In vitro biotransformation of pharmaceuticals and pesticides by
trout liver S9 in the presence and absence of carbamazepine

Junho Jeon^{a, b, c*} and *Juliane Hollender*^{c, d}

^a Graduate School of FEED of Eco-Friendly Offshore Structure, Changwon National
University, Changwon, Gyeongsangnamdo, 51140, Korea

^b School of civil, environmental and chemical engineering, Changwon National
University, Changwon, Gyeongsangnamdo, 51140, Korea

^c Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf,
Switzerland

^d Institute of Biogeochemistry and Pollutant Dynamics, ETH Zürich, CH-8092,
Zürich, Switzerland

* Corresponding author: Junho Jeon

Tel: +82 55 213 3748

Fax: +82 55 281 3011

E-mail: jjh0208@changwon.ac.kr

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Abstract

The aim of the present study was to develop (i) a technique for identifying metabolites of organic contaminants by using an *in vitro* system of trout S9 and liquid chromatography-high-resolution mass spectrometry-based identification method and (ii) to apply this technique to identify the interactive potential of carbamazepine on the formation rate of other metabolites. The pharmaceuticals carbamazepine and propranolol and the pesticides azoxystrobin, diazinon, and fipronil were selected as test contaminants. As a result, a total of ten metabolites were identified for the five parent substances, six of which were confirmed using reference standards. Metabolic reactions included hydroxylation, epoxidation, S-oxidation, and dealkylation. The metabolic transformation rate ranged from 0.2 to 3.5 pmol/mg protein/min/ μ mol substrate. In the binary exposure experiment with increasing carbamazepine concentration, the formation rates of diazinon and fipronil metabolites (MDI2 and MFP2, respectively) increased, while formation of metabolites of propranolol and azoxystrobin (MPR1, MPR2, MPR3, and MAZ1) slowed down. Meanwhile, S9 pre-exposed to carbamazepine produced diazoxon, a toxic metabolite of diazinon, and pyrimidinol, a less toxic metabolite, more rapidly. These results suggest that carbamazepine, a perennial environmental pollutant, might modulate the toxicity of other substances such as diazinon but further *in vivo* studies are needed.

Keywords: trout liver S9 fraction, carbamazepine, biotransformation, liquid chromatography-high resolution mass spectroscopy, suspect/non-target screening, diazinon

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50 **1. Introduction**

51 While the use of pharmaceuticals as well as pesticides has improved the quality of
52 human by enhancing medical practices and agricultural productivities, respectively,
53 their frequent occurrences in the environment are critical issues with respect to public
54 and environmental health (Al Aukidy et al., 2012; Löffler et al., 2005).

55 Pharmaceuticals and pesticides have often been found in surface waters (Choi et al.,
56 2008; Huntscha et al., 2012; Moschet et al., 2014; Sim et al., 2011) and aquatic
57 organisms (Konwick et al., 2006; Valdés et al., 2014). Because pharmaceuticals and
58 pesticides are designed to be biologically active, there may be a risk to non-target
59 organisms accumulating these agents.

60 Biotransformation, a subsequent process of bioaccumulation, modifies the structure of
61 xenobiotic compounds mainly through enzymatic reactions. The resulting products,
62 metabolites, are generally less toxic than their parent compounds and readily excreted
63 from the biota. However, in some cases, xenobiotics such as pharmaceuticals can be
64 bio-activated through biotransformation, leading to unexpected enhancement of
65 toxicity (Cashman et al., 1989; Li et al., 2011; Obach et al., 2008). Thus,
66 understanding the metabolic pathway and its kinetics is critical for toxicity evaluation
67 and risk assessment of environmental contaminants (Ashauer et al., 2012; Jeon et al.,
68 2013a). Despite their ecotoxicological importance, information on the metabolism of
69 pharmaceuticals in diverse aquatic organisms is lacking and limited to certain species
70 (e.g., fish) (Connors et al., 2013b; Jones et al., 2012; Mazur and Kenneke, 2008;
71 Mehinto et al., 2010). The data scarcity is attributable to numerous reasons: the
72 difficulty in culturing or maintaining test organism and biological sample treatment,
73 the lack of analytical tools for detecting metabolites without standards, and ethical

74 issues related to animal sacrifice. To explore biotransformation in aquatic organisms
75 using an *in vivo* approach, a relatively high volume of exposure solution and a large
76 number of organisms are required to obtain chemical extracts for analysis. In addition,
77 *in vivo* experiments require a long time to reveal a distinguishable profile of
78 metabolites because of sufficient bioaccumulation, which can take hours to days
79 (Abbas et al., 1996; Ashauer et al., 2012; Kretschmann et al., 2011b), before
80 metabolic reactions take place in a sufficient amount. In contrast, *in vitro* tests require
81 fewer organisms to be sacrificed and are relatively easy in terms of sample handling
82 for chemical extraction because of the lower sample volume in most cases. The
83 development and optimization of *in vitro* screening techniques are reasonable first tier
84 approaches for evaluating the risks of environmental contaminants in the environment
85 (Juhasz et al., 2013; Mazzoleni et al., 2009). For example, supernatant liver
86 homogenate obtained by centrifugation at $9000 \times g$ (S9) and microsomes are widely
87 used *in vitro* systems because they are commercially available, cost-effective, and
88 suitable as screening tools for environmental risk assessment (Connors et al., 2013b;
89 Nichols et al., 2007). As the fish liver is the primary organ of xenobiotic
90 biotransformation, *in vitro* liver systems are widely used for studies of
91 bioaccumulation, metabolic pathways, and detoxification following chemical
92 exposures (Han et al., 2009; Lee et al., 2014; Nabb et al., 2007; Schmieder et al.,
93 2000; Smith et al., 2010).

94 In pharmacological study, *in vitro* experiments can serve as a screening mechanism
95 for identifying not only metabolic pathways (Abdallah et al., 2014; Nabb et al., 2007),
96 but also drug-drug interactions (Keubler et al., 2012; Wang et al., 2010). Interaction
97 studies typically involve inhibitions of specific enzymes such as cytochrome P450
98 (Huang et al., 2008; Shou et al., 1994; Wang et al., 2010), which plays crucial roles in

detoxification and overall toxicity of contaminants. Therefore, it is possible to confirm the potential mixture toxicity of pollutants accumulated in aquatic organisms through *in vitro* inhibition experiments. Considering that surface waters are contaminated with various biologically active pollutants including pharmaceuticals and pesticides, the chemical interactions between toxicants, even at trace levels, may occur in aquatic organisms. For example, the pharmaceutical carbamazepine (CMZ) is present at relatively high frequencies and concentrations in surface waters (Sim et al., 2010; Valdés et al., 2014; Yoon et al., 2010), which is mainly attributable to the low removal efficiency in wastewater treatments (Aymerich et al., 2016; Bahlmann et al., 2014; Buchicchio et al., 2016; Zhang et al., 2008). The anticonvulsant agent is known to interact with other chemicals during cytochrome P450 (CYP)-associated reactions (Chen et al., 2015; Tateishi et al., 1999). As many pesticides are metabolized and thereby detoxified by CYP isoenzymes (Fabrizi et al., 1999; Kretschmann et al., 2011a), carbamazepine may modulate the toxicity of pesticides in this enzymatic reaction.

The inhibition or enhancement of enzyme activity can be quantified by measuring resulting metabolites, which is difficult in metabolic studies mainly due to lack of reference standards for the metabolites. Recently, high-resolution mass spectrometry (HRMS) has been shown to be a reliable instrumental tool for tentatively identifying metabolites. Because of the sufficient sensitivity and selectivity of mass detection, HRMS enables identification of unpredicted metabolites at trace levels even without the reference standards (Jeon et al., 2013b; Leblanc and Sleno, 2011).

The present study aimed to establish an *in vitro* system for deriving xenobiotic metabolism using trout liver S9, one of the representative freshwater biological system (Han et al., 2009), and a novel analytical method for identifying formed

metabolites. We applied a suspect and non-target screening method using liquid chromatography (LC)-HRMS/MS for the identification of metabolites which has been a major obstacle to metabolism study. Two pharmaceuticals and 3 pesticides were selected for the tests by considering their occurrences and concentrations in environments (Konwick et al., 2006; Park et al., 2018) and ecotoxicological importance (Ferrari et al., 2004; Kostich et al., 2014). For the identified metabolites formed in the *in vitro* system, metabolic pathways are suggested for the parent compounds. Finally, the effect of carbamazepine, known as a CYP regulator, on the formation of substrate metabolites was evaluated via *in vitro* binary exposure experiments with carbamazepine and selected substrates.

2. Materials and methods

2.1 Chemicals and S9 fraction

Information on the selected compounds, carbamazepine(CMZ), azoxystrobin(AZX), diazinon(DIZ), fipronil(FPN), and propranolol(PRN) are shown in SI, Table S1. A list of all chemicals used, their sources, and purity are given in Table S2. Chemicals of the highest available purity were used. Stock solutions of target compound and internal standards were dissolved in methanol at concentrations of 1,000 mg/L. For exposure tests and mass analysis, stock solutions and internal standards were diluted with methanol to lower their concentrations (100, 10, 1.0 mg/L).

Commercialized trout liver S9 fraction (Gibco, Grand Island, NY, USA) was purchased from Life Technologies (Carlsbad, CA, USA). The fraction at 20 mg/mL protein concentration was delivered frozen and then stored at -80°C until use.

2.2 In vitro formation of metabolites

In vitro incubation method using the S9 fraction was established with minor modifications of previous methods described elsewhere(Connors et al., 2013b; Gomez et al., 2010). The frozen S9 was thawed and diluted with 0.05 M phosphate (PP) buffer (pH 7.2) to 3.0 mg/mL of nominal protein concentration immediately before incubation with target substances to induce metabolite formation. Chemical stock solutions (100 mg/L in methanol) were diluted with buffer containing 1 mM EDTA to achieve a final concentration of 500 µg/L. The diluted chemical solution (100 µL) was then combined with S9 (100 µL of 3 mg protein/mL) and 1 mM NADPH in buffer (100 µL) in a 2-mL tube and thoroughly mixed by vortexing. The mixture

solutions were prepared in triplicate. After incubation for 1 h at 13°C, 300 µL of methanol was added to the mixtures to terminate the reactions. For chemical analysis with LC-HRMS/MS, the mixtures were filtered through a 0.2-µm nylon syringe filter. The filter was rinsed with 400 µL of methanol and the filtrates were combined to a total volume of 1.0 mL. Two different controls, one without chemical (chemical-negative) and one without S9 (S9-negative), were also performed. Protein contents of the solutions were measured using the Lowry method (Everette et al., 2010) with bovine serum albumin as a reference protein.

2.3 Biotransformation kinetic experiment

In vitro kinetic experiments were conducted to estimate biotransformation rates for propranolol. The experimental procedure was analogous to the *in vitro* exposure test described above, with slight modifications. Briefly, 200 µL of S9 fractions at 3 mg protein/mL were mixed with 200 µL of chemical solution (3 µM, prepared in 0.05 M phosphate buffer). The reaction was initiated by adding 200 µL of NADPH (3 mM) dissolved in buffer. During incubation at 13°C, 50 µL of the solution was collected at 0, 5, 10, 20, 30, 40, and 60 min and directly mixed with 150 µL of methanol containing isotopic-labeled-internal standard to terminate the reaction. Additionally, the linear increase in formation of metabolites were examined by quantifying metabolites at 0, 15, 30, 45, and 60 min. As a control, heat-treated S9 was incubated in parallel. All samples were evaluated in triplicate. The collected samples were centrifuged at 200,000 ×g for 10 min. Supernatants were collected and filtered (0.2-µm) for chemical analysis by LC-HRMS/MS and the measurement of protein.

2.4 Inhibition/induction of biotransformation by carbamazepine

S9 biotransformation of substrate compounds in the presence of carbamazepine was performed to determine the potential of carbamazepine as an inhibitor/inducer of metabolic reactions. Pesticides, i.e., azoxystrobin, diazinon, and fipronil, and the pharmaceutical, propranolol, were used as substrates. Forty microliters of S9 at 4 mg/mL (triplicate) was mixed with 40 μ L of substrate at 4 μ M and 40 μ L of carbamazepine at concentrations of 0, 0.04, 0.2, 2, 10, 20, 100, and 200 μ M. The incubation was initiated by adding 40 μ L of NADPH (4 μ M). After 30 min incubation at 13°C, 200 μ L of methanol was added to terminate the reactions. In parallel, carbamazepine pre-incubation regime tests as described elsewhere (Fowler and Zhang, 2008; Silverman, 1995) were conducted to examine time-dependent changes in metabolite formation of diazinon. For pre-incubation, 80 μ L of S9 at 20 mg/mL (triplicate) was mixed with 40 μ L of NADPH (4 μ M) and 40 μ L of carbamazepine at concentrations of 0, 0.4, 2, 20, 100, 200, 1000, and 2000 μ M. After 30 min incubation, 16 μ L of the CMZ-S9 mixture was diluted with 40 μ L of S9 4 mg/mL and 24 μ L of PP buffer. The diluted mixture was then combined with 40 μ L of substrate at 4 μ M and 40 μ L of NADPH (4 μ M) for a 30-min incubation. For termination, 200 μ L of methanol containing isotopic-labeled-internal standard was injected into each tube. In addition, the adequacy of the protein concentration used in pre-incubation test was evaluated by measuring the metabolite formed with different amounts of S9 protein. The controls, protein content measurement, and post-treatments for chemical analysis were conducted as described above.

2.5 Metabolite prediction

Metabolite prediction was performed to calculate the exact mass of suspected metabolites prior to mass spectrometry analysis. The calculation was aimed at

facilitating the screening of chromatographic peaks for identification of metabolites. Therefore, the change in the exact mass by the gain or loss of molecules in metabolic reactions was determined regardless of the modification site of the reaction. Prediction was performed in two steps based on common metabolic reactions. In the first step, universal oxidative/reductive reactions such as addition of oxygen (e.g., oxidation, hydroxylation, and epoxidation), methylation, and hydrogenation were considered. In the second step, structure specific reactions that have been reported in the literature (e.g., N- or O-dealkylation) were included in the prediction. For example, deisopropylation was included for propranolol, which has an N-isopropyl moiety, whereas the breakage of ether was predicted for AZX and DIZ. All reactions considered in the predictions and the resulting exact mass changes are noted in Table S3. Based on the predicted metabolic mechanisms, all possible combinations of reactions were established by forming a matrix equation to calculate the exact mass of metabolites formed through one or two consecutive reactions. The calculated results were used to build a mass list of suspected metabolites, which was inserted into the instrumental method file for data-dependent MS/MS acquisition as described below.

2.6 LC-high-resolution mass spectrometry

The extracts from *in vitro* experiments and exposure solutions were analyzed by liquid chromatography electrospray ionization-tandem mass spectrometry (Q Exactive Orbitrap LC-ESI-MS/MS, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The analytical procedure has been described by Jeon et al (Jeon et al., 2013b). Briefly, automated online solid phase extraction was applied to avoid contamination of the mass analyzer by matrix components. A mixed bed multilayer cartridge (10 mg Oasis HLB (Waters, Milford, MA, USA) above a 10 mg mixture of ENV+ (International

235 Sorbent Technology, UK), Strata X-AW, and X-CW (Phenomenex, UK)) was used to
236 enrich the broad range of parent compounds as well as small and ionic metabolites.
237 For online enrichment, 100 μ L of the extract from *in vitro* assays was injected into 20-
238 mL headspace glass vials (Infochroma AG, Zug, Switzerland) which were then filled
239 with deionized water. A loading solution conditioned with 2 mM ammonium acetate
240 was used for sample enrichment. The mobile phase (HPLC-grade water and methanol
241 with 0.1% (vol.) formic acid) was pumped into a Waters Atlantis T3 3 mm column
242 (3.0×150 mm) at 300 μ L/min. Before sample injection, the loop was rinsed with
243 HPLC-grade acetonitrile at a flow rate of 2000 μ L/min. Next, the samples were
244 injected together with the loading solution as described in Table S4. Detection of
245 analytes was performed with the QExactive using electrospray ionization interface in
246 positive and negative mode. High-resolution mass spectra with a resolution of 70,000
247 and mass accuracy of <3 ppm were recorded. The QExactive operation method was
248 designed to perform data-dependent MS/MS at a resolution of 35,000 for the five
249 most intense ions among the mass list of parents and metabolites for each scan event.
250 If no mass from the list of predicted metabolites was found, the most intense ions
251 were fragmented. Detailed information regarding setting parameters for the ionization
252 and data acquisition of mass spectrometry are provided in SI (SI. A). Parent
253 compounds and their metabolites whose reference standards were available were
254 quantified using internal standard calibration methods with the area response ratio of
255 the reference standard to the respective internal standard. Recovery and limit of
256 quantification for online-SPE method over S9 matrix is described in Table S7. For
257 identified metabolites without reference standards, the amounts of products were
258 estimated from the response area ratio adjusted by ionization efficiency (IE), which
259 can be predicted from the physicochemical properties of molecules and

chromatographic retention times (Jeon et al., 2013a; Leito et al., 2008). The resulting adjusted conversion factors for metabolites of propranolol are presented in Table S5. The adjusted response area ratios were obtained by dividing the response area ratio of the metabolites with the corresponding conversion factors.

2.7 Suspect/non-target screening

Data from HRMS analysis were evaluated to identify metabolite peaks using commercial software, SIEVE (v. 2.1, Thermo Fisher Scientific, Inc.), which enables concurrent suspect and non-target screening. The software carries out comparative analysis for HRMS spectra of the control (chemical-negative and S9-negative samples) and treatment sample groups. For comparison, SIEVE gradually performs chromatogram alignment, frame generation (corresponding to peak picking), and peak identification (with the help of the database). Appropriate threshold values were set in frame generation step to reduce the number of peaks to be evaluated, but not to miss any major metabolites: ion range: 100–1000 m/z; retention time range: from RT of parent compound - 5 min to RT of parent compound + 2; m/z isolation width: 5 ppm; peak intensity: >500,000 for positive ion, >300,000 for negative ion. The generated frames (i.e., peaks) are characterized using numerous parameters including exact mass (m/z), retention time (RT), charge state of ion (charge), peak area ratio between the control and treatment group (ratio), p-value from the statistical test for the difference (p-value), and covariant value for each data group (CV_treatment or _control), among others. For suspect screening where peaks of the predicted metabolites were isolated, a list of exact masses of predicted metabolites was inserted into the program prior to data processing. If ions in the list were identified, the software output a report for identification. For the remaining generated peaks, non-target screening was conducted.

The parameters were used as filters to narrow down candidate peaks. Among them, ratio, charge, and CV_treatment were the most effective parameters for isolating the interesting peaks. The threshold values for screening were as follows: Ratio >5, Charge = 1, and CV_treatment <100. Detailed information on parameters used in SIEVE is provided in Table S6. All peaks exceeding all threshold values remained after filter screening. Next, the shape and intensity of resulting peaks were visually evaluated. All peaks isolated from suspect/non-target screening with appropriate peak shape were re-evaluated using MS/MS fragment information in the Qual Browser of Xcalibur (Thermo Scientific, Inc.). If the fragment data were not available, the samples were re-measured using a targeted MS/MS method. The structures of the identified metabolites were elucidated based on MS/MS fragment interpretation using fragment prediction tools such as MassFrontier (Thermo Scientific, Inc.) and MetFrag (Wolf et al., 2010) and the database, MassBank (Horai et al., 2010). Finally, a confidence level (CFL) for the identified metabolite was assigned according to criteria described by Schymanski et al. (2014).

3. Results and discussion

3.1 Validation of S9 fraction activity

Metabolic activity of the S9 fraction used in the present study was validated by propranolol depletion, which has been successfully demonstrated using the trout liver S9 fraction in previous studies (Connors et al., 2013b; Gomez et al., 2010). In our kinetic study, the propranolol concentration was sufficiently decreased by approximately 53% of the initial concentration (i.e. 0.73 μ M, measured) after 1-h incubation with S9 fraction, while no significant depletion was observed in the S9-negative (heat-treated) control (Fig. S1). The resulting clearance rate (CL) was 0.83 (± 0.04) mL/h/mg protein, which is greater than the estimates obtained by Gomez et al. (2010) (0.3 – 0.5 mL/h/mg protein). Our estimate, however, is lower than the CL estimates from 2 separate bioassays obtained by Connors et al. (2013b, 2013a) (2.0 and 2.74 mL/h/mg protein) and very recently by Baron et al. (2017) (1.57 mL/h/mg protein, converted from 78.5 mL/h/g liver by a factor 50 mg protein/g liver). The varying estimates may indicate that differences in physiological status of sacrificed organisms, protein concentrations, and starting substrate concentrations as reasons for the different estimates. For comparison, another clearance rate for PRN was estimated from the carbamazepine interaction experiments in the present study, which was calculated using two points of PRN concentration data (one from the S9-negative (denatured)/CMZ-negative control and the other from the S9-positive/CMZ-negative incubation for 30 min), as approximately 0.97 mL/h/mg protein. Considering the identical experimental conditions but with different S9 stocks purchased and a greater initial concentration (1.15 μ M, measured) for the second estimate, the slightly increased CL value was observed likely because of the different, but more active pool

of the S9 fraction used in the CMZ interaction experiment. Nevertheless, the standard error for these variable estimates was 0.11 mL/h/mg protein, which is reasonable for common *in vitro* kinetic estimations (Connors et al., 2013b). As another indication for the activity of the S9 fraction, transformation products formed via oxidative reactions were tentatively identified as N-hydroxy (MPR1), ring-hydroxy (MPR2), and desisopropyl product (MPR3) (chromatograms shown in Fig. S2 and MS/MS fragments and suggested structures shown in Fig. 1). Additional details are discussed below.

3.2 Identification of metabolites formed in S9 fraction

Qualitative chemical analysis with the HRMS-based target/suspect/non-target screening approach provided chromatograms and MS/MS fragment information, revealing the presence of metabolites of the test compounds. To reliably identify metabolites, byproducts or physically degraded products identified in the inactive (heat-treated) S9 fraction were excluded from metabolite analysis. The metabolites remarked with confidence level are shown in Table 1. In total, 10 metabolites were evident for 5 parent substances. Among them, 6 metabolites were orthogonally confirmed with reference standards acquiring CFL1 whereas the remaining 4 metabolites were tentatively identified at CFL 2 to 4. Their structures and MS/MS spectra are shown in Fig. S3–S6. As an active/major metabolite of carbamazepine, the formation of carbamazepine-10,11-epoxide (MCM) was successfully confirmed using the reference standard. In a previous study of the *in vitro* clearance of pharmaceuticals, a decrease in the concentration of carbamazepine and formation of metabolites was not evident in the rainbow trout liver S9 fraction (Connors et al., 2013b). This is likely either because the clearance study did not focus on metabolite identification and/or

the quantity of the epoxy metabolite was below limit of detection of the analytical method. Although CYP3A4, which is responsible for oxidative metabolism of carbamazepine in the human microsome (Hata et al., 2008; Kang et al., 2008), has not been clearly identified in trout liver (Buhler and Wang-Buhler, 1998; Connors et al., 2013b), an equivalent isozyme metabolism of human CYP3A4 was observed in trout in *in vitro* assays of conazoles (Mazur and Kenneke, 2008). Consistently, our results suggest the presence of subcellular enzymes involved in the epoxidation of carbamazepine. For azoxystrobin, demethylation resulting in the formation of azoxystrobin acid (MAZ1) was confirmed as a primary metabolite. MAZ1 is recognized as a major transformation product in aquatic (Rodrigues et al., 2013; Singh et al., 2010), soil (Adetutu et al., 2008; Ghosh and Singh, 2009) environments, as well as recently in invertebrates through *in vivo* exposure test (Rösch et al., 2017). MAZ1 has been detected as a phase 1 metabolite in rats (Laird et al., 2003). Interestingly, another product, MAZ2 with an exact mass of 362.1135 and formula of C₂₀H₁₅N₃O₄ (suggested structure depicted in Fig. 2) was tentatively identified only in the pre-incubation test regime with carbamazepine, indicating time-dependent activation by CMZ or its metabolite to form MAZ2. This metabolite has recently also been detected in invertebrates (Rösch et al., 2017); additional studies are needed for structure confirmation at a higher confidence level.

Two major metabolites of diazinon, diazoxon (MDI1) and pyrimidinol (MDI2), which are responsible for the bioactivation and detoxification of diazinon, respectively (Kretschmann et al., 2011a; Sams et al., 2004), were also confirmed. Although diazinon is a prodrug and designed to be activated via CYP-mediated transformation to diazoxon (Mutch and Williams, 2006), the responsible CYPs are known to be species-specific. For example, in a human metabolism study, CYP1A1, -

2C19, and -2B6 for MDI1, but CYP2C19 for MDI2 were revealed as major enzymes (Ellison et al., 2012), while both metabolites were formed efficiently by CYP2D6, -2C19, -3A5, and -3A4 in another study (Mutch and Williams, 2006). A thorough study of the activation of DIZ confirmed that 2C19 is a major enzyme (Kappers et al., 2001). In contrast, in rat, CYP2B1/2 was predominantly involved in the formation of diazoxon (Fabrizi et al., 1999). These previous studies which identified the CYP enzymes responsible for the metabolism indicate that DIZ is extensively engaged in oxidative reactions to form MDI1 or MDI2, which involves various CYP isoenzymes. For aquatic species, several studies (Ashauer et al., 2010; Keizer et al., 1993; Kretschmann et al., 2011b) also identified MDI1 and MDI2 which are consistent with our results.

Fipronil sulfide (MFP1) and fipronil sulfone (MFP2) have been identified as major mammalian metabolites and were also found in the present study. MFP1 is likely formed by reduction, while MFP2 is a product of oxidative dealkylation and is mainly responsible for CYP3A4 in human (McMahan et al., 2015; Tang et al., 2004). Both metabolites are known to be more active than (or comparable to) the parent (Romero et al., 2016; Roques et al., 2012), which have also been detected in aquatic environments (Gan et al., 2012; Wu et al., 2015), but no studies found the metabolites in wildlife.

In a dietary bioaccumulation experiment using rainbow trout (Konwick et al., 2006), MFP2 was identified as a major metabolite and remained 3-fold longer in the biota than its parent. This active metabolite formation and the slow excretion characteristics may considerably attribute to its high toxicity in rainbow trout (US EPA. Office of Pesticide Programs, 1996).

Two structural isomers formed via hydroxylation and one oxidative dealkylation product were tentatively identified for propranolol. Although the suggested metabolites of PRN were not orthogonally confirmed because of the non-availability of commercial standard materials, numerous diagnostic/deterministic MS/MS structural data support the depicted structures for the metabolites in Fig. 1. For example, fragment m/z 74.0606 of precursor m/z 276.16 for MPR1 indicates the addition of one oxygen molecule to the N-isopropyl moiety corresponding to a molecular formula of C_3H_8ON . The neutral loss of H_2O from the previous fragment resulting in an m/z 56.0503 (C_3H_6N) often occurs on N-oxide/hydroxylation products (Jeon et al., 2013b), MPR1 was predicted as an N-hydroxy product. Fragment m/z 173.0600, suggesting $C_{11}H_9O_2$, is strong evidence for hydroxylation of the naphthol moiety of PRN to form MPR2. Additionally, the MS/MS fragment pattern for MPR2 is considerably similar to that of 4-hydroxypropranolol recorded in the MS/MS database MassBank (Horai et al., 2010), leading to CFL 2. MS/MS information for MPR3 also includes characteristic fragments such as 74.0607 (C_3H_8ON) resulting from a breakage of ether, indicating de-isopropylation. Except for the N-oxidation product, MPR2 and 3 are known as predominant metabolites in human, rat, or fish microsomes (Barnes et al., 2008; Masubuchi et al., 1994; Narimatsu et al., 1997; Partani et al., 2009; Yoshimoto et al., 1995), strongly suggesting the presence of equivalent CYP isoenzymes in the S9 fraction used.

Overall, the identified formation of transformation products indicates that the S9 fraction is able to transform selected organic compounds to metabolites which have been identified in mammalian (e.g., human, rat, etc) and/or aquatic organisms (daphnia, trout, etc) and so possesses active CYP isoenzymes comparable to those listed in table 1 as responsible CYP.

3.3 Comparison of normalized transformation rates

In conventional *in vitro* enzyme kinetic experiments, the Michaelis–Menten equation is applied to determine the relationship between the enzymatic transformation rate and substrate concentrations, which typically ranges from μM up to mM levels (Hutzler et al., 2002; Keubler et al., 2012; Shou et al., 1994). The present study, however, focused on metabolic kinetics at environmentally relevant substrate concentrations, and thus formation rates were estimated at a range covering sub- μM substrate concentrations, CMZ, AZX, DIZ, FPN, and PRN were 1.16, 0.53, 0.52, 0.32, and 0.87 μM , respectively. For this concentration range, the formation of all transformation products increased linearly within 30 min of incubation (Fig. S7). Assuming that the substrate concentrations are below Michaelis constant (K_m), initial formation rates depend on the substrate-concentration. Thus, concentration-normalized formation rates were estimated for inter-chemical comparison as described in Table 1 and ranged from 0.2 to 5.5 pmol/mg protein/min/ μmol substance. CMZ epoxidation and PRN N-hydroxylation showed the lowest formation rates. In contrast, fipronil metabolites, MFP1 and MFP2, show substantial formation rates (1.7 and 1.6 pmol/mg protein/min/ μmol substance), leading to approximately 8.5% depletion of the parent compound within 30 min (Table 1). A comparable study estimated the formation rate for MFP2. The calculated rates from human/rat liver microsome experiments were 1.9/9.8 pmol/mg protein/min/ μmol substance (Tang et al., 2004). Upon comparison, human liver microsome and S9 showed similar activity for the formation of MFP2, but faster formation in the rat liver microsome was expected.

Because reference standards for metabolites of PRN were not commercially available, the formation rates were estimated using a semi-quantitative method based on the adjusted response area ratio as described in section 2.6. Moreover, information on the metabolic kinetics of propranolol, to our knowledge, has not been reported yet, and thus the estimated formation rates cannot be compared. Nevertheless, the overall transformation rate appeared to be considerable based on the greatest depletion of PRN among the 4 test compounds (approximately 30% after 30-min reaction). This indicates either underestimation of the rates or the presence of other metabolic pathways not identified in the present study.

Azoxystrobin acid, a major metabolite of AZX, was formed at a relatively low rate (1.0 pmol/mg protein/min/ μ mol substance). Unfortunately, comparable in vitro data was not available, and an in vivo transformation rate is recently estimated, but in different species, i.e., *Gammarus pulex* (Rösch et al., 2017). In contrast, DIZ metabolites, diazoxon and pyrimidinol, formed relatively more rapidly. Sams et al. (2004) compared the biotransformation of diazinon by the human liver microsome and estimated the desulfuration and dearylation characteristics as $K_m = 45 \mu\text{M}$, $V_{max} = 776 \text{ pmol/min/mg}$ and as $K_m = 28 \mu\text{M}$, $V_{max} = 1,186 \text{ pmol/min/mg}$, respectively. The calculated normalized rates ($V_{max}/2K_m$) based on these estimates were 8.6 and 21.2 pmol/min/mg/ μM substrate, respectively. The faster detoxification than bioactivation in human metabolism is consistent with our results, whereas both human transformation rates were 2.5- to 4-fold greater than those of the S9 fraction.

3.4 Inhibition/Activation of metabolite formation by carbamazepine

The formation of MPR1, MPR2, MPR3, and MAZ1 was negatively affected by increasing CMZ concentrations (Fig. 3). The inhibitory effects of CMZ have been

reported for CYP2D6 (Ono et al., 2002; Spina et al., 2001), which is known to be involved in the metabolic pathway of propranolol (Masubuchi et al., 1994). Although this inhibition was observed in clinical studies, the result is consistent with our *in vitro* results showing the retarded formation of MPR1 and MPR2. The inhibition can be explained by competitive binding or occupation because CYP2D6 is responsible for both CMZ and PRN. The relatively low transformation rate of MCM might indicates that CYP-CMZ binding persists for longer, decreasing the availability of free CYP. It remains unclear whether the activity of CYP1A2 responsible for MPR3 is inhibited by CMZ exposure in trout S9. Instead, there is evidence for the induction of CYP1A2 activity by CMZ in human (Magnusson et al., 2008), which indicates that unknown and complex metabolic mechanisms involving CYP species may occur for MPR3. No enzymes responsible for the formation of MAZ1 were revealed in our literature survey. However, considerable clearance of AZX was recently observed in enzyme incubation with CYP3A4 and 2C19 (Wetmore et al., 2014). Thus, the dramatic reduction in the acid product was likely because of the competition of CMZ and AZX to CYP3A4.

Additionally, in the presence of carbamazepine, the production of MDI2 and MFP2 seems slightly enhanced in a concentration-dependent manner. The enhancement was more significant when diazinon was incubated with the pre-exposed S9 to carbamazepine (Fig. S8), which is in accordance with time-dependent changes demonstrated elsewhere (Riley et al., 2007). The levels of both diazoxon and pyrimidinol were approximately 1.8-fold higher in the pre-incubated S9 fraction at different carbamazepine concentrations. As there was no significant changes in the formation of the metabolites with two different S9 protein concentrations (1 and 10 mg/mL) (Fig. S9), the enhanced formations were hardly attributable to the protein

500 contents of the pre-incubated mixture. Instead, this might indicate that not only CMZ,
501 but also metabolites such as the epoxide product, MCM1, play a role in the
502 enhancement. The increased formation is possibly attributable either to the inhibition
503 of the formations of secondary/tertiary metabolites or the activation of enzymatic
504 metabolism by carbamazepine and/or the metabolite. The former case is hardly
505 identifiable due to lack of information on downstream metabolites in the present study
506 whereas there has been a number of relevant studies demonstrating *in vitro* activation
507 of enzyme as a result of drug-drug interaction. For example, the formation of fipronil
508 sulfone (i.e., MFP2) in the human liver microsome was enhanced by up to 2.3-fold
509 with increasing testosterone concentrations (Tang et al., 2004), which is a comparable
510 result to ours. In addition, a hydroxylated product of flurbiprofen from *in vitro*
511 CYP2C9 incubation was amplified in the presence of an antibiotic, dapsone, and its
512 analogue, phenylsulfone, whereas naproxen demethylation was enhanced by p-
513 tolylsulfone and sulfamethazine at high concentrations (Hutzler et al., 2002; Korzekwa
514 et al., 2002). CYP3A4 activation has been also widely investigated for diverse
515 substances (Galetin et al., 2002; Shou et al., 1994; Tang et al., 1999). Among these,
516 Shou et al. (1994) conducted structure-activity relationship studies to identify the
517 effect of functional groups on CYP activation and found that naphthalene substituted
518 with a ketone group such as 7,8- benzoflavone activated CYP3A4-related metabolism.
519 This is consistent with our results showing increased levels of MFP2, known as
520 CYP3A4-mediated product (Tang et al., 2004), in the presence of carbamazepine
521 possessing a ketone. This suggests that the ketone moiety is involved in the activation
522 mechanism. As a possible explanation for the mechanism, Keubler et al. (2012)
523 proposed enhanced coupling between P450 and NADPH-P450 reductase by activating
524 compounds. Hutzler et al. (2003) also suggested that coupling can be increased if

water molecules potentially causing uncoupling at active sites are replaced with certain compounds. This was partially supported by the nearly 2-fold higher product:NADPH ratio for CYP2C9 in the presence of dapson, indicating more efficient coupling formation. Another mechanism, also suggested by Hutzler et al. (2003) occurs through the reduced volume of the active site by the occupation of activating molecules, which likely pushes substrates closer to the active oxidant and/or catalytic site, resulting in a fast turnover rate.

Although the *in vitro* activation mechanism remains unclear, our results and those of others support that the activation is highly correlated with the change in the steric conformation in the presence of carbamazepine.

3.5 Environmental and ecotoxicological implications

Information on the biotransformation of environmental contaminants provides important clues for understanding the environmental fate and toxicity of a substance. Identification of structure-toxicity relationships for metabolites with confirmed structures can broaden the understanding of how a substance is metabolized in the body of a fish. However, the metabolites identified *in vitro* are only some of the metabolites that can be produced *in vivo*, and the *in vitro* studies can only be a first tier. In the S9 exposure experiment for the binary mixture (substrate-carbamazepine), the interactive potential of carbamazepine for the metabolism of organic pollutants such as pesticides was confirmed. Considering that the surface water concentration of carbamazepine is on the sub-microgram per liter level and that the expected maximum concentration of biomass is on the micromole level per kg (Valdés et al., 2016; Vernouillet et al., 2010), the *in vitro* system concentration of carbamazepine used in the experiment reflects the actual exposure situation. Thus, the activity of the

identified carbamazepine suggests that it may be involved in the toxicity of substrate compounds. For example, changes in the production rate of the toxic metabolite diazoxon with varying concentrations of carbamazepine indicate that fish such as trout can alter the vulnerability to diazinon depending on the presence or absence of carbamazepine. Changes in the formation of pyrimidinol, a low toxic metabolite, may contribute to toxicity reduction. Particularly, the time-dependent effect of carbamazepine in pre-incubation experiments showed that the degree of change can vary with pre-exposure time.

In general, pharmaceuticals such as carbamazepine are recognized as continuously present organic pollutants because of their origin from human wastewater while pesticides such as diazinon are diffuse contaminants present in the application period. Thus, the pre-exposure (incubation) experiments adequately represent exposure conditions in the actual stream with low-but-continuously present concentrations of carbamazepine and with pulse exposures of pesticides. Thus, the results from the experiment design are not negligible. The whole organism is a much more complex response system, and the results from *in vitro* experiments only partially reflect this situation. Nonetheless, *in vitro* studies that are faster, easier, and free from ethical concerns related to animal use have confirmed that they can produce meaningful results regarding ecotoxicological consequences, and thus serve as useful first tools for future ecotoxicological studies.

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