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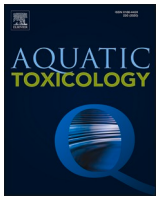

Comparative in vitro hepatic clearances of commonly used antidepressants, antipsychotics, and anti-inflammatory agents in rainbow trout liver S9 fractions

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ABSTRACT

Residues of human pharmaceuticals are widely detected in surface waters and can be taken up by and bioaccumulate in aquatic organisms, especially fish. One of the key challenges in assessing the bioaccumulation potential of ionizable organic compounds, such as the pharmaceuticals, is the lack of empirical data for biotransformation. In the present study, we assessed the in vitro intrinsic clearances (CL_{INT}) of twelve pharmaceuticals, individually and some additionally as mixtures, in rainbow trout (*Oncorhynchus mykiss*) liver S9 fractions (RT-S9) adhering to the OECD test guidance 319B. The test substances included four anti-inflammatory agents (diclofenac, ibuprofen, ketoprofen, naproxen), seven antidepressants/antipsychotics (citalopram, haloperidol, levomepromazine, mirtazapine, risperidone, sertraline, venlafaxine) and the O-desmethyl metabolite of venlafaxine. Quantifiable intrinsic clearances were detected for diclofenac, ibuprofen, naproxen, levomepromazine, and sertraline. Apart from diclofenac, the in vitro clearances of the other four pharmaceuticals were shown to be critically dependent on the cytochrome P450 (CYP) metabolism. Therefore, we also determined the half-maximal inhibitory concentrations (IC_{50}) of the same twelve pharmaceuticals toward CYP1A-like (7-ethoxyresorufin-O-deethylation, EROD) and CYP3A-like (benzyloxy-4-trifluoromethylcoumarin-O-debenzyloxylation, BFCOD) activities in RT-S9 using IC_{50} shift assay. As a result, levomepromazine and sertraline were identified as the most potent inhibitors of both EROD and BFCOD activity (unbound $IC_{50} < 10$ μM each), followed by citalopram and haloperidol (10 μM $< IC_{50} <$ 100 μM). Additionally, mirtazapine was a selective EROD inhibitor ($IC_{50} \sim 30$ μM). The inhibitory impacts of haloperidol and sertraline were indicatively time dependent. Finally, we carried out intrinsic clearance assays with mixtures of diclofenac, ibuprofen, naproxen, levomepromazine, and sertraline to examine the impacts of EROD and BFCOD inhibitions on their in vitro CL_{INT} in RT-S9. Our in vitro data suggests that the intrinsic clearances of ibuprofen, levomepromazine, and sertraline in rainbow trout can be significantly reduced as the result of P450 inhibition by pharmaceutical mixtures, whereas the clearances of diclofenac and naproxen are less impacted.

1. Introduction

Pharmaceutical residues are abundant in surface waters all around the globe (Aus der Beek et al., 2016; Wilkinson et al., 2022) posing ecotoxicological risks to both aquatic and terrestrial ecosystems. Fish are particularly sensitive to the pharmaceuticals' adverse effects, owing to their high evolutionary conservation of human drug targets (Gunnarsson et al., 2019). Besides acute toxicity, pharmaceuticals' bioaccumulation in fish can potentially increase their chronic effects outcomes even at trace level environmental concentrations (Gómez-Regalado et al., 2023). The OECD test guidance 305 lays the basis for standardized assessment of bioaccumulation in fish. It is commonly used for the regulatory environmental risk assessment (ERA) of pharmaceuticals, which is mandatory for the marketing authorization in the EU, US, and Canada (Lee and Choi, 2019). This standardized test is, however, time-consuming and costly, and requires ca. hundred fish per substance. Therefore, it is not feasible for large-scale screening of the thousandfold (legacy) pharmaceuticals registered prior to adaptation of the regulatory

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ERA and lacking environmental fate data. To address this gap, several computational tools have been developed for assessment of the bioconcentration factor (BCF) in fish, including quantitative structure-activity relationship (QSAR) (Dimitrov et al., 2012; Garg and Smith, 2014), read-across (Rovida et al., 2020), mass balance (Arnot and Gobas, 2003), and physiologically based toxicokinetic models (Wang et al., 2022). However, one of the greatest uncertainties of computational methods relates to the impact of hepatic biotransformation on the intrinsic clearance rates of pharmaceuticals in fish. The rate of hepatic biotransformation can strongly influence xenobiotic elimination/bioaccumulation and is thus a critical input parameter for the model development. However, empirical data for ionizable organic compounds, such as most pharmaceuticals, are presently very limited (Armitage et al., 2017).

In the recent decade, sustained research efforts have gone on collateral development of in vitro biotransformation assays for fish liver (initially using freshly isolated fish hepatocytes and liver S9 fractions) as well as cryopreservation techniques enabling long-term storing and shipping of cells and subcellular fractions between laboratories. This has eventually led to adoption of standardized in vitro assays for determination of the in vitro intrinsic clearance rate, using cryopreserved rainbow trout hepatocytes (RT-HEP, OECD 319A) or subcellular liver S9 fractions (RT-S9, OECD 319B). The intra- and interlaboratory validation of these assays has readily demonstrated good reproducibility (Fay et al., 2014; Nichols et al., 2018). Currently, further evaluation of their applicability domain, including different organisms and structural and physicochemical diversity of the test substances, is actively pursued (Connors et al., 2013; Kropf et al., 2020; Kosfeld et al., 2020) and is also an important aim of the present study. Here, we assessed the intrinsic clearances of a total of twelve human pharmaceuticals in RT-S9 according to the OECD test guidance 319B, including four anti-inflammatory agents (diclofenac, ibuprofen, ketoprofen, and naproxen), seven antidepressant or antipsychotic drugs (citalopram, haloperidol, levomepromazine, mirtazapine, risperidone, sertraline, venlafaxine) and a metabolite (O-desmethyl venlafaxine). These pharmaceuticals were selected based on their high consumption volumes in human medicine, and thus expectedly high environmental exposures arising from human excretions to the sewage systems. The O-desmethyl metabolite of venlafaxine was included in the study, because it is, along with the parent venlafaxine, on the EU's Watch list (EU 2015/495) of chemical substances that may pose a significant risk to water bodies across the EU (WFD, 2000/60/EC).

Besides intrinsic clearance prediction, the subcellular liver S9 fractions also enable detailed, mechanism-based studies of dedicated xenobiotic clearance routes, such as the cytochrome P450 system, which catalyzes the majority (>70 %) of pharmaceuticals' biotransformation reactions in humans (Zanger and Schwab, 2013). Owing to the high evolutionary conservation of P450 genes in fish (Uno et al., 2012), substantial efforts have gone on investigating the functional activity (Burkina et al., 2021) as well as the inhibition of fish P450 system. Several studies have reported broad, nonselective inhibition of the P450 activities by pharmaceuticals in rainbow trout in vitro (Miranda et al., 1998; Smith et al., 2012; Burkina et al., 2013; Pihlaja et al., 2022; Pihlaja et al., 2024) and in vivo (Hasselberg et al., 2005). The inhibitory concentrations of individual pharmaceuticals toward fish P450 in vitro (Burkina et al., 2013; Pihlaja et al., 2022; Pihlaja et al., 2024) are typically thousand folds higher than their average reported concentrations in the environment (Aus der Beek et al., 2016; Wilkinson et al., 2022) or in fish plasma (Muir et al., 2017; Cerveny et al., 2021). However, if the inhibitory substances themselves are bioaccumulative (BCF > 2000), their concentrations in fish tissues may arise close to the threshold concentration that triggers inhibition in vitro. This kind of broad inhibition of the P450 system can have an unexpected impact on pharmaceuticals' intrinsic clearances in wild fish that are simultaneously exposed to mixtures of several different pharmaceuticals and other alike chemical residues, which compete for the same P450 clearance route. Furthermore, pharmaceuticals can potentially trigger synergistic P450 inhibition in fish, even at individual no-effect concentrations (Hasselberg et al., 2005; Pihlaja et al., 2022). Clearly, such combined effects are not revealed in the standardized tests (e.g., OECD 305), when the fish are exposed to one substance at a time. In fact, not many pharmaceuticals are deemed bioaccumulative (BCF > 2000), when assessed individually (Gómez-Regalado et al., 2023). However, the BCFs measured in laboratory conditions do not always match with bioaccumulation factors (BAF) in field conditions (Gómez-Regalado et al., 2023). For example, bioaccumulation (BAF > 2000) of many antimicrobials and antipsychotics has been reported in wild fish exposed to pharmaceutical mixtures (Muir et al., 2017; Cerveny et al., 2021; Grabicova et al., 2017). In addition, contradictory results have been reported regarding the bioaccumulation of, e.g., ibuprofen in fish exposed to sewage effluents containing residues of commonly used anti-inflammatory agents at different locations (Brown et al., 2007). Thus, we hypothesize that P450 inhibition could be a mechanism to explain these kind of contradictory data.

A subsequent aim of the present study was to test the validity of this hypothesis using RT-S9 assays. For this purpose, we determined the half-maximal inhibitory concentrations (IC_{50}) of the same twelve pharmaceuticals toward selected P450 activities in RT-S9 to facilitate systematic design of mixture assays. Finally, we tested the hypothesis by assessing the in vitro intrinsic clearances of some of the test pharmaceuticals in mixtures with and without P450 inhibitors.

2. Materials and methods

2.1. Rainbow trout liver S9 fractions

Rainbow trout (*Oncorhynchus mykiss*) was chosen as the model organism, because it is one of the recommended fish species for exposure assessment owing to its sensitivity to chemical contaminants (OECD 203). Two different batches of commercially available rainbow trout liver S9 fractions (RT-S9, Primacyt Cell Culture Technology GmbH, total protein concentration 20 mg/mL) were used as the enzyme sources in the present study. The in vitro intrinsic clearances of the test pharmaceuticals (individually and in mixtures) were determined using a mixed gender pool of seven (five female, two male) sexual immature fish (lot #RTL-S9 200629-3). The intrinsic clearances of diclofenac, levomepromazine, and sertraline were additionally determined using a mixed gender pool of six (three female, three male) sexual immature fish (lot #RTL-S9 180216). The cytochrome P450 inhibition assays were also performed using the pool of six fish (lot #RTL-S9 180216). The lot-specific information, including their enzyme activities provided by the supplier are given in the Supplementary material, Table S1.

2.2. Chemicals and reagents

The test pharmaceuticals (Table 1) were purchased from either Toronto Research Chemicals (citalopram, haloperidol, levomepromazine, mirtazapine, risperidone, sertraline, and venlafaxine) or Sigma-Aldrich (diclofenac, ibuprofen, ketoprofen, naproxen, and O-desmethyl venlafaxine). The prefluorescent CYP1A and CYP3A model substrates 7-ethoxyresorufin (ER) and 7-benzyloxy-4-trifluoromethyl coumarin (BFC) were purchased from Toronto Research Chemicals (TRC) and Apollo Scientific Ltd, respectively. The corresponding metabolite standards resorufin and 7-hydroxy-4-trifluoromethyl coumarin were from Sigma Aldrich and TRC, respectively.

Potassium dihydrogen phosphate, acetonitrile, methanol, and hydrochloric acid were from Riedel-de-Haën. Formic acid was from VWR International and dipotassium hydrogen phosphate from Amresco. Trizma® base (trishydroxy methylamino methane), ammoniumhydroxide (25 %, v/v, aqueous), dimethyl sulfoxide (DMSO), and the cofactors β-nicotinamide-adenindinucleotide-2-phosphate (NADPH, reduced tetrasodium salt hydrate, >93 %), uridine 5′-

Table 1
Physicochemical properties and human metabolism routes of the test pharmaceuticals. Acronyms: pKa = acid dissociation constant at logarithmic scale. $LogK_{OW}$ = (logarithm of) n-octanol-water partition coefficient. LogD = (logarithm of) distribution coefficient. CYP = cytochrome P450 (main CYP isoenzyme bolded). GST = glutathione S-transferase. MAO-A/MAO-B = monoamine oxidase (A or B).

Test pharmaceutical molecular mass	Water solubility * (mg/L, pH 7.8)	pKa * acid (a) basic (b)	LogKOW * experimental (e) predicted (p)	LogD * (pH 7.8)	Human CYP metabolism	Human Phase II conjugation	Other known human metabolism routes
Anti-inflammatory drugs							
Diclofenac [1] 296,148 g/mol	95.09	4.00(a)	4.51(e)	0.91	CYP2C9	direct acyl glucuronidation/ sulfatation	GST
Ibuprofen [2] 206,29 g/mol	52.52	4.85(a)	3.97(e)	0.996	CYP2C8, **CYP2C9**, CYP2C19	direct acyl glucuronidation	–
Ketoprofen [3] 254,281 g/mol	254.29	3.88(a)	3.12 (e)	0.23	CYP2C9, CYP3A4	direct acyl glucuronidation	–
Naproxen [4] 230,26 g/mol	230.26	4.19(a)	3.18(e)	−0.28	CYP1A2, CYP2C8, **CYP2C9**	direct acyl glucuronidation/ sulfatation	–
Antidepressant/antipsychotic drugs							
Citalopram [5] 324,392 g/mol	4.45	9.78(b)	3.76(e)	1.79	**CYP2C19**, CYP2D6, CYP3A4	not known	MAO-A, MAO-B
Haloperidol [6] 3759 g/mol	0.04	13.96(a) 8.05(b)	4.3(e)	3.22	CYP2D6, **CYP3A4**	direct O-glucuronidation	–
Levomepromazine [7] 328,473 g/mol	0.385	9.42(b)	4.68(e)	2.63	CYP1A2, **CYP3A4**	not known	–
Mirtazapine [8] 265,36 g/mol	0.873	6.67(b) 5.36(b)	2.9(e)	3.18	CYP1A2, **CYP2D6**, CYP3A4	subsequent glucuronidation	–
Risperidone [9] 410,485 g/mol	9.297	8.76(b) 4.65(b)	3.49(p)	1.62	**CYP2D6**, CYP3A4	not known	–
Sertraline [10] 306,229 g/mol	0.257	9.56(b)	5.51(e)	3.39	CYP2B6, CYP2C19, CYP2D6. **CYP3A4**	N-carbamoyl glucuronidation	MAO-A, MAO-B
Venlafaxine [11] 277,402 g/mol	10.058	14.42(a) 8.91(b)	3.2(e)	1.6	CYP2C19, **CYP2D6**, CYP3A4	subsequent glucuronidation	–
O-Desvenlafaxine [12] 263,375 g/mol	23.139	10.11(a) 8.87(b)	2.72(p)	1.45	CYP2C19, CYP2D6, CYP3A4	direct O-glucuronidation	–

* Water solubility and LogD values from Chemaxon (https://chemaxon.com/). Predicted LogKOW values from Comptox (https://comptox.epa.gov/) and experimental from PubChem. Human metabolism data collected from: [1]King et al., 2001; [2]Hamman et al., 1997; [3]Kuehl et al., 2005; [4]Tracy et al., 1997; [5]Von Moltke et al., 2001; [6]Pan et al., 1998; [7]Wójcikowski et al., 2014; [8]Dodd et al., 2001; [9]Yasui-Furukori et al., 2001; [10]Xu et al., 1999; [11]Otton et al., 1996; [12]PharmGKB, 2023, http://pharmgkb.org.

diphosphoglucuronic acid (UDPGA, trisodium salt), L-Glutathione (GSH, reduced), and adenosine 3-phosphate 5-phosphosulfate (PAPS, lithium salt hydrate, >60 %) were from Sigma-Aldrich. Alamethicin was purchased from A.G. Scientific Inc. All reagents and solvents used were of HPLC, LC-MS or analytical grade (≥98.0 %) unless otherwise stated. Water was purified with a Milli-Q water purification system (Merck Millipore).

2.3. Determination of the in vitro intrinsic clearances of individual pharmaceuticals

The intrinsic clearances of the test pharmaceuticals were determined using substrate depletion approach, in accordance with the OECD test guidance no. 319B (OECD 2018). The starting concentration of the test substance was set at 1 μM for all pharmaceuticals, based on the sensitivity of the analytical methods (see Supplementary material) and assuming that the substrate depletion at this concentration follows first-order kinetics (i.e., K_M >1 μM). Each test pharmaceutical was incubated separately with RT-S9 (1 mg/mL total S9 protein) in 100 mM potassium phosphate buffer (pH 7.8 ± 0.1) at 11 ± 1 °C, using an initial volume of 1000 μL (one vial approach). The incubations were performed in duplicate with both active RT-S9 and inactive RT-S9, using cofactor concentrations of 2 mM NADPH, 2 mM UDPGA, 0.1 mM PAPS, and 5 mM GSH. The inactive RT-S9 was prepared by denaturing the RT-S9 stock (20 mg/mL) in boiling water (100 °C) bath for 15 min or at room temperature for at least 5 days, after which it was diluted with the incubation buffer (10 mg/mL) and aliquoted for storing at −20 °C until use.

Before initiation of the enzymatic reactions, the RT-S9 fractions were preincubated with alamethicin (25 μg/mL) at 11 ± 1 °C for 15 min. Next, the clearance assays were initiated by the addition of the cofactors and the test pharmaceutical. A total of nine samples (each 100 μL) were withdrawn from the incubation vial at sampling times of 2, 10, 20, 30, 60, 90, 120, 150 and 180 min. The enzyme activity of these aliquots was quenched by adding 100 μL (1:1) or 200 μL (1:2) ice-cold acetonitrile and incubating the samples on ice for at least 30 min, before centrifugation (15,000 g, 20 min). To quantify the amount of the remaining (nonmetabolized) pharmaceutical, the supernatants were analyzed by liquid chromatography.

The substrate depletion rates of the test pharmaceuticals were determined using log_{10}-linear transformation of the pharmaceutical concentration (μM) over incubation time (h^{-1}). The slope of the log_{10}-linear decline was determined using Graphpad Prism software (version 9.0.0). Finally, the slope of the active RT-S9 (average of two statistically identical series) was used for the calculation of the in vitro intrinsic clearance (CL_{INT}; mL/h/mg protein) according to Eq. (1):

$$CL_{INT,in\ vitro} = \frac{k_e}{C_{S9}} = \frac{-2.3 \times slope}{C_{S9}} \quad (1)$$

where k_e is the depletion rate constant (h^{-1}), and C_{S9} is the total S9 protein concentration used in the incubation (here, 1 mg/mL). The extrapolated in vivo CL_{INT} values were derived by multiplying the in vitro CL_{INT} values with a scaling factor of 163 mg microsomal protein/g liver, as instructed by Nichols et al. (2013). The depletion rate constants (k_e) were further exploited to calculation of the bioconcentration factors (BCF) of the test substances as instructed in the OECD guidance document no. 280.

Those substances, which exhibited substrate depletion rates significantly different from zero (diclofenac, ibuprofen, levomepromazine, naproxen, and sertraline), were additionally incubated individually (1

μM) with RT-S9 and all other cofactors, but not NADPH, to examine the importance of P450 metabolism on their overall clearance rates. These incubations were also performed in duplicate both with active and inactive RT-S9, and the samples were prepared and analysed similarly by liquid chromatography.

2.4. Determination of the half-maximal inhibitory concentrations of the test pharmaceuticals

The half-maximal inhibitory concentrations (IC_{50}) of each test pharmaceutical were determined separately toward two different CYP marker activities, 7-ethoxyresorufin O-deethylation (EROD; CYP1A) and benzyloxy-4-trifluoromethylcoumarin-O-debenzyloxylation (BFCOD; CYP3A). The enzyme-specificity of these model reactions has been established in previous literature (Hegelund et al., 2004; Jönsson et al., 2006; Christen et al., 2009). In the IC_{50} assays, each test pharmaceutical was incubated at six different concentrations (between 0.1 and 500 μM) with active RT-S9 (0.5 mg/mL; #RTL-S9 180216), alamethicin (25 μg/mL), and cofactors (1 mM NAPDH, 1 mM UDPGA, 2.5 mM GSH, and 0.05 mM PAPS) in 100 mM potassium phosphate buffer (pH 7.8 ± 0.1) at 11 ± 1 °C. In these reactions, the concentration of the marker substrate was adjusted close to its K_M value reported for rainbow trout liver microsomes in a previous study (Pihlaja et al., 2022), and was 1 μM for 7-ethoxyresorufin (ER; CYP1A) and 75 μM for benzyloxy-4-trifluoromethylcoumarin (BFC; CYP3A). The incubation times of both EROD (10 min) and BFCOD (20 min) reactions were also pre-optimized to ensure that the basal activities (without inhibitor) follow first order kinetics (see Supplementary material, Fig. S1).

The IC_{50} values of the test pharmaceuticals were determined using the IC_{50} shift method (Obach et al., 2007; Berry and Zhao, 2008; Grimm et al., 2009) and two different preincubation (30 min) protocols before initiation of the EROD or BFCOD reaction. In the first protocol (Cofactors-), the RT-S9 was preincubated with only the test pharmaceutical, and the marker reaction was initiated by the addition of the marker substrate (ER or BFC) and the cofactors. In the second protocol (Cofactors+), the RT-S9 was preincubated with both the test pharmaceutical and the cofactors, and the marker reaction was initiated by the addition of the marker substrate. The incubations (both series, all concentrations) were done in duplicate in a total reaction volume of 100 μL and terminated by the addition of 37.5 μL of ice-cold acetonitrile/-Trizma base (0.5 M) 4:1 to the incubation mixture. The samples were then incubated on ice for at least 30 min and centrifuged (15,000 g, 15 min). The supernatants (100 μL) were analyzed for fluorescence with Varioskan LUX microplate reader (Thermo Fisher Scientific) to quantitate the respective marker metabolites, resorufin (ex/em 570 nm/595 nm) or 7-hydroxy-4-trifluoromethyl coumarin (ex/em 419 nm/501 nm) at different concentrations of the test substance, including control (zero pharmaceutical concentration).

The test pharmaceuticals' IC_{50} values toward EROD and BFCOD activities were determined separately for both differently preincubated series (i.e., Cofactor- and Cofactor+) using the GraphPad Prism software and a nonlinear regression, without weightings, according to Eq. (2):

$$y = \frac{100}{1 + 10^{(LogIC50 - x) \times HillSlope}} \quad (2)$$

where y is the relative activity (%) of the marker reaction compared with control incubation performed at zero pharmaceutical concentration, x is the concentration of the test pharmaceutical (μM), and Hill Slope is the steepness of the curve (constant value of −1). Owing to the limited water solubility of the test pharmaceuticals, all stock solutions were prepared in appropriate organic solvent and diluted so that the residual solvent concentration was constant between all incubations (2 %, v/v, DMSO in EROD incubations or 1 %, v/v, DMSO and 1 %, v/v, acetonitrile in BFCOD incubations). Thus, the impact of the residual solvent on the relative EROD or BFCOD activities could be considered negligible. To account for the impact of microsomal binding, the nominal IC_{50} values were multiplied by the unbound (free) fraction of the test substance in RT-S9 ($f_{U,RT\text{-}S9}$), to calculate the unbound IC_{50}. The IC_{50} shifts were calculated by dividing the IC_{50} values obtained from Cofactor(−) series with that of Cofactor(+) series, according to Eq. (3):

$$IC_{50\ shift} = \frac{IC_{50\ Cofactor(-)}}{IC_{50\ Cofactor(+)}} \quad (3)$$

The test compound was considered as a time-dependent inhibitor, if the IC_{50} shift was ≥1.5.

2.5. Determination of the unbound fractions of test pharmaceuticals in rainbow trout S9 fractions

The unbound (free) fractions of the test pharmaceuticals in RT-S9 ($f_{U,RT\text{-}S9}$) were determined using rapid equilibrium dialysis assay (RED, Thermo Fisher Scientific), which exploits two-compartment inserts separated by a dialysis membrane with a molecular weight cut-off of 8000 Da. In the RED assay, the test pharmaceutical (50 μM) was incubated with RT-S9 (1 mg/mL; #RTL-S9 200629-3) in 100 mM potassium phosphate buffer (pH 7.8 ± 0.1) in a total volume of 100 μL on one side of the membrane (sample chamber), and 300 μL of the phosphate buffer on the other side (buffer chamber). To reach equilibrium, the samples were incubated on an orbital shaker (LLG UniThermix 2, 300 rpm) at 11 °C for 4 h. Next, an aliquot of 50 μL was withdrawn from sample chamber and diluted with 50 μL of the phosphate buffer. Similarly, an aliquot of 50 μL was withdrawn from the buffer chamber and mixed with 50 μL of inactive RT-S9 (1 mg/mL) solution. Finally, 200 μL acetonitrile was added to both aliquots separately to precipitate the proteins, the samples were centrifuged (15,000 g, 20 min), and the supernatants were analyzed by liquid chromatography to quantitate the pharmaceuticals concentrations on both sides of the membrane at equilibrium. All test pharmaceuticals were assessed in triplicate and the average $f_{U,RT\text{-}S9}$ was calculated by dividing the pharmaceutical's concentration in the buffer chamber ($c_{protein\text{-}free}$) with that of the sample chamber ($c_{protein\text{-}containing}$), according to Eq. (4):

$$f_{U,\ RT-S9} = \frac{C_{protein-free}}{C_{protein-containing}} \quad (4)$$

2.6. Determination of the in vitro intrinsic clearances of the test pharmaceuticals in mixtures

In addition to individual incubations, the intrinsic clearance rates of selected test pharmaceuticals were determined by incubating them as mixtures, and as mixtures together with selected EROD inhibitors (haloperidol and mirtazapine), to evaluate the impact of CYP inhibition on their overall clearance rates in RT-S9. For this purpose, four different mixture assays were designed, including a binary mixture of diclofenac and naproxen (each 1 μM) and a quinary mixture of diclofenac, ibuprofen, levomepromazine, naproxen, and sertraline (each 1 μM). The binary mixture of diclofenac and naproxen was additionally conducted with sub-inhibitory concentrations (0.01 μM) each of haloperidol, levomepromazine, mirtazapine, and sertraline, and the quinary mixture with inhibitory concentrations of haloperidol and mirtazapine (each 37.5 μM). In each case, the substrate depletion rates of the selected test substances (1 μM) were determined by liquid chromatography and the in vitro intrinsic clearances calculated based on the slope of the $\log_{10}$-linear regression plot, according to Eq. (1). The theoretical combined inhibitory impacts (%) of the test substances included in the mixture assays, toward EROD and BFCOD activities, were calculated following the concentration addition model (Loewe and Muischnik, 1926), using Eq. (5):

$$\sum Inhibition\ (\%) = 100\% \times \sum_{i=1}^{n} \frac{C_i}{IC50_i} \quad (5)$$

where c_i is the concentration of substance i in the mixture and $IC_{50,i}$ (μM) is its half-maximal inhibitory concentration toward EROD or BFCOD activity in RT-S9 (determined in this study).

2.7. Liquid chromatography

The pharmaceuticals' concentrations in RT-S9 incubation matrices (clearance and $f_{U,RT\text{-}S9}$ determinations) were determined using high performance liquid chromatography (HPLC) with UV, fluorescence, or mass spectrometric detection. The instrumentation and the methods used are described in detail in Supplementary material, Tables S2-S4. For the clearance determination, the standards were prepared in a 1:1 (v/v) mixture of the incubation buffer (100 mM potassium phosphate, pH 7.8 ± 0.1) and acetonitrile, and the LC methods were validated with respect to lower limit of quantitation (LLOQ), linearity (between LLOQ and 2 μM concentration), precision, accuracy, and recovery, by adhering to the FDA Bioanalytical Method Validation Guidance for Industry (FDA, 2018). The LLOQ was defined as the lowest concentration of the standard curve that was within ± 20 % (precision and accuracy). The precision and accuracy within run ($n = 5$) and between runs ($n = 3$) were additionally assessed on three different concentration levels (low, mid, high) using the validation criteria within ±15 %. The recovery of each test pharmaceutical from the matrix ($n = 5$) was also determined on three different concentration levels. For this purpose, blank matrix was prepared by incubating inactive RT-S9 (1 mg/mL) with alamethicin (25 μg/mL) and all cofactors (NADPH 2 mM, UDPGA 2 mM, GSH 5 mM, PAPS 0.1 mM), and spiked separately with each test substance. The proteins were precipitated by adding 100 μL of cold acetonitrile and incubating on ice for 30 min followed by centrifugation (15,000 g, 20 min). The validation data is presented in Supplementary material, Table S5. For the $f_{U,RT\text{-}S9}$ determination, the standards were prepared in a 1:2 (v/v) mixture of the incubation buffer (100 mM potassium phosphate, pH 7.8 ± 0.1) and acetonitrile. The calibration curves were established at higher concentration ranges (Supplementary material, Table S5) and run on the same day with samples and appropriate quality controls at low, mid and high concentrations including blanks.

2.8. Statistical analyses

The statistical analysis of the slopes of the $\log_{10}$-linear decline of the test pharmaceuticals in the individual incubations as well as in the mixture assays was determined using Graphpad Prism software (version 9.0.0). First, the statistical similarity of the slopes of two parallel actives (or inactives) was evaluated using the analysis of covariance (ANCOVA) to calculate a P value (two-tailed), which tests if the slopes are identical and parallel (null hypothesis). In addition, the F-test was used to compare if the slopes are significantly different from zero. Finally, to distinguish metabolic clearance from abiotic loss, the slopes of the active RT-S9 (average of two statistically similar series) were compared with inactive RT-S9 using the ANCOVA test. The threshold for statistically significant difference was $p < 0.05$.

3. Results and discussion

3.1. In vitro intrinsic clearance rates of the test pharmaceuticals in rainbow trout liver S9 fractions

The intrinsic clearances of altogether twelve pharmaceuticals in RT-S9 fractions were assessed in the present study according to the OECD test guidance 319B. The $\log_{10}$-linear regression plots of all test substances, established based on the substrate depletion assays, are given in Fig. 1. On the basis of statistical analysis (ANCOVA), the substrate depletion rates of six out of twelve substances assessed in this study were significantly different from zero in RT-S9, including those of diclofenac, ibuprofen, ketoprofen, naproxen, levomepromazine, and sertraline. The corresponding in vitro intrinsic clearances (CL_{INT}) calculated based on the slopes of the $\log_{10}$-linear plots, as well as the extrapolated in vivo CL_{INT} obtained using an average scaling factor of 163 mg microsomal

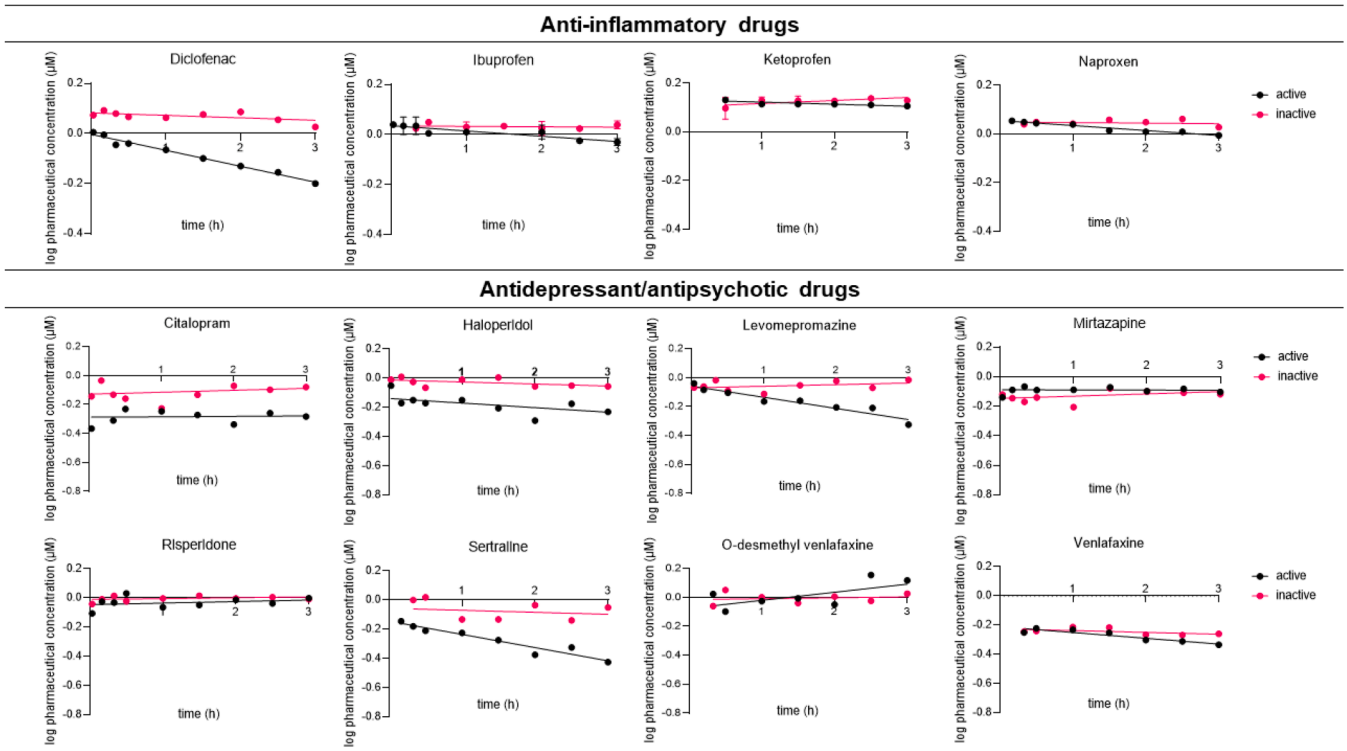

Fig. 1. Hepatic biotransformation rates of test pharmaceuticals. The $\log_{10}$-linear regression plots of the test pharmaceuticals incubated individually with RT-S9 at 1 μM concentration (each) with all cofactors, including NADPH (2 mM), UDPGA (2 mM), PAPS (0.1 mM), and glutathione (5 mM), as well as alamethicin (25 μg/mL). All incubations were performed in duplicate both with active and inactive RT-S9 using the RT-S9 pool of seven fish (#RTL-S9 200629-3).

protein/g liver (Nichols et al., 2013), are given in Table 2. For the other test substances, no hepatic clearances were observed or the substrate depletion rates in RT-S9 were too slow to be reliably quantified (i.e., not significantly different from zero). The intrinsic clearances of all twelve test substances were determined using the same commercial RT-S9 pool of seven fish (lot #RTL-S9 200629-3).

To examine the possible RT-S9 lot-to-lot variation in the in vitro clearances, the substrate depletion rates of the three substances exhibiting the most extensive metabolism in the pool of seven fish (diclofenac, levomepromazine, sertraline) were additionally determined using another commercial RT-S9 pool of six fish (lot #RTL-S9 180216). The comparison of the $\log_{10}$-linear regression plots between the two different RT-S9 lots is given in Fig. 2 and the corresponding in vitro and in vivo CL_{INT} in Table 2. The data demonstrates statistically similar substrate depletion rates between the two RT-S9 pools for diclofenac ($p = 0.1609$) and sertraline ($p = 0.2617$). However, the substrate depletion rates of levomepromazine were statistically different ($p < 0.0001$) between the RT-S9 pools. These results preliminarily suggest that the biotransformation of levomepromazine in rainbow trout is likely catalyzed by different enzymes than those responsible of the biotransformation of diclofenac and sertraline. This kind of uneven variation in the enzyme activities between RT-S9 pools can arise from, e.g., seasonal variation or other phenotype differences between individual fish. Our results emphasize that these factors need to be accounted for when selecting fish for cell harvesting. If the primary aim is to predict the average clearance rates for a number of pharmaceuticals, like in this study, increasing the pool size (number of fish) could help reduce lot-to-to variation. This is also the accustomed practice in human in vitro biotransformation assays, where the pool size for subcellular liver fractions typically varies between at least twenty and up to a hundred donors.

Compared with previous literature, the extrapolated in vivo CL_{INT} of diclofenac (19–24 mL/h/g liver) was about two-fold higher than that reported earlier (9.5 mL/h/g liver) by Connors et al. (2013). However, in the previous work, a different scaling factor (50 mg microsomal protein/g liver), derived from in-house characterization data, was used for the in vitro-in vivo extrapolation. By using this same scaling factor (50 mg microsomal protein/g liver), the extrapolated in vivo CL_{INT} of diclofenac derived from the in vitro data of the present study is very close (5.9–7.3 mL/h/g liver) to that reported by Connors et al. (2013) and likely falls within the lot-to-lot variation between RT-S9 preparations.

Besides in vivo CL_{INT} extrapolation, the in vitro elimination rate constants were also exploited to calculation of the predicted bioconcentration factors (BCFs), according to the OECD guidance 280. Table 2 gives an overview of the calculated BCFs with and without incorporation of the in vitro CL_{INT}. This comparison illustrates the impact of hepatic biotransformation on the calculated BCFs, which

Table 2
Hepatic biotransformation of test pharmaceuticals. The slopes of the $\log_{10}$-linear regressions and of those test substances, for which the substrate depletion rate (h^{-1}) was significantly different from zero ($p < 0.05$) as well as the in vitro intrinsic clearances (CL_{INT}) calculated according to Eq. (1) and the in vivo CL_{INT} and bioconcentration factor (BCF) extrapolations calculated according to OECD guidances 319B and 280, respectively. BCF_{TOT} = calculated on a total conc basis, without lipid normalization (mL/g). $BCF_{FD,L}$ = calculated on freely dissolved basis, normalized for fish lipid (mL/g lipid). Lot numbers #RTL-S9 180216 and #RTL-S9 200629-3 refer to the pools of six and seven fish, respectively. BAF = Bioaccumulation factor. (p) = predicted. <LOQ = Below lower limit of quantitation.

Test substance	RT-S9 lot	Slope (R^2) (h^{-1})	In vitro CL_{INT} [a] (mL/h/mg protein)	In vivo CL_{INT} [b] (mL/h/g liver)	Calculated BCF_{TOT} / $BCF_{FD,L}$ [c]		Predicted BCF (p) [d] /Empirical BCF or BAF (*species*)
					w/o metabolism	w/ $CL_{INT,in\ vitro}$	
Anti-inflammatory drugs							
Diclofenac	*#RTL-S9 180216*	−0.051 ± 0.007 (0.78)	0.118 ± 0.016 0.147 ± 0.012	19 ± 3 24 ± 2	1561/ 31,939	460/ 9410 414/ 8479	15.2 (p) / 2730 (*Oncorhynchus mykiss* (*liver*)) [d] <11 (*Oncorhynchys mykiss*, wild fish) [e]
	#RTL-S9 200629-3	−0.064 ± 0.005 (0.90)					
Ibuprofen	*#RTL-S9 200629-3*	−0.021 ± 0.005 (0.61)	0.049 ± 0.011	8.0 ± 1.8	462/ 9297	309/ 6222	46.1 (p) / 14,100–49,000 (*Oncorhynchus mykiss* (*bile*), *0.630 Pimephales promelas* (*muscle*), *1.57 Pimephales notatus* (*gills*), *0.51 Ictalurus punctatus* (*liver*)) [d] <63…18,667 (*Oncorhynchys mykiss*, wild fish) [e]
Ketoprofen	*#RTL-S9 200629-3*	−0.008 ± 0.002 * (0.68)	(0.019 ± 0.004) *	3.2 ± 0.7	66/ 1317	62/ 1247	7.21 (p) / <29 (*Oncorhynchys mykiss*, wild fish) [e]
Naproxen	*#RTL-S9 200629-3*	−0.021 ± 0.003 (0.82)	0.047 ± 0.006	7.7 ± 1.1	76/ 1513	66/ 1330	5.65 (p) / <2–56 (*Oncorhynchys mykiss*, wild fish) [e]
Antidepressant/antipsychotic drugs							
Levomepromazine	*#RTL-S9 180216*	−0.218 ± 0.011 (0.96)	0.502 ± 0.026 0.177 ± 0.026	82 ± 4 29 ± 4	2269/ 46,950	292/ 6036 444/ 9189	126 (p) / <LOQ (*Salmo trutta m. fario*, wild fish) [f]
	#RTL-S9 200629-3	−0.077 ± 0.011 (0.76)					
Sertraline	*#RTL-S9 180216*	−0.062 ± 0.009 (0.93)	0.143 ± 0.020 0.208 ± 0.039	23 ± 3 34 ± 6	11,615/ 286,275	826/ 20,358 667/ 16,437	990 (p) / 418 (*Pimephales promelas* (*muscle*)) [d] 680…4400 (*Salmo trutta m. fario*, wild fish) [f]
	#RTL-S9 200629-3	−0.091 ± 0.017 (0.69)					

[a] Calculated from the slope according to Eq. (1).
[b] Calculated from the in vitro CL_{INT} using a scaling factor of 163 mg S9 protein per g liver (OECD 319B).
[c] Calculated using the MS Excel spreadsheet provided in the OECD guidance no. 280, using $\log K_{OW}$ values from Table 1 and assuming unbound fraction (f_U) equal to one.
[d] US EPA, 2023.
[e] Brown et al., 2007.
[f] Grabicova et al., 2017.
* The reaction rate (k_e), defined as −2.3 × slope, below the minimum reliably detectable range of 0.05–0.14 h^{-1} recommended by the OECD 319B.

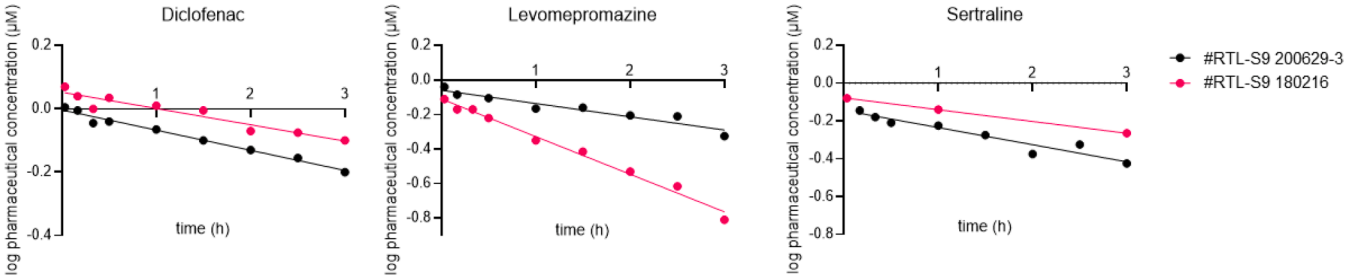

Fig. 2. Lot-to-lot variation in hepatic biotransformation rates between RT-S9 pools of six (#RTL-S9 180216) and seven (#RTL-S9 200629-3) sexual immature fish. Comparison of the $\log_{10}$-linear regression plots of diclofenac, levomepromazine, and sertraline, incubated individually at 1 µM concentration (each) with the RT-S9 pools of seven fish (sacrificed in the summer) and six fish (sacrificed in the winter). All incubations were performed in duplicate both with active and inactive (data not shown) RT-S9 and including all cofactors (NADPH 2 mM, UDPGA 2 mM, PAPS 0.1 mM, and glutathione 5 mM) and alamethicin (25 µg/mL).

typically results in a fold-decrease in the BCF of fairly lipophilic ($\log K_{OW} > 4.5$) and extensively metabolized substances, like diclofenac, levomepromazine and sertraline (Table 2). As a result, the calculated BCFs of all four anti-inflammatory agents (diclofenac, ibuprofen, ketoprofen, naproxen) are well below the threshold for bioaccumulation (BCF < 2000). This result is well in line with a previous study reporting insignificant bioaccumulation (BCF < 100) of the same set of anti-inflammatory agents in trout exposed to sewage effluents at ≤1 µg/L pharmaceutical concentrations (Brown et al., 2007). In another study, progressive increase in the BAF (kidney) of citalopram and mirtazapine over exposure time was observed in trout exposed to treated wastewater containing residues of antidepressant and antipsychotic drugs (Grabicova et al., 2017). This is also well in line with the fact that these pharmaceuticals had insignificant hepatic clearance rate in RT-S9 (this study). On the other hand, the BAF of venlafaxine in fish in vivo was insignificant (Grabicova et al., 2017), even if it is not effectively metabolized in RT-S9 (this study). This is likely because of ionization at physiological pH, which effectively decreases the lipophilicity of many pharmaceuticals (log K_{OW} vs. logD_{OW}, Table 1), and consequently, their bioaccumulation in fish tissues. Nevertheless, previous literature has also reported in vivo BCFs or BAFs >2000 for some of the substances that were significantly depleted in RT-S9 (e.g., diclofenac, ibuprofen, sertraline) in fish in vivo (Table 2). This warrants for further research of their clearance routes and possible mixture effects in fish.

3.2. *In vitro intrinsic clearance routes of the test pharmaceuticals in rainbow trout*

To further examine the hepatic clearance routes in rainbow trout, those pharmaceuticals that exhibited quantifiable substrate depletion in RT-S9 were additionally incubated without NADPH, the cofactor of P450 system. In humans, all four anti-inflammatory agents (diclofenac, ibuprofen, ketoprofen, naproxen) are principally CYP2C substrates, although they may also undergo direct (acyl) glucuronidation to the carboxylic acid moiety by human UGTs (Table 1). In analogy, a previous study has reported upon the formation of both 2-hydroxyibuprofen (a CYP metabolite) and ibuprofen glucuronide (a UGT metabolite) in liver S9 fractions of rainbow trout (Gomez et al., 2011). In the present study, when incubated without NADPH, the substrate depletion rates of ibuprofen and naproxen zeroed and that of diclofenac decreased by ca. one-third compared with the basal clearances determined with all cofactors (Fig. 3). The substrate depletion rates of ibuprofen and naproxen

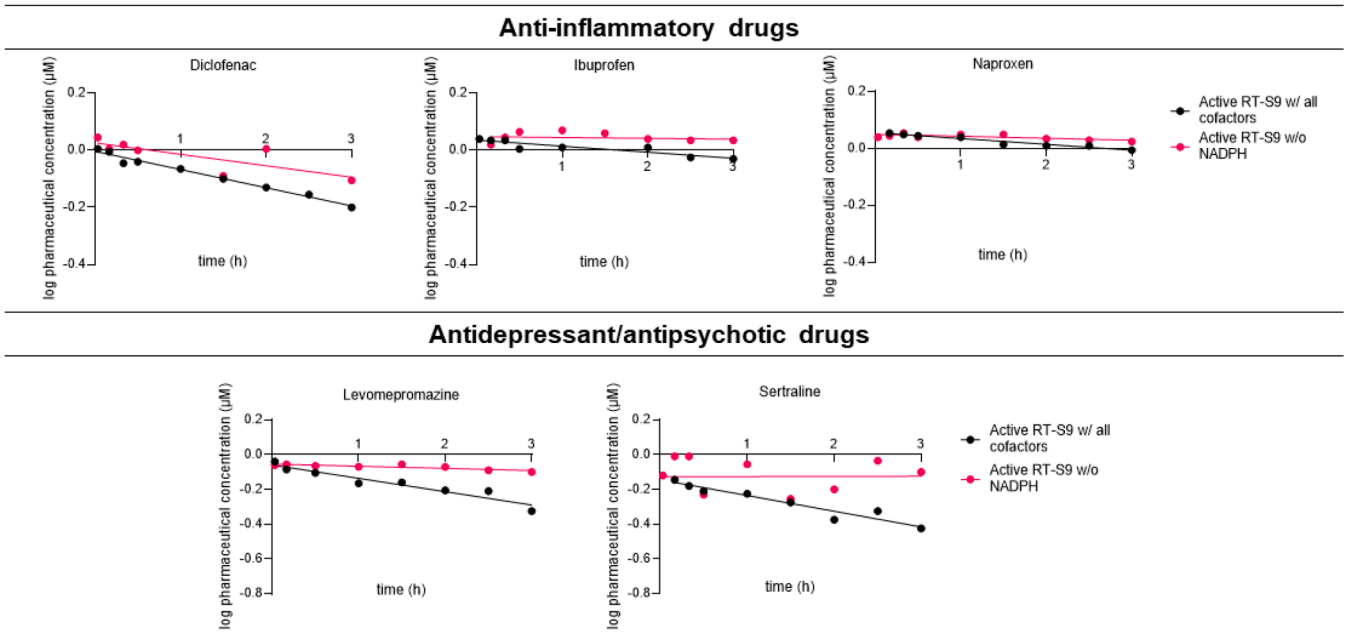

Fig. 3. Hepatic biotransformation rates of test pharmaceuticals with and without cytochrome P450 metabolism. Comparison of the $\log_{10}$-linear regression plots of diclofenac, ibuprofen, naproxen, levomepromazine, and sertraline, incubated individually (1 µM) with RT-S9 (pool of seven fish; #RTL-S9 200629-3) with and without NADPH (2 mM), the crucial cofactor of P450 metabolism. All incubations were performed in duplicate both with active and inactive (data not shown) RT-S9 and including all other cofactors (UDPGA 2 mM, PAPS 0.1 mM, and glutathione 5 mM) and alamethicin (25 µg/mL).

without NADPH were statistically different ($p < 0.05$) from those determined with all cofactors included, suggesting that these substances are likely to undergo substantial P450 metabolism in rainbow trout. Instead, the substrate depletion rates of diclofenac with and without NADPH were not statistically different from each other ($p = 0.0622$), suggesting that diclofenac is at least partly biotransformed by phase II transferase enzymes in rainbow trout, presumably via UGTs like in human. For ketoprofen, the elimination rate constant with all cofactors included ($k_e = 0.019\ h^{-1}$) was below the recommended minimum of in vitro activity, which can be reliably quantified (i.e., 0.05 to 0.14 h^{-1}; Nichols et al., 2013; Chen et al., 2016), and thus comparison against the substrate depletion rate without NADPH could not be performed.

The antidepressants and antipsychotics assessed in this study are all human CYP3A4 substrates, although some are principally metabolized via human CYP2C19 or CYP2D6 enzymes (Table 1). Additionally, haloperidol and the O-desmethyl metabolite of venlafaxine can undergo direct O-glucuronidation in human, and sertraline can form N-carbamoyl glucuronides, without prior phase I metabolism (Table 1). For all other antidepressants/antipsychotics assessed in this study, the metabolic clearance in human is predominantly dependent on phase I (P450) metabolism prior to further phase II conjugation reactions (Table 1). Similar to mammalian CYP3A, rainbow trout CYP3A27 is highly abundant in liver and displays 54.9 % sequence homology to human CYP3A4 (Lee et al., 1998). Nevertheless, only levomepromazine and sertraline were extensively metabolized in RT-S9 in the present study. When incubated without NADPH, the substrate depletion rates of both levomepromazine ($p = 0.0003$) and sertraline ($p = 0.0073$) zeroed and were statistically different from those determined with all cofactors included (Fig. 2). This indicates that their intrinsic clearances in RT-S9 are critically dependent on P450 metabolism. Similar to our findings, a previous study by Burkina et al. (2020) has reported about hepatic metabolism of sertraline, but not venlafaxine, in S9 fractions of common carp (*Cyprinus carpio*). In the previous study, the biotransformation of sertraline in common carp in vitro yielded pharmacologically active metabolite, N-desmethylsertraline, which is primarily produced via CYP3A4 or CYP2 isoenzymes in human. This gives reason to believe that CYP3A-like activity is the likely clearance pathway for sertraline in RT-S9 as well. However, contrary to RT-S9 (this study), hepatic metabolism of citalopram was detected in S9 fractions of common carp, yielding N-desmethyl metabolite, also a CYP3A4 catalyzed pathway in human (Burkina et al., 2020).

The results of the present study are well in line with the findings by Connors et al. (2013), who screened the intrinsic clearances of diclofenac and a total of eleven other pharmaceuticals in RT-S9. The majority of these pharmaceuticals were also principally human CYP2C9, CYP2D6 or CYP3A4 substrates, but quantifiable intrinsic clearances were only detected for propranolol, a human CYP1A2 substrate, as well as diclofenac. Among the antidepressants/antipsychotics assessed in this study, levomepromazine and mirtazapine are also substrates of human CYP1A2, which has an abundant sequence homologue, CYP1A1, expressed by most rainbow trout tissues, including liver (Burkina et al., 2021). In this study, quantifiable substrate depletion was not detected for mirtazapine in RT-S9, but the results suggest that CYP1A-like activity could be an important pathway for the clearance of levomepromazine in rainbow trout in addition to CYP3A-like activity.

3.3. Cytochrome P450 inhibition by the test pharmaceuticals in rainbow trout liver S9 fractions

Overall, the CYP1A (EROD) and CYP3A-like (BFCOD) activities are known to be inducible in rainbow trout by many xenobiotics (Burkina et al., 2021), thus representing the most likely primary clearance routes for pharmaceuticals. However, the CYP1A and CYP3A-like activities are also inhibited by many pharmaceuticals in rainbow trout in vitro and in vivo (Miranda et al., 1998; Hasselberg et al., 2005; Smith et al., 2012; Burkina et al., 2013; Pihlaja et al., 2022; Pihlaja et al., 2024). Therefore, the inhibitory impacts of the test substances toward the EROD and BFCOD activities in RT-S9 were also assessed in this study using the IC_{50} shift approach. When preincubated together with the cofactors (Cofactor(+) series), the test substance can readily metabolize in RT-S9 before the marker substrate is added. This can increase the inhibitory impact of the test substance, if biotransformation yields metabolites that are more potent inhibitors than the parent form or react with and irreversibly inhibit the monitored enzyme system. As a result, a fold-increase in the IC_{50} value is observed compared with preincubation without the cofactors (Cofactor(-) series). If the IC_{50} shift is $\geq$1.5 (Eq. (3)), the test substance is considered a time-dependent inhibitor (TDI), which is a warning flag in human drug discovery and development for possibly clinically important drug-drug interactions arising from long-lasting enzyme inactivation (Grimm et a. 2009). In the present study, we evaluated the risk of long-lasting enzyme inhibition in rainbow trout through identification of possible TDIs for EROD and BFCOD activities in RT-S9.

The nonlinear regression analyses of the residual EROD and BFCOD activities at different pharmaceutical concentrations are given in the Supplementary materials (Supplementary material, Figs. S2 and S3, respectively) and the IC_{50} values obtained from these analyses are summarized in Table 3. On the basis of our results, none of the anti-inflammatory agents inhibited either EROD or BFCOD at nominal concentrations <500 μM, with the exception of ketoprofen, which resulted in apparent, very weak EROD inhibition ($IC_{50} > 200$ μM), when preincubated with the cofactors. This result correlates well with human P450 interactions (Preissner et al., 2010), as none of the anti-inflammatory agents are inhibitors of human CYP1A or CYP3A either. The nonspecific binding of the anti-inflammatory drugs to RT-S9 was also generally low (Table 3). Instead, the majority of the antidepressants and antipsychotics bound more extensively to the RT-S9 and also inhibited both marker activities. The strongest inhibitory impacts were observed toward the EROD activity by levomepromazine and sertraline, each with unbound $IC_{50} < 1$ μM. These substances were also the strongest inhibitors of BFCOD activity, with slightly higher IC_{50} values and extensive microsomal binding to RT-S9 with free fraction as small as 3 % (Table 3). Additionally, sertraline was indicatively a TDI of BFCOD (CYP3A-like) activity, but not EROD activity in RT-S9. This suggests that its biotransformation in rainbow trout may result in formation of metabolites that inhibit or react with CYP3A-like enzymes. These results correlate well with human P450 interactions, as levomepromazine is a known inhibitor of both human CYP1A2 and CYP3A4 (Basińska-Ziobroń et al., 2015) and sertraline a TDI of human CYP3A4 (Masubuchi and Kawaguchi, 2013), but not human CYP1A2 (Preissner et al., 2010).

Among the other test substances, haloperidol was also identified as a TDI (IC_{50} shift > 1.5) of both marker activities (Table 3), exhibiting moderate EROD inhibition ($IC_{50} < 10$ μM), weak BFCOD inhibition ($IC_{50} < 100$ μM), and strong microsomal binding to RT-S9 (free fraction ca. 0.14). In human, haloperidol is not known to inhibit neither CYP1A2 nor CYP3A4 (Preissner et al., 2010). Thus, it may be hypothesized that its inhibitory impacts toward the corresponding enzyme activities in RT-S9 could result from its strong, nonselective binding to the microsomal proteins rather than formation of a reactive or an inhibitory metabolite. This conclusion is also supported by the fact that haloperidol was not effectively metabolized in the substrate depletion assays (Fig. 1). Besides haloperidol, none of the other test substances were identified as TDIs of neither EROD nor BFCOD activities in RT-S9, even if some of them, such as citalopram (free fraction ca. 0.35) and risperidone (free fraction ca. 0.19), were strongly bound to the RT-S9 fractions. Risperidone and venlafaxine or its O-desmethyl metabolite were not inhibiting either one of the marker activities at nominal concentrations <100 μM (Table 3), but citalopram was a weak inhibitor of both EROD and BFCOD ($IC_{50} \sim 100$ μM) and mirtazapine the only selective inhibitor of EROD ($IC_{50} \sim 30$ μM), but not BFCOD. These results correlate well with human P450 interactions, since neither citalopram, mirtazapine, risperidone nor

Table 3
Nonspecific binding of and enzyme inhibition by test pharmaceuticals. The free (unbound) fractions of the test substances in rainbow trout (RT) liver S9 fractions ($f_{U,RT\text{-}S9}$) and their half-maximal inhibitory concentrations (IC_{50}) toward the ethoxyresorufin-O-deethylase (EROD) and benzyloxy-4-trifluoromethylcoumarin-O-debenzyloxylase (BFCOD) activities in RT-S9 incubations, when preincubated with (+) and without (−) cofactors before initiation of the marker reactions. The unbound IC_{50} values and IC_{50} shifts were calculated according to Eqs. (4) and (3), respectively. IC_{50} shifts ≥1.5 are bolded. n.s. = not significant. n/a = not applicable.

Test substance	Unbound fraction $f_{U,RT\text{-}S9}$ (at 50 μM)	Inhibition of the EROD activity in RT-S9			Inhibition of the BFCOD activity in RT-S9		
		Nominal IC_{50} (μM) Cofactors(−) Cofactors(+)	Unbound IC_{50} (μM) Cofactors(−) Cofactors(+)	IC_{50} shift	Nominal IC_{50} (μM) Cofactors(−) Cofactors(+)	Unbound IC_{50} (μM) Cofactors(−) Cofactors(+)	IC_{50} shift
Anti-inflammatory drugs							
Diclofenac	0.63 ± 0.01	n.s.	n.s.	n/a	n.s.	n.s.	n/a
Ibuprofen	0.96 ± 0.18	n.s.	n.s.	n/a	n.s.	n.s.	n/a
Ketoprofen	0.74 ± 0.03	–	–	n/a	n.s.	n.s.	n/a
		289 ± 97	214 ± 72				
Naproxen	0.88 ± 0.11	n.s.	n.s.	n/a	n.s.	n.s.	n/a
Antidepressant/antipsychotic drugs							
Citalopram	0.35 ± 0.13	286 ± 72	100 ± 45	0.80 ± 0.56	287 ± 74	100 ± 45	1.2 ± 0.7
		356 ± 137	125 ± 67		231 ± 72	81 ± 30	
Haloperidol	0.14 ± 0.05	36 ± 12	5.1 ± 2.4	3.0 ± 2.4	529 ± 217	74 ± 40	1.6 ± 1.1
		12 ± 6	1.7 ± 1.0		321 ± 168	45 ± 16	
Levomepromazine	0.03 ± 0.00	24 ± 7	0.72 ± 0.21	0.93 ± 0.40	103 ± 23	3.1 ± 0.7	0.92 ± 0.21
		26 ± 8	0.77 ± 0.24		112 ± 46	3.4 ± 0.2	
Mirtazapine	0.60 ± 0.02	49 ± 11	30 ± 7	0.67 ± 0.33	n.s.	n.s.	n/a
		73 ± 19	44 ± 12				
Risperidone	0.19 ± 0.09	n.s.	n.s.	n/a	n.s.	n.s.	n/a
Sertraline	0.03 ± 0.00	10 ± 3	0.30 ± 0.08	0.44 ± 0.14	51 ± 11	1.5 ± 0.3	1.5 ± 0.4
		23 ± 4	0.69 ± 0.13		33 ± 8	1.0 ± 0.1	
Venlafaxine	0.60 ± 0.17	n.s.	n.s.	n/a	n.s.	n.s.	n/a
O-Desmethyl venlafaxine	0.90 ± 0.05	n.s.	n.s.	n/a	n.s.	n.s.	n/a

venlafaxine are significant inhibitors of human CYP1A2 or CYP3A4 (Preissner et al., 2010). Thus, the selective inhibition of EROD by mirtazapine in RT-S9 is likely explained by the fact that mirtazapine is a human CYP1A2 substrate (Table 1) and may thereby compete for the same binding site as the ER substrate in the corresponding rainbow trout homologue, even if it is not extensively metabolized in RT-S9 based on the substrate depletion assays (Fig. 1).

3.4. *In vitro intrinsic clearances of the test pharmaceuticals in mixtures with P450 inhibitors*

In the previous literature, P450 inhibition has been shown to increase the bioavailability and, consequently bioaccumulation, of CYP1A substrates, such as benzo(a)pyrene (Levine et al., 1997; Celander, 2011) or estrogens (Hasselberg et al., 2008) in the presence of fish P450 inhibitors. In the present study, a series of mixture assays were designed based on the IC_{50} data (Supplementary material, Table S6).

For one part, the aim of the mixture assays was to test our hypothesis regarding the impact of P450 inhibition on the intrinsic clearances of such pharmaceuticals that are effectively metabolized via the P450 system, like ibuprofen, naproxen, levomepromazine, and sertraline (Fig. 3). For the other part, the aim was to further elaborate and shed light on the plausible clearance routes of these pharmaceuticals in rainbow trout. For this purpose, diclofenac and naproxen, neither of which were inhibitors of EROD or BFCOD, were first incubated as a binary mixture with each other (each 1 μM). This resulted in ca. one-third decrease in the in vitro CL_{INT} of diclofenac, although the difference was not statistically significant ($p = 0.1401$) compared with its

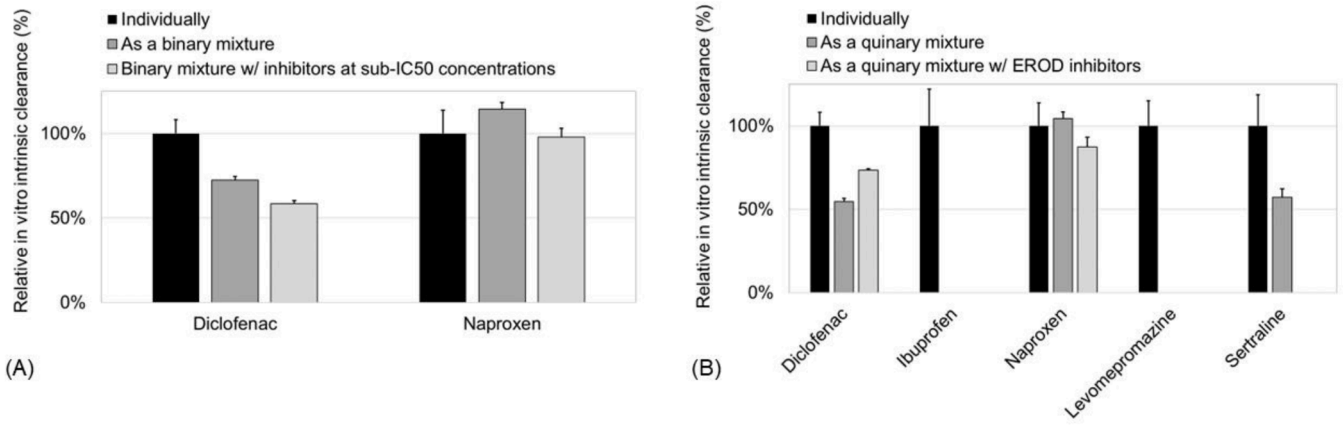

Fig. 4. Hepatic in vitro intrinsic clearances of test pharmaceuticals in mixtures. (A) Relative in vitro intrinsic clearances (%) of diclofenac and naproxen (each 1 μM each) incubated as a binary mixture with and without CYP inhibitors (haloperidol, levomepromazine, mirtazapine, and sertraline, each 0.01 μM) and including all cofactors (NADPH 1 mM, UDPGA 2 mM, PAPS 0.1 mM, glutathione 5 mM) and alamethicin (25 μg/mL). (B) Relative in vitro intrinsic clearances (%) of diclofenac, ibuprofen, naproxen, levomepromazine, and sertraline (each 1 μM each) incubated as a quinary mixture with and without EROD inhibitors (haloperidol and mirtazapine, each 37.5 μM) and including all cofactors (NADPH 1 mM, UDPGA 2 mM, PAPS 0.1 mM, glutathione 5 mM) and alamethicin (25 μg/mL). All incubations were performed in duplicate both with active and inactive RT-S9 using the pool of seven fish (#RTL-S9 200629-3). The relative intrinsic clearances (%) were calculated in comparison to the in vitro clearances determined for each substance individually.

substrate depletion rate in individual assays (Fig. 4A). Instead, no change was observed in the CL_{INT} of naproxen. This result suggests that these two substances likely compete for the same binding site, with naproxen exhibiting higher enzyme affinity. As expected, addition of the EROD and BFCOD inhibitors (haloperidol, mirtazapine, levomepromazine, and sertraline) to the binary mixture, at individual no-effect concentration of 0.01 μM each, did not change the result of the binary assay.

Next, diclofenac, ibuprofen, naproxen, levomepromazine, and sertraline were incubated as a quinary mixture, each at 1 μM. As a result, the intrinsic clearances of ibuprofen and levomepromazine zeroed and those of diclofenac and sertraline halved, each with statistically significant difference ($p < 0.05$), compared with their intrinsic clearances determined based on individual substrate depletion assays (Fig. 4B). These results further confirmed that the metabolism of ibuprofen and levomepromazine in RT-S9 is effectively catalyzed by the P450 system (in line with the data presented in Fig. 3), and that their P450 interactions can interfere with the intrinsic clearances of one another in vitro. According to the concentration addition approach (Eq. (5)), the theoretical combined inhibitory impact of the quinary mixture, at 1 μM concentration each, is only about 14 % and 3 % toward EROD and BFOCD activity, respectively (Supplementary material, Table S6). Thus, the in vitro data suggests that the combined inhibitory impact is synergistic.

On the basis of the IC_{50} data, sertraline has the highest affinity toward CYP1A-like (EROD) activity. It can thereby prevent the binding of other substrates, such as levomepromazine and ibuprofen to this enzyme. Thus, the data indirectly suggests that CYP1A-like activity could be the most important clearance pathway for these two substances in RT-S9. Similarly, the inhibitory impact of levomepromazine toward the CYP3A-like (BFCOD) activity detected in the IC_{50} shift assays, can interfere with the binding of sertraline to this enzyme. This mode of action could explain the observed ca. 40 % decrease in the CL_{INT} of sertraline in the quinary mixture (Fig. 4B). Also this data suggests that the combined inhibitory impacts of these five pharmaceuticals toward the EROD and BFCOD activities in RT-S9 are likely synergistic ($1 + 1 > 2$), rather than additive, as the measured reduction in CL_{INT} was clearly greater than the theoretical inhibitory effect (Table 1). Similar synergistic inhibition by pharmaceuticals toward P450 inhibition in fish have also been reported in other studies (Fallahi et al., 2020; Pihlaja et al., 2022).

Interestingly, the CL_{INT} of naproxen in the quinary mixture was statistically similar ($p = 0.8360$) to that determined individually, even if its metabolism was effectively catalyzed by the P450 system (Fig. 3). In a subsequent mixture assay, the same five pharmaceuticals (each 1 μM) were additionally incubated together with haloperidol and mirtazapine (each 37.5 μM), to intentionally inhibit the CYP1A-like (EROD) activity entirely. This simultaneously reduced the nominal BFCOD activity by ca. 10 % (Supplementary material, Table S6). In this case, the intrinsic clearance of sertraline zeroed ($p = 0.0044$ compared with individual incubation), confirming that its biotransformation in RT-S9 is likely catalyzed by CYP1A or CYP3A isoenzymes, or both. Still, no statistically significant difference ($p = 0.6141$) in the intrinsic clearance of naproxen or no further decrease in the intrinsic clearance of diclofenac were observed compared with individual incubations (Fig. 4B). Thus, the data suggests that the biotransformations of diclofenac and naproxen are potentially catalyzed by phase II enzyme systems or other P450 isoenzymes, respectively.

Altogether the in vitro results of the present study may explain the mechanistic basis of the bioaccumulation of pharmaceuticals reported for fish exposed to sewage effluents containing residues of anti-inflammatory or psychoactive drugs. In field-testing, the bioaccumulation (BCF) of ibuprofen in juvenile rainbow trout exposed to sewage effluents from three treatment plants varied considerably between test sites (Brown et al., 2007). The measured plasma levels were >200 times higher than modelled for the largest plant, yielding BCF as high 18,667. The BCFs of ibuprofen in fish exposed to effluent of the other two plants were negligible (Brown et al., 2007). This suggests that the inconsistent findings for ibuprofen could result from the combined inhibitory effects of other chemical contaminants in the sewage effluent. On the other hand, the BCFs of diclofenac or naproxen were not significantly affected by the effluents nor different between the sites (Brown et al., 2007). This is well in line with the findings on biotransformation routes of the present study. In another previous study (Grabicova et al., 2017), the BAFs of sertraline varied upon exposure period, between <1000 and >4000, which suggests that its hepatic P450 interactions, demonstrated in vitro in the present study, may affect its intrinsic clearance rate in fish exposed to pharmaceutical mixtures.

4. Conclusions

The first aim of the present study was to evaluate the validity of the OECD test guidance 319B for prediction of the intrinsic hepatic clearances of ionizable organic compounds, including four commonly used anti-inflammatory agents and eight antidepressants/antipsychotics. Our results show that only six out of twelve test substances (diclofenac, ibuprofen, ketoprofen, naproxen, levomepromazine, and sertraline) had quantifiable depletion kinetics in RT-S9. The hepatic clearances of the other six substances in rainbow trout are likely very low or negligible, which may elevate their bioaccumulation in fish tissues. Accounting for the in vitro hepatic clearance in the calculated BCF yielded results that were generally well in line with the BCFs and BAFs reported in fish in vivo. Those substances that were effectively cleared in RT-S9 have not been reported to bioaccumulate in fish in most cases, whereas some of the substances lacking hepatic metabolism in RT-S9 (e.g., citalopram and mirtazapine) are reportedly bioaccumulative (BCF > 2000) in fish in vivo (Brown et al., 2007; Grabicova et al., 2017). However, the ionization state of the pharmaceutical at the physiological pH of fish is also a critical factor besides hepatic clearance.

Overall, our data suggests that hepatic biotransformation routes in fish cannot be explicitly predicted based on human metabolism data alone, but the in vitro intrinsic clearance assays (OECD 319B) can preliminarily advise about the bioaccumulation risk of likewise new drug candidates and legacy pharmaceuticals lacking environmental fate data. As such, the results of this study provide new in vitro data on the intrinsic clearances of the test pharmaceuticals in rainbow trout, which can be used to refine the computational BCF models for fish as well as the calculated BCF predictions (OECD 280). In this regard, however, attention should be paid on the impacts of, e.g., seasonal variation or individual phenotype differences on the enzyme activities in RT-S9 preparations. Our results show that these factors may result in an uneven lot-to-lot variation in the in vitro clearance rates between pharmaceuticals. As a result, the biotransformation rates of some pharmaceuticals (like levomepromazine) derived from RT-S9 assays can be significantly different between different subcellular preparations, while others (like diclofenac and sertraline) exhibit statistically similar depletion kinetics. Thus, our results emphasize that including appropriate reference chemicals, ideally two or more differently metabolized substances, as positive controls is important to account for the biological variation between individual fish in the RT-S9 clearance (OECD319B) assays.

The second aim of this study was to evaluate the impact of P450 inhibition on the intrinsic clearance rates of pharmaceuticals. Based on unbound IC_{50} concentrations, sertraline and levomepromazine are strong inhibitors of both EROD and BFCOD in RT-S9, while haloperidol, mirtazapine (only EROD), and citalopram are moderate or weak inhibitors. The results suggest that if these substances are also very bioaccumulative in fish tissues, they may interfere with the clearance of any other co-existing chemical contaminant that is effectively eliminated via the P450 system. According to our results, the in vitro clearances of ibuprofen, naproxen, levomepromazine, and sertraline were significantly dependent on the P450 metabolism. As a consequence, the intrinsic clearances of ibuprofen, levomepromazine, and sertraline were

significantly and synergistically inhibited in mixtures. The anti-inflammatory drugs (diclofenac, ibuprofen, ketoprofen, naproxen) as such were shown to be noninhibitors of CYP1A- or CYP3A-like activities in RT-S9 and thus, they are not likely to potentiate the bioaccumulation of other substances in rainbow trout. Apart from ibuprofen, the hepatic clearances of anti-inflammatory drugs were not affected by other P450 inhibitors in the same way as those of levomepromazine and sertraline. Overall, the in vitro data of the present study confirmed our hypothesis on the interfering impact of P450 inhibition on hepatic clearance in fish in vitro. Furthermore, this in vitro data may shed light on the previously found discrepancies between BAF and BCF of some of the same substances in trout in vivo, suggesting that P450 inhibition may be a biologically relevant factor behind the mixture effects in wild fish. With the introduction of new pharmaceuticals, the diversity of the chemical contaminants entering the environment as a result of human use is likely to increase, which warrants new strategies to address complex mixture effects.

Overall, our data demonstrates that the subcellular RT-S9 assays can provide detailed mechanistic information of the biotransformation pathways in fish and the interlinked bioaccumulation and hepatotoxicity risks. In this regard, the time-dependent inhibition of the BFCOD activity by sertraline in RT-S9, as well as that of haloperidol toward both EROD and BFCOD, could warrant for further research. This is because time-dependent inhibition is often indicative of irreversible inactivation of the P450 detoxification route (Obach et al., 2007) and may result from the formation of reactive metabolites that trigger hepatotoxic effects (Li, 2002) and elevate the bioaccumulation of other substances in fish in vivo. Thus, further information of the environmental fate and exposure profiles of sertraline and haloperidol could help evaluate the true risks of these substances to aquatic wildlife (e.g., under the regional water monitoring programs, such as the EU's Watch list mechanism). Overall, this in vitro enzyme inhibition data can shed light on the substrate specificities of rainbow trout CYP1A1 and CYP3A27 homologs, and consequently help predict the combined effects of pharmaceutical mixtures arising from P450 interactions. Contrary to hepatic clearance predictions, the inhibitory impacts of the twelve test pharmaceuticals toward rainbow trout CYP1A and CYP3A-like in vitro activities were shown to correlate well with those of the corresponding human P450 homologs.

CRediT authorship contribution statement

Tea L.M. Pihlaja: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Jade Pätsi:** Formal analysis, Investigation, Writing – review & editing. **Elisa Ollikainen:** Methodology, Writing – review & editing. **Tiina M. Sikanen:** Conceptualization, Data curation, Supervision, Writing – review & editing, Funding acquisition, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2024.107048.

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