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The Anti-Inflammatory Drugs Diclofenac, Naproxen and Ibuprofen are found in the Bile of Wild Fish Caught Downstream of a Wastewater Treatment Plant

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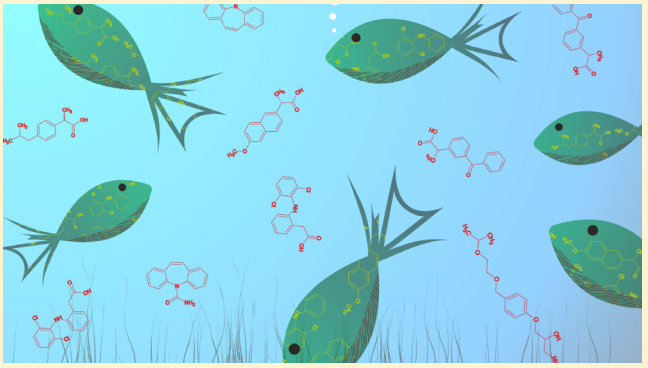
Supporting Information

ABSTRACT: Pharmaceutical residues are ubiquitous in rivers, lakes, and at coastal waters affected by discharges from municipal wastewater treatment plants. In this study, the presence of 17 different pharmaceuticals and six different phase I metabolites was determined in the bile of two wild fish species, bream (*Abramis brama*) and roach (*Rutilus rutilus*). The fish were caught from a lake that receives treated municipal wastewater via a small river. Prior to analyses, the bile content was enzymatically hydrolyzed to convert the glucuronide metabolites into the original pharmaceuticals or phase I metabolites. The solid phase extracts of hydrolyzates were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in the multiple reaction monitoring mode. The anti-inflammatory drug naproxen could be detected in all the six bream and roach bile samples. Diclofenac was found in five of the bream and roach samples, while ibuprofen was detected in three bream and two roach samples. The observed bile concentrations of diclofenac, naproxen, and ibuprofen in bream ranged from 6 to 95 ng mL^{-1}, 6 to 32 ng mL^{-1}, and 16 to 34 ng mL^{-1}, respectively. The corresponding values in roach samples ranged from 44 to 148 ng mL^{-1}, 11 to 103 ng mL^{-1} and 15 to 26 ng mL^{-1}, respectively. None of the other studied compounds could be detected. The study shows that pharmaceuticals originating from wastewater treatment plant effluents can be traced to the bile of wild bream and roach living in a lake where diclofenac, naproxen, and ibuprofen are present as pollutants.



INTRODUCTION

Pharmaceuticals detected in the environment are classified as emerging pollutants. Due to the continuous and increasing consumption and their incomplete elimination in wastewater treatment plants (WWTP), pharmaceuticals are ubiquitous compounds in rivers, lakes, and coastal waters (e.g., refs 1−7). Commonly, the concentration of pharmaceuticals range from ng to low $\mu g\ L^{-1}$ levels. Since pharmaceuticals are designed to be biologically active, the concern of their possible risks to aquatic ecosystems has been raised.

Our group has previously exposed rainbow trout (*Oncorhynchus mykiss*) to anti-inflammatory drugs diclofenac, naproxen and ibuprofen in aquaria at low $\mu g\ L^{-1}$concentrations, and determined their uptake and metabolism by studying blood plasma and bile samples.[8−11] The pharmaceuticals could be detected in the bile mainly as different metabolites. The main metabolites were the phase II glucuronide conjugates of the parent drugs and of their phase I metabolites. In studies performed on fish in aquaria[11] and on caged fish in the field,[12] we found that the bioconcentration of pharmaceuticals in the bile was several orders of magnitude higher than in the plasma. Previously, bile metabolites have been used to monitor xenobiotics present in water, such as chlorophenols,[13,14] synthetic and natural hormones[15−17] and polycyclic aromatic hydrocarbons.[18]

It has been reported that pharmaceuticals such as diclofenac, naproxen, ibuprofen, ketoprofen, and carbamazepine can be detected in plasma of fish exposed to treated wastewater in aquaria.[19,20] The antidepressant drugs fluoxetine and sertraline and their metabolites norfluoxetine and desmethylsertraline have been found in the liver, muscle, and brain of fish caught from rivers that receive discharges from WWTPs.[21−23]

The aim of the current study was to determine the presence of 17 different pharmaceuticals and six different phase I metabolites in the bile of two wild fish species. The fish were caught from a lake, which receives discharges from a municipal WWTP. The bile samples were enzymatically hydrolyzed, solid phase extracted and analyzed by liquid chromatography-tandem

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mass spectrometry (LC-MS/MS) in the multiple reaction monitoring mode (MRM).

MATERIAL AND METHODS

Chemicals. The analytical standards (purity ≥98%) atenolol, bezafibrate, carbamazepine, citalopram, diclofenac, fluoxetine, ibuprofen, ketoprofen, naproxen, sotalol, and internal standards alprenolol, 2-acetamidophenol, 10,11-dihydrocarbamazepine and d5-fluoxetine were purchased from Sigma Aldrich Inc. (St. Louis, MO). Bisoprolol hemifumarate was obtained from Heuman Pharma GmbH (Nürnberg, Germany) and d3-ibuprofen from Fluka (Seelze, Germany). Sertraline, d3-sertraline, venlafaxine, d4-bezafibrate, d4-diclofenac, 1-β-O-acyl glucuronide of diclofenac, 13C-d3-ketoprofen, d3-naproxen, 1-β-O-acyl glucuronide of naproxen, 4′-hydroxydiclofenac, d4−4′-hydroxydiclofenac, 6-O-desmethylnaproxen, d3−6-O-desmethylnaproxen, carboxy-ibuprofen, 2-hydroxyibuprofen, 1-hydroxyibuprofen and 1-β-O-acyl glucuronide of ibuprofen were purchased from Toronto Research Chemicals (North York, ON, Canada). The chemical purities of all deuterated standards were 98% and isotopic purities were 99%. Amitriptyline, citalopram, cyclophospamide, fluoxetine, paracetamol, and propranolol were gifts from pharmaceutical companies. Taurine conjugate of ibuprofen was synthesized at Åbo Akademi University, Laboratory of Organic Chemistry.

For quantification of diclofenac, naproxen and ibuprofen in the bile, their corresponding deuterated analogues were used as internal standards, that is, d4-diclofenac, d3-naproxen, and d3-ibuprofen, respectively. For the determination of the recoveries of pharmaceuticals and some of the metabolites from the bile, deuterated analogues and surrogate standards were used (Supporting Information Table S2). The quantification of the pharmaceuticals in lake water was performed as described by Daneshvar et al.,[6,7] Lindqvist et al.[3] and Vieno et al.[4,5] The water used in the LC-MS analysis was purified using a Millipore Simplicity 185 system (Millipore S.A.S., Molsheim, France), and the solvents were HPLC or LC-MS grade.

Sample Collection. The wild fish were caught with bag-shaped nets (fyke netting) in May 24, 2011 from Lake Haapajärvi (220 ha, average depth 1.4 m), Southeastern Finland. The lake receives treated wastewater from the WWTP (daily influent flow 15 000 m^3 d^{-1}) of the city of Lappeenranta (population 60 000) via the shallow (average depth 0.5−1 m and width 2 m) River (creek) Rakkolanjoki. The wastewater treatment consists of primary clarification, biomaterial removal by the conventional activated sludge process and phosphorus removal by coagulation with ferric salts. The distance between the wastewater discharge point and the lake is 19 km, and the drainage basin of the river-and-lake system before Lake Haapajärvi is 92 km^2. At the time of the sampling, the flow rate at the river mouth was approximately 0.44 m^3 s^{-1} and the percentage of treated wastewater was approximately 50% (Marja Kauppi, Centre for Economic Development, Transport and the Environment for Southeastern Finland, personal communication). The calculated average residence time of the water in Lake Haapajärvi is approximately one month.

Ten breams (*Abramis brama*; four males, five female, one juvenile) and 25 roaches (*Rutilus rutilus*; 6 males, 19 females) were caught from the lake. The average lengths and weights of the breams were 43 ± 6 cm (average ± SD) and 922 ± 398 g, respectively, and the corresponding values for the roaches were 15 ± 1 cm and 29 ± 8 g, respectively. In July 1, 2011, the reference fish, six breams (three males, three females; length 30 ± 6 cm) and eight roaches (seven males, one female; length 18 ± 2 cm) were caught by fyke netting from Lake Saimaa, Southeastern Finland. The reference fish were caught at a location which is not influenced by municipal wastewater.

The bile samples were collected immediately after the fish were caught, frozen in liquid nitrogen and stored at −80 °C. The bile samples were collected and pooled until approximately 100 to 150 μL of bile had been collected. Blank bile samples, used in the determination of the matrix matched standard curves, were obtained from a one year-old rainbow trout (*Oncorhynchus mykiss*) purchased from a hatchery (Savon Taimen Inc., Rautalampi, Finland).

For the determination of pharmaceuticals in Lake Haapajärvi, one liter water samples were collected from sampling sites A and B (Figure 1) in February, 22; May, 25; July, 7 and November, 18 during the year 2010. The samples were collected by the use of a Limnos sampler at the depth of 1 m. The samples were stored at −18 °C until sample preparation and analyses.

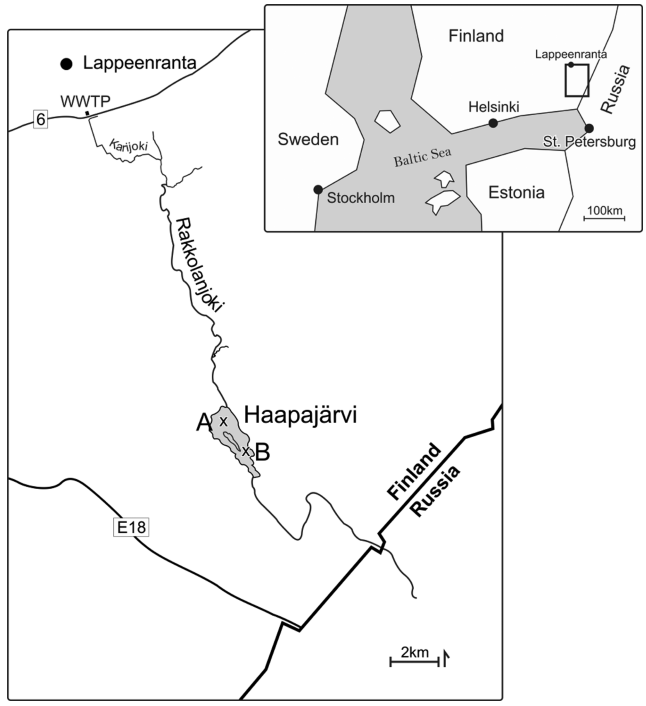

Figure 1. Map of River Rakkolanjoki and Lake Haapajärvi. Sampling sites A and B in Haapajärvi.

Sample Preparation. The glucuronide metabolites that could be present in the bile were hydrolyzed by 600 U (diluted to 200 μL with 1 M acetate buffer, pH 5) of β-glucuronidase/aryl-sulfatase isolated from *Helix pomatia* (obtained from Sigma Aldrich, cat. no. G7770) back to their phase I metabolites or to the parent compounds. The bile samples (30−100 μL) and 200 μL of 1 M acetate buffer (pH 5) containing 250 ng of each internal standard were added to the acetate solution of the enzymes. The obtained solution was incubated for 4 h at 37 °C. In addition, a blank rainbow trout bile sample to which 1-β-O-acyl glucuronides of diclofenac, naproxen, and ibuprofen had been added was hydrolyzed in the same way as the bile of wild fish.

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Table 1. Concentration of Pharmaceuticals (ng L^{-1}) in the Water of Lake Haapajärvi Sampled in February 22, May 25, July 7, and November, 18, 2010[a]

	February		May		July		November	
acidic pharmaceutical	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[b]	10	16
Ketoprofen	85	106	30	nd[b]	32	36	24	19
Naproxen	210	98	61	89	54	43	43	40
	February		May		July		November	
basic pharmaceutical	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[b]	nd[b]	nd[b]	nd[b]	4	3
Fluoxetine	nd[b]	nd[b]	nd[b]	nd[b]	nd[b]	nd[b]	nd[b]	nd[b]
Setraline	nd[b]	nd[b]	nd[b]	nd[b]	nd[b]	nd[b]	nd[b]	nd[b]
Venlafaxine	2	2	1	1	1	nd[b]	14	15
Carbamazepine	355	318	109	114	216	212	111	103

[a]The sampling sites A and B are depicted in Figure 1. nd = not detected, LOD = limit of detection. [b]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}.

Following hydrolysis, the samples were diluted with 1 mL water (pH 2) and purified by solid phase extraction. Oasis HLB (3 cm^2, 60 mg; Waters, Milford, MA) cartridges were used for the purification of acidic pharmaceuticals, while Oasis MCX (3 cm^2, 60 mg; Waters, Milford, MA) cartridges were used for the basic pharmaceuticals. The cartridges were conditioned with 3 mL methanol, 3 mL water (only for basics) and 3 mL water (pH 2). After sample loading, the cartridges were washed with 1 mL water (pH 2) and 1 mL of 20% methanol. The acidic pharmaceuticals were eluted with 1 mL of 2% NH_4OH in 80% methanol and the basics with 1 mL of 100% methanol followed by 1 mL of 2% NH_4OH in methanol. The extracts were evaporated to dryness under a stream of nitrogen, redissolved in 300 μL of 0.01 M ammonium hydroxide in 2% acetonitrile (acidic pharmaceuticals) or 0.5% acetic acid in 3% acetonitrile (basic pharmaceuticals), and analyzed immediately by LC-MS/MS.

Acidic pharmaceuticals were determined in bile samples of six bream, three reference bream, six pooled roach and five pooled reference roach. Basic pharmaceuticals were determined in bile samples of five bream, three reference bream, four pooled roach, and three pooled reference roach.

The lake water samples (1 L) were split into two 400 mL subsamples, one for the analysis of the acidic pharmaceuticals and one for the basic ones. The procedures for the extraction and sample preparation were the same as reported in Daneshvar et al.[6,7]

LC-MS/MS Analysis. The quantitative analysis of 17 different pharmaceutical and six different phase I pharmaceutical metabolite residues in fish bile, and 13 pharmaceuticals in Lake Haapajärvi water samples, was performed with the Quattro Micro triple-quadrupole mass analyzer equipped with electrospray ionization source (Waters, Milford, MA). The mass analyzer was operated in the multiple reaction monitoring mode (MRM), and negative ions were recorded for acidic pharmaceuticals and positive ions for basic pharmaceuticals. Nitrogen was used as a desolvation gas with a temperature of 325 °C and flow rate of 630 L h^{-1} and 640 L h^{-1} for acidic and basic pharmaceuticals, respectively. Argon was used as the collision gas with a collision cell pressure 5.1×10^{-3} mbar and 5.5×10^{-3} mbar for acidic and basic pharmaceuticals, respectively. The source block temperature was 120 °C and the capillary voltage was set to 3.2 kV (acidics) and 3.8 kV (basics). Quattro Micro was connected to an Agilent 1100 series HPLC consisting of a binary pump, a vacuum degasser, an autosampler and a thermostatted column oven set to 30 °C.

The chromatographic separations were carried out on an XBridge C18 analytical column (3.5 μm, 2.1 × 50 mm; Waters, Milford, MA) equipped with a guard column of the same brand (3.5 μm, 2.1 × 10 mm). To ensure the correct identification of diclofenac and ibuprofen, the chromatographic retention of the compounds was determined on an XBridge C8 column (3.5 μm, 2.1 × 50 mm, equipped with a C18 guard column; Waters, Milford, MA) and on an ACE Phenyl 5 column (5 μm, 2.1 × 125 mm, equipped with a guard column from the same brand; Advanced Chromatography Technologies, Aberdeen, Scotland).

The mobile phase used in the analysis of acidic pharmaceuticals consisted of 0.01 M ammonium hydroxide (A) and 0.01 M ammonium hydroxide in 90% acetonitrile (B). The columns were eluted isocratically for 2 min with 2% of B and then with a gradient to 33% of B over the course of 14 min. In the analysis of basic pharmaceuticals, the mobile phase consisted of 0.5% acetic acid (A) and 0.5% of acetic acid in acetonitrile (B). The XBridge C18 column was eluted isocratically for 1 min with 3% of B and then with a gradient to 65% of B in 14 min. The XBridge columns were eluted at a flow rate of 300 μL min^{-1} and the ACE Phenyl column was eluted at a flow rate of 350 μL min^{-1}. The injection volume was 30 μL.

The XBridge C18 column was used for the analysis pharmaceuticals in lake water. The mobile phase consisted of 0.01 M ammonium hydroxide (A) and acetonitrile (B) (acidic pharmaceuticals) and 0.5% acetic acid (A) and 0.5% of acetic acid in acetonitrile (B) (basic pharmaceuticals). The column was eluted at a flow rate of 300 μL min^{-1}. For the analysis of

Table 2. Concentrations (ng mL^{-1}) of Diclofenac, Naproxen and Ibuprofen in the Bile of the Breams and Roaches Caught from Lake Haapajärvi[a]

fish species	bile number	Diclofenac ng mL^{-1}	S/N	naproxen ng mL^{-1}	S/N	ibuprofen ng mL^{-1}	S/N
bream	1	56	10.9	32	11.7	34	15.7
	2	6	11.7	18	41.0	24	14.8
	3	95	42.6	31	82.9	nd	nd
	4	67	14.0	21	74.5	nd	nd
	5	nd	nd	6	10.2	nd	nd
	6	27	10.7	15	15.9	16	14.2
roach[b]	1	88	53.8	42	14.4	<LOQ	8.4[c]
	2	44	28.4	36	81.9	26	14.6
	3	46	26.3	19	56.7	nd	nd
	4	148	73.1	16	38.1	nd	nd
	5	nd	nd	11	64.1	15	16.5
	6	116	29.1	103	182.7	nd	nd

[a]nd = not detected, S/N = signal to noise, LOQ = limit of quantification, LOD = limit of detection. LOD (diclofenac): 1.1−3.5 ng mL^{-1}; LOD (ibuprofen): 1.5−4.9 ng mL^{-1}. [b]Roach samples 2−6 are pooled. One pooled sample corresponds to bile from two to five individuals. [c]S/N lower than 10 and thus not quantified. The ion chromatogram is depicted in Figure 2.

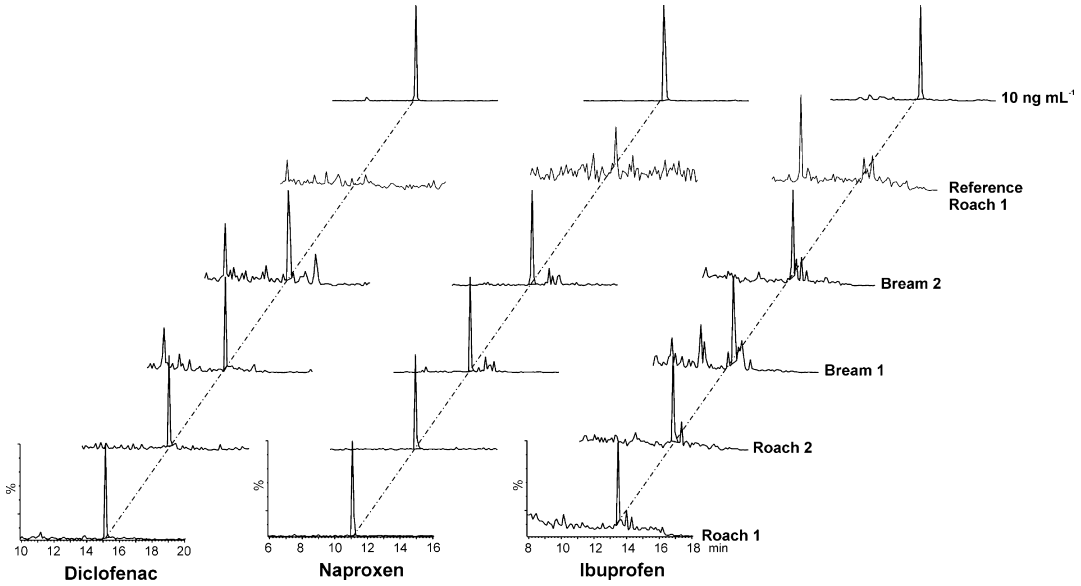

Figure 2. LC-MS/MS ion chromatograms representing MRM transitions of diclofenac, naproxen, and ibuprofen and obtained from the analyses of four wild fish bile samples (roach bile samples 1−2 and bream bile samples 1−2, Table 2), of a reference roach and of 10 ng mL^{-1} of diclofenac, naproxen and ibuprofen added to a blank bile of rainbow trout.

acidic pharmaceuticals, the column was eluted isocratically for 1 min with 3% of B and then with a gradient to 65% of B over the course of 9 min. The gradient for basic pharmaceuticals was the same as used for the bile samples. The injection volume was 30 μL.

The MassLynx 4.0 sofware was used for data collection and handling. The cone voltages, the collision energies, the precursor and product ions (Supporting Information Table S1) were optimized by direct infusion of the pure standards and internal standards to the mass spectrometer. Samples were quantified by using internal standard method. Matrix matched calibration curves ranging from 1 ng mL^{-1} to 1000 ng mL^{-1} were prepared in blank rainbow trout bile, which were hydrolyzed and extracted as described above. Calibration curves (1−1000 ng mL^{-1}) for the analysis of pharmaceuticals in lake water were prepared in distilled water. The limit of quantification (LOQ) was defined as the concentration of the analyte in bile having a signal-to-noise (S/N) value of 10, and limit of detection (LOD) an S/N value of 3. The extraction recoveries were calculated from the results of five to nine injections and ranged from 72−136% (Supporting Information Table S2). The repeatability (standard deviation) was affected by the complicated sample matrix (bile). The LOQ's were 1.1−76.6 ng mL^{-1} and were dependent on the volume of bile analyzed. Blank solvent samples were injected between sample series and calibration curves to prevent possible carryover from previous samples.

RESULTS AND DISCUSSION

The studied lake (Lake Haapajärvi, Figure 1) receives wastewater effluents via River Rakkolanjoki. In February, May, July and November 2010, samples of the lake water were collected from sites A and B (Figure 1) and the presence of 13 different pharmaceuticals were determined by LC-MS/MS analysis. At site A 10 to 11 and at site B 8 to 11 different pharmaceuticals could be detected and quantified (Table 1).

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The antiepileptic drug carbamazepine was the major pharmaceutical in all samples. The anti-inflammatory drugs diclofenac, naproxen, and ibuprofen 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.

In May 2011, wild bream and roach were caught from the lake and their bile was collected and subjected to LC-MS/MS analysis. In all, the presence of 17 different pharmaceuticals and six different phase I metabolites was determined (Supporting Information Table S1). The same analyses were performed on the bile of bream and roach caught from the reference site, Lake Saimaa.

Previously, we have found that diclofenac, naproxen, and ibuprofen are mainly present in the fish bile as phase II metabolites and that the glucuronides are the major conjugates.[8−11] To enhance the detectability of the pharmaceuticals in wild fish bile, the glucuronides were subjected to enzymatic deconjugation. Prior to the deconjugation of the bile samples, the outcome of the hydrolysis was determined by the addition of 1-*β*-*O*-acyl glucuronides of diclofenac, naproxen, and ibuprofen to the bile collected from rainbow trout not exposed to pharmaceuticals. Following the enzymatic treatment and solid phase extraction, the LC-MS/MS analysis showed that the chromatographic peaks of diclofenac, naproxen, and ibuprofen appeared, whereas the peaks corresponding to the glucuronides disappeared (data not shown). Thus it could be concluded that the conjugates seemed to be completely hydrolyzed and that the detectability of the drugs was improved.

In the next step, the wild fish bile samples were enzymatically hydrolyzed, the hydrolyzates purified by solid phase extraction and finally, the extracts were subjected to LC-MS/MS analyses. Based on the matching retention times of the analytes and deuterated internal standards on the C18 chromatographic column, and of MRM transitions, it can be concluded that anti-inflammatory drugs diclofenac, naproxen, and ibuprofen were present in the bile of bream and roach (Table 2 and Figure 2). The S/N ratio of diclofenac ranged from 10.7 to 73.1, of naproxen from 10.2 to 182.7 and of ibuprofen from 8.4 to 16.5 (Table 2). In the case of naproxen, the correct identification could also be established by observing the product ion of a second transition (229 → 185) (Supporting Information Table S1). This transition was observed in the ion chromatograms of MRM transitions in all bile samples. Due to the low intensity of the ions produced during the second transition of diclofenac (294 → 214) and the lack of a second transition of ibuprofen, additional transitions could not be applied for further verification of the occurrence of diclofenac and ibuprofen.

To obtain additional proof for the correct identity of the peaks representing diclofenac and ibuprofen in the ion chromatograms of the bile samples, the retention of the peaks and the standards were observed on columns with different mechanisms of separation, i.e. different stationary phases. It was found that upon injection of the bile samples on the C8 and the Phenyl columns, the ion chromatograms of the bile samples exhibited peaks at exactly the same retention time as the diclofenac and ibuprofen standards (Figure 3). This finding is a further verification of the correct identity of the peaks due to diclofenac and ibuprofen. In all, the MRM

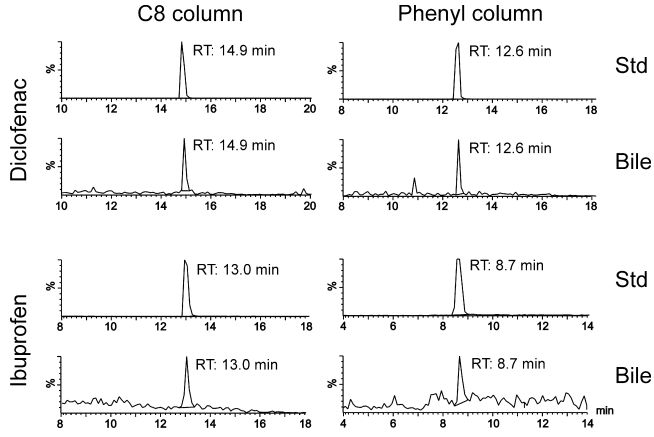

Figure 3. LC-MS/MS ion chromatograms of MRM transitions of diclofenac and ibuprofen in roach bile samples and the ion chromatograms of d4-diclofenac (std) and d3-ibuprofen (std). The analyses were performed on C8 and Phenyl columns.

transitions together with the chromatographic characteristics of the compounds provide a very strong evidence for the occurrence of diclofenac, naproxen and ibuprofen in the bile of the fish.

Although a wide range of pharmaceuticals were present in Lake Haapajärvi, only diclofenac, naproxen, and ibuprofen 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} in bile and in roach from 11 to 103 ng mL^{-1} in bile (Table 2). 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 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).

The feeding status of the fish has a pronounced effect on the concentration of chemicals and metabolites in the bile.[8−14] Following feeding, the fish will empty the gall bladder to the intestine and consequently the bile volume and the content of foreign chemicals will be low. In laboratory, the feeding can be controlled, but in studies using wild fish, the feeding status is unknown and a wide variation in the bile content of chemicals and metabolites will be observed.

In a previous work, we analyzed pharmaceuticals in the bile hydrolysates collected from rainbow trout kept in cages close to the discharge point of wastewater treatment plants for 10 days.[12] 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 (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 and naproxen ranged from 29−194 ng mL^{-1}, 11−84 ng mL^{-1} and not detected−71 ng mL^{-1}, respectively.[12] 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, although phase I metabolites are known to be formed by fish.[8−11] 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 (Supporting Information Table S2). This may explain why low amounts of phase I metabolites possibly present in the hydrolyzates could not be detected.

Carbamazepine was the dominating pharmaceutical in the water samples collected in 2010 and was most likely present in significant concentrations also at the time when the wild fish bile samples were collected. In a preliminary study, we have found that carbamazepine is excreted only to a limited degree into the bile of rainbow trout.[10] Also, Togunde et al.[24] reported negligible uptake of carbamazepine in the bile of rainbow trout exposed to the compound in aquaria. In other studies, antidepressant drugs and their metabolites have been detected in wild fish tissues such as liver, muscle and brain.[21−23] In Lake Haapajärvi, the antidepressant drugs citalopram and setraline were not detected and venlafaxine was detected at only marginal concentrations (1 ng L^{-1}). Therefore, these drugs are not likely to be found in the fish bile.

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.[12,22] At present, nothing seems to be known about the pharmacokinetics of β-blockers in fish,[25] which means that also the extent of uptake and the route of excretion (renal or bile) remains to be clarified. The analytical method allowed for the detection of ketoprofen and bezafibrate in the hydrolysates, but neither of the compounds was detected in the bile samples.

This work shows that the anti-inflammatory drugs diclofenac, naproxen, and ibuprofen, which originate from WWTP effluents, can be found in the bile of two wild fish species living in the recipient water. The concentration of diclofenac, naproxen, and ibuprofen in the bile was roughly 1000 times higher than the concentration found in the lake water (Tables 1 and 2). Since the bioconcentration factors (concentration in tissue/concentration in water) in blood plasma and muscle tissue are for many pharmaceuticals, including diclofenac, naproxen and ibuprofen, reported to be in the range of 0.01 to 9 (e.g., refs 11,19, and 26), it is evident that bile is a much more suitable matrix for monitoring acidic anti-inflammatory drugs in the aquatic environment. All the year, the lake water is contaminated with pharmaceuticals, which implies that the fish are steadily accumulating mixtures of pharmaceuticals in the bile, which in turn means that the mixtures are also present in the blood of fish. High bile concentrations of pharmaceuticals do neither prove that the compounds cause health effects nor that the plasma concentrations are at a level that has an impact on the biological functions of the fish. On the other hand, the knowledge of both bile and plasma concentrations of the compounds in question would be of significant value in biological effect studies. Previous studies have shown that diclofenac at near environmental concentration (approximately 1 μg L^{-1}) interfere with the biological functions of rainbow trout following exposure to the pure compound for 21 days.[27] As far as we know, the impact on the biological functions of fish chronically exposed to mixtures of pharmaceuticals has not been studied. Further work is urgently needed where the effect on fish of individual pharmaceuticals and of their mixtures is determined. Only when such studies have been performed, it is possible to evaluate whether pharmaceutical residues in the aquatic environment pose risk on wild fish populations and on the aquatic environment as a whole.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Tables S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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