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Organ-specific biotransformation in salmonids: Insight into intrinsic enzyme activity and biotransformation of three micropollutants

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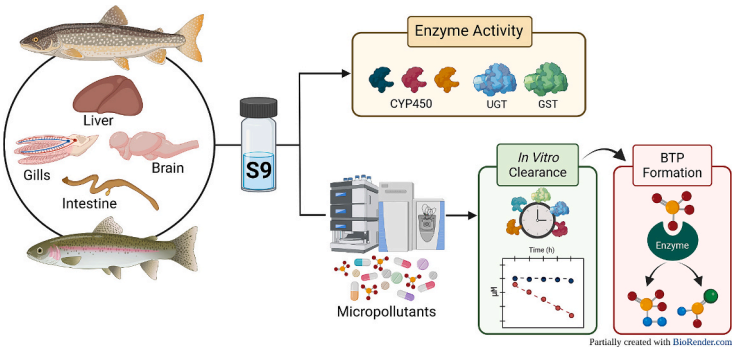
HIGHLIGHTS

- An integrative, organ-specific biotransformation assessment for fish is reported.
- Phase I and II biotransformation enzymes displayed hepatic and extrahepatic activity.
- Extrahepatic organs are important contributors of chemical clearance.
- Hepatic biotransformation product profiles followed species-specific trends.

GRAPHICAL ABSTRACT



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ABSTRACT

Aquatic ecosystems continue to be threatened by chemical pollution. To what extent organisms are able to cope with chemical exposure depends on their ability to display mechanisms of defense across different organs. Among these mechanisms, biotransformation processes represent key physiological responses that facilitate detoxification and reduce the bioaccumulation potential of chemicals. Biotransformation does not only depend on the ability of different organs to display biotransformation enzymes but also on the affinity of chemicals towards these enzymes. In the present study, we explored the ability of different organs and of two freshwater fish to support biotransformation processes through the determination of in vitro phase I and II biotransformation enzyme activity, and their role in supporting intrinsic clearance and the formation of biotransformation products. Three environmentally relevant pollutants were evaluated: the polycyclic aromatic hydrocarbon (PAH) pyrene (as recommended by the OECD 319b test guideline), the fungicide azoxystrobin, and the pharmaceutical propranolol. Comparative studies using S9 sub-cellular fractions derived from the liver, intestine, gills, and brain of brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) revealed significant phase I and II enzyme activity in all organs. However, organ- and species-specific differences were found. In brown trout, significant extrahepatic biotransformation was observed for pyrene but not for azoxystrobin and propranolol. In rainbow

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trout, the brain appeared to biotransform azoxystrobin. In this same species, propranolol appeared to be biotransformed by the intestine and gills. Biotransformation products could be detected only from hepatic biotransformation, and their profiles and formation rates displayed species-specific patterns and occurred at different magnitudes. Altogether, our findings further contribute to the current understanding of organ-specific biotransformation capacity, beyond the expression and activity of enzymes, and its dependence on specific enzyme-chemical interactions to support mechanisms of defense against exposure.

1. Introduction

Aquatic ecosystems represent important sinks for chemical pollutants (Bertram et al., 2022), potentially leading to important losses in aquatic biodiversity (Sigmund et al., 2023). In this context, determining chemically driven alterations to the integrity of individuals and populations is critical for proper environmental risk assessment and management of the aquatic environment. In this regard, it is important to recognize that organisms possess biological traits and processes that allow them to cope with chemical pollution while minimizing adverse effects. For example, detoxification mechanisms including biotransformation pathways, ATP-binding cassette (ABC) transporters, and antioxidant systems (Eide et al., 2021). Such collections of response mechanisms correspond to the chemical defensome (Goldstone et al., 2006).

Among the multiple defense mechanisms displayed by organisms, biotransformation pathways are key in directly influencing the toxicokinetics of chemicals, particularly those processes associated with their detoxification and bioaccumulation (Strobel et al., 2015). In general, different biotransformation pathways support the excretion of exogenous compounds by increasing their polarity (Schlenk et al., 2008). In studies with fish, biotransformation has been recognized as a fundamental aspect of the physiological responses to pollution and it has been suggested to influence species sensitivity to chemical exposure (Lech and Bend, 1980). Hence, the biotransformation ability of an organism is ecologically relevant — modifications to important biotransformation pathways resulting from chemical exposure have been described as evolutionary mechanisms allowing individuals to maintain fitness (Franco et al., 2022b; Oziolor et al., 2019; Strobel et al., 2022). The role of biotransformation processes in influencing the bioaccumulation potential of chemicals has also been highlighted in recently adopted guidelines for bioaccumulation assessment, namely the test guidelines 319 a and b from the Organization for Economic Cooperation and Development (OECD, 2018b, 2018c).

Yet, biotransformation studies, including the above mentioned OECD test guidelines, have largely focused on the liver as the organ with the highest metabolic activity. The liver is an essential organ for the detoxification of endogenous and exogenous molecules, making it a point of interest when exploring how organisms deal with chemical pollution. Nonetheless, physiological responses to chemical exposure often involve a collection of reactions that are differentially displayed across different organs (Heath, 2018). In the context of biotransformation of pollutants, a few studies using S9 fractions from fish have pointed out significant extrahepatic biotransformation of polycyclic aromatic hydrocarbons (PAHs), particularly in the intestine (Butt et al., 2010; Saunders et al., 2020) and the gills (Gomez et al., 2010). Similarly, PAH biotransformation has been described in cell-based models derived from different fish organs, including intestinal and gill cell lines (Stadnicka-Michalak et al., 2018). Such observations may be directly related to the uptake of chemicals, since the intestine and the gills are of relevance when evaluating exposure via diet and respiration (i.e. aqueous exposure), respectively. As such, these organs may display biotransformation ability as one of the first lines of defense when encountering xenobiotics.

Other previous studies have provided insightful contributions into the existing knowledge of biotransformation capacity of different organs in fish at the enzyme activity level. For example, significant CYP1A activity was measured in the intestine, gills, kidney, and brain of the gild-head bream (*Sparus aurata*) (Ortiz-Delgado et al., 2008) and the streaked prochilod (*Prochilodus lineatus*) (Santos and dos Reis Martinez, 2020) under basal conditions and upon exposure to legacy pollutants (e.g. PAHs). In addition, the activity of other CYP1, CYP2, and CYP3 isoforms was measured extrahepatically in the Chinese rare minnow (*Gobiocypris rarus*) (Yuan et al., 2013), the Barbel (*Barbus callensis*) (Habila et al., 2017), and the rainbow trout (*Oncorhynchus mykiss*) (Burkina et al., 2021), all showing organ-specific patterns. Phase II biotransformation enzymes have also been suggested to be differentially expressed across organs in zebrafish (*Danio rerio*) (Christen and Fent, 2014). However, while elucidating the presence and activity of specific enzymes provides important insight into organ-specific biotransformation, this information alone may not be fully representative of an organ's ability to interact with chemicals and their contribution towards chemical clearance.

This knowledge gap becomes larger when considering different classes of pollutants, such as pharmaceuticals and pesticides. Many of these substances display different ways in which they are absorbed, distributed and metabolized (Chang et al., 2021). Indeed, a large proportion of pollutants may target specific organs, and whether they are biotransformed and eventually excreted may not only depend on organ-specific activity of biotransformation enzymes but also on the chemicals' ability to interact with them. It is important, however, to state that biotransformation processes may also lead to more active compounds that display higher toxicity than the parent compounds, as it is often observed for PAHs and some pesticides (e.g. malathion) (Cook et al., 1976).

On this background, the present study evaluated the activity of different biotransformation pathways across fish organs and whether they contributed to micropollutant biotransformation in support of detoxification mechanisms. These evaluations included: I) the characterization of phase I and II biotransformation enzyme activity, II) the estimation of intrinsic clearance of pyrene (PYR), azoxystrobin (AZO) and propranolol (PRO), and III) the identification and quantification of their biotransformation products (BTPs). These chemicals were selected based on their current status regarding their use in Switzerland and the European Union (ECHA, 2023), on monitoring studies reporting their presence in surface waters (Spycher et al., 2018; Wilkinson et al., 2022), on previous studies suggesting elevated biotransformation in trout (Connors et al., 2013; Kosfeld et al., 2020; Nichols et al., 2023), as well as on their structural affinity to more than one biotransformation enzyme (Masubuchi et al., 1994; Rösch et al., 2017) and their recommended use in biotransformation studies (PYR; OECD (2018a)). All biotransformation studies employed S9 sub-cellular fractions isolated from the liver, intestine, gills, and brain of two salmonid species: the brown trout (*Salmo trutta*) and the rainbow trout (*Oncorhynchus mykiss*). We hypothesized that phase I and II biotransformation pathways are differentially displayed across organs and that their magnitude of activity could translate into the organs' capacity to support micropollutant clearance and the formation of BTPs. Moreover, while both fish species used in the present study are closely related (family Salmonidae), we suspected that interspecific differences may also influence their biotransformation ability. Overall, the integrative assessment performed here contributes to the understanding of biotransformation processes in fish beyond traditional approaches and highlights the need to evaluate mechanisms by which organisms cope with contaminants in the aquatic environment.

2. Materials and methods

2.1. Chemicals and reagents

The standard enzyme substrates, 7-ethoxyresorufin (7-ER), 7-pentoxyresorufin (7-PR), 7-methoxy-4-(trifluoromethyl)coumarin (MFC), and 7-benzyloxy-4-(trifluoromethyl)coumarin (BFC), and the standard 7-hydroxy-4-(trifluoromethyl)coumarin were obtained from Chemodex (St. Gallen, CH). Resorufin sodium salt, 1-chloro-2,3-dinitrobenzene (>99 %; CAS: 97-00-7), p-nitrophenol (>99 %; CAS: 100-02-7), pyrene (>99 %; CAS: 129-00-0), and the internal standard azoxystrobin-D4 (98%; CAS: 1346606-39-0), as well as the enzyme co-factors reduced β-nicotinamide adenine dinucleotide 2′-phosphate, tetrasodium salt (NADPH; 96 %), L-glutathione reduced (GSH), uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA), and adenosine 3′-phosphate 5′-phosphosulfate lithium salt hydrate (PAPS) were purchased from Merck (Darmstadt, DE). Alamethicin from *Trichoderma viride* was from Cayman Chemical and purchased from AdipoGen (Liestal, CH). Azoxystrobin (AZO; >98 %; CAS: 131860-33-8), propranolol (PRO; >98 %; CAS: 318-98-9), and propranolol-D7 (>98 %; CAS: 344298-99-3) were obtained from Toronto Research Chemicals Inc. (Toronto, CA). Azoxystrobin acid (99 %; CAS: 1185255-09-7) was purchased from HPC standards GmbH (Borsdorf, DE). HPLC-grade methanol, acetonitrile, and formic acid were purchased from Fisher Scientific AG (Reinach, CH).

2.2. Isolation of S9 sub-cellular fractions

Brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) were obtained from the Bremgarten fish farm (www.fischzucht-bremgarten.ch) on April 7th 2022 and processed on-site. A total of 9 brown trout, grouped into 3 pools of 3 individuals each, and 12 rainbow trout, grouped into 3 pools of 4 individuals each, were used in the preparation of S9 fractions. Fish were fasted for 24 h prior to euthanasia by electric shock, following the farms' in-house procedures. Average length (cm), weight (g), condition factor (*K*), and fractional organ weights (g/g whole body) are provided in Table S1. Specific details about the buffer solutions and procedures for the isolation of S9 fractions from each organ are included as supplemental information (section 1.1).

Briefly, the isolation of liver S9 sub-cellular fractions followed standard preparation guidelines reported by OECD (2018a) and Johanning et al. (2012). Upon dissection and perfusion, pooled livers were minced and placed in 2 volumes of ice-cold homogenization buffer. Intestinal S9 fractions were prepared following the protocols described in McElroy and Kleinow (1992) and Saunders et al. (2020). The intestinal mucosa was obtained by scraping the lumen of the anterior intestine, an area with high enterocyte density, and placed into 15 mL conical tubes containing ice-cold homogenization buffer. The resulting suspension was centrifuged (2000*g* for 5 min at 4 °C), and the cell pellet was resuspended in 5 mL of homogenization buffer. For the gills, the preparation of S9 fractions was based on the protocol by Gomez et al. (2010). Clean arches from both sides were minced into 2–3 mm pieces, and pooled into conical tubes with two volumes of ice-cold homogenization buffer. Lastly, brain S9 sub-cellular fractions were isolated from whole brains. Brains were pooled in 15 mL conical tubes containing two volumes of homogenization buffer.

Upon transport to the laboratory, all organ suspensions in homogenization buffer were further homogenized using a digital ULTRA-TURRAX® homogenizer (IKA®-Werke GmbH & Co. KG, Staufen, DE). The resulting homogenates were transferred to a high-speed centrifuge (UniCn MR, Herolab GmbH, Wiesloch, DE) and spun (13,000*g* for 25 min at 4 °C). Supernatants (i.e. S9 fractions) were then collected and transferred to cryogenic vials in aliquots of 0.25 or 0.5 mL, depending on the resulting S9 volume and to minimize repetitive thawing that could compromise the integrity of the S9 preparations. Total protein content in the S9 fractions was quantified via the Coomassie blue assay (Bradford, 1976), using bovine serum albumin (BSA) as a standard (Table S2). All S9 sub-cellular fractions were stored at −80 °C until assays were performed.

Prior to obtaining fish from the Bremgarten farm, initial evaluations were conducted with juvenile rainbow trout from the Blausee Organic Trout Farm (www.blausee.ch) in Bern, CH, on March 16th 2022. A total of 15 rainbow trout were grouped into 3 pools of 5 fish each for the isolation of S9 fractions. Upon conducting enzyme activity bioassays, it was determined that CYP1A activity was rather low in comparison to values reported in the literature (Nichols et al., 2018), hence the motivation to obtain fish from a different source and use rainbow trout from the Blausee farm for comparative purposes.

In addition, rainbow trout liver S9 fractions (lot: 200629-3, 20 mg/mL) were also purchased from PRIMACYT Cell Culture Technology GmbH (Schwerin, DE). These were used as reference material for substrate depletion experiments.

2.3. Biotransformation enzyme activity

Phase I and II biotransformation activity was measured via standardized fluorescence- or absorbance-based bioassays in micro-well plates, and included four cytochrome P450 isoforms (phase I): CYP1A, CYP2B, CYP2C9, and CYP3A4, and two conjugation (phase II) enzymes: glutathione-S-transferase (GST) and UDP-glucuronosyltransferase (UGT). Michaelis-Menten kinetic experiments were conducted by evaluating the activity of each enzyme at different substrate concentrations to determine the lowest substrate concentration that resulted in the highest reaction rate (*Vmax*; data not shown), thus avoiding saturating conditions. These concentrations were then selected for subsequent bioassays to compare organ-specific activity magnitudes of each enzyme. Details pertaining the bioassays for each biotransformation enzyme are included as supplemental information (section 1.2).

Briefly, CYP1A activity was measured via the ethoxyresorufin-*O*-deethylase (EROD) bioassay using 10 μM 7-ethoxyresorufin (7-ER). CYP2B-like activity was evaluated under similar conditions as EROD but by using the pentoxyresorufin-*O*-deethylase (PROD) bioassay with 10 μM of 7-pentoxyresorufin (7-PR) as substrate. CYP2C9-like and CYP3A4-like activities were measured from the production of 7-hydroxy-4-(trifluoromethyl)coumarin following the dealkylation of 50 μM 7-methoxy-4-trifluorocoumarin (MFC) and of 50 μM 7-benzyloxy-4-trifluoromethylcoumarin (BFC), respectively.

The activity of GST was evaluated colorimetrically following the conjugation of 1-chloro-2,3-dinitrobenzene (CDNB; Habig et al. (1974)) at 5 mM. The resulting GST activity was calculated with a conjugated CDNB molar extinction coefficient of 9.6 mM^{-1}, adapted for 96-well plates (5.03 mM^{-1}). Finally, UGT activity was monitored following the protocol by Ladd et al. (2016), adjusted for 96 micro-well plates and using *p*-nitrophenol at 50 μM.

2.4. Substrate depletion bioassays

The depletion of PYR, used as a reference chemical as recommended by the OECD 319b test guideline (OECD, 2018a), by liver S9 was monitored for 10 min, whereas for the other organs the reaction was conducted for 30 min. Additionally, depletion assays were conducted for AZO and PRO; enzymatic depletion of these chemicals by all organs was monitored for 90 min.

For all chemicals, duplicate experiments were established for each S9 pool derived from each organ and species, and following the OECD 319b test guideline (OECD, 2018a). Reactions were conducted at a chemical concentration of 0.05 μM, selected based on relevant levels detected in the environment and on recent reports suggesting higher biotransformation rates at low chemical concentrations (0.025–0.2 μM; Laue et al. (2020); Saunders et al. (2018)). Further details about substrate depletion experiments are included as supplemental information (section 1.3).

2.5. Instrumental analysis

Concentrations of PYR were determined via high-performance liquid chromatography coupled with a fluorescence detector (HPLC-FLD; Agilent Technologies HP1200 Series). Chromatographic separation was achieved with a NUCLEODUR 100-3 C18ec column (3 μm, 250 × 4.6 mm; Macherey Nagel AG) and a mobile phase gradient of nanopure water and methanol. Excitation and emission wavelengths were 335 and 388 nm, respectively. Quantification was done by integrating peak areas and based on external calibration.

Chemical analyses for the identification and quantification of AZO, PRO, and their BTPs followed the methods by Jeon et al. (2013) and Lauper et al. (2022), after online solid phase extraction (SPE; refer to section 1.4 of SI for details on SPE procedures). The analyses were conducted via high-performance liquid chromatography (Ultimate, Thermo Fisher Scientific) coupled with a hybrid quadrupole Orbitrap high-resolution tandem mass spectrometry system (HR-MS/MS; Q-Exactive, Thermo Fisher Scientific) with electrospray ionization (ESI) in switch (positive and negative) mode. Chromatographic separation was achieved with an Atlantis® T3 (5 μm, 3.0 × 150 mm) column and a mobile phase gradient of nanopure water and methanol, each with 0.1 % formic acid. The identification and quantification of the target chemicals was based on internal standard calibration with isotope-labelled standards using Freestyle 1.7 and TraceFinder 5.1 (Thermo Fisher Scientific). Suspect screening (Table S3) was conducted for the identification of BTPs from AZO and PRO biotransformation using Compound Discoverer 3.3 (Thermo Fisher Scientific), and based on the reports from Rösch et al. (2017) and Jeon and Hollender (2019).

2.6. Statistical analyses and calculation of in vitro intrinsic clearance

Datasets corresponding to enzyme activity magnitude for each organ were evaluated for normality via the Shapiro-Wilk normality test. As the datasets did not follow a normal distribution, significant differences were evaluated via generalized linear models (GLM), following a gamma distribution, and by considering organ and species as categorical variables. p-values <0.05 were denoted as significant. In cases were significant differences were observed, post-hoc pairwise comparisons were conducted to depict significantly different groups. These analyses were done using R version 4.2.2. and the RStudio platform.

The depletion of test chemicals was evaluated by combining the results from independent experiments conducted with S9 pools derived from each organ and from each fish species. Linear regression analyses were employed to corroborate that chemical depletion followed first-order kinetics (log-linear decrease of chemical concentrations over time) by plotting average log-transformed measured concentrations against time (h). The resulting slopes were then multiplied by −2.3 to obtain the natural log (Ln) and determine the first-order depletion rate constant (k_{DEP}; 1 h^{-1}) for each test chemical. In vitro intrinsic clearance ($CL_{IN\ VITRO}$) was then estimated by dividing k_{DEP} by the concentration of S9 protein in the depletion bioassays. Significant differences between depletion patterns were determined by direct comparison of the resulting slopes from each regression line in GraphPad Prism 9.3.1.

3. Results and discussion

3.1. Phase I biotransformation enzyme activity

The characterization of enzymatic activity revealed that CYP1A displayed the highest levels in liver S9 from both species, but activity was significantly lower in rainbow trout (0.9 ± 0.3 pmol·mg protein^{-1} min^{-1}) than in brown trout (8.7 ± 1.3; $t = 11.2$, $p < 0.05$; Fig. 1A). This level of intrinsic CYP1A activity was also observed in rainbow trout from the Blausee fish farm (0.4 ± 0.2 pmol·mg protein^{-1} min^{-1}). Contrarily, commercially available rainbow trout liver S9 displayed CYP1A activity (7.4 ± 0.9) close to that observed for brown trout and reported elsewhere (Nichols et al., 2013). Such intraspecific differences for CYP1A may be related to strains from different regions of the world, although environmental factors (e.g. temperature), biological factors (e.g. sex and reproductive cycles), and environmental exposure and evolutionary histories have been shown to modify biotransformation processes in fish (Cerveny et al., 2021; Figueiredo-Fernandes et al., 2006; Franco et al., 2022a).

Despite these intraspecific differences for intrinsic hepatic CYP1A activity, an important finding in our study corresponds to the CYP1A activities measured in extrahepatic organs. In brown trout, CYP1A activities were 0.3 ± 0.0, 1.0 ± 0.1, and 0.3 ± 0.0 pmol·mg protein^{-1} min^{-1} for the intestine, gills, and brain, respectively. In rainbow trout, all organs displayed CYP1A activity, but only the liver and intestine S9 followed Michaelis-Menten kinetics (data not shown). This observation may indicate differential enzyme-substrate interactions when the enzymes are found at low levels. CYP1A activities in the rainbow trout intestine, gills and brain were 0.5 ± 0.1, 0.3 ± 0.0, and 0.1 ± 0.0 pmol·mg protein^{-1} min^{-1}, respectively. These results are in line with previous reports pointing out differential CYP1A expression and activity among different fish organs, including the brain, intestine, and gills (Burkina et al., 2021; Ortiz-Delgado et al., 2008; Santos and dos Reis Martinez, 2020).

CYP2B-like activity was detected in liver, intestine and gill S9 from both species (Fig. 1B). Liver and intestinal S9 displayed similar activity in brown trout (both at 0.8 ± 0.1 pmol·mg protein^{-1} min^{-1}), but the activity was higher in the liver (1.0 ± 0.1) than in the intestine (0.6 ± 0.3) in rainbow trout. Moreover, gill S9 fractions from rainbow trout displayed the highest activity of this isoform across organs and species

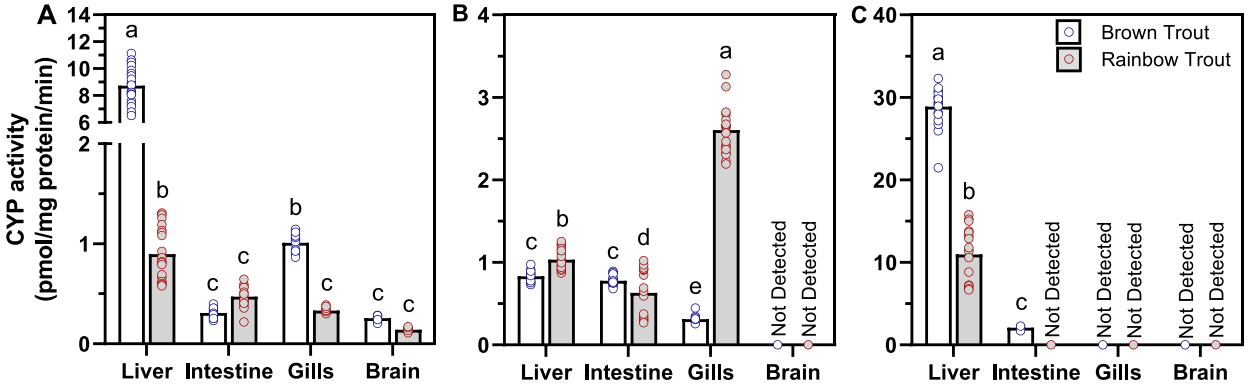

Fig. 1. Activity of A) CYP1A, B) CYP2B-like, and C) CYP3A4-like in S9 sub-cellular fractions isolated from the liver, intestine, gills, and brain of brown trout and rainbow trout. Each bar corresponds to the mean activity of three different S9 pools, with dots representing individual data points from technical replicates (n = 15–24). Different lower-case letters denote significant differences among organs and species.

(2.6 ± 0.3). This observation was further corroborated with gill S9 fractions isolated from rainbow trout from the Blausee fish farm, as CYP2B-like activity (1.9 ± 0.4) was higher than in the liver and intestine S9 (1.2 ± 0.1 and 0.5 ± 0.0, respectively). These activity levels placed rainbow trout as the species with a better ability to display CYP2B-like activity ($t = -22.9$, $p < 0.05$).

To date, only a few studies have evaluated CYP2B-like activity in fish, and their findings often present uncertainty regarding its presence and inducibility across organs and species (Burkina et al., 2012; Iwata et al., 2002; Koenig et al., 2012; Nabb et al., 2006). It is well-established that the constitutive androstane receptor (CAR) pathway is responsible for the regulation of CYP2B isoforms (Iwata et al., 2002). Thus, in fish, whether organs and species display CYP2B-like activity directly depends on the presence and functionality of the CAR pathway. Based on our observations, brown trout and rainbow trout appear to be equipped with functional CAR, both hepatically and extrahepatically. The elevated CYP2B-like activity in the gills of rainbow trout is of particular interest, and potentially places this organ as a major contributor for the biotransformation of chemicals that interact with CYP2B isoforms (e.g. barbiturates). Studies in mammalian models have suggested that CYP2B enzymes are significantly expressed in the lungs (Chirulli et al., 2005; Lee and Dinsdale, 1995). This could explain the observation for elevated CYP2B-like activity in the rainbow trout gills, given the physiology and close functionality of both organs and the shared evolutionary trajectory of both organs as oxygen transport systems, driven by the need to maintain oxygen homeostasis in aquatic and terrestrial environments (Hsia et al., 2013). However, further evaluations would be necessary to determine why brown trout did not display a similar trend, and experimentation considering interspecific traits at the genetic level and the presence of CYP2B-regulating pathways in brown trout organs would be necessary to explain the low CYP2B-like activity displayed by the gills.

Even though CYP1A enzymes are denoted as gold-standard biomarkers of biotransformation in fish (Goksøyr, 1995), CYP3A isoforms have also been elucidated as key factors for the biotransformation of pollutants, particularly pharmaceuticals (Burkina et al., 2021; Habila et al., 2017). Evaluations of CYP3A4-like activity also presented organ-specific differences, with the liver displaying the highest activity in both species: 28.9 ± 2.1 pmol·mg protein^{-1} min^{-1} for brown trout and 11.0 ± 3.1 for rainbow trout (Fig. 1C). In extrahepatic tissues, CYP3A4-like activity was only detected in the intestine of brown trout (2.1 ± 0.2 pmol·mg protein^{-1} min^{-1}). While our observations would place brown trout as the species with a higher ability to display CYP3A4-like activity ($t = 15.5$, $p < 0.05$), evaluations in brown trout are scarce. On the other hand, previous studies have reported significant hepatic and intestinal activity of CYP3A4 enzymes in rainbow trout (Lee and Buhler, 2003; Saunders et al., 2020) and other teleosts (Habila et al., 2017; Oziolor et al., 2017). Yet, further comparative studies between salmonids in the context of CYP3A4 activity would help in deciphering how this enzyme is regulated among species. CYP3A isoforms are highly involved in the biotransformation of a large proportion of pharmaceuticals, and while previous studies have suggested a low importance of CYP3A relative to CYP1A enzymes in fish (Nabb et al., 2006), organs and species lacking significant CYP3A4-like activity may be at a higher risk of accumulating drugs that display affinity for such CYP isoforms and of experiencing pharmaceutical-driven toxicity. Nonetheless, it is important to point out that CYP3A enzymes are highly inducible (Creusot et al., 2015), and while constitutive CYP3A4 activity may be low, this isoform could display a significant contribution towards biotransformation upon induction.

3.2. Phase II biotransformation enzyme activity

Besides the fundamental role of CYP450 and other NADPH-independent enzymes in biotransformation, phase II biotransformation processes are also critical in supporting the elimination of xenobiotics (Schlenk et al., 2008). In the present study, phase II biotransformation was assessed by estimating the activity of two conjugation enzymes: glutathione-S-transferase (GST; Fig. 2A) and UDP-glucuronosyltransferase (UGT, Fig. 2B). In general, brown trout displayed the highest GST activity in the liver S9 (955 ± 86 nmol·mg protein^{-1} min^{-1}), followed by the gills (589 ± 96), intestine (424 ± 62), and brain (333 ± 55). In rainbow trout, liver S9 also displayed the highest activity (602 ± 47), with the gills and intestine displaying similar but lower activity levels (389 ± 40 and 379 ± 71, respectively), and the brain showing the lowest activity (185 ± 26). It was observed that the overall magnitude of GST activity in brown trout was significantly higher than in rainbow trout when directly comparing the same organs ($t = 12.9$, $p < 0.05$). Nonetheless, the commercially available liver S9 from rainbow trout displayed GST activity of 804 ± 58 nmol·mg protein^{-1} min^{-1}, comparable to that of brown trout.

Similarly, UGT activity was observed in both the liver and intestine S9 of the two species. Activities in brown trout were 239 ± 76 pmol·mg protein^{-1} min^{-1} for the liver and 218 ± 69 for the intestine, whereas for rainbow trout they were 379 ± 77 and 371 ± 120 for the liver and intestine, respectively. Contrarily to GST, UGT activity was significantly higher in the rainbow trout organs ($t = -5.7$, $p < 0.05$). No UGT activity was detected in gills nor in brain S9 of neither species.

In the context of biotransformation, GST and UGT isoforms are responsible for catalyzing conjugation reactions by the addition of glutathione and glucuronic acid, respectively (Hayes and Pulford, 1995; Meech et al., 2019). Different studies have pointed out the presence of GST and UGT in the liver (Dominey et al., 1991; Ladd et al., 2016), intestine (Van Veld et al., 1991), gills (Espinoza et al., 2012; Rodrigues et al., 2019), and brain (Cazenave et al., 2008) of teleost fish. However, besides the tissue-specific evaluations of UGT in zebrafish performed by Christen and Fent (2014), integrative and simultaneous evaluations of organ-specific expression and activity of conjugation enzymes in fish are scarce. Thus, the observations from our study further support that different organs display phase II biotransformation ability, albeit at different magnitudes. Additionally, GST appears to be more widespread than UGT, as the latter was not detected in the gills nor the brain. These organ-specific patterns may also dictate the degree of phase II biotransformation activity, and could be of particular importance in the detoxification of chemicals that target specific organs. Indeed, as with phase I biotransformation, the distribution of chemicals following uptake and the organ-specific activity of GST and UGT may be the determining factors influencing mechanisms of defense at the organ level.

The activity of both phase II enzymes also displayed species-specific patterns, with brown trout having overall higher GST activity than rainbow trout, but with the latter displaying slightly higher UGT activity. Whether these differences in activity may translate into lesser ability to support chemical clearance is unclear, yet these observations may indicate important implications for other biological responses involving phase II enzymes. For example, given the major role of GST in redox pathways and in the regulation of antioxidant responses (Lenartova et al., 2008), organisms with lower GST activity may face important challenges in coping with chemical exposure. However, previous studies on salmonids have indicated that exposure to xenobiotics could also trigger the upregulation of both GST and UGT enzyme families (Almli et al., 2002; Ladd et al., 2016; Petřivalský et al., 2009), thus reducing the risk for adverse effects.

3.3. Intrinsic clearance of micropollutants

The characterization of biotransformation enzymes revealed organ-specific patterns in both species. However, our study addresses the relationship between enzymatic activity, chemical clearance, and BTP formation across organs in an effort to depict whether enzymatic activity is directly translatable to pollutant biotransformation, as this corresponds to an understudied aspect of biotransformation in fish.

Our results from substrate depletion bioassays support the traditional perception of the liver as the organ with the highest metabolic

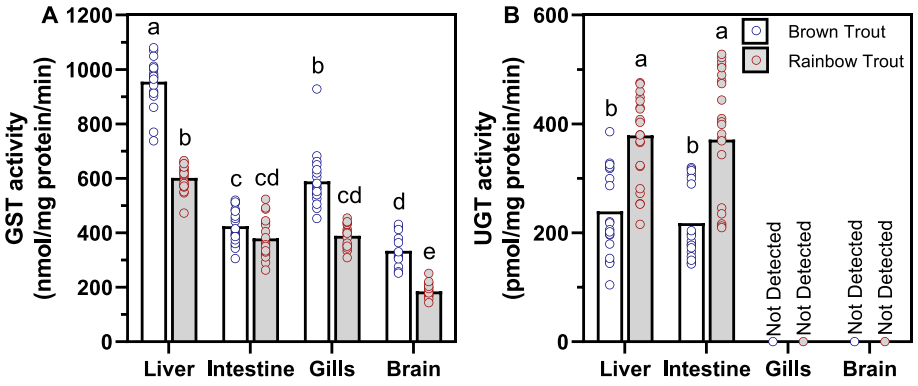

Fig. 2. Activity of A) glutathione-S-transferase, B) UDP-glucuronosyltransferase in S9 sub-cellular fractions isolated from the liver, intestine, gills, and brain of brown trout and rainbow trout. Each bar corresponds to the mean activity of three different S9 pools, with dots representing individual data points from technical replicates (n = 21–24). Different lower-case letters denote significant differences among organs and species.

potential, as pointed out by the significant hepatic $CL_{IN\ VITRO}$ of PYR (Fig. 3), as well of AZO (Figs. 4 and S1), and PRO (Figs. 5 and S2) in both species. Brown trout displayed hepatic $CL_{IN\ VITRO}$ of 6.1 mL·mg protein^{-1} min^{-1} for PYR, 0.5 for AZO, and 1.2 for PRO. Moreover, rainbow trout presented $CL_{IN\ VITRO}$ of 4.9, 0.4, and 1.1 mL·mg protein^{-1} min^{-1} for PYR, AZO, and PRO, respectively. These trends were also observed when employing the commercially available liver S9 (Fig. S3). While neither of the micropollutants evaluated in our study displayed extrahepatic clearance higher than those observed for the liver, we demonstrated that extrahepatic organs also contributed towards clearance (Table 1). Specifically, the brain of brown trout displayed the highest clearance for PYR, followed by the intestine and the gills in that order. Similarly, in rainbow trout, brain S9 appeared to biotransform AZO (0.2 mL·mg protein^{-1} min^{-1}), while PRO was depleted by intestine (0.1 mL·mg protein^{-1} min^{-1}) and gill S9 (0.2 mL·mg protein^{-1} min^{-1}).

First, it is important to point out that the enzyme characterization component of the present study relied on a targeted analysis for enzymes that are often reported as major contributors towards biotransformation (Schlenk et al., 2008), similar to what most studies have conducted in the past. To an extent, this targeted approach allows for an estimation of the biotransformation ability of organisms. However, biotransformation enzymes are quite diverse among different taxonomic groups. For example, a total of 137 genes encoding CYP450 enzymes have been identified across different fish species (Uno et al., 2012). Therefore, such diversity is likely to be responsible for supporting chemical clearance of a broad variety of chemicals with different functional groups.

Additionally, the general notion regarding the need for phase I biotransformation enzymes to be active in order for phase II biotransformation to occur may lead to incorrect assumptions about how chemical pollutants undergo biotransformation. In many cases indeed, the role of phase II biotransformation enzymes is highly dependent on

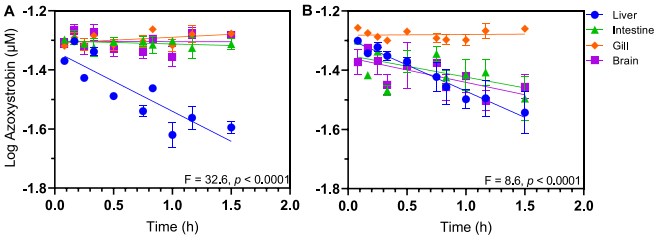

Fig. 4. Azoxystrobin (AZO) depletion by liver, intestine, gill, and brain S9 sub-cellular fractions from A) brown trout and B) rainbow trout. Data points correspond to the mean Log_{10} concentrations (μM) ± SEM of two independent experiments with each S9 pool (n = 6).

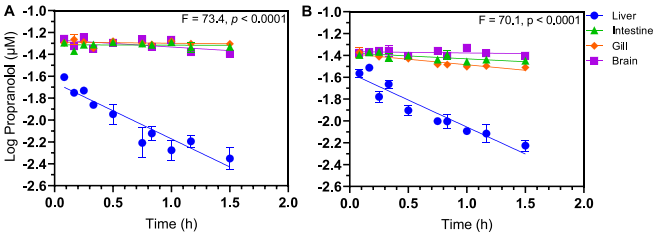

Fig. 5. Propranolol (PRO) depletion by liver, intestine, gill, and brain S9 sub-cellular fractions from A) brown trout and B) rainbow trout. Data points correspond to the mean Log_{10} concentrations (μM) ± SEM of two independent experiments with each S9 pool (n = 6).

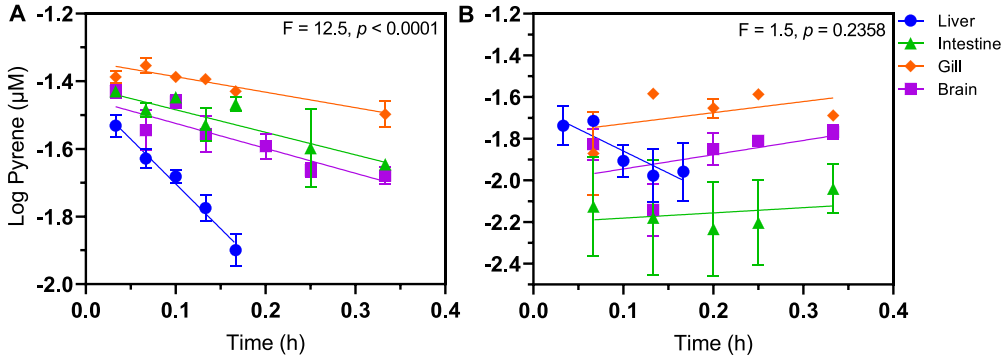

Fig. 3. Pyrene (PYR) depletion by liver, intestine, gill, and brain S9 sub-cellular fractions from A) brown trout and B) rainbow trout. Data points correspond to the mean Log_{10} concentrations (μM) ± SEM of two independent experiments with each S9 pool (n = 6).

Table 1
In vitro intrinsic clearance ($CL_{IN\ VITRO}$; $mL \cdot h^{-1}$ mg $protein^{-1}$) and linear regression coefficients for PYR, AZO and PRO by liver, intestine, gill, and brain S9 sub-cellular fractions from brown trout and rainbow trout. ND: not detected depletion of the test compound.

	Liver				Intestine				Gills				Brain			
	Brown Trout (*S. trutta*)		Rainbow Trout (*O. mykiss*)		Brown Trout (*S. trutta*)		Rainbow Trout (*O. mykiss*)		Brown Trout (*S. trutta*)		Rainbow Trout (*O. mykiss*)		Brown Trout (*S. trutta*)		Rainbow Trout (*O. mykiss*)	
	$CL_{IN\ VITRO}$	R^2	$CL_{IN\ VITRO}$	R^2	$CL_{IN\ VITRO}$	R^2	$CL_{IN\ VITRO}$	R^2	$CL_{IN\ VITRO}$	R^2	$CL_{IN\ VITRO}$	R^2	$CL_{IN\ VITRO}$	R^2	$CL_{IN\ VITRO}$	R^2
Pyrene (PYR)	6.09	0.98	4.86	0.80	1.62	0.85	ND	ND	1.02	0.89	ND	ND	1.85	0.83	ND	ND
Azoxystrobin (AZO)	0.47	0.76	0.41	0.97	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.19	0.51
Propranolol (PRO)	1.20	0.90	1.13	0.90	ND	ND	0.12	0.62	ND	ND	0.23	0.88	ND	ND	ND	ND

the activity of CYPs and other, NADPH-independent enzymes during phase I biotransformation (Schlenk et al., 2008). However, a variety of chemical compounds display functional groups with direct affinity for phase II biotransformation enzymes. Hence, the contribution of e.g. CYP450 enzymes towards intrinsic clearance may not be as significant. This scenario is reflected by the $CL_{IN\ VITRO}$ of PRO in rainbow trout, which displays a OH^- functional group that facilitates glucuronidation and sulfation according to predictive models (BioTransformer 3.0). The rather low phase I biotransformation activity in rainbow trout may have been compensated by e.g. the elevated UGT activity, leading to similar depletion rates as the ones observed for brown trout (Table 1) and the commercially available liver S9 (Fig. S3C).

Furthermore, whether organs display biotransformation ability at the expression and activity levels does not necessarily imply their contribution towards the clearance of chemicals. Systemic responses to chemical exposure may be influenced by where chemicals are absorbed and distributed, as they could target specific organs. Our findings suggested the brain of rainbow trout to biotransform AZO, a broad-spectrum systemic fungicide. Pesticides have been shown to cause alterations to the nervous system upon increasing brain barrier permeability and eventually going through (Cresto et al., 2023). Moreover, studies in mammalian models have indicated that different regions of the brain, including the blood-brain barrier, display biotransformation capacity (Agúndez et al., 2014). Under this scenario, the brain's biotransformation ability could play a significant role in detoxification and bioaccumulation processes related to chemicals that interact and potentially cross the blood-brain barrier.

The intestine and gills have also received attention regarding their biotransformation capacity (Gomez et al., 2010; Leguen et al., 2000; Lo et al., 2015; Saunders et al., 2020). Both organs are of particular interest due to being primary points of contact with pollutants via diet and aqueous exposure. Thus, these organs could be equipped with different biotransformation pathways as mechanisms of first defense against chemical exposure. In our study, the intestine's and gills' biotransformation potential was shown by the PRO clearance in rainbow trout. For different chemicals, however, these organs may display a higher contribution towards clearance. For example, for chemicals like octocrylene, a common ingredient of sunscreen, intestinal biotransformation measured in substrate depletion experiments was shown to occur at higher rates (Saunders et al., 2020). This observation suggested that the liver may not represent the primary organ contributing to the overall intrinsic clearance of this particular chemical. Similarly, Gomez et al. (2010) reported slightly higher ibuprofen clearance in the gills of rainbow trout and catfish compared to those of the liver. Such observations, as well as those from the present study, are evidence of the importance of enzyme-chemical affinities, as it is not enough for organs to display biotransformation pathways if the involved enzymes are unable to transform the pollutants. Therefore, specific enzyme-chemical interactions, or lack thereof, are critical for organisms to successfully biotransform chemicals, and may be an important factor for the variability observed among different organs and species.

In the particular case of the intestine, the contribution of the intestinal microbiome towards biotransformation represents another key factor when considering the role of the intestine in displaying defense mechanisms against pollution. The extent at which intestinal microbiota supports chemical clearance is yet to be thoroughly investigated, although previous studies with fish have indicated a significant role of gut bacteria in influencing responses to metal and PAH exposure (DeBofsky et al., 2020; Yang et al., 2021). While not directly considered in the present study (due to the intestinal S9 preparation that could have affected the integrity of the microbiome), it is important to acknowledge a potential influence of the gut microbiome in the observed clearance for the tested chemicals.

3.4. Biotransformation product profiles

The identification of four AZO and three PRO BTPs (Figs. S4, S5, Table S3) presented important differences related to interspecific biotransformation ability. Even though our results indicated a degree of extrahepatic clearance of the test chemicals in both species, no BTPs were detected from extrahepatic biotransformation nor from phase II biotransformation, potentially due to falling below the limits of detection. The hepatic formation of a major AZO BTP, azoxystrobin acid (MAZ1), and two BTP resulting from the removal of a C_2H_2O group (MAZ2 and MAZ3; Table S3), displayed a time-dependent increase in both salmonid species. In brown trout, the formation rate was 7.4 ± 1.6 pmol·mg $protein^{-1}$ h^{-1} for MAZ1, 1.9 ± 0.6 for MAZ2, and 0.9 ± 0.4 for MAZ3, whereas in rainbow trout, formation rates were 4.5 ± 2.4, 0.7 ± 0.4, and 0.6 ± 0.1 pmol·mg $protein^{-1}$ h^{-1} for MAZ1, MAZ2, and MAZ3, respectively. A fourth BTP resulting from GSH conjugation and subsequent enzymatic cleavage to a cysteine product (MAZ4) was also detected in both species (Table S3), but no time-dependent increases were observed. Altogether, the formation of AZO BTPs was significantly higher in brown trout than in rainbow trout (Fig. 6A, C, E), despite the similar $CL_{IN\ VITRO}$ between the two species.

Studies in mammalian models (Yamazoe et al., 2020) and in aquatic organisms (Kosfeld et al., 2020; Rösch et al., 2017) have suggested that AZO is biotransformed by different CYPs, primarily by isoforms of CYP1A, CYP3A, and CYP2C. While CYP2C9-like activity was not detected in neither of the species evaluated in the present study, likely due to significantly low constitutive levels in trout compared to other fish species (e.g. mummichogs) (Franco et al., 2022a; Smith and Wilson, 2010), the elevated hepatic CYP1A and CYP3A4-like activities in brown trout may have accounted for the higher production of MAZ1, MAZ2 and MAZ3 as potentially major BTPs produced by these isoforms. In rainbow trout, it is possible that other CYP isoforms participated in AZO biotransformation, leading to similar $CL_{IN\ VITRO}$ but potentially resulting in higher production rates of other BTPs that were not detected. Further transformation may have also occurred for which products were not screened.

Similarly, three PRO BTPs were identified in both species (Table S2), following a suspect analysis based on Jeon and Hollender (2019).

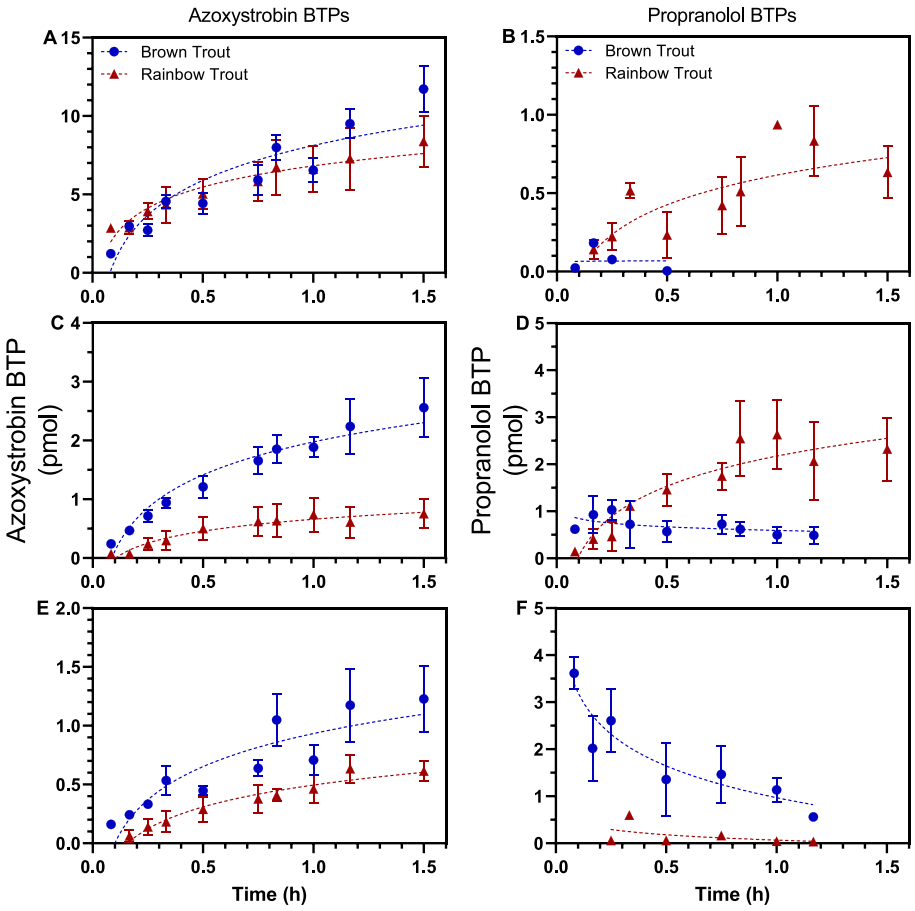

Fig. 6. Time-dependent formation of AZO BTPs: A) azoxystrobin acid (MAZ1), C) MAZ2, and E) MAZ3, and of PRO BTPs: B) propranolol-N-hydroxyl (MPR1), D) 4-hydroxypropranolol (MPR2), and F) propranolol-desisopropyl (MPR3) by liver S9 sub-cellular fractions from brown trout and rainbow trout. BTP formation rates were estimated from fitting semilog lines, and data points correspond to mean pico-moles ± SEM of two independent experiments with each S9 pool (n = 6).

However, time-dependent production was only observed in rainbow trout. In this species, the major BTP corresponded to 4-hydroxypropranolol-hydroxyl (MPR2; Fig. 6D), displaying a production rate of 2.1 ± 0.4 pmol·mg protein^{-1} h^{-1}, followed by propranolol-N-hydroxyl (MPR1; Fig. 6B), at a rate of 0.6 ± 0.5 pmol·mg protein^{-1} h^{-1}. The third BPT, propranolol-desisopropyl (MPR3; Fig. 6F), was detected but did not present time-dependent formation. Mammalian studies have previously suggested CYP1A2 and CYP2D6 as the major enzymes responsible for the biotransformation of PRO (Masubuchi et al., 1994). However, even when displaying significantly higher hepatic CYP1A activity, BTPs in brown trout did not follow a time-dependent increase. Based on these observations, two scenarios may be responsible for the PRO BTP differences among species. First, a rapid transition from phase I to phase II biotransformation in brown trout may have occurred, which could have resulted in the low detection of phase I BTPs. This scenario could have been linked to the observed time-dependent reduction of MPR3 at a rate of 2.2 ± 0.6 pmol·mg protein^{-1} h^{-1}. Alternatively, significant hepatic activity of other biotransformation enzymes (e.g. CYP2D6) in rainbow trout that were not targeted in our study, could have led to the time-dependent production of other propranolol BTPs.

In our study, the lack of evidence related to BTPs resulting from the extrahepatic biotransformation of AZO and PRO, and from phase II biotransformation, are likely the result of lower production rates in relation to the ones observed for the liver and from the activity of different CYP isoforms. These lower rates could have translated into to low BTP concentrations that were below detection limits. However, the continuous advances in analytical technologies open the opportunity to consider in the future the identification of BTPs resulting from extrahepatic biotransformation as a critical component of biotransformation studies. This is of particular interest in ecotoxicology because active transformation products may display the potential to cause adverse effects at higher magnitudes than their parental compounds. Such scenario has been extensively exemplified with phase I metabolites from the biotransformation of PAHs (Franco and Lavado, 2019) and from other pollutants, such as the pesticide malathion and its transformation product malaoxon (Cook et al., 1976). Thus, obtaining knowledge about biotransformation products could also help in characterizing risk from exposure to different classes of pollutants.

3.5. *The concept of inducibility*

It is important to point out that we based our experimentation on the OECD 319b guideline (OECD, 2018b), which implied the use of fish kept under controlled conditions and the consideration of basal biotransformation activity. While this conservative aspect of the test guideline is important for the identification of potentially bioaccumulative substances, it ignores a fundamental aspect of biotransformation pathways: inducibility. In the environment, organisms are subject to different natural and anthropogenic factors that trigger biotransformation processes. For example, exposure to chemicals that act as inducers or inhibitors of biotransformation enzymes may modify the bioaccumulation potential of other substances, thus facilitating or reducing their elimination (Franco et al., 2022b). It is also likely that biotransformation pathways not often considered within traditional bioaccumulation assessments do not display significant constitutive activity across all organs and that their activity is subject to inducibility to support

extrahepatic chemical clearance. Exploring the dynamics of biotransformation processes in environmentally relevant scenarios is a major avenue of research that must be addressed in order to further understand the magnitude of chemical biotransformation within and across species.

4. Conclusion

Existing knowledge about the contribution of different organs towards biotransformation in fish is often limited to measurements of the expression and activity of biotransformation enzymes, particularly CYP1A. Our observations in the present study indicate that, while different biotransformation pathways display organ-specific activity, as initially hypothesized, their contribution to chemical clearance could be significantly dependent on whether biotransformation enzymes display affinity for the pollutants. In addition, chemicals that have similar structures could display similar enzymatic affinities and biotransformation rates, yet important aspects of absorption and distribution must be considered if read-across approaches are employed for compounds that share similarities in their chemical structures. The influence of species-specific factors may have also accounted for how the biotransformation ability of organs influenced intrinsic clearance and BTP formation. Indeed, even when biotransformation-associated genes are conserved among species, their regulation may not display the same magnitude even when species are closely related. This became apparent for brown trout and rainbow trout used in the present study. Therefore, the integration of targeted enzyme activity measurements, substrate depletion experiments, and identification of BTPs represents a thorough approach to address research gaps associated with biotransformation processes in fish. Comprehensive biotransformation assessments, as the ones reported in our study, provide solid evidence for the need to go beyond the traditional evaluation of expression and activity of biotransformation enzymes, as their direct interaction with pollutants may represent the most significant factor to support chemical clearance. Further exploring such interactions, in conjunction with evaluations of the inducibility of biotransformation pathways, are fundamental to better understand organ-specific responses that govern chemical-organism interactions and the overall role of defense mechanisms against pollution.

CRediT authorship contribution statement

Marco E. Franco: Writing – original draft, Investigation, Funding acquisition, Formal analysis, Conceptualization. **René Schönenberger:** Investigation. **Juliane Hollender:** Writing – review & editing, Supervision. **Kristin Schirmer:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.171769.

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