How Biotransformation influences Toxicokinetics of Azole Fungicides in the Aquatic Invertebrate *Gammarus pulex*

Andrea Rösch^{1,2}, Sabine Anliker^{1,2}, Juliane Hollender^{1,2 *}

¹Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland

²Institute of Biogeochemistry and Pollutant Dynamics, ETH Zürich, 8092 Zürich, Switzerland

Abstract:

Biotransformation is a key process that can greatly influence the bioaccumulation potential and toxicity of organic compounds. In this study biotransformation of seven frequently used azole fungicides (triazoles: cyproconazole, epoxiconazole, fluconazole, propiconazole, tebuconazole and imidazoles: ketoconazole, prochloraz) was investigated in the aquatic invertebrate *Gammarus pulex* in a 24 h exposure experiment. Additionally, temporal trends of the whole body internal concentrations of epoxiconazole, prochloraz, and their respective biotransformation products (BTPs) were studied to gain insight into toxicokinetic processes such as uptake, elimination and biotransformation. By the use of high resolution tandem mass spectrometry in total 37 BTPs were identified. Between one (ketoconazole) and six (epoxiconazole) BTPs were identified per parent compound except for prochloraz, which showed extensive biotransformation reactions with 18 BTPs detected that were mainly formed through ring cleavage or ring loss. In general, most BTPs were formed by oxidation and conjugation reactions. Ring loss or ring cleavage was only observed for the imidazoles as expected from the general mechanism of oxidative ring openings of imidazoles, likely affecting

the bioactivity of these BTPs. Overall, internal concentrations of BTPs were up to three orders of magnitude lower than that of the corresponding parent compound. Thus, biotransformation did not dominate toxicokinetics and only played a minor role in elimination of the respective parent compound, with the exception of prochloraz.

1. Introduction

Aquatic organisms are exposed to a mixture of numerous pollutants originating from industrial, agricultural and domestic activities. One of the most widely used classes of antifungal agents are azole fungicides, which include among others triazoles and imidazoles (see Figure 1). These fungicides are applied in agriculture as well as in pharmacology against various fungal infections.¹ Azoles enter the aquatic environment via runoff from agricultural areas and effluents from wastewater treatment plants. Hence, azoles are frequently detected in surface waters with concentrations ranging from low ng L⁻¹ to low µg L⁻¹.²⁻⁴ In a recent pesticide screening in Swiss surface waters several azoles have been detected up to a concentration of 100 ng L⁻¹.⁵ Azole fungicides are moderately toxic toward aquatic invertebrates with LC₅₀ values in the low mg L⁻¹ range.⁶⁻¹⁰

The anti-fungal activity of azoles results from their ability to inhibit the cytochrome P450 (CYP) catalyzed ergosterol biosynthesis in fungi. Binding between the azole and the enzyme occurs via the nucleophilic nitrogen in the specific azole moiety and the iron of the enzyme which is responsible for converting lanosterol to ergosterol. This leads to a depletion of ergosterol, which is essential for the structure and functioning of fungal cell membranes.¹¹⁻¹² Thus, biotransformation reactions taking place at the active azole moiety of the fungicide can affect its antifungal activity. The huge enzyme class of CYP monooxygenases catalyzes a wide variety of oxidative reactions in metabolism and in non-target organisms CYPs are an important enzyme class active in the detoxification of xenobiotics. In aquatic species, such as in daphnids it has been observed that azole fungicides can enhance the effect of other pesticides such as of pyrethroids.¹³⁻¹⁷ This synergistic effect is supposed to be caused by the inhibition of CYP, and hence by less biotransformation of the toxic pesticide.

Gammarids are small crustaceans belonging to the order of amphipoda and are an important keystone species in lotic aquatic ecosystems. They represent one of the most sensitive aquatic invertebrate species and therefore they are often used as model organism for ecotoxicological studies and for biomonitoring.¹⁸⁻²¹ Chemical exposure concentrations are closely related to

internal concentrations inside organisms and can determine the effect of a chemical. Key factors regulating the internal chemical concentration are uptake and elimination processes including biotransformation – also termed toxicokinetics – which describe the time course of the toxicant concentration inside the organism.²²⁻²³

Biotransformation within an organism determines the bioaccumulation potential of a specific azole fungicide as well as the bioaccumulation of co-occurring substances. To date, very little information is available about the biotransformation of azoles in aquatic invertebrates. Therefore, the goals of this study were first to investigate biotransformation of seven selected azole fungicides in *Gammarus pulex* (*G. pulex*) and second to get insight into toxicokinetic processes, *i.e.* the time course of chemical uptake, biotransformation, and elimination. Five frequently used triazole fungicides (cyproconazole: CP, epoxiconazole: EP, fluconazole: FLU, propiconazole: PRP, and tebuconazole: TEB) and two imidazole fungicides (ketoconazole: KET and prochloraz: PRZ) with different physicochemical properties ($\log D_{ow}$ from 0.6 to 4.3, see Table 1) were chosen to explore the role of structural similarity in relation to biotransformation. Since the azole ring represents the active moiety of these fungicides it was of interest if this part of the molecule remains intact during biotransformation and can therefore still act as CYP inhibitor and maintain its antifungal activity. By determining the kinetic rate constants of uptake, biotransformation, and elimination we aimed to investigate the role of biotransformation in relation to the reduction of bioaccumulation of the respective parent compounds.

2. Material and Methods

Chemicals, Solutions and Test Organisms

Detailed information about all chemicals and solutions used during experiments and instrumental analysis are provided in the SI A.

Adult *G. pulex* were collected from a small creek near Zürich, Switzerland (E 702150, N 2360850) between October and December 2014. The sampling area is characterized by relatively little agricultural use and is located in a small nature reserve. Male and female

G. pulex were not differentiated since a reliable assignment of sex is only possible with an examination under a microscope using inactive and therefore dead organisms.²⁴ Prior to the experiments, organisms were acclimatized for 3-5 days to the test conditions (11 °C, 12 h light / 12 h dark cycle) in an aquarium with aerated artificial pond water (APW)²⁵ composed of 0.12 g L⁻¹ MgSO₄ · 7 H₂O, 0.065 g L⁻¹ NaHCO₃, 0.0058 g L⁻¹ KCl, and 0.29 g L⁻¹ CaCl₂ · 2 H₂O. *G. pulex* were fed with horse chestnut (*Aesculus hippocastanum*) leaves inoculated with *Cladosporium herbarum* to facilitate the decomposition of the leaves, thereby making them more edible.²⁵

Experimental Design

Biotransformation Product Screening Experiment

Biotransformation product (BTP) screening experiments were carried out separately for each selected azole fungicide. Exposure solutions were prepared in APW at nominal concentrations of 200 µg L⁻¹. 600 mL-glass beakers were filled in duplicate with 500 mL exposure solution and four organisms were added to each beaker. To provide food and shelter and to avoid cannibalism, four horse chestnut leaf discs (diameter: 2 cm) were added to each beaker. After incubation for 24 h at test conditions, the organisms were sieved, rinsed with nanopure water, blotted dry on tissue, transferred into 2 mL-microcentrifuge tubes, and weighed. After addition of 100 µL isotopic labelled internal standard mix solution (100 µg L⁻¹), 500 µL MeOH, and 300 mg of 1 mm zirconia/silica beads (BioSpec Products, Inc., USA), the extraction and homogenization was carried out with a FastPrep bead beater (MP Biomedicals, Switzerland) in two cycles of 15 sec at 6 m s⁻¹ (cooling on ice in between). Afterwards, samples were centrifuged (6 min, 10'000 rpm, 20 °C) and filtered through 0.45 µm regenerated cellulose filters (BGB Analytic AG, Switzerland). Filters were washed with 400 µL MeOH and the filtrate pooled with the extract. Additionally, different controls were performed, *i.e.* without target chemicals ("chemical negative, food and organism positive"), without organisms ("organism negative, chemical and food positive") and one with only chemicals ("organism and food negative, chemical positive"). To quantify the aqueous concentrations the exposure medium

was sampled at the beginning and the end of the experiments. All samples were stored at -20 °C until chemical analysis.

Toxicokinetic Experiment

G. pulex were exposed separately to epoxiconazole and prochloraz at concentrations of 100 µg L⁻¹ for 24 h, and were afterwards transferred to clean media for a 120 h depuration phase. During both the exposure and depuration phase *G. pulex* (four organisms per beaker) were sampled in duplicate at different time points (see SI F). Immediately after sampling 200 µL of isotopic labelled internal standard mix solution (100 µg L⁻¹) and 400 µL of MeOH were added.

Unless stated otherwise, the sample preparation was similar to the one described above.

Chemical Analysis

All samples were analysed by online solid phase extraction coupled to liquid chromatography high resolution tandem mass spectrometry (online-SPE-LC-HRMS/MS).²⁶ Automated online-SPE served for sample clean-up and enrichment. By using a mixed bed multilayer cartridge a broad range of compounds with different polarities, molecular sizes, and charges was retained and enriched. Detection of analytes was performed by using a quadrupole-orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific Inc.) with electrospray ionization (ESI). Full scan acquisition for a mass range of 100 – 1000 m/z with a resolution of 70 000 (at m/z 200) was performed in polarity-switching mode followed by five (positive mode) and two (negative mode) data-dependent MS/MS scans with a resolution of 17 500 (at m/z 200) and an isolation window of 1 m/z. For triggering data-dependent MS/MS acquisition a mass list with suspected BTPs (between 450 and 1300 masses per parent compound) was set up based on *in silico* pathway prediction and scientific literature (see SI B).

Quantification was based on internal standard calibration (Trace Finder software 3.1 (Thermo Scientific)). Amounts of BTPs were estimated based on the calibration curve of their parent compound since no reference standards could be obtained. For more details about the analytical procedure, quantification, and quality control refer to SI B and SI C.

Biotransformation Product Identification by Suspect and Non-target Screening

SIEVE software version 2.1 and 2.2 (Thermo Scientific) for differential expression analysis was used to compare treatment and control samples (see SI D).

As a first step a suspect screening was carried out. Therefore, the mass list with the suspected BTPs was uploaded to SIEVE and used for peak identification. Peaks only detected in the treatment samples with a peak intensity $>10^6$ in at least one replicate sample and with a minimum of three scans in the extracted ion chromatogram were regarded as possible BTPs. For prochloraz and epoxiconazole, where kinetic data were available, the concentrations of the suspected BTPs should increase in the uptake phase and decrease in the depuration phase.

As a second step a non-target screening was conducted to discover additional not predicted BTPs. Therefore, in addition to the above mentioned criteria two extra criteria were used – a new intensity threshold of 0.1% of the intensity of the parent compound and an intensity ratio between treatment and control samples of more than 10.

Structure Elucidation

Structure elucidation was based on the interpretation of (1) the exact mass and the isotopic pattern to assign molecular formulas and of (2) MS/MS spectra to identify diagnostic fragments or losses characteristic for one specific structure (D) or for several positional isomers (d).

Structure elucidation was assisted by the fragmentation prediction tool Mass Frontier (version 7.0, HighChem, Slovakia) and by molecular structure generation including MS and MS/MS information with Molgen-MSMS.²⁷

For some tentatively identified BTPs additional evidence was given by enzyme deconjugation experiments (e), by biotransformation pathway information (p), or by the same structure and/or MS/MS spectra already reported in scientific literature (l, m). Enzymatic sulfate deconjugation was carried out experimentally analogous to Kukkonen and Oikari (1988)²⁸ (see SI G). The letters in parentheses (D, d, e, p, l, m) indicate the identification confidence of the BTPs shown in Table 1. For triazoles the presence of the ionized triazole moiety ($m/z = 70.0400$) in the MS/MS spectra provided further evidence that identified BTPs were actually formed from a

triazole parent compound (see SI I). Furthermore confidence levels proposed by Schymanski et al. (2014)²⁹ were assigned to communicate identification confidence.

Modelling Bioaccumulation and Biotransformation Kinetics

Model Structure

To estimate the rate constants for the toxicokinetic processes a one-compartment first-order model was applied. Build Your Own Model (BYOM) version 1.5, a set of multiple scripts and functions, programmed by Tjalling Jager (<http://debtox.nl/about.html>) was used for modelling bioaccumulation and biotransformation kinetics in Matlab R2012b/R2015b. Although the term BTP is preferred, M for metabolite is used within the equations for simplicity.

The time courses of the parent compounds are described by the following ordinary differential equation:

$$\frac{dC_{in,p}(t)}{dt} = C_{water}(t) \cdot k_u - C_{in,p}(t) \cdot k_e - C_{in,p}(t) \cdot (k_{M1,1st} + \dots + k_{Mx,1st}) \quad (1)$$

where $C_{in,p}(t)$ [nmol kg_{wet weight (ww)}⁻¹] is the time course of the whole body internal concentration of the parent compound in *G. pulex* and $C_{water}(t)$ is the time course of the parent compound concentration in the medium [nmol L⁻¹]. Uptake of the parent compound via food, dermal and respiratory surfaces is described by the uptake rate constant k_u [L kg_{ww}⁻¹ d⁻¹], whereas direct elimination of the parent compound via excretion through dermal and respiratory surfaces is described by the elimination rate constant k_e [d⁻¹]. $k_{Mx,1st}$ and $k_{Mx,2nd}$ [d⁻¹] are the rate constants for biotransformation for the primary and secondary BTPs, respectively.

We differentiate between primary BTPs, that are formed directly from the parent compound in separate independent pathways, and secondary BTPs, where a direct precursor BTP was identified. The time courses of the BTPs are described as follows:

For the primary BTPs:

$$\frac{dC_{in, M1, 1st}(t)}{dt} = C_{in, p}(t) \cdot k_{M1, 1st} - C_{in, M1, 1st}(t) \cdot k_{eM1, 1st} - C_{in, M1, 1st}(t) \cdot (k_{M1, 2nd} + \dots + k_{Mx, 2nd}) \quad (2)$$

For the secondary BTPs:

$$\frac{dC_{in, M1, 2nd}(t)}{dt} = C_{in, M1, 1st}(t) \cdot k_{M1, 2nd} - C_{in, M1, 2nd}(t) \cdot k_{eM1, 2nd} - C_{in, M1, 2nd}(t) \cdot (k_{M1, further} + \dots + k_{Mx, further}) \quad (3)$$

where $C_{in, Mx, 1st}(t)$ and $C_{in, Mx, 2nd}(t)$ [nmol kg_{ww}⁻¹] are the time courses of the whole body internal concentration of the primary and secondary BTPs, respectively, and $k_{eMx, 1st}$ and $k_{eMx, 2nd}$ are the elimination rate constants of the primary and secondary BTPs, respectively. For BTPs where no successors were identified, the elimination rate $k_{eMx, 1st \text{ or } 2nd}$ includes direct excretion and elimination due to further biotransformation. Otherwise, an additional elimination term was added depending on the number of successors (see equations 2 and 3).

Model Calibration and Parameter Estimation

For model calibration the equations were solved numerically (Runge-Kutta method) and fitted to the measured internal concentrations of the parent compounds and their BTPs. All parameters were fitted simultaneously by minimizing the sum of squares (Nelder-Mead Simplex method) between measured and simulated internal concentration. All parameters were restricted to positive values and no weighting of particular data sets was applied. As exposure concentration the average measured medium concentration during the uptake phase was used. During the depuration phase the medium concentration was set to zero, which was confirmed by the analysis of the exposure medium. For simulating the time courses of the internal concentrations of the parent compounds and their BTPs the best fit parameters were used. 95% confidence intervals were calculated for all parameters using profile likelihoods.

Bioaccumulation Factor and Elimination Half-lives

Bioaccumulation factors (BAFs) [L kg⁻¹] can either be calculated at steady state at a specific time point based on the ratio of the internal concentration of a parent compound and the water concentration

$$BAF = \frac{C_{in,p}(t)}{C_{water}(t)} \quad (4)$$

or based on the kinetic rate constants

$$\text{kinetic BAF (BAF}_k) = \frac{k_u}{k_e + k_{M1,1st} + \dots + k_{Mx,1st}} \quad (5)$$

Ashauer et al. (2012)³⁰ showed that the dietary uptake of 15 organic chemicals (log K_{ow} from 0.33 to 5.18) in *G. pulex* contributed to less than 1% of the total uptake. Thus, the measured BAFs are close to the bioconcentration factors (BCFs).

Elimination half-lives ($t_{1/2}$) [h] were calculated based on the total elimination for parent compounds and BTPs. For parent compounds these calculations are valid under the assumption of constant water concentrations (C_{water}). For BTPs, even though BTP formation is considered during both the uptake and depuration phase (see equations 2 and 3), for the calculation of half-lives we have to assume that no further BTP formation occurs during the depuration phase (reasonable assumption in the case of a fast elimination of the parent compounds).

For parent compounds:

$$t_{1/2,p} = \frac{\ln 2}{k_e + k_{M1,1st} + \dots + k_{Mx,1st}} \quad (5)$$

For BTPs where one or more direct successor is known:

$$t_{1/2, M1, 1st \text{ or } 2nd} = \frac{\ln 2}{k_{eM1, 1st \text{ or } 2nd} + k_{M1, 2nd \text{ or further}} + \dots + k_{Mx, 2nd \text{ or further}}} \quad (6)$$

For BTPs where no direct successor is known:

$$t_{1/2, M1, 1st \text{ or } 2nd} = \frac{\ln 2}{k_{eM1, 1st \text{ or } 2nd}} \quad (7)$$

3. Result and Discussion

Identified Biotransformation Products and Biotransformation Reactions

Exposure medium concentrations were measured at the beginning and at the end of the experiments. Medium concentrations were in general less than 20% lower than nominal concentrations and were comparable to “chemical positive” control samples. Medium concentrations declined during the 24 h exposure phase on average by 3% in the BTP screening experiment and by 14% in the kinetic experiment (see SI E). Mortality was only observed during the exposure to PRP in the BTP screening experiment. Exposure times of 24 h were regarded as sufficient to gain an understanding about predominant biotransformation processes, as toxicokinetic studies in gammarids³⁰⁻³¹ have shown that steady state was reached after 24 h exposure for chemicals with comparable log D_{ow} values.

In total 37 BTPs were tentatively identified in *G. pulex* for six out of the seven selected azole fungicides. The identification confidence for each BTP as described in the section *Structure Elucidation* is stated in Table 1. Even though no reference standards could be obtained for the detected BTPs, we are often able to report only one structure based on diagnostic fragments for one structure (D) or based on diagnostic fragments for positional isomers in combination with pathway information (metabolic logic) (d, p). According to the identification confidence levels proposed by Schymanski et al. (2014)²⁹ 17 BTPs match level 2b (*probable structure: diagnostic evidence for one structure*) and 20 BTPs match level 3 (*tentative candidates: e.g. positional isomers*). Annotated MS/MS spectra for each BTP are reported in the SI I and are available electronically in the MassBank database.³² Mass errors between theoretical and measured mass were generally < 3 ppm.

The naming of BTPs is as follows: e.g. CP_M308a at which the first letters (CP) represent the abbreviation of the parent compound, M stands for metabolite, the following number represents m/z, and the final letter differentiates between isobaric BTPs.

Fluconazole was the only parent compound where no BTPs were detected. This is in accordance with its low bioaccumulation caused by its much higher polarity (log D_{ow} = 0.7,

Table 1) compared to the other selected azoles and with previous studies in humans and wastewater treatment.³³⁻³⁵ Brammer et al. (1991)³⁵ found 80% of fluconazole unchanged in human urine and identified only two minor BTPs, a glucuronide conjugate and an N-oxide.

For the six remaining azoles between one (KET) and 18 (PRZ) BTPs were identified per parent compound. Out of the 37 detected BTPs, 29 were predicted with *in silico* prediction and by literature research (see SI B). The remaining eight BTPs were found with the non-target screening approach.

Biotransformation of azoles took place via different reactions. The most common biotransformation reactions observed were oxidations and conjugations as well as the combination of the two. An overview of all biotransformation reactions identified for each azole, with differentiation between reactions occurring at the active moiety or at the remaining molecule, is depicted in Figure 1. Hydroxylation was the most prevalent reaction observed for all azoles and occurred at the aliphatic part of the molecule, the chlorophenyl moiety, and at the azole ring. In some cases hydroxylation products were further oxidized to ketones.

Different conjugation reactions were identified including those with sulfate (for EP and PRZ), with glutathione resulting in cysteine products (for EP, PRZ), with glucose-sulfate (for PRZ), with phosphate (for TEB), and with acetate (for PRZ). Sulfate and glucose-sulfate conjugation products were further confirmed by enzymatic deconjugation experiments. Several studies report sulfate, glucose and glucose-sulfate conjugates being major BTPs for different organic contaminants in aquatic invertebrates.^{28, 30, 36-37} The glutathione conjugates and resulting cysteine products confirmed the recent results of Jeon et al. (2013)²⁶, where glutathione conjugates and cysteine products have been detected for the first time in freshwater crustaceans. Nucleophilic addition of glutathione to electrophiles (e.g. epoxides) is in agreement with common observations of glutathione conjugation during biotransformation.³⁸ To date phosphate conjugation has been only described for insects and has been rarely observed in vertebrate species.³⁹⁻⁴⁰ Acetylations are known for the mammalian metabolism of xenobiotics⁴¹ and have been observed in algae, macrophytes and bivalves⁴² as well as during

microbial biotransformation⁴³. However, phosphate conjugation and acetylation have not been detected in aquatic crustaceans previously.

For the two compounds epoxiconazole and prochloraz showing the highest BAFs (see Table 1) most diverse biotransformation reactions including conjugations were detected. Conjugation products might have also been formed for the other studied azoles but at concentrations below LOQ. Nevertheless, due to the enrichment capability of the online-SPE method, the sensitivity and broad scan range (100-1000 m/z) of the LC-HRMS/MS method, the low limits of quantification (LOQ) for all azoles and BTPs ($\text{LOQ} < 0.5 \text{ nmol kg}_{\text{ww}}^{-1}$, see SI C), and the combined suspect and non-target screening approaches, we assume not to have missed any relevant BTP.

Figure 1: Overview of identified biotransformation products in *G. pulex* after 24 h exposure to 200 µg L⁻¹ of the respective azole fungicide.

The structural changes between the biotransformation products (BTPs) and its parent compound are highlighted in red. In case the exact position of structural change is not known the effected moiety is marked with red brackets and the change is labelled. Below each reaction the BTPs are listed, where this reaction happened as well (imidazoles: marked in green, triazoles: marked in blue). The active moiety (imidazole or triazole ring) is highlighted in yellow. The larger font size indicates the name of the depicted structure.

Comparison of Triazole and Imidazole Biotransformation

Large differences in biotransformation reactions were present between triazoles and imidazoles. Ring cleavages or ring losses were only observed for imidazoles. For prochloraz most biotransformation reactions (13 out of 18) (see Figure 2) took place at the azole functional moiety. For ketoconazole only one imidazole ring oxidation product was observed (KET_M565). These results also fit within expectations since the imidazole ring cleavage is known to be initiated by formation of an epoxide⁴⁴⁻⁴⁵, which is not possible for triazoles due to a lack of two adjacent carbon atoms in the triazole ring (see Figure 1). In the case of triazoles, ring hydroxylation (for CP, EP and TEB) represented the only change occurring at the active moiety.

Conservation of the Ring Functional Moiety

As mentioned above, for prochloraz most biotransformation reactions resulted in a cleavage or loss of the imidazole ring. In contrast, for the remaining azoles biotransformation reactions rarely occurred at the active azole moiety, whereby their antifungal activity and their CYP inhibitory potential may be conserved. But even changes at the active moiety can be regarded as negligible, since the internal concentrations of the respective parent compounds were much higher than those of the BTPs, with the exception of prochloraz (see section *Influence of Azole Biotransformation on Bioaccumulation*).

Crucial for azole-CYP complex formation are steric and electronic effects of the substituents, the position of substitution at the ring, and hydrophobic interactions between the azole and the CYP.⁴⁶ Therefore, it remains unclear whether ring hydroxylation hinders one of the nitrogen atoms to coordinate with the heme active site.

Since azoles are known to act as CYP inhibitors one could hypothesize that they might be able to inhibit their own biotransformation. However, prochloraz which is known to be a strong CYP inhibitor in other aquatic species^{14, 16-17, 47}, showed the highest biotransformation potential. Prochloraz can be an inhibitor and a substrate at the same time⁴⁶ and the internal concentration of prochloraz determines the proportion of inhibited CYP. Therefore, knowledge

of the inhibition potency would provide information about the strength of inhibition and thus the amount of available active CYP.⁴⁸

Comparison with Literature Data for other Species

To the best of our knowledge no study exist about the biotransformation of azole fungicides in aquatic crustaceans. Furthermore, there is also no research which compares biotransformation of imidazoles and triazoles.

For the imidazole fungicides prochloraz and ketoconazole various studies support our findings about the frequent ring opening. In *G. pulex* oxidation of the imidazole ring was observed resulting in the aldehyde product PRZ_M353 and the thereof formed BTP PRZ_M325. These BTPs were also observed in rat (PRZ_353/325)⁴⁹⁻⁵⁰, rainbow trout (PRZ_M353)⁵¹, and plants (PRZ_M353/325)⁵².

Conjugation reactions showed some differences between aquatic invertebrates and the other organisms. Glucuronic acid (rainbow trout, rat) and sulfate (rat) conjugations were identified in the afore mentioned studies. This is consistent with the findings of James (1987)⁵³ that glucuronidations are more common in fish, whereas glucosidations may represent a more important pathway in invertebrates.

The microsomal metabolism of ketoconazole has been studied extensively due to its importance as antifungal drug and its ability to act as specific inhibitor of CYP3A4.⁵⁴⁻⁵⁷ Biotransformation of ketoconazole in human and rat microsomes led to the oxidation of the imidazole and/or piperazine moiety. KET_M565 identified in this study was also the most abundant BTP in rat and human microsomes.⁵⁷

According to literature, biotransformation of triazoles is not that conserved over different organisms as it seems for imidazoles. In contrast to our findings, microbial biotransformation of tebuconazole led to ring cleavages.⁵⁸⁻⁵⁹ In rats extensive biotransformation of cyproconazole was observed with the major BTP being formed by oxidative removal of the triazole ring.⁵² In human urine hydroxyl and carboxyl products of tebuconazole were identified as major BTPs, both free and as glucuronide conjugates.⁶⁰ For propiconazole the large majority of BTPs identified in mammals contained the unchanged triazole ring which is in line with our results.⁵²

Little data is available for epoxiconazole. Contrary to our results, in *in vitro* experiments with rat and trout hepatic microsomes, and with human CYP3A4 no BTPs were identified for epoxiconazole.⁶¹ Despite the diversity of reactions, BTPs resulting from the cleavage of the triazole ring were rarely observed, which is in agreement with expectations since no epoxide can be formed on the ring to initiate cleavage (see above).

In conclusion, we detected several BTPs in *G. pulex* that have been previously identified in other organisms such as mammals or rats. However, cross-species extrapolation of biotransformation reactions is precarious without a thorough understanding of the underlying mechanisms and enzymes.

Table 1: Overview of parent compounds and respective biotransformation products formed in *G. pulex*.

Biotransformation products are listed according to their relative peak intensity. Mean values for the retention time (RT) and the bioaccumulation factor (BAF) are provided for the replicate samples. Below each biotransformation product the abbreviation (S) stands for “identified by suspect screening”, whereas (N) stands for “identified by non-target” screening. The asterisk marks biotransformation products where the active azole moiety was altered.

Compound	Formula [M] Exact mass of [M+H] ⁺ / [M-H] ⁻ ⁱⁱⁱ⁾	RT [min]	Elemental change ^{iv)}	Description	Log D _{ow} ^{v)}	Identification confidence ^{vi)} /level according to Schymanski et al. (2014) ^{29, vii)}
Cyproconazole (CP)	C ₁₅ H ₁₈ ClN ₃ O 292.1211	15.7		parent compound	2.9	/1/
BAF (t₂₄) ⁱ⁾: 12 L kg_{ww}⁻¹						
CP_M308a (S)	C ₁₅ H ₁₈ ClN ₃ O ₂ 308.1160	13.5	+ O	aliphatic hydroxylation	1.5-1.9	I ⁵² , /3/
CP_M308b * (S)	C ₁₅ H ₁₈ ClN ₃ O ₂ 308.1160	14.1	+ O	triazole ring hydroxylation	3.1	d, /3/
CP_M308c * (S)	C ₁₅ H ₁₈ ClN ₃ O ₂ 308.1160	14.7	+ O	triazole ring hydroxylation	3.2	d, /3/
Epoxiconazole (EP)	C ₁₇ H ₁₃ ClFN ₃ O 330.0804	16.0		parent compound	3.7	/1/
BAF (t₂₄) ⁱ⁾: 49 L kg_{ww}⁻¹ BAF_k ⁱⁱ⁾: 33 L kg_{ww}⁻¹						
EP_M346 * (S)	C ₁₇ H ₁₃ ClFN ₃ O ₂ 346.0753	15.0	+ O	triazole ring hydroxylation	4.0-4.1	d, /3/
EP_M449 (S)	C ₂₀ H ₁₈ ClFN ₄ O ₃ S 449.0845	13.9	+ C ₃ H ₇ NO ₂ S - H ₂	cysteine product, further oxidation	0.8	/3/
EP_M451 (S)	C ₂₀ H ₂₀ ClFN ₄ O ₃ S 451.1001	12.6	+ C ₃ H ₇ NO ₂ S	cysteine product	0.4	D, /2b/
EP_M467 (S)	C ₂₀ H ₂₀ ClFN ₄ O ₄ S 467.0951	11.5	+ O + C ₃ H ₇ NO ₂ S	hydroxylation, cysteine product	0.1	d, /3/
EP_M424 (S)	C ₁₇ H ₁₃ ClFN ₃ O ₅ S 424.0176	12.2	+ O + SO ₃	aliphatic hydroxylation, sulfate conjugation	0.8	e, /3/
EP_M637 (S)	C ₂₇ H ₃₀ ClFN ₆ O ₇ S 637.1642	12.4	+ °C ₁₀ H ₁₇ N ₃ O ₆ S	glutathione conjugation	-5.1	D, /2b/
Fluconazole (FLU)	C ₁₃ H ₁₂ F ₂ N ₆ O 307.1113	7.5		parent compound	0.6	/1/
BAF (t₂₄) ⁱ⁾: 0.4 L kg_{ww}⁻¹						

Compound	Formula [M] Exact mass of [M+H] ⁺ / [M-H] ⁻ ⁱⁱⁱ⁾	RT [min]	Elemental change ^{iv)}	Description	Log D _{ow} ^{v)}	Identification confidence ^{vi)} /level according to Schymanski et al. (2014) ^{29/ vii)}
Propiconazole (PRP)	C ₁₅ H ₁₇ Cl ₂ N ₃ O ₂ 342.0771	17.0		parent compound	4.3	/1/
BAF (t₂₄) ⁱ⁾: 27 L kg_{ww}⁻¹						
PRP_M358a (S)	C ₁₅ H ₁₇ Cl ₂ N ₃ O ₃ 358.0720	13.9	+ O	aliphatic hydroxylation	2.8-4.0	d, l ⁶¹ , m ⁶¹ , /3/
PRP_M358b (S)	C ₁₅ H ₁₇ Cl ₂ N ₃ O ₃ 358.0720	14.5	+ O	aliphatic hydroxylation	2.8-4.0	d, l ⁶¹ , m ⁶¹ , /3/
PRP_M258 (N)	C ₁₀ H ₉ Cl ₂ N ₃ O 258.0195	12.3	- C ₅ H ₈ O	partial loss of dioxolane containing the propyl moiety (ether cleavage)	2.0	D, l ⁵² , /2b/
PRP_M256 (S)	C ₁₀ H ₇ Cl ₂ N ₃ O 256.0039	11.6	-C ₅ H ₁₀ O	partial loss of dioxolane containing the propyl moiety (ether cleavage), further oxidation	2.2	D, l ⁵² , /2b/
Tebuconazole (TEB)	C ₁₆ H ₂₂ ClN ₃ O 308.1524	16.8		parent compound	3.7	/1/
BAF (t₂₄) ⁱ⁾: 31 L kg_{ww}⁻¹						
TEB_M324a (S)	C ₁₆ H ₂₂ ClN ₃ O ₂ 324.1473	15.1	+ O	aliphatic hydroxylation	2.4	d, l ⁶² , /2b/
TEB_M388 (N)	C ₁₆ H ₂₃ ClN ₃ O ₄ P 388.1187	15.8	+ HPO ₃	phosphate conjugation	-0.2	D, /2b/
TEB_M404 (N)	C ₁₆ H ₂₃ ClN ₃ O ₅ P 404.1137	12.6	+ O + HPO ₃	aliphatic hydroxylation, phosphate conjugation	-1.5	D, /2b/
TEB_M324b * (S)	C ₁₆ H ₂₂ ClN ₃ O ₂ 324.1473	15.9	+ O	triazole ring hydroxylation	4.0-4.1	d /3/
TEB_M324c (S)	C ₁₆ H ₂₂ ClN ₃ O ₂ 324.1473	16.3	+ O	hydroxylation	2.4	d, l ⁶² , /3/
Ketoconazole (KET)	C ₂₆ H ₂₈ Cl ₂ N ₄ O ₄ 531.1560	13.6		parent compound	4.2	/1/
BAF (t₂₄) ⁱ⁾: 9.2 L kg_{ww}⁻¹ ^{viii)}						
KET_M565 * (S)	C ₂₆ H ₃₀ Cl ₂ N ₄ O ₆ 565.1615	14.6	+ H ₂ O ₂	imidazole ring oxidation	2.2	d, l ^{55, 57} , m ⁵⁷ , /3/

Compound	Formula [M] Exact mass of [M+H] ⁺ / [M-H] ⁻ ⁱⁱⁱ⁾	RT [min]	Elemental change ^{iv)}	Description	Log D _{ow} ^{v)}	Identification confidence ^{vi)} /level according to Schymanski et al. (2014) ^{29/ vii)}
Prochloraz (PRZ)	C ₁₅ H ₁₆ Cl ₃ N ₃ O ₂ 376.0381	16.2		parent compound	3.6	/1/
BAF (t₂₄) ⁱ⁾: 57 L kg_{ww}⁻¹ BAF_k ⁱⁱ⁾: 50 L kg_{ww}⁻¹						
PRZ_M282 * (N)	C ₁₁ H ₁₄ Cl ₃ ON 282.0214	13.2	- C ₄ H ₂ N ₂ O	loss of imidazole ring and CO	2.4	D, p, /2b/
PRZ_M353 * (S)	C ₁₃ H ₁₅ Cl ₃ O ₃ N ₂ 353.0221	16.9	- C ₂ HN + O	partial loss of hydroxylated imidazole ring, aldehyde formation	3.4	D, p, l ^{49, 51-52} , m ⁵¹ , /2b/
PRZ_M323b * (S)	C ₁₂ H ₁₂ Cl ₃ O ₃ N 323.9956	16.0	- C ₃ H ₄ N ₂ + O	imidazole ring loss, aliphatic hydroxylation and further oxidation to a ketone	2.6-3.2	d, /3/
PRZ_M323a * (S)	C ₁₂ H ₁₂ Cl ₃ O ₃ N 323.9956	15.7	- C ₃ H ₄ N ₂ + O	imidazole ring loss, aliphatic hydroxylation and further oxidation to a ketone	2.3-2.6	d, /3/
PRZ_M392b * (S)	C ₁₅ H ₁₆ Cl ₃ N ₃ O ₃ 392.0330	15.3	+ O	imidazole ring hydroxylation	2.3	d, p, /3/
PRZ_M392a (S)	C ₁₅ H ₁₆ Cl ₃ N ₃ O ₃ 392.0330	13.9	+ O	aliphatic hydroxylation	2.1-2.5	d, /3/
PRZ_M325 * (S)	C ₁₂ H ₁₅ Cl ₃ O ₂ N ₂ 325.0272	17.1	- C ₃ HN	partial loss of imidazole ring	3.4	D, p, l ^{49-50, 52} , /2b/
PRZ_M298 * (N)	C ₁₁ H ₁₄ O ₂ NCl ₃ 298.0163	12.9	- C ₄ H ₂ N ₂ O + O	loss of imidazole ring and CO, hydroxylation	1.4-2.9	/3/
PRZ_M429 * (S)	C ₁₅ H ₁₉ Cl ₃ O ₄ N ₂ S 429.0204	16.6	- C ₃ H ₂ N ₂ + C ₃ H ₅ NO ₂ S	loss of imidazole ring, cysteine product	1.4	D, /2b/
PRZ_M239 * (N)	C ₈ H ₈ ONCl ₃ 239.9744	12.6	- C ₇ H ₈ N ₂ O	remaining chlorophenyl moiety and C ₂ H ₅ NO	1.4	D, /2b/
PRZ_M435 * (S)	C ₁₇ H ₂₁ Cl ₃ N ₄ O ₃ 435.0752	14.9	+ C ₂ H ₂ O	acetylation at CO-imidazole ring moiety; NH ₄ ⁺ adduct	-	d, /3/
PRZ_M382 * (S)	C ₁₄ H ₁₈ Cl ₃ N ₃ O ₃ 382.0487	16.6	- CH ₂ + °O	partial loss of hydroxylated imidazole ring	2.2-3.2	d, p, /2b/
PRZ_M632a (N)	C ₂₁ H ₂₆ Cl ₃ N ₃ O ₁₁ S 632.0281	10.4	+ O + C ₆ H ₁₀ O ₈ S	aromatic hydroxylation, glucose and sulfate conjugation	-1.3	D, e, /2b/
PRZ_M469 (S)	C ₁₅ H ₁₆ Cl ₃ N ₃ O ₆ S 469.9753	11.1	+ O + SO ₃	aromatic hydroxylation, sulfate conjugation	0.5	D, e, /2b/
PRZ_M310 * (S)	C ₁₂ H ₁₄ O ₂ NCl ₃ 310.0163	16.8/17.5	- C ₃ H ₂ N ₂	loss of imidazole ring	3.7	D, /2b/
PRZ_M632b (N)	C ₂₁ H ₂₆ Cl ₃ N ₃ O ₁₁ S 632.0281	11.1	+ O + C ₆ H ₁₀ O ₈ S	aromatic hydroxylation, glucose and sulfate conjugation	-1.3	D, e, /2b/
PRZ_M477 (S)	C ₁₈ H ₂₂ Cl ₂ N ₄ O ₅ S 477.0761	11.2	+ C ₃ H ₆ NO ₂ S + O - Cl	cysteine product, hydroxylation, dehalogenation	-	d, /3/
PRZ_M573 * (S)	C ₁₉ H ₂₃ Cl ₃ O ₈ N ₄ S 573.0375	13.8	- C ₃ H ₂ N ₂ - C ₃ H ₆ + C ₁₀ H ₁₅ N ₃ O ₆ S	loss of imidazole ring, loss of propyl side chain, glutathione conjugation	-1.9	/3/

ⁱ⁾ See Equation 4 in section *Modelling Bioaccumulation and Biotransformation Kinetics* for the calculation of BAFs at steady state.

ⁱⁱ⁾ See Equation 5 in section *Modelling Bioaccumulation and Biotransformation Kinetics* for the calculation of kinetic BAFs (BAF_ks).

ⁱⁱⁱ⁾ [M-H]⁻ only for the sulfate-containing BTPs because they are more sensitive in negative ionization mode. However, they were quantified in positive ionization mode because the respective parent compounds were also detected in positive ionization mode.

^{iv)} The elemental change refers to the change in the molecular formula of the BTP in comparison with the parent compound.

^{v)} Log D_{ow} values were predicted by MarvinSketch version 14.10.20.0 at pH 7.9 and 25 °C. Log D_{ow} values correspond to corrected log K_{ow} values to account for pH-dependent dissociation. At pH 7.9 all selected target compounds are neutral thus log D_{ow} is equal to log K_{ow}. If different positional isomers are possible for one BTP, a range of log D_{ow} values is given.

^{vi)} D: diagnostic fragment for one structure; d: diagnostic fragment for positional isomers; e: enzyme deconjugation; l: structure reported in literature; m: MS/MS data from literature; p: biotransformation pathway information; d, p: diagnostic fragment for positional isomers (d) in combination with pathway information (p) give evidence for one possible structure.

^{vii)} Levels are defined as follows: 5 (*exact mass*), 4 (*unequivocal molecular formula*), 3 (*tentative candidates: e.g. positional isomers*), 2 (*probable structure: library spectrum match (a) or diagnostic evidence for one structure (b)*) and 1 (*confirmed structure*).

^{viii)} No mean value is reported due to inconsistent medium concentrations in the replicate sample. For details refer to SI I.

Influence of Azole Biotransformation on Bioaccumulation

Toxicokinetic Modelling of Epoxiconazole and Prochloraz: Assumptions, Difficulties, Parameter Estimation, and Parameter Correlation

Epoxiconazole and prochloraz were selected to investigate toxicokinetic processes since they exhibited highest bioaccumulation and most diverse biotransformation reactions in the screening experiment. To avoid potential toxic effects caused by bioaccumulation lower exposure concentrations ($100 \mu\text{g L}^{-1}$) were chosen compared to those used in the screening experiment ($200 \mu\text{g L}^{-1}$). Therefore, three BTPs each for epoxiconazole and prochloraz were not detected during the kinetic experiment, as they already showed low peak intensities in the screening experiment ($1 \cdot 10^6 - 2 \cdot 10^6$).

All three BTPs detected for epoxiconazole were modelled as primary BTPs because no direct precursor was observed. Obviously, some BTPs have formed in several consecutive steps, yet we did not observe the intermediate products.

The estimation of kinetic rate constant was challenging for prochloraz due to the high number of identified BTPs (15). The number of BTPs defines the number of parameters ($k_{M1, 1st / 2nd} - k_{Mx, 1st / 2nd}$ and $k_{eM1, 1st / 2nd} - k_{eMx, 1st / 2nd}$) built-into the model equations necessary to describe the time series of the internal concentration of each BTP. If too many parameters have to be fitted based on the number of underlying data the model gets less reliable. In order to decrease the number of parameters it was considered shifting to reaction types rather than single BTPs. But this adjustment did not help to decrease the number of parameters because BTPs were predominantly formed by more than one reaction (e.g. PRZ_M353: partial loss of the hydroxylated imidazole ring and further oxidation to an aldehyde).

As a first step we tried to elucidate the biotransformation pathway, to identify primary and secondary BTPs and thereby define the model equations. BTPs were formed without delay, i.e. no chronology of formation was observed, which would have assisted in pathway elucidation. We propose the biotransformation pathway of prochloraz depicted in Figure 2 based on common drug biotransformation reactions, metabolic logic and known reactions occurring at the imidazole ring.⁴⁴⁻⁴⁵ The cascade of reactions taking place at the imidazole ring

is described to start via an epoxide formation at the double bond between C4-C5. The proposed pathway was reviewed by model calibration and by profiling likelihoods to calculate robust confidence intervals for each parameter. In general the proposed pathway is supported by the model results and the modelled outcome is depicted in Figure 3 (see SI H, Figure S1). However, the elimination rate constants of two secondary BTPs ($k_{eM, PRZ_M325, 2nd}$, $k_{eM, PRZ_M310, 2nd}$) resulted in confidence intervals that hit the limits of the parameter values. Moreover, the elimination rate constants of four BTPs ($k_{eM, PRZ_M298, 2nd}$, $k_{eM, PRZ_M382, 2nd}$, $k_{eM, PRZ_M392a, 1st}$, $k_{eM, PRZ_M392b, 1st}$) exhibited large confidence intervals, where the limits of the parameter values ranged up to four orders of magnitude (see SI H, Table S12). Reasons for unidentified parameters or large confidence intervals might be the not fully elucidated model structure with its high number of parameters, or possible correlation of some parameters to one or more others. A Bayesian correlation analysis was conducted that confirmed the latter assumption (see SI H, Table S13). Experimentally determined rate constants of BTPs would have helped to decrease the model uncertainty but this was not possible since the BTPs were not commercially available as reference standards.

elimination ($k_e + k_{M1, 1st} + \dots + k_{Mx, 1st}$) resulted in bioaccumulation for prochloraz and epoxiconazole. BAF_k s derived from the kinetic rate constants are in good agreement with $BAFs$ based on steady state calculated from the internal concentration of the parent compound and the concentration of the exposure medium after 24 h exposure (see Table 1). If the assumption of steady state was inaccurate, rather lower $BAFs$ based on internal concentrations would be expected compared to BAF_k s. Our kinetic and steady state $BAFs$ (approx. $9 - 50 \text{ L kg}_{ww}^{-1}$) are in the same range to $BAFs$ obtained in gammarids in previous studies (approx. $1 - 400 \text{ L kg}_{ww}^{-1}$) for micropollutants with similar $\log K_{ow}$ values.^{6, 30-31, 63} As defined in REACH, Annex XIII compounds are considered as bioaccumulative with $BAFs > 2000 \text{ L kg}^{-1}$.⁶⁴ Thus, azoles showed low bioaccumulation compared to the REACH criteria. Azoles potentially adsorbed to the exoskeleton of gammarids can influence the determination of $BAFs$ by overestimating internal concentrations and uptake rates. Miller et al. (2016)⁶⁵ analysed the exoskeletons of gammarids that moulted during the exposure period. They observed that the percentage of the adsorbed fraction on the total chemical mass in the animal was between 3 and 24% for five out of eight pharmaceuticals studied. As these pharmaceuticals are ionic, they will more easily adsorb to the animal cuticle composed of calcium carbonate and chitin than the neutral azole fungicides. Consequently, we believe that the amount of adsorbed azoles does not significantly influence our determined $BAFs$. At the end of the 5 d depuration phase both parent compounds were almost completely eliminated to 1-2% of the highest internal concentration measured.

For epoxiconazole biotransformation contributed little to the elimination of the parent compound and thus to the reduction of bioaccumulation. This is expressed by around three orders of magnitude smaller biotransformation rate constants $k_{Mx, 1st}$ in comparison to the direct elimination k_e of epoxiconazole (for exact values refer to SI_H).

For prochloraz a different pattern was observed. Primary biotransformation rate constants $k_{Mx, 1st}$ of BTPs that are not transformed further were also around three orders of magnitude smaller than the direct elimination k_e of prochloraz. However, especially the secondary BTPs PRZ_282, PRZ_M325, and PRZ_M353, each characterized by ring cleavage, were formed

with biotransformation rate constants $k_{Mx, 2nd}$ that are one order of magnitude higher than the direct elimination of prochloraz. The major BTP PRZ_M282 reached mean internal concentrations of approx. 40% of those of the parent compound after 24 h exposure.

For the remaining studied azoles, where no kinetic study was performed, internal concentrations of the parent compound and their corresponding BTPs were compared after 24 h. Internal BTP concentrations of cyproconazole, propiconazole, tebuconazole and ketoconazole were up to three orders of magnitude lower than the concentrations of the appropriate parent compounds ($2 \cdot 10^3$ - $2 \cdot 10^4$ nmol kg_{ww}⁻¹). These low internal concentrations indicate that biotransformation only plays a minor role regarding bioaccumulation and the elimination of the parent compounds. Consequently, prochloraz is the only studied azole fungicide where biotransformation (mainly hydroxylation and ring loss) adds to the reduction of bioaccumulation.

Only one study on toxicokinetics including biotransformation of azole fungicides in aquatic organisms was found. Konwick et al. (2006)⁶⁶ have shown that biotransformation highly contributes to the elimination of selected azoles in rainbow trout.

Biotransformation of azoles resulted in more polar compounds (see log D_{ow} values in Table 1) that were mostly retained longer in the organism in comparison with the parent compound (see $t_{1/2}$ depicted in Figure 3; for details on polarity of BTPs refer to SI J). These findings are in agreement with the results of other studies, that did not observe a simple relationship between elimination kinetics and hydrophobicity either.^{30-31, 63, 66} The slower elimination of hydrophilic molecules can be explained by the impaired transport across hydrophobic cell membranes, although their intracellular mobility is increased.⁶⁷

Biotransformation kinetics of organic compounds in gammarids differ widely among compounds. Ashauer et al. (2012)³⁰ have observed that biotransformation dominated toxicokinetics (log K_{ow} of the 15 studied compounds from 0.33-5.18). Richter and Nagel (2007)⁶⁸ have also found higher BTP concentrations compared to the two investigated parent compounds (log K_{ow} 2.9 and 5.3, respectively), whereas our results are comparable with the findings of Jeon et al. (2013)³¹ for irgarol (log D_{ow} : 2.9).

Figure 3: Measured (dots) and modelled (lines) time-series of internal concentrations of epoxiconazole (a) and prochloraz (b) and their biotransformation products in *G. pulex* in the uptake and depuration phase as well as modelled elimination half-lives ($t_{1/2}$).

The dashed vertical line indicates the change from uptake (1 d) to depuration (5 d). In the panels on the right the y-axis has been expanded to show the kinetics of the less concentrated BTPs.

Environmental Relevance

Our results confirm that the extent of biotransformation of organic xenobiotics is highly substance- and species-specific.^{30-31, 42, 69-70} Predicting BTPs based on common drug biotransformation reactions such as oxidations by CYP enzymes is feasible and was successfully applied in this study, assuming CYPs to be present among different organismal levels and species.^{42, 61, 71-72} However, toxicokinetic processes that determine the internal concentration quantitatively and thus the effect of a chemical are extremely difficult to predict. Uptake rate constants for small aquatic invertebrates seem to be mainly diffusion driven and dependent on the physico-chemical properties as well as on the individual test species, whereas no simple relationship exists between elimination rate constants and hydrophobicity.

30-31, 63, 66, 73-74

We have shown that aside from prochloraz, azole BTPs were formed at low concentrations and most biotransformation reactions did not take place at the active moiety, indicating that the antifungal activity as well as the CYP inhibitory capacity of azoles is preserved. This can be of concern for organisms that are exposed to chemical mixtures containing an azole fungicide, as azoles might influence the bioaccumulation potential of other chemicals by inhibiting the CYP catalysed biotransformation reactions. Therefore, a better understanding of inhibition, induction, expression and activity of CYPs towards chemicals in aquatic organisms is necessary to improve the link between exposure concentration, internal concentration at the target site, and effects – also in terms of environmental risk assessment.

Associated content

Supporting Information

Details on experimental conditions, analytical methods, quality control, SIEVE parameter settings, toxicokinetics, Bayesian statistics and structure elucidation evidence for all BTPs including MS/MS spectra. This material is available free of charge via the internet at <http://pubs.acs.org>.

Author Information

Corresponding Author

* Phone: +41 58 765 5493. Fax: +41 58 765 5893. e-mail: juliane.hollender@eawag.ch

Notes

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