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Thiacloprid-Induced Toxicity Influenced by Nutrients: Evidence from In Situ Bioassays in Experimental Ditches

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Abstract: Many studies show that neonicotinoid insecticides cause toxicity to aquatic invertebrates. Some studies report that insecticide toxicity may differ in combination with other agrochemicals under realistic field conditions. To explore such altered toxicity further, we aimed to determine the single and combined effects of environmentally relevant levels of the neonicotinoid thiacloprid and nutrients on different endpoints of 4 aquatic invertebrate species. Animals were exposed to these agrochemicals using a caged experiment within experimental ditches. We observed thiacloprid-induced toxicity for 2 crustaceans, *Daphnia magna* and *Asellus aquaticus*, and for 1 out of 2 tested insect species, *Cloeon dipterum*. We observed no toxic effects for *Chironomus riparius* at the time-weighted average test concentration of 0.51 μg thiacloprid/L. For *D. magna*, the observed toxicity, expressed as the lowest-observed-effect concentration (LOEC), on growth and reproduction was present at thiacloprid concentrations that were 2456-fold lower than laboratory-derived LOEC values. This shows that these species, when exposed under natural conditions, may exhibit neonicotinoid-induced toxic stress. Contrary to the low nutrient treatment, such toxicity was often not observed under nutrient-enriched conditions. This was likely attributable to the increased primary production that allowed for compensatory feeding. These findings warrant the inclusion of different feeding regimes in laboratory experiments to retrieve the best estimates of neonicotinoid-induced toxicity in the natural environment. *Environ Toxicol Chem* 2018;37:1907–1915. © 2018 SETAC

Keywords: Agrochemical; Crustacean; Fertilizer; Insect; Multiple stressors; Neonicotinoid

INTRODUCTION

The field of ecotoxicology has a long history of attempting to extrapolate results between laboratory and field. Field surveys of indigenous biota are used to estimate overall ecological water quality as impacted by all stressors present at the sampling site. Relating these ecological monitoring data to environmental chemical monitoring data has many challenges and gives at best a statistically based indication of stress (Vijver and van den Brink 2014; Hallmann et al. 2017). This can be explained by the many confounding factors present that may alter toxicity or species fitness, for example, nutrient loadings (Alexander et al. 2013), competition (Liess 2002; Kattwinkel and Liess 2014), and predation (Schulz and Dabrowski 2001). In comparison, a laboratory approach to studying toxicity shows direct effects of chemical compounds on an organism with high experimental control of possible confounding factors. However, this approach lacks ecological realism because it standardizes or does not replicate additional natural stressors such as the confounding variables mentioned before (Burton et al. 2005; Crane et al. 2007). An approach that combines the influence of natural stressors with an experimental control is through the use of caged experiments: a method of studying toxicity under increased ecological realism while being less subject to sample collection (Crane et al. 2007).

Caged experiments allow for testing additional anthropogenic alterations to the environment that do not necessarily induce stress but can indirectly affect species' fitness. An example thereof is increased reproductive output of zooplankton species attributable to increased nutrient levels (Ieromina et al. 2014b) through increased primary production (Jak et al. 1998). Such elevated nutrient levels are commonly found in agricultural drainage ditches because of fertilizer runoff, drift, or leakage (Janse and Puijenbroek 1998). These emissions often include other agrochemicals such as neonicotinoids, insecticides that are known to cause toxicity to aquatic invertebrate species and communities (Beketov et al. 2008; Roessink et al. 2013; Van den Brink et al. 2016; Vijver et al. 2017). Alexander et al. (2013) showed that nutrient-enriched conditions can "mask" the toxicity of insecticides to invertebrate communities. However, experimental data on whether nutrients can reduce

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neonicotinoid toxicity of different single invertebrate species' fitness, and if fitness is reduced at a similar rate, are lacking.

This data gap on combined effects is not surprising because of the difficulty of experimental approaches capable of dealing with the indirect effects of nutrients and monitoring population responses in a similar fashion as a laboratory experiment. To reduce this data gap one needs an experimental setup including multiple trophic levels while still controlling the confounding factors mentioned before. To reduce the data gap and to determine if neonicotinoids and nutrients potentially affect a single species' fitness, we aimed to determine the single and combined effects of environmentally relevant levels of the neonicotinoid thiacloprid and nutrients on the fitness of 4 aquatic invertebrate species. Our overarching hypothesis was that thiacloprid-induced toxicity would be reduced under nutrient-enriched conditions. Baas and Kooijman (2015) showed that somatic maintenance—being a parameter in dynamic energy budget theory—correlates with species sensitivity to toxicants. This was confirmed for 50 different animal species for which chemical effects on survival were investigated for 4 different pesticides. To this end, we tested the effects of these agrochemicals on 3 different endpoints of 2 crustaceans (proposed less susceptible organisms for neonicotinoid insecticides) and 2 insect species (proposed more susceptible species) using a cage setup in ecologically and chemically similar experimental ditches.

MATERIALS AND METHODS

Test species and culture conditions

Test species used in the present study were juveniles of 2 crustacean species, *Asellus aquaticus* Linnaeus (<2 wk old) and *Daphnia magna* Straus (<24 h old), and 2 insect species, *Cloeon dipterum* Linnaeus (4–5 instar nymphs) and *Chironomus riparius* Meigen (<9 d old). All species were indigenous at the site of the in situ experiment.

The crustacean species *A. aquaticus* and *D. magna* originated from long-standing cultures present in the laboratory of Leiden University (Leiden, The Netherlands). *Asellus aquaticus* was cultured at room temperature on fine-grained, ignited quartz sand in 50% demineralized water and 50% filtered (106 μm sieve) ditch water and fed with "decomposition and consumption tablets" (Decotabs, consisting of 2% agar, 6% finely ground hay, and demineralized water; Kampfraath et al. 2012). *Daphnia magna* was reared at 18 °C with a 16:8-h light:dark cycle in Elendt m4 medium and fed triweekly with fresh algae (*Pseudokirchneriella subcapitata*; Organisation for Economic Co-operation and Development 2004a). At intervals of approximately 4 mo, the sensitivity of the *D. magna* culture was investigated with the reference toxicant $K_2Cr_2O_7$. These tests showed that the sensitivity of the daphnid culture was well within limits set by the Organisation for Economic Co-operation and Development (OECD) guideline (24-h 50% effective concentration for mobility = 0.6–2.1 mg/L $K_2Cr_2O_7$; Organisation for Economic Co-operation and Development 2004a).

The insect species either were caught from a nonagricultural site (*C. dipterum*; <500 m away from the test site) or originated from long-standing cultures from the University of Amsterdam (*C. riparius*; Amsterdam, The Netherlands). *Cloeon riparius* was reared at 22 °C with a 16:8-h light:dark cycle (Organisation for Economic Co-operation and Development 2011) in Dutch Standard Water (200 mg/L $CaCl_2 \times 2H_20$, 180 mg/L $MgSO_4 \times 7 H_2O$, 100 mg/L $NaHCO_3$, and 20 mg/L $KHCO_3$) on fine-grained, ignited quartz sand and fed triweekly with fish flakes (20:1; Trouvit:Tetraphyll).

Exposure conditions

The caged experiments were performed in outdoor experimental ditches at the Living Lab facility of Leiden University, Oegstgeest, The Netherlands. The site is not located in any close proximity to agricultural practices and is adjacent to grassland. The 36 ditches used in the present study have a length of 10 m, a width of 0.8 m at the surface level, and 0.4 m width at the bottom at a depth of 0.3 m. The ditches are adjoining to a water level compensation reservoir that is characterized by low levels of soluble nutrients and is connected to the watershed of the Old Rhine. At the end of April 2017, after leaving the ditches connected to the reservoir for 6 mo to form natural ecological conditions (fauna, flora, etc.), each ditch was hydrologically isolated from the reservoir to avoid cross-contamination of the added agrochemicals (thiacloprid and nutrients; see later in this section) between ditches. We isolated the ditches by placing a 1000 × 500 × 2 mm acrylic plate firmly into the ditch banks and 15 cm deep into the sediment at the end of every ditch. After isolation, half of the ditches were continuously enriched with nutrients by hanging 3 sachets containing slow-releasing fertilizer granulates (75 g Osmocote per sachet; N:P:K = 15:9:11 combined with microelements) into each ditch equally divided over the ditch length. Two weeks later, following a block design, 9 ditches that did and did not receive additional nutrients were spiked with an environmentally relevant nominal concentration of thiacloprid. Because in the present study, thiacloprid is subject to processes such as degradation and adsorption, and we do not refresh exposure "media" (ditchwater); we provide our exposure concentration (see later in this section for the method on measuring thiacloprid concentrations) as a time-weighted average (TWA), calculated as in the following equation:

$$C_{TWA} = \frac{\sum_{n-1}^{N} \left(\Delta t_n \frac{c_{n-1}+c_n}{2}\right)}{\sum_{n-1}^{N} \Delta t_n}$$

where Δt is the time interval between measurements, c is the concentration (in nanograms per liter) at time interval n (in days), and N the total number of intervals. Our nominal time-weighted average was 400 ng/L thiacloprid (99.9% purity; purchased from Sigma–Aldrich) over the period of 1 mo. We based this concentration on thiacloprid concentrations residing in surface waters of The Netherlands over a 5-yr period (2011–2015, 11 751 observations; Leiden University, Rijkswaterstaat-WVL 2017). Of all observations, 608 unique observations showed a concentration of thiacloprid above the detection limit (10 or 20 ng/L in most cases), with a maximum observed concentration of 12 000 ng/L (for details see Supplemental Data, Figure A1).

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Including both the nutrient and thiacloprid additions, the experiment entailed a full factorial design of 4 different treatments with 9 replicates per treatment: 1) control (no added substances), 2) thiacloprid addition, 3) nutrient addition, and 4) thiacloprid and nutrient addition. To maintain experimental concentrations, 2 wk after the first thiacloprid spike, a second spike was performed in equal nominal concentrations. Spiking was conducted by diluting a 25 mg/L stock concentration of thiacloprid in a glass bottle with 10 L of ditchwater originating from the ditch to which the thiacloprid was added. Subsequently, the bottle was mounted with a watering can head, and the mixture was spread evenly over the ditch to simulate spray drift and to stimulate homogenization of the compound.

Once a week, the experimental ditches were monitored for pH, dissolved oxygen, temperature, and conductivity using a portable hq 40 d electronic multiparameter meter (Hach). In addition, water samples were collected 5 cm below the surface level in the middle of each ditch each day in the first week after the first thiacloprid spike and thereafter twice per week. One hour after the first as well as after the second spike, we analyzed all collected samples for thiacloprid concentration. Subsequent samples were randomly selected blocks within the block design. These samples were then analyzed for their thiacloprid concentration at Wageningen University using liquid chromatography–tandem mass spectrometry (Agilent Technologies; see Roessink et al. 2013 for the detailed procedure). From these data, we calculated the average 50% and 90% dissipation times (DT50 and DT90) for both spikes individually. Biweekly, dissolved nitrate (NO_3^-) and phosphate (PO_4^{2-}) concentrations were determined colorimetrically using a Nova 60 Spectroquant® photometer (Merck). Finally, right before nutrient addition, 4 d after nutrient addition, and 3 d prior to the end of the experiment we collected, extracted, and analyzed water samples to determine chlorophyll *a* concentrations using the method described by Arar and Collins (1997) on a F900 fluorescence spectrometer (Edinburgh Instruments).

Experimental setup

Three days prior to the first thiacloprid spike, test species were entered in enclosures and placed into each ditch to let the animals acclimatize to the present water conditions. *Daphnia magna* acclimatized for only 1 d prior to the thiacloprid spike, to prevent substantial unexposed growth. Before inoculation, animals were allowed to slowly acclimatize to the ditch temperature (which was 20 °C at the time of inoculation), to prevent heat shock. After the acclimatization period, the animals were, if possible, checked for survival (>98% for *C. dipterum* and *D. magna*). For the test species *A. aquaticus* and *C. dipterum*, 150-mL high-density polyethylene enclosures with a 3.75-cm opening on one side covered with mesh (mesh size 700 μm) were prepared. Equal enclosures were prepared for *D. magna* except that we used a mesh size of 150 μm to prevent escaping neonates. *C. riparius* enclosures were prepared out of 250-mL glass jars containing 60 g of fine-grained, ignited quartz sand (grain size 0.1–0.5 mm) as sediment and closed with 150-μm mesh.

Each 150-mL enclosure held 5 juveniles per test species and was placed horizontally in the ditch by sticking a ring of PVC steel line wire into the sediment. The *C. riparius* enclosures held 10 juveniles and were dug roughly 5 cm vertically into the sediment. Each ditch received one enclosure of *A. aquaticus*, *C. dipterum*, and *D. magna* and two enclosures of *C. riparius*. Right before placing the enclosures, we fed each cultured species according to its culture conditions (see *Test species and culture conditions*). For *C. dipterum* we provided 0.1 mg wet weight of live periphyton of the ditch in which the enclosure was inoculated. Generally, enclosures were retrieved 21 d after the first thiacloprid spike. The first of the 2 *C. riparius* enclosures, however, was retrieved after 14 d of exposure, to measure several larval endpoints (explained in *Test species endpoints*) before emergence.

Test species endpoints

Survival of *C. dipterum* and *D. magna* species was recorded every 3 d by bringing the enclosures to the surface, carefully opening them, and counting the mobile animals. Animals were considered immobile if they did not respond within 15 s of gentle stimulation by stirring (Organisation for Economic Co-operation and Development 2004a). During inspection, we removed and counted the number of juveniles produced by *D. magna* and the number of emerged *C. dipterum* individuals to score reproduction and emergence respectively. The emerged *C. dipterum* individuals were easily collected because they were trapped in a small air pocket within the enclosure (<5% of enclosure total volume). Survival of *A. aquaticus* and *C. riparius* was estimated after enclosure retrieval because it proved unfeasible to accurately estimate survival of these species as a result of water clarity and *C. riparius*' natural tendency to bury itself in the sediment. These animals were therefore collected by filtering the contents of the enclosure over a sieve (106 μm), after which survival was scored.

After 18 d of exposure, emergence was scored for *C. riparius* by placing an emergence trap (60 × 60 cm) directly above the remaining enclosure that was connected to a 1-mm mesh tunnel leading directly into the trap. We did not use the same method to score emergence for *C. riparius* and for *C. dipterum* because of the high tendency of degradation of *C. riparius*.

Directly prior to inoculation of the test species, 20 animals per species were photographed using an eScope DP-M17 USB-microscope camera. After 14 d of exposure for *C. riparius* (because of very fast growth in the nutrient addition treatments) and 21 d for the remaining species, enclosures were retrieved and surviving animals were collected, shock-preserved in 96% ethanol for 15 s and stored in 80% ethanol to be photographed 1 wk later. Image J (Ver 1.48f) was then used to determine the initial and final body lengths of each species. *Asellus aquaticus* was measured from the tip of the head to the end of the pleotelson, *C. riparius* from the tip to the head to the anal tubules, and *C. dipterum* and *D. magna* from the rostral end to the attachment of the tail. Subsequently, for each species, daily growth was calculated by subtracting the initial body length from the final body length and dividing by the number of days the

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organism was exposed. Finally, to determine differences in the consumption of *A. aquaticus*, we loaded each enclosure with one Decotab (0.3 g wet wt consisting of 2% agar and 6% finely ground hay). We also loaded 3 test vessels with one Decotab but no animals, per treatment to correct for possible microbial decomposition. After test vessel retrieval, we dried the Decotabs for 3 d at 60 °C and weighed them subsequently using a microbalance.

Statistical analyses

Chlorophyll *a* concentration and the endpoints survival, consumption, emergence, and reproduction were analyzed by means of factorial analysis of variance (ANOVA; type 3 sums of squares) to explore the effects of thiacloprid addition (2 levels: yes/no) and nutrient addition (2 levels: yes/no) and their possible combined effects. Similarly, we explored the possible effects of thiacloprid and nutrients on the water chemistry parameters water temperature, pH, dissolved oxygen concentration, and conductivity. To further investigate possible differences between treatments (4 levels: control, thiacloprid, nutrients, thiacloprid and nutrients), we performed one-way ANOVAs followed by Tukey post hoc tests. Because we measured multiple individuals per ditch to determine the daily growth rate, we used linear mixed effects models (function lme of package nlme) and nested individuals in their respective experimental ditch to account for possible effects of the ditch. For each model, we tested for homogeneity of variances using Levene's tests and for normality of the random factor and model residuals using quantile–quantile plots. If either of these assumptions were not met, data were either log10- or square root–transformed to improve their fit. Because data transformation did not improve the normality of the survival data for *C. riparius*, we tested these data with Kruskal–Wallis tests. Statistical analyses were performed using R-studio, Ver 1.0.153 (R Ver 3.4.1; R Development Core Team 2017).

RESULTS

The average ditch temperature was between 18 °C (standard deviation [SD] 0.33) and 24 °C (SD 0.54) during the exposure period of the animals and did not differ between treatments ($p > 0.05$). Nutrient addition significantly increased the pH over time, with a time-weighted average of 0.27 (SD 0.21) during the exposure period ($F_{1,29} = 18.7$, $p < 0.001$). Nutrient addition also significantly increased dissolved oxygen concentrations at $t = 7$ ($F_{1,32} = 46.9$, $p < 0.001$) compared with the treatments that did not receive additional nutrients. In addition, long-term monitoring (9 mo; data not shown) of the experimental ditches never showed dissolved oxygen concentrations <9.5 mg/L, meaning there was no oxygen deficiency. Unexpectedly, nutrient addition also significantly decreased conductivity interacting with time with an average of 57 μS/cm (SD 34) during the exposure period ($F_{1,29} = 5.56$, $p = 0.020$), likely ascribed to the depletion of bicarbonate (HCO_3^-) because of increased algal photosynthesis (see next paragraph). There was no observed effect of thiacloprid on the tested water chemistry parameters. The actual time-weighted average (3 wk) exposure concentration of thiacloprid was 0.51 μg/L (SD 0.08). The average DT50 and DT90 (calculated for both spikes individually and averaged subsequently) of thiacloprid in water was 3.3 d (SD 0.1) and 11.1 d (SD 0.4), respectively. Our observed DT50 and DT90 values were comparable to those summarized by the European Chemicals Agency (2015), which reported DT50 and DT90 values of 2.9 and 9.7 d in a pond setup. There was no effect of nutrient enrichment on thiacloprid concentration at a given time point ($p > 0.05$). Ditches without added thiacloprid showed no presence of the compound (all values below detection limit). As expected, both time-weighted averages of nitrate and phosphate concentrations significantly increased by nutrient addition ($F_{1,32} = 10.6$, $p = 0.002$; $F_{1,32} = 28.8$, $p < 0.001$, respectively) over the course of the experiment. On average, time-weighted average concentrations of nitrate and phosphate increased by a factor of 1.5 (SD 0.5) and 2.2 (SD 0.9), respectively. There was no effect of thiacloprid on the measured nutrient concentrations ($p > 0.05$ for all comparisons). All time-weighted averages of these exposure conditions are provided in Table 1.

Chlorophyll *a* concentrations did not differ between treatments before the addition of the agrochemicals ($p > 0.05$; see Supplemental Data, Table A1). Four days after nutrient addition, chlorophyll *a* concentrations in the water significantly increased in nutrient-enriched ditches by a factor of 1.9 (SD 0.9) on average ($p < 0.001$) compared with ditches that had not received additional nutrients. Three days before enclosure retrieval, chlorophyll *a* concentrations were significantly lowered

TABLE 1: Time-weighted averages (±standard error; 21 d) of the water chemistry parameters per treatment during the exposure period[a]

	Treatment			
Parameter	C	T	N	TN
Conductivity (μS/cm)	783 (±11.6)	760 (±8.08)	727 (±8.36)	702 (±13.3)
Oxygen (mg/L)	12.4 (±0.62)	12.1 (±0.37)	13.8 (±0.59)	14.2 (±0.31)
Nitrate (mg/L)	1.28 (±0.18)	1.04 (±0.15)	1.81 (±0.23)	1.70 (±0.17)
pH	8.07 (±0.03)	8.03 (±0.02)	8.35 (±0.08)	8.31 (±0.06)
Phosphate (mg/L)	0.17 (±0.06)	0.13 (±0.03)	0.38 (±0.06)	0.27 (±0.03)
Temperature (°C)	21.9 (±0.01)	21.9 (±0.01)	22.0 (±0.02)	21.9 (±0.01)
Thiacloprid (μg/L)	<DL	0.49 (±0.03)	<DL	0.53 (±0.03)

[a]For nitrate and phosphate, time-weighted averages are given since the start of enrichment (35 d).
C = control; <DL = below detection limit (100 ng/L); N = nutrients; T = thiacloprid; TN = thiacloprid and nutrients.

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TABLE 2: The overall effects of thiacloprid and nutrient addition on the different endpoints of the test species[a]

Species	Endpoint	Treatment		
		T	N	TN
Asellus aquaticus	Survival	0.442	0.251	0.415
	Growth	0.324	0.785	0.361
	Consumption	0.029*	0.644	0.340
Daphnia magna	Survival	0.635	0.645	0.844
	Growth	0.017*	0.530	0.179
	Reproduction	<0.001*	0.710	0.092
Cloeon dipterum	Survival	0.002*	0.708	0.064
	Growth	0.691	0.016*	0.339
	Emergence	0.234	0.002*	0.703
Chironomus riparius	Survival	0.771	0.031*	n.d.
	Growth	0.549	0.052*	0.422
	Emergence	n.d.	n.d.	n.d.

[a]Shown are the *p* values of the comparisons assessed with (nested) factorial analysis of variance or Kruskal–Wallis.
*$p \leq 0.05$.
N = nutrient addition; n.d. = not determined; T = thiacloprid addition; TN = thiacloprid and nutrient addition.

by thiacloprid addition ($F_{1,32} = 13.7$, $p < 0.001$) and were significantly lowest in the mixture treatment compared with the control and nutrient treatments ($p = 0.019$ and $p = 0.011$ respectively) but not the thiacloprid treatment ($p > 0.05$).

After 21 d of exposure, thiacloprid or nutrients did not significantly affect the survival or growth of the crustacean species *A. aquaticus* (Table 2). Survival of *A. aquaticus* was 31% (SD 23) in the control treatment (Figure 1A), and these animals grew on average 0.12 mm per day (SD 0.06, Figure 1B). In contrast to the absence of any treatment effect on these endpoints, consumption significantly decreased by thiacloprid addition ($F_{1,27} = 5.33$, $p = 0.029$; Table 2). Decotab consumption was 73% lower in the thiacloprid treatment and 45% in the thiacloprid and nutrient mixture compared with the control treatment (Figure 1C); however, this difference was not confirmed statistically by one-way ANOVA ($p = 0.08$ and $p = 0.09$, respectively). We recognize that Decotab consumption is likely affected by the number of animals within the enclosure, which was severely reduced in all treatments irrespective of the treatment. However, because we have no knowledge of when mortality occurred (and thus feeding of an individual stopped), we chose to not standardize Decotab consumption to the number of animals remaining in the enclosure at $t = 21$ d. We believe this to be a valid method because the survival between treatments was not statistically different at $t = 21$ d. For the crustacean species *D. magna*, survival was 59% (SD 37) in the control treatment, and we did not detect any differences between treatments after 21 d of exposure ($p > 0.05$; Figure 1D). However, both growth ($F_{1,61} = 6.00$, $p = 0.017$) and reproduction ($F_{1,26} = 16.5$, $p < 0.001$) were significantly reduced by thiacloprid addition (Table 2). Growth was significantly reduced in the thiacloprid treatment compared with the control ($p = 0.028$) and nutrient treatment ($p = 0.027$) but not compared with the thiacloprid and nutrient mixture ($p > 0.05$; Figure 1E). At the end of the experiment, *D. magna* females within the control treatment produced an average of 58 (SD 11) neonates per female while not being fed by the researchers, meaning that food was consumed only as they could find it in the experimental ditches. The average reproduction of 58 neonates approached the recommended 60 neonates (under ad libitum food conditions in the laboratory) as stated by the OECD guideline (Organisation for Economic Co-operation and Development 2012). In the thiacloprid treatment, reproduction was significantly lowered compared with all other treatments ($p < 0.05$) with an average reduction of 51% (SD 19) compared with the control (Figure 1F). In addition, we observed and collected ephippia (resting eggs) in all treatments, but these were not found in the control. In total, 1 ephippium was found in the nutrient treatment, 14 in the thiacloprid treatment, and 3 in the mixture treatment. A presence/absence analysis (glm) showed that the number of *D. magna* enclosures where ephippia were observed was significantly higher in the thiacloprid treatment compared with the control ($p = 0.002$) and nutrient treatment ($p = 0.010$). We also found a marginally higher number of enclosures with ephippia in the thiacloprid treatment compared with the mixture treatment ($p = 0.061$).

In contrast to the crustacean species, the agrochemicals did affect the survival of both insect species. The survival of the insect *C. dipterum* was significantly decreased by thiacloprid ($F_{1,32} = 10.9$, $p = 0.002$). We also observed a marginally significant interaction effect of thiacloprid and nutrients ($F_{1,32} = 3.68$, $p = 0.064$). Survival in the thiacloprid treatment was significantly reduced by 51 to 56% (SD 24–26) compared with the other treatments ($p < 0.05$ for all comparisons; Figure 1G). Survival in the mixture treatment did not differ from that in either the control or the nutrient treatment ($p > 0.05$). Toxicity of thiacloprid was not observed for the growth and emergence of *C. dipterum*; however, these endpoints were significantly increased by nutrient addition ($F_{1,30} = 6.49$, $p = 0.016$ and $F_{1,32} = 11.8$, $p = 0.002$, respectively). Growth significantly increased in the nutrient and mixture treatment compared with the control treatment ($p \leq 0.05$ for both comparisons) and compared with the thiacloprid treatment ($p < 0.001$ for both comparisons; Figure 1H). After 21 d of exposure, on average 12% (SD 21) of animals within the control treatment emerged (Figure 1I). Emergence in the thiacloprid treatment was lower, on average 2% (SD 6), represented by a single emerged individual; but we could not confirm this statistically ($p > 0.05$). The nutrient treatment showed a significantly higher emergence of 41% (SD 23) compared with both the control and thiacloprid treatment ($p = 0.008$ and $p < 0.001$ respectively). Emergence was also increased in the mixture treatment, 20% (SD 16) but only deviated statistically from the thiacloprid treatment ($p = 0.033$; Figure 1I; Supplemental Data, Figure A2).

There was no observed effect of thiacloprid on the different endpoints of the insect species *C. riparius*. However, nutrients showed significant effects on both survival (χ^2 1,38 = 4.67, $p = 0.030$) and growth ($F_{1,32} = 4.06$, $p = 0.052$; Table 2). Average survival in the control and thiacloprid treatment was 70% (SD 24) and 69% (SD 26), respectively, and increased to 88% (SD 17) and 90% (SD 20) for the nutrient and mixture treatment, although no significant differences between individual treatments were

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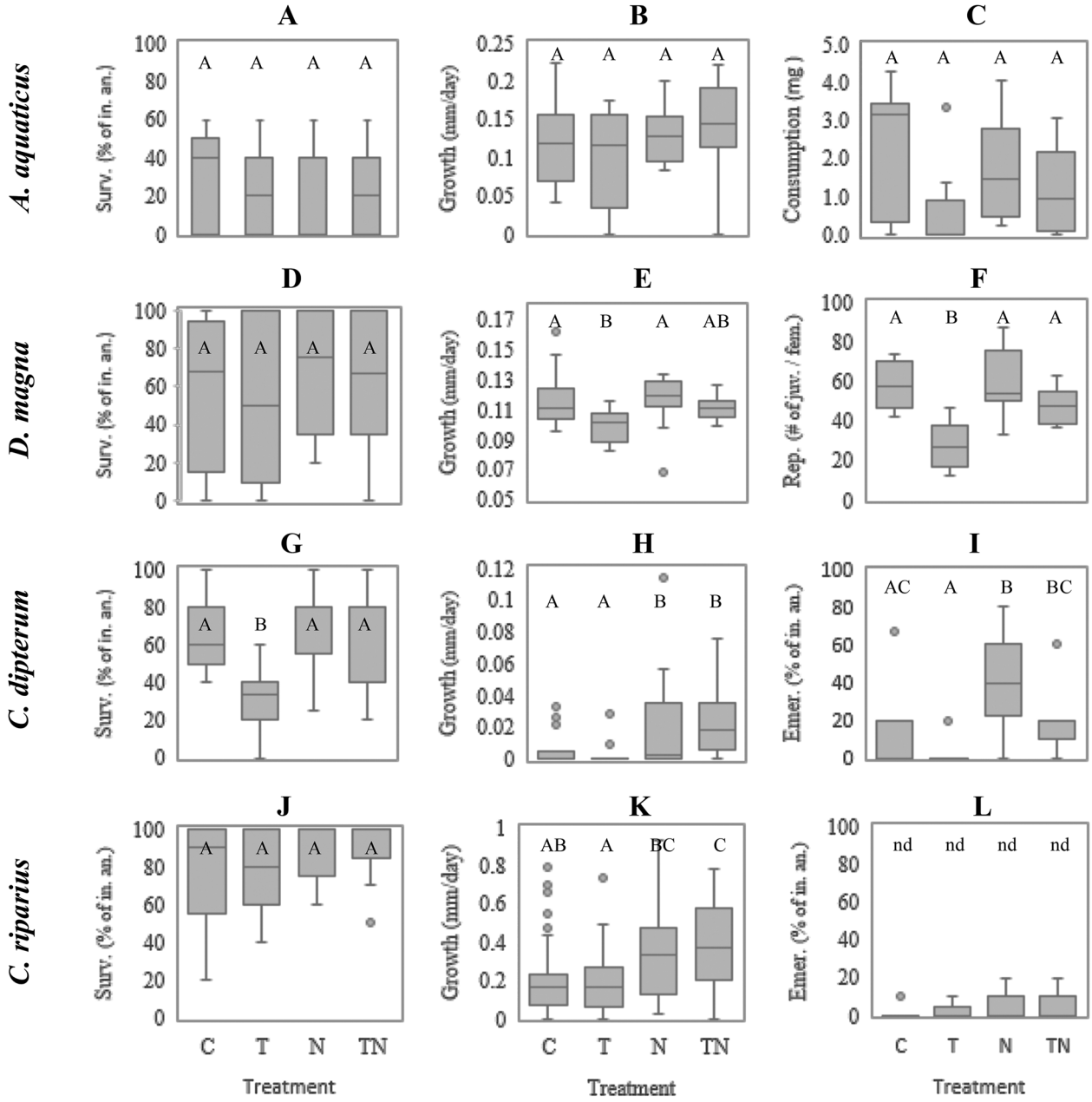

FIGURE 1: Box and whisker plots of the survival (percentage of initial animals), growth (millimeters per day), and consumption (milligrams) divided by cumulative emergence (percentage of initial animals) divided by cumulative reproduction (number of juveniles per female) for *Asellus aquaticus* (A–C), *Daphnia magna* (D–F), *Cloeon dipterum* (G–I), and *Chironomus riparius* (J–L) after 21 d (14 d for *C. riparius* survival and growth) of exposure to thiacloprid and/or nutrient addition. Different letters (A, B, C) indicate significant differences (one-way analysis of variance + Tukey's post hoc) between treatments at significance level $p \leq 0.05$. C = control; Emer. = emergence; in. an. = initial animals; juv. = juveniles; N = nutrients; nd = not determined; Rep. = reproduction; Surv. = survival; T = thiacloprid; TN = thiacloprid and nutrients.

observed ($p > 0.05$; Figure 1J). Growth was highest in the mixture treatment and deviated statistically from both the control ($p = 0.004$) and thiacloprid treatment ($p < 0.001$) but not from the nutrient treatment ($p > 0.05$; Figure 1K). Similarly, growth was increased in the nutrient treatment but only differed significantly from the thiacloprid treatment ($p = 0.017$) and not the control ($p = 0.096$). Although many *C. riparius* individuals appeared nearly ready to emerge after retrieval of the first enclosure, we only recorded a low number of emergence (on average ≤7% per treatment; Figure 1L). This was attributable to unfortunate escape of emerged individuals as the emergence traps were blown away. We therefore excluded these data from further analysis.

DISCUSSION

The present study aimed to quantify the possible interactions between nutrients and thiacloprid on specific endpoints of 4 aquatic invertebrate species under semifield conditions. We showed toxicity of thiacloprid to different endpoints for 2

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crustacean and one insect species. We observed no toxicity to the insect species *C. riparius*. Overall insect fitness increased as a result of nutrient enrichment for the endpoints survival, growth, and emergence (dependent on species). More importantly, we observed reduced thiacloprid toxicity under nutrient-enriched conditions.

Both crustacean species were significantly impacted by exposure to thiacloprid. Thiacloprid exposure significantly reduced (45–73% compared with the control) the consumption of *A. aquaticus* but not its survival or growth. To our knowledge, chronic toxicity data for *A. aquaticus* are currently lacking, except for postexposure toxicity effects provided by Beketov and Liess (2008), who reported an 18-d lowest-observed-effect concentration (LOEC) for survival of 287 μg/L, a concentration that is out of limits for environmental relevance. Thus, the present study is the first to show that the consumption of *A. aquaticus* may be significantly impacted at thiacloprid levels currently residing in surface waters. Although we observed no effects on either survival or growth, longer-term experiments might show reduced fitness because of a thiacloprid-induced lack of nutrition for this species. More substantial toxicity data for *D. magna* were available in the literature: Pavlaki et al. (2011) report 21-d LOECs of 1250 μg/L for both reproduction and body length, whereas Beketov and Liess (2008) report a postexposure 21-d LOEC for survival of 4740 μg/L. As expected, we observed no effect of thiacloprid on the survival of *D. magna*. However, we did observe significant thiacloprid-induced toxicity for both growth (15% reduction compared with the control) and reproduction (51% reduction compared with the control) at environmentally relevant concentrations that are 2456-fold lower than the LOEC values reported by Pavlaki et al. (2011). In addition, in the thiacloprid treatment we observed significant ephippium production, which is a behavior of *D. magna* to endure unfavorable environmental conditions (Ebert 2005). These toxic effects observed for *D. magna* can be direct or indirect toxicity or a combination thereof. Direct toxicity would mean that thiacloprid would directly reduce the fitness of *D. magna*, but such toxicity at our experimental concentration would be absent when animals are exposed under stable lab conditions (see Pavlaki et al. 2011). This can be explained by the fact that the experimental ditch is a multistress environment, which may add to the observed toxicity (Clements et al. 2012). Indirect effects would mean that the aquatic invertebrate communities residing in the ditches possibly depressed algae concentrations (food quantity) and/or altered the algal community composition (food quality). Our observed toxic effects of thiacloprid (being direct or indirect) were absent under the nutrient-enriched condition. This indicates the importance of nutrients that led to increased food availability and/or quality for *D. magna*, enabling the species to cope with neonicotinoid-induced toxicity. We strongly suspect that this lack of toxicity is a result of compensatory feeding (Alexander et al. 2007; Goedkoop et al. 2010) on free-floating algae because chlorophyll *a* concentrations were lowest in the mixture treatment. Such chlorophyll *a*–depressing effects have been suggested by Alexander et al. (2013). Although high nutrient loadings in agricultural ditches are a common ecosystem property (Janse and Puijenbroek 1998), *D. magna* populations residing in environments with lower nutrient loadings may be currently at risk when exposed to thiacloprid. Such apparent risks are likely to be missed by common laboratory approaches because they rely on static test conditions with regular high-quality feeding regimes (see Organisation for Economic Co-operation and Development 2012). These tests are missing the mechanism that poor food quality can reduce the fecundity of *Daphnia* (Van Donk et al. 1997), which may add to toxicity. This is illustrated by Ieromina et al. (2014a), who showed that poor food quality as measured on P-contents of algae indeed increased the sensitivity of nontarget grazing species to the neonicotinoid insecticide imidacloprid. Thus, the common laboratory tests might severely underestimate neonicotinoid-induced toxicity in the long term.

The insect species *C. dipterum* showed strongly reduced survival in the thiacloprid treatment (53% compared with the control), confirming previous results on the severe toxicity of neonicotinoids to this mayfly species (Roessink et al. 2013; Van den Brink et al. 2016). However, when *C. dipterum* was exposed to thiacloprid under nutrient-enriched conditions, this toxicity was not detected. Such absence of toxicity can be comparable to the argument made for *D. magna*, attributable to stimulated primary production that allows for compensatory feeding (Alexander et al. 2007; Goedkoop et al. 2010). Nutrient enrichment stimulated the growth and emergence of *C. dipterum*, thus completing its aquatic life stage more quickly. We found no significant effects of thiacloprid on these endpoints. However, emergence in the thiacloprid treatment was represented by a single emerged individual, whereas all other treatments showed increasing emergence over time (Supplemental Data, Figure A2). Thus, it is likely that the effects of thiacloprid on emergence would become apparent with a longer test duration. Similar to *C. dipterum*, nutrient enrichment also increased the growth of *C. riparius* as well as its survival. However, we found no effects of thiacloprid on either of these measured endpoints. This was unexpected because Langer-Jaesrich et al. (2010) report a 17-d LOEC for survival of 0.5 μg/L, which is equal to the time-weighted average water concentration used in the present study. A possible explanation for the absence of toxicity is that we did not actively spike thiacloprid into the sediment (as we simulated spray drift), thus lowering the actual exposure compared with Langer-Jaesrich et al. (2010), who did spike the (comparable) sediment (Organisation for Economic Co-operation and Development 2004b).

Survival within our control treatment for each test species was never 100%. This is a clear indication that there were additional environmental stressors within the test setup. This was expected because we tested the species in experimental ditches that yielded full ecosystem complexity (although we excluded biotic processes such as predation in our cage setup). Numerous environmental factors might have reduced our control survival (weather patterns, fluctuating water chemistry, etc.). Such additional stress probably added to toxicity, which was likely the case for *D. magna* because we observed far lower toxicity values compared with laboratory studies (Pavlaki et al. 2011).

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This is in accordance with many aquatic toxicology studies that showed altered toxicity of chemicals when additional environmental factors were assessed (Clements et al. 2012). Overall, we showed toxicity induced by environmentally relevant concentrations of the neonicotinoid thiacloprid to 3 aquatic invertebrate species. For the insect species *C. riparius*, we observed no toxic effects on the measured endpoints. Sensitivity of the organisms to thiacloprid often deviated from laboratory-derived toxicity values, especially for *D. magna*.

CONCLUSIONS

We showed the importance of nutrient enrichment (and the resulting increase in primary production) for coping with neonicotinoid-induced toxicity. It is likely that such neonicotinoid-induced toxicity is often not observed in laboratory experiments because of high-quality feeding regimes that allow for compensatory feeding. Similar to laboratory-derived data, this might explain why this toxicity is not observed in agricultural ditches because the common high nutrient loadings allow for compensatory feeding as well. Thus, of all the natural stressors and abiotic fluctuations that are abundant in the field, ad libitum feeding, as often done in the laboratory, can explain much of the discrepancies between laboratory- and field-derived ecotoxicity data observed in oligotrophic conditions. This warrants the inclusion of different feeding regimes in laboratory experiments to retrieve the best estimates of neonicotinoid-induced toxicity in the natural environment. Often, many water quality management actions focus either on a reduction of nutrients or on a reduction of pesticide emissions for the protection of biodiversity. Because the present data show that the relative pressure of neonicotinoids to aquatic biodiversity may become relatively more pronounced when nutrient emissions are reduced, we argue that the greatest protection of aquatic biodiversity is achieved by reducing the emissions of both agrochemicals.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.4142.

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Data Availability—Additional data are available in the Supplemental Data, and the basic data can be obtained from the first author via e-mail (s.h.barmentlo@cml.leidenuniv.nl).

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