



Article

Polystyrene Microplastics Modulate the Toxicity of the Hydrophilic Insecticide Thiacloprid for Chironomid Larvae and Also Influence Their Burrowing Behavior

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Abstract: As there is still little knowledge of interactions between microplastics (MP) and hydrophilic compounds, we propose ways the toxicity of hydrophilic pesticides can be modulated by MP, when sorption can be excluded. Larvae of *Chironomus riparius* were exposed to thiacloprid (TH, 1 µg/L) and polystyrene microplastic particles (PS; <50 µm; 150,000 and 1,000,000 particles/L) for 96 h, solely or in co-exposure. Burrowing behavior and mortality were observed. Larvae in treatments containing PS established themselves quicker in the sediment and kept the ability to rebury for a longer time compared to control and TH, respectively. While TH elevated the mortality, exposure to PS alone did not affect the survival of the larvae. In co-exposure of TH and PS, a concentration of 150,000 particles/L significantly reduced the toxicity of 1 µg/L TH after 96 h, an effect that was not observed at 1,000,000 particles/L. Therefore, we hypothesize that this modulation of the toxicity of TH eventually may have resulted from a combination of a ‘protective MP layer’ in the gut and a higher retention time of particles in larvae exposed to 150,000 particles/L than in those exposed to 1,000,000 particles/L due to the lower number of ingestible particles in the former.

Keywords: pesticide; neonicotinoid; ecotoxicology; microplastics; chironomid; polystyrene

1. Introduction

Although the first sightings of small plastic particles in oceans were already described in the 1970s [1], intense research on so-called microplastics (MP, plastics 1 to <1000 µm in size [2]) has started just about 15–20 years ago and initially focused on marine ecosystems. However, in recent years, the number of studies on MP in freshwater and even in terrestrial ecosystems has steadily increased [3–8]. Today, MP can be found ubiquitously distributed in aquatic and terrestrial environments around the world, even at the most remote locations like Arctic waters [9], lakes in northern Tibet [10], the deep sea [11], and the Austrian Alps [12]. In order to assess the potential risks of MP to humans and the environment, it must be emphasized that the term ‘MP’ does not stand for a type of stressor as such but covers many different types of polymers in various sizes, shapes, and colors, and with different chemical and physical properties. For this reason, its effects on biota can also be as diverse as its properties. Studies showed that MP can be ingested by many

aquatic organisms across different trophic levels due to their small sizes, from fish [13–15] to filter-feeders [16–18], deposit-feeders [17,19], or even by zooplankton [20,21]. Physical effects such as blockage of the digestive tract [20], accumulation [22], or tissue translocation of very small particles [23,24] are conceivable. Hazardous polymer additives like endocrine-disrupting chemicals or pigments leaching from MP might also pose a potential threat to exposed organisms [25–27]. Various toxicity studies demonstrated effects of MP on different endpoints like reproduction [28,29], growth [28–32], emergence [31–33], oxidative stress [34], and assimilation efficiency [35] in different aquatic invertebrates. In addition to the direct effects of MP, interactions with other substances have received increasing attention. One hypothesis proposes that MP serve as vectors to transfer hydrophobic organic chemicals (HOCs) into biota. While MP can adsorb and desorb HOCs, there are many physicochemical conditions that can influence these processes like changes in the pH-level, concentration of the pollutant, or the polymer type [36,37], but there are also gaps in knowledge that need to be addressed further. However, the importance of this conceivable path of exposure is currently considered to be rather low compared to exposure to HOCs via natural particles, food, prey, or water [38,39]. Nevertheless, numerous studies showed that the adsorption of HOCs to MP could both increase and decrease the bioavailability of chemicals and thus modulate their effects [40–43], whereas interactions of MP and hydrophilic substances are still rarely examined [33]. Therefore, the aim of this study was to investigate whether the toxicity of a hydrophilic pesticide can also be modulated by MP.

Due to its emerging importance, worldwide application and the rising ecological concerns regarding neonicotinoids, we used the highly selective insecticide thiacloprid ($\log P = 1.26$; [44]) which belongs to the second generation of neonicotinoids. It interferes with the nervous system of insects, acting as nicotinic acetylcholine receptor (nAChR) agonist and is not supposed to adsorb to polystyrene microplastic particles (PS).

As test organisms, we chose the non-biting midge *Chironomus riparius* Meigen, 1804 (syn. *Chironomus thummi* Kieffer, 1911), which occurs in high numbers in freshwater communities [45]. This species is widely distributed in European waters and of fundamental importance in aquatic food webs. It passes three aquatic stages (egg, four larval stages, pupa) before the adult midge emerges into the air for mating [46]. *C. riparius* serves as a well-examined and widely used standard test organism in ecotoxicology to examine the toxicity of sediments and water [47,48], and has been proven to be very sensitive to thiacloprid exposure [49–51]. Thus, in our study, we characterized the sorption properties of thiacloprid to PS and the effects of both compounds, isolated and in combination, on the mortality and the burrowing behavior of 3rd to 4th instar larvae (L3–L4) of *C. riparius*.

2. Materials and Methods

2.1. *Chironomus Riparius*

Harlequin flies used for this study were cultured in a thermostat-controlled chamber (20 °C; 12 h/12 h light/dark regime). They were kept in tubs, containing annealed quartz sand (grain size 0.2–0.6 mm; filling height: 4 cm) and a mixture of filtered tap water (filtered with an iron- and activated carbon filter; Filwatec, Bad Liebenzell, Germany) and deionised water, which was constantly aerated. All containers were covered with fly screens (65 × 55 × 120 cm) to enable emerged adults to swarm and mate. Larvae were fed thrice a week with Tetra Min fish flakes (Tetra GmbH, Melle, Germany), and 50% of the water was exchanged once a week.

2.2. Microplastics

2.2.1. Pre-Experiment with Fluorescent PS Particles

In a pre-experiment, chironomid larvae (L3–L4) were exposed to fluorescent spherical PS smaller than 50 µm (PS-FluoRot-50, mean diameter 48.2 µm, microParticles GmbH, Berlin, Germany, Figure S1), in order to verify whether the test animals are able to ingest the particle fraction. After several hours of exposure, we recorded light microscopy images of living larvae (Axioscope 2, Carl Zeiss Microscopy Deutschland GmbH, Oberkochen,

Germany) in a bright-field and with a DAPI-filter. As in the gut contents of *C. riparius*, large silt particles have been found [52]; Scherer, et al. [53] described that *C. riparius* larvae can ingest particles between 1–90 µm depending on the mentum size at a given head capsule width defining the respective life stage, and suggested that large L4-larvae may be able to ingest even larger particles. Vos [54] claimed that L4-larvae were able to ingest particles up to 150 µm.

2.2.2. Polystyrene Particles Used in Main Experiments

Since larvae of *C. riparius* feed mainly on surficial sediment [52], sedimentation of PS was ensured by choosing a particle density >1 g/mL.

For the main experiments, colorless polystyrene granules (Polystyrol 158 K, BASF, Ludwigshafen, Germany) were cryogenically milled (CryoMill, Retsch, Haan, Germany) to obtain irregularly shaped PS (density 1.05 g/mL). Additional information on particle preparation is provided by Eitzen, et al. [55].

PS were suspended in ultra-pure water (without addition of surfactants), filtered with a micro-sieve (50 µm nominal mesh-size, polyamide monofilament, Figure S2) and particle concentrations in the permeate were analyzed with a particle counter (SVSS, PAMAS, Rutesheim, Germany) by light extinction in a laser-diode sensor (type HCB-LD-50/50). Exemplary particle size numbers, distributions and an SEM image are provided in Figure S3 and by Schmieg et al. [56,57]. Defined volumes of the highly concentrated stock suspensions were then diluted for experiments.

2.3. Sediment

The quartz sand used as test sediment (Aquarium sand, Eggert Luchterhand GmbH, Achim, Germany) was washed several times with deionized water and heat-treated at 250 °C for 8 h. The grain size was specified as 0.2–0.6 mm according to the manufacturer, which was confirmed by microscopic images (stereomicroscope ‘Stemi 2000-C’, Carl Zeiss Microscopy Deutschland GmbH, Oberkochen, Germany, Figure S4). Due to this size, the sediment cannot be ingested by the larvae.

2.4. Test Solutions

Before each test run, a stock solution containing 5 mg/L thiacloprid (CAS no. 111988-49-9; analytical standard, Sigma Aldrich, Germany) was prepared with distilled deionised water and stirred for 24 h in the dark. All treatments containing thiacloprid were diluted from this stock accordingly with aerated filtered tap water to a target concentration of 1 µg/L thiacloprid. This concentration was chosen on the basis of the results of Lorenz, et al. [51].

PS were pre-portioned in suspensions and added to the test solutions to obtain the final concentration of 150,000 or 1,000,000 particles/L. Besides the control treatment, 1 µg/L thiacloprid and PS were tested both alone and in co-exposure (control, TH, PS 150,000, PS 1,000,000, MIX 150,000, MIX 1,000,000).

2.5. Acute Toxicity Test

Generally, tests were conducted according to OECD 235 [58], however, modified in respect to the use of sediment, the age of larvae and exposure time. To ensure that all larvae used in this study were at the same age at the start of the experiment, fresh egg clutches (<24 h) were taken out of the breed and reared in a separate container for 23 days prior to the start of the test. Age stages were visually determined according to Day, et al. [59] using a stereomicroscope, and only instars L3 and L4 were used. All test runs were conducted in a thermostat-controlled chamber at 20 °C and test solutions were tempered and aerated to avoid stress. Depending on the number of larvae that could be obtained for the individual test runs, 10–48 replicates were tested for each treatment. A total of seven test runs were performed. All details of the experimental design are provided in the Supplementary Materials (Table S1).

Prior to the experiments, test vessels (glass cylinders, diameter 7 cm, height 6.5 cm) were filled with 30 g of annealed quartz sand (0.2–0.6 mm) and saturated with the corresponding test solutions for at least two days. Mixed treatments (MIX 150,000 and MIX 1,000,000) were only saturated with thiacloprid, and PS-treatments only with filtered tap water, to ensure accurate particle concentration. Subsequently, the solutions were carefully removed, leaving the sediment in the glass cylinders.

Starting the experiment, all vessels were refilled with 100 mL of filtered and aerated tap water (control) or the corresponding test solutions. We waived aerating the test solutions during exposure to avoid turbulences, and because chironomids are regarded as highly tolerant of hypoxic conditions [46]. Five larvae of *C. riparius* were added to each vessel using a blunt glass pipette. Subsequently, the glasses were covered with parafilm and placed randomly on a table in the thermostat-controlled chamber at 20 °C. Throughout the test, larvae were not fed to avoid interactions between food, PS and the pesticide, and to prevent oxygen depletion. Larvae were exposed for 96 h, and mortality of each individual was checked every 24 h. For this purpose, burrowed animals were carefully manually excavated. Larvae were assigned ‘dead’ either after remaining immobile for over 30 s despite mechanical stimulation and removed from the vessels, or when not being recovered from the sediment.

2.6. Burrowing Behavior

Since Pestana, et al. [60] showed the influence of imidacloprid on the burrowing behavior of chironomid larvae, which was confirmed by our own observations during the initial few test runs, we visually recorded the behavior of surviving larvae as ‘burrowed’ or ‘not burrowed’. Therefore, the vials were carefully surveyed daily in advance of checking mortality.

2.7. Chemical Analyses

2.7.1. Analyses of Thiacloprid

Composite samples of the respective treatments containing thiacloprid were collected at the beginning and end of the experiment and stored at −20 °C in PE-centrifuge tubes (Sarstedt AG & Co. KG, Nuembrecht, Germany) prior to chemical analysis. Thiacloprid concentrations in the samples were measured via LC-MS/MS. Instrument operation, acquisition and evaluation of the acquired LC-MS/MS, and further detailed information are described in the Supplementary Materials (Tables S3–S5). Maximum possible recovery rate was determined for samples from a 1 µg/L thiacloprid solution, which served as a point of reference for the recovery rates of thiacloprid measured in the experimental samples.

2.7.2. Sorption Behavior of Thiacloprid to PS

In a batch experiment, sorption behavior of thiacloprid to PS in synthetic freshwater as a sample matrix was chosen. For the determination of sorption isotherms, an indirect method (extraction of aqueous phase) was applied and subsequent measurements were performed by means of the LC-MS/MS system. See supplements for further details on methodology.

2.8. Statistical Analyses

To describe changes in burrowing behavior and mortality rates across exposure treatment and exposure duration, we performed generalized linear mixed models (GLMM) with the glmmTMB package [61] in R (version 4.0.3, [62]). Predictor variables were the exposure *treatment* with six levels (control, PS 150,000, PS 1,000,000, MIX 150,000, MIX 1,000,000, and TH), and *time*, since exposure as a gradient along the four measured time points (24 h, 48 h, 72 h, 96 h) was implemented as a z-standardized numeric predictor. We added the *treatment-by-time* interaction to characterize differences in temporal responses between treatments. Given that each replicate was measured at multiple exposure durations, we added *vial-ID* as a random intercept to avoid pseudoreplication.

We used variograms as implemented in the R package geoR [63] to check for temporal autocorrelation across the four consecutive measurements but did not detect any. To assess model performance and optimize final models, we explored family-standardized residuals extracted with the R package DHARMa [64] within and among covariate levels, and conducted posterior predictive checks on simulated data following the procedure outlined in Korner-Nievergelt, et al. [65].

Our first model investigated variation in the proportion of buried chironomids, which derived from the raw counts of burrowed and non-burrowed individuals per replicate vial. A binomial model suffered from overdispersion and zero-inflation, and thus generated overly confident coefficient estimates. We successfully accounted for these by instead using the betabinomial family and adding a zero-inflation term that modelled the *treatment*-specific access of zero counts [65].

The second analysis checked variation in mortality rates, again derived from counts of dead vs. alive animals per replicate. We here stayed with a binomial family, despite modest signs of underdispersion, which could not be solved by changing to a betabinomial family or adding a zero-inflation formula. We accepted the resulting overestimation of confidence intervals (CI), because this leads to more conservative interpretation of results.

We provide coefficient and effect size estimates and their confidence intervals, but refrain from presenting *p*-values and their associated evaluation of binary null hypotheses in accordance with current recommendations for unbiased statistical reporting e.g., Halsey, et al. and Berner and Amrhein [66,67].

3. Results

3.1. Pre-Experiment with Fluorescent PS

We confirmed that the larvae were able to ingest the fluorescent PS, as they could be detected in the gut as well in the bright field as under fluorescent light using a DAPI filter (Axioscope 2, Carl Zeiss Microscopy Deutschland GmbH, Oberkochen, Germany, Figure 1).

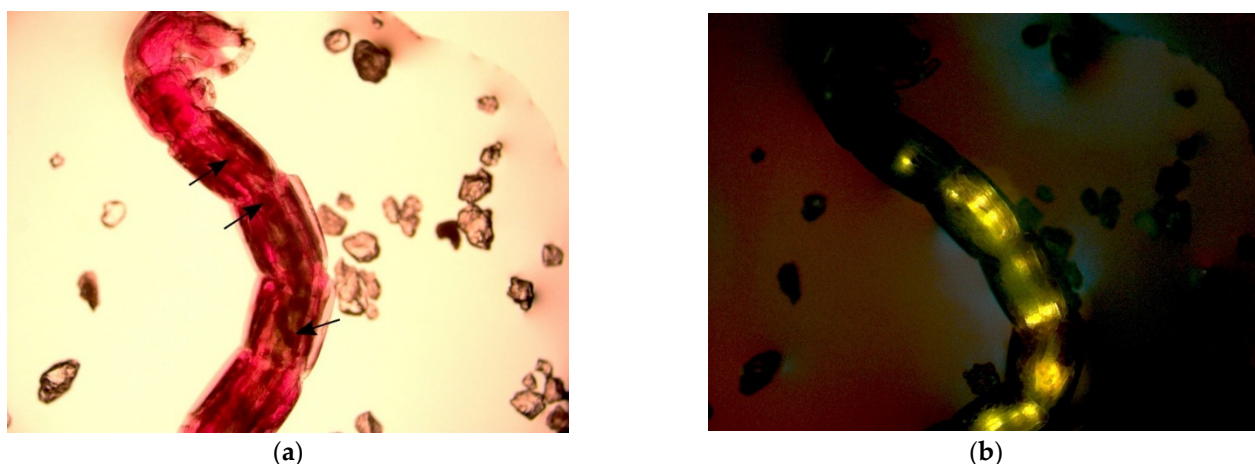


Figure 1. Living *C. riparius* larva with ingested fluorescent PS (48.2 μm); (a) bright-field image; (b) corresponding fluorescence image (DAPI). Fluorescence is localized in distinct spots in (b) that can be homologized with the particles in the gut lumen, visible in the bright-field LM image (a) (a selection of particles is marked with arrows). The halos around the fluorescence spots are of optical origin and derive from overshadowing and most likely not from leaching as reported e.g., by Catarino, et al. [68]. Non-fluorescent particles are quartz sand particles from the sediment.

3.2. Acute Toxicity Test

In all test runs, mortality in controls remained well below 10% thus corresponding to the natural mortality rate. Therefore, all tests can be considered as valid according to the criteria of OECD 235 [58]. Remarkably, in six out of seven test runs, mean mortality after 96 h was lower for larvae in MIX 150,000 than for those in TH. Consistently, a

substantial fraction of the observed variation in the proportion of dead chironomids could be attributed to experimental treatments and exposure duration (GLMM $R^2_{\text{marginal}} = 0.439$, $R^2_{\text{conditional}} = 0.481$). Mortality was close to zero in all treatments after 24 h exposure, but further rates of change strikingly varied. Changes in mortality over exposure time were close to zero in the absence of thiacloprid, i.e., in control and both PS treatments (Figure 2, Table 1), resulting in very low and broadly overlapping average mortalities close to 2% even after 96 h exposure (Table 1). In contrast, exposure to thiacloprid in the TH and both MIX treatments resulted in substantial mortality increases across exposure durations, with a 4–6% increase in the odds of being dead per hour of exposure. As a result, all three treatments reached average mortalities well above 25% after 96 h exposure (Table 1). Here, mortality was similarly high in the TH treatment and in MIX 1,000,000 (note similar ranges in Table 1), while mortality in MIX 150,000 was at the lower end of the other two treatments. There was no overlap in 95% CI with TH and only mild overlap with MIX 1,000,000 (Table 1). In summary, the concentration of 150,000 PS particles/L clearly reduced the toxicity of 1 µg/L thiacloprid on *C. riparius*, but this effect was not concentration-dependent as it vanished at the higher particle concentration of MIX 1,000,000.

Table 1. Regression coefficients for the change in the proportion of dead chironomids per treatment over exposure durations, and the resulting estimates for the final exposure timepoint (96 h).

Exposure Treatment	Regression Estimates for Exposure Duration					% Dead After 96 h	
	Coefficient Estimate *	SE	Lower 95% CI	Upper 95% CI	Odds-Ratio *	Estimate	95% CI
Control	0.0234	0.0054	0.0128	0.0341	1.024	2.4	1.5–3.9
PS 150,000	0.0159	0.0065	0.0031	0.0287	1.016	1.8	0.9–3.1
PS 1,000,000	0.0434	0.0221	>−0.001	0.0868	1.044	1.3	0.4–3.7
MIX 150,000	0.0449	0.0031	0.0388	0.0511	1.046	27.9	22.5–33.8
MIX 1,000,000	0.0596	0.0067	0.0465	0.0727	1.061	37.7	26.5–50.2
TH	0.0553	0.0027	0.0499	0.0608	1.057	45.3	39.4–51.6

* Coefficient estimates of the betabinomial GLMM display the predicted change in log-odds per hour, their exponent the odds-ratios. From odds-ratios, we can derive the proportional change in the odds of being dead per unit time. To exemplify, the odds-ratio of 1.057 for the TH treatment implies that the proportional change in odds is $1.057 - 1 = 0.057$. Hence, the odds of being dead increased by approx. 5.7% per hour exposure.

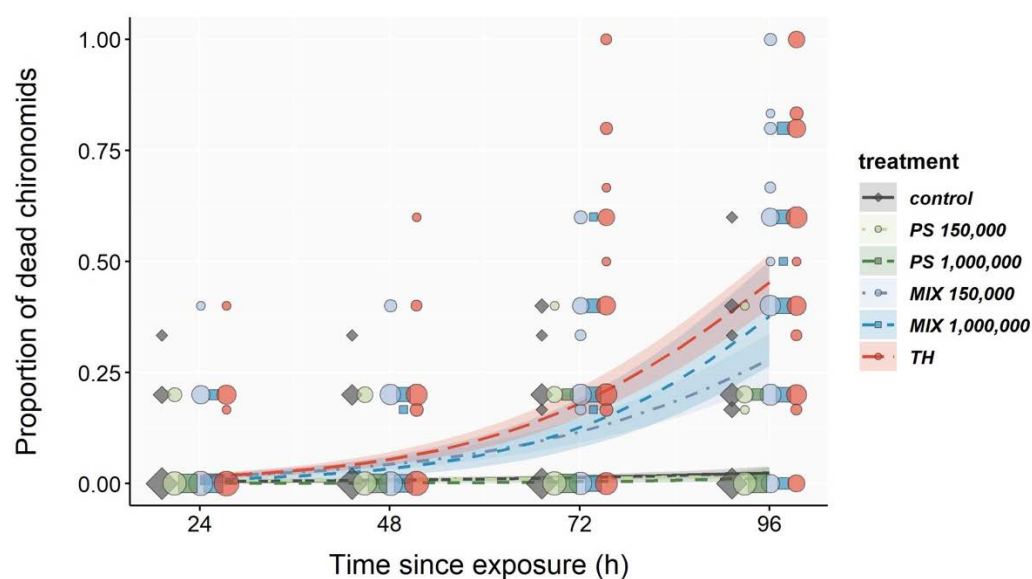


Figure 2. Proportion of dead chironomids, as given in the raw data (points) and predicted by the binomial GLMM (dashed curves with their 95% CI). Point size scales with the number of replicates that share identical values (range: $n = 1$ to $n = 115$ from smallest to largest dot size).

3.3. Burrowing Behavior

Similar to mortality, experimental exposure treatments substantially varied in the temporal development of chironomid burrowing behavior (GLMM $R^2_{\text{marginal}} = 0.509$, $R^2_{\text{conditional}} = 0.579$, Figure 3). In the absence of thiacloprid, i.e., in the control and both PS treatments, the proportion of burrowed chironomids remained rather high and almost stable across exposures, with estimated regression coefficients close to zero (Table 2, Figure 3). Burrowing activity in solutions containing PS initially even exceeded that observed in ‘clean’ control water, and this difference was maintained under exposure to 150,000 particles/L until the end of the experiment (Table 2, Figure 3). In contrast, exposure to thiacloprid, i.e., in the TH and both MIX treatments, generally induced a rapid decline in the odds of being buried (Table 2, Figure 3).

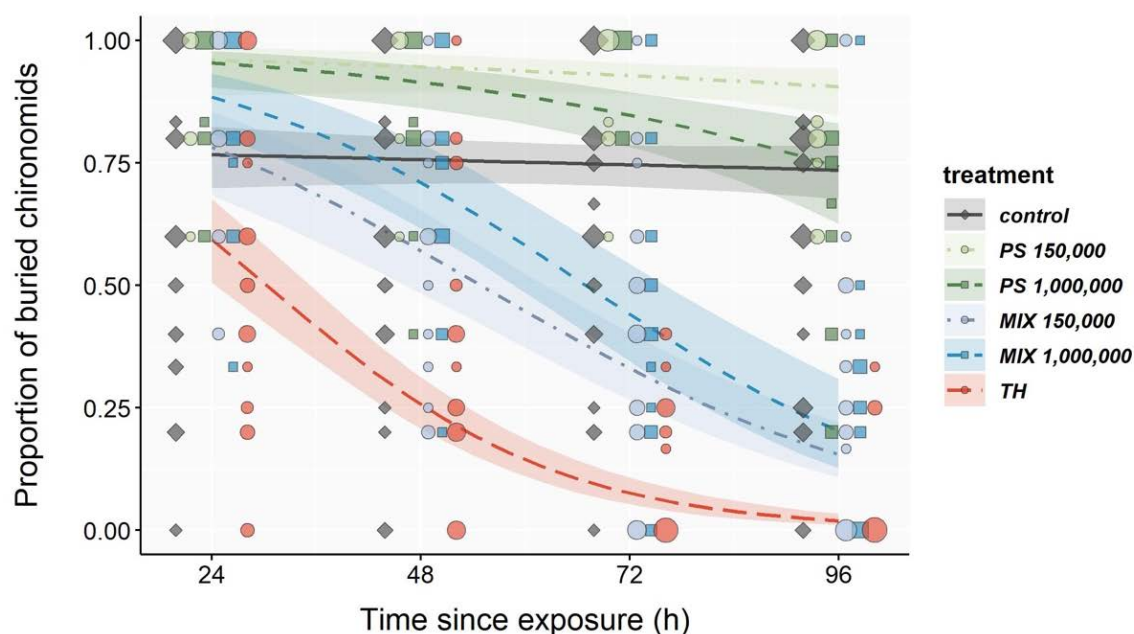


Figure 3. Proportion of buried chironomids as given in the raw data (points) and predicted by the betabinomial zi-GLMM (dashed curves with their 95% CI). Point size scales with the number of replicates that share identical values (smallest dot size corresponds to $n = 1$, the largest to $n = 61$).

Table 2. Regression coefficients for the change in the proportion of buried chironomids per treatment over exposure durations, and the resulting estimates for the final exposure timepoint (96 h). For further explanations see Table 1.

Exposure Treatment	Regression Estimates for Exposure Duration					% Buried After 96 h	
	Coefficient Estimate	SE	Lower 95% CI	Upper 95% CI	Odds-Ratio	Estimate	95% CI
Control	−0.0064	0.0034	−0.0131	0.0001	0.994	73.1	67.6–78.1
PS 150,000	−0.0128	0.0092	−0.0308	0.0053	0.987	89.7	83.9–93.5
PS 1,000,000	−0.0266	0.0069	−0.0401	−0.0130	0.974	73.5	62.4–81.9
MIX 150,000	−0.0428	0.0050	−0.0527	−0.0328	0.959	16.0	11.4–22.1
MIX 1,000,000	−0.0452	0.0060	−0.0569	−0.0334	0.956	21.4	14.1–31.4
TH	−0.0575	0.0055	−0.0684	−0.0466	0.944	2.3	1.3–4.0

However, this decline was clearly lower under a combined exposure to thiocloprid and PS in both MIX treatments compared to exposure to identical thiocloprid concentrations in the absence of PS (TH) (Table 2, Figure 3).

These differentiated temporal patterns resulted in a clear separation among treatments at the end of the exposure treatment (96 h, Table 2, Figure 3). Only around 2% of the chironomids still exhibited burrowing behavior under TH exposure, and around 15–20% after exposure to both MIX treatments. In contrast, proportions of burrowed individuals remained high in a 70–90% range in both PS treatments as well as in the control (Table 2). For details on experimental design see Table S2 in the Supplementary Materials.

We observed that the larvae re-burrowed faster and to a larger extent in the presence of PS. This pattern was also evident in the treatments containing thiocloprid (MIX 150,000, MIX 1,000,000).

3.4. Chemical Analyses

3.4.1. Thiocloprid

Chemical analysis showed only slight variations in the thiocloprid concentrations during the course of the experimental period. Analysis of a thiocloprid solution (nomi-

nally 1 µg/L), stored in PE-centrifuge tubes at −20 °C, showed an average recovery of 0.770 ± 0.022 µg/L ($n = 3$). Relative to this value, all measurements of thiacloprid samples from the experiments were also converted into relative average recovery rates, which varied between 66 and 88% (Table 3).

Table 3. Results of thiacloprid analysis: n = number of tests runs where composite samples were taken at the beginning (top row) and at the end (bottom row) of a test; third column of table: mean values + standard deviation of samples taken at the beginning and at the end of different test runs. Fourth column: mean values + standard deviation of relative average recovery rates in percentages; TH = 1 µg/L thiacloprid; MIX 150,000 = 150,000 PS/L + 1 µg/L thiacloprid; MIX 1,000,000 = 1,000,000 PS /L + 1 µg/L thiacloprid.

Treatment	Sample Size (n_{Start}) (n_{End})	Thiacloprid Conc. [µg/L]; (MV + SD)	Thiacloprid Rel. Recovery Rate [%]; (MV + SD)
TH	($n = 4$)	0.675 ± 0.170	87.66 ± 22.08
	($n = 5$)	0.552 ± 0.223	71.69 ± 28.96
MIX 150,000	($n = 4$)	0.505 ± 0.241	65.58 ± 31.17
	($n = 5$)	0.610 ± 0.101	79.22 ± 13.12
MIX 1,000,000	($n = 2$)	0.555 ± 0.025	72.08 ± 3.25
	($n = 2$)	0.625 ± 0.075	81.17 ± 9.74

3.4.2. Sorption

For the PS in synthetic freshwater, no sorption of thiacloprid could be determined (Figure 4). This is most likely caused by the rather polar properties of thiacloprid and thus its higher affinity to the polar aqueous phase compared to the non-polar polymer surface.

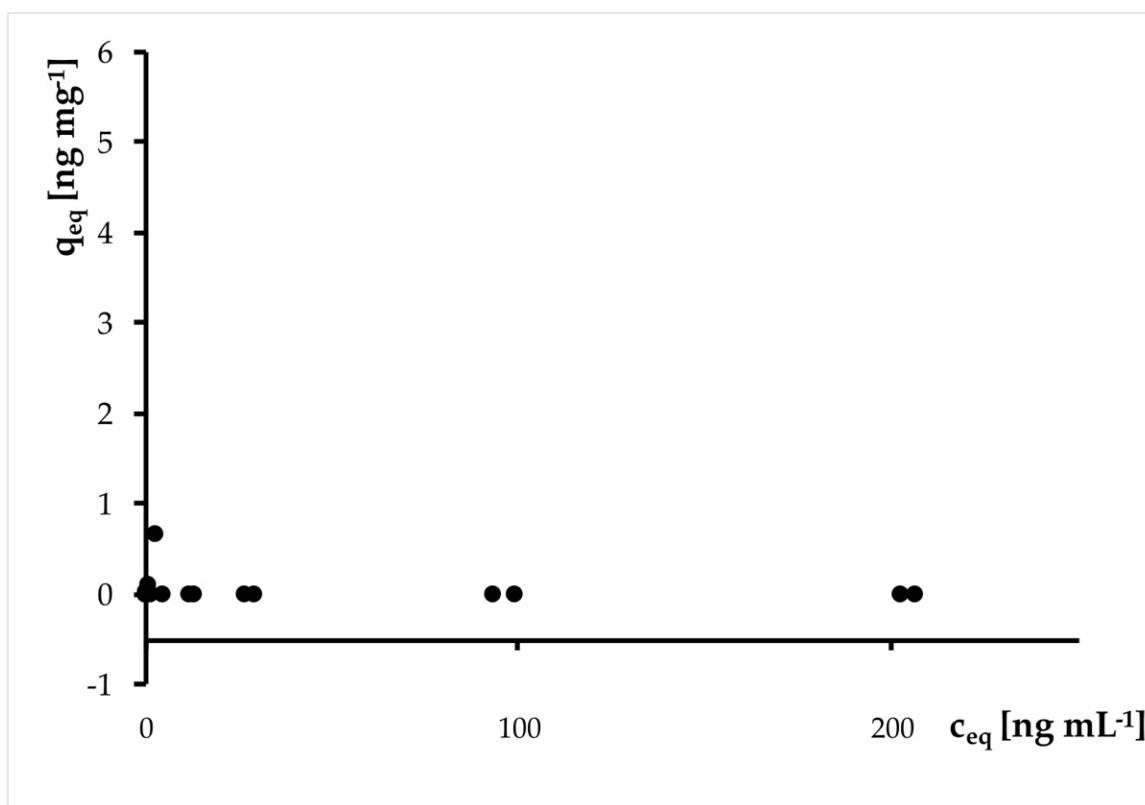


Figure 4. Sorption dynamics of thiacloprid for the system thiacloprid/PS in synthetic freshwater. Loading of thiacloprid to PS is stated as q_{eq} in ng mg^{-1} and the remaining aqueous concentration of thiacloprid is stated as c_{eq} in ng mL^{-1} . Loading and aqueous thiacloprid concentrations were determined in the equilibrium state (30 days).

4. Discussion

The modulation of the toxicity of hydrophobic chemicals by MP has been described in numerous publications and, in most cases, the bioavailability and thus toxicity was altered by adsorption [41,42,69,70]. However, in the current study, sorption processes cannot explain the observed results on thiacloprid toxicity modulation by PS, as sorption of thiacloprid to the PS was excluded. The results of our study lead to the question of which relationship there might be between burrowing behavior and mortality that could explain the strikingly lower mortality in MIX 150,000.

Naylor and Rodrigues [71] observed that *C. riparius* larvae are more efficient at tube building when they encounter a wider size range of particles, and only start foraging on the sediment surface when they have established themselves in their tube. In our study, this is reflected in the behavioral differences when comparing the treatments with and without PS addition: generally more animals were found burrowed in the treatments containing PS than in the treatments without PS. Halpern, et al. [72] demonstrated that tubes of *C. luridus* protected larvae from copper or chloramine exposure depending on the grain size used for building the tubes, as tubes made of silt showed a higher protective effect than sand tubes. Although the concentration of chloramine decreased with an increasing amount of silt in that study, the copper concentration remained the same, showing that in both cases, silt tubes protected the larva better than sand tubes, despite the different modes of action of the chemicals. Since we used PS belonging to the same size category as silt (4–63 μm , according to the Wentworth [73] scale), it would be conceivable that larvae in tubes that were built not only from sand but also contained silt-sized PS were better protected since the tube walls presumably are more compact and tighter in structure. However, this effect alone cannot entirely explain our results for two reasons; first, when we checked

mortality, we forced the larvae to leave this ‘protective case’ on a daily basis; second, this protective effect should increase—or at least remain—with increasing PS concentrations, which contrasts our observation that elevated survival compared to TH occurred exclusively in MIX 150,000, but not in the MIX 1,000,000 treatment. Therefore, we suggest another scenario as an explanation for the observed results: previous work described that a lack of nutritious particles leads to a higher uptake of polyamide MP into the intestinal lumen of *C. riparius* [74]. Since larvae were not fed during the experiment and the quartz sand could not be ingested given its large grain size, only the added PS were available for ingestion. Considerations that a biofilm on the PS could serve as food source for larvae in the particulate treatments were discarded, because of short exposure time and the use of filtered water and heat-treated sand. Ben-Dov, et al. [75] observed reduced toxicity of *Bacillus thuringiensis* ssp. in larvae of mosquitoes (*Aedes aegypti*) and moths (*Spodoptera littoralis*) in the presence of non-nutritional particles. They explained this effect by the attachment of the particles to the peritrophic membrane, a chitinous and proteinous sheath which protects the midgut epithelium of *C. riparius* from abrasion [76], and thus reduces and delays the binding of *B. thuringiensis* δ -endotoxins to the corresponding receptors. This protective mechanism was also considered by Lorenz, et al. [51], who found that Al_2O_3 -nanoparticles significantly reduced the toxicity of thiacloprid in *C. riparius* larvae, although no sorption of the pesticide to the particle was detected. In contrast to our observations, here, the protective effect of particles increased with increasing nanoparticle concentration. We therefore suggest not that only the formation of a protective layer on the peritrophic membrane helps to delay the effect of the toxin, but also that its retention time in the gut could play an important role in modulating its toxicity. It has been shown, that the residence time of food particles in the gut of *C. riparius* is prolonged when the animal is starving or when no further adequate particles for ingestion are available [77]. Dadd [78,79] also found that *Culex pipiens* larvae must continuously ingest particles in order to excrete others that are already present in the gut. Because *C. riparius* larvae feed only on ingestible particles available from the sediment surface near their tube [71], we hypothesize that the number of particles in MIX 150,000 was sufficient to form a protective layer that remained longer in the gut than in the MIX 1,000,000 treatment, because further ingestible particles were limited in number. In contrast, larvae in MIX 1,000,000 had a higher number of PS available for ingestion over a longer period of time, potentially resulting in a faster and also continuous intestinal passage, which may have weakened the protective binding of particles to the peritrophic membrane and thus led to higher mortality. It can be assumed that both, the formation of the protective layer and the velocity of the particle transport out of the intestine, the latter corresponding to their retention time in the gut, are strongly dependent on the size, shape, amount, and surface characteristics of the MP. The chemical properties and concentration of a pesticide also certainly play a role in how efficiently this barrier may shield and delay toxicity. A non-destructive technique recently published by Nigamatzyanova and Fakhrullin [80] may serve as a valuable tool to provide in situ information on the distribution of particles in organs and tissues, probably also in chironomid larvae.

5. Conclusions

We demonstrated that assessing the burrowing behavior of *C. riparius* serves as a good sublethal endpoint for neurotoxic effects and also to determine how efficiently larvae can build tubes and thus establish themselves in different sediments. Whether this method can also be used to assess the effects of other stressors requires further research. This artificial experimental approach, which employed high PS numbers, and no other particles suitable for ingestion like fine sediment or food, may not be directly transferable to natural conditions. However, using it unravels a potential mechanism behind the observed modulation of the toxicity of a hydrophilic chemical by MP which is not based on adsorption of the toxicant.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/microplastics1030036/s1>, Figure S1: Spherical PS particles (PS-FluoRot-50, mean diameter 48.2 µm, microParticles GmbH, Berlin, Germany). (a) bright-field image and (b) corresponding fluorescence image (DAPI). Figure S2: Image obtained by scanning electron microscopy of the micro-sieve used for particle fractionation; Figure S3: Particle numbers and size distribution in an exemplary stock suspension; Figure S4: Microscopic image of quartz sand used as test sediment; Table S1: Experimental design and mortality of different test runs at each timepoint in percent; Table S2: Burrowing behavior; percentage of living larvae burrowed at the respective time points; Table S3: ESI-parameter for LC-MS/MS analysis of thiacloprid; Table S4: Chromatographic gradient for LC-MS/MS analysis of thiacloprid; Table S5: MS settings used for the analysis of thiacloprid. Transitions used as quantifier are stated in bold.

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