WWTPs varies depending on the pharmaceutical, the treatment applied, and the season of the year (3, 8−12). Although the compounds are detected at low concentrations (ng to low μg L^{-1}), as being biologically active molecules they are considered potentially hazardous for aquatic organisms and health of the ecosystem (13).

After the first reports on the detection of pharmaceuticals in surface waters, research has focused on their fate in the aquatic environment (14−16) and on their possible effects to aquatic organisms (17−23). Many of the pharmaceuticals have been shown to have a rather low acute lethality (i.e. mg L^{-1} range) towards aquatic animals and other organisms (13, 24−25). Despite this, chronic and even life-long exposure has to be considered as a risk factor not only for single species, but for the ecosystem as well. However, data on the chronic effects of pharmaceuticals on aquatic organisms are scarce and the environmental risk assessment is commonly based on the acute and short-term lethality data of single compounds on certain test organisms (13). A further complication to the risk assessments is the occurrence of transformation products of pharmaceuticals, whose identities at present are largely unknown. For instance, phototransformation has been shown to produce compounds that can be more harmful for aquatic organisms than the parent compound (26−27). In addition, pharmaceuticals do not occur as single compounds in the environment. The effects caused by pharmaceutical mixtures to aquatic animals might differ from the effects caused by a single pharmaceutical, i.e. the toxicity of the mixture can cause effects at concentrations, when the single pharmaceutical has little or no effect (13).

1.2 Diclofenac, naproxen and ibuprofen

The non-steroidal anti-inflammatory drugs diclofenac (**1**), naproxen (**2**) and ibuprofen (**3**) (Figure 1) belong to the most commonly used pharmaceuticals worldwide. During year 2010, 1100 kg of diclofenac, 6200 kg of naproxen and 113 000 kg of ibuprofen were consumed in Finland (28). The consumed amounts correspond to 200 mg of diclofenac, 1.2 g of naproxen and 21 g of ibuprofen per inhabitant per year. Diclofenac, naproxen and ibuprofen are polar compounds, with high water solubility and low potential to volatilize. They are all weak acids with pK_a values ranging from 4.15 to 4.91 (Table 1).



Diclofenac, 1 **Naproxen, 2** **Ibuprofen, 3**

Figure 1. Chemical structures of diclofenac (**1**), naproxen (**2**) and ibuprofen (**3**).

Diclofenac, naproxen and ibuprofen are used to reduce pain and inflammatory disorders, and they are available in a number of formulations with the generic names e.g. Diclometin, Diclomex and Voltaren (diclofenac), Miranax, Naprometin and Pronaxen (naproxen) and Burana, Ibumax and Ibumetin (ibuprofen). In Finland, only naproxen is a prescription drug, while ibuprofen is marketed as a prescription and as an over-the-counter drug. Diclofenac is marketed as a prescription drug, although diclofenac containing gels and sprays are marketed over-the-counter. Naproxen is marketed as a single enantiomer (*S*-naproxen) and ibuprofen as a racemate (mixture of *S*- and *R*-ibuprofen).

The elimination of diclofenac in WWTP treatment processes has been reported to be generally poor or even negligible due to possible reformation of the parent compound, i.e. deconjugation of the human glucuronide metabolites during the treatment processes (3, 12, 29). In receiving waters, the main mechanism of elimination of diclofenac is argued to be phototransformation (16, 30−31). Naproxen is also considered as a recalcitrant compound, although higher elimination rates (55−98 %) than for diclofenac have been reported (3, 8). Naproxen is eliminated mainly *via* biotransformation in WWTP (10) and the main mechanisms of elimination in receiving waters are phototransformation and

biotransformation (32−35). The elimination of ibuprofen in WWTP treatment processes is typically efficient (>90 %) (3, 8, 10, 30). Ibuprofen is eliminated in WWTP mainly through biotransformation (9, 10) and it is considered as the main elimination mechanism in receiving waters (32−34).

Table 1. Physicochemical properties of diclofenac (**1**), naproxen (**2**) and ibuprofen (**3**).

	Diclofenac	**Naproxen**	**Ibuprofen**
CAS number	15307-86-5	22204-53-1	15687-27-1
MW ($g\ mol^{-1}$)[1]	296.2	230.3	206.3
Water solubility ($mg\ L^{-1}$)[1]	2.4	15.9	21
Henry's law constant ($atm\ m^3\ mol^{-1}$)[1]	4.73×10^{-12}	3.39×10^{-10}	1.50×10^{-7}
pK_a[1]	4.15	4.15	4.91
log K_{OW}[1]	4.51	3.18	3.97
log D[2]	1.77	0.73	0.19

[1] SRS Phys Prop database, 2012.

[2] Calculated with ADC/Labs V10.02, pH 7.

Some studies have reported physiological and behavioral effects on fish exposed to environmental or near environmental concentrations of pharmaceuticals. In the studies of Schwaiger et al. (17), Triebskorn et al. (18−19) and Hoeger et al. (20), diclofenac was found to induce cytological changes in rainbow and brown trout tissues (kidney and gills) at environmental concentrations. More recently, Mehinto et al. (21) found that exposure of rainbow trout to diclofenac can interfere with the biochemical functions of the fish and lead to tissue damage. In addition, diclofenac has been found to affect the gene expression in fish (21, 23). Diclofenac has been recently proposed to be added into the list of priority substances in the EU's Water Framework Directive (36). The compound has also been connected with the serious decline of the *Gyps* vultures in Asia due to renal failure and visceral gout, and ultimately death (37−40). Diclofenac is suspected to also threaten the *Gyps* vultures in South Africa (41).

Ibuprofen has been found to cause behavioral changes in fish and other aquatic organisms. Low concentrations of ibuprofen have been found to significantly decrease the activity of crustacean *Gammarus pulex* (42). Besides changes in e.g. spawning behavior and hatching

and development of embryos (43−45) in several aquatic organisms, relatively low amounts of ibuprofen has been found to disrupt the cellular stress response in rainbow trout (46).

1.3 Uptake, biotransformation and excretion of pharmaceuticals in fish

The majority of pharmaceuticals and a high number of chemicals in general preregistered for REACH are either weak organic acids and/or bases (47−48). The degree of ionization of a chemical depends on its acid dissociation constant (i.e. the pK_a value of the dissolved compound) and pH of the surrounding medium. When pK_a of the compound equals the surrounding pH, a 50:50 mixture of ionized and non-ionized species of the compound exists. Weak acids are increasingly neutral at pH conditions below compound's pK_a value and weak bases at pH conditions above compound's pK_a value. Therefore, diclofenac, naproxen and ibuprofen (weak acids, Table 1) and antidepressant fluoxetine (a weak base, pK_a 10.1) as well as the majority of other pharmaceuticals, occur mainly in their ionized form (> 99 %) in natural waters with pH conditions ranging from 6 to 8, and in fish blood (pH 7.6).

Bioconcentration of a neutral compound is considered a partitioning process between the aqueous and organic phase (mainly lipids), and it depends mostly on the compound's liphophilicity (hydrophobicity) (49−50). In addition to experimental measurements, a compound's bioconcentration potential can be estimated from the octanol-water partition coefficient (K_{OW}). For weak acids and bases, K_{OW} needs to be corrected for the compound's ionized forms, and it is named distribution coefficient (D). In general, the bioconcentration potential of a neutral compound is dramatically higher than its ionic form (51).

The pH of the surrounding water, and therefore the proportion of the neutral compound and its ionized form, has been shown to influence the uptake, bioconcentration and toxicity of weak acids and bases (51−55). In general, increasing pH can decrease the toxicity of weak acids and increase the toxicity of weak bases. For example, Nakamura et al. (54) found the bioconcentration of antidepressant drug fluoxetine (a weak base, pK_a 10.1) in Japanese medaka (*Oryzias latipes*) to be lower at lower exposure pH and higher at higher exposure pH. Supporting observation was made by Valenti et al. (55), who exposed fathead minnows (*Pimephales promelas*) to antidepressant drug sertraline (also a weak base, pK_a 9.5). The mortality of fathead minnows occurred more rapidly when the exposure was performed at

higher pH conditions (pH 8.5) than at lower pH conditions (pH 6.5 and 7.5). In summary, compound's physicochemical properties (log K_{OW} and p*K*a) and ambient pH can affect the compound's bioconcentration and effect in fish (51). Because diclofenac, naproxen and ibuprofen are mainly in their deprotonated form in natural waters, the compounds are not expected to be readily bioconcentrated. However, generalizations for all the pharmaceuticals cannot be made (56).

The mechanisms of the uptake of pharmaceuticals into fish are not exactly known. To some extent the uptake is taking place through gills, but it can also occur *via* skin or intestine. In gills, compounds may be taken up by diffusion through epithelial cells. The proportion of the neutral compound and its ionized form in the uptake surface will affect the uptake rate, since the membrane permeation through the phospholipid matrix of neutral compounds is easier than for ions (53, 57). The roles of transporters specific to anionic and cationic pharmaceuticals are essentially unidentified in gills. Following the uptake, the compounds are distributed into tissues in the first stage *via* the blood stream. In tissues, ionized pharmaceuticals can be actively taken up by a variety of anionic and cationic xenobiotic transporters (58−59).

The uptake and distribution of pharmaceuticals and other xenobiotics is followed by various biotransformation reactions. In general, in biotransformation the compounds are modified to their less toxic and more water soluble forms, which can be excreted more readily from the body *via* hepatobiliary route (feces) or urine. At the same time, the pharmacological potency of the compound is generally lost, although the formed metabolites might still have some pharmaceutical activity. Biotransformation of pharmaceuticals is divided into two enzymatically catalyzed steps: phase I and phase II reactions (Table 2). In general, phase I reactions introduce a reactive group (e.g. –OH, $–NH_2$, –SH or –COOH) into the molecule and in phase II reactions various conjugates are formed through reactions with these nucleophilic groups (e.g. glucuronide, sulfate, taurine or glutathione). (60)

In vertebrate animals, the major site for biotransformation reactions is the liver and high concentrations of the formed metabolites are excreted from the liver to urine and feces. Enzymes belonging to cytochrome P450 (CYP) superfamily are widely involved in the oxidation of pharmaceuticals (60). Several CYP isoforms have been detected also in fish

tissues (61). Glucuronidation and sulfation reactions and conjugation with glutathione are catalyzed by UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs) and glutathione transferases (GSTs), respectively (60).

Table 2. Phase I and II biotransformation reactions in vertebrate animals (60).

Phase I	Phase II
Oxidation	Glucuronidation
Hydrolysis	Sulfation
Reduction	Acetylation
	Methylation
	Glutathione conjugation
	Amino acid (e.g. taurine, glycine) conjugation

As an example of biotransformation, the phase I and II transformation reactions of diclofenac (**1**) in mammals are shown in Figure 2 (all the possible metabolites or biotransformation routes are not depicted). Diclofenac (**1**) is oxidized to 4'-hydroxydiclofenac (**4**), 5-hydroxydiclofenac and 3'-hydroxydiclofenac (phase Imetabolites) (only 4'-hydroxydiclofenac is depicted in Figure 2) (62). Diclofenac (**1**) can be conjugated with glucuronic acid or with taurine, and form an acyl glucuronide (**5**) and a taurine conjugate (**8**), respectively. Further, 4'-hydroxydiclofenac (**4**) can be conjugated with glucuronic acid and with sulfate, and form an acyl glucuronide (**6**), an ether glucuronide (**7**) and a sulfate conjugate (**9**), respectively (63−65).

The tripeptide glutathione forms with diclofenac (**1**) a glutathione conjugate (**10**), and with 4'-hydroxydiclofenac (**4**), glutathione (**11**) and quinone imine glutathione conjugates (**12**) (66−68). The first step in the formation of glutathione conjugate (**10**) is the formation of acyl glucuronide (**5**) (67). The conjugation proceeds *via* transacylation of glutathione with **5** or through a similar reaction with other potential metabolite, e.g. diclofenac-*S*-acyl-CoA thioester (67). The other glutathione conjugates have been proposed to be formed *via* oxidation of 5-hydroxydiclofenac (**13**) to a benzoquinone imine (**14**) (Figure 3) and its conjugation to glutathione reacting *via* the strong nucleophilic –SH group in the glutathione (66). In addition, the glutathione conjugate of 4-hydroxydiclofenac has been identified in

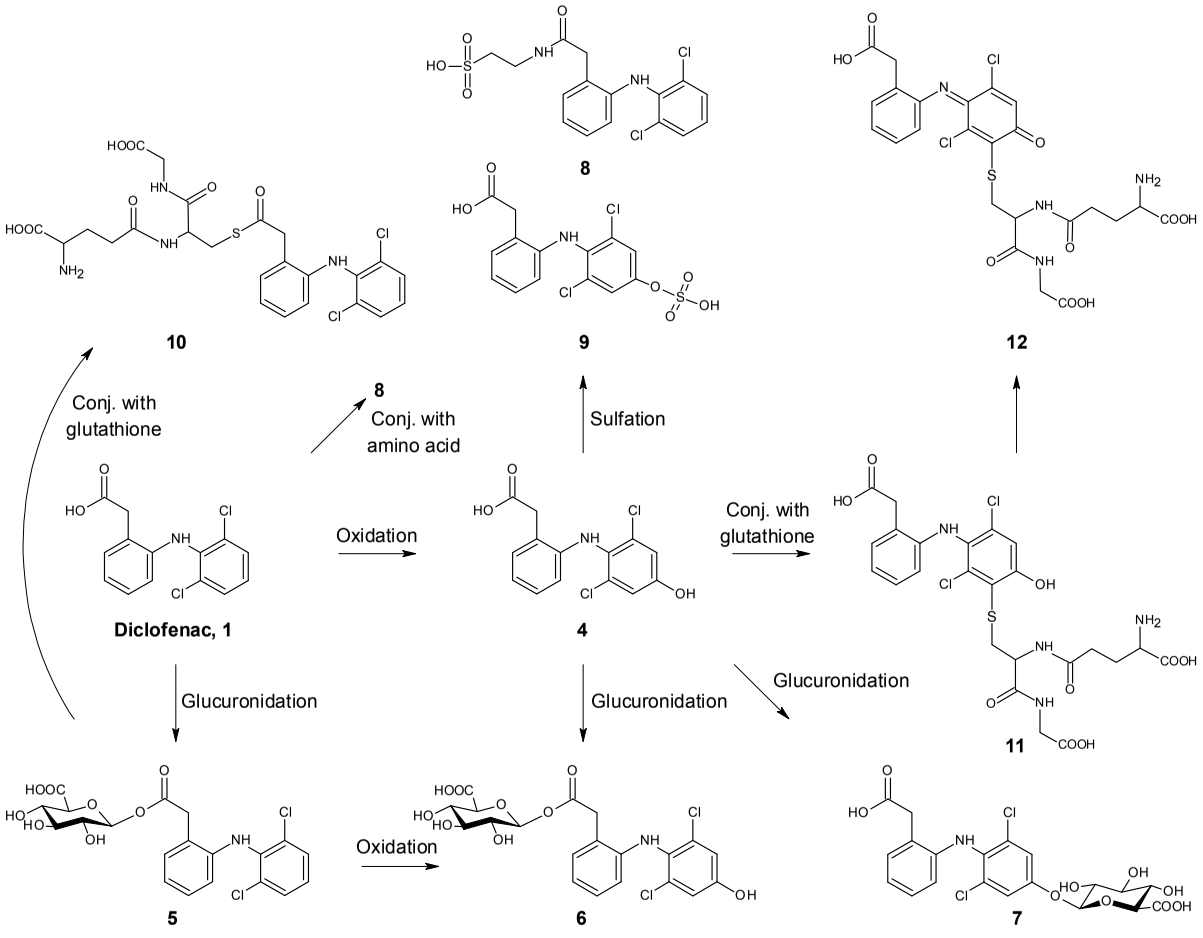


Figure 2. Mammalian biotransformation of diclofenac (**1**). Names of the metabolites are depicted in the text.

previous studies (66). Other quinones originating from diclofenac have been found to form in microbial processes in river sediments (**14**) (69) and under UV-light (**15, 16**) (16) (Figure 3).

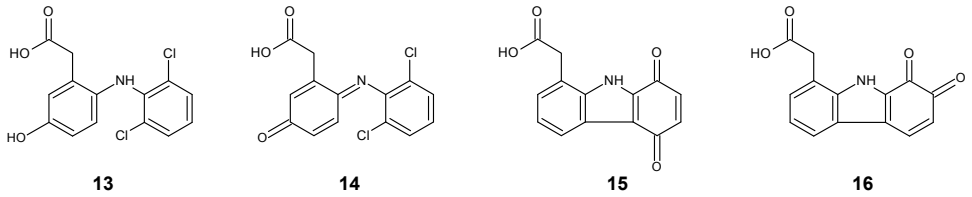


Figure 3. Chemical structures of 5-hydroxydiclofenac (**13**), *para*-benzoquinone imine of 5-hydroxydiclofenac (**14**), *para*- (**15**) and *ortho*- (**16**) carbazole quinones.

Similar phase I and II reactions as shown for diclofenac are also taking place for naproxen and ibuprofen in mammals. Naproxen (**2**) is *O*-demethylated (*O*-dealkylation) to its phase I metabolite, 6-*O*-desmethylnaproxen (**17**) (Figure 4). The major phase II metabolites are the acyl and ether glucuronides and sulfate conjugates of naproxen and 6-*O*-desmethylnaproxen (70). The major mammalian metabolites of ibuprofen (**3**) are the phase I metabolites 2-hydroxyibuprofen (**18**) and carboxyibuprofen (**19**), and their phase II glucuronide conjugates (71). In humans, the taurine conjugation of ibuprofen has been shown to be a minor (1.5 % of the oral dose over 24 h) metabolic pathway (72).

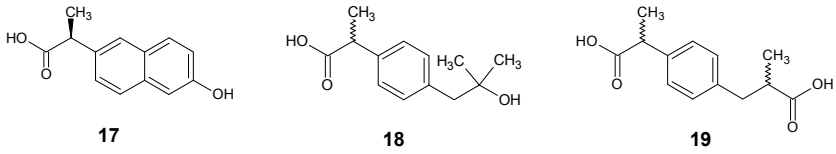


Figure 4. Major phase I metabolites of naproxen (**2**) and ibuprofen (**3**): 6-*O*-desmethylnaproxen (**17**), 2-hydroxyibuprofen (**18**) and carboxyibuprofen (**19**).

The phase I metabolites of diclofenac, naproxen and ibuprofen have also been detected in WWTP and in surface water samples (9, 12, 73−75). In addition to biotransformation reactions in humans, the phase I metabolites may be formed *via* microbial transformation processes in WWTPs (10, 69, 76−78).

Although biotransformation is generally considered as a detoxifying pathway, in some cases, the formed metabolites might be more reactive and therefore more toxic than the parent compound. For example, the covalent protein and DNA nucleoside adducts formed *via* the acyl glucuronide and quinone imine conjugates of diclofenac and hydroxylated diclofenacs

have been connected to the rare, but severe adverse effects (e.g. cellular toxicity and drug hypersensitivity) observed in humans after diclofenac treatment (79−82). The reactivity and toxicity have also been connected to a metabolite of paracetamol (**20**), i.e. containing a quinone moiety *N*-acetyl-*para*-benzoquinone imine (**23**) (Figure 5) and the formation of protein and DNA adducts. Normally, analgesic and antipyretic paracetamol is biotransformed to its ether glucuronide (**21**), sulfate (**22**) and glutathione (**24**) conjugates. When the capacity of biotransformation is saturated (e.g. due to overdose) and when the cells experience a shortage of glutathione, the quinone metabolite (**23**) may accumulate and bind covalently to DNA (83). The mode of toxicity of acyl glucuronides has been suggested to proceed after the formation of adducts v*ia* direct or indirect disruption of the cell function (e.g. glutathione depletion) or through antigen formation and hypersensitivity (81). The reactive metabolites (i.e. acyl glucuronides) have also been postulated to cause the physiological changes in rainbow trout after long-term exposure to diclofenac (21).

Currently, the data of biotransformation of pharmaceuticals in fish is very scarse, even though the knowledge of uptake and biotransformation is important for the ecotoxicological risk assessment (84). Most of the published scientific literature concern antidepressants. For example, fish have been shown to biotransform antidepressant drugs fluoxetine (**25**) and sertraline (**26**) to their pharmacologically active metabolites, norfluoxetine (**27**) and norsertraline (**28**) (Figure 6) (54, 85−86). In addition, few studies have reported on the biotransformation of diclofenac and ibuprofen in fish (21, 87−89), but the biotransformation products were seldom properly identified or quantified.

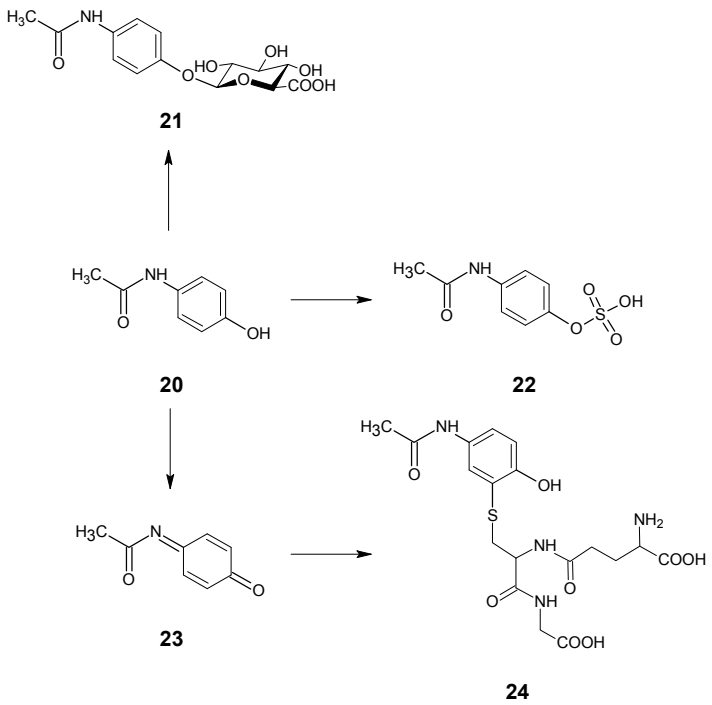


Figure 5. Paracetamol (**20**) is biotransformed in mammals to its ether glucuronide (**21**), sulfate (**22**) and glutathione (**24**) conjugates. Glutathione conjugate is formed *via* a reactive metabolite, *N*-acetyl-*para*-benzoquinone imine (**21**), which form adducts with proteins and DNA.

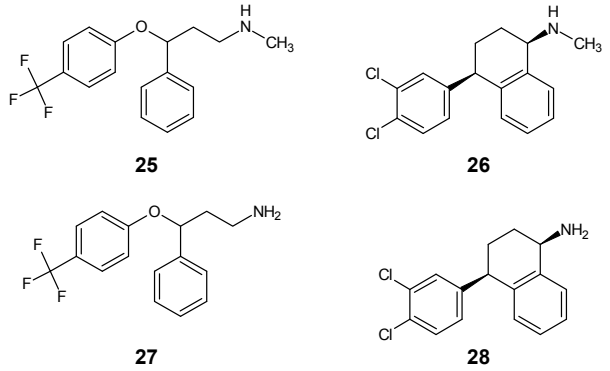


Figure 6. Chemical structures of fluoxetine (**25**), sertraline (**26**) and their metabolites, norfluoxetine (**27**) and norsertraline (**28**).

1.3.1 Acyl migration

The 1-*β*-*O*-acyl glucuronide conjugates are the major mammalian metabolites of pharmaceuticals containing a carboxylic acid group. The conjugates are unstable and reactive in physiological conditions (e.g. in plasma, liver, bile and intestine). Acyl glucuronides can form isomers by a non-enzymatic process called acyl migration (i.e. an intramolecular transesterification) or be hydrolyzed back to the parent compound (reversible metabolism). The acyl migration takes place through the 2-OH, 3-OH, and 4-OH groups of the glucuronic acid yielding 2-*β*-*O*-, 3-*β*-*O*-, and 4-*β*-*O*-acyl glucuronides (Figure 7). The reaction mechanism of acyl migration involves nucleophilic attack by the hydroxyl group on the adjacent carbon and formation of a cyclic *ortho*-ester intermediate. Furthermore, the anomerization of the hydroxyl group at the C-1 position of the *β*-*O*-acyl glucuronides will produce the *α*-isomers *via* the ring opened aldehyde form of the glucuronides. Conversion of the initially formed 1-*β*-*O*-acyl glucuronide to the 2-*β*-*O*-acyl glucuronide has been found to be unidirectional. Review articles of acyl glucuronides and acyl migration have been written by Spahn-Langguth and Benet (90), Shipkova et al. (81) and Regan et al. (82).

Several pharmaceuticals containing a carboxylic acid group, e.g. ibufenac (**29**) and alclofenac (**30**) (Figure 8), have been withdrawn from the marked due to severe adverse effects. The mechanism behind the adverse effects has been connected to acyl migration and formation of adducts with proteins and DNA (81, 90). For the drugs still on market, severe adverse effects have been reported following e.g. diclofenac (**1**) (Chapter 1.3) and ketoprofen (**31**) (Figure 8) treatment (81).

Besides the potential toxicological aspects, acyl migration may complicate the identification and the quantitative analysis of the metabolites. Following chromatographic separation, the isomers are presented by several peaks in the chromatograms (91). Since acyl migration leads to the formation of more hydrolysis resistant isomers, they are more likely to travel further down in the intestinal system and lead to the formation of adducts (81).

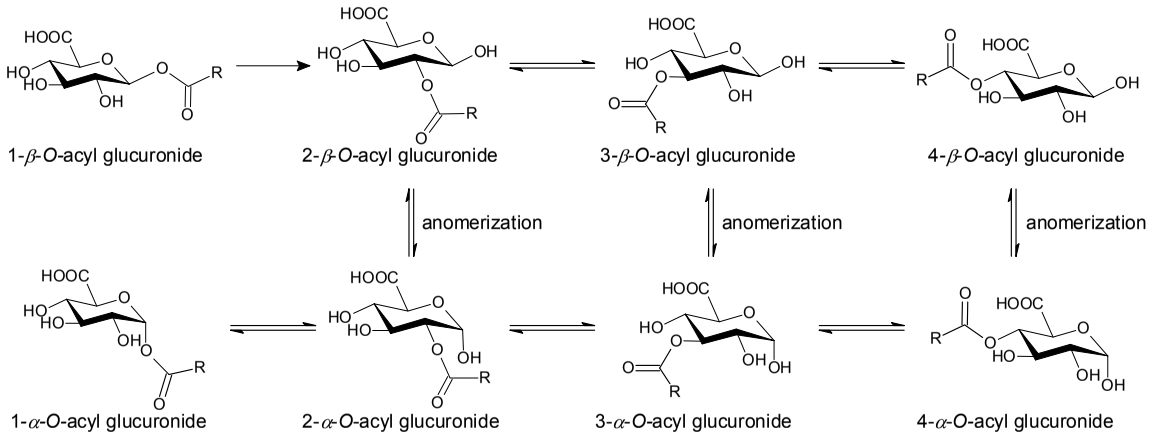


Figure 7. Acyl migration and anomerization of 1-*β*-*O*-acyl glucuronide of a carboxylic acid group containing pharmaceutical (R = e.g. diclofenac, naproxen or ibuprofen). Anomerization is the process of conversion of the cyclic sugar anomer to the other (*α* to *β* or vice versa).

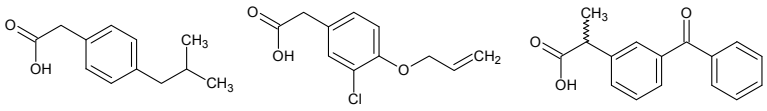


Figure 8. Chemical structures of ibufenac (**29**), alclofenac (**30**) and ketoprofen (**31**).

Acyl migration and its rate and the reactivity of the isomer depend on several factors: e.g. pH conditions, temperature and the parent molecule the glucuronide is conjugated to. In general, acyl glucuronides are most stable in acidic conditions and at low temperature. Neutral or slightly basic pH conditions in many physiological compartments (e.g. bile and intestine) will favor the acyl migration (81). Acyl migration rates of *S*- and *R*-enantiomers of acyl glucuronide conjugates of naproxen (92) and ibuprofen (93) have been found to differ. 3-*O*-acyl glucuronides of naproxen were the most stable isomers of acyl glucuronide of *S*-naproxen, and 3-*O*- and 4-*O*-acyl glucuronides of acyl glucuronide of *R*-naproxen (92). Besides the acyl glucuronide of the parent compound, also the acyl glucuronides of phase I metabolites of ibuprofen have been found to undergo acyl migration (93).

1.4 Analysis of pharmaceutical residues in environmental samples

Liquid chromatography-mass spectrometry (LC-MS) is the mostly used method for qualitative or quantitative analysis of low concentrations of pharmaceuticals and their transformation products (and many other xenobiotics) in environmental samples due to its superior sensitivity and selectivity. Solid phase extraction (SPE) and other isolation techniques are routinely used for the sample clean-up and concentration prior to LC-MS analysis. The compounds of interest are then chromatographically separated on the LC column, ionized in the ionization source and detected in the mass spectrometer by their mass-to-charge ratio (*m/z*). (94) Review articles have been published discussing the state-of-the-art environmental mass spectrometry (95−97) and of the identification of biotransformation products of mammalian pharmaceuticals (98−100) by LC-MS methods.

Another important analytical tool is gas chromatography-mass spectrometry (GC-MS), but it often lacks the much needed sensitivity for the analysis of pharmaceuticals in environmental samples. In GC-MS analysis, the analyzed compounds typically need to be derivatized prior to analysis to improve their volatility.

The mostly used ionization techniques applied in the LC-MS analysis of pharmaceuticals and their biotransformation products are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). ESI is considered as a soft ionization technique, since relatively low amount of fragment ions are produced in relation to the precursor ion. APCI is used for the ionization of usually more polar compounds. The mass spectrometer can be run on negative (ESI−) or positive (ESI+) ionization mode depending on the charge of the pharmaceutical studied.

Depending on whether qualitative or quantitative data is needed, different types of mass analyzers can be applied. Structural elucidation of a compound can be achieved with ion trap and time-of-flight (ToF) type of mass analyzers. Ion traps allow the structural elucidation of a compound in unit resolution by the use of the multiple tandem mass spectrometry (MS^n) feature. Collision induced dissociation (CID) experiments are used to create fragment ions from the compound of interest in the trap, and these fragments can be further isolated and fragmented. (94, 101) For example, first the MS spectrum is recorded for the parent ion, i.e. the deprotonated phase II metabolite acyl glucuronide conjugate of 4'-hydroxydiclofenac (**6**) at *m/z* 486. Then, the parent ion at *m*/*z* 486 is isolated, and fragmented (MS/MS) (Figure 9A) in the trap, and a fragment ion corresponding to phase I metabolite 4'-hydroxydiclofenac (**4**) is formed at *m*/*z* 310. The formed fragment ion at *m*/*z* 310 can be further isolated and fragmented (Figure 9B), and in this was structural information of the phase I metabolite may be obtained.

ToF and quadrupole (Q)-ToF mass analyzers are able to measure the compound's mass with only a few ppms difference to the exact mass (Figure 10, error −0.4 ppm) in the full scan mode. The measurement is based on the flight time of the ions in the mass analyzer given an energy pulse. The data obtained from the measurement enables the determination of the elemental composition of the compound. An important feature of the Q-ToF measurements is the possibility to create, filter, and measure the exact masses of produced fragments. (94, 101)

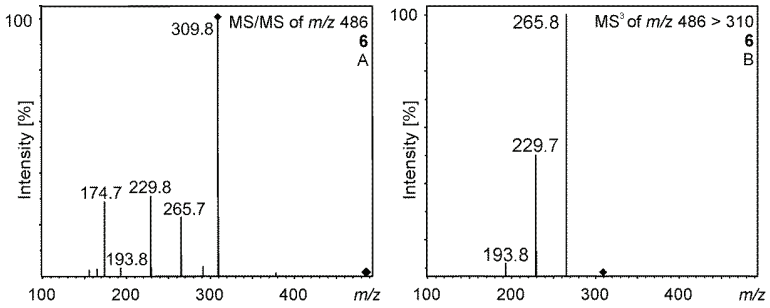


Figure 9. Example of MS/MS (A) and MS^3 (B) mass spectra of acyl glucuronide of 4′-hydroxydiclofenac (**6**) recorded by ion trap type of mass analyzer. A: an ion at *m/z* 486 is isolated and fragmented and product ions are detected. B: an ion at *m/z* 310 is isolated and fragmented and product ions are detected.

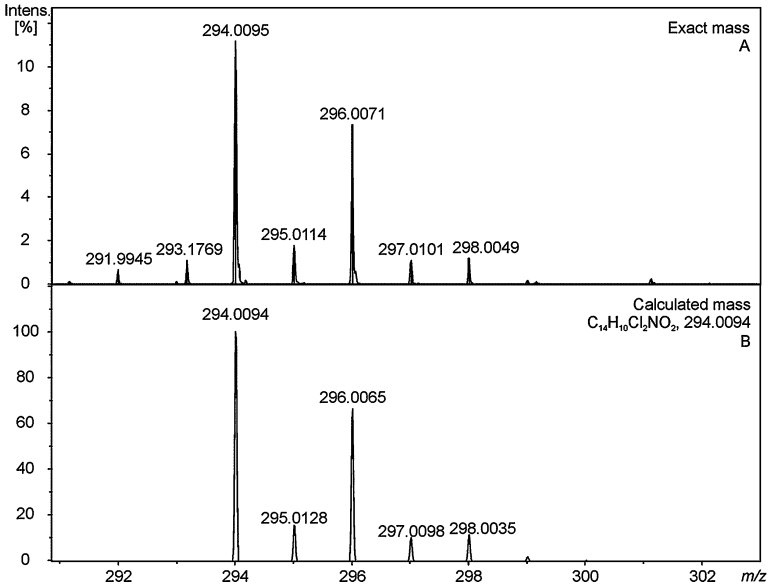


Figure 10. The exact mass (A) and calculated mass (B) of diclofenac (**1**) recorded by the high resolution mass instrument, quadrupole time-of-flight (Q-ToF) mass analyzer. The typical isotopic pattern of a compound containing two chlorine atoms (ion peaks M, M+2 and M+4 with intensities of 100%, 64% and 10%) is evident.

Triple quadrupole (QqQ) type mass analyzers are most often used in the quantitation of low concentrations of pharmaceuticals in environmental samples. QqQs consist of two sets of quadrupoles and a collision cell between the quadrupoles. In comparison to ion trap and ToF type of mass analyzers, QqQs are more sensitive, when operated in the multiple reaction monitoring (MRM) mode. In an MRM experiment, compound specific precursor-product transitions are recorded. The first quadrupole is used as a mass filter to select the ion of interest (e.g. deprotonated diclofenac at *m/z* 294), the second as a collision cell to fragment the selected precursor ion, and the third as a mass filter for the specific product ion (*m/z* 250, formed after a loss of CO_2 from the precursor ion) (Figure 11). Other operating modes are also available (e.g. neutral loss scan). In neutral loss scan, the instrument is scanning e.g. for the loss of anhydroglucuronic acid from the molecular ions, and all the glucuronide conjugates would be seen as peaks in the obtained chromatogram. (94)

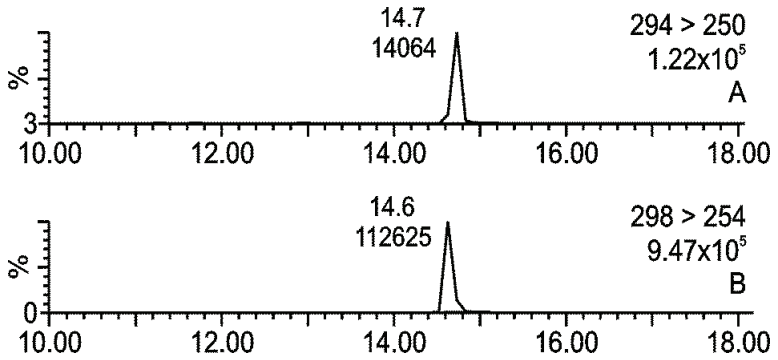


Figure 11. LC-triple quadrupole ion chromatograms of MRM transition of deprotonated diclofenac (**1**) (A) and its internal standard, deprotonated deuterated diclofenac (B).

The main disadvantages of the QqQs in general are the matrix suppression, and limited sensitivity of the instrument in the full scan mode, when compared to MRM mode and ion trap and ToF type of mass analyzers. The matrix suppression can be neglected by the use of isotopically labeled compounds. (94)

1.4.1 Characteristic fragmentation of phase II metabolites in the mass analyzer

The knowledge of fragmentation patterns of the parent ion in the mass analyzer is important for the qualitative and quantitative LC-MS/MS analysis. When QqQ mass analyzers are operated in the MRM mode, compound specific precursor-product ion transitions are monitored (see above). In Figure 12, some examples of characteristic collision induced fragments formed in the mass analyzer for five phase II metabolites of diclofenac are given. The fragments shown in Figure 12 and described in the text are recorded either in the ESI− (**6**−**8**) or in the ESI+ (**10**) mode.

In general, upon fragmentation the acyl glucuronide conjugates (**6**) tend to lose a major fragment having a mass of 176 mass units (i.e. anhydroglucuronic acid, *m*/*z* 175) (70, 65, 98). In contrast to acyl glucuronides, ether conjugates of carboxylic acid group containing pharmaceuticals (**7**) can lose initially a CO_2 from the free carboxylic acid group, and subsequently the formed fragment ion can lose the glucuronide unit (65, 70). Fragments (e.g. *m*/*z* 113, losses of CO_2 and H_2O from anhydroglucuronic acid, *m*/*z* 175) formed from the anhydroglucuronic acid can sometimes be detected (98).

Sulfate conjugates (**9**) are characterized by the loss of SO_3 from the sulfate group (70, 98). Sometimes a less abundant fragment at a mass of 98 mass units lower (i.e. the loss of H_2SO_4) than the deprotonated precursor molecule can be seen (98). Taurine conjugates (**8**) form deprotonated taurine (*m*/*z* 124), and can lose SO_3 from the deprotonated precursor molecule (65, 72, 98). The glutathione conjugate (**10**) will likely cleave between the conjugation site in the glutathione (cysteine) and the compound it is conjugated to, and form a fragment at *m/z* 308 (98). The losses of anhydroglutamic acid (the loss of 129 mass units) and glutamine (the loss of 146 mass units) from the glutathione are often recorded (98).

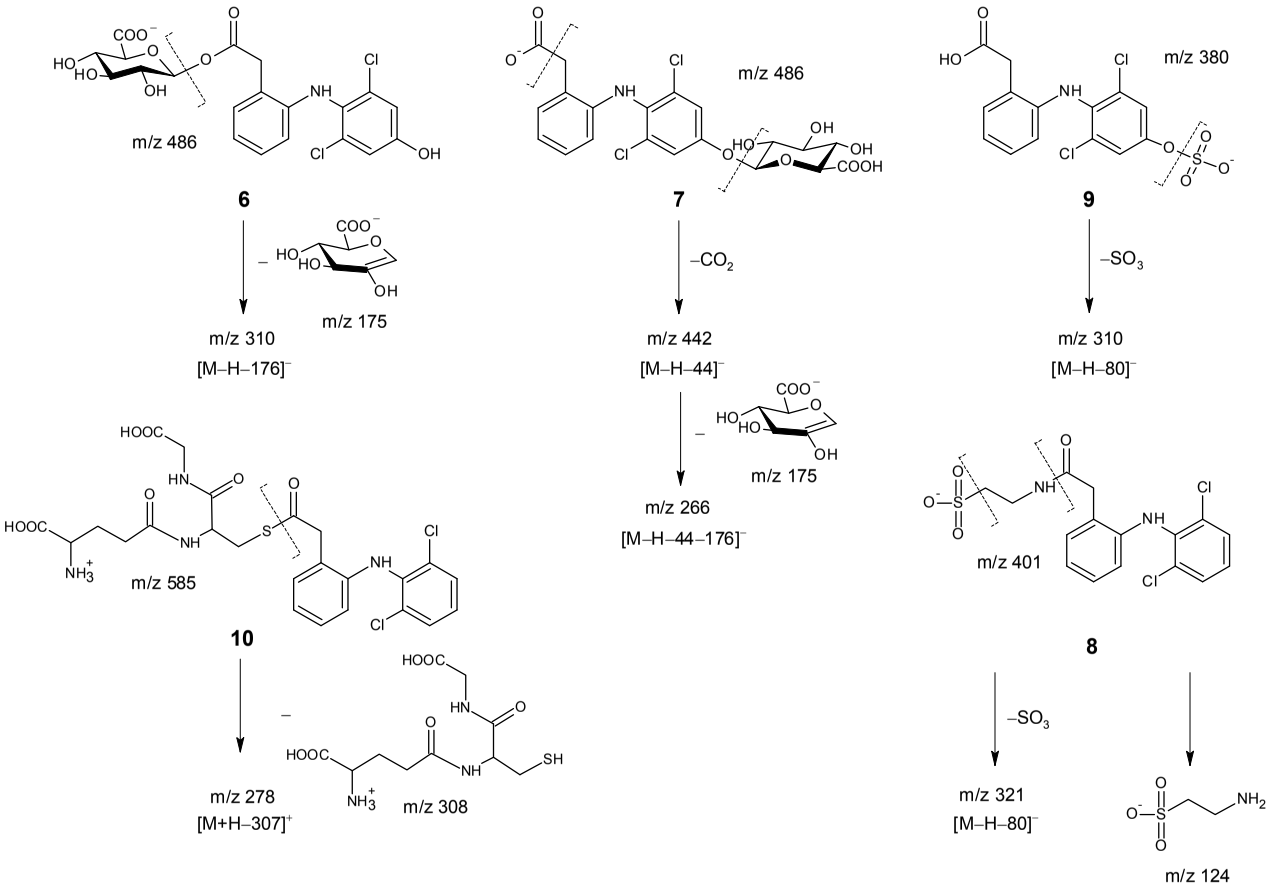


Figure 12. Examples of characteristic fragments formed in the mass analyzer of acyl (**6**) and ether (**7**) glucuronide ions, taurine (**8**), sulfate (**9**) and glutathione (**10**) conjugate ions. The metabolites of diclofenac are used as model compounds.

2 OBJECTIVES

The objective of this thesis was to gain knowledge on the possible uptake, biotransformation and excretion of pharmaceuticals into fish bile. This was done by identifying bile metabolites formed in the fish liver and by assessing the dependency of exposure pathway (*via* intraperitoneal injection, *i.p.* or *via* water) and the exposure concentration of the pharmaceutical in aquaria. Finally, it was studied, if exposure takes place in wild fish populations living in a lake contaminated with WWTP effluents.

The more specified objectives were:

- to study the biotransformation of diclofenac (**I**), naproxen (**II**) and ibuprofen (**III**) in rainbow trout (*Oncorhynchus mykiss*) exposed *via* intraperitonel injections or *via* aquaria water
- to determine the presence of 17 pharmaceuticals and six phase I metabolites in the bile of two native fish species, bream (*Abramis brama*) and roach (*Rutilus rutilus*), caught from a lake receiving wastewater effluents (**IV**)

3 MATERIALS AND METHODS

The following section is a summary of the materials and methods. A more detailed description of the experiments and analysis is presented in the original papers **I** to **IV**.

3.1 Fish experiments and sampling

3.1.1 Exposure to pharmaceuticals via *i.p.* injection

One- (**III**) and 1.5-year-old (**I–II**) rainbow trout (*Oncorhynchus mykiss*) were purchased from the Finnish Game and Fisheries Research Institute (Laukaa, Finland). Before the experiments, trouts were acclimatized to laboratory conditions for 1 (**III**) or 2 weeks (**I–II**). The average weights of the fish were 249 g (**I–II**) and 58 g (**III**).

The fish were anesthetized with MS222, and subsequently 2.5 mg kg^{-1} of diclofenac (**I**), 5 mg kg^{-1} of naproxen (**II**) or 5.2 mg kg^{-1} of ibuprofen (**III**) was intraperitoneally (*i.p.*) injected to the peritoneal cavity of the fish. The injections were repeated after 24 h and the bile was sampled after 48 h of the first administration. Three (**III**) to four (**I–II**) control fish were injected with 1 mL kg^{-1} of 1:1 ethanol/0.7% NaCl solution.

3.1.2 Exposure to pharmaceuticals in water

Half-a-year-old (**III**) and one-year-old (**I–II**) rainbow trout (*Oncorhynchus mykiss*) were purchased from the hatchery of Hanka Taimen Oy (Hankasalmi, Finland, 0.5-yearl-old) and Savon-Taimen Oy (Rautalampi, Finland, 1-year-old). Before the experiments, fish were acclimatized to laboratory conditions for 1 (**III**) or 2 weeks (**I–II**). The average weights of the fish were 169 g (**I–II**) and 9 g (**III**).

Four fish were exposed to 1.8 μg L^{-1} of diclofenac (**I**) or 1.6 μg L^{-1} of naproxen (**II**) for 10 days in a flow-through system. Thirty six fish were exposed to 0.17, 1.9, 13 and 145 μg L^{-1} of ibuprofen (nine fish per concentration) for four days (**III**) in a periodic replacement system. Nine (**III**) or four (**I–II**) control fish were kept under similar conditions as fish exposed to diclofenac, naproxen or ibuprofen. The concentrations of the pharmaceuticals in aquaria were monitored daily.

3.1.3 Population sampling

Ten breams (*Abramis brama*) and 25 roaches (*Rutilus rutilus*) were caught by fyke netting in late May 2011 from Lake Haapajärvi, SE Finland (**IV**). The average lengths and weights of the breams were 43 cm and 922 g, and those of the roaches were 15 cm and 29 g, respectively. In early July 2011, six breams (average length 30 cm) and eight roaches (average length 18 cm) were caught from a reference site, Southern Lake Saimaa, SE Finland. The fish were sampled on site.

For the determination of pharmaceuticals in the water of Lake Haapajärvi, one liter water samples were collected from two sampling sites in February, May, July and November 2010 using a Limnos sampler.

3.2 Sample preparation

Bile samples (50 μL) obtained from the *i.p.* injection experiments (**I–III**) were diluted with 500 μL of 5% acetonitrile in 0.01 M ammonium acetate and analyzed by different LC-MS methods (Chapter 3.3.2). Bile samples (20–100 μL) from the diclofenac and naproxen exposures *via* water (**I–II**) were solid phase extracted as described in Chapter 3.2.2.

Due to the small size of rainbow trout in ibuprofen exposures *via* water, nine biles per exposure concentration were pooled to a one analytical sample (60−100 μL) (**III**). Some of the roach bile samples were also combined so that one sample (30−100 μL) was made up from two to five individuals of the same gender (**IV**). Biles from ibuprofen exposures (**III**) and from wild breams and roaches (**IV**) were treated enzymatically and further extracted (Chapters 3.2.1 and 3.2.2).

Aquarium water samples (25−100 mL) (**I–III**) and Lake Haapajärvi water samples (400 mL) (**IV**) were solid phase extracted as depicted in Chapter 3.2.2.

3.2.1 Hydrolysis

In order to enhance the detectability of the analytes, the bile samples obtained from ibuprofen exposures *via* water (**III**) and from wild fish (**IV**), were hydrolyzed enzymatically by *β*-glucuronidase/aryl-sulfatase enzyme isolated from *Helix pomatia* (obtained from Sigma Aldrich, cat. no. G7770). The enzyme cleaves glucuronide (optimal pH: 4.5−5.0) and sulfate (optimal pH: 6.2) conjugates back to the phase I metabolites or to the parent compounds. The hydrolysis conditions were optimized prior to the treatment of the bile samples by the addition of 1-*β*-*O*-acyl glucuronides of diclofenac, naproxen and ibuprofen to the bile collected from control rainbow trout.

The bile samples, internal standards (250 ng) and enzyme were incubated in acetate solution (pH 5) for 4 h at 37 °C.

3.2.2 Solid phase extraction

The biles (**I–II**) and their hydrolyzates (**III–IV**) were diluted with acidified water (pH 2) and internal standard (100 ng D3-ibuprofen) was added (**I–II**). Oasis HLB (Waters, Milford, MA, USA) cartridges were used for the purification of acidic pharmaceuticals (**I–IV**), while Oasis MCX (Waters, Milford, MA, USA) cartridges were applied for the basic pharmaceuticals (**IV**). After conditioning, sample loading and wash, the acidic pharmaceuticals were eluted with 1 mL 2% NH_4OH in 80% methanol (**I–IV**) and the basics with 1 mL 100% methanol and 1 mL 2% NH_4OH in methanol (**IV**). The extracts were evaporated to dryness, re-dissolved in 200 μL of 5% acetonitrile in 0.01 M ammonium acetate (acidic pharmaceuticals, **I–II**), 300 μL of 2% acetonitrile in 0.01 M ammonium hydroxide (acidic pharmaceuticals, **III–IV**) or 300 μL of 3% acetonitrile in 0.5% acetic acid (basic pharmaceuticals, **IV**), and analyzed immediately by LC-MS/MS.

After the acidification of water samples (**I–III**) to pH 2, internal standard (53 ng fenoprop or 200 ng D3-ibuprofen, respectively for **I–II** and **III**) was added and the samples were passed through Oasis HLB cartridges. The compounds of interest were eluted with 4 × 0.5 mL of methanol, evaporated to dryness and re-dissolved in 500 μL of 30% acetonitrile in 0.01 M ammonium acetate and analyzed by LC-MS/MS. Lake Haapajärvi water samples (**IV**) were extracted as reported in Daneshvar et al. (6, 7) for acidic and basic pharmaceuticals.

3.3 LC-MS analysis

3.3.1 Chromatographic methods

The chromatographic separations for the identification of the biliary metabolites of diclofenac (**I**) and naproxen (**II**) were performed on a Zorbax Eclipse C18 analytical column (5 μm, 2.1 × 50 mm column; Agilent Technologies, Palo Alto, CA). For the identification of metabolites of ibuprofen (**III**), quantification of pharmaceuticals and their metabolites in wild fish bile (**IV**), in experimental aquaria water (**I–III**) and in water of Lake Haapajärvi (**IV**), an XBridge C18 column (3.5 μm, 2.1 × 50 mm; Waters, Milford, MA, USA) was used. In addition, the chromatographic retention of diclofenac, naproxen and ibuprofen in wild fish bile were studied on an XBridge C8 column (3.5 μm, 2.1 × 50 mm; Waters, Milford, MA, USA) and on an ACE Phenyl 5 column (5 μm, 2.1 × 125 mm; Advanced Chromatography Technologies, Aberdeen, Scotland) (**IV**).

The columns were eluted with different gradients of acetonitrile in 0.01 M ammonium acetate (**I–II**), in 0.01 M ammonium hydroxide (**III–IV**) or in 0.5% of acetic acid (**IV**).

3.3.2 Identification of the metabolites

The identification of the metabolites of diclofenac (**I**), naproxen (**II**) and ibuprofen (**III**) in fish bile was based on the exact mass determinations on a Bruker electrospray ionization quadrupole-time-of-flight mass analyzer (Q-ToF-MS; Bruker Daltonics, Bremen, Germany) and on a collision induced dissociation (CID) experiments coupled with multiple tandem mass spectrometry (MS^n) on an Agilent 1100 Series LC/MSD Trap SL mass analyzer (IT-MS; Agilent Technologies, Palo Alto, CA, USA).

The analytes were transferred to the mass analyzers by an Agilent 1200 Series LC system (Q-ToF-MS) or by an Agilent 1100 Series LC system (IT-MS). The systems consisted of a binary pump, a vacuum degasser, an autosampler, a thermostated column and an UV detector. The obtained data was collected and handled with the Bruker Compass DataAnalysis 4.0 software (Q-ToF-MS) or Agilent ChemStation data system (IT-MS). Both of the mass analyzers applied nitrogen as a nebulizing gas and as a drying gas. Helium was used as a collision gas for the CID experiments.

3.3.3 Quantification of pharmaceuticals and their metabolites

The quantitative analysis of pharmaceuticals and their metabolites in fish bile (**I–IV**), in experimental water (**I–III**) and in water of Lake Haapajärvi (**IV**) were performed on an electrospray Quattro Micro triple-quadrupole mass analyzer (QqQ-MS; Waters, Milford, MA, USA) operated in a multiple reaction monitoring (MRM) mode. The chemical structures of the compounds analyzed in **IV** are presented in Figures S1 and S2. The mass analyzer was connected either to a Waters Alliance 2795 LC system (Waters, Milford, MA, USA) (**I–II**) or to an Agilent 1100 series LC system (Agilent Technologies, Palo Alto, CA, USA) (**III–IV**). The LC systems consisted of tertiary (Waters) or binary (Agilent) pump, a vacuum degasser, an autosampler and a thermostated column. Nitrogen was used as a desolvation gas and argon as a collision gas. The cone voltages, the collision energies, the precursor and product ions were optimized by direct infusion of the pure standards and internal standards to mass spectrometer. The MRM parameters are presented in the original papers **I** to **III** and in Table S1 (**IV**). The data was collected and handled with MassLynx 4.0 software.

4 RESULTS AND DISCUSSION

In this section, the results of the study are briefly summarized. A more detailed presentation of the results is available in the original papers **I** to **IV**.

4.1 Identification of the metabolites of diclofenac, naproxen and ibuprofen in fish bile

The bile metabolites of rainbow trout treated intraperitoneally (*i.p.*) were used for the identification of the biotransformation pathways of diclofenac (**I**), naproxen (**II**) and ibuprofen (**III**). Although *i.p.* dosing is an unnatural way of exposure, it is useful for the purpose of identification of the metabolites, since the studied compounds directly enter into the extracellular space of the fish from where they are taken up in organs, e.g. the liver. For comparison with *i.p.* dosing, fish were exposed *via* water (Chapter 4.2).

4.1.1 Diclofenac

Based on the data obtained by LC-UV, LC-IT-MS and LC-Q-ToF-MS, *i.p.* dosed rainbow trout metabolized diclofenac to several phase I and phase II metabolites (**I**) (Table 3). Also unmetabolized diclofenac (**1**) was detected in the bile. The structures of the parent compound diclofenac and the metabolites are depicted in Figure 13. Two hydroxylated phase I metabolites (4'-hydroxydiclofenac, **4**; 5-hydroxydiclofenac, **13**), their sulfate conjugates (sulfate conjugate of 4'-hydroxydiclofenac, **9**; sulfate conjugate of 5-hydroxydiclofenac, **32**) and acyl glucuronides (acyl glucuronide of 4'-hydroxydiclofenac, **6**; acyl glucuronide of 5-hydroxydiclofenac, **33**) were detected in the trout bile. In addition, a sulfate conjugate of dihydroxylated diclofenac (**34**), an ether glucuronide of 4'-hydroxydiclofenac (**7**) and an acyl glucuronide of 3'-hydroxydiclofenac (**35**) were found. Also, diclofenac (**1**) was found to form a acyl glucuronide conjugate (**5**). Diclofenac or its metabolites were not detected in any of the control rainbow trout.

Previously known mammalian metabolites of diclofenac (Chapter 1.3), benzoquinone imines (e.g. **14**), glutathione (e.g. **10**, **11** and **12**) or taurine conjugates (**8** and taurine conjugate of hydroxylated diclofenac) of diclofenac, hydroxylated diclofenacs or benzoquinone imines (66−67) were not detected in fish bile. After the publication of the paper **I**, two additional

metabolites could be identified, i.e. acyl glucuronide-sulfate conjugate of hydroxylated diclofenac (Q-ToF-MS, *m/z* 565.9907, 4.5 ppm error) and diglucuronide conjugate of hydroxylated diclofenac (Q-ToF-MS, *m/z* 662.0675, 1.5 ppm error) (chemical structures of the metabolites are not shown).

The chromatograms, mass spectra and schemes of the fragmentation patterns of the metabolites in the mass spectrometer are found in the original publication (**I**). In general, the acyl glucuronides **6**, **33** and **35** lost initially 176 mass units [M−H−anhydroglucuronic acid]$^-$ and it was followed by a loss of CO_2 [M−H−anhydroglucuronic acid−CO_2]$^-$. The initial loss from the ether glucuronide **7** was 44 mass units and subsequent to this loss, the glucuronide unit was split off. The sulfate conjugates **9**, **32** and **34** showed a characteristic loss of 80 mass units [M−H−SO_3]$^-$. The phase I metabolites **4** and **13** were possible to distinguish by observing the fragments generated in the MS^2 spectra. Both compounds readily produced a fragment ion at *m/z* 266 due to the loss of CO_2 (*m/z* 310 → *m/z* 266), but **4** produced additionally intense ions at *m/z* 230 and 194 through losses of CO_2 + HCl and CO_2 + 2 × HCl, respectively.

Some reactive metabolites of diclofenac (i.e. acyl glucuronides and quinone imine conjugates of diclofenac and hydroxylated diclofenac, Chapter 1.3) have been found to form covalent protein and DNA adducts (79, 80−82). Formation of these adducts have been connected e.g. with cellular toxicity in humans. Mehinto et al. (21) postulated that acyl glucuronides of hydroxylated diclofenac detected in the rainbow trout bile could be related with a tissue damage found in trout's intestine following long-term exposure.

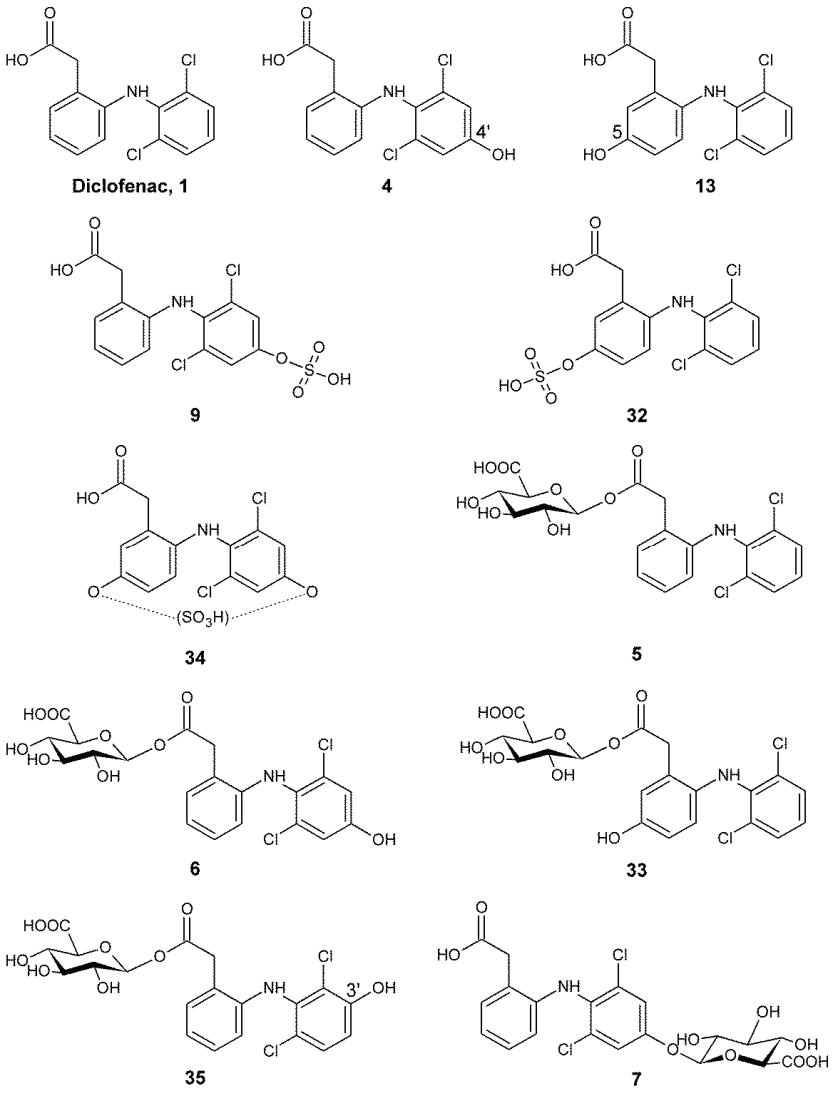


Figure 13. Chemical structures of diclofenac (**1**) and its identified metabolites: 4′-hydroxydiclofenac (**4**), 5-hydroxydiclofenac (**13**), sulfate conjugate of 4′-hydroxydiclofenac (**9**), sulfate conjugate of 5-hydroxydiclofenac (**32**), sulfate conjugate of 4′,5-dihydroxydiclofenac (**34**), acyl glucuronide of diclofenac (**5**), acyl glucuronide of 4′-hydroxydiclofenac (**6**), acyl glucuronide of 5-hydroxydiclofenac (**33**), acyl glucuronide of 3′-hydroxydiclofenac (**35**), and ether glucuronide of 4′-hydroxydiclofenac (**7**).

Table 3. Diclofenac (**1**) and its identified metabolites in the bile of rainbow trout: the exact mass data recorded by the Q-ToF mass analyzer, and major fragment ions observed in the spectra recorded by the IT mass analyzer.

No	Name	t_R (min)	$[M-H]^-$ calculated	$[M-H]^-$ exper.	Error (ppm)	Formula $[M-H]^-$	ESI− fragment ions, IT-MS
1	diclofenac	18.7	294.0094	294.0095	−0.4	$C_{14}H_{10}Cl_2NO_2$	250
4	4′-hydroxydiclofenac	15.4	310.0043	310.0048	−1.7	$C_{14}H_{10}Cl_2NO_3$	266, 230, 194
13	5-hydroxydiclofenac	16.1	310.0043	310.0032	-	$C_{14}H_{10}Cl_2NO_3$	266, 230
9	sulfate conjugate of 4′-hydroxydiclofenac	11.6	389.9611	389.9609	0.6	$C_{14}H_{10}Cl_2NO_6S$	310
32	sulfate conjugate of 5-hydroxydiclofenac	12.0	389.9611	389.9611	0.1	$C_{14}H_{10}Cl_2NO_6S$	310, 266, 230
34	monosulfate conjugate of dihydroxydiclofenac	12.7	405.9561	405.9557	1.0	$C_{14}H_{10}Cl_2NO_7S$	370, 326, 246
5	acyl-migrated isomer of acyl glucuronide of diclofenac	15.6, 16.0	470.0415	470.0418, 470.0424	−0.7, −1.9	$C_{20}H_{18}Cl_2NO_8$	294, 250
6	acyl glucuronide of 4′-hydroxydiclofenac	5.1	486.0364	486.0361	0.8	$C_{20}H_{18}Cl_2NO_9$	310, 266, 230, 194, 175
33	acyl glucuronide of 5-hydroxydiclofenac	8.3	486.0364	486.0362	0.4	$C_{20}H_{18}Cl_2NO_9$	310, 266, 230, 193, 175
35	acyl-migrated isomers of acyl glucuronide of 3′-hydroxydiclofenac	15.4−16.5	486.0364	486.0355a	1.9	$C_{20}H_{18}Cl_2NO_9$	310, 266, 175
7	ether glucuronide of 4′-hydroxydiclofenac	9.9	486.0364	486.0356	1.7	$C_{20}H_{18}Cl_2NO_9$	442, 266, 230, 175

4.1.2 Naproxen

Based on the data obtained by LC-UV, LC-IT-MS and LC-Q-ToF-MS, *i.p.* dosed rainbow trout metabolized naproxen to one phase I and to two phase II metabolites (**II**) (Table 4). The structures of the parent compound naproxen and the metabolites are depicted in Figure 14. The phase I metabolite, 6-*O*-desmethylnaproxen (**17**), was found in bile. Naproxen (**2**) and 6-*O*-desmethylnaproxen (**17**) were further conjugated with glucuronic acid, and formed an acyl glucuronide of naproxen (**36**) and an acyl glucuronide of 6-*O*-desmethylnaproxen (**37**). In addition, unmetabolized naproxen (**2**) was detected in fish bile. Previously known mammalian phase II metabolites of naproxen, sulfate conjugate of 6-*O*-desmethylnaproxen, ether glucuronide of 6-*O*-desmethylnaproxen or acyl glucuronide-sulfate conjugate of 6-*O*-desmethylnaproxen (70) were not found in fish bile.

The chromatograms, mass spectra and schemes of the fragmentation patterns of the metabolites in the mass spectrometer are found in the original publication (**II**). Naproxen or its metabolites could not be detected in any of the control rainbow trout. The same major fragments were found for acyl glucuronides **36** and **37** as for acyl glucuronide conjugates of diclofenac (**I**), i.e. the initial loss of anhydroglucuronic acid [M−H–anhydroglucuronic acid]$^-$ followed by the loss of CO_2 from the carboxylic acid group.

The acyl glucuronides of naproxen (**36**) and 6-*O*-desmethylnaproxen (**37**) were represented in the obtained chromatograms by several peaks due to different geometric isomers of the conjugates. The isomers are formed *via* acyl migration (Chapter 1.3.1). On the basis of LC-^{1}H NMR studies, Mortensen et al. (91) was able to deduce the order of retention of the acyl-migrated isomers on a C18 reversed phase column. The peak profiles in the obtained LC-UV chromatograms of fish bile (Figure 15) were essentially identical to that of Mortensen et al. (91). Thus it was possible assign the order of elution of the isomers of acyl glucuronide of naproxen and of 6-*O*-desmethylnaproxen, i.e., 1-*α*-, 1-*β*- (**36a**, **37a**), 3-*α*- and 3-*β*-, 2-*α*- (**36b**, **37b**), and 2-*β*-*O*-acyl (**36c**, **37c**) glucuronides.

Further, on the basis of the MS/MS spectra of the reference compound (**36**), it was possible to differentiate the 1-*β*-*O*-acyl glucuronides from the other acyl-migrated isomers in the bile samples. The former gave a rise to abundant fragment ions at *m*/*z* 193 (deprotonated

glucuronic acid), whereas the other isomers gave abundant ions at *m*/*z* 175 (deprotonated anhydroglucuronic acid). In addition, upon incubation of the trout bile sample with *β*-glucuronidase enzyme, 1-*β*-*O*-acyl glucuronide peaks (**36a**, **37a** in Figure 15) were found to disappear due to deconjugation, and the peaks of naproxen (**2**) and 6-*O*-desmethylnaproxen (**37**) were found to increase in abundance. In addition, the observed broadening of the chromatographic peak of acyl glucuronide of 4'-hydroxydiclofenac (**6**), two peaks of acyl glucuronide of diclofenac (**5**) and several peaks of acyl glucuronide of 3'-hydroxydiclofenac (**35**) are due to acyl migration (**I**) (Chapter 4.1.1).

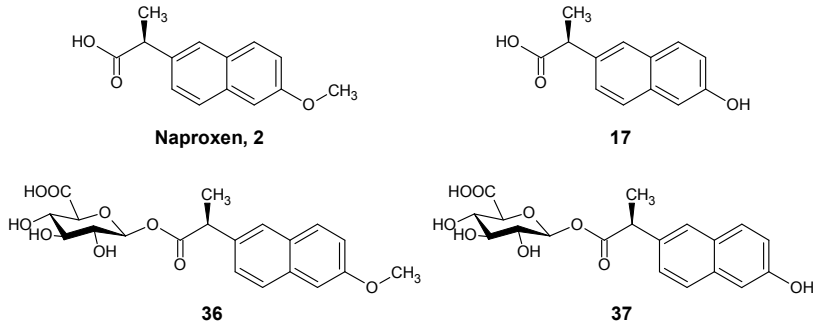


Figure 14. Chemical structures of naproxen (**2**) and its identified metabolites: 6-*O*-desmethylnaproxen (**17**), acyl glucuronide of naproxen (**37**) and acyl glucuronide of 6-*O*-desmethylnaproxen (**36**).

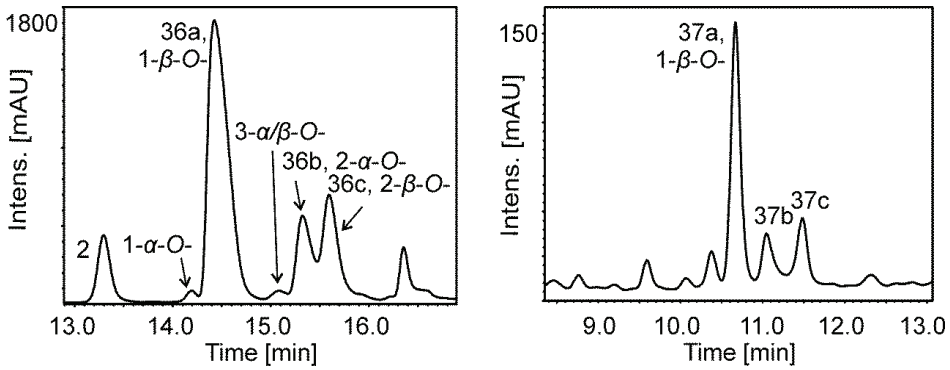


Figure 15. LC-UV chromatograms of acyl migrated isomers of acyl glucuronide of naproxen (**36**) and acyl glucuronide of 6-*O*-desmethylnaproxen (**37**). The numbers above the peaks correspond to 1-*α*-, 1-*β*- (**36a**, **37a**), 3-*α*- and 3-*β*-, 2-*α*- (**36b**, **37b**), and 2-*β*-*O*-acyl (**36c**, **37c**) glucuronides of naproxen or 6-*O*-desmethylnaproxen, respectively.

Table 4. Naproxen (**2**) and its identified metabolites in the bile of rainbow trout: the exact mass data recorded by the Q-ToF mass analyzer, and major fragment ions observed in the spectra recorded by the IT mass analyzer.

No	Name	t_R (min)	$[M-H]^-$ calculated	$[M-H]^-$ exper.	Error (ppm)	Formula $[M-H]^-$	ESI– fragment ions, IT-MS
2	naproxen	13.9	229.0870	229.0877	−2.9	$C_{14}H_{13}O_3$	185, 170
17	6-*O*-desmethylnaproxen	8.4	215.0714	215.0725	−5.4	$C_{13}H_{11}O_3$	171
36	acyl-migrated isomers of acyl glucuronide of naproxen	14.7–16.0	405.1191	405.1197	−1.5	$C_{20}H_{21}O_9$	229, 193, 185, 175, 170, 131, 113
37	acyl-migrated isomers of acyl glucuronide of 6-*O*-desmethylnaproxen	8.5–11.8	391.1035	391.1046	−2.8	$C_{19}H_{19}O_9$	215, 193, 175, 171, 131, 113

4.1.3 Ibuprofen

Based on the data obtained by LC-IT-MS and LC-Q-ToF-MS, *i.p.* dosed rainbow trout metabolized ibuprofen (**3**) to numerous phase I and phase II metabolites (**III**) (Table 5). The structures of the parent compound ibuprofen and the metabolites are depicted in Figure 16. Ibuprofen (**3**) was metabolized in the fish liver to two hydroxylated metabolites (2-hydroxyibuprofen, **18**; 3-hydroxyibuprofen, **38**) and to their acyl glucuronide (**39**), ether glucuronide (**40**) and taurine (**41**–**43**) conjugates. Besides the detection of unconjugated ibuprofen (**3**) in the bile, also its acyl glucuronide (**44**) and taurine conjugate (**45**) were detected. In addition, rainbow trout metabolized ibuprofen to three taurine-ether glucuronide diconjugates of hydroxylated ibuprofens (**46**–**48**). Ibuprofen and its metabolites were not detected in any of the control fish. The ibuprofen phase I metabolite (carboxyibuprofen, **19**), earlier found in our studies (102), was not detected. The position of hydroxylation in the aliphatic chain of ibuprofen in metabolites **39**, **40** and **41**–**43** and **46**–**48** could not be determined.

The obtained chromatograms and more detailed information of the fragmentation patterns are found at the original publication (**III**). The same major fragments were recorded for **44** and **39** as for acyl glucuronide conjugates of diclofenac (**I**) and naproxen (**II**), and for **40** as for ether glucuronide of 4'-hydroxydiclofenac (**I**). The taurine conjugates **41**–**43** and **45** formed a characteristic fragment at *m/z* 124 $[H_2N\text{-}CH_2\text{-}CH_2\text{-}SO_3]^-$. The loss of sulfate group from taurine conjugates was not detected.

Since seven taurine conjugates (**41**–**43**, **45, 46**–**48**) were detected in the rainbow trout bile samples taurine conjugation must present important metabolic pathway in fish. In humans, taurine conjugation of ibuprofen has been shown to be a minor (1.5 % of the dose) metabolic pathway, and other taurine conjugates than **45** have not been detected (72). Recently however, Sarda et al. (65) detected taurine conjugates of diclofenac and hydroxylated diclofenacs, and taurine-ether glucuronide diconjugates of hydroxylated diclofenac in mouse urine and fecal extracts.

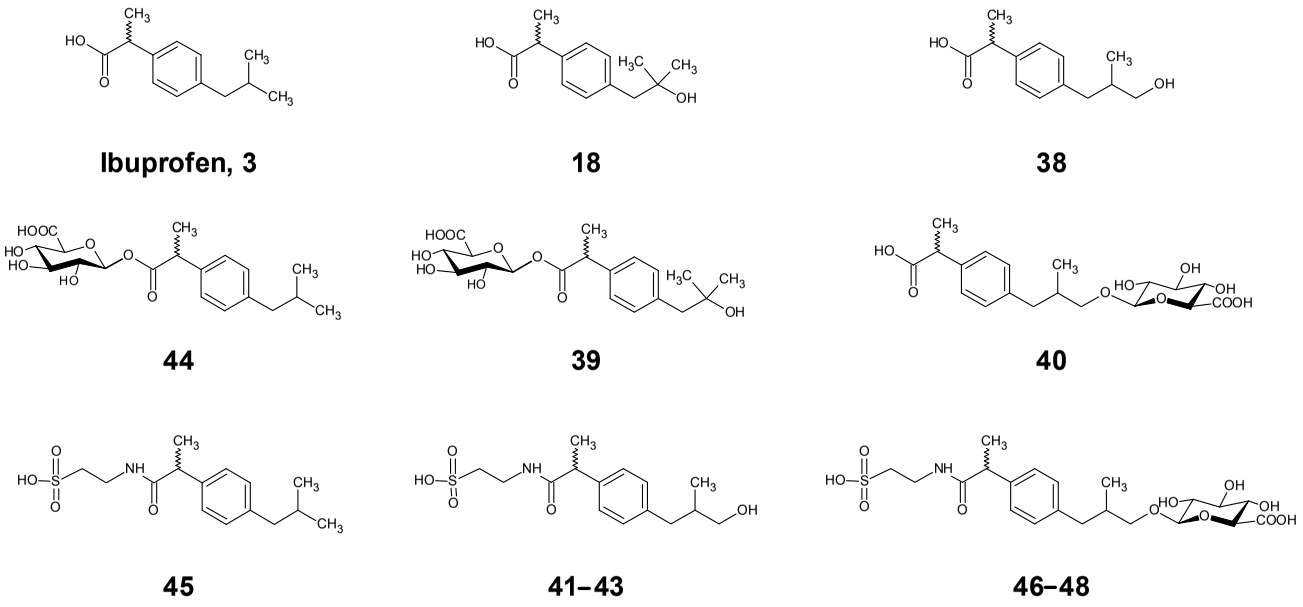


Figure 16. Chemical structures of ibuprofen (**3**) and its identified metabolites in the bile of rainbow trout: 2-hydroxyibuprofen (**18**), 3-hydroxyibuprofen (**38**), acyl glucuronide of ibuprofen (**44**), acyl glucuronide of 2-hydroxyibuprofen (**39**), ether glucuronide of hydroxylated ibuprofen (**40**), taurine conjugate of ibuprofen (**45**), taurine conjugate of hydroxylated ibuprofens (**41**–**43**) and taurine-ether glucuronide diconjugate of hydroxylated ibuprofens (**46**–**48**). The position of hydroxylation in metabolites **39**, **40**, **41**–**43** and **46**–**48** and the stereochemistry is unknown.

Table 5. Ibuprofen (**3**) and its identified metabolites in the bile of rainbow trout: the exact mass data recorded by the Q-ToF mass analyzer, and major fragment ions observed in the spectra recorded by the IT mass analyzer.

No	Name	t_R (min)	$[M-H]^-$ calculated	$[M-H]^-$ exper.	Error (ppm)	Formula $[M-H]^-$	ESI− fragment ions, IT-MS
3	ibuprofen	14.5	205.1234	205.1237	−1.6	$C_{13}H_{17}O_2$	161, 159
18	2-hydroxyibuprofen	8.3	221.1183	221.1183	0	$C_{13}H_{17}O_3$	177, 133
38	3-hydroxyibuprofen	7.4	221.1183	221.1197	−6.3	$C_{13}H_{17}O_3$	177, 133
44	acyl glucuronide of ibuprofen	16.0	381.1555	381.1556	−0.3	$C_{19}H_{25}O_8$	205, 193, 175, 161, 159, 131, 113
39	acyl-migrated isomers of acyl glucuronide of hydroxylated ibuprofen	10.0–11.9	397.1504	397.1511–397.1512	−1.7–(−)1.9	$C_{19}H_{25}O_9$	221, 193, 177, 175, 133, 131, 113
40	ether glucuronide of hydroxylated ibuprofen	1.6	397.1504	397.1501	0.9	$C_{19}H_{25}O_9$	353, 177, 175, 113
45	taurine conjugate of ibuprofen	16.1	312.1275	312.1276	−0.2	$C_{15}H_{22}NO_4S$	269, 124, 107
41–43	taurine conjugates of hydroxylated ibuprofens	1) 10.2 2) 10.7 3) 11.6	328.1224	1) 328.1225 2) 328.1224 3) 328.1227	1) −0.3 2) 0.1 3) −0.9	$C_{15}H_{22}NO_5S$	269, 124, 107
46–48	taurine-ether glucuronide diconjugates of hydroxylated ibuprofens	1) 2.5 2) 4.0 3) 5.4	504.1545	1) 504.1536 2) 504.1563 3) 504.1563	1) 1.8 2) −3.6 3) −3.6	$C_{21}H_{30}NO_{11}S$	328

4.2 Presence of diclofenac, naproxen and ibuprofen and their metabolites in bile of fish exposed to pharmaceuticals in water

While the previous chapter (4.1) discusses the identification of the metabolites of diclofenac (**I**), naproxen (**II**) and ibuprofen (**III**) in fish bile, this chapter describes the presence of pharmaceuticals in bile of fish exposed to the pharmaceuticals *via* water. Rainbow trout were experimentally exposed in laboratory to water containing diclofenac, naproxen and ibuprofen at various concentrations for 10 (**I**–**II**) or 4 days (**III**).

The average concentrations (± standard deviation, SD) of diclofenac, naproxen and ibuprofen in aquaria measured daily were 1.8 ± 0.2 $\mu g\ L^{-1}$of diclofenac (nominal concentration 1.9 $\mu g\ L^{-1}$), 1.6 ± 0.1 $\mu g\ L^{-1}$ of naproxen (nominal concentration 1.4 $\mu g\ L^{-1}$) and 0.17 ± 0.04 $\mu g\ L^{-1}$ of ibuprofen (nominal concentration 0.1 $\mu g\ L^{-1}$). In addition, fish were exposed to three higher concentrations of ibuprofen: 1.9 ± 0.3 $\mu g\ L^{-1}$ (nominal concentration 1.0 $\mu g\ L^{-1}$), 13.0 ± 1.6 $\mu g\ L^{-1}$ (nominal concentration 10 $\mu g\ L^{-1}$) and 145 ± 28 $\mu g\ L^{-1}$ (nominal concentration 100 $\mu g\ L^{-1}$).

The same metabolites were present in rainbow trout bile in aquarium exposures as in the *i.p.* experiments. Large variations (RSD 37–87 %) in the metabolite concentrations between the individuals were found, possible due to individual variations in the uptake, biotransformation and hepatobiliary transport of the compounds (**I**–**II**). Anyhow, these findings prove that diclofenac, naproxen and ibuprofen present in water at the exposure concentrations can be taken up by fishes.

Acyl glucuronides of diclofenac (**5**), of hydroxylated diclofenacs (**6**, **33** and **35**) and acyl glucuronides of naproxen (**36**) and of 6-*O*-desmethylnaproxen (**37**) accounted for in total around 80 to 90 % of the metabolites of diclofenac and naproxen, respectively. The contribution from the phase I metabolites (hydroxylated diclofenacs **4** and **13**, and 6-*O*-desmethylnaproxen, **17**), sulfate conjugate of 5-hydroxydiclofenac (**32**) and unmetabolized diclofenac (**1**) and naproxen (**2**) were low (in total, ca. 20 and 10 %, respectively for diclofenac and naproxen). Since the bile samples from ibuprofen exposures were enzymatically hydrolyzed before the subsequent sample treatment and LC-MS/MS analysis, it was impossible to quantify, how much ibuprofen (**3**) was present in its

unconjugated form or as the different acyl glucuronides or taurine conjugates. Based on the data obtained from the *i.p.* experiments, only trace amounts of ibuprofen were present, and the acyl glucuronides of ibuprofen (**44**) and 2-hydroxyibuprofen (**39**) were the major metabolites.

In order to investigate the relation between the ambient water and the metabolic fate of a pharmaceutical in fish, the total bioconcentration factors (BCF_{bile}) were calculated according to equation (1) for the three pharmaceuticals and their metabolites (Tables 6 and 7).

$$BCF_{bile} = \frac{total\ concentration\ of\ pharmaceutical\ and\ its\ metabolites\ in\ bile\ (\mu g\ L^{-1})}{concentration\ of\ parent\ pharmaceutical\ in\ water\ (\mu g\ L^{-1})} \quad (1)$$

BCF_{bile} is considered to be a practical tool to assess the internal exposure of fish to chemicals, which are readily biotransformed and excreted (103−106), such as pharmaceuticals (102). Ibuprofen and its metabolites was found to be most readily accumulated in bile (BCF_{bile} ranging from 14 000 to 49 000 depending on the exposure concentration) (**III**). The measured BCF_{bile} for naproxen and its metabolites was one order of magnitude lower than for ibuprofen (on average 1170) (**II**), and the BCF_{bile} for diclofenac and its metabolites (on average 520) (**I**) was half of the BCF_{bile} for naproxen. The BCF_{bile} for ibuprofen appeared to vary significantly when the exposure concentration varied. An inverse dependency of ibuprofen BCF_{bile} and exposure concentration was observed, i.e. the highest BCF_{bile} value was found at the lowest exposure concentration. The reason for this might be related to the limited capacity of the system involved in the hepatobiliary secretion at the highest exposures or enhanced performance at the lowest one. A strong positive linear correlation (R^2 = 0.99) between the sum of hydrolyzed ibuprofen and its metabolites in fish bile and the exposure concentration was found (Figure 17). To summarize, a high-capacity of biotransformation and excretion in rainbow trout liver is evident.

Table 6. Total amount of diclofenac (**1**) and naproxen (**2**) and their metabolites (mean ± SD, μg L^{-1}) in the bile of rainbow trout exposed to mean concentrations of 1.8 μg L^{-1} of diclofenac and 1.6 μg L^{-1} of naproxen in experimental aquaria for 10 days at 14 °C (**I**–**II**). Total bioconcentration factors (BCF_{bile}) were calculated according to equation (1). n = number of animals or water samples.

	n	Diclofenac	Naproxen
Total bile concentration, μg L^{-1}	4	915 ± 512	1 873 ± 1 255
Exposure conc. in water, μg L^{-1}	11	1.8 ± 0.2	1.6 ± 0.1
BCF_{bile}	4	520 ± 291	1 170 ± 784

Table 7. Total amount of hydrolyzed ibuprofen (**3**) its metabolites (μg L^{-1}) in the bile of rainbow trout exposed to four concentrations (mean ± SD, μg L^{-1}) of ibuprofen in experimental aquaria for 4 days at 14 °C (**III**). Nine biles were pooled to one sample per exposure concentration due to the low amount of bile. Total bioconcentration factors (BCF_{bile}) were calculated according to equation (1).

	Ibuprofen			
Total bile concentration, μg L^{-1}	8 330	37 700	221 800	2 034 0800
Exposure conc. in water μg L^{-1}	0.17 ± 0.04	1.9 ± 0.3	13 ± 1.6	145 ± 28
BCF_{bile}	49 000	19 900	17 100	14 100

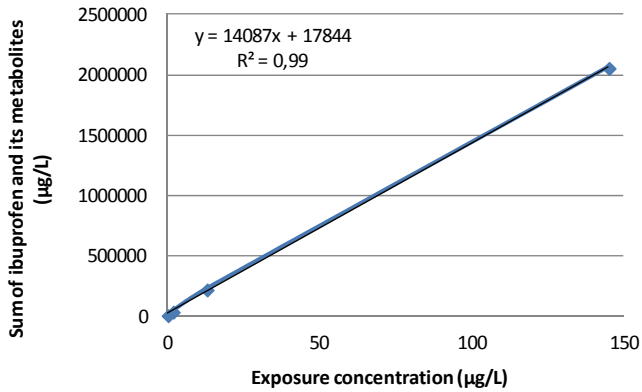


Figure 17. Sum of hydrolyzed ibuprofen (**3**) and its metabolites (**18**, **38** and **45**) (μg L^{-1}) in the bile of rainbow trout plotted against exposure concentration (μg L^{-1}) of ibuprofen in the experimental aquaria (**III**). The exposure time was four days. Each value represents nine animals analyzed in combination.

The measured BCF_{bile} for diclofenac and its metabolites (Table 6) is in the same order as reported by Mehinto et al. (21). Her group exposed rainbow trout to 0.5, 5.0 and 25 $\mu g\ L^{-1}$ of diclofenac for 21 days in aquaria, and BCFs for the parent diclofenac in bile ranged between 509 and 657. The highest BCF was found at the lowest exposure concentration. Schwaiger et al. (17) found that the bioconcentration of diclofenac in rainbow trout liver correlated negatively with the concentration of diclofenac in 28 days exposure in aquaria. The highest BCF_{liver} (2732) of the parent diclofenac was measured at the lowest exposure concentration, 1 $\mu g\ L^{-1,}$ and the lowest BCF_{liver} (12) at the highest exposure concentration, 500 $\mu g\ L^{-1}$. In contrast to Mehinto et al. (21) and Schwaiger et al. (17), Cuklev et al. (23) found the bioconcentration of diclofenac in rainbow trout liver to be fairly stable (2.54 ± 0.36) between the three diclofenac exposure levels (1.6, 11.5 and 81.5 $\mu g\ L^{-1}$) when rainbow trout were exposed to diclofenac for 14 days. Our results from the ibuprofen exposures are in accordance with Schwaiger et al. (17) and Mehinto et al. (21), since the highest BCF_{bile} value was found at the lowest exposure concentration. The finding is also supported by our other study, where higher BCF_{bile} values of diclofenac, naproxen and ibuprofen were found in the bile of rainbow trout at the lower exposure levels (102).

In theory, the BCF of an organic compound should not be much affected by exposure concentration, since the bioconcentration is a results of unsaturated uptake and elimination processes under dynamic equilibrium, unless the concentration impacts the organism in some physiological manner (e.g. enzyme saturation or toxic effect) (107). Decreasing BCFs with increasing water concentration of the studied compounds has also been reported e.g. for chlorophenols (108), perfluorinated chemicals (109) and a synthetic steroid, levonorgestrel (110). Cuklev et al. (111) speculated that the observation could be explained by increased activity of detoxification system at higher exposure concentrations.

4.3 Fish bile metabolites as biomarkers for the exposure to environmental pharmaceuticals

Traditionally, the exposure of fish to environmental organic contaminants has been monitored by analyzing tissue residues, since e.g. hydrophobic chemicals will likely accumulate in tissues with high lipid content (Chapter 1.3). However, the exposure of fish to compounds which are readily metabolized, like many of the pharmaceuticals, cannot be

assessed by measuring their tissue levels. In addition, due to the differences in chemical structures and properties between and within the different therapeutic groups (e.g. anti-inflammatory drugs, β-blockers, antidepressants and antibiotics), uptake and bioconcentration of pharmaceuticals cannot be predicted in general by classical bioaccumulation models (56).

This far, only a limited amount of data exists on the bioconcentration of pharmaceuticals in fish tissues. In general, a fairly low bioconcentration of diclofenac (2.5−29), naproxen (1.4−56) and ibuprofen (2−58) in fish tissues is reported (Table 8). In the study of Brown et al. (112), however, a much higher BFC (18667) for ibuprofen was found in rainbow trout. The method of determining the bioconcentration (i.e. exposure to a single compound or to a mixture of compounds in aquaria vs. exposure to WWTP effluent) can affect the results. Cuklev et al. (23, 111) pointed out that the bioconcentration of diclofenac, naproxen, ibuprofen and ketoprofen measured in fish exposed to WWTP effluent is higher than when the fish are exposed in pure water (i.e. in laboratory).

Besides pharmaceuticals, bile metabolites have been used to monitor xenobiotics present in water, such as chlorophenols (103−104), resin adics (105−106), synthetic and natural hormones (113−115) and polycyclic aromatic hydrocarbons (117). Up to 10^6 fold accumulation of the parent compound from water to fish bile have been reported.

Table 8. Bioconcentration factors (BCF) of parent pharmaceuticals, exposure types (laboratory water exposure or exposure to wastewater treatment plant effluent), exposure concentration and concentration of diclofenac, naproxen and ibuprofen in rainbow trout.

Pharmaceutical	Exposure	Exposure conc. $\mu g\ L^{-1}$	Body fluid or tissue	Conc. $\mu g\ L^{-1}$	BCF	Reference
Diclofenac	Effluent	2.32	Plasma	12	5	(112)
Diclofenac	Effluent	0.7–0.8	Plasma	2.2–20	2.5–29	(116)
Diclofenac	Lab. water exposure	1.8, 43	Plasma	10, 210	5.7, 4.9	(102)
Diclofenac	Lab. water exposure	1.6–81.5	Plasma		4	(23)
Diclofenac	Lab. water exposure	1.6–81.5	Liver		2.5	(23)
Naproxen	Effluent	0.25	Plasma	14	56	(112)
Naproxen	Effluent	1.2–1.8	Plasma	33–46	26–28	(116)
Naproxen	Lab. water exposure	1.6, 40	Plasma	2.7, 55	1.6, 1.4	(102)
Ibuprofen	Effluent	0.0045	Plasma	84	18667	(112)
Ibuprofen	Effluent	0.1–2.2	Plasma	5.5–102	21–58	(116)
Ibuprofen	Lab. water exposure	1.0, 25	Plasma	4.2, 88	4.3, 3.3	(102)
Ibuprofen	Lab. water exposure	3	Muscle		2	(85)
Ibuprofen	Lab. water exposure	3	Adipose fin		24	(85)

4.4 Pharmaceuticals in a lake receiving municipal effluents and exposure of wild fish populations

Lake Haapajärvi (Figure 15) receives municipal wastewater effluents *via* River Rakkolanjoki. In February, May, July and November 2010, lake water samples from two sampling sites (A and B, Figure 18) were collected and the presence of 13 pharmaceuticals was determined (**IV**). At site A, 10 to 11 and at site B, 8 to 11 different pharmaceuticals could be detected and quantified (Table 9). The antiepileptic drug carbamazepine was the major pharmaceutical in all samples, but also diclofenac (**1**), naproxen (**2**) and ibuprofen (**3**) were detected at concentrations ranging from 10 to 302 ng L^{-1} with the exception of ibuprofen, which was not detected at site B in July. Since the water is flowing from sampling point A to B, a distance of 2 km, the finding of pharmaceuticals at both points shows that the compounds are spread over large areas of the lake. The found concentrations (i.e. low ng L^{-1}) are in accordance with earlier results obtained from rivers receiving WWTP effluents in Finland and Sweden (3, 5, 6–7).

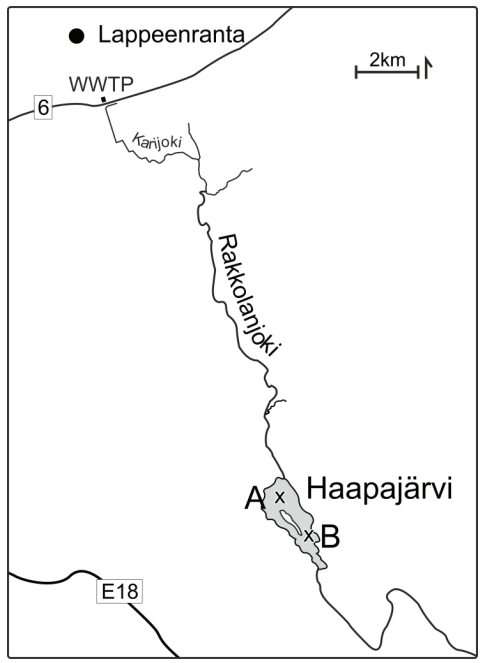


Figure 18. The river-lake investigated in this study (**IV**): River Rakkolanjoki and Lake Haapajärvi. Water sampling sites A and B in Lake Haapajärvi.

Table 9. Concentration of pharmaceuticals (ng L^{-1}) in Lake Haapajärvi water sampled at four times (February 22; May 25; July 7 and November 18) during 2010. nd = not detected above limit of detection, LOD. Chemical structures of the analyzed pharmaceuticals are depicted in Figures S1 and S2.

Acidic pharmaceutical	**February**		**May**		**July**		**November**	
	Site A	Site B	Site A	Site B	Site A	Site B	Site A	Site B
Bezafibrate	22	24	8	9	7	7	6	6
Diclofenac	302	231	33	60	22	22	168	145
Ibuprofen	69	55	17	39	18	nd*	10	16
Ketoprofen	85	106	30	nd*	32	36	24	19
Naproxen	210	98	61	89	54	43	43	40
Basic pharmaceutical	**February**		**May**		**July**		**November**	
	Site A	Site B	Site A	Site B	Site A	Site B	Site A	Site B
Atenolol	98	93	38	38	23	25	43	91
Bisoprolol	104	195	47	63	92	58	2	3
Sotalol	55	53	18	23	23	23	4	4
Citalopram	1	1	nd*	nd*	nd*	nd*	4	3
Fluoxetine	nd*	nd*	nd*	nd*	nd*	nd*	nd*	nd*
Setraline	nd*	nd*	nd*	nd*	nd*	nd*	nd*	nd*
Venlafaxine	2	2	1	1	1	nd*	14	15
Carbamazepine	355	318	109	114	216	212	111	103

* LOD (ibuprofen): 5 ng L^{-1}; LOD (ketoprofen): 12.5 ng L^{-1}; LOD (citalopram): 0.1 ng L^{-1}; LOD (fluoxetine): 12.5 ng L^{-1}; LOD (sertraline): 1.0 ng L^{-1}; LOD (venlafaxine): 1 ng L^{-1}

To study whether the pharmaceuticals found in the water of Lake Haapajärvi are also present in fish, wild bream and roach bile were collected on May 2011. Prior to analyses, the bile content was enzymatically treated to convert the glucuronide and sulfate conjugates into the original pharmaceuticals or phase I metabolites (Chapter 4.3). In all, the presence of 17 pharmaceuticals and six phase I metabolites was monitored and compared to reference bile of bream and roach from the Southern Lake Saimaa. The chemical structures of the monitored compounds are presented in Figures S1 and S2.

Although a wide range of pharmaceuticals were present in Lake Haapajärvi, only diclofenac (**1**), naproxen (**2**) and ibuprofen (**3**) could be detected in the bile samples. Naproxen was detected in all bream and all roach bile samples. In bream, the concentration ranged from 6 to 32 ng mL^{-1} and in roach from 11 to 103 ng mL^{-1} (Table 10). Diclofenac was found in all but one bream and one roach samples and the observed bile concentrations were 6–95 ng mL^{-1} and 44–148 ng mL^{-1} in bream and roach, respectively. Ibuprofen was present in three of the bream bile samples at concentrations of 16–34 ng mL^{-1} and in two of the roach bile samples at concentrations of 15–26 ng mL^{-1}. In bream and roach, the mean concentration of diclofenac was 2–3 times higher than the concentration of naproxen or ibuprofen. Pharmaceuticals were not found in wild fish bile samples caught from Lake Saimaa (reference lake).

Table 10. The mean concentrations (ng mL^{-1}) of diclofenac, naproxen and ibuprofen after enzymatic hydrolysis in the bream and roach biles caught from Lake Haapajärvi. n = number of samples where the compound was detected out of six samples. Five out of the six roach samples are pooled. One pooled sample corresponds to bile from two to five individuals.

Fish species	Diclofenac ng mL^{-1}	n/6	Naproxen ng mL^{-1}	n/6	Ibuprofen ng mL^{-1}	n/6
Bream	50 ± 35	5/6	21 ± 10	6/6	25 ± 9	3/6
Roach	88 ± 45	5/6	38 ± 34	6/6	21 ± 7	2/6

In a previous work, we analyzed pharmaceuticals in the bile hydrolysates collected from rainbow trout kept in cages close to the discharge point of WWTPs for 10 days (118). In one of the sample points of the study, the concentrations of diclofenac, naproxen and ibuprofen have been reported to be close to those we found in Lake Haapajärvi (i.e. diclofenac 20–44 ng L^{-1}, naproxen 22–129 ng L^{-1} and ibuprofen 7–82 ng L^{-1}) (4). At this site, we found that in the caged fish, the bile content of diclofenac, naproxen and ibuprofen ranged from 29–194 ng mL^{-1}, 11–84 ng mL^{-1} and not detected–71 ng mL^{-1}, respectively (118). The concentrations are close to those observed in the current study. However, this comparison is based on the assumption that pharmaceuticals are taken up and metabolized in the same way and to the same extent in various fish species (rainbow trout, roach and bream). This

may not be the case and therefore a strict comparison of the results of the two studies cannot be made.

Phase I metabolites were not detected in the hydrolyzates. The phase I metabolites are more polar than the parent compounds and therefore their retention on the chromatographic column is poor. The metabolites elute together with the numerous other highly polar bile constituents and consequently, their signals may be subjected to ion suppression. Further, the ion signals of the phase I metabolites are weaker in intensity than the signals of the parent compounds. This may explain why low amounts of phase I metabolites possibly present in the hydrolyzates could not be detected.

Neither ketoprofen nor bezafibrate was detected in the bream and roach biles. The result is in accordance with Brown et al. (112), who found a very low proportion of uptake of ketoprofen to trout plasma after exposure to high concentration (490 μg L^{-1}) of ketoprofen. When fish were exposed to WWTP effluent with ketoprofen concentration ranging from 0.1 to 0.28 μg L^{-1}, ketoprofen was not detected in blood plasma (112). In a recent study of the group, BCF_{plasma} of ketoprofen in fish was found to vary between 3.5 and 48 at higher effluent ketoprofen concentrations (ranging from 1.3 to 4.3 μg L^{-1}) (116). Fick et al. (116) was unable to detect bezafibrate in trout blood plasma, although the concentration in WWTP effluents was very low (ranging from 3 to 9 ng L^{-1}).

As carbamazepine was the dominating pharmaceutical in the water samples collected in 2010, it was most likely present in significant concentrations also at the time when the natural fish populations were sampled. In a preliminary study, we have found that carbamazepine is excreted only to a lower degree, when compared e.g. with diclofenac, into the bile of laboratory exposed rainbow trout. Togunde et al. (86) also reported negligible bioconcentration (0.06–0.26) of carbamazepine in rainbow trout bile exposed to 2.9, 24.7 and 175.3 μg L^{-1} of carbamazepine in aquarium. In addition, the BCFs of carbamazepine in fish plasma, muscle and adipose fin was found to be low (i.e. 0.3–0.4, 0.5 and 4, respectively for plasma, muscle and adipose fin) (85, 102). Ramirez et al. (119) were able to detected low amounts of carbamazepine in wild fish fillet (mean of 2.3 ng g^{-1}) and liver (mean of 6 ng g^{-1}) in one of the five study sites in USA.

Antidepressant drugs and their major phase I metabolites have been detected in wild fish tissues such as liver, muscle and brain (119−121). In Lake Haapajärvi, the antidepressant drugs fluoxetine and setraline were not detected above their LODs (12.5 and 1.0 ng L^{-1}, respectively) and citalopram and venlafaxine were found at only very low concentrations. Therefore these drugs are not likely to be found in the fish bile. Togunde et al. (86) was unable to detect carbamazepine or fluoxetine in fathead minnow (*Pimephales promelas*) bile exposed to WWTP effluent in cages, but low concentrations (0.22−2.8 ng mL^{-1}) of venlafaxin, norfluoxetine and sertraline were detected. In earlier laboratory studies, fluoxetine and its metabolite norfluoxetine has been shown to bioconcentrate in Japanese medaka (*Oryzias latipes*) (BCFs up to 3100 and 3700 for fluoxetine and norfluoxetine in the liver, respectively, and 260 and 650 in the whole body) and in rainbow trout (BCF 143 for fluoxetine in adipose fin and 59 in muscle) tissues with exposure concentrations ranging between 3 and 10 μg L^{-1} (54, 85).

The β-blockers (atenolol, bisoprolol and sotalol) were found at the same concentration range as diclofenac, naproxen and ibuprofen in the lake water, but they were not found in the bile. To our knowledge, β-blockers have not been detected in fish tissues in previous studies (118, 120). At present, nothing seems to be known about the pharmacokinetics of β-blockers in fish (122), which means that also the extent of uptake and the route of excretion (renal or bile) remains to be clarified. On the other hand, Lahti and Oikari (123−124) found fairly high concentrations of citalopram, bisoprolol and acebutolol in settleable particulate matter and in sediment cores collected from several sites in Finland, including Lake Haapajärvi and Lake Päijänne. These findings reflect different environmental fate scenarios of the given pharmaceuticals rather than the uptake to fish and excretion in bile (118, 123−124). In summary, the study (**IV**) shows that pharmaceuticals originating from WWTP effluents can be traced to populations of wild bream and roach living in a lake where, at least diclofenac, naproxen and ibuprofen are present as pollutants.

4.5 The usefulness of fish bile as a tissue for monitoring pharmaceuticals in the environment

Since liver is the primary site of biotransformation in fish, and the formed metabolites are released to the intestine *via* the common bile duct, the bile metabolite levels can be used as a reliable biomarker of exposure of fish to xenobiotics. Although the transportation of metabolites to bile is an elimination pathway, the drugs or their metabolites might enter the enterohepatic circulation, which may result in a prolonged exposure of the fish (20). The longer residence time in the body promotes a higher degree of exposure and may thus enhance toxic effects.

Because bile metabolites are good biomarkers of the internal exposure of the readily metabolized compounds, technically a fairly low volume (20–100 μL) of fish bile is needed for the trace analysis of the metabolites (**I–IV**, 102). In experiments performed in the laboratory (102) and in field (118), the concentration of pharmaceutical and its metabolites in bile has been reported to be several orders of magnitude higher than the concentration of the parent pharmaceutical in the plasma. To summarize, a lower amount of pharmaceutical, or xenobiotic in general, present in water can be more easily detected in fish bile than in plasma.

Although bile can be a good matrix to study pharmaceuticals present in fish tissues, the bile analysis has several uncertainties, which are mainly connected to bile secretion and the physicochemical properties of the compound of interest. Feeding status of the fish will affect the metabolite level and bile volume, since it is commonly suggested that the fish will empty the gall bladder to the intestine soon after feeding (125). In laboratory exposure studies or in studies with caged fish, the fullness of gall bladder can be to some extent controlled, e.g. by not feeding the animals during the short term exposures (**I–III**, 102) or for a few days prior the caging (118, 126). It should be borne in mind that pharmaceutical levels in WWTP effluent might also vary from day to day and the metabolites in the fish bile presents the exposure fish was subjected to during the last few days.

The analysis of bile metabolites requires knowledge of the metabolism on the studied compounds in fish. The metabolism and biliary excretion might vary between fish species (103, 105). To simplify and sensitize the analytical procedure, phase II conjugates in bile are

often hydrolyzed enzymatically (e.g. by *β*-glucuronidase) or chemically (by alkaline or acidic solution) back to phase I compounds or parent compounds (**III–IV**, 103, 105, 118, 127). Therefore all the pure metabolite standards are not necessarily needed for the quantitative analysis. In rainbow trout, acyl glucuronides were the major bile metabolites of diclofenac (**I**), naproxen (**II**) and ibuprofen (**III**). These conjugates, however, are reactive and can therefore form isomers at physiological pH or during sample preparation through acyl migration (Chapter 1.3.1). Acyl migration will complicate the analysis, since one compound is presented by several chromatographic peaks in the chromatograms. Also, the acyl migrated and anomerizated isomers are resistant against stereospecific *β*-glucuronidase (81−82). In addition, due to the interindividual physiological variation, the bile analyses have to be performed on a sufficient number of fish.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

The objective of this thesis was to gain knowledge on the uptake and biotransformation of pharmaceuticals in fish. Initially, the biotransformation and excretion patterns of three anti-inflammatory drugs in rainbow trout liver were studied. The summary of the biotransformation pathways observed is presented in Figure 19. Secondly, the uptake, biotransformation and biliary excretion of diclofenac, naproxen and ibuprofen in fish exposed to the drugs *via* water were shown. In addition, a high capacity system for elimination of pharmaceuticals in rainbow trout liver was evident. The highest bioconcentration factors (BCF_{bile}, the ratio of the sum of parent compound and its metabolites in bile and the concentration of the parent compound in water) was found for ibuprofen followed by diclofenac and naproxen. The glucuronidation and taurine conjugation were the main biotransformation pathways of the parent compound and their phase I metabolites, and the same metabolites were present in fish bile in exposures *via* ambient water as in *i.p.* experiments.

Following the laboratory exposures, it was studied, whether the exposure also takes place in wild fish populations living in water contaminated with WWTP effluents. The outcome of the study clearly showed that diclofenac, naproxen and ibuprofen were present in bream and roach bile. In the bream and roach bile, the concentrations of diclofenac, naproxen and ibuprofen were roughly 1000 times higher than those in the lake water. In the laboratory exposures, the bioconcentration of the compounds and their metabolites in rainbow trout bile ranged from the same level as in wild fish to a magnitude higher. Thus, the parent compound and its metabolites in fish bile can be used as a reliable biomarker to monitor the exposure of fishes to environmental pharmaceuticals present in waters receiving discharges from WWTPs.

Several studies have reported physiological and behavioral effects on fish exposed to a certain pharmaceutical or their mixtures at near environmental concentrations. Since acyl glucuronides are connected to adverse effects in humans and they present the main biotransformation pathway in fish, the reactive metabolites have been postulated to cause the physiological changes also found in rainbow trout after laboratory exposure. The detection of pharmaceutical residues in wild fish bile implies that fish are internally

exposed to mixtures of pharmaceuticals over their whole lifespan due to chronic exposure to WWTP effluents. This means that the mixtures are also present physiologically uphill, i.e. in fish blood. Very little, if anything is known about the toxicities and about the biological effects of fish chronically exposed to mixtures of pharmaceuticals. Further work is urgently needed where the effect of an individual pharmaceutical and of their mixtures on fish can be determined. Only when such studies have been performed, it is possible to evaluate whether pharmaceutical residues in the aquatic environment pose a risk to wild fish populations and on the aquatic environment as a whole. In addition, the biotransformation of other pharmaceuticals than the three compounds studied, diclofenac, naproxen and ibuprofen, in fish should be researched. Wild fish population sampling should be performed in other locations with a higher number of fish individuals and species.

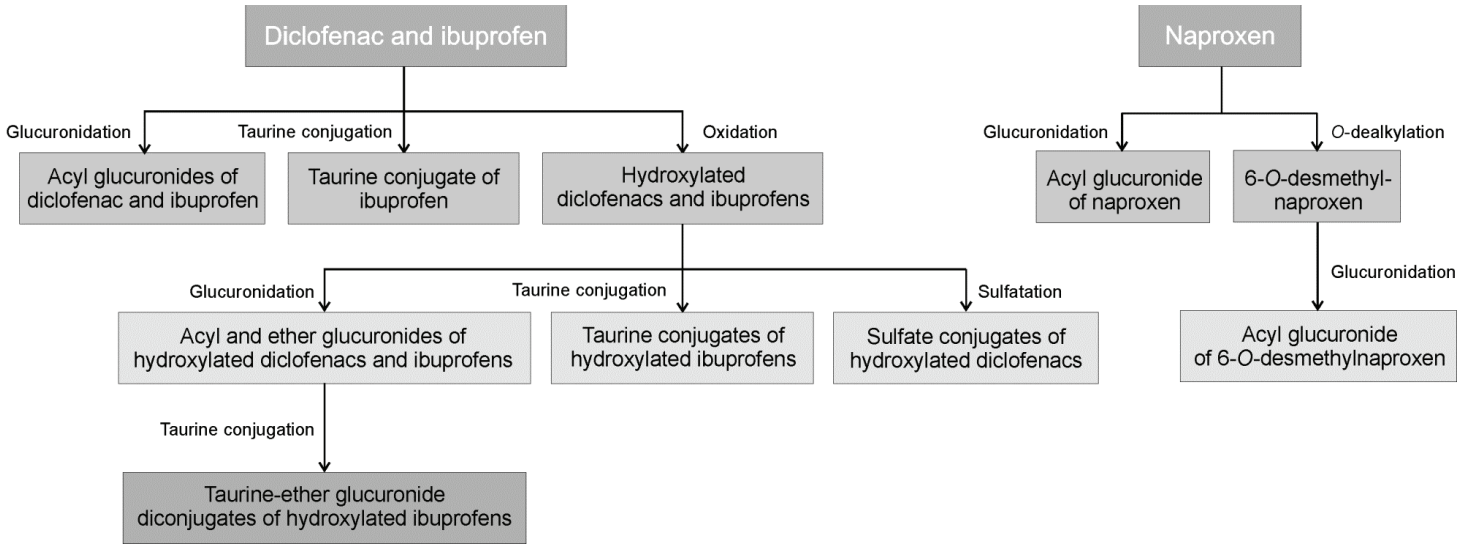


Figure 19. A summary of biotransformation of diclofenac, naproxen and ibuprofen in rainbow trout.

REFERENCES

(1) Halling-Sørensen, B.; Nors Nielsen, S.; Lanzky, P.F.; Ingerslev, F.; Holten Lützhøft, H.C.; Jørgensen, S.E. Occurrence, fate and effects of pharmaceutical substances in the environment – A review. *Chemosphere* **1998**, *36,* 357–393.

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

(3) Lindqvist, N.; Tuhkanen, T.; Kronberg, L. Occurrence of acidic pharmaceuticals in raw and treated sewages and in receiving waters. *Water Res.* **2005**, *39,* 2219–2228.

(4) Vieno, N.M.; Tuhkanen, T.; Kronberg, L. Seasonal variation in the occurrence of pharmaceuticals in effluents from a sewage treatment plant and in the recipient water. *Environ. Sci. Technol.* **2005**, *39,* 8220–8226.

(5) Vieno, N.M; Tuhkanen, T.; Kronberg, L. Analysis of neutral and basic pharmaceuticals in sewage treatment plants and in recipient rivers using solid phase extraction and liquid chromatography–tandem mass spectrometry detection. *J. Chromatogr. A.* **2006**, *1134*, 101–111.

(6) Daneshvar, A.; Svanfelt, J.; Kronberg, L.; Weyhenmeyer, G.A. Winter accumulation of acidic pharmaceuticals in a Swedish river. *Environ. Sci. Pollut. Res.* **2010**, *17*, 908–916.

(7) Daneshvar, A.; Svanfelt, J.; Kronberg, L.; Prévost, M.; Weyhenmeyer, G.A. Seasonal variations in the occurrence and fate of basic and neutral pharmaceuticals in a Swedish river-lake system. *Chemosphere* **2010**, *80*, 301–309.

(8) Ternes, T.A. Occurrence of drugs in German sewage treatment plants and rivers. *Wat. Res.* **1998**, *32*, 3245–3260.

(9) Buser, H.-R.; Poiger, T.; Müller, M.D. Occurrence and environmental behavior of the chiral pharmaceutical drug ibuprofen in surface waters and in wastewater. *Environ. Sci. Technol.* **1999**, *33*, 2529–2535.

(10) Quintana, J.B.; Weiss, S.; Reemtsma, T. Pathways and metabolites of microbial degradation of selected acidic pharmaceutical and their occurrence in municipal wastewater treated by a membrane bioreactor. *Water Res.* **2005**, *39,* 2654–2664.

(11) Vieno, N.M; Tuhkanen, T.; Kronberg, L. Elimination of pharmaceuticals in sewage treatment plants in Finland. *Wat. Res.* **2007**, *41*, 1001–1012.

(12) Perez, S.; Barcelo, D. First evidence for occurrence of hydroxylated human metabolites of diclofenac and aceclofenac in wastewater using QqLIT-MS and QqTOF-MS. *Anal. Chem.* **2008**, *80,* 8135–8145.

(13) Fent, K.; Weston, A.A.; Caminada, D. Ecotoxicology of human pharmaceuticals. *Aquat. Toxicol.* **2006**, *76,* 122–159.

(14) Agüera, A.; Estrada, L.A.P.; Ferrer, I.; Thurman, E.M.; Malato, S.; Fernández-Alba, A.R. Application of time-of-flight mass spectrometry to the analysis of phototransformation products of diclofenac in water under natural sunlight. *J. Mass Spectrom.* **2005**, *40,* 908–915.

(15) Chiron, S.; Minero, C.; Vione, D. Photodegradation processes of the antiepileptic drug carbamazepine, relevant to estuarine waters. *Environ. Sci. Technol.* **2006**, *40,* 5977–5983.

(16) Eriksson, J.; Svanfelt, J.; Kronberg, L. A Photochemical study of diclofenac and its major transformation products. *Photochem. Photobiol.* **2010**, *86,* 528–532.

(17) Schwaiger, J.; Ferling, H.; Mallow, U.; Wintermayr, H.; Negele, R.D. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac: Part I: histopathological alterations and bioaccumulation in rainbow trout. *Aquat. Toxicol.* **2004**, *68,* 141-150.

(18) Triebskorn, R.; Casper, H.; Heyd, A.; Eikemper, R.; Köhler, H.-R.; Schwaiger, J. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac: Part II. Cytological effects in liver, kidney, gills and intestine of rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* **2004**, *68,* 151–166.

(19) Triebskorn, R.; Casper, H.; Scheil, V.; Schwaiger, J. Ultrastructural effects of pharmaceuticals (carbamazepine, clofibric acid, metoprolol, diclofenac) in rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*). *Anal. Bioanal. Chem.* **2007,** *387,* 1405–1416.

(20) Hoeger, B.; Köllner, B.; Dietrich, D.R.; Hitzfeld, B. Water-borne diclofenac affects kidney and gill integrity and selected immune parameters in brown trout (*Salmo trutta* f. *fario*). *Aquat. Toxicol.* **2005**, *75,* 53–64.

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

(22) Han, S.; Choi, K.; Kim, J.; Ji, K.; Kim, S.; Ahn, B.; Yun, J.; Choi, K.; Khim, J.S.; Zhang, X.; Giesy, J.P. Endocrine disruption and consequences of chronic exposure to ibuprofen in Japanese medaka (*Oryzias latipes*) and freshwater cladocerans *Daphnia magna* and *Moina macrocopa. Aquat. Toxicol.* **2010**, *98*, 256–264.

(23) Cuklev, F.; Kristiansson, E.; Fick, J.; Asker, N.; Förlin, L.; Larsson, D.G.J. Diclofenac in fish: blood plasma levels similar to human therapeutic levels affect global hepatic gene expression. *Environ. Toxicol. Chem.* **2011**, *30*, 2126–2134.

(24) Ferrari, B.; Paxeus, N.; Lo Giudice, R.; Pollio, A.; Garric, J. Ecotoxicological impact of pharmaceuticals found in treated wastewaters: study of carbamazepine, clofibric acid, and diclofenac. *Ecotoxicol. Environ. Saf.* **2003,** *55,* 359–370.

(25) Haap, T.; Triebskorn, R.; Köhler, H. Acute effects of diclofenac and DMSO to *Daphnia magna*: Immobilisation and hsp70-induction. *Chemosphere* **2008,** *73,* 353–359.

(26) Isidori, M.; Lavorgna, M.; Nardelli, A.; Parrella, A.; Previtera, L.; Rubino, M. Ecotoxicity of naproxen and its phototransformation products. *Sci. Tot. Environ.* **2005**, *348*, 93–101.

(27) Schmitt-Jansen, M.; Bartels, P.; Adler, N.; Altenburger, R. Phytotoxicity assessment of diclofenac and its phototransformation products. *Anal. Bioanal. Chem.* **2007**, *387*, 1389–1396.

(28) Finnish Medicines Agency FIMEA **2012**. Lääkekulutus vuosina 2007–2010 (Finnish drug consumption during years 2007–2010). http://www.fimea.fi/laaketieto/kulutustiedot.

(29) Lishman, L.; Smyth, S.A.; Sarafin, K.; Kleywegt, S.; Toito, J.; Peart, T.; Lee, B.; Servos, M.; Beland, M.; Seto, P. Occurrence and reductions of pharmaceuticals and personal care products and estrogens by municipal wastewater treatment plants in Ontario, Canada. *Sci. Tot. Environ.* **2006**, *367*, 544–558.

(30) Buser, H.-R.; Poiger, T.; Müller, M.D. Occurrence and fate of the pharmaceutical drug diclofenac in surface waters: rapid photodegradation in a lake. *Environ. Sci. Technol.* **1998**, *32*, 3449–3456.

(31) Poiger, T.; Buser, H.-R.; Müller, M.D. Photodegradation of the pharmaceutical drug diclofenac in a lake: pathway, field measurements, and mathematical modeling. *Environ. Toxicol. Chem.* **2001**, *20*, 256–263.

(32) Lin, A.Y.-C.; Reinhard, M. Photodegradation of common environmental pharmaceuticals and estrogens in river water. *Environ. Toxicol. Chem.* **2005,** *24*, 1303–1309.

(33) Fono, L.J.; Kolodziej, E.P.; Sedlak, D.L. Attenuation of wastewater-derived contaminants in an effluent-dominated river. *Environ. Sci. Technol.* **2006**, *40*, 7257–7262.

(34) Lin, A.Y.-C.; Plumlee, M.H.; Reinhard, M. Natural attenuation of pharmaceuticals and alkylphenol polyethoxylate metabolites during river transport: photochemical and biological transformation. *Environ. Toxicol. Chem.* **2006**, *25*, 1458–1464.

(35) Radke, M.; Ulrich, H.; Wurm, C.; Kunkel, U. Dynamics and attenuation of acidic pharmaceuticals along a river stretch. *Environ. Sci. Technol.* **2010**, *44*, 2968–2974.

(36) European Union **2012.** Proposal for a directive of the European parliament and of the council amending directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. http://ec.europa.eu/environment/water/water-dangersub/pdf/com_2011_876.pdf.

(37) Oaks, J.L.; Gilbert, M.; Virani, M.Z.; Watson, R.T.; Meteyer, C.U.; Rideout, B.A.; Shivaprasad, H.L.; Ahmed, S.; Iqbal Chaudhry, M.J.; Arshad, M.; Mahmood, S.; Ali, A.; Ahmed Khan, A. Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature* **2004**, *427,* 630–633.

(38) Swan, G.E.; Cuthbert, R.; Quevedo, M.; Green, R.E.; Pain, D.J.; Bartels, P.; Cunningham, A.A.; Duncan, N.; Meharg, A.A.; Lindsay Oaks, J.; Parry-Jones, J.; Shultz, S.; Taggart, M.A.; Verdoorn, G.; Wolter, K. Toxicity of diclofenac to *Gyps* vultures. *Biol. Lett.* **2006**, *2,* 279–282.

(39) Swan, G.; Naidoo, V.; Cuthbert, R.; Green, R.E.; Pain, D.J.; Swarup, D.; Prakash, V.; Taggart, M.; Bekker, L.; Das, D.; Diekmann, J.; Diekmann, M.; Killian, E.; Meharg, A.; Patra, R.C.; Saini, M.; Wolter, K. Removing the threat of diclofenac to critically endangered Asian vultures. *PLoS Biol.* **2006**, *4,* 395–402.

(40) Taggart, M.A.; Cuthbert, R.; Das, D.; Sashikumar, C.; Pain, D.J.; Green, R.E.; Feltrer, Y.; Shultz, S.; Cunningham, A.A.; Meharg, A.A. Diclofenac disposition in Indian cow and goat with reference to *Gyps* vulture population declines. *Environ. Pollut.* **2007**, *147,* 60–65.

(41) Naidoo, V.; Wolter, K.; Cuthberg, R.; Duncan, N. Veterinary diclofenac threatens Africa's endangered vulture species. *Reg. Toxicol. Pharmacol.* **2009**, *53,* 205–208.

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

(43) Flippin, J.L.; Huggett, D.; Foran, C.M. Changes in the timing of reproduction following chronic exposure to ibuprofen in Japanese medaka, *Oryzias latipes*. *Aquat. Toxicol.* **2007**, *81,* 73–78.

(44) David, A.; Pancharatna, K. Developmental anomalies induced by a non-selective COX inhibitor (ibuprofen) in zebrafish (*Danio rerio*). *Environ. Toxicol. Pharmacol.* **2009**, *27*, 390–395.

(45) Han, S.; Choi, K.; Kim, J.; Ji, K.; Kim, S.; Ahn, B.; Yun, J.; Choi, K.; Khim, J.S.; Zhang, X.; Giesy, J.P. Endocrine disruption and consequences of chronic exposure to ibuprofen in Japanese medaka (*Oryzias latipes*) and freshwater cladocerans *Daphnia magna* and *Moina macrocopa. Aquat. Toxicol.* **2010**, *98,* 256–264.

(46) Gravel, A.; Vijayan, M.M. Non-steroidal anti-inflammatory drugs disrupt the heat shock response in rainbow trout. *Aquat. Toxicol.* **2007**, *81,* 197–206.

(47) Manallack, D.T. The pK_a distribution of drugs: application to drug discovery. *Perspect. Medicin. Chem.* **2007**, *1,* 25–38.

(48) Franco, A.; Ferranti, A.; Davidsen, C.; Trapp, S. An unexpected challenge: ionizable compounds in the REACH chemical space. *Int. J. Life Cycle Assess.* **2010**, *15,* 321–325.

(49) Mackay, D.; Fraser, A. Bioaccumulation of persistent organic chemicals: mechanisms and models. *Env. Pollut.* **2000**, *110,* 375–391.

(50) Schwarzenbach, R.P.; Gschwend P.M.; Imboden D.M. *Environmental Organic Chemistry*, 2 ed.; Wiley: New Jersey, USA, **2003**.

(51) Fu, W.; Franco, A.; Trapp, S. Methods for estimating the bioconcentration factor of ionizable organic chemicals. *Environ. Toxicol. Chem.* **2009**, *28*, 1372–1379.

(52) Saarikoski, J.; Viluksela, M. Influence of pH on the toxicity of substituted phenols to fish. *Arch. Environm. Contam. Toxicol.* **1981**, *10*, 747–753.

(53) Erickson, R.J.; McKim, J.M.; Lien, G.J.; Hoffman, A.D.; Batterman, S.L. Uptake and elimination of ionizable organic chemicals at fish gills: II. Observed and predicted effects of pH, alkalinity, and chemical properties. *Environ. Toxicol. Chem.* **2006,** *25*, 1522–1532.

(54) Nakamura, Y.; Yamamoto, H.; Sekizawa, J.; Kondo, T.; Hirai, N.; Tatarazako, N. The effects of pH on fluoxetine in Japanese medaka (*Oryzias latipes*): Acute toxicity in fish larvae and bioaccumulation in juvenile fish. Chemosphere **2008**, *70*, 865–873.

(55) Valenti, T.W.; Perez Hurtado, P.; Chambliss, C.K.; Brooks, B.W. Aquatic toxicity of sertraline to *Pimephales promelas* at environmentally relevant surface water pH. *Environ. Toxicol. Chem.* **2009**, *28*, 2685-2694.

(56) Daughton, C.G.; Brooks, B.W. Active pharmaceutical ingredients and aquatic organisms. In *Environmental contaminants in biota: interpreting tissue concentrations*, 2nd ed.; Nelson Beyer W.; Meador, J.P. Eds.; CRC Press, Taylor and Francis, Boca Raton, FL, USA, **2011**; pp 286−347.

(57) Erickson, R.J.; McKim, J.M.; Lien, G.J.; Hoffman, A.D.; Batterman, S.L. Uptake and elimination of ionizable organic chemicals at fish gills: I. Model formulation, parameterization, and behavior. *Environ. Toxicol. Chem.* **2006**, *25*, 1512–1521.

(58) Lehman-McKeeman, L.D. Absorption, distribution, and excretion of toxicants. In *Casarett & Doull's toxicology: the basic science of poisons*, 7 ed.; Klaassen, C.D. Ed.; McGraw-Hill Medical, New York, NY, USA, **2008**, pp. 131–159.

(59) Miller, D.S. Cellular transport and elimination. In *Molecular and Biochemical Toxicology*, 4 ed.; Smart, R.C.; Hodgson E. Eds.; John Wiley & Sons, Inc, Hoboken, NJ, USA, **2008**, pp. 273–285.

(60) Parkinson, A.; Ogilvie, B.W. Biotransformation of xenobiotics. In *Casarett & Doull's toxicology: the basic science of poisons*, 7 ed.; Klaassen, C.D. Ed.; McGraw-Hill Medical, New York, NY, USA, **2008**, pp. 161–304.

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

(62) Bort, R.; Macé, K.; Boobis, A.; Gómez-Lechón, M.-J.; Pfeifer, A.; Castell, J. Hepatic metabolism of diclofenac: role of human CYP in the minor oxidative pathways. *Biochem. Pharmacol.* **1999**, *58,* 787–796.

(63) Corcoran, O.; Nicholson, J.K.; Lenz, E.M.; Abou-Shakra, F.; Castro-Perez, J.; Sage, A.B.; Wilson, I.D. Directly coupled liquid chromatography with inductively coupled plasma mass spectrometry and orthogonal acceleration time-of-flight mass spectrometry for the identification of drug metabolites in urine: application to diclofenac using chlorine and sulfur detection. *Rapid Commun. Mass Spectrom.* **2000,** *14*, 2377–2384.

(64) Kenny, J.R.; Maggs, J.L.; Meng, X.; Sinnott, D.; Clarke, S.E.; Park, B.K.; Stachulski, A.V. Syntheses and characterization of the acyl glucuronide and hydroxy metabolites of diclofenac. *J. Med. Chem.* **2004,** *47,* 2816–2825.

(65) Sarda, S.; Page, C.; Pickup, K.; Schultz-Utermoehl, T.; Wilson, I. Diclofenac metabolism in the mouse: novel *in vivo* metabolites identified by high performance liquid chromatography coupled to linear ion trap mass spectrometry. *Xenobiotica* **2012**, *42*, 179–194.

(66) Tang, W.; Stearns, R.A.; Bandiera, S.M.; Zhang, Y.; Raab, C., Braun, M.P.; Dean, D.C.; Pang, J.; Leung, K.H.; Doss, G.A.; Strauss, J.R.; Kwei, G.Y.; Rushmore, T.H.; Chiu, S.-H.L.; Baillie, T.A. Studies on cytochrome P-450-mediated bioactivation of diclofenac in rats and in human hepatocytes: identification of glutathione conjugated metabolites. *Drug Metab. Dispos.* **1999**, *27*, 365–372.

(67) Grillo, M.P.; Knutson, C.G.; Sanders, P.E.; Waldon, D.J.; Hua, F.; Ware, J.A. Studies on the chemical reactivity of diclofenac acyl glucuronide with glutathione: identification of diclofenac-*S*-Acyl-glutathione in rat bile. *Drug Metab. Dispos.* **2003**, *31*, 1327–1336.

(68) Waldon, D.J.; Teffera, Y.; Colletti, A.E.; Liu, J.; Zurcher, D.; Copeland, K.W.; Zhao, Z. Identification of quinone imine containing glutathione conjugates of diclofenac in rat bile. *Chem. Res. Toxicol.* **2010**, *23*, 1947–1953.

(69) Gröning, J.; Held, C.; Garten, C.; Claußnitzer, U.; Kaschabek, S.R.; Schlömann, M. Transformation of diclofenac by the indigenous microflora of river sediments and identification of a major intermediate. *Chemosphere* **2007**, *69*, 509–516.

(70) Jaggi, R.; Addison, R.S.; King, A.R.; Suthers, B.D.; Dickinson, R.G. Conjugation of desmethylnaproxen in the rat – a novel acyl glucuronide-sulfate diconjugate as a major biliary metabolite. *Drug Metab. Dispos.* **2002**, *30*, 161–166.

(71) Tan, S.C.; Patel, B.K.; Jackson, S.H.D.; Swift, C.G.; Hutt, A.J. Stereoselectivity of ibuprofen metabolism and pharmacokinetics following the administration of the racemate to healthy volunteers. *Xenobiotica* **2002**, *32*, 683–697.

(72) Shirley, M.A.; Guan, X.; Kaiser, D.G.; Halstead, G.W.; Baillie, T.A. Taurine conjugation of ibuprofen in humans and in rat liver *in vitro*. Relationship to metabolic chiral inversion. *J. Pharmacol. Exp. Ther.* **1994**, *269*, 1166–1175.

(73) Scheurell, M.; Franke, S.; Shah, R.M.; Hühnerfuss, H. Occurrence of diclofenac and its metabolites in surface water and effluent samples from Karachi, Pakistan. *Chemosphere* **2009**, *77*, 870–876.

(74) Selke, S.; Scheurell, M.; Shah, M.R.; Hühnerfuss, H. Identification and enantioselective gas chromatographic mass-spectrometric separation of *O*-desmethylnaproxen, the main metabolite of the drug naproxen, as a new environmental contaminant. *J. Chromatorg. A.* **2010**, *1217*, 419–423.

(75) Langford, K.; Thomas, K.V. Input of selected human pharmaceutical metabolites into the Norwegian aquatic environment. *J. Environ. Monit.* **2011**, *13*, 416–421.

(76) Zwiener, C.; Seeger, S.; Glauner, T.; Frimmel, F.H. Metabolites from the biodegradation of pharmaceutical residues of ibuprofen in biofilm reactors and batch experiments. *Anal. Bioanal. Chem.* **2002**, *372*, 569–575.

(77) Lahti, M.; Oikari, A. Microbial transformation of pharmaceuticals naproxen, bisoprolol, and diclofenac in aerobic and anaerobic environments. *Archiv. Environ. Contam. Toxicol.* **2011**, *61*, 202–210.

(78) Borges, K.B.; de Oliveira, A.R.M.; Barth, T.; Jabor, V.A.P.; Pupo, M.T.; Bonato, P.S. LC–MS–MS determination of ibuprofen, 2-hydroxyibuprofen enantiomers, and carboxyibuprofen stereoisomers for application in biotransformation studies employing endophytic fungi. *Anal. Bioanal. Chem.* **2011**, *399*, 915–925.

(79) Kretz-Rommel, A.; Boelsterli, U.A. Diclofenac covalent protein binding is dependent on acyl glucuronide formation and is inversely related to P450-mediated acute cell injury in cultured rat hepatocytes. *Toxicol. Appl. Pharmacol.* **1993**, *120*, 155–161.

(80) Boelsterli, U.A. Diclofenac-induced liver injury: a paradigm of idiosyncratic drug toxicity. *Toxicol. Appl. Pharmacol.* **2003**, *192*, 307–322.

(81) Shipkova, M.; Armstrom, V.W.; Oellerich, M.; Wieland, E. Acyl glucuronide drug metabolites: toxicological and analytical implications. *Ther. Drug Monit.* **2003**, *25*, 1–16.

(82) Regan, S.L.; Maggs, J.L.; Hammond, T.G.; Lambert, C.; Williams, D.P.; Park. B.K. Acyl glucuronides: the good, the bad and the ugly. *Biopharm. Drug Dispos.* **2010**, 31, 367–395.

(83) Bergman, K.; Müller, L.; Weberg Teigen, S. The genotoxicity and carcinogenicity of paracetamol: a regulatory (re)view. *Mutat. Res.* **1996**, *349*, 263–288.

(84) European Medicines Agency EMEA **2008**. Revised guideline on environmental impact assessment for veterinary medicinal products in support of the VICH guidelines GL6 and GL38, EMEA/CVMP/ERA/418282/2005-Rev.1 consultation. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/10/WC500004389.pdf.

(85) Zhang, X.; Oakes, K.D.; Cui, S.; Bragg, L.; Servos, M.R.; Pawliszyn, J. Tissuespecific *in vivo* bioconcentration of pharmaceuticals in rainbow trout (*Oncorhynchus mykiss*) using space-resolved solid-phase microextraction. *Environ. Sci. Technol.* **2010**, *44*, 3417–3422.

(86) Togunde, O.P.; Oakes, K.D.; Servos, M.R.; Pawliszyn, J. Determination of pharmaceutical residues in fish bile by solid-phase microextraction couple with liquid chromatography-tandem mass spectrometry (LC/MS/MS). *Environ. Sci. Technol.* **2012**, *46*, 5302–5309.

(87) Hoeger, B.; Dietrich, D.R.; Schmid, D.; Hartmann, A.; Hitzfeld, B. Distribution of intraperitoneally injected diclofenac in brown trout (*Salmo trutta* f. *fario*). *Ecotoxicol. Environ. Saf.* **2008**, *71*, 412–418.

(88) Gomez, C.F.; Constantine, L.; Moen, M.; Vaz, A.; Wang, W.; Huggett, D.B. Ibuprofen metabolism in the liver and gill of rainbow trout, *Oncorhynchus mykiss. Bull. Environ. Contam. Toxicol.* **2011**, *86*, 247–251.

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

(90) Spahn-Langguth, H; Benet, L.Z. Acyl glucuronides revisited: is the glucuronidation process a toxication as well as a detoxification mechanism? *Drug Metab. Rev.* **1992**, *24*, 5–47.

(91) Mortensen, R.W.; Corcoran, O.; Cornett, C.; Sidelmann, U.G.; Troke, J.; Lindon, J.C.; Nicholson, J.K.; Hansen, S.H. LC-^{1}H NMR used for determination of the elution order of S-naproxen glucuronide isomers in two isocratic reversed-phase LC-systems. *J. Pharm. Biomed. Anal.* **2001,** *24,* 477–485.

(92) Mortensen, R.W.; Sidelman, U.G.; Tjørnelund, J.; Hansen, S.H. Stereospecific pH-dependent degradation kinetics of R- and S-naproxen-β-1-O-acyl-glucuronide. Chirality **2002**, *14*, 305–312.

(93) Johnson, C.H.; Wilson, I.D.; Harding, J.R.; Stachulski, A.V.; Iddon, L.; Nicholson, J.K.; Lindon, J.C. NMR spectroscopic studies on the *in vitro* acyl glucuronide migration kinetics of ibuprofen ((±)-(*R,S*)-2-(4-isobutylphenyl) propanoic acid), its metabolites, and analogues. *Anal. Chem.* **2007**, *79*, 8720–8727.

(94) Thurman, E.M.; Ferrer, I. Comparison of quadrupole time-of-flight, triple quadrupole, and ion-trap mass spectrometry/mass spectrometry for the analysis of emerging contaminants. In *Liquid chromatography/mass spectrometry, MS/MS and time-of-flight MS: analysis of emerging contaminants*; Ferrer, I.; Thurman, E.M. Eds. American Chemical Society, Oxford University Press, Washington, DC, USA **2003**; pp. 14–31.

(95) Petrovic, M.; Farré, M.; de Alda, M.L.; Perez, S.; Postigo, C.; Köck, M.; Radjenovic, J.; Gros, M.; Barcelo, D. Recent trends in the liquid chromatography–mass spectrometry analysis of organic contaminants in environmental samples. *J. Chromatorg. A.* **2010**, *1217*, 4004–4017.

(96) Richardson, S.D. Environmental mass spectrometry: emerging contaminants and current issues. *Anal. Chem.* **2010***, 82,* 4742–4774.

(97) Richardson, S.D. Environmental mass spectrometry: emerging contaminants and current issues. *Anal. Chem.* **2012***, 84,* 747–778.

(98) Levsen, K.; Schiebel, H.-M.; Behnke, B.; Dötzer, R.; Dreher, W.; Elend, M.; Thiele, H. Structure elucidation of phase II metabolites by tandem mass spectrometry: an overview. *J. Chromatogr. A.* **2005,** *1067,* 55–72.

(99) Holcapek, M.; Kolarova, L.; Nobilis, M. High-performance liquid chromatography–tandem mass spectrometry in the identification and determination of phase I and phase II drug metabolites. *Anal. Bioanal. Chem.* **2008,** *391,* 59–78.

(100) Niessen, W.M.A. Fragmentation of toxicologically relevant drugs in positive-ion liquid chromatography-tandem mass spectrometry. *Mass Spectrom. Rev.* **2011**, *30*, 626–663.

(101) Gross, J.H. *Mass spectrometry – A text book*; Springer-Verlag Berlin Heidelberg, Germany, **2004.**

(102) Lahti, M.; Brozinski, J.-M.; Jylhä, A.; Kronberg, L.; Oikari, A. Uptake from water, biotransformation, and biliary excretion of pharmaceuticals by rainbow trout. *Environ. Toxicol. Chem.* **2011**, *30*, 1403–1411.

(103) Oikari, O.A.J. Metabolites of xenobiotics in the bile of fish in waterways polluted by pulp mill effluents. *Bull. Environ. Contam. Tocixol.* **1986**, *36*, 429–436.

(104) Oikari, O.; Kukkonen, J.V.K.; Vuorikari, P. Can biliary chlorophenolics represent their tissue residues in fish? *Toxicol. Environ. Chem.* **1999**, *69*, 49–60.

(105) Meriläinen, P.S.; Krasnov, A.; Oikari, A. Time- and concentration-dependent metabolic and genomic responses to exposure to resin acids in brown trout (*Salmo trutta* m. *lacustris*) *Environ. Tox. Chem.* **2007**, *26*, 1827–1835.

(106) Meriläinen, P.; Oikari, A. Exposure assessment of fishes to modern pulp and paper mill effluents after black liquour spill. *Environ. Monit. Assess.* **2008**, *144*, 419–435.

(107) Arnot, J.A.; Gobas, F.A.P.C. A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organism. *Environ. Rev.* **2006**, *14*, 257–297.

(108) Kondo, T.; Yamamoto, H.; Tatarazako, N.; Kawabe, K.; Koshio, M.; Hirai, N.; Morita, M. Bioconcentration factor of relatively low concentrations of chlorophenols in Japanese medaka. *Chemosphere* **2005**, *61,* 1299–1304.

(109) Liu, C.; Gin, K.Y.H.; Chang, V.W.C.; Goh, B.P.L.; Reinhard, M. Novel perspectives on the bioaccumulation of PFCs – the concentration dependency. *Environ. Sci. Technol.* **2011**, *45*, 9758–9764.

(110) Contardo-Jara, V.; Lorenz, C.; Pflugmacher, S.; Nützmann, G.; Kloas, W.; Wiegand, C. Molecular effects and bioaccumulation of levonorgestrel in the non-target organism *Dreissena polymorpha*. *Environ. Polutt.* **2011**, *159,* 38–44.

(111) Cuklev, F.; Fick, J.; Cvijovic, M.; Kristiansson, E.; Förlin, L.; Larsson, D.G.J. Does ketoprofen or diclofenac pose the lowest risk to fish? *J. Haz. Mater.* **2012**, *229–230,* 100–106.

(112) Brown, J.N.; Paxéus, N.; Förlin, L.; Larsson, D.G.J. Variations in bioconcentration of human pharmaceuticals from sewage effluents into fish blood plasma. *Environ. Toxicol. Pharmacol.* **2007**, *24,* 267–274.

(113) Larsson, D.G.J.; Adolfsson-Erici, M.; Parkkonen, J.; Pettersson, M.; Berg, A.H.; Olsson, P.-E.; Förlin, L. Ethinyloestradiol – an undesired fish contraceptive? *Aquat. Toxicol.* **1999**, *45*, 91–97.

(114) Fenlon, K.A.; Johnson, A.C.; Tyler, C.R.; Hill, E.M. Gas–liquid chromatography–tandem mass spectrometry methodology for the quantitation of estrogenic contaminants in bile of fish exposed to wastewater treatment works effluents and from wild populations *J. Chromatogr. A.* **2010**, *1217*, 112–118.

(115) Rostkowski, P.; Horwood, J.; Shears, J.A.; Lange, A.; Oladapo, F.O.; Besselink, H.T.; Tyler, C.R.; Hill, E.M. Bioassay-directed identification of novel antiandrogenic compounds in bile of fish exposed to wastewater effluents. *Environ. Sci. Technol.* **2011**, *45*, 10660–10667.

(116) Fick, J.; Lindberg, R.H.; Parkkonen, J.; Arvidsson, B.; Tysklind, M.; Larsson, D.G.J. Therapeutic levels of levonorgestrel detected in blood plasma of fish: results from screening rainbow trout exposed to treated sewage effluents. *Environ. Sci. Technol.* **2010**, *44*, 2661–2666.

(117) Beyer, J.; Jonsson, G.; Porte, C.; Krahn, M.M.; Ariese, F. Analytical methods for determining metabolites of polycyclic armatic hydrocarbon (PAH) pollutants in fish bile: A review. *Environ. Toxicol. Pharmacol*. **2010**, *30*, 224–244.

(118) Lahti, M.; Brozinski, J.-M.; Segner, H.; Kronberg, L.; Oikari, A. Bioavailability of pharmaceuticals in waters close to wastewater treatment plants - use of fish bile for exposure assessment. *Environ. Toxicol. Chem.* **2012**, *31*, 1831–1837.

(119) Ramirez, A.J.; Brain, R.A.; Usenko, S.; Mottaleb, M.A.; O´Donnell, J.G.; Stahl, L.L.; Wathen, J.B.; Snyder, B.D.; Pitt, J.L.; Perez-Hurtado, P.; Dobbins, L.L.; Brooks, B.W.; Chambliss, C.K. Occurrence of pharmaceuticals and personal care products in fish: results of a national pilot study in the United States. *Environ. Toxicol. Chem.* **2009**, *28*, 2587–2597.

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

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

(122) Owen, S.F.; Giltrow, E.; Huggett, D.B.; Hutchinson, T.H.; Saye, J.; Winter, M.J.; Sumpter, J.P. Comparative physiology, pharmacology and toxicology of β-blockers: mammals versus fish. *Aquat. Toxicol.* **2007**, *82*, 145–162.

(123) Lahti, M.; Oikari, A. Pharmaceuticals in settleable particulate material in urban and non-urban waters. *Chemosphere* **2011**, *85*, 826–831.

(124) Lahti, M.; Oikari, A. Vertical distribution of pharmaceuticals in lake sediments – citalopram as potential chemomarker. *Environ. Toxicol. Chem.* **2012**, *31*, 1738–1744.

(125) Talbot, C.; Higgins, P.J. Observations on the gall bladder of juvenile Atlantic salmon *Salmo salar* L., in relation to feeding. *J. Fish Biol.* **1982**, *21*, 663–669.

(126) Oikari, A. Caging techniques for field exposures of fish to chemical contaminants. *Aquat. Toxicol.* **2006**, *78*, 370–381.

(127) Legler, J.; Jonas, A.; Lahr, J.; Vethaak, A.; Brouwer, A.; Murk, A.J. Biological measurement of estrogenic activity in urine and bile conjugates with the in vitro ER-CALUX reporter gene assay. *Environ. Toxicol. Chem.* **2002**, *21*, 473–479.

APPENDICES

Figure S1. Chemical structures of the analyzed acidic pharmaceuticals. Internal standards are not depicted.

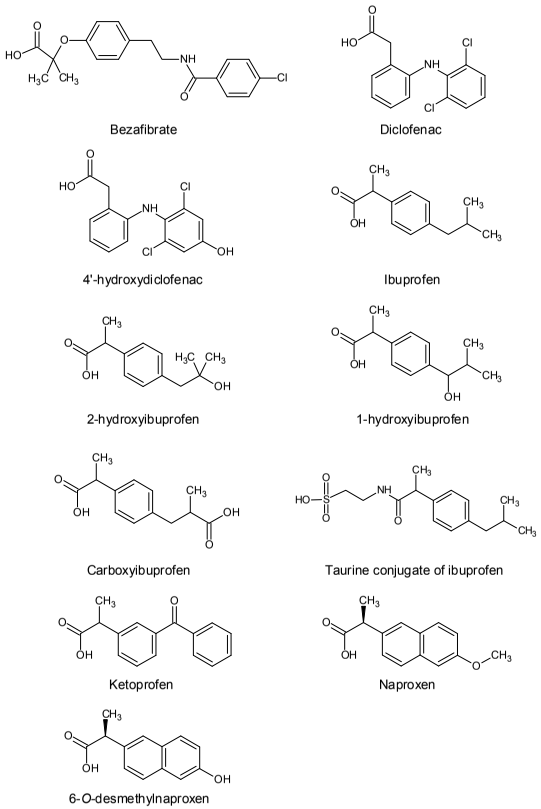


Figure S2. Chemical structures of the analyzed basic pharmaceuticals. Internal standards are not depicted.

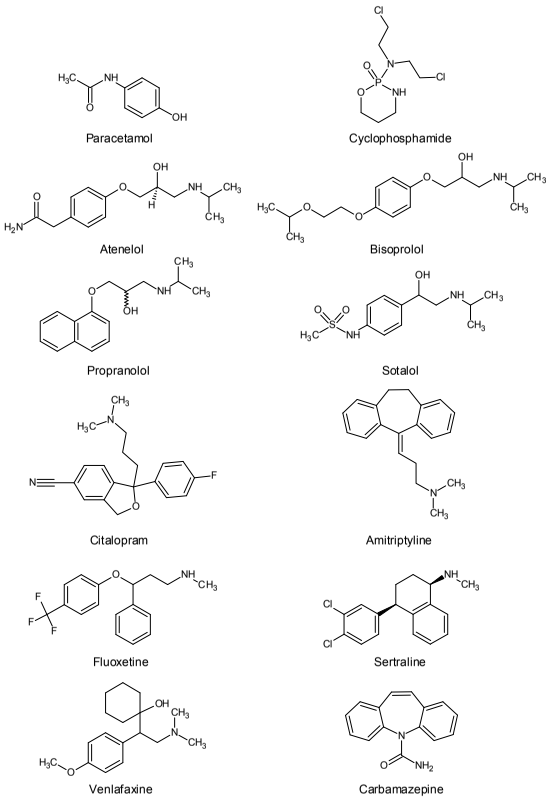


Table S1. Ionization mode, retention times and the multiple reaction monitoring (MRM) parameters applied. IS = internal standard, ESI = electrospray ionization.

Name	Ionization mode	t_R (min)	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (eV)
Bezafibrate	ESI-	13.2	360	274	23	18
D4-Bezafibrate (IS)	ESI-	13.2	364	278	25	17
Diclofenac	ESI-	15.0	294	250	15	12
D4-Diclofenac (IS)	ESI-	15.0	298	254	18	13
4'-Hydroxydiclofenac	ESI-	1.5	310	266	15	12
D4-4'-Hydroxydiclofenac (IS)	ESI-	1.5	314	270	17	14
Ibuprofen	ESI-	13.5	205	161	15	8
2-Hydroxyibuprofen	ESI-	7.2	221	177	17	11
1-Hydroxyibuprofen	ESI-	9.3	221	177	17	11
Carboxyibuprofen	ESI-	0.7	235	191	15	9
Taurine-conj. of ibuprofen	ESI-	15.2	312	124	21	26
D3-Ibuprofen (IS)	ESI-	13.5	208	164	17	9
Ketoprofen	ESI-	11.9	253	209	14	8
C13-D3-Ketoprofen (IS)	ESI-	11.9	257	213	14	9
Naproxen	ESI-	10.9	229	170	11	16
Naproxen	ESI-	10.9	229	185	9	11
D3-Naproxen (IS)	ESI-	10.9	232	173	11	16
6-*O*-Desmethylnaproxen	ESI-	1.5	215	171	12	13
D3-6-*O*-Desmethylnaproxen (IS)	ESI-	1.5	218	174	15	10
Paracetamol	ESI+	2.5	152	110	35	19
2-Acetamidophenol (IS)	ESI+	5.5	152	110	35	19
Cyclophospamide	ESI+	12.9	260	116	35	22
Alprenolol (IS)	ESI+	13.0	250	116	30	20
Atenolol	ESI+	5.0	267	145	33	25
Bisoprolol	ESI+	12.5	326	116	33	21
Propranolol	ESI+	10.1	261	140	38	22
Sotalol	ESI+	4.0	273	255	19	12
Citalopram	ESI+	13.5	325	109	20	23
Amitriptyline	ESI+	14.9	278	233	35	19
Fluoxetine	ESI+	15.2	310	148	13	10
D5-Fluoxetine (IS)	ESI+	15.2	315	153	11	10
Sertraline	ESI+	15.4	306	275	23	13
D3-Sertraline (IS)	ESI+	15.4	309	275	23	13
Venlafaxine	ESI+	12.5	278	121	27	20
Carbamazepine	ESI+	12.0	237	194	30	19
Dihydrocarbamazepine (IS)	ESI+	12.1	239	194	36	23

ISBN 978-952-12-2875-9