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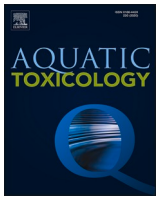

Sediment characteristics mediate mixture effect of metconazole and thiacloprid on the activity behavior of the amphipod *Hyalella azteca*

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ABSTRACT

Pesticide mixtures occur frequently in freshwaters. Here, pesticides can persist over long periods and alter aquatic communities and ecosystems by causing chronic indirect effects. Particularly effects on activity behavior of organisms can be considered as starting points of cascading effects as they provide the basis for further sublethal responses such as reproduction or feeding. Therefore, the impact of two pesticides in combination, the fungicide metconazole and the insecticide thiacloprid, was evaluated on the immobilization and activity behavior of *Hyalella azteca* with varying sediment conditions. The results showed a change from additive effects to synergism in the mobility tests for sediment with higher contents of total carbon but not for the activity behavior tests using a Multispecies Freshwater Biomonitoring system. However, sediments with high carbon, nitrogen and phosphorous contents led to comparable activity behavior of *H. azteca* to control conditions after three days of contaminant exposure which was not the case in all other treatments. The autoregressive integrated moving average (ARIMA) forecast approach used showed that this activity behavior remained constant after recovery to pre-exposure levels at least for a time period of 16 h. This study showed that mobility and activity of *H. azteca* are largely affected by the exposure to pesticides, which is mediated by the structure of the sediment. However, further studies are needed that test activity behavior impairments in environments where the individuals are in direct contact with the sediment that may buffer the pesticide exposure from the water column.

1. Introduction

Mixtures of pesticides are commonly detected in freshwaters (Ippolito et al., 2015; Liess et al., 2021; Schreiner et al., 2016) and are reported to alter aquatic biota (e.g., Neale et al., 2020; Rodney et al., 2013; Weisner et al., 2021). The reasons for the occurrence of pesticide mixtures in freshwaters are manifold, but in general agriculture within the catchments uses different pesticides throughout the year with sometimes several active ingredients to protect field crops (Rossberg, 2007). Pesticides then enter freshwaters via transport routes such as spray drift, run-off and drainage waters (Kreuger, 1998; Schulz, 2004; Wauchope, 1978; Wolters et al., 2008). Thereby, the smallest water bodies suffer from the largest risks from agricultural pesticides (Halbach et al., 2021; Szöcs et al., 2017; Weisner et al., 2022) due to their intimate connection to adjacent agricultural fields (Lorenz et al., 2017; Meinikmann et al., 2021).

Pesticides can persist in freshwater ecosystems for large time periods (Schulz and Liess, 2001). They profoundly change aquatic invertebrate communities and hence aquatic ecosystems (Liess et al., 2021; Schäfer et al., 2011) by particularly affecting sensitive species that may contribute to ecosystem functioning (Fernandez et al., 2015; Schäfer et al., 2012). Pesticides may rarely cause acute toxic effects in freshwater ecosystems but rather long lasting indirect chronic effects (Cedergreen and Rasmussen, 2017). Chronic pesticide effects may occur for different species traits, such as feeding behavior (Agatz et al., 2014; De Castro-Catala et al., 2017; Feckler et al., 2016) or locomotion/activity (De Castro-Catala et al., 2017; Rodrigues et al., 2016) amongst others. However, behavioral endpoints are only rarely assessed in ecotoxicological studies, presumably due to a lack of standardized toxicity methods that allow for repeatability (Ford et al., 2021). Effects on activity or locomotion provide the basis to the understanding of other sublethal responses to toxic stress, such as reproduction or feeding. Hence, their assessment is pivotal as the information gathered by such kind of study may help to classify results from accompanying sublethal tests (De Castro-Catala et al., 2017; Ford et al., 2021).

The aim of this study is to evaluate the impact of two pesticides in combination, the fungicide metconazole and the insecticide thiacloprid, on the immobilization and activity of the amphipod shredder *Hyalella*

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azteca under two different sediment conditions. Both pesticides are frequently applied on agricultural farms, i.e., 64.9 % (metconazole) and 70.8 % (thiacloprid) of German oilseed rape farmers applied the respective compound in 2014 (Rossberg, 2016). Therefore, co-occurrence of these substances in freshwaters is not implausible as pesticide mixtures are frequently found in freshwaters surrounded by agricultural land (Liess et al., 2021; Trau et al., 2023). The objectives were (i) to test if the toxicity of pesticide mixtures is altered by sediment condition by changing mixture effect magnitudes (e.g. from additive effects to antagonistic effects), and (ii) to assess if short-term adaption in behavior to non-lethal environmental concentrations mediated by sediment condition may occur. We hypothesized that sediment with larger content of organic matter would lead to antagonistic effects of the pesticide mixture. Further, we hypothesized that the activity of *H. azteca* would be significantly reduced under the tested non-lethal pesticide concentration. We also expected a recovery of activity behavior with increasing time of the experiment due to sediment adsorption of the pesticides.

2. Material and methods

2.1. Hyalella azteca

All individuals of *H. azteca* were derived from the breeding stock of the Julius Kühn Institute. The population has not been exposed to pesticides since 2014 but a theoretical possible insecticide resistance could not be tested prior to the experiments. Individuals were kept in the breeding stock at 22 °C in aquaria and fed daily with dried *Spirulina* algae and dried oak or alder leaves. The test individuals have been selected two days prior to the experiments from the breeding stock and were transferred to a climate chamber to adapt to the test temperature of 20 °C. The individuals were constantly exposed to a 16/8 h light/darkness cycle. The individuals were kept in culture media using distilled water and Tropic Marin Sea Salt adjusted for a conductivity of 1200 µS cm^{-1}.

2.2. Pesticide selection and analysis

The fungicide metconazole and the neonicotinoid insecticide thiacloprid were applied as commercial pesticide products. All concentration calculations are based on active substance (a.s.) content and not on the amount of pesticide product. The pesticides Caramba (60 g L^{-1} metconazole, BASF) and Calypso (480 g L^{-1} thiacloprid, BAYER) were purchased from the experimental field sites of the Julius Kühn Institute. Water samples were analyzed using liquid chromatography with tandem mass spectrometry (LC MS/MS). All samples were filtered prior to analysis (2 µm syringe filter) and were stored at 4 °C until analysis. Water samples for analysis of metconazole were diluted 1:10. Chlorflurazone and isoproturone were used as surrogates. 50 µl internal standard isoproturone D6 (company LGC, purity 98,61 %, 1 mg/ml) was added to 950 µl water sample prior to LC MS/MS measurement. LC measurement (5 µl injection) was performed using Phenomenex KINETEX Biphenyl columns (50×2.1 mm, 2.6 µm) on a Dionex UltiMate 3000 LC system. Methanol with 0.5 % formic acid and 5 mmol ammonium formate was used as solvent to account for the mobile phase A, while methanol was replaced by water in the solvent to account for mobile phase B. An AB SCIEX QTRAP 5500 system was used for MS measurements using Analyst 1.6.3 software. The limit of detection was 0.001 µg/L and the limit of quantification was 0.002 µg/L.

2.3. EC_{50} tests

Half maximal effective concentration (EC_{50}) tests were performed using the a.s. metconazole and thiacloprid. EC_{50} were determined using eight adult individuals of *H. azteca* in 80 ml test media spiked with the nominal a.s. concentrations given in Table 1. Individuals of 3 mm size (defined as adults) were selected using a hand net with the respective mesh size. Each EC_{50} test was performed in 4 replicates. The tests were conducted following OECD (2004a; b) and USEPA (2000) guidelines. Temperature was kept at 22 °C and air humidity at 85 %. The daily lighting regime followed a cycle of 16/8 h light/dark periods. The beaker were gently aerated and no substrate or food was provided. Mobile and immobile individuals were counted after 48 h of exposure. Individuals were classified as immobile when no physical reaction was recorded within the next 15 s after gently moving the test beaker.

Table 1
Nominal test concentrations for the EC_{50} determination of *Hyalella azteca*.

metconazole (mg/L)	thiacloprid (mg/L)
0.0	0.0
0.263	0.0012
0.525	0.0037
1.05	0.0111
2.1	0.0333
4.2	0.1
	0.3

2.4. Mobility tests

Mobility test were performed based on the EC_{50} test results using the a.s. metconazole and thiacloprid alone or in combination. Additionally, different sediment types (one with low and one with high organic matter content) have been tested for their effects on *H. azteca* mobility (Table 2). The sediment characteristics have been determined according to the guidelines DIN ISO 13,878: 1998–11, DIN ISO 10,694: 1996–08, DIN ISO 11,260: 1997–05, DIN ISO 19,683–2: 04.97, DIN ISO 19,684–3, DIN ISO 13,805: 2014–12. EC_{50} values have been converted to toxic units (TU) (von der Ohe and de Zwart, 2013) to account for comparability of effects. Each EC_{50} concentration was set to 1 TU, and a sum of 0, 0.25, and 0.5 TU was added in all tests. In the tests using the a.s. combination (metconazole + thiacloprid), 0 TU, 0.125 TU and 0.25 TU of each a.s. was added to account for the aforementioned total sums of TU (sumTU), respectively. The respective sumTU was added to 177 ml of culture media of which 2 ml were taken immediately for a.s. analysis. The tests using sediment type A and B (Table 2) were performed using 50 g dried sediment of the respective type moistened with 10 ml of culture media additional to the175 ml test solution. Ten individuals of *H. azteca* were transferred to each test beaker and stored in a climate chamber at 20 °C with 16/8 h light/darkness cycle for 10 days. Individuals were daily fed with dried *Spirulina* algae (50 mg) during the test and a second 2 ml water sample for a.s. analysis was taken at day 10. After 10 days, all individuals were counted and classified as mobile/immobile following the aforementioned criteria. Each test was performed in 4 replicates.

Table 2
Sediment characteristics of sediment type A and B used in mobility and activity tests. TC = total carbon, TN = total nitrogen, OS = organic substance, CEC_{eff} = effective cation exchange capacity, WRC = water retention capacity. Sand = 0.063 – 0.2 mm, silt = 0.002 – 0.063 mm, clay = < 0.002 mm.

	Sediment A	Sediment B
pH	4.7	7.2
TC [%]	0.07	0.24
TN [%]	0.7	3.58
OS [%]	1.55	7.57
CEC_{eff} [$cmol_c$/kg]	1.34	25.58
WRC [%]	20.0	43.1
sand [%]	87.0	27.0
silt [%]	6.0	45.0
clay [%]	7.0	27.0

2.5. Activity tests

A Multispecies Freshwater Biomonitor (MFB) (Gerhardt and Schmidt, 2002) was used to detect non-lethal effects of a 0.25 sumTU combination of metconazole (0.125 TU) and thiacloprid (0.125 TU) with different sediment types. The MFB detects the amplitude and temporal pattern of movement activity of individuals that were placed inside a measurement chamber (2 cm diameter, 3 cm length). Two opposing pairs of stainless steel electrode plates are fixed in each measurement chamber. One electrode pair generates a high frequency alternating current over the measurement chamber while the other electrode pair senses impedance changes resulting from movements of the test individuals inside the measurement chamber (Gerhardt and Schmidt, 2002).

One specimen of *H. azteca* (size: 3 mm) was transferred to a MFB measurement chamber one day prior to the start of the test. The measurement chambers (3 control chambers, 3 treatment chambers) were placed in 437.5 ml of culture media at 22 °C and recording of MFB measurements started immediately after placement. After 24 h, the 0.25 sumTU was added to 437.5 ml of culture media in separate beakers of which 2 ml were taken immediately for a.s. analysis. The tests using sediment type A and B were performed using 125 g dried sediment of the respective type moistened with 25 ml of culture media additional to the test solution. With addition of 0.25 sumTU, the measurement chambers were transferred to the new test beakers and MFB recording was continued for the following three days. A second 2 ml water sample for a.s. analysis was taken at day 3.

Activity behavior was recorded as an average of 300 s of individual activity data records. Raw data of MFB records are automatically processed by the MFB software using Fast Fourier Transformation resulting in a histogram of Hz signal frequencies that occurred during the whole 300 s recording time (Gerhardt and Schmidt, 2002). According to MFB calibration towards the use of further amphipod species (Gerhardt et al., 2007), activity classification was set as 0.5–2.0 Hz for locomotion (swimming and crawling activity) and as 2.5–8.0 Hz for inactivity. MFB threshold level was set as 112 mV and noise level was set as 21 mV. Locomotion and inactivity were recorded as percentages in the respective Hz class.

2.6. Statistical analysis

All statistical analyses were performed using the statistical language R (R Core Team, 2018). EC_{50} concentrations were determined using the drc package (Ritz et al., 2015). Two-parametric Weibull functions have been selected as the best fitting dose-response models based on the lowest Akaikes Information Criteria (AIC). Analysis of variance (ANOVA) was performed on square root-transformed data to compare a.s. effects on *H. azteca* mobility. Data were checked for homogeneity of variances using Brown-Forsysthe test (Hui et al., 2008) and for normality using qreference-plot comparisons to randomly drawn data from normal distributions (Maindonald and Braun, 2015). Tukey post hoc tests using Bonferroni correction (Hothorn et al., 2008) were performed to account for sumTU effects within sediment types. Two-factorial ANOVA was performed to test for interaction between TU and sediment type.

Locomotion and inactivity data recorded by the MFB were averaged over their respective Hz range. Subsequently, the % inactivity average value was subtracted from the respective % locomotion average value to account for comparability of baseline activity. Following this procedure, the resulting data were normalized in the range of 0, 1. Data were split in the segments 'before start' and 'after start' and piecewise regressions were calculated using the R package SiZer (Sonderegger et al., 2009) to identify significant breaking points in activity behavior. According to the breaking points identified by the piecewise regression analysis, Welch *t*-tests were performed comparing the activity after the breaking points between control and treatment 'before start' and 'after start'. Time series analysis was used on data. Activity data were decomposed into trend activity, periodic activity and random activity using the stl function (Cleveland et al., 1990). Trend activity and random activity were extracted and compared between control and treatment using Welchs *t*-test. Data were used to check for activity recovery using ARIMA (autoregressive integrated moving averages) models within the following 16 h after Day 3 calculated using the forecast package (Hyndman, 2016; Hyndman and Khandakar, 2008). The best-fitting ARIMA models were selected based on the lowest AIC.

3. Results

3.1. EC_{50} tests

EC_{50} concentrations of 0.9732 mg a.s. L^{-1} and 0.0117 mg a.s. L^{-1} causing 50% immobilization in *H. azteca* were determined for metconazole and thiacloprid, respectively (Table 3, Fig. 1). These EC_{50} values were considered as 1 TU in the subsequent mobility and activity tests.

3.2. Mobility tests

Concentrations of metconazole dropped remarkably in the single substance trials from day 0 to day 10 independently of sediment presence or sediment type (Table 4). Concentrations of thiacloprid remained stable in the single substance trials without sediment, but decreased slightly over test duration for sediment type A and decreased strongly for sediment type B (Table 4). In the test system with both a.s. (metconazole + thiacloprid), metconazole concentrations remained stable or decreased slightly in general. Contrary, thiacloprid remained stable in the tests with both a.s. without sediment and with sediment type A, but decreased strongly over the test duration using sediment type B (Table 4).

There was a significant impact of TU on the mobility of *H. azteca* in the metconazole treatment without sediment (ANOVA, $p<0.0001$) and in the treatment with sediment type B (ANOVA, $p<0.0001$). Similarly, there was a significant impact of TU on mobility in the combination treatment (metconazole + thiacloprid) without sediment (ANOVA, $p<0.001$) and in the treatment with sediment type B (ANOVA, $p<0.01$). However, in the thiacloprid treatment, a significant impact of TU on the mobility of *H. azteca* was only found in the treatment without sediment (ANOVA, $p<0.001$). A significant effect of sumTU interaction with sediment treatment was found for all pesticide treatments (single and combination treatments, two-way ANOVA, $p<0.0001$). Strong synergistic effects were found for the combination of metconazole and thiacloprid in the treatment with sediment type B, while additive effects were observed in the other treatments (Table 5).

3.3. Activity tests

Activity measurements on *H. azteca* showed a distinct phase of adaptation to the measurement chamber environment with high activity prior to the beginning of metconazole + thiacloprid addition (Fig. 2). Averaged over the six test series (of the period before experimental start) the mean breaking point of adaptation end identified by piecewise regressions was 13.6 ± 8.8 h (SD). Maximum adaptation time to the measurement chambers was 24.7 h (time point −1483) in the control of the sediment type B treatment while minimum adaption time was 3.3 h

Table 3
Results of the EC_{50} concentration tests on metconazole and thiacloprid for *Hyalella azteca*. a.s. = active substance, EC_{50} = half maximal effective concentration, SD = standard deviation, CI = confidence intervall.

a.s.	EC_{50}	SD	CI (lower / upper)	model
metconazole	0.9732	0.1795	0.6215 / 1.3249	Weibull (type 2)
thiacloprid	0.0117	0.0029	0.0059 / 0.0174	Weibull (type 1)

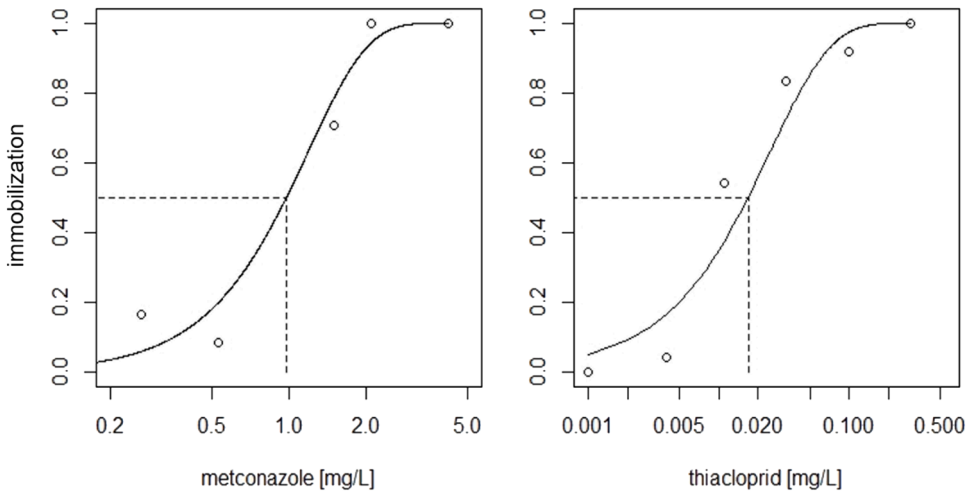

Fig. 1. Dose-response-curves on the immobilization of *Hyalella azteca* under increasing nominal concentrations of metconazole (left panel) and thiacloprid (right panel). Dashed lines indicate the respective half maximal effective concentration (EC_{50}).

Table 4
Toxic units (TU) based on the EC_{50} test results of metconazole and thiacloprid from day 0 (start) to day 10 (end) in the mobility tests with *Hyalella azteca* using different sediment types. metc. = meconazole, thiac. = thiacloprid, sumTU = sum of toxic units.

	without sediment				sediment type A				sediment type B			
	metc.	thiac.	combination		metc.	thiac.	combination		metc.	thiac.	combination	
			metc.	thiac.			metc.	thiac.			metc.	thiac.
0.0 sumTU												
start	0.0	0.0	0.0	0.0	0.01	0.0	0.01	0.0	0.0	0.0	0.0	0.0
end	0.0	0.0	0.0	0.0	0.01	0.0	0.01	0.0	0.01	0.0	0.01	0.0
0.25 sumTU												
start	0.29	0.33	0.14	0.08	0.34	0.18	0.19	0.10	0.24	0.28	0.12	0.14
end	0.18	0.39	0.10	0.10	0.14	0.12	0.15	0.06	0.20	0.04	0.13	0.03
0.5 sumTU												
start	0.61	0.66	0.28	0.16	0.65	0.44	0.33	0.21	0.57	0.50	0.26	0.27
end	0.38	0.85	0.20	0.20	0.22	0.27	0.34	0.15	0.33	0.08	0.20	0.06

Table 5
Mobility reduction (%) of *Hyalella azteca* after 10 days of treatment with meconazole, thiacloprid or the combination of both depending on sums of toxic units (sumTU) and sediment type. One sumTu corresponds to the half maximal effective concentration EC_{50} of *Hyalella azteca*.

		% reduction of mobility				
	sumTU	metconazole	thiacloprid	metconazole + thiacloprid	combined effect size	effect type
no sediment					**Ø 1.2**	**additive - slight synergistic**
	0	0	0	0	–	
	0.25	3	47	30	1.2	
	0.5	17	73	60	1.3	
	1	87	90	100	1.1	
sediment A					**Ø 1.2**	**additive - slight synergistic**
	0	0	0	0	–	
	0.25	3	10	7	1.1	
	0.5	17	17	20	1.2	
sediment B					**Ø 2.4**	**synergistic**
	0	3	3	3	–	
	0.25	3	0	7	2.3	
	0.5	47	7	67	2.5	

(time point −195) in the control treatment without sediment.

Following the time point of addition of metconazole + thiacloprid in the treatments, the control test series for the without sediment and with sediment type A dropped slightly in activity for 26.6 h (without sediment, time point 1595) and 43.3 h (sediment type A, time point 2595) and remained subsequently stable (ESM Fig. 1). In the corresponding metconazole + thiacloprid treatments, activity immediately dropped after pesticide addition and slightly recovered over the test duration for 50.7 h (without sediment, time point 3040) and 19.0 h (sediment type A, time point 1290) and subsequently remained stable at this level of activity below the control activity level (ESM Fig. 1). The test series with sediment type B showed both in the control and the treatment a constant increase in activity over the test duration (ESM Fig. 1). However, there is a visual drop in activity in the first 6 h that quickly recovers to control

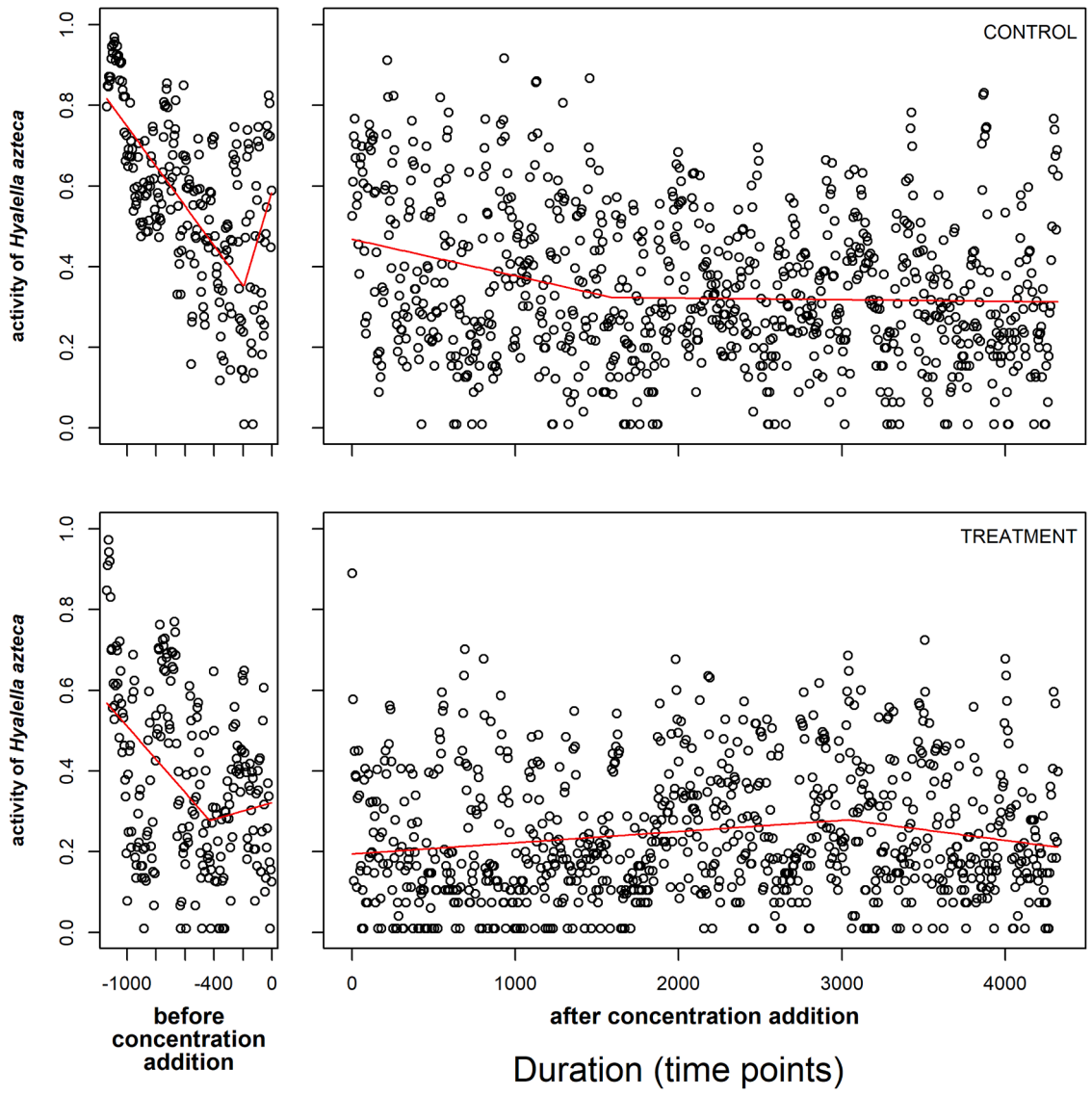

Fig. 2. Activity of *Hyalella azteca* over the duration of the experiment in the treatment without sediment before (left) and after (right) addition of the metconazole/thiacloprid mixture. Each time point corresponds to accumulated 5 min measurement of the average activity of three individuals. r^2-values: 0.33 (control before addition), 0.04 (control after addition), 0.14 (treatment before addition), 0.007 (treatment after addition). The results for sediment type A and B are presented in the Electronic Supplement.

activity level. Several strong periodic decreases are observable in the activity of the sediment type B treatment.

Trend activity was extracted by time series analysis from the raw data of the activity measurements (ESM Fig. 2). Trend activity was significantly lower in treatment than in the control for the test series without sediment and with sediment type A (Fig. 3). Trend activity was similar between control and treatment in the test series with sediment type B (Fig. 3). All comparisons of random activity between control and treatment for all test series were not significant (data not shown, Welch *t*-test, smallest $p>0.8$). Smoothed trend activities of the treatments with no sediment and with sediment type A relative to their controls showed, that the treatment effect occurred over the total duration of the experiment and only slowly recovered towards control activities to the end of the experiment, but still stayed below control activity (Fig. 4). The smoothed trend activity of the treatments sediment type B relative to control showed a consistent periodic behavior with times of higher and lower activity compared to control in the range of ± 10 % (Fig. 4).

ARIMA models of the extracted trend activity showed no increase in activity for the forecasted 16 h time period following the experiments (ESM Fig. 3). Note, however, that despite forecasted trend activity remained at the same level as the measured data, trend activity was already at a high level in the test series with sediment type B and further increases may not occur.

4. Discussion

4.1. Test system suitability

Amphipod species are sensitive to nutrient and pesticide pollution (Gerhardt et al., 2012). Particularly gammarid and hyalellid species have been proven to be more sensitive for neonicotid exposure than the daphnid standard test species in ecotoxicology (Morrissey et al., 2015). The water concentrations of the tested pesticide metconazole dropped in all treatments independently of sediment type. This indicates well known relocation of this substance to the sediment or moderately fast dissipation in water only systems (European Food Safety, 2006; Lewis et al., 2016). Contrary, water concentrations of thiacloprid remained stable in the no-sediment test system and only dropped when sediment

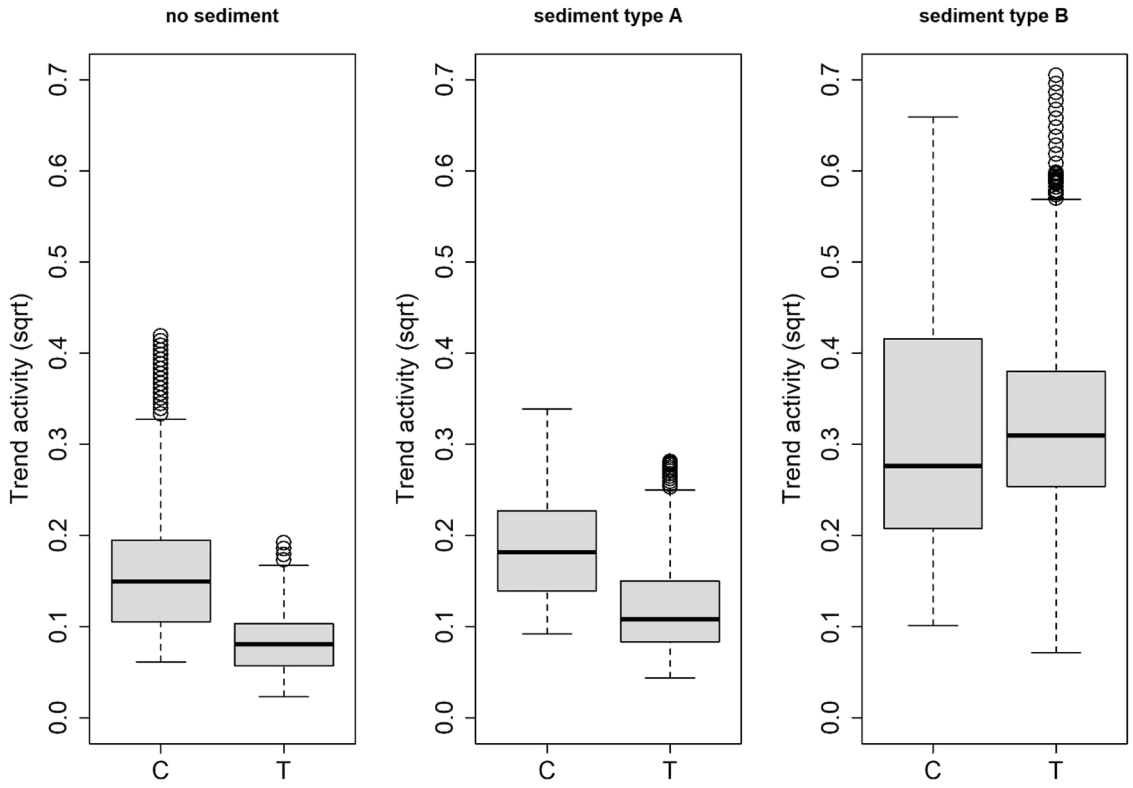

Fig. 3. Trend activity of *Hyalella azteca* between control (C) and treatment (T) for the different sediment types. The description of sediment types is given in Table 2.

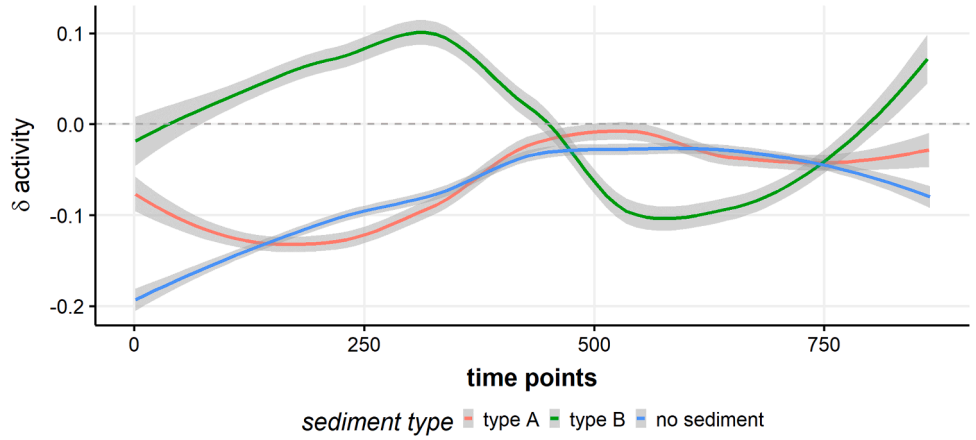

Fig. 4. Trend activity of *Hyalella azteca* relative to control over the duration of the experiment for the different sediment types. Each time point corresponds to accumulated 5 min measurement of the average activity of three individuals. Activity of zero represents no differences between treatment and control.

was present (European Food Safety et al., 2019). The test system used in this study only assessed overlying water concentrations but not pore-water concentrations, as this was analytically not possible. Therefore, it was also not possible to take in to account the time it takes for both compounds to equilibrate with sediment during the experiments. The observed differences in toxicity thus can also result from different equilibration dynamics of metconazole and thiacloprid in both sediment types, as particularly hydrophobic pesticides tend to partition between the aqueous phase of water and organic carbon in sediment. With this equilibration unknown in this test system, the current experimental design might more closely match a point-source runoff contamination event at a pristine or previously uncontaminated site for example in streams, where contaminants can be transported downwards with the flowing water.

Recent studies showed that pore water concentrations could be more representative for the toxicity of *H. azteca* than concentrations of the overlaying water (Hiki et al., 2021). However, it is well known that *H. azteca* changes its burrowing behavior depending on sediment or toxicant type (Doig and Liber, 2010; Whiteman et al., 1996), and hence overlaying water exposure can become a more relevant exposure route. Additionally, the MFB system (which only assessed toxicity resulting from concentrations in the overlyaing water) yielded traceable results in terms of activity behavior changes using the test species *H. azteca* as shown before for other amphipod species (Felten et al., 2008; Gerhardt, 2020; Gerhardt et al., 2012).

The EC_{50} of metconazole and thiacloprid for *H. azteca* in this study are considerably lower than reported elsewhere for *Daphnia magna* (Lewis et al., 2016), and for thiacloprid is slightly lower compared to

other results on this amphipod species (0.024 mg / L (Bartlett et al., 2019) and 0.027 mg / L (Raby et al., 2018)). However, the regulatory acceptable concentration derived during the approval process of this pesticide (0.004 µg/L, (UBA, 2020)) is still considerably lower than the EC_{50} derived here or the non-lethal 0.125 TU concentration of 0.0015 mg/L used in the mobility tests. Therefore, environmental risks of the concentrations used still cannot be excluded despite immobilization was not observed. Reports of metconazole EC_{50} to *H. azteca* could not be found in literature but the regulatory acceptable concentration of 0.219 µg/L (BVL, 2017) similarly indicates environmental risks of the metconazole concentrations lower than EC_{50} used in the mobility tests. Additionally, 48-h EC_{50} values were used to determine the equipotent mixture concentrations for the 3-day (activity) and 10-day (mobility) tests. Due to potential differences in toxicokinetics of metconazole and thiacloprid, 48-h exposure doesnt predict directly toxicity in longer exposure. However, as the aim was to detect potential effects on *H. azteca* behavior at non-lethal concentrations and no test organisms died during the activity tests, the test system can be seen suitable in general to detect behavioral changes over periods of several days but would require validation if long-term behavioral changes need to be detected.

4.2. Impact of sediment characteristics on the test results

The results showed a change from additive effects to synergism in the mobility tests for sediment B. Combined synergistic effects have been shown in crustacean species before but using daphnids as test species (Cedergreen et al., 2006; Shahid et al., 2019). Azolic fungicides, such as metconazole, have been shown to produce synergistic effects in toxicant mixtures (Cedergreen, 2014). Additionally, environmental stressors may act highly synergistically with toxic substances (Liess et al., 2019). The higher contents of total carbon in sediment B compared to sediment A may have contributed to the synergistic effects of sediment B as sediment carbon content has been shown to affect pesticide toxicity to *Hyalella* sp. (Nebeker et al., 1989). However, this increase in effect size to synergism was not visible in the results of the activity behavior tests. Here in turn activity in sediment type B increased in both the control and the treatment while the activity in the treatment without sediment and sediment A decreased significantly. The positive effect of sediment B might be due to the high organic content and the resulting buffering of pesticide concentrations in the water column. Additional to such buffering effect, an "indirect feeding effect" could also explain the increase in activity as it was observed both in the control and the treatment. The organic suspended particles in the sediment B may have provided an additional food source, albeit only to a limited extent, that in contrast to the other test series may have increased the activity of the individuals. For example, the burrowing behavior of *H. azteca* was affected by the addition of food to the sediment of contaminant test systems (Sheahan and Fisher, 2012).

Our results on the increased effect size shifting from additive to synergistic effects are based on the exposure of the individuals to spiked overlaying water while pore-water concentrations have not been measured and burrowing of the individuals was either not observed (mobility test system) or not possible (activity test system). Burrowing of the test individuals would potentially offer a further exposure pathway to them, by either exposing them to dissolved concentrations of toxicants in pore-water or to concentrations sorbed to suspended particles (Doig and Liber, 2010). However, particularly sandy sediments could result in reduced sediment and sediment pore-water contact by *H. azteca* (Doig and Liber, 2010) and *H. azteca* was shown to limit its exposure to sediment toxicants when pore-water concentrations exceed the concentrations of the overlaying water (Whiteman et al., 1996). Therefore, our result concerning the shift from additive effects to synergism may hold only true for exposure via overlaying water concentrations, whereas effect sizes of mixture contaminants in pore-water may potentially be altered differently.

Impacts of sediment characteristics on activity behavior may also impair the results of standardized test systems used for this organism (Doig and Liber, 2010). Tests using *H. azteca* normally follow guidelines such as (Ingersoll et al., 2005; Novak and Taylor, 2017; USEPA, 2000). While a large part of tests with *Hyalella* sp. conducted in the past used spiked or environmental sediments, also overlying water and water only exposure tests have been performed (Dutra et al., 2009). Mainly tests using natural soils may suffer from deviations due to variable organic contents (Doig and Liber, 2010), and here, particularly results from water sediment test systems using spiked water should be interpreted with caution when used in a behavioral activity context.

4.3. Adaption in behavior

Hyalella azteca is not a single species but a highly diverse, cryptic species complex (Witt and Hebert, 2000; Witt et al., 2006). It is well known that species of the *H. azteca* complex have developed resistance to certain pesticides of the group of pyrethroid or organophosphate insecticides (Gamble et al., 2023; Major et al., 2018). Pyrethroid resistant individuals of *H. azteca* are known to survive at pyrethroid concentrations of two orders of magnitude higher than non-resistant wild-type individuals (Gamble et al., 2023; Muggelberg et al., 2017; Weston et al., 2013). Currently, it is unknown if such selective pressure for insecticide resistance could also apply to other pesticides such as azolic fungicides, but for the neonicotinoid insecticide imidacloprid no resistance pattern was found in populations that showed pyrethroid or organophosphate resistance (Gamble et al., 2023). Also, it has been shown that pyrethroid resistant individuals of *H. azteca* show greater sensitivity to other chemical stressors such as carbaryl and DDT (Gamble et al., 2023) or copper (II) sulfate and sodium chloride (Heim et al., 2018). The sensitivity in activity behavior towards metconazole and thiacloprid exposure may thus be an effect related to unknown pyrethroid resistance of the studied individuals and not visible in wild-type populations of *H. azteca*.

4.4. Further research needs

The above described resistance patterns of *H. azteca* show the capacity of this species to adapt to anthropogenic contamination of its habitat. Such adaption could also occur in activity behavior under constant and chronic exposure to chemical stressors. In our tests, particularly the presence of sediment containing high carbon, nitrogen and phosphorous contents enabled *H. azteca* individuals to show again activity behavior comparable to control conditions after three days of contaminant exposure. The results of our ARIMA modeling approach show that this activity behavior will remain constant after recovery to pre-exposure levels at least for a time period of 16 h following 72 h of exposure. However, our results are not suitable to assess any potential changes in activity behavior over longer periods or within the life cycle of *H. azteca*. The positive effect of the sediment B on activity behavior might result from a transfer of the metconazole and thiacloprid to the sediment. Therefore, further studies are needed that test potential activity behavior impairments in environments where the individuals are in direct contact with the sediment and their movement space is not restricted to measurement chambers located above the sediment.

Additionally, shredders such as *H. azteca* break up leaves and other plant parts into smaller pieces which is an important ecosystem function and part of the nutrient cycling in freshwaters. The rate at which this process occurs is often interpreted as an indicator of the ecological balance of a water body (Clarke et al., 2008; Kominoski et al., 2013). If mobility and activity are lowered, shredding rate is likely lowered as well and the ecosystem functioning may suffer. Such relationship has not yet been investigated, but toxicity approaches such as GamTox (Gerhardt, 2011) might provide a suitable framework to test such simultaneous impairments of activity and shredding behavior in future.

Mobility and activity of *H. azteca* are largely affected by the exposure

to pesticides, which is mediated by the structure of the sediment. Sediment type may compensate for a certain amount of shredding activity loss induced by pesticide exposure when activity behavior of individuals is affected to a lower extent. Hence, effects of agricultural pesticide usage on freshwater ecosystems may differ largely between water bodies, particularly in freshwater types characterized by a huge variability in sediment characteristics such as small lentic water bodies (Lischeid et al., 2017; Nitzsche et al., 2017; Reverey et al., 2016). Additionally, the type of pesticide exposure also affects the behavior of shredders in terms of food consumption. Azolic fungicides are known to influence the degradation of leaves in various ways. Either leaf degradation by microorganisms was slowed under propiconazole exposure which in turn led to increased shredding and food consumption by the amphipod *Gammarus pulex* and the caddisfly *Halesus radiatus* as a result of lowered leave nutrient contents due to decreased microbial biomass (Rasmussen et al., 2012). On the other hand, food intake of the amphipod *Gammarus fossarum* and thus the shredding of plant biomass was either reduced (Zubrod et al., 2014) or avoided (Bundschuh et al., 2011) under tebuconazole exposure which limits ecosystem functioning. Hence, sediment type and contaminant type (even within the same group of e.g. azolic fungicides) in combination may have the potential to either produce antagonistic, additive or synergistic effects on shredding behavior of aquatic organisms, which needs further studies in future to provide clarification on effect sizes.

5. Conclusion

The current study showed that mobility and activity of *H. azteca* are largely affected by the exposure to single and multiple pesticides, which is mediated by the structure of the sediment. Environmental risk assessment during pesticide authorization is performed only for single active ingredient toxicity and in tiered approaches, with higher tier studies, such as species sensitivity distributions or aquatic mesocosm tests, conducted under stable conditions. However, ecosystems can show an enormous variability in sediment and water biogeochemistry. Particularly small lentic water bodies show high dynamics in biogeochemical processes with resulting fast transformation processes of the involved chemical compounds (Lischeid et al., 2017; Onandia et al., 2018; Reverey et al., 2018). Pesticide entries in systems with such high dynamics and variability may therefore direct different ecological reactions depending on the spatial and temporal location of contamination. However, similar to behavioral endpoints in ecotoxicological assessments, current pesticide regulation for example in the European Union does not account for such unpredictability.

Author declaration

I wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

I confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed.

I confirm that I have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In doing so I confirm that I have followed the regulations of my institution concerning intellectual property.

I understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. I confirm that I have provided a current, correct email address which is accessible by the Corresponding Author and which has been configured to accept email from stefan.lorenz@julius-kuehn.de.

CRediT authorship contribution statement

Stefan Lorenz: Writing – review & editing, Writing – original draft, Visualization, Validation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

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