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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 LC_{50}s 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,

Invertebrates, *Hyalella azteca*, Cytochrome P450 inhibition.

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.[1–3] 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,[4,5] have been detected in surface waters at high frequencies with concentration ranging from low ng/L to several tens of µg/L.[3,6–8] Azoxystrobin and prochloraz inhibit the respiratory chain and cytochrome P450 enzymes (CYPs) in fungi, respectively. They are both toxic toward aquatic invertebrates. [6,9,10] The acute toxicity (LC_{50}s, 96h) of azoxystrobin and prochloraz on *Gammarus pulex* as single substances has been determined to be 270 and 2180 µg/L, respectively.[11] In addition, prochloraz has high chronic toxic potency toward *Daphnia magna*, with an EC_{50} for fecundity reduction (measure of reproductive success) of 286 µg/L.[12]

The toxicity of substance mixtures is often well estimated using concentration addition or independent action models.[13,14] However, the interaction of co-occurring organic micropollutants can produce synergistic effects that alters contaminant fate and toxicity in non-target organisms.[15] Indeed, recent studies have shown that azole fungicides significantly enhance the toxicity of other pesticides (e.g. strobilurins and pyrethroids) toward terrestrial [16,17] and aquatic invertebrates.[18,19] Although the precise mechanisms are not clear in all species, we have recently shown that the inhibition of CYPs,[18] 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.[20] As a result, the internal concentration of the parent compound azoxystrobin increases following the CYPs inhibition and thus increases toxicity.[18] 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*.[19] 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.[21] *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.[22,23] As a result of its widespread occurrence, ease of culture, environmental relevance and sensitivity towards chemicals, *H. azteca* has been used as test species for sediment and water quality assessment predominantly in North America,[24–27] whereas in Europe amphipods from the genus *Gammarus* are often used for biomonitoring or toxicity tests.[28–30] However, since culturing of *Gammarus spp*. is challenging, most laboratory studies that employ *Gammarus spp*. for toxicity testing typically, collected them from uncontaminated stream sites with an exception of a few studies that used lab-cultures.[31,32] In addition to the easier cultivation of a homogenous test population, the genomes of several *H. azteca* strains have been sequenced and their genomes and transcripts have been annotated to identify the responsive genes associated with micropollutant exposure.[33] However, there is still more information needed if *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 *H. azteca* with our previous results obtained in *G. pulex*.[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

106 constants. Second, we elucidated the potential synergistic effects caused by
107 prochloraz and thereof resulting altered toxicity of azoxystrobin in the two species.

108 ## Materials and Methods

109 All experiments concerning *H. azteca* were performed in this study. The lipid
110 content and the internal concentrations of azoxystrobin at LC_{50}s of *G. pulex* were
111 measured in this study, while the remaining data on *G. pulex* were obtained from our
112 previous studies.[19,34] In general, experiments for *G. pulex* were performed in a similar
113 way compared to *H. azteca*. Main differences are the optimal culturing conditions for
114 *G. pulex*, i.e., 11 ± 2 °C and a 12 h/12 h light/dark cycle and the medium composition,
115 i.e., aerated artificial pond water (APW).[35] Details on experiments concerning *G.*
116 *pulex* are given elsewhere.[19]

117 ### Chemicals, Solutions and Test Organisms

118 Detailed information on chemicals and solutions used in this study are provided
119 in the Supporting Information (**SI. A**). *H. azteca* were cultured in aerated Borgmann
120 water (BW) in the lab,[36] whereas *G. pulex* were collected from uncontaminated creeks
121 in Switzerland and acclimatized in an aquarium with APW.[35] More details are given
122 in **SI. B**.

123 ### Experimental Design for Screening Biotransformation Products (BTPs) and
124 ### Determining Toxicokinetic (TK) Rate Constants

125 *H. azteca* (number of organisms n=30) were introduced into 600 mL-glass
126 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-d_4 (0.2

148 μM) and prochloraz-d_7 (0.3 μM), 500 μL of pure methanol and 300 mg of 1-mm
149 zirconia/silica beads (BioSpec Products, Inc.). The samples were homogenized with a
150 FastPrep bead beater (MP Biomedicals) in two cycles of 15 s at 6 m s^{-1} (cooling on ice
151 in between). The homogenate was centrifuged (10 000 rpm × 6 min, 20 °C) and
152 filtered through 0.45 μm regenerated cellulose filters (BGB Analytic AG). The filters
153 were washed with 400 μL methanol. Afterwards, the filtrate and the wash solution
154 were combined. The exposure media (500 μL) were sampled in 2 mL LC-vials at 0,
155 24, and 120 h, spiked with 100 μL of methanol containing azoxystrobin-d_4 (0.2 μM)
156 and prochloraz-d_7 (0.3 μM), and 500 μL pure methanol, and mixed evenly. All
157 samples were stored at -20 °C until chemical analysis.

158 **Chemical Analysis**

159 All samples were cleaned up and enriched with an automated online solid phase
160 extraction (SPE) system and further analyzed by reversed phase liquid
161 chromatography coupled to a high resolution tandem mass spectrometer (LC-
162 HRMS/MS) (Q Exactive, Thermo Fisher Scientific Inc.) through an electrospray
163 ionization interface. Full scan acquisition with a resolution of 70000 (at m/z 200) was
164 conducted in polarity switching mode followed by data-dependent MS/MS scans (five
165 scans at positive mode, and two at negative mode) with a resolution of 17500 (at m/z
166 200) and an isolation window of 1 m/z. Further details are described elsewhere[19] and
167 information on quality control and quantification are given in **SI. D**. The mass lists
168 used for triggering data-dependent MS/MS scans of BTPs were obtained from

169 literature and *in silico* prediction (**SI. E**).

170 **Biotransformation Products Identification**

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

182 **Toxicokinetic Modeling**

183 To determine toxicokinetic rate constants of uptake, elimination and
184 biotransformation, toxicokinetic rate constants for both parent compounds and their
185 BTPs were estimated using a first-order compartment kinetic model with Matlab
186 R2015b (http://www.debtox.info/byom.html). The model is based on the
187 biotransformation pathways of azoxystrobin and prochloraz in *H. azteca* and in *G.*
188 *pulex*, respectively. In this model, we distinguish between the time courses of the
189 parent compounds, the time courses of the sum of all detected primary BTPs directly

190 formed from the parent compound, and the time courses of the sum of all detected
191 secondary BTPs, where a direct precursor BTP was detected. This model is called
192 "summed model", because no rate constants for single BTPs are modeled.
193 The first-order ordinary differential equations employed in the model are
194 described as follows. Details on the raw data and the performance evaluation are
195 available in **SI. F**.
196 Parent compound:

197 $$\frac{dC_{in,p}(t)}{dt} = C_{water}(t) \cdot k_u - C_{in,p}(t) \cdot k_e - C_{in,p}(t) \cdot k_{m,1st,total}$$

198 (1)

199 Primary BTPs:

200 $$\frac{dC_{in,m,1st,total}(t)}{dt} = C_{in,p}(t) \cdot k_{m,1st,total} - C_{in,m,1st,total}(t) \cdot k_{em,1st,total} - C_{in,m,1st,total}(t) \cdot k_{m,2nd,total}$$

201 (2)

202 Secondary BTPs:

203 $$\frac{dC_{in,m,2nd,total}(t)}{dt} = C_{in,m,1st,total}(t) \cdot k_{m,2nd,total} - C_{in,m,2nd,total}(t) \cdot k_{em,2nd,total}$$ (3)

204 where $C_{in,\,p}(t)$, $C_{in,\,m,\,1st\,,total}(t)$ and $C_{in,\,m,\,2nd,\,total}(t)$ [µmol $kg_{wet\ weight\ (ww)}^{-1}$] are the
205 whole body internal concentrations of the parent compound, the sum of all primary
206 BTPs and the sum of all secondary BTPs, respectively in *H. azteca* or *G. pulex*. C_{water}
207 (t) [µM] describes the time course of the parent compound in the exposure medium.
208 Measured exposure medium concentrations during the uptake and depuration phase
209 were used as input for C_{water}. Uptake of the parent compound via food, dermal and
210 respiratory surfaces is described by the uptake rate constant k_u [L kg_{ww}^{-1} d^{-1}], whereas

211 k_e [d^{-1}] is the direct elimination of the parent compound via passive (respiratory and
212 dermal surfaces) and active (excretion of faeces) processes. $k_{m,\ 1st,\ total}$ and $k_{m,\ 2nd,\ total}$
213 and $k_{em,\ 1st,\ total}$ and $k_{em,\ 2nd,\ total}$ are the biotransformation rate constants [d^{-1}] and
214 elimination rate constants [d^{-1}] for the sum of primary BTPs and the sum of secondary
215 BTPs, respectively. $k_{em,\ 2nd,\ total}$ is a lumped rate constant that includes direct excretion
216 of secondary BTPs as well as elimination due to further biotransformation. All
217 parameters were fitted simultaneously.

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

222 $$\mathrm{BAF} = \frac{C_{in,p}(t)}{C_{water}(t)} \quad (4)$$

223 or based on the kinetic rate constants:

224 $$\mathrm{BAF_k\ (kinetic\ BAF)} = \frac{k_u}{k_e + k_{m,1st,total}} \quad (5)$$

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

227 $t_{1/2}$ of parent compound:

228 $$t_{1/2,p} = \frac{\ln 2}{k_e + k_{m,1st,total}}$$

229 (6)

230 $t_{1/2}$ of primary BTPs:

231 $$t_{1/2,1st,total} = \frac{\ln 2}{k_{em,1st,total} + k_{m,2nd,total}}$$

232 (7)

233 $t_{1/2}$ of secondary BTPs:

234 $$t_{1/2,2nd,total} = \frac{\ln 2}{k_{em,2nd,total}}$$

235 (8)

236 **Acute Toxicity with and without Prochloraz**

237 To evaluate the influence of prochloraz on the acute toxicity of azoxystrobin, the
238 LC_{50}s of azoxystrobin with and without prochloraz were determined in *H. azteca*
239 similar to our study with *G. pulex*.[19] Briefly, animals (n=10) were pre-exposed to 0.2
240 µM (74 µg/L) prochloraz or to clean medium for 18 h, followed by a 24-h co-
241 exposure to increasing concentrations of azoxystrobin (0 - 1.5 µM) in duplicates.
242 Azoxystrobin concentrations were chosen based on a range-defining test (**SI. G**).
243 Survival was monitored directly after the 24h exposure phase to azoxystrobin to
244 determine the survial rate. A glass rod was used to prod immobile organisms. The
245 organism was defined as "dead" when no movement of the appendices was observed.
246 The LC_{50}s were determined by fitting a two-parameter log-logistic model available in
247 the Graphpad Prism (v. 5.02, GraphPad Software Inc., USA). The 5%-response
248 benchmark dose (BMD5) was calculated with PROAST version 38.9 in R by
249 following the manual provided by European Food Safety Authority (EFSA).[38]
250 Subsequently, the internal concentrations of azoxystrobin in the organisms were
251 determined at the estimated LC_{50}s for azoxystrobin in the presence and absence of
252 prochloraz in both test species under the same exposure conditions (see sections

253 "Sample Preparation" and "Chemical Analysis").

254 **Half-maximal Inhibitory Concentration (IC_{50}) of Prochloraz for CYP-**

255 **mediated Biotransformation**

256 To determine the half-maximal inhibitory concentration of prochloraz based on

257 CYP-mediated azoxystrobin biotransformation reactions ($IC_{50,\ PRZ,\ AZ}$s), the internal

258 concentrations of azoxystrobin and its BTPs in *H. azteca* were monitored in the

259 presence of varying prochloraz concentrations (**SI. H** and **I**) similar to *G. pulex*.[19]

260 Briefly, animals (n=30) were pre-exposed to prochloraz at different concentrations (0,

261 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

262 24-h co-exposure to azoxystrobin (0.1 μM). Internal concentrations of azoxystrobin

263 and associated BTPs were measured using the above described online SPE LC-

264 HRMS/MS method. The $IC_{50,\ PRZ,\ AZ}$ was determined by fitting a four-parameter log-

265 logistic model (**SI. J**).

266 **Lipid Content Determination**

267 The average lipid content of *H. azteca* and *G. pulex* was determined in unexposed

268 organisms by gravimetric measurement of the lipid extract. The lipid extraction was

269 based on the method developed by Kretschmann[39] with a mixture of isopropanol-

270 cyclohexane-water (4 : 5 : 5.5, *v*/*v*/*v*).

271 **Locomotory Behavior**

272 The locomotory behavior of *H. azteca* (n=17) was recorded via video-tracking in

273 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*.[19] 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_{ww}^{-1}, which is similar to the BAF found in *G. pulex* (5 ± 0.5 L kg_{ww}^{-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.[40] By contrast, BAFs of prochloraz in *H. azteca* (110 ± 22 L kg_{ww}^{-1}) were doubled compared to *G. pulex* (57 ± 4 L kg_{ww}^{-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_{ow} of 4.1[41] and 2.5,[42] respectively). The observed BAFs were similar to the BAFs of micropollutants with similar log K_{ow} in *H. azteca* and *G. pulex*.[43–45] Nevertheless, azoxystrobin and prochloraz are considered as lowly bioaccumulative in both species according to the threshold provided by the REACH criteria,[46] i.e. substances with BAFs > 2000 L kg^{-1} are considered as bioaccumulative.

294 **Biotransformation Products of Azoxystrobin and Prochloraz in *H. azteca* and *G.***
295 ***pulex* and its Relevance in Invertebrates**

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

315 in general *H. azteca* comprises similar transforming enzymes compared to *G. pulex*.

316 The major oxidation BTPs of prochloraz resulting from hydroxylation

317 (PRZ_M392), ring cleavage (PRZ_M353/325), and ring loss (PRZ_M282) were also

318 detected in other species such as Sprague-Dawley rat,[47,48] and rainbow trout

319 (*Oncorhynchus mykiss*).[49] The de-methylation product of azoxystrobin (AZ_M390a)

320 was also observed in bacteria,[50] urine and feces of Wistar rat,[51] and plant (lettuce,

321 pack choi, and broccoli).[52,53] The ester hydrolysis products (AZ_M390b) was also a

322 major BTP of other strobilurin fungicides such as trifloxystrobin[54,55] and kresoxim-

323 methyl,[55] presumably mediated by methyl esterase acitivities.[50] Furthermore, the

324 formation of these BTPs are likely detoxification processes for this fungicide class,

325 because the ester moiety of the methyl β-methoxyacrylate group of azoxystrobin is

326 crucial for its binding at the respiration complex III and therefore for the inhibition of

327 mitochondrial respiration.[56–58] This suggests that these reactions and responsible

328 enzymes are conserved across species.

329 The conjugation reactions with glutathione (PRZ_M573 (*G. pulex* (G), *H. azteca*

330 (H))/M615 (H)) and the subsequent further transformation is an important pathway

331 for both azoxystrobin and prochloraz in both species. The BTPs resulting from the

332 breakdown of glutathione are varied (AZ_M328 (G), AZ_M525 (G, H), AZ_M493

333 (G, H), AZ_M541 (H), PRZ_M558 (H), PRZ_M386 (H), and PRZ_M429 (H)).

334 Glutathione conjugation and thereof formed degradation products were found in many

335 species across invertebrates (e.g. *D. magna* and *G. pulex*)[19,34,45,59] and vertebrates,[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.[62] 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*,[34,43,45,59] *D. magna*,[59,63,64] and other aquatic invertebrates[65] as well as vertebrates[47] and plants.[66,67] 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.[62,68]

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,[69,70] fish,[62,71,72] rodents,[73,74] birds,[73] mammals,[73,75] and humans.[75,76] 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

357 biotransformation leads to a BTP with a carboxylic acid moiety. In addition to taurine
358 conjugation, malonyl conjugation of glucose conjugates was another unique reaction
359 observed in *H. azteca* for both compounds. Malonyl-glucose conjugates have been
360 observed in higher plants,[77,78] phytoplankton,[79] and terrestrial invertebrates.[80]
361 Although glucoside is the natural substrate of the *O*-malonyltransferase,[79,81] and
362 malonyl conjugation serves as a general biotransformation pathway in plant, this
363 pathway was only observed in *H. azteca* and not *G. pulex*, suggesting malonyl-
364 glucose conjugation is not a general biotransformation pathway in invertebrates.

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

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

375 To compare toxicokinetics between the two test species, a first-order
376 compartment model with a reduced biotransformation pathway was used to
377 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 $k_{m,\ 1st,\ total}$ 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.[19] 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 $k_{e,\ \text{total or parent}}$) 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_ks 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 ($k_e + k_{m,\ 1st,\ total}$) 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*.[82] 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 ($k_{m,\ 1st,\ total}$ and $k_{m,\ 2nd,\ total}$) of azoxystrobin were up to 3 times higher in *H. azteca* than in *G. pulex* (**Figure 2**). $k_{m,\ 1st,\ total}$ 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, $k_{m,\ 2nd,\ total}$ of both compounds were 4-17 times higher than $k_{m,\ 1st,\ total}$ 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*.[19] 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

441 prochloraz (i.e. $IC_{50, PRZ, AZ}$) was 0.1 μM (95% confidence interval (CI): 0.08 - 0.15
442 μM) and 0.02 μM (95% CI: 0.01 - 0.04 μM) for *H. azteca* and *G. pulex,* respectively,
443 indicating that *G. pulex* is 2-15 times significantly more sensitive than *H. azteca*
444 towards prochloraz induced CYP-inhibition (**Figure 3A**). Correspondingly, the
445 internal concentration of AZ_M390a decreased when *H. azteca* or *G. pulex* were co-
446 exposed to prochloraz. The IC_{50, PRZ, AZ_390a} based on the dose-response curve of
447 AZ_M390a gave similar values to that of $IC_{50, PRZ, AZ}$ in *H. azteca* but about 2.5-fold
448 higher values in *G. pulex* (see **Figure 3B** and **SI. J)**), which may be explained by the
449 increased uptake of azoxystrobin. The IC_{50}s of prochloraz on CYP-mediated
450 azoxystrobin biotransformation in *H. azteca* is in the same range of IC_{50s} of
451 prochloraz and other azole fungicides determined for other substrates in
452 invertebrates.[83–85]

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

455 To investigate whether the synergistic effects of prochloraz contribute to the acute
456 toxicity of azoxystrobin in *H. azteca* similar as in *G. pulex*,[19] the lethal toxicity of
457 azoxystrobin for *H. azteca* was studied in the presence and absence of a nonlethal
458 prochloraz concentration (**Figure 4A**). In the absence of prochloraz, the LC_{50} was
459 0.51 μM (95% CI: 0.48 - 0.55 μM) in *H. azteca*, whereas it substantially decreased to
460 0.15 μM (95% CI: 0.13 - 0.16 μM) in the presence of 0.2 μM prochloraz (fold change
461 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[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_{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* ,[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_{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^{-1}) of cypermethrin,[87,88] and also recently for *G. pulex* exposed to 0.1 μM prochloraz.[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 azoxystrobin 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-

504 occurrence of azoxystrobin and prochloraz induced synergistic effects in both species,
505 but *H. azteca* was about five times less sensitive than *G. pulex*. The importance of
506 these species' sensitivity differences regarding ecotoxicological risk assessment
507 depends on the quality of the toxicity data and the related assessment factors.
508 However, both species can be used for toxicity tests in risk assessment frameworks
509 and would deliver results in the same order of magnitude. Nevertheless, *H. azteca*
510 might be preferred as test species in the future because *H. azteca* can provide a
511 homogenous test population throughout the whole year, as indicated previously,
512 several strains of *H. azteca* were sequenced and the genomes and transcripts have
513 been annotated to identify toxicant responsive genes.[33] Indeed, *H. azteca* is already in
514 use for measuring toxicity and bioaccumulation of sediment-associated contaminants
515 in North America.[25]

516 The synergistic effects of prochloraz and azoxystrobin were observed at
517 concentrations 10-1000 folds higher than concentration ranges found in the
518 environment.[10] However, these findings are still relevant because, considering a
519 realistic exposure situation, aquatic organisms are exposed to a mixture of synergists
520 such as to different azoles (e.g., prochloraz, epoxiconazole, tebuconazole, and
521 propiconazole) that have the same mode of action. [6,8,9] They may add up to a total
522 exposure concentration that exceeds the threshold where synergism - in this case CYP
523 inhibition - starts. Indeed, a mixture of epoxiconazole and propiconazole enhanced the
524 toxicity of pyrethroids in *D. magna*.[6,8,89] Nevertheless, whether such synergistic

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

527 **Supporting Information**

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

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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_u, k_e, k_m, k_{em}), half-lives ($t_{1/2}$), 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_{50} in medium.

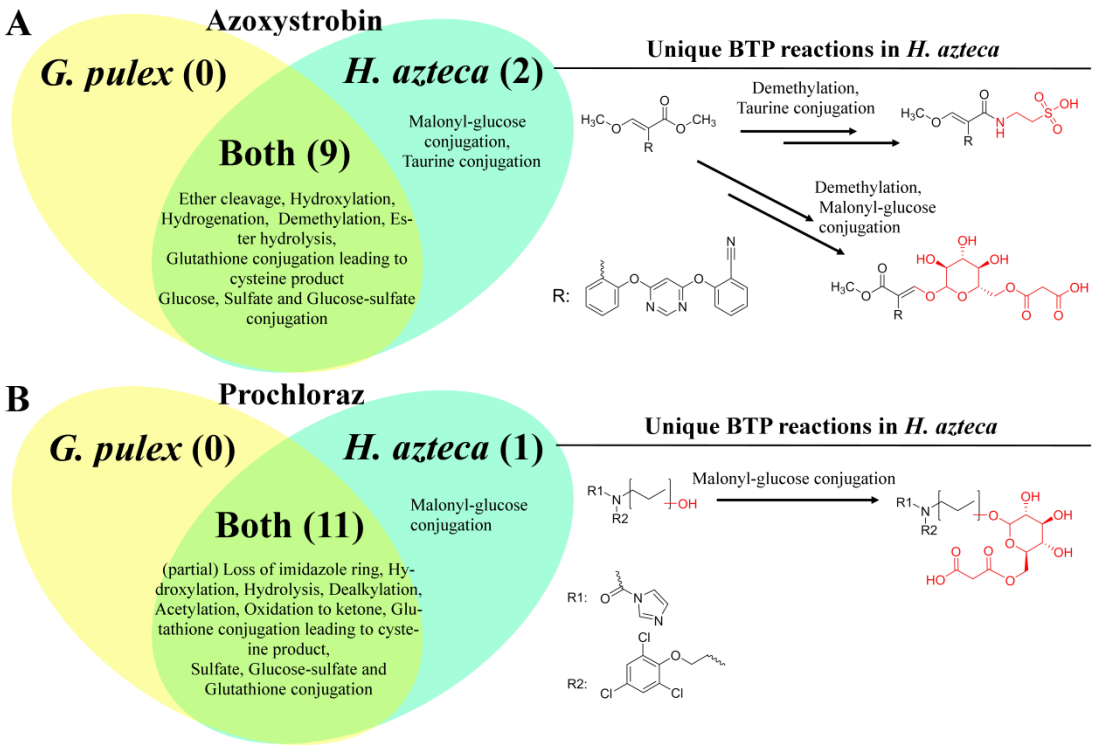
A
Azoxystrobin
G. pulex (0)
H. azteca (2)
Both (9)
Malonyl-glucose conjugation, Taurine conjugation
Ether cleavage, Hydroxylation, Hydrogenation, Demethylation, Ester hydrolysis, Glutathione conjugation leading to cysteine product Glucose, Sulfate and Glucose-sulfate conjugation
Unique BTP reactions in H. azteca
Demethylation, Taurine conjugation
Demethylation, Malonyl-glucose conjugation
R:
B
Prochloraz
G. pulex (0)
H. azteca (1)
Both (11)
Malonyl-glucose conjugation
(partial) Loss of imidazole ring, Hydroxylation, Hydrolysis, Dealkylation, Acetylation, Oxidation to ketone, Glutathione conjugation leading to cysteine product, Sulfate, Glucose-sulfate and Glutathione conjugation
Unique BTP reactions in H. azteca
Malonyl-glucose conjugation
R1:
R2:

Figure 1

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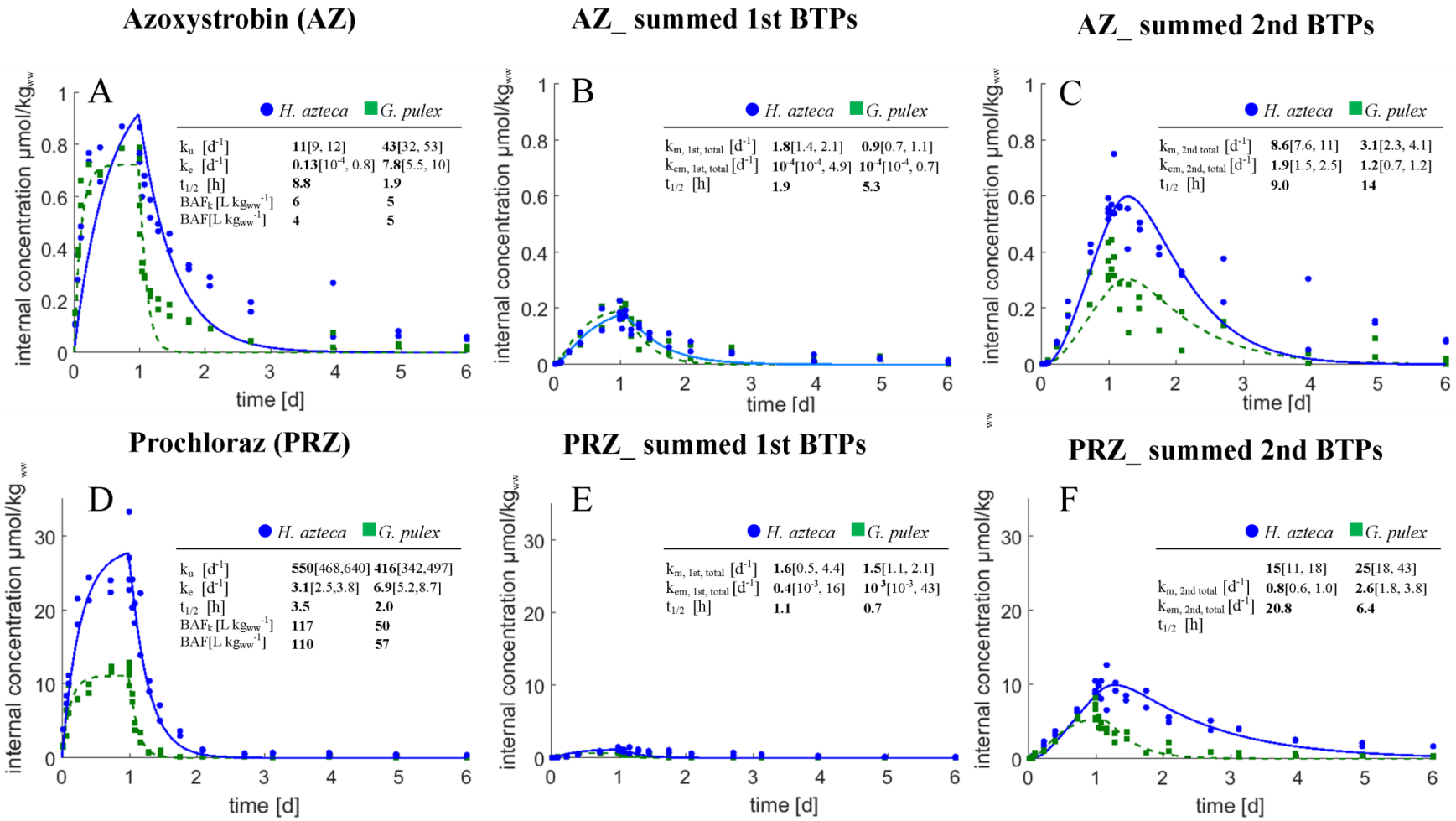

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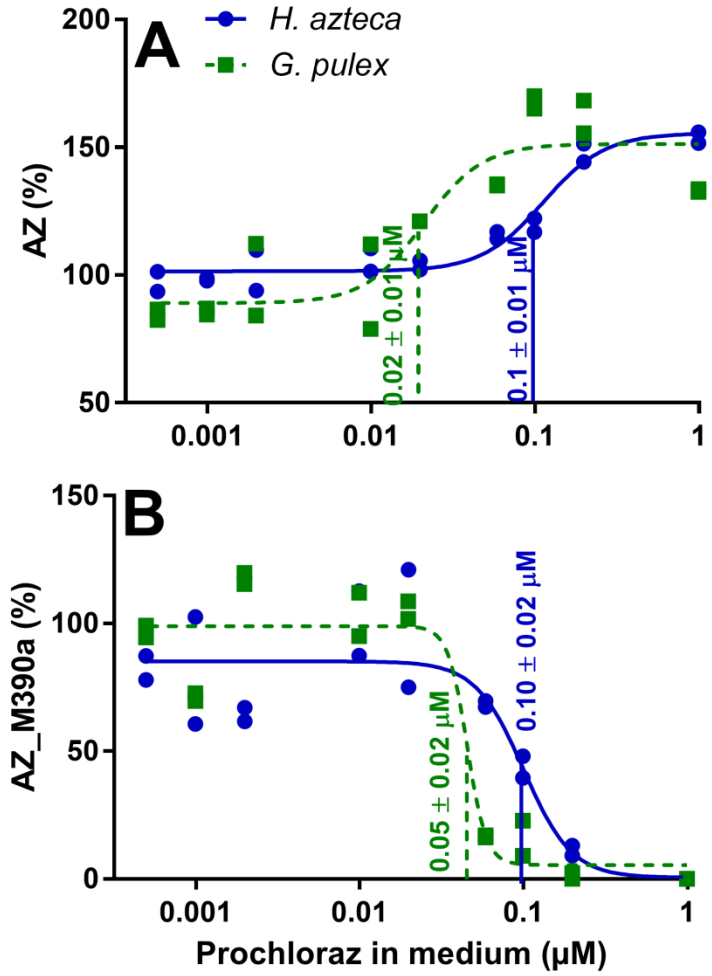

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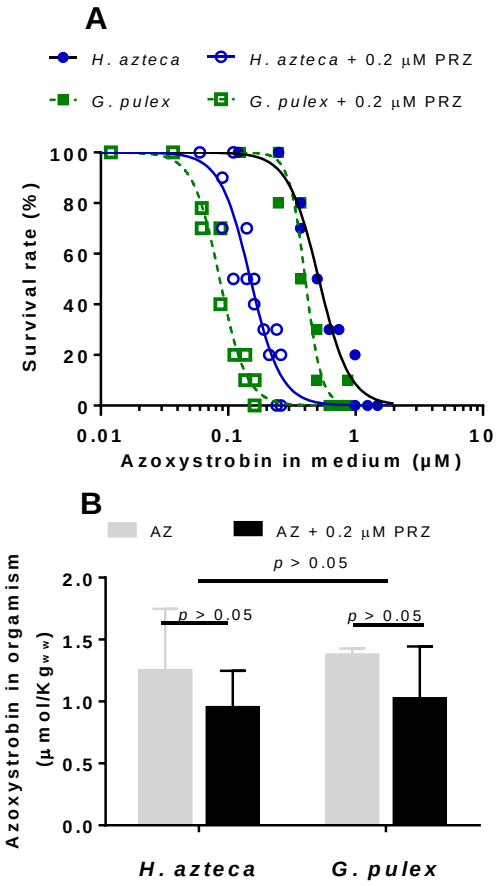
889 **Figure 3**



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891 **Figure 4**

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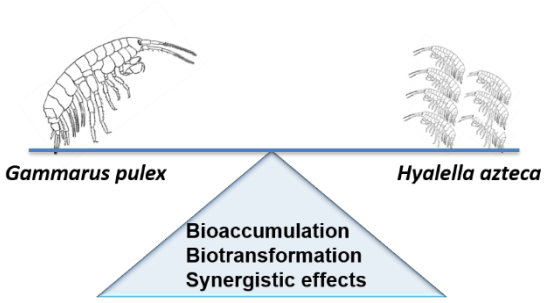
Table of Content



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