Mechanistic Understanding of the Synergistic Potential of Azole Fungicides in the Aquatic Invertebrate *Gammarus pulex*

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Abstract

Azole fungicides are known inhibitors of the important enzyme class cytochrome P450 monooxygenases (CYPs), thereby influencing the detoxification of co-occurring substances via biotransformation. This synergism in mixtures containing an azole has mostly been studied by effect measurements, while the underlying mechanism has been less well investigated. In this study, six azole fungicides (cyproconazole, epoxiconazole, ketoconazole, prochloraz, propiconazole and tebuconazole) were selected to investigate their synergistic potential and their CYP inhibition strength in the aquatic invertebrate *Gammarus pulex*. As co-occurring substrate, the strobilurin fungicide azoxystrobin was chosen and the synergistic potential was measured in terms of internal concentrations of azoxystrobin and associated biotransformation products (BTPs). Azoxystrobin is biotransformed by various reactions and 18 BTPs were

identified. By measuring internal concentrations of azoxystrobin and its BTPs with high resolution tandem mass spectrometry in the presence and absence of azole fungicides followed by toxicokinetic modelling, we showed that inhibition of CYP-catalyzed biotransformation reactions indeed played a role for the observed synergism. However, synergism was only observed for prochloraz at environmentally realistic concentrations. Increased uptake rate constants, an increase in the total internal concentration of azoxystrobin and its BTPs, *in vivo* assays for measuring CYP activities, and *G. pulex* video-tracking suggested that the twofold increase in bioaccumulation and thereby raised toxicity of azoxystrobin in the presence of prochloraz is not only caused by inhibited biotransformation but even more by increased azoxystrobin uptake induced by hyperactivity.

Graphical Abstract

1. Introduction

Mixture effects of environmental contaminants, such as pesticides, have been discussed for a long time and a better understanding of the mechanisms behind these mixture effects is often desired.¹⁻³ Recently, inclusion of mixtures into risk assessment in the framework of the European REACH regulation and the Water Framework Directive (WFD) has been proposed.⁴⁻⁵ Within the European Legislation on Plant Protection Products (EC 1107/2009⁶) only intentional mixtures of co-formulated products are considered in risk assessment. Concentration addition and independent action models have been developed to estimate the toxicity of mixtures on the basis of the toxicity of the single compounds. Often these models give accurate estimations of the toxicity of mixtures.⁷ However, synergistic interactions do occur, *i.e.*, some compounds can enhance the toxicity of other compounds. Two reviews reported that in approximately 5% of pesticide mixtures that were investigated, the observed effects were more than twofold greater than estimated from concentration addition.⁷⁻⁸ In general, the underlying mechanisms causing synergy are diverse and interactions between chemicals can influence several processes such as bioavailability, uptake, internal transport, biotransformation, binding at the target site, and excretion.⁸ According to a recent review by Cedergreen (2014)⁸, one of the most relevant processes causing synergy is altered enzyme activity that subsequently affects biotransformation. Cytochrome P450 monooxygenases (CYPs) are one of the most important enzyme classes present in all kingdoms of life⁹⁻¹² and play a significant role in the detoxification of xenobiotics, their main function being oxidation of a large number of endogenous and exogenous compounds. Increased biotransformation usually leads to decreased toxicity, if the toxicity stems from the parent compound. However, in some cases biotransformation leads to bioactivation by enzymatically introducing an active group or by modifying an inactive molecule to an active molecule.¹³ Strobilurin and azole fungicides are two of the most important fungicide classes that are frequently applied worldwide against various fungal diseases.¹⁴⁻¹⁵ In aquatic ecosystems azole

66 and strobilurin fungicides have been measured at concentrations between low ng L⁻¹ and low
67 µg L⁻¹.¹⁶⁻²² One representative of strobilurin fungicides is the broad-spectrum agricultural
68 fungicide azoxystrobin. It acts by inhibiting mitochondrial respiration in fungi^{15, 23} and exhibits a
69 generally high toxicity towards aquatic invertebrates with median-lethal concentrations (LC₅₀s)
70 ranging between 150 and 350 µg L⁻¹ determined in short-term acute toxicity tests.²⁴⁻²⁷
71 Copepods and cladocerans have been shown to be the most sensitive species towards
72 azoxystrobin.²⁸⁻³⁰ The Maximum Allowable Concentration Environmental Quality Standard
73 (MAC-EQS) for azoxystrobin proposed by the Swiss Centre for Applied Ecotoxicology (the
74 Ecotox Centre Eawag-EPFL) is 0.55 µg L⁻¹.³¹ No EQSs for azoxystrobin and azole fungicides
75 are given in the WFD, which only lists EQSs for a limited number of priority substances.³²
76 Azole fungicides, including the triazoles and the imidazoles, are frequently applied in
77 agriculture as well as in human and veterinary medicine and are well-known to interfere with
78 the fungal cell membrane by inhibiting CYPs.¹⁵ They belong to the class of ergosterol-
79 biosynthesis-inhibitors which inhibit the specific CYP isoform – the lanosterol-14 α-
80 demethylase – that catalyzes the reaction from lanosterol to ergosterol, which is an essential
81 constituent of fungal cell membranes.³³⁻³⁴ Azoles can interact with the CYP in the following
82 ways: like a substrate via hydrophobic interactions in the binding cavity of the enzyme and by
83 strongly coordinating to the active site – the heme-iron – thereby hindering the binding of
84 molecular oxygen and interrupting the CYP catalytic cycle.³⁵⁻³⁷ Thus, azole fungicides can
85 affect the biotransformation and bioaccumulation of other chemicals by inhibiting CYP-
86 catalyzed biotransformation reactions. As single compounds, they exhibit moderate acute
87 toxicity towards small aquatic organisms (LC₅₀s: low mg L⁻¹).^{24, 38-41} MAC-EQSs proposed by
88 the Ecotox Centre Eawag-EPFL are in the low µg L⁻¹ range (0.24-1.4 µg L⁻¹) for selected azole
89 fungicides (cyproconazole, epoxiconazole and tebuconazole). For prochloraz only an ad hoc
90 MAC-EQS of 1.6 µg L⁻¹ is available (see Supporting Information (SI) Q).³¹ Several studies have
91 shown that azole fungicides can enhance the toxicity of other pesticides, such as of
92 pyrethroids, towards aquatic species.⁴²⁻⁴⁶ One study by Cedergreen et al. (2006)⁴⁵ has shown
93 that prochloraz strongly synergized the effect of azoxystrobin towards *Daphnia magna*. It is

likely that a threshold for synergistic interactions exists for most synergists, below which no effects on the metabolic processes are observed.⁸ Whether such a threshold is above or below environmental realistic concentrations (ng L^{-1} to low $\mu\text{g L}^{-1}$) is not always clear. To date, most studies have been conducted at concentrations much higher than what is observed in the environment and only the effect, such as mortality or immobilisation, has been documented. Studies with lower exposure concentrations^{1, 44} and which include the measurement of internal concentrations of parent compounds and associated biotransformation products (BTPs) are rare (one example is Belden and Lydy (2000)⁴⁷) and are needed to further understand the proposed mechanism of synergism.

In this study, we mechanistically investigated whether the observed synergism of azoles in mixtures is caused by the inhibition of CYPs and thus by the inhibition of biotransformation reactions. Six azole fungicides – four triazoles (cyproconazole, epoxiconazole, propiconazole, tebuconazole) and two imidazoles (ketoconazole, prochloraz) – were selected to test their synergizing potential at a range of concentrations by measuring the internal concentrations of azoxystrobin and its biotransformation products (BTPs) in the test species *Gammarus pulex*. Azoxystrobin was selected as it was strongly biotransformed to various products and CYPs were most probably responsible for several but not for all biotransformation reactions. To enable the detection of BTPs present at low concentrations, azoxystrobin concentrations were chosen in the μM range, located within one order of magnitude below acute LC_{50}s . Gammarids are small aquatic invertebrates that as shredders are of great relevance in freshwater ecosystems and they exhibit a high sensitivity towards a vast range of stressors.⁴⁸⁻⁵¹ Additionally, we aimed to determine the strengths of CYP inhibition of the selected azoles in terms of half maximal inhibitory concentrations (IC_{50}s) to determine and compare their CYP inhibition potencies – also in terms of relevance of synergism at environmentally realistic concentrations. As it was recognized throughout the study that uptake might be influenced by specific prochloraz concentrations, video-tracking of gammarids was used to test if prochloraz can induce hyperactivity.

2. Material and Methods

2.1 *Chemicals, Solutions and Test Organisms*

Detailed information about all chemicals and solutions used during experiments and instrumental analysis are provided in SI A. Depending on the experiment, male and female gammarids (length: 1.5 ± 0.5 cm) were collected at uncontaminated creeks in Switzerland and Denmark (see SI B). Gammarids were kept in aerated artificial pond water (APW)⁵² at pH of ~ 7.9 (11 ± 1 °C, 12 h light/12 h dark cycle) and were fed with horse chestnut (*Aesculus hippocastanum*) leaves inoculated with *Cladosporium herbarum*⁵² or degraded leaves collected at the sampling site. Experiments were performed at the above mentioned conditions, and organisms were acclimatized to these test conditions for at least three days.

2.2 *Whole Body Internal Concentration Measurements*

2.2.1. General Design of Exposure Experiments to determine Whole Body Internal Concentrations

If not stated otherwise in the experimental description, the following specifications are valid for all experiments dealing with internal concentration measurements of parent substrates (azoxystrobin, 7-ethoxycoumarin and tramadol) and associated BTPs. Experiments were performed in 600 mL-glass beakers filled with 500 mL exposure medium prepared in APW at test conditions. Duplicate samples were prepared for each treatment. Four gammarids and four horse chestnut leaf discs inoculated with *Cladosporium herbarum* (diameter: 2 cm) were added to each beaker. Leaf discs provided food and shelter during the experiment. Different controls were performed during each experiment, *i.e.*, “organism controls” (chemical negative, organism and food positive), “chemical controls” (organism and food negative, chemical positive), and “food controls” (organism negative, food and chemical positive). Exposure media were sampled at the beginning and end of the experiments to determine exposure concentrations.

At the end of the exposure phase, organisms were shortly rinsed with nanopure water, blotted dry on tissue, transferred to 2 mL-microcentrifuge tubes, and weighed. The homogenisation and extraction was carried out with a FastPrep bead beater (MP Biomedicals, Switzerland) in two cycles of 15 s at 6 m s⁻¹ (cooling on ice in between). Prior to that 500 µL of methanol, 100 µL of isotopically labelled internal standard mix solution composed of azoxystrobin-d4, propiconazole-d5, prochloraz-d7, tebuconazole-d6, 7-ethoxycoumarin-d5 and tramadol-d6 (each 100 µg L⁻¹), and 300 mg of 1 mm zirconia/silica beads (BioSpec Products, Inc., U.S.A.) were added. Subsequently, samples were centrifuged (6 min, 10 000 rpm, 20 °C), filtered (0.45 µm regenerated cellulose filters, BGB Analytic AG, Switzerland), and the filters were washed with 400 µL methanol. Filtrate and extract were merged and the samples were stored at -20 °C until chemical analysis.

2.2.2. Biotransformation Product Identification of Azoxystrobin

To screen for BTPs of azoxystrobin, gammarids were exposed for 24 h to 0.5 µM of azoxystrobin. SIEVE software version 2.2 (Thermo Scientific) was used for conducting a suspect screening of predicted BTP candidates (see SI C) and a nontarget screening. Details about the criteria, such as (i) peak intensity thresholds, (ii) peak shape, (iii) kinetic pattern of increase and decrease in the uptake and depuration phase, respectively, and (iv) integrated intensity ratio between treatment and control samples that had to be fulfilled for both screening approaches, are found in the SI E.

Structure elucidation of azoxystrobin BTPs was carried out similar to the procedure described in our previous publication⁵³ based on (1) the exact mass and isotopic pattern analysis to propose molecular formulas and on (2) the interpretation of MS/MS spectra to identify diagnostic fragments and losses either specific for only one structure or for several positional isomers.

Biotransformation pathway information, sulfate and glucose enzymatic deconjugation experiments according to Kukkonen and Oikari (1988)⁵⁴ (see SI G), as well as the same

structure and/or MS/MS spectra reported in scientific literature provided additional evidence for some tentatively identified BTPs.

2.2.3. Exposure to Binary Mixtures

To investigate the effect of the selected azole fungicides on the internal azoxystrobin concentrations, gammarids were exposed to binary mixtures. Each mixture was composed of the substrate azoxystrobin (0.1 or $0.2 \mu\text{M} \triangleq 40$ or $80 \mu\text{g L}^{-1}$) and of similar molar concentration of the selected azole fungicide. Gammarids were pre-exposed separately for 4 h to each azole fungicide until azoxystrobin was added for a 24 h exposure phase. For prochloraz, which had the largest detected effect on the internal azoxystrobin concentration, additional exposure concentrations (0.15 and $0.5 \mu\text{M}$ of azoxystrobin and similar molar concentrations of prochloraz) and pre-exposure times (12 and 18 h) were tested. For comparison, piperonyl butoxide ($1.5 \mu\text{M}$, pre-exposure 4 h), a known CYP inhibitor, was also tested in combination with azoxystrobin ($0.5 \mu\text{M}$).

To test the effect of prochloraz on further substrates and to provide a link to CYP activities described in section 2.4, 7-ethoxycoumarin ($0.5 \mu\text{M}$) and tramadol ($0.4 \mu\text{M}$) were used in combination with two different prochloraz concentrations (0.1 and $1 \mu\text{M}$) and two different pre-exposure times (4 and 18 h). For each treatment triplicates were prepared and incubation with the substrate lasted 24 h.

2.2.4. Toxicokinetics of Azoxystrobin with and without Prochloraz

To determine internal concentrations over time, gammarids were exposed to $0.2 \mu\text{M}$ azoxystrobin for up to 24 h and were sampled at 7 time points during the uptake phase. For the 120 h depuration phase, gammarids were exposed for 24 h to $0.2 \mu\text{M}$ azoxystrobin, were subsequently transferred to clean medium and were sampled at 13 time points. Simultaneously, in a separate uptake experiment, gammarids were pre-exposed to $0.2 \mu\text{M}$ prochloraz for 4 h before the substrate azoxystrobin ($0.2 \mu\text{M}$) was added. After substrate

addition, gammarids were sampled at 7 time points during 24 h. Exact sampling time points are given in SI F.

2.2.5. Modeling Bioaccumulation and Biotransformation Kinetics

Toxicokinetic rate constants for azoxystrobin - alone and in the presence of prochloraz - were estimated with a first-order compartment model using Matlab R2015b (BYOM: Build Your Own Model, <http://www.debtox.nl/about.html>). The toxicokinetic model is described by ordinary differential equations (ODEs) at which we differentiate between the time course of the parent compound, the time courses of primary BTPs (1st BTPs) that are directly formed from the parent compound, and the time courses of secondary BTPs (2nd BTPs), where a direct precursor BTP was detected. All parameters were fitted simultaneously.

Bioaccumulation factors were either calculated at a specific time point based on the ratio of the concentration of the parent compound in the organisms and of the concentration of the parent compound in the exposure medium with the requirement of steady state (BAF), or kinetically based on the ratio of the uptake rate constant and of the total elimination rate constant of the parent compound (BAF_k). Full details including ODEs, equations to calculate $BAF_{(k)}$ s and elimination half-lives ($t_{1/2}$) are described in SI H.

2.2.6. Half Maximal Inhibitory Concentrations of Prochloraz ($IC_{50, PRZ, AZS}$) based on Internal Concentrations of Azoxystrobin and Associated BTPs

To investigate where synergism starts, $IC_{50, PRZ, AZS}$ were determined by pre-exposing gammarids for 18 h to varying prochloraz concentrations (0 (control), 0.0005, 0.001, 0.002, 0.01, 0.02, 0.06, 0.1, 0.2 and 1 μ M) before the substrate azoxystrobin (0.1 μ M) was added and gammarids were exposed to the substrate for 24 h. The internal concentrations of azoxystrobin and associated BTPs in the treatment samples were compared to those in the control samples and the $IC_{50, PRZ, AZS}$ were determined by fitting a four-parameter log-logistic model (see SI L) available in the R⁵⁵ package “drc” from Ritz and Streibig (2005)⁵⁶.

2.2.7. Chemical Analysis

Directly before analysis, 200 µL (100 µL for BTP screening experiment) gammarid extract or exposure medium were added to 20 mL-headspace glass amber vials filled with 20 mL nanopure water. For chemical analysis, automated online solid phase extraction reverse-phase liquid chromatography coupled to a high resolution quadrupole-orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific Inc.) was used (online-SPE-LC-HRMS/MS).⁵³ Full scan data were acquired in polarity switching mode (electrospray ionization) for a mass range of 100-1000 m/z with a resolution of 70000 (at m/z 200). For triggering data-dependent MS/MS scans with a resolution of 17500 (at m/z 200) a mass list with suspected BTPs for azoxystrobin was included based on *in silico* pathway prediction and scientific literature (see SI C). Internal standard calibration was used for quantification (Trace Finder software 3.1 and 3.3, Thermo Scientific). Due to the lack of reference standards, most BTPs were semi-quantified based on the calibration curve of the parent compound. Further details about chemical analysis, quantification and quality control can be found in SI C and SI D.

2.3 Median-Lethal Concentrations of Azoxystrobin (LC_{50} s) in the Presence and Absence of Prochloraz

LC_{50} s (24 h) were determined for azoxystrobin alone and in the presence of two different prochloraz concentrations (0.001 and 0.2 µM) in APW at test conditions. Azoxystrobin concentrations were chosen based on a range-defining test (see SI O). Each concentration was tested in duplicate by adding 10 organisms and one leaf collected at the sampling site to each beaker. Gammarids were pre-exposed for 18 h to one of the selected prochloraz concentrations or to APW, until azoxystrobin was added. Mortality was monitored 24 h after substrate addition. If no moving of any appendices was observed by prodding immobile gammarids with a glass rod, gammarids were defined as dead. To determine the aqueous concentrations, the exposure medium was sampled at the beginning and at the end of the experiment. "Organism controls", "chemical controls", "food controls" (see section 2.2.1), "solvent controls" (APW plus maximal ethanol concentration of 0.04% used in the treatments

due to chemical spiking), and “prochloraz controls” (prochloraz, organisms and food positive) were performed. For estimating LC_{50} s a two-parameter log-logistic model was applied (see SI L) assuming binomially distributed data available in the R⁵⁵ package “drc”⁵⁶.

2.4 Half Maximal Inhibitory Concentrations based on ECOD in vivo

Activity ($IC_{50, ECODS}$)

To determine CYP activities and their inhibition by azoles in a fast way, the transformation of the substrate 7-ethoxycoumarin to its fluorescent product 7-hydroxycoumarin (ECOD: 7-ethoxycoumarin-O-dealkylation) was measured spectrophotometrically. Therefore, IC_{50} s for ECOD *in vivo* activity ($IC_{50, ECODS}$) were determined according to Gottardi et al. (2015)⁵⁷ at test conditions. Two azole concentrations (1 and 10 μ M, 3 replicates with 2 organisms in 3 mL substrate/azole solution) were tested for all selected azoles in initial experiments and the resulting 7-hydroxycoumarin fluorescence in the medium was compared to the fluorescence in the medium of the control samples.

More detailed investigations were conducted for 0 (control), 0.02, 0.1, 0.2, 1, 2 and 10 μ M prochloraz, and 0 (control), 0.1, 0.2, 1, 2, 10 and 20 μ M epoxiconazole in 250 mL-glass flasks filled with 200 mL exposure solution (prepared in APW) and 23 gammarids. Gammarids were pre-exposed to the different prochloraz/epoxiconazole concentrations for 18 h and were fed with degraded leaves collected at the sampling site. For prochloraz, additional pre-exposure times of 0 and 4 h were investigated. After pre-exposure, 7-ethoxycoumarin was added to a final concentration of 20 μ M (3.8 mg L⁻¹). Five replicates per concentration were prepared by filling 5 mL of substrate/azole solution into 10 mL-glass vials and by each adding four gammarids. During an incubation period of 6 h, 100 μ L aliquots were sampled hourly from the substrate/azole exposure media and were directly added to a black microwell plate (BRANDplates® pureGrade™, Brand, Germany). The fluorescence of 7-hydroxycoumarin (excitation: 380 nm, emission: 480 nm) was measured with a multimode microplate reader (SpectraMax M5 Microplate Reader, Molecular Devices, U.S.) at room temperature. The

exposure media were sampled at the beginning and end of the pre-exposure phase to determine the azole concentration.

Treatment samples were compared to the control samples (see SI M for details) and $IC_{50, ECODS}$ were determined by applying a Brain-Cousens⁵⁸ four parameter hormesis model (see SI L), which is a modification of the three-parameter log-logistic model accounting for hormesis available in the R⁵⁵ package “drc”⁵⁶.

2.5 Video-Tracking of the Locomotory Behaviour of Gammarids in the Presence of Prochloraz

Locomotion was recorded at 12 °C with the Noldus software (Media recorder and analysed with Ethovision XT10) and the camera was placed above the infrared floor. Gammarids were individually placed in glass petri dishes filled with 40 mL APW and acclimated overnight. The next morning, prochloraz was added in 10 mL APW to obtain the targeted concentrations (0 (control), 0.02, 0.1, 0.2, 1 and 2 μ M). After 30 min, gammarids were recorded for 18 h with constant light. Three runs of 18 gammarids were performed (three gammarids per treatment in each run). Exposure media were sampled at the beginning and end of the experiment. Distance moved was analysed with a general linear model in Statistica 9.0 with treatment and time as fixed factors, and run and gammarid number as random one.

3. Results and Discussion

3.1 Bioaccumulation and Biotransformation of the Substrate Azoxystrobin

Bioaccumulation of azoxystrobin with BAFs and BAF_{ks} of 5 L kg_{wet weight (ww)}⁻¹ on average (see Figure 2a, for exact values refer to SI H and SI I) was low in gammarids compared to the threshold of 2000 L kg⁻¹ given in the European REACH regulation, Annex XIII for bioaccumulative substances.⁵⁹ Azoxystrobin medium concentrations, important for the calculation of BAFs, varied on average by 10% from nominal concentrations and declined during the 24 h exposure phase on average by 6% (see SI I).

In total, 18 BTPs were tentatively identified for azoxystrobin with the suspect and nontarget screening approaches, revealing a complex biotransformation pathway. Figure 1 shows the structures of the single BTPs and displays the proposed biotransformation pathway. Since no reference standards were available for most BTPs, their structures are mainly based on the identification of diagnostic fragments to propose one specific structure or to propose tentative candidates where several positional isomers exist. The identification confidence of each BTP is stated in detail in SI R. Biotransformation predominantly took place via oxidation and/or conjugation reactions and all BTPs (except AZ_M214 and AZ_M328a,b) were characterized by changes at the active (*E*)-methyl β-methoxyacrylate group. These changes mainly included demethylation, hydrolysis, hydroxylation, different conjugations, reduction of the acrylate double bond, or combinations of these. The hydrolysis of the methyl ester resulting in the acid derivative (AZ_M390b) is well-known and has been detected in natural aquatic environments⁶⁰, at varying abiotic laboratory conditions⁶¹⁻⁶², as well as during biotransformation across different organismal levels⁶³⁻⁶⁵. Since the ester structure of strobilurins is required to maintain their antifungal activity, ester hydrolysis contributes to the detoxification of azoxystrobin.^{64, 66} The major BTP AZ_M392, which is formed via reduction of the acrylate double bond of the ester hydrolysis product AZ_M390b, reached mean internal concentration of around 35% of those of the parent compound after 24 h exposure (see SI H). This unusual reduction of azoxystrobin acid has been previously detected in plants.⁶⁴ Conjugation reactions with glutathione (resulting

in cysteine products), with sulfate, or with glucose-sulfate were detected for azoxystrobin, confirming the importance of conjugation reactions for aquatic invertebrates.^{53-54, 67-70}

Primary biotransformation rate constants $k_{Mx,1st}$ that directly contributed to the reduction of bioaccumulation of the parent compound were between one and three orders of magnitude lower than the direct elimination k_e of the parent compound (see SI H). Therefore, the percentage of the sum of $k_{Mx,1st}$ on the total elimination ($k_e + \text{sum of } k_{Mx,1st}$) was only approximately 10%. Thus, biotransformation contributed to the elimination of the parent compound azoxystrobin, but did not play a major role in terms of reduction in the bioaccumulation of azoxystrobin.

Figure 1: Proposed biotransformation pathway of azoxystrobin in *G. pulex*. Structural modifications of the BTPs are highlighted in red. The colour and shape of the arrows distinguishes between biotransformation reaction types: black and continuous: reactions influenced by prochloraz; black and dashed: secondary reactions influenced by prochloraz only due to previous reactions being influenced by prochloraz; green: reactions not influenced by prochloraz; grey: alternative pathway used for the kinetic comparison of single exposure to azoxystrobin and mixture exposure to azoxystrobin and prochloraz since for the mixture exposure AZ_M552 was not detected.

BTPs marked in grey were not included in the kinetic model for comparing the kinetic rate constant in the presence and absence of prochloraz because they were not detected in the mixture exposure or only in the screening experiment using a higher exposure concentration (italic).

3.2 Influence of co-occurring Azoles on the Bioaccumulation and Biotransformation of Azoxystrobin

Out of the six tested binary fungicide mixtures composed of 0.1 or 0.2 μM ($\pm$ 40 or 80 $\mu\text{g L}^{-1}$) azoxystrobin and similar molar concentrations of one of the selected azoles, only prochloraz showed a strong inhibitory effect measured in terms of internal concentrations of the substrate azoxystrobin and associated BTPs (see SI J). Therefore, only the synergistic potential of prochloraz was investigated in more detail. The increase in the internal azoxystrobin concentration of approximately 50% in the presence of prochloraz was similar to the increase observed in the presence of the known CYP inhibitor piperonyl butoxide (see SI J).

Pre-exposure to the inhibitor instead of simultaneous exposure to the inhibitor and the substrate facilitates the investigation of single processes, such as binding to the enzyme, and ensures that azoles can display their synergistic potential. Different pre-exposure times to prochloraz (4, 12 and 18 h) were tested for the binary mixtures composed of prochloraz and azoxystrobin but no differences were observed in internal concentrations of azoxystrobin (see SI J). In previous work⁵³ it was determined that prochloraz is taken up fast and steady-state is reached after 24 h. After 5.5 h and 17.5 h exposure, 65% and nearly 100% of the maximal internal concentration after 24 h exposure were reached, respectively. Apparently, the internal concentration reached after 4 h pre-exposure is sufficient to cause distinct inhibition.

All tested prochloraz concentrations in the binary mixtures (0.1, 0.15, 0.2 and 0.5 μM) lead to clear CYP inhibition. Nevertheless, 100% inhibition was not reached with any of the prochloraz concentrations tested, since low concentrations of azoxystrobin BTPs (< limit of quantification (LOQ) up to 5% compared to the control) likely to be CYP-catalyzed were still detected after 24h exposure. These detections were a result of the high sensitivity of the LC-HRMS/MS method with LOQs for azoxystrobin and associated BTPs of < 3 nmol kg_{ww}⁻¹ (see SI D). In addition, the azoxystrobin and prochloraz concentrations used were mostly below the levels of acute toxicity, therefore still enabling biotransformation in the organisms. Since the substrate azoxystrobin is highly toxic towards *G. pulex* (LC₅₀ (24 h): 0.4 $\pm$ 0.02 μM $\pm$ 157 $\pm$ 3 $\mu\text{g L}^{-1}$, see

below) the applied azoxystrobin and prochloraz concentrations are a compromise to avoid visible synergistic effects, such as mortality, while having sufficiently detectable internal concentrations.

3.3 Toxicokinetics of Azoxystrobin with and without Prochloraz and changed Toxicity

Due to the observed CYP inhibition with prochloraz, a kinetic experiment was performed for azoxystrobin alone and in combination with prochloraz for the determination of toxicokinetic rate constants. The implemented model equations were based on the depicted biotransformation pathway in Figure 1. For consistency, the same biotransformation pathway was used for single and mixture exposure, focusing on BTPs still present in the mixture exposure (see Figure 1).

Figure 2: panels (a), (c) and (d): 24 h uptake kinetic for the single exposure to 0.2 μM azoxystrobin (black) and for the 4 h pre-exposure to 0.2 μM prochloraz and the subsequent addition of 0.2 μM azoxystrobin (red). Shown are the measured (symbols) and modelled (lines) time-courses for (a) the parent compound azoxystrobin, (c) the BTP AZ_M390a and (d) the BTP AZ_M390b. Blue crosses mark sampled time points where all gammarids died during exposure. In panel (a) bioaccumulation factors ($\text{BAF}_{(k)}$ s) are listed for the single exposure to azoxystrobin and for the mixture exposure to azoxystrobin and prochloraz in black and red, respectively, whereas in panels (c) and (d) primary biotransformation rate constants ($k_{M, 1st}$) are displayed. Panel (b) shows the total internal concentration of azoxystrobin and associated BTPs after 24 h exposure in black for the single exposure to 0.2 μM azoxystrobin (AZ) and in red for the 4 h pre-exposure to 0.2 μM prochloraz and the subsequent addition of 0.2 μM azoxystrobin (AZ + PRZ). Standard deviations are given for the total internal concentrations.

381

382 Figure 2a shows the internal concentrations over time of the substrate azoxystrobin during the

383 24 h uptake phase. There are significant differences concerning the internal concentrations of

384 azoxystrobin between single and mixture exposure. The internal concentration of azoxystrobin

385 after 24 h exposure was approximately twice as high in gammarids being co-exposed to

386 prochloraz compared to the internal concentration of gammarids being exposed to

387 azoxystrobin only. This result is also reflected in the calculated BAFs and BAF_k s for

388 azoxystrobin that double in the presence of prochloraz (see Figure 2a as well as SI H and SI I

for exact values). Consequently, co-exposure to prochloraz leads to increased mortality (see blue crosses in Figure 2), indicating a higher concentration of azoxystrobin at the target site and thus raised toxicity around three times higher than what is expected from the model of concentration addition (see SI K). As the organisms started to die after steady-state was reached, toxicokinetic rate constants should not be strongly affected by the observed mortality. In separate toxicity tests, LC_{50} s were determined for azoxystrobin alone and in combination with prochloraz (0.2 μ M), confirming the increased toxicity as the LC_{50} was reduced by a factor of 4.5 (see Figure 3).

Figure 3: Concentration-response (mortality) curves for the LC_{50} determination of azoxystrobin (AZ) alone and in combination with two different prochloraz (PRZ) concentrations (0.2 and 0.001 μ M, respectively) after 18 h pre-exposure to prochloraz.

As expected, mainly oxidative biotransformation reactions likely to be CYP-catalyzed were influenced by prochloraz (see Figure 1). This result is also illustrated in Figure 2c which shows exemplary the internal concentration over time of the demethylation product AZ_M390a during the 24 h uptake phase. In the presence of prochloraz nearly no demethylation product ($< LOQ$) was formed after 24 h, pointing towards the inhibition of the respective CYP isoform. In contrast, no inhibitory effect was observed for direct conjugation reactions and ester hydrolyses such as azoxystrobin acid (AZ_M390b) (see Figure 2d, reactions are shown in Figure 1), for which similar internal concentrations were reached after 24 h. These observations fit within expectations since conjugation reactions and ester hydrolyses are catalyzed by different enzymes, such as transferases or esterases. Moreover, these

differences in the amount of BTPs formed between single and mixture exposure are also visible in the estimated biotransformation rate constants $k_{Mx, 1st\ or\ 2nd}$ (see $k_{Mx, 1st}$ displayed in Figure 2c and 2d as well as in Table 1). Rate constants for the single exposure and for the mixture exposure were defined as significantly different when their respective 95% confidence intervals (CIs) did not overlap (see Table 1). In general, biotransformation rate constants influenced by prochloraz showed 2 to 70 times smaller $k_{Mx, 1st\ or\ 2nd}$ (true for 6 out of 7 reactions) and non-influenced reactions exhibited similar $k_{Mx, 1st\ or\ 2nd}$ (true for 4 out of 6 reactions) compared to the $k_{Mx, 1st\ or\ 2nd}$ estimated from the single exposure to azoxystrobin (see Table 1). Yet, there are some exceptions, such as the modelled $k_{M, 1st}$ for azoxystrobin acid (AZ_M390b), which is significantly lower in the presence of prochloraz. This difference might be due to the lack of isotopically labelled internal standards for BTPs and/or slightly different biological activity of gammarids (see Figure 2d and Table 1).

Surprisingly, the hydroxylation product AZ_M420 was not influenced by prochloraz (see Figure 1), although hydroxylation is a typically CYP-catalyzed biotransformation reaction. Different enzyme classes apart from CYPs might be involved in this hydroxylation reaction, such as flavin-containing monooxygenases (FMOs). However, FMOs are more likely to catalyze the formation of N- and S-oxides, whereas C-hydroxylations are rarely reported.⁷¹ Several secondary reactions are indirectly influenced by prochloraz, such as the glucose (AZ_M552) or glucose-sulfate (AZ_M630, AZ_M618) conjugation products. Only their precursor (AZ_M390a) is influenced and the actual conjugation reaction is unaffected.

Using a mass balance after 24 h, the difference in the internal concentrations of the parent compound azoxystrobin in the single and mixture exposure (see Figure 2a) can only partially be explained by inhibited biotransformation reactions that lead to an accumulation of azoxystrobin. Contrary to expectations, most BTPs were not excreted faster compared to the parent compound azoxystrobin (see elimination half-lives in Table 1). Therefore, the sum of the internal concentrations of the parent compound azoxystrobin and its formed BTPs should stay constant, if prochloraz only affects biotransformation. However, the total internal concentration increased in the presence of prochloraz (see Figure 2b), pointing towards additional processes

being influenced. This effect varied in its potency among the different tested prochloraz concentrations. The strongest increase in the total internal concentration compared to the control sample was observed with a concentration of $0.1 \mu\text{M}$ ($\pm 37 \mu\text{g L}^{-1}$) prochloraz (see SI J).

This effect was confirmed by the simultaneous fitted kinetic rate constants. The sum of the primary biotransformation rate constants $k_{Mx, 1st}$ decreased by approximately 80% in the presence of prochloraz. However, the sum of $k_{Mx, 1st}$ only contributed to about 10% to the total elimination in the single exposure to azoxystrobin and was reduced to 2% in the mixture exposure. In contrast, the fitted uptake rate constant k_u increased by a factor of about 1.4 in the presence of prochloraz (see SI H). When first only fitting the uptake rate k_u and one elimination rate k_e to the total internal concentration, and in a second step fitting all other parameters for the single BTPs, stronger weight is given to the uptake rate. Thereby, the difference in the uptake rates becomes even more distinct as the uptake rate increases by a factor of 3.2 in the presence of prochloraz (see SI H).

Table 1: Elimination half-lives ($t_{1/2}$) for azoxystrobin and associated BTPs. Additionally, biotransformation rate constants (k_{Mx}) for the single exposure to azoxystrobin and for the mixture exposure to azoxystrobin and prochloraz are displayed (lower and upper 95% confidence intervals are given in brackets; if two confidence intervals are displayed, then the confidence interval from the likelihood profile is a broken set). $t_{1/2}$ and k_{Mx} are based on the reduced azoxystrobin biotransformation pathway for comparing rate constants between single and mixture exposure displayed in Figure 1. BTPs are listed with increasing $t_{1/2}$. Toxicokinetic rate constants for uptake and elimination are reported in SI H.

	$t_{1/2}$ [h]	k_{Mx} [d ⁻¹] single exposure to azoxystrobin	k_{Mx} [d ⁻¹] mixture exposure to azoxystrobin and prochloraz
Azoxystrobin	1.9		
AZ_M525 (1 st BTP)	2.0	0.037 [0.030; 0.060]	0.0067 [0.0051; 0.0089] [0.011; 0.021]
AZ_M390b (1 st BTP)	2.1	0.44 [0.38; 0.61]	0.073 [0.061; 0.087]
AZ_M498 (2 nd BTP)	3.1	1.9 [1.4; 3.0]	1.0 [0.30; 2.7]
AZ_M378 (2 nd BTP)	5.8	0.79 [0.19; 0.44] [0.62; 1.3]	0.46 [0.33; 0.66] 0.97; 4.8]
AZ_M420 (1 st BTP)	8.9	0.045 [0.036; 0.069]	0.039 [0.030; 0.062]
AZ_M630 (2 nd BTP)	12	1.4 [1.1; 1.8]	15 [4.8; 94]
AZ_M390a (1 st BTP)	12	0.14 [0.12; 0.19]	0.018 [0.0054; 0.049]
AZ_M214 (1 st BTP)	13	0.052 [0.040; 0.081]	0.0011 [0.00082; 0.0014]
AZ_M328a (1 st BTP)	14	0.063 [0.049; 0.090]	0.00095 [0.00062; 0.0096]
AZ_M493 (2 nd BTP)	18	8.3 [6.3; 18]	0.93 [0.45; 1.7]
AZ_M392 (2 nd BTP)	21	8.1 [6.8; 13]	2.2 [1.7; 2.7]
AZ_M362a (1 st BTP)	16000	0.0015 [0.00055; 0.0026]	0.00096 [0.00039; 0.0016] [0.0019; 0.095]
AZ_M362b (1 st BTP)	16000	0.032 [0.025; 0.052]	0.014 [0.0083; 0.023]

3.4 Azole CYP Inhibition Strength Determined via the ECOD Assay and by Internal Concentration Measurements

The ECOD assay according to Gottardi et al. (2015)⁵⁷ has been applied as it is described as a fast tool for measuring CYP activities in various aquatic invertebrates⁵⁷, mammals⁷²⁻⁷⁴, fish⁷⁵, molluscs⁷⁶, nematodes⁷⁷, and insects⁷⁸⁻⁷⁹ *in vivo* and *in vitro*.

In a range-defining test on the selected azoles using up to 10 μM , only prochloraz and epoxiconazole inhibited CYP activity in *G. pulex*. Since the concentrations tested were well above environmentally realistic concentrations – 10 μM equates to 2.9 (cyproconazole) to 5.3 (ketoconazole) mg L^{-1} – further experiments were only done with prochloraz. For epoxiconazole no $\text{IC}_{50, \text{ECOD}}$ could be determined as the organisms started to die at 20 μM before ECOD activity was severely inhibited.

Figure 4a shows the concentration-response curve for the $\text{IC}_{50, \text{ECOD}}$ determination of prochloraz (18 h pre-exposure) based on the ECOD assay ($\text{IC}_{50, \text{PRZ, ECOD}}$). The determined $\text{IC}_{50, \text{PRZ, ECOD}}$ was approximately $0.5 \pm 0.1 \mu\text{M}$ ($200 \pm 60 \mu\text{g L}^{-1}$). The relatively large variations among the sample replicates (coefficient of variation: 0.23-0.85) are most likely caused by biological diversity among the single gammarids which were collected in the field. At low prochloraz concentrations (0.02 μM and 0.1 μM) increased ECOD activity was observed, being statistically significant for 0.1 μM prochloraz ($p < 0.05$) compared to the control (see SI M). Different pre-exposure times (0, 4 and 18 h) all revealed the same pattern of stimulated ECOD activity at low doses (see SI M). Hormesis, a stimulation of response at low doses and inhibition of response at high doses, is well-known and can be induced by organic or inorganic compounds as well as by radiation across different organismal levels.⁸⁰⁻⁸² This hormetic effect of increased ECOD activity can be caused by the following processes: the induction of specific CYP isoforms responsible for the deethylation reaction of 7-ethoxycoumarin or the influence of prochloraz on other processes, such as on the uptake of 7-ethoxycoumarin.

As it was reported that the O-deethylation reaction of 7-ethoxycoumarin is catalyzed by a broad spectrum of CYP isoforms⁷³⁻⁷⁴ in various organisms^{57, 72-79}, it seems likely that some of these CYP isoforms can also catalyze oxidative biotransformation reactions of azoxystrobin.

Therefore, we expected to observe the same pattern with the ECOD assay as well as with the internal concentration measurements, *i.e.*, that low concentrations of prochloraz increase the response (ECOD activity and amount of oxidative formed azoxystrobin BTPs, respectively) and high doses inhibit the response. However, hormesis was not found for the substrate azoxystrobin, as no BTP of azoxystrobin that is likely to be CYP-catalyzed showed a higher internal concentration compared to the control in the presence of low prochloraz concentrations (see Figure 4b, exemplary BTP_M390a).

Figure 4: Dose-response curves for the IC₅₀ determination based on ECOD activity (a), or based on internal concentration measurements of azoxystrobin and associated BTPs (exemplary AZ_M390a is shown) (b) with 18 h pre-exposure to prochloraz. The dashed lines mark the determined and displayed IC₅₀s. The bar plot in panel (a) shows the results of the video-tracking experiment and displays the effect of varying prochloraz concentrations on the locomotory behaviour, *i.e.*, on the distance gammarids moved per 1 h during 18 h exposure. Asterisks (black: ECOD activity, blue: video-tracking) mark treatment samples that are significantly different from the control.

499 To address this discrepancy, internal concentrations of 7-ethoxycoumarin and its BTPs were
500 measured with LC-HRMS/MS in the presence of two prochloraz concentrations, one where
501 clearly increased ECOD activity was observed (0.1 μ M) and one where clearly decreased
502 ECOD activity was observed (1 μ M). Surprisingly, 7-hydroxycoumarin (7-Etc_M161), which is
503 the deethylation product measured in the ECOD assay, was hardly detectable in gammarids.
504 Instead, two conjugation products were identified that were formed via sulfate (7-Etc_M240) or
505 glucose-sulfate (7-Etc_M403) conjugation of 7-Etc_M161 (see SI J). 7-Ethoxycoumarin was
506 mainly present in its unchanged form after 24 h exposure and total BTP concentrations of 7-
507 ethoxycoumarin were low, reaching at maximum 6% of those of the parent compound.
508 However, low prochloraz concentrations (0.1 μ M) lead to slightly higher concentrations of 7-
509 Etc_M240 compared to the control, confirming the hormesis observed in the ECOD assay (see
510 Figure 4a). Moreover, significantly higher ($p < 0.05$) internal 7-ethoxycoumarin concentrations
511 (approximately 20%) were obtained in the presence of low prochloraz concentrations (0.1 μ M)
512 compared to the controls (see SI J). This increase in the total internal concentrations of the
513 parent compounds 7-ethoxycoumarin and azoxystrobin (see section 3.2 and 3.3) in the
514 presence of low prochloraz concentrations indicates that uptake is influenced and no induction
515 of specific CYP isoforms occurs. For further confirmation, internal concentrations of tramadol
516 and its BTPs were measured in the presence and absence of prochloraz (0.1 and 1 μ M).
517 Tramadol – a pharmaceutical with known BTPs and corresponding elimination half-lives in
518 *G. pulex*⁶⁷ – showed the same trend in increasing total internal concentrations for the 0.1 μ M
519 exposure to prochloraz as the substrates azoxystrobin and 7-ethoxycoumarin (see SI J).
520 Overall, the *in vivo* ECOD assay does not only include primary biotransformation reactions
521 such as the formation of 7-hydroxycoumarin – which would be desired if we want to compare
522 CYP activities across different species. Secondary biotransformation reactions including
523 conjugation reactions as observed in this study, uptake, and excretion can strongly affect the
524 actual concentration of the measured fluorescent BTP 7-hydroxycoumarin. Since toxicokinetic
525 processes are extremely difficult to predict and can differ strongly among species, CYP
526 activities based on the detection of only one primary BTP should only be compared across

different species with great care. Nevertheless, the ECOD assay is suitable to compare the potencies of different chemicals, e.g., azoles in one species. However, transferring the IC_{50} for prochloraz determined via ECOD activity ($IC_{50, PRZ, ECOD}$: $0.5 \pm 0.1 \mu M \triangleq 200 \pm 60 \mu g L^{-1}$) to another substrate is not feasible since we have seen considerable inhibition already at much lower prochloraz concentrations (see section 3.2 and 3.3 as well as Figure 4b) when measuring internal concentrations of azoxystrobin and associated BTPs. These differences indicate that 7-ethoxycoumarin is not as broad a CYP substrate as it is described in humans.⁷³⁻

⁷⁴ To identify when actually synergisms starts between azoxystrobin and prochloraz an additional IC_{50} for prochloraz was determined via internal concentration measurements using azoxystrobin as a substrate ($IC_{50, PRZ, AZ}$). Figure 4b depicts the accumulation of the parent compound azoxystrobin and the formation of one exemplary BTP, its demethylation product AZ_M390a, in the presence of different prochloraz concentrations. The $IC_{50, PRZ, AZ}$ was $0.02 \pm 0.01 \mu M$ ($8 \pm 3 \mu g L^{-1}$), and the $IC_{10, PRZ, AZ}$ was $0.009 \pm 0.005 \mu M$ ($4 \pm 2 \mu g L^{-1}$) based on the dose-response curve of the parent compound azoxystrobin. The $IC_{50/10, PRZ, AZ}$ s based on the dose-response curves of the associated primary CYP-catalyzed BTPs were in the same range (see SI N). It is likely that the $IC_{50/10, PRZ, AZ}$ s determined via the substrate azoxystrobin are transferable to other environmental contaminants, since several different biotransformation reactions were observed for azoxystrobin, with many reactions probably catalyzed by CYPs (see Figure 1). Moreover, one of the structural features of azoxystrobin, the alkyl group attached to an oxygen, is widespread among chemicals and CYP-catalyzed O-dealkylation reactions are frequent reactions in drug metabolism.⁷¹

3.5 Locomotory Behaviour of Gammarids in the Presence of Prochloraz

To test whether increased uptake is caused by increased movement of pleopods due to hyperactivity which results in increased ventilation, the locomotory behaviour of gammarids in the presence of different prochloraz concentrations was investigated by video-tracking. Hyperactivity has been reported for gammarids being exposed to sublethal insecticide concentrations and has been shown to be linked to drift behaviour.⁸³⁻⁸⁴ In *Chironomus* larvae,

increased uptake rates have been observed in the presence of pesticide mixtures.⁴⁷ Figure 4a shows the results of the video-tracking experiment and displays the effect of prochloraz on gammarids' locomotory behaviour. At 0.1 μM prochloraz, the total distance gammarids moved during 18 h was greatest (almost double compared to control) and significantly different from the control (see SI P). The behavioural data are in line with the increase in total internal concentrations of several different substrates, the *in vivo* ECOD assay, and the modelled uptake rate constants k_u (see section 3.3), thereby providing strong evidence for elevated uptake induced by hyperactivity.

3.6 Environmental Relevance

All six azoles apart from prochloraz showed no synergistic effects measured in terms of internal azoxystrobin concentrations and ECOD activity using exposure concentrations in the low μM range (40 and 80 $\mu\text{g L}^{-1}$). Azole concentrations measured in Swiss surface waters are between approximately 0.06-0.3 $\mu\text{g L}^{-1}$ suggesting that synergism is not relevant at environmental concentrations in Swiss surface waters.^{19, 22} However, the $\text{IC}_{10, \text{PRZ, AZ}}$ for prochloraz ($4 \pm 2 \mu\text{g L}^{-1}$) is only around 10 times higher than what was found in Swiss monitoring data.²² Strobilurin and azole fungicides are two of the most important fungicide classes applied worldwide¹⁴⁻¹⁵ and are detected in considerably higher concentrations in surface waters strongly influenced by agriculture and/or wastewater^{17, 23, 85}. The LC_{50} of azoxystrobin in the presence of Swiss environmental prochloraz concentrations ($0.001 \mu\text{M} \pm 0.37 \mu\text{g L}^{-1}$) was not significantly reduced (see Figure 3). However, LC_{50} s refer to acute toxicity and no conclusions can be made about chronic effects over a longer time period. In addition, further investigations are needed, to determine whether species more sensitive towards azoxystrobin, such as copepods and cladocerans, show synergistic effects already at lower exposure concentrations.²⁸⁻³⁰

We showed that the synergism by prochloraz is not only caused by CYP inhibition but also by increased substrate uptake. Independent simulations of both processes with the developed toxicokinetic model showed that increased substrate uptake contributed significantly more to

the observed synergism compared to CYP inhibition (see SI H). The combination of both processes might be the reason why out of the selected azoles only the co-exposure to prochloraz produced such a pronounced increase in internal azoxystrobin concentrations leading to increased toxicity. This is in line with other studies where prochloraz was also found to be a strong synergist.⁴²⁻⁴⁶ Moreover, hydrophobic interactions of the azole ring substituents of the selected azoles most likely differ, which thereby influence the complex formation of azole and CYP. Needing to account for both processes, CYP inhibition and increased uptake, further complicates mechanistic based toxicokinetic and toxicodynamic modelling that would allow for the prediction of internal concentrations of a chemical and its effect, also in the presence of varying inhibitor/substrate ratios, for a proper mixture risk assessment. In the future, hopefully more information will be gathered about chemical induced behavioural changes, as well as about the enzyme composition and kinetics in invertebrates. This would support more accurate predictive modelling of synergistic effects and thereby enable a better evaluation of the importance of synergy.

Associated Content

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: Details on experimental conditions, analytical methods, exposure medium concentrations and calculated $BAF_{(k)}$ s, quality control, parameter settings for BTP identification with SIEVE software, toxicokinetics, dose-response fitting, structure elucidation evidence for all BTPs including MS/MS spectra (PDF)

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Notes

The authors declare no competing financial interest.

Acknowledgment

Special thanks go to Emma Schymanski (Eawag) for assistance with computational MS/MS spectra annotation. Furthermore, we thank Roman Ashauer (University of York) and Tjalling Jager (DEBtox Research) for helpful discussion and advice with toxicokinetic modelling, Rebecka Hischier (Eawag) for acute toxicity testing, Jennifer Schollée (Eawag) for reading the manuscript.

This research was funded by the Swiss National Science Foundation, grant number 315230141190 and 205320165935.

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