Bioaccumulation, Biotransformation and Synergistic Effects of Binary Fungicide Mixtures in *Hyalella azteca* and *Gammarus pulex*: How Different/Similar are the Two Species?

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Abstract

Aquatic organisms are consistently exposed to a mixture of micropollutants that can bioaccumulate, undergo biotransformation, and may exert mixture effects. However, little is known on the underlying mechanisms and species-specificity. Herein we investigated bioaccumulation, biotransformation and synergistic effects of azole (i.e. prochloraz) and strobilurin (i.e. azoxystrobin) fungicides in the two aquatic invertebrate species, *Hyalella azteca* and *Gammarus pulex*. Bioaccumulation of azoxystrobin was similar whereas bioaccumulation of prochloraz was slightly different in the two species but was still significantly below the REACH criteria for bioaccumulative substances. Similar biotransformation patterns were observed in both species, and only a few unique biotransformation reactions were detected in *H. azteca* such as malonyl-glucose and taurine conjugation. Toxicokinetic modeling additionally indicated that biotransformation is a more important elimination pathway in *H. azteca*. In mixtures, no-observed-adverse-effect levels of prochloraz decreased the LC₅₀s of azoxystrobin in both species which correlated well with increased internal azoxystrobin concentrations. This synergistic effect is partly due to the inhibition of cytochrome P450 monooxygenases by prochloraz which subsequently triggered the reduced biotransformation of azoxystrobin (lower by 5 folds in *H. azteca*). The largely similar responses in both species suggest that the easier-to-cultivate *H. azteca* is a promising representative of invertebrates for toxicity testing.

**Key words:** Fungicides, Synergistic effect, Mixture toxicity, Biotransformation,

Introduction

The use of synthetic chemicals is increasing as a result of the combined global population and economic growths in many regions. These chemicals are typically introduced into aquatic ecosystems via household activities, agricultural run-off, industrial wastewater emissions, and effluent discharges from wastewater treatment plants. Hence, numerous chemicals have been concurrently detected in the aquatic environment with concentrations ranging from ng/L to µg/L. Some of these chemicals (i.e. pesticides, pharmaceuticals) are designed to be biologically active and may exert acute or chronic toxic effects to exposed aquatic organisms, especially towards highly sensitive species such as macroinvertebrates. For example, strobilurin (e.g. azoxystrobin) and azole fungicides (e.g. prochloraz), two classes of widely used agrochemicals that are often applied together, have been detected in surface waters at high frequencies with concentration ranging from low ng/L to several tens of µg/L. Azoxystrobin and prochloraz inhibit the respiratory chain and cytochrome P450 enzymes (CYPs) in fungi, respectively. They are both toxic toward aquatic invertebrates. The acute toxicity (LC₅₀, 96h) of azoxystrobin and prochloraz on *Gammarus pulex* as single substances has been determined to be 270 and 2180 µg/L, respectively. In addition, prochloraz has high chronic toxic potency toward *Daphnia magna*, with an EC₅₀ for fecundity reduction (measure of reproductive success) of 286 µg/L.
The toxicity of substance mixtures is often well estimated using concentration addition or independent action models. However, the interaction of co-occurring organic micropollutants can produce synergistic effects that alters contaminant fate and toxicity in non-target organisms. Indeed, recent studies have shown that azole fungicides significantly enhance the toxicity of other pesticides (e.g. strobilurins and pyrethroids) toward terrestrial and aquatic invertebrates. Although the precise mechanisms are not clear in all species, we have recently shown that the inhibition of CYPs, the main detoxification enzymes present across all kingdoms of life, contributes to the synergistic effects observed in gammarids. Azole fungicides are known to inhibit CYPs by strongly coordinating to the active sites, the heme iron, thereby interrupting the CYP catalytic cycle. As a result, the internal concentration of the parent compound azoxystrobin increases following the CYPs inhibition and thus increases toxicity. In addition, we have previously demonstrated that specific prochloraz concentrations increase the uptake of azoxystrobin by inducing hyperactivity and thereby enhancing its toxicity to G.pulex. These synergistic effects on toxicokinetic processes such as uptake, biotransformation, and elimination of compounds can overall influence the sensitivity of different aquatic species.

The freshwater amphipods G. pulex and H. azteca play an important ecological role in the production, decomposition, and translocation of organic matter in aquatic ecosystems. G. pulex is found across Europe and Northern Asia, while H. azteca is widespread in Central and North America. These species are highly sensitive towards
a wide range of chemicals and have been extensively used for biomonitoring and ecotoxicological testing.\textsuperscript{22,23} As a result of its widespread occurrence, ease of culture, environmental relevance and sensitivity towards chemicals, \textit{H. azteca} has been used as test species for sediment and water quality assessment predominantly in North America,\textsuperscript{24–27} whereas in Europe amphipods from the genus \textit{Gammarus} are often used for biomonitoring or toxicity tests.\textsuperscript{28–30} However, since culturing of \textit{Gammarus spp.} is challenging, most laboratory studies that employ \textit{Gammarus spp.} for toxicity testing typically, collected them from uncontaminated stream sites with an exception of a few studies that used lab-cultures.\textsuperscript{31,32} In addition to the easier cultivation of a homogenous test population, the genomes of several \textit{H. azteca} strains have been sequenced and their genomes and transcripts have been annotated to identify the responsive genes associated with micropollutant exposure.\textsuperscript{33} However, there is still more information needed if \textit{H. azteca} and related aquatic invertebrates exhibit similar sensitivities towards chemicals.

The objectives of this study were to compare bioaccumulation, biotransformation patterns and the importance of biotransformation in reducing bioaccumulation, as well as mixture effects of azoxystrobin and prochloraz in \textit{H. azteca} with our previous results obtained in \textit{G. pulex}.\textsuperscript{19} Our hypothesis was that the inhibition of CYP-mediated biotransformation reactions is similar in both species and results in synergistic toxicity. First, we compared the biotransformation patterns in the two species by determining the routes of biotransformation and the toxicokinetic rate
constants. Second, we elucidated the potential synergistic effects caused by prochloraz and thereof resulting altered toxicity of azoxystrobin in the two species.

**Materials and Methods**

All experiments concerning *H. azteca* were performed in this study. The lipid content and the internal concentrations of azoxystrobin at LC$_{50}$s of *G. pulex* were measured in this study, while the remaining data on *G. pulex* were obtained from our previous studies. In general, experiments for *G. pulex* were performed in a similar way compared to *H. azteca*. Main differences are the optimal culturing conditions for *G. pulex*, i.e., 11 ± 2 °C and a 12 h/12 h light/dark cycle and the medium composition, i.e., aerated artificial pond water (APW). Details on experiments concerning *G. pulex* are given elsewhere.

**Chemicals, Solutions and Test Organisms**

Detailed information on chemicals and solutions used in this study are provided in the Supporting Information (SI. A). *H. azteca* were cultured in aerated Borgmann water (BW) in the lab, whereas *G. pulex* were collected from uncontaminated creeks in Switzerland and acclimatized in an aquarium with APW. More details are given in SI. B.

**Experimental Design for Screening Biotransformation Products (BTPs) and Determining Toxicokinetic (TK) Rate Constants**

*H. azteca* (number of organisms n=30) were introduced into 600 mL-glass beakers filled with 500 mL BW. A piece of cotton gauze (6 × 8 cm) was added into
each beaker for animals to perch and hide. Experiments were performed in a climate incubator (Binder KB 115) while maintaining the optimal conditions for *H. azteca* (23 ± 1 °C and a 16 h/8 h light/dark cycle). For BTP screening experiments, azoxystrobin and prochloraz were separately spiked into the different beakers to yield a nominal initial concentration of 100 (0.25) and 100 (0.27) µg/L (µM), respectively. Animals were collected after 24 h exposure. In parallel, different control experiments were performed, including organism controls (chemical negative, organism and cotton gauze positive), chemical controls (organism and cotton gauze negative, chemical positive) and cotton gauze controls (organism negative, cotton gauze and chemical positive).

For the determination of toxicokinetic rate constants, animals were exposed to 80 µg/L (0.20 µM) azoxystrobin or 100 µg/L (0.27 µM) prochloraz for 24 h, and were sampled at 7 different intervals during the uptake phase. For the depuration phase, the animals were pre-exposed to the test chemicals for 24 h and then shortly rinsed with nanopure water, followed by transferring them into clean BW medium for depuration. The animals were sampled at 12 different intervals during the 120 h depuration phase (SI. C).

**Sample Preparation**

The collected animals were shortly rinsed with nanopure water, blotted dry using tissue paper, transferred into 2-mL centrifuge tubes, and weighed. The sampled organisms were then spiked with 100 µL of methanol containing azoxystrobin-d₄ (0.2
µM) and prochloraz-d₇ (0.3 µM), 500 µL of pure methanol and 300 mg of 1-mm zirconia/silica beads (BioSpec Products, Inc.). The samples were homogenized with a FastPrep bead beater (MP Biomedicals) in two cycles of 15 s at 6 m s⁻¹ (cooling on ice in between). The homogenate was centrifuged (10 000 rpm × 6 min, 20 °C) and filtered through 0.45 µm regenerated cellulose filters (BGB Analytic AG). The filters were washed with 400 µL methanol. Afterwards, the filtrate and the wash solution were combined. The exposure media (500 µL) were sampled in 2 mL LC-vials at 0, 24, and 120 h, spiked with 100 µL of methanol containing azoxystrobin-d₄ (0.2 µM) and prochloraz-d₇ (0.3 µM), and 500 µL pure methanol, and mixed evenly. All samples were stored at -20 °C until chemical analysis.

**Chemical Analysis**

All samples were cleaned up and enriched with an automated online solid phase extraction (SPE) system and further analyzed by reversed phase liquid chromatography coupled to a high resolution tandem mass spectrometer (LC-HRMS/MS) (Q Exactive, Thermo Fisher Scientific Inc.) through an electrospray ionization interface. Full scan acquisition with a resolution of 70000 (at m/z 200) was conducted in polarity switching mode followed by data-dependent MS/MS scans (five scans at positive mode, and two at negative mode) with a resolution of 17500 (at m/z 200) and an isolation window of 1 m/z. Further details are described elsewhere¹⁹ and information on quality control and quantification are given in SI.D. The mass lists used for triggering data-dependent MS/MS scans of BTPs were obtained from
Biotransformation Products Identification

To identify BTP candidates, a suspect and non-target screening was performed by analyzing the acquired HRMS/MS raw data using Compound Discoverer software 2.1 (CD2.1) (Thermo Scientific, criteria and parameter settings in SI. E). BTP candidates were identified based on their unique presence in the treatment and absence in all controls, peak intensity > 10^5, and ≥ 3 scans in the extracted ion chromatograms. Structure elucidation was based on (1) the exact mass and the isotopic pattern to assign molecular formulas, (2) MS/MS spectra information to identify diagnostic fragments or losses either specific for one structure or for several positional isomers, (3) fragmentation patterns reported in literature, databases or predicted with Mass Frontier (version 7.0, HighChem), and (4) reference standards. Finally, the confidence levels of the BTP identification were proposed according to Schymanski et al.\textsuperscript{37}

Toxicokinetic Modeling

To determine toxicokinetic rate constants of uptake, elimination and biotransformation, toxicokinetic rate constants for both parent compounds and their BTPs were estimated using a first-order compartment kinetic model with Matlab R2015b (http://www.debtox.info/byom.html). The model is based on the biotransformation pathways of azoxystrobin and prochloraz in \textit{H. azteca} and in \textit{G. pulex}, respectively. In this model, we distinguish between the time courses of the parent compounds, the time courses of the sum of all detected primary BTPs directly
formed from the parent compound, and the time courses of the sum of all detected secondary BTPs, where a direct precursor BTP was detected. This model is called “summed model”, because no rate constants for single BTPs are modeled.

The first-order ordinary differential equations employed in the model are described as follows. Details on the raw data and the performance evaluation are available in SI. F.

Parent compound:

\[
\frac{dC_{\text{in,p}}(t)}{dt} = C_{\text{water}}(t) \cdot k_u - C_{\text{in,p}}(t) \cdot k_e - C_{\text{in,p}}(t) \cdot k_{m,1st,\text{total}}
\]  

(1)

Primary BTPs:

\[
\frac{dC_{\text{in,m,1st,\text{total}}}(t)}{dt} = C_{\text{in,p}}(t) \cdot k_{m,1st,\text{total}} - C_{\text{in,m,1st,\text{total}}}(t) \cdot k_{e,m,1st,\text{total}} - C_{\text{in,m,1st,\text{total}}}(t) \cdot k_{m,2nd,\text{total}}
\]  

(2)

Secondary BTPs:

\[
\frac{dC_{\text{in,m,2nd,\text{total}}}(t)}{dt} = C_{\text{in,m,1st,\text{total}}}(t) \cdot k_{m,2nd,\text{total}} - C_{\text{in,m,2nd,\text{total}}}(t) \cdot k_{e,m,2nd,\text{total}}
\]  

(3)

where \(C_{\text{in,p}}(t)\), \(C_{\text{in,m,1st,\text{total}}}(t)\) and \(C_{\text{in,m,2nd,\text{total}}}(t)\) [\(\mu\text{mol kg}_{\text{ww}}^{-1}\)] are the whole body internal concentrations of the parent compound, the sum of all primary BTPs and the sum of all secondary BTPs, respectively in \(H. \text{azteca}\) or \(G. \text{pulex}\). \(C_{\text{water}}\) describes the time course of the parent compound in the exposure medium.

Measured exposure medium concentrations during the uptake and depuration phase were used as input for \(C_{\text{water}}\). Uptake of the parent compound via food, dermal and respiratory surfaces is described by the uptake rate constant \(k_u [L \text{kg}_{\text{ww}}^{-1} \text{d}^{-1}]\), whereas
\( k_e \text{ [d}^{-1}] \) is the direct elimination of the parent compound via passive (respiratory and dermal surfaces) and active (excretion of faeces) processes. \( k_{m, \text{1st, total}} \) and \( k_{m, \text{2nd, total}} \) and \( k_{em, \text{1st, total}} \) and \( k_{em, \text{2nd, total}} \) are the biotransformation rate constants [d\(^{-1}\)] and elimination rate constants [d\(^{-1}\)] for the sum of primary BTPs and the sum of secondary BTPs, respectively. \( k_{em, \text{2nd, total}} \) is a lumped rate constant that includes direct excretion of secondary BTPs as well as elimination due to further biotransformation. All parameters were fitted simultaneously.

Bioaccumulation factors (BAFs) [L kg\(_{\text{ww}}^{-1}\)] were either calculated based on the ratio of the internal concentration of the parent compound in the organisms and the concentration of the parent compound in the exposure medium with the requirement of steady-state:

\[
BAF = \frac{C_{\text{in,p}}(t) \text{ (t)}}{C_{\text{water}}(t) \text{ (t)}}
\] (4)

or based on the kinetic rate constants:

\[
BAF_k (\text{kinetic BAF}) = \frac{k_u}{k_e + k_{m, \text{1st,total}}}
\] (5)

Elimination half-lives (t\(_{1/2}\)) [h] were calculated based on the total elimination for azoxystrobin, primary BTPs and secondary BTPs:

\[
t_{1/2, \text{p}} = \frac{\ln 2}{k_e + k_{m, \text{1st, total}}}
\] (6)

\[
t_{1/2, \text{1st, total}} = \frac{\ln 2}{k_{em, \text{1st, total}} + k_{m, \text{2nd, total}}}
\]
\[ t_{1/2} \text{ of secondary BTPs:} \]
\[ t_{1/2,2\text{nd},\text{total}} = \frac{\ln 2}{k_{\text{em,2\text{nd},total}}} \]  

(7)

(8)

**Acute Toxicity with and without Prochloraz**

To evaluate the influence of prochloraz on the acute toxicity of azoxystrobin, the LC$_{50}$s of azoxystrobin with and without prochloraz were determined in *H. azteca* similar to our study with *G. pulex*. Briefly, animals (n=10) were pre-exposed to 0.2 µM (74 µg/L) prochloraz or to clean medium for 18 h, followed by a 24-h co-exposure to increasing concentrations of azoxystrobin (0 - 1.5 µM) in duplicates. Azoxystrobin concentrations were chosen based on a range-defining test (SI. G). Survival was monitored directly after the 24h exposure phase to azoxystrobin to determine the survival rate. A glass rod was used to prod immobile organisms. The organism was defined as “dead” when no movement of the appendices was observed. The LC$_{50}$s were determined by fitting a two-parameter log-logistic model available in the Graphpad Prism (v. 5.02, GraphPad Software Inc., USA). The 5%-response benchmark dose (BMD5) was calculated with PROAST version 38.9 in R by following the manual provided by European Food Safety Authority (EFSA). Subsequently, the internal concentrations of azoxystrobin in the organisms were determined at the estimated LC$_{50}$s for azoxystrobin in the presence and absence of prochloraz in both test species under the same exposure conditions (see sections
“Sample Preparation” and “Chemical Analysis”).

**Half-maximal Inhibitory Concentration (IC<sub>50</sub>) of Prochloraz for CYP-mediated Biotransformation**

To determine the half-maximal inhibitory concentration of prochloraz based on CYP-mediated azoxystrobin biotransformation reactions (IC<sub>50, PRZ,AZs</sub>), the internal concentrations of azoxystrobin and its BTPs in *H. azteca* were monitored in the presence of varying prochloraz concentrations (SI. H and I) similar to *G. pulex*. Briefly, animals (n=30) were pre-exposed to prochloraz at different concentrations (0, 0.0005, 0.001, 0.002, 0.01, 0.02, 0.06, 0.1, 0.2 and 1.0 µM) for 18 h, followed by a 24-h co-exposure to azoxystrobin (0.1 µM). Internal concentrations of azoxystrobin and associated BTPs were measured using the above described online SPE LC-HRMS/MS method. The IC<sub>50, PRZ,AZ</sub> was determined by fitting a four-parameter log-logistic model (SI. J).

**Lipid Content Determination**

The average lipid content of *H. azteca* and *G. pulex* was determined in unexposed organisms by gravimetric measurement of the lipid extract. The lipid extraction was based on the method developed by Kretschmann with a mixture of isopropanol-cyclohexane-water (4 : 5 : 5.5, v/v/v).

**Locomotory Behavior**

The locomotory behavior of *H. azteca* (n=17) was recorded via video-tracking in the presence of different prochloraz concentrations (0, 0.02, 0.1, 0.2, 1 and 2 µM).
according to our previous study in *G. pulex*. Details on video-tracking and data analysis are described in the SI. K.

**Results and Discussion**

**Bioaccumulation of Azoxystrobin and Prochloraz in *H. azteca* compared to *G. pulex***

After 24 h exposure, the medium concentrations of azoxystrobin and prochloraz, important for the calculation of BAFs, decreased by less than 7% (SI. H). The BAF based on internal and exposure concentrations of azoxystrobin in *H. azteca* was 4 ± 0.2 L kg<sub>ww</sub>⁻¹, which is similar to the BAF found in *G. pulex* (5 ± 0.5 L kg<sub>ww</sub>⁻¹), indicating comparable bioaccumulation potential of azoxystrobin in these two species. This is in line with the similar lipid contents in *H. azteca* (1.9 ± 0.7% of ww) and *G. pulex* (2.6 ± 0.3% of ww) measured in this study, as well as lipid contents reported in other studies. By contrast, BAFs of prochloraz in *H. azteca* (110 ± 22 L kg<sub>ww</sub>⁻¹) were doubled compared to *G. pulex* (57 ± 4 L kg<sub>ww</sub>⁻¹). The higher BAFs of prochloraz compared to those of azoxystrobin in both species can be explained by the higher hydrophobicity of prochloraz (log *K*<sub>ow</sub> of 4.1<sup>41</sup> and 2.5<sup>42</sup> respectively). The observed BAFs were similar to the BAFs of micropollutants with similar log *K*<sub>ow</sub> in *H. azteca* and *G. pulex*. Nevertheless, azoxystrobin and prochloraz are considered as lowly bioaccumulative in both species according to the threshold provided by the REACH criteria, i.e. substances with BAFs > 2000 L kg⁻¹ are considered as bioaccumulative.
Biotransformation Products of Azoxystrobin and Prochloraz in *H. azteca* and *G. pulex* and its Relevance in Invertebrates

To compare biotransformation pathways of azoxystrobin and prochloraz in *H. azteca* and *G. pulex*, target, suspect, and non-target screening approaches were used to identify BTPs in the test species 24 h after exposure. Both compounds were extensively transformed in both species. In total, 29 BTPs were identified for azoxystrobin and 30 BTPs for prochloraz in *H. azteca*, whereas 18 BTPs were identified for each compound in *G. pulex* (SI. L and M). Despite the differences in the number of identified BTPs in *H. azteca* and *G. pulex*, the biotransformation reactions of each compound were to a large extent similar (Figure 1). For azoxystrobin, the main biotransformation reactions took place at the active (E)-methyl β-methoxyacrylate group in both species. These reactions were mainly ether cleavage, hydroxylation, demethylation, glucose and/or sulfate conjugation as well as glutathione conjugation-derived cysteine products (Figure 1A). Two unique new reactions, i.e. malonyl-glucose conjugation and taurine conjugation were only observed in *H. azteca*, but not in *G. pulex* (Figure 1A). For prochloraz, main biotransformation reactions took place at the fungicidal active moiety, i.e. the imidazole ring. These reactions include ring cleavage or ring loss, de-methylation, hydrolysis, oxidation, acetylation, sulfate conjugation, glucose-sulfate conjugation, and glutathione conjugation-derived BTPs (Figure 1B). Malonyl-glucose conjugation of prochloraz was only observed in *H. azteca* (Figure 1B). These results suggest that
in general *H. azteca* comprises similar transforming enzymes compared to *G. pulex*.

The major oxidation BTPs of prochloraz resulting from hydroxylation (PRZ_M392), ring cleavage (PRZ_M353/325), and ring loss (PRZ_M282) were also detected in other species such as Sprague-Dawley rat,\textsuperscript{47,48} and rainbow trout (*Oncorhynchus mykiss*).\textsuperscript{49} The de-methylation product of azoxystrobin (AZ_M390a) was also observed in bacteria,\textsuperscript{50} urine and feces of Wistar rat,\textsuperscript{51} and plant (lettuce, pack choi, and broccoli).\textsuperscript{52,53} The ester hydrolysis products (AZ_M390b) was also a major BTP of other strobilurin fungicides such as trifloxystrobin\textsuperscript{54,55} and kresoxim-methyl,\textsuperscript{55} presumably mediated by methyl esterase activities.\textsuperscript{50} Furthermore, the formation of these BTPs are likely detoxification processes for this fungicide class, because the ester moiety of the methyl β-methoxyacrylate group of azoxystrobin is crucial for its binding at the respiration complex III and therefore for the inhibition of mitochondrial respiration.\textsuperscript{56–58} This suggests that these reactions and responsible enzymes are conserved across species.

The conjugation reactions with glutathione (PRZ_M573 (*G. pulex* (G), *H. azteca* (H))/M615 (H)) and the subsequent further transformation is an important pathway for both azoxystrobin and prochloraz in both species. The BTPs resulting from the breakdown of glutathione are varied (AZ_M328 (G), AZ_M525 (G, H), AZ_M493 (G, H), AZ_M541 (H), PRZ_M558 (H), PRZ_M386 (H), and PRZ_M429 (H)). Glutathione conjugation and thereof formed degradation products were found in many species across invertebrates (e.g. *D. magna* and *G. pulex*)\textsuperscript{19,34,45,59} and vertebrates,\textsuperscript{60,61}
suggesting that glutathione conjugation is a common xenobiotic defense mechanism in invertebrates and vertebrates.

Sulfate conjugation and glucose conjugation are also important detoxification pathways. In this study, BTPs resulting from these conjugations and the combination of both were observed for prochloraz and azoxystrobin in *H. azteca* and *G. pulex*.

Especially sulfate conjugations were well observed for several compounds in many species such as *G. pulex*, *D. magna*, and other aquatic invertebrates as well as vertebrates and plants. In contrast, glucuronide conjugation was not observed in both *H. azteca* and *G. pulex*, which is in line with previous observations that glucoside conjugation is more common in invertebrates, whereas glucuronide conjugation is mainly found in vertebrates.

Taurine conjugation of azoxystrobin was identified for the first time in small aquatic invertebrates, such as *H. azteca* (*Figure 1*). The taurine conjugate was likely derived from the ester hydrolysis product AZ_M390b, which has a carboxylic acid group. This is in agreement with many other studies that identified taurine conjugation for compounds carrying a carboxylic acid group in large crustacean, fish, rodents, birds, mammals, and humans. These results suggest that *H. azteca* may also transform other xenobiotic carboxylic acids to taurine conjugates. The specificity of taurine conjugation for biotransformation in *H. azteca* compared to *G. pulex* needs to be confirmed by testing more substrates that contain a carboxylic acid moiety (e.g. acidic pharmaceuticals) or substrates where
biotransformation leads to a BTP with a carboxylic acid moiety. In addition to taurine conjugation, malonyl conjugation of glucose conjugates was another unique reaction observed in *H. azteca* for both compounds. Malonyl-glucose conjugates have been observed in higher plants,77,78 phytoplankton,79 and terrestrial invertebrates.80 Although glucoside is the natural substrate of the O-malonyltransferase,79,81 and malonyl conjugation serves as a general biotransformation pathway in plant, this pathway was only observed in *H. azteca* and not *G. pulex*, suggesting malonyl-glucose conjugation is not a general biotransformation pathway in invertebrates.

**Toxicokinetics of Azoxystrobin and Prochloraz in *H. azteca* and *G. pulex***

To quantitatively compare the kinetics of bioaccumulation, biotransformation, and elimination between the two species, internal concentrations of azoxystrobin, prochloraz, and their BTPs were determined during a 24-h uptake phase and a 120-h depuration phase. In the uptake phase, the internal concentrations of azoxystrobin and prochloraz quickly increased up to a maximum of 0.77 µM and 28.0 µM in *H. azteca* and 0.75 µM and 12.2 µM in *G. pulex*, respectively (**Figure 2A and 2D**). In the depuration phase (24-144 h), the levels of azoxystrobin and prochloraz decreased to negligible levels in *H. azteca* (0.06 and 0.4 µM) and *G. pulex* (0.02 and 0.1 µM) at the end of the 120-h depuration phase (**Figure 2A and 2D**).

To compare toxicokinetics between the two test species, a first-order compartment model with a reduced biotransformation pathway was used to simultaneously fit the time course of the internal concentration of the parent
compound, the time course of the sum of all primary BTPs and the sum of all secondary BTPs. This process allows the quantification of rates regardless of detailed knowledge on the biotransformation pathway. The number of parameters is also reduced in the modeling, thereby decreasing model uncertainties. Consequently, no biotransformation rate constant of single BTPs ($k_{mx, 1st or 2nd}$) can be compared, but the summed model still allows for an estimation of the importance of biotransformation since $k_{mx, 1st, total}$ indicates how much biotransformation adds to the reduction of parent compound bioaccumulation. A comparison of the summed azoxystrobin kinetic model of *G. pulex* with the detailed modelling of kinetic rate constants of single BTPs in *G. pulex* carried out in our previous study revealed similar results. In general, the summed model with simultaneous fitting of all parameters was able to describe the experimental data (Figure 2). However, the modeled time courses of the parent compound for both species did not perfectly reflect the measured internal concentrations. For *H. azteca*, the experimental data hinted a more rapid uptake than was predicted, whereas for *G. pulex* uptake was well captured by the model but simulated elimination during the depuration phase was much faster than proposed by the experimental data (Figure 2A). Thus, these rate constants should be carefully interpreted. We further applied a stepwise fitting approach to initially determine the uptake and elimination rate of the parent compound by fitting the experimental data with the simplest compartment model (see SI. F) comprising with only two parameters ($k_u$ and $k_e, total or parent$) to the internal concentration of the parent compound.
In a second step, $k_u$ was fixed, BTPs were included and the remaining rate constants were fitted simultaneously. This stepwise approach ensures that stronger weight is given to the uptake rate, since in the first step only two parameters are fitted at once. Overall, the simultaneously fitting approach of all rate constants and the stepwise fitting approach showed similar results (SI. F).

BAF$_k$s of azoxystrobin and prochloraz derived from the kinetic rate constants were comparable and in accordance with BAFs determined from experimentally derived internal and external concentrations in both species. A fourfold lower uptake rate of azoxystrobin was estimated for *H. azteca* than for *G. pulex*. In contrast, similar uptake rates of prochloraz were observed in both species (Figure 2A and 2D). For the direct excretion rates of the parent compounds, the difference was higher between the species than for the compounds. They were much lower in *H. azteca* compared to *G. pulex*. The total elimination rates ($k_e + k_{m, \text{1st, total}}$) of both compounds were lower in *H. azteca* (azoxystrobin, 1.9 d$^{-1}$; prochloraz, 4.7 d$^{-1}$) compared to *G. pulex* (azoxystrobin, 8.7 d$^{-1}$; prochloraz, 8.4 d$^{-1}$). The uptake rate for azoxystrobin in *H. azteca* was similar to uptake rates for other neutral organic chemicals with similar log $K_{ow}$ determined in *H. azteca*. However, these results were against our initial expectation, since *H. azteca* exhibits a greater surface area to volume ratio compared to *G. pulex*, and with decreasing body size, the ventilation volume and gill surface area per unit body weight usually increases. Other factors such as biotransformation might play a role for the uptake and elimination.
The total primary or secondary biotransformation rate constants ($k_{m, \text{1st, total}}$ and $k_{m, \text{2nd, total}}$) of azoxystrobin were up to 3 times higher in *H. azteca* than in *G. pulex* ([Figure 2]). $k_{m, \text{1st, total}}$ contributed approximately 93% (34%) to the total elimination of azoxystrobin (prochloraz) in *H. azteca* but only 10% (18%) in *G. pulex*, suggesting that biotransformation adds more to the total elimination of the parent compounds in *H. azteca* compared to *G. pulex*. Moreover, $k_{m, \text{2nd, total}}$ of both compounds were 4-17 times higher than $k_{m, \text{1st, total}}$ in both species ([Figure 2C and 2F]), indicating that the primary BTPs of both azoxystrobin and prochloraz quickly underwent further biotransformation. These results indicate the relevance of secondary biotransformation reactions such as conjugation reactions in aquatic invertebrates.

**Inhibition of Prochloraz on Azoxystrobin Biotransformation and Species Differences**

We have recently observed that prochloraz inhibits the CYP-catalyzed biotransformation of azoxystrobin and decreases the levels of CYP-catalyzed BTPs in *G. pulex*. To test whether this process occurs in *H. azteca*, the internal concentrations of azoxystrobin and its primary CYP-catalyzed de-methylation product AZ_M390a were monitored in the presence of varying prochloraz concentrations. The presence of prochloraz increased the internal concentration of azoxystrobin in a concentration-dependent manner, indicating that also in *H. azteca* prochloraz inhibited the biotransformation of azoxystrobin ([Figure 3A]). Based on the dose-response curve of the parent compound azoxystrobin, the half maximal inhibition concentration of
prochloraz (i.e. IC$_{50, PRZ, AZ}$) was 0.1 µM (95% confidence interval (CI): 0.08 - 0.15 µM) and 0.02 µM (95% CI: 0.01 - 0.04 µM) for *H. azteca* and *G. pulex*, respectively, indicating that *G. pulex* is 2-15 times significantly more sensitive than *H. azteca* towards prochloraz induced CYP-inhibition (Figure 3A). Correspondingly, the internal concentration of AZ_M390a decreased when *H. azteca* or *G. pulex* were co-exposed to prochloraz. The IC$_{50, PRZ, AZ, 390a}$ based on the dose-response curve of AZ_M390a gave similar values to that of IC$_{50, PRZ, AZ}$ in *H. azteca* but about 2.5-fold higher values in *G. pulex* (see Figure 3B and SI. J)), which may be explained by the increased uptake of azoxystrobin. The IC$_{50}$s of prochloraz on CYP-mediated azoxystrobin biotransformation in *H. azteca* is in the same range of IC$_{50}$s of prochloraz and otherazole fungicides determined for other substrates in invertebrates.$^{83-85}$

**Impact of Prochloraz on Lethal Toxicity of Azoxystrobin in *H. azteca* compared to *G. pulex***

To investigate whether the synergistic effects of prochloraz contribute to the acute toxicity of azoxystrobin in *H. azteca* similar as in *G. pulex*, the lethal toxicity of azoxystrobin for *H. azteca* was studied in the presence and absence of a nonlethal prochloraz concentration (Figure 4A). In the absence of prochloraz, the LC$_{50}$ was 0.51 µM (95% CI: 0.48 - 0.55 µM) in *H. azteca*, whereas it substantially decreased to 0.15 µM (95% CI: 0.13 - 0.16 µM) in the presence of 0.2 µM prochloraz (fold change of 3-4). Similar synergistic effects had been observed for *G. pulex*, with LC$_{50}$ of
azoxystrobin of 0.4 µM (95% CI: 0.37 - 0.43 µM) and 0.1 µM (95% CI: 0.08 - 0.09 µM) in the absence and presence of prochloraz, respectively (fold change of 4-5).

These results suggest that prochloraz can greatly enhance the toxicity of azoxystrobin in both species and *G. pulex* appeared to be slightly more sensitive. The BMD\textsuperscript{38} were also lower when the animals were co-exposed to azoxystrobin and prochloraz (SI. J).

To further confirm our hypothesis that prochloraz increases the internal azoxystrobin concentration by inhibiting CYP-catalyzed biotransformation and thus enhances the toxicity, the internal concentrations at the LC\textsubscript{50} were determined in both species.

Indeed, they were not significantly different (p > 0.05) in the presence and absence of prochloraz, i.e. a lower external exposure concentration of azoxystrobin was required in the presence of prochloraz compared to the single exposure to azoxystrobin, to reach the same internal concentrations of azoxystrobin (Figure 4B). Our results are in agreement with a study on *D. magna*,\textsuperscript{86} with the same binary mixture but in this study, we provided insights on the synergistic mechanism for the first time by comparing the internal concentration at the LC\textsubscript{50}.

**Influence of Prochloraz on Locomotory Behavior and Species Differences**

Hyperactivity can lead to increased uptake of chemicals and subsequently higher toxicity. It has been previously observed for several invertebrates (*G. pulex*, *Leuctra nigra*, and *Heptagenia sulphurea*) when being exposed to environmental relevant concentrations (low ng L\textsuperscript{-1}) of cypermethrin,\textsuperscript{87,88} and also recently for *G. pulex* exposed to 0.1 µM prochloraz.\textsuperscript{19} For *H. azteca* no information about the locomotory
behavior has been reported so far. To test if hyperactivity contributes also to the
observed synergistic effects of prochloraz towards *H. azteca* the locomotory behavior
of the organisms was recorded during 18 h exposure of prochloraz (SI. K). At the
tested concentrations, ranging from 0.02 to 2.0 µM of prochloraz, the total distance *H.
azteca* moved during 18 h was not substantially different from the control, suggesting
that prochloraz did not induce hyperactivity in *H. azteca* and hence, does not
contribute to the synergistic effect. This might explain the stronger decrease of the
LC$_{50}$ in the presence of prochloraz for *G. pulex* (4 fold) in comparison to *H. azteca*
(2.5 fold) (Figure 4A).

**Environmental Implications**

Our findings highlight that organic micropollutants can be extensively
biotransformed in aquatic organisms, and that biotransformation influences the
bioaccumulation and the subsequent toxicity of these compounds toward freshwater
crustaceans.$^{34,43,45,59,82}$ Aside from a few unique BTPs observed in *H. azteca*, *H.
azteca* and *G. pulex* exhibit comparable biotransformation capacities on both
azonystrobin and prochloraz. Toxicokinetic modeling indicated that biotransformation
is more important for the reduction of bioaccumulation in *H. azteca* compared to *G.
pulex*. The summed modeling of BTPs could be used as a promising approach to
include biotransformation into toxicokinetic modeling without specifically identifying
the biotransformation pathway in detail. Hence, the importance of biotransformation
regarding the reduction of bioaccumulation can be evaluated directly. The co-
occurrence of azoxystrobin and prochloraz induced synergistic effects in both species, but *H. azteca* was about five times less sensitive than *G. pulex*. The importance of these species’ sensitivity differences regarding ecotoxicological risk assessment depends on the quality of the toxicity data and the related assessment factors. However, both species can be used for toxicity tests in risk assessment frameworks and would deliver results in the same order of magnitude. Nevertheless, *H. azteca* might be preferred as test species in the future because *H. azteca* can provide a homogenous test population throughout the whole year, as indicated previously, several strains of *H. azteca* were sequenced and the genomes and transcripts have been annotated to identify toxicant responsive genes. Indeed, *H. azteca* is already in use for measuring toxicity and bioaccumulation of sediment-associated contaminants in North America.

The synergistic effects of prochloraz and azoxystrobin were observed at concentrations 10-1000 folds higher than concentration ranges found in the environment. However, these findings are still relevant because, considering a realistic exposure situation, aquatic organisms are exposed to a mixture of synergists such as to different azoles (e.g., prochloraz, epoxiconazole, tebuconazole, and propiconazole) that have the same mode of action. They may add up to a total exposure concentration that exceeds the threshold where synergism - in this case CYP inhibition - starts. Indeed, a mixture of epoxiconazole and propiconazole enhanced the toxicity of pyrethroids in *D. magna*. Nevertheless, whether such synergistic

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effects occur for environmental mixtures in agriculture-impacted streams, for example after rain events in the pesticide application period, needs to be confirmed.

Supporting Information

Additional details are available free of charge via the Internet at http://pubs.acs.org/.

Acknowledgments

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References


(11) Beketov, M. A.; Liess, M. Potential of 11 pesticides to initiate


(20) de Montellano, P. R. O.; Correia, M. A. Inhibition of cytochrome P450 enzymes. In *Cytochrome P450*; Springer, 1995; pp 305–364.


(29) Gerhardt, A.; Kienle, C.; Allan, I. J.; Greenwood, R.; Guigues, N.; Fouillac, A. M.; Mills, G. A.; Gonzalez, C. Biomonitoring with *Gammarus pulex* at the Meuse (NL), Aller (GER) and Rhine (F) rivers with the online Multispecies Freshwater Biomonitor. *J. Environ. Monit.* 2007, 9 (9), 979–985.

(30) Bundschuh, M.; Pierstorf, R.; Schreiber, W. H.; Schulz, R. Positive


(34) Rösch, A.; Anliker, S.; Hollender, J. How Biotransformation Influences
663 Toxicokinetics of Azole Fungicides in the Aquatic Invertebrate
665
666 (35) Naylor, C.; Malby, L.; Callow, P. Scope for growth in Gammarus pulex,
668
669 (36) Borgmann, U. Systematic analysis of aqueous ion requirements of
670 Hyalella azteca: A standard artificial medium including the essential
672
673 (37) Schymanski, E. L.; Jeon, J.; Gulde, R.; Fenner, K.; Ruff, M.; Singer, H.
674 P.; Hollender, J. Identifying small molecules via high resolution mass
676 48, 2097–2098.
677
678 (38) EFSA Scientific Committee. Guidance of the scientific committee on use
679 of the benchmark dose approach in risk assessment. EFSA J. 2009, 1150,
680 1–72.
681
682 (39) Kretschmann, A.; Ashauer, R.; Preuss, T. G.; Spaak, P.; Escher, B. I.;
683 Hollender, J. Toxicokinetic model describing bioconcentration and
685 2011, 45 (11), 4995–5002.
686
687 (40) Katagi, T. Bioconcentration, bioaccumulation, and metabolism of
688 pesticides in aquatic organisms. In Reviews of environmental
689 contamination and toxicology; Springer, 2010; pp 1–132.
688
33


(59) Jeon, J.; Kurth, D.; Hollender, J. Biotransformation pathways of biocides and pharmaceuticals in freshwater crustaceans based on structure
elucidation of metabolites using high resolution mass spectrometry.


(64) Ikenaka, Y.; Eun, H.; Ishizaka, M.; Miyabara, Y. Metabolism of pyrene by aquatic crustacean, Daphnia magna. Aquat. Toxicol. 2006, 80 (2), 158–165.


(66) Fu, Q.; Ye, Q.; Zhang, J.; Richards, J.; Borcherdt, D.; Gan, J. Diclofenac...


2012, 243, 250–256.


(69) James, M. O. Disposition and taurine conjugation of 2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid, bis(4-chlorophenyl)acetic acid, and phenylacetic acid in the spiny lobster, *Panulirus argus*. *Drug Metab. Dispos.* 1982, 10 (5), 516–522.


(72) James, M. O.; Bend, J. R. Taurine conjugation of 2,4-dichlorophenoxyacetic acid and phenylacetic acid in two marine species.


Petroutsos, D.; Katapodis, P.; Samiotaki, M.; Panayotou, G.; Kekos, D.

Detoxification of 2,4-dichlorophenol by the marine microalga Tetraselmis marina. *Phytochemistry* **2008**, *69* (3), 707–714.


Beijer, K.; Jönsson, M.; Shaik, S.; Behrens, D.; Brunström, B.; Brandt, I. Azoles additively inhibit cytochrome P450 1 (EROD) and 19 (aromatase)


Figure Captions

Figure 1. Common or different biotransformation reactions of azoxystrobin (A) and prochloraz (B) between *H. azteca* and *G. pulex*. The number of reactions is indicated in parentheses. The pathways that involve unique biotransformation products are displayed on the right side of each panel.

Figure 2. Toxicokinetics of azoxystrobin (A) and prochloraz (D), their respective summed 1st (B, E) and 2nd (C, F) biotransformation products (BTPs). The toxicokinetics rate constants with respective 95% confidence intervals are displayed in brackets. The kinetic rate constants (*k*<sub>u</sub>, *k*<sub>e</sub>, *k*<sub>m</sub>, *k*<sub>em</sub>), half-lives (t<sub>1/2</sub>), bioaccumulation factors (BAFs) for both *H. azteca* and *G. pulex* are displayed on the right side of each panel.

Figure 3. Inhibition effects of prochloraz on the biotransformation of azoxystrobin in *H. azteca* (blue, filled circle and solid line) and *G. pulex* (green, square and dashed line). (A) Relative internal concentrations of azoxystrobin, and (B) relative internal concentrations of oxidative transformation product AZ_M390a in *H. azteca* or *G. pulex* pre-exposed to increasing concentrations of prochloraz. Concentrations of azoxystrobin or its transformation product AZ_M390a were normalized to those in animals that are not pre-exposed to prochloraz.

Figure 4. Lethal toxicity of azoxystrobin (AZ) to *H. azteca* and *G. pulex* pre-exposed to prochloraz (PRZ). (A) dose (concentrations in medium) −response (survival rate) curves of AZ; (B) internal concentration of azoxystrobin (AZ) in *H. azteca* and *G. pulex* pre-exposed to 0 or 0.2 µM of PRZ at the LC<sub>50</sub> in medium.
**Figure 1**

A. **Azoxystrobin**
- *G. pulex (0)*
- *H. azteca (2)*
- Both (9)

Either cleavage, Hydroxylation, Hydrogenation, Demethylation, Ester hydrolysis, Glutathione conjugation leading to cysteine product.

Glucose, Sulfate and Glucose-sulfate conjugation

B. **Prochloraz**
- *G. pulex (0)*
- *H. azteca (1)*
- Both (11)

(partial) Loss of imidazole ring, Hydroxylation, Hydrolysis, Deacetylation, Acetylation, Oxidation to ketone, Glutathione conjugation leading to cysteine product.

Sulfate, Glucose-sulfate and Glutathione conjugation

**Unique BTP reactions in H. azteca**
- Demethylation, Taurine conjugation
- Demethylation, Malonyl-glucose conjugation
**Azoxystrobin (AZ)**

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**AZ_summed 1st BTPs**

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**Prochloraz (PRZ)**

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**PRZ_summed 1st BTPs**

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**PRZ_summed 2nd BTPs**

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**Figure 2**
Figure 3
Figure 4