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Citation

Barmantlo, S. H., Schrama, M. J. J., Snoo, G. R. de, Bodegom, P. M. van, Nieuwenhuijzen, A. van, & Vijver, M. G. (2021). Experimental evidence for neonicotinoid driven decline in aquatic emerging insects. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 118(44). doi:10.1073/pnas.2105692118

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Note: To cite this publication please use the final published version (if applicable).



Experimental evidence for neonicotinoid driven decline in aquatic emerging insects

S. Henrik Barmantlo^{a,1,2}, Maarten Schrama^a, Geert R. de Snoo^{a,b}, Peter M. van Bodegom^a, André van Nieuwenhuijzen^c, and Martina G. Vijver^a

^aInstitute of Environmental Sciences, Leiden University, 2300 RA Leiden, The Netherlands; ^bNetherlands Institute of Ecology, 6700 AB Wageningen, The Netherlands; and ^cAdviesbureau Haloplus, 378 46 Rosec, Czech Republic

Edited by Bruce G. Hammock, University of California, Davis, CA, and accepted by Editorial Board Member David Zilberman August 16, 2021 (received for review March 24, 2021)

There is an ongoing unprecedented loss in insects, both in terms of richness and biomass. The usage of pesticides, especially neonicotinoid insecticides, has been widely suggested to be a contributor to this decline. However, the risks of neonicotinoids to natural insect populations have remained largely unknown due to a lack of field-realistic experiments. Here, we used an outdoor experiment to determine effects of field-realistic concentrations of the commonly applied neonicotinoid thiacloprid on the emergence of naturally assembled aquatic insect populations. Following application, all major orders of emerging aquatic insects (Coleoptera, Diptera, Ephemeroptera, Odonata, and Trichoptera) declined strongly in both abundance and biomass. At the highest concentration (10 µg/L), emergence of most orders was nearly absent. Diversity of the most species-rich family, Chironomidae, decreased by 50% at more commonly observed concentrations (1 µg/L) and was generally reduced to a single species at the highest concentration. Our experimental findings thereby showcase a causal link of neonicotinoids and the ongoing insect decline. Given the urgency of the insect decline, our results highlight the need to reconsider the mass usage of neonicotinoids to preserve freshwater insects as well as the life and services depending on them.

biodiversity | field experiment | insect decline | insecticide | toxicity

Insects represent the most diverse class of animals on our planet and outnumber all other animal species (1). They play key roles in crucial ecosystem services including nutrient cycling, pollination, decomposition, and pest control (2, 3). In addition, many vertebrate animals, such as freshwater fish, birds, and bats, rely on insects as their main or only source of diet, illustrating their pivotal role in ecosystems. However, several studies now suggest that insects are declining at an alarming rate both in terms of species richness and biomass, even of common species (4–10), likely with negative repercussions for natural and human ecosystems (11).

A number of key human-induced activities related to intensive land use have been suggested to underlie this decline, of which the most important are 1) alterations in temperature and drought/wet periods, 2) habitat loss and fragmentation, and 3) chemical crop protection (2, 6, 10, 12–14). Inferring causality on the contribution of each of these and other drivers is challenging and is commonly addressed using large datasets that describe local population dynamics. Such approaches (4, 12, 15) contain a number of confounding factors that limit the understanding of individual drivers. As a result, studies addressing potential drivers of decline are often inconclusive because of their correlative nature, possible occurrence of confounding factors, and the fact that most data are local instead of global (4, 10). The absence of clear proof for the potential and relative contribution of each of the drivers has led to a polarized debate to such an extent that some find the narrative of “insect apocalypse or demise” exaggerated (16, 17), while others state that we already know enough to act now (13, 14, 18). To reconcile this debate, well-controlled experiments with

complete insect communities under realistic ecosystems conditions are needed to determine causality of the proposed drivers (12).

The impact of insecticides, most notably neonicotinoids, on natural insect populations has often been mentioned as one of the most important drivers for insect decline as these substances are meant to kill insects (6, 19–22). Neonicotinoids were first introduced in 1991 and are currently the most widely used class of insecticides globally (23, 24). The main difference with previous classes of insecticides (e.g., persistent organic pollutants) is that they have a relatively lower environmental persistence but can still persist in soils in measurable concentrations after months or years (25). Neonicotinoids also have a specific mode-of-action that is specifically aimed to target insects (26) and a lower tendency to bioaccumulate (27). However, it has been observed that neonicotinoids have high leaching and runoff potential, and consequently, they are now found in soil and surface waters across the globe (28–32). Their potential hazard to nontarget invertebrates has been presented in a relatively large body of literature based on laboratory experiments (see <https://cfpub.epa.gov/ecotox/>) as well as some field observations (33). These studies indicate that a variety of insect species

Significance

Survey data show a large-scale decline in insects. This global decline is often linked to human actions in intensive agricultural areas. To investigate whether this decline has a causal relationship with neonicotinoid insecticides, we performed an outdoor experiment with representative surface water concentrations of the neonicotinoid thiacloprid. We exposed naturally formed aquatic communities to increasing neonicotinoid concentrations and monitored insect emergence during a 3-mo period. We show that increasing neonicotinoid concentrations strongly decreased the abundance and biomass of five major insect orders that together comprised >99% of the 55,574 collected insects as well as the diversity of the most species-rich freshwater family, thus showing a causal relation between insect decline and neonicotinoids.

Author contributions: S.H.B., M.S., G.R.d.S., P.M.v.B., and M.G.V. designed research; S.H.B., M.S., A.v.N., and M.G.V. performed research; S.H.B., M.S., G.R.d.S., P.M.v.B., and M.G.V. analyzed data; and S.H.B., M.S., G.R.d.S., P.M.v.B., A.v.N., and M.G.V. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission. B.G.H. is a guest editor invited by the Editorial Board.

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¹Present address: Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Science Park 904, 1090 GE Amsterdam, The Netherlands.

²To whom correspondence may be addressed. Email: S.H.Barmantlo@uva.nl.

This article contains supporting information online at <http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2105692118/-DCSupplemental>.

Published October 25, 2021.

might be at risk (34), including populations of pollinators (26, 33) as well as other nontarget organisms such as freshwater insects (35). Nonetheless, experimental studies that evaluate the potential causal relationship between environmental levels of neonicotinoids and decreasing natural insect abundances are lacking. Moreover, most of the risk assessment focuses on short-term experiments that are performed with a few species of insect larvae (36), while mid- to long-term exposures to the whole life cycle of natural insect communities are less often assessed (but see refs. 37 and 38) even though imagoes (i.e., the adult life stage of emerging insects) are crucial for species propagation. Although there is evidence that connects the neonicotinoid imidacloprid to declines in dragonflies (22), there remains a major knowledge gap on the causality between the widespread occurrence of neonicotinoid insecticides and the ongoing decline of insects (39, 40).

Here, we tested the hypothesis that field-realistic surface water concentrations of neonicotinoids cause a decline in abundance, species richness, and biomass of aquatic emerging insects. We explored the effects of a range of concentrations of a model neonicotinoid, thiacloprid, on insect emergence of naturally assembled populations residing in experimental freshwater ecosystems (Fig. 1). To this end, we applied two biweekly spikes of 0, 0.1, 1.0, or 10 $\mu\text{g/L}$ to 9 experimental ditches per concentration and surveyed insect emergence from these, in total, 36 ditches for a duration of 3 mo. Using this experimental approach, we investigated the relationship between thiacloprid concentration and the diversity of the most species-rich family of freshwater insects as well as the abundance and biomass of five orders of insects, Coleoptera, Diptera, Ephemeroptera, Odonata, and Trichoptera, which together comprised >99% of the total emerged insects.

Results

Insects Collected and Exposure Conditions. During a 3-mo time-span, we collected insects twice a week from emergence traps that were placed over 36 experimental ditches. In total, 55,574 emerged aquatic insects were caught, which included 51,458 flies and midges (Diptera), 1,842 mayflies (Ephemeroptera), 1,088 beetles (Coleoptera), 919 caddisflies (Trichoptera), 232 dragonflies and damselflies (Odonata), 30 hymenopterans (Hymenoptera), three true bugs (Hemiptera), and two alderflies (Megaloptera). Fourteen percent of the insects were trapped in the first month before the first neonicotinoid spike was applied (i.e., before the May 18, 2018), and 86% were caught during the experimental period (i.e., May 18 to July 12, 2018). The number of trapped insects is dependent on the increasing spring and summer temperatures, and as such, this pattern of higher insect emergence later in the season

coincided with a steady increase of 7 °C in water temperature during the first month of trapping (SI Appendix, Fig. S1).

Neonicotinoid-Driven Decline in Insect Abundance. Total insect emergence was significantly affected by neonicotinoid concentrations in interaction with time (adj. $R^2 = 0.67$, $\chi^2 [34, n = 864] = 6,107$, $P < 0.001$) but did not follow a dose–response relationship (Fig. 2 “Total”). Compared to the control, we observed a temporal increase in emergence at a nominal spike concentration of 0.1 $\mu\text{g/L}$ (52% at $t = 24$ d). A decrease of emergence was observed at 10 $\mu\text{g/L}$ (25% at $t = 55$ d). This pattern was strongly driven by Diptera, as they made up 93% (on average, SD = 5.4%) of the total abundance and their emergence mirrored the patterns for total abundances (adj. $R^2 = 0.64$, $\chi^2 [34, n = 864] = 6,050$, $P < 0.001$, Fig. 2 “Diptera”). Effects of the neonicotinoid on the other four insect orders—Coleoptera, Odonata, Trichoptera, and Ephemeroptera—were more pronounced; shortly after applying thiacloprid, we observed large reductions in the number of emerging insects for all four orders. For Coleoptera, we observed strong declines over time, resulting in 61 and 91% reduced emergence at 1.0 and 10 $\mu\text{g/L}$ thiacloprid on the final day of insect collection (adj. $R^2 = 0.58$, $\chi^2 [34, n = 864] = 368$, $P < 0.001$, Fig. 2 “Coleoptera”), while we observed no effects at the lowest test concentration. Similar patterns were found for Odonata [which were strongly dominated by *Ischnura elegans* (41)] and Trichoptera, as they also consistently decreased over time relative to the control (adj. $R^2 = 0.61$, $\chi^2 [34, n = 864] = 28$, $P < 0.001$ and adj. $R^2 = 0.42$, $\chi^2 [34, n = 864] = 47$, $P < 0.001$, respectively). At the final day of collection, we observed decreases of 37, 53, and 61% in Odonata with increasing neonicotinoid concentrations, respectively (Fig. 2 “Odonata”). The number of emerged Trichoptera, like Coleoptera, came to an abrupt near full stop within 10 d after application (Fig. 2 “Trichoptera”), leading to a decline in abundance of 74% at the final day of collection at 10 $\mu\text{g/L}$ thiacloprid. Ephemeroptera emergence only started 1 mo (mid-June) after the initial spike but was immediately affected by the neonicotinoid application, albeit not in a dose–response manner (adj. $R^2 = 0.43$, $\chi^2 [34, n = 864] = 1,109$, $P < 0.001$). We found a sharp average decline of 98% relative to the control in the cumulative number of emerged Ephemeroptera at a concentration of 10 $\mu\text{g/L}$ thiacloprid (Fig. 2 “Ephemeroptera”). At 1.0 $\mu\text{g/L}$, however, the cumulative number of Ephemeroptera increased by 138% relative to the control on the final day of insect collection.

Effects on Biomass of Emerging Insects. The total collected insect biomass significantly declined with increasing neonicotinoid concentration (adj. $R^2 = 0.92$, $F_{1,34} = 0.84$, $P < 0.001$; Fig. 3). At the final day of sampling, average cumulative biomass had



Fig. 1. Overview of the experimental site (Left). Shown are individual experimental ditches adjoining to a small lake. Each ditch was fitted with one emergence trap from which insects are caught and directly stored (Right).

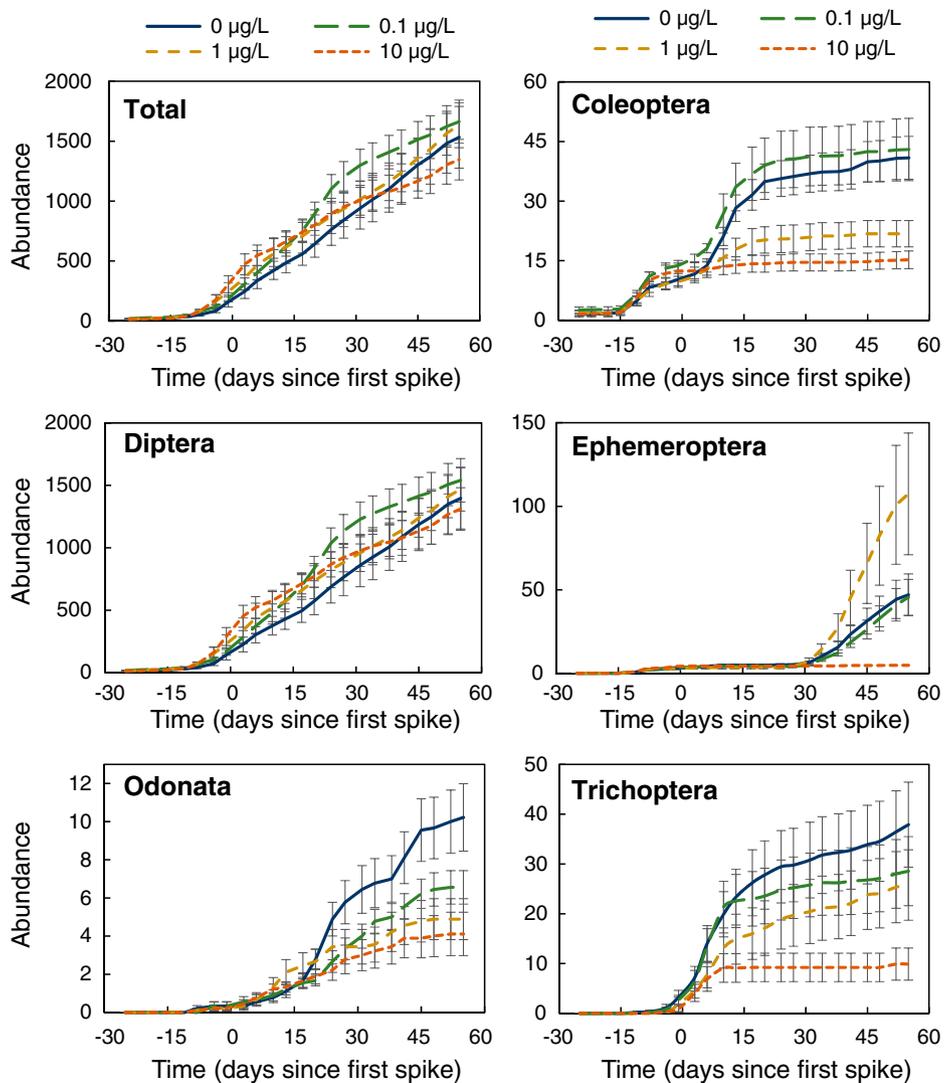


Fig. 2. Average cumulative number of emergent insects (per 60-cm² water surface level, \pm SEM, $n = 9$) of the total emergence and that of the five major taxonomic orders over time per nominal spike concentration of the neonicotinoid thiacloprid.

declined during the exposure period by 11, 4, and 50% with increasing neonicotinoid concentrations, respectively (Table 1). The decline in median cumulative biomass was 18, 27, and 48% with increasing neonicotinoid concentration, respectively (*SI Appendix*, Fig. S2). This deviance between average and median biomass is explained by some high Ephemeroptera counts at a nominal concentration of 1.0 $\mu\text{g/L}$, skewing down the decrease in average biomass (Fig. 2 “Ephemeroptera” and Fig. 3). The order of Diptera made up the bulk of the biomass collected, constituting on average 52% of the control biomass on the final day of collection. Cumulative Diptera biomass decreased with increasing thiacloprid concentrations (adj. $R^2 = 0.91$, $F_{1,34} = 0.59$, $P < 0.001$), by 7, 17, and 25% at the final day of collection, respectively (Table 1). Despite these losses, the relative contribution of Diptera to total biomass increased to 82% at 10 $\mu\text{g/L}$ ($F_{1,34} = 18.9$, $P < 0.001$) as the contribution of the other taxonomic orders decreased more strongly (Fig. 3). Coleopterans accounted for 10.5% of the total control’s biomass but decreased to 9.5, 3.3, and 1.9% with increasing neonicotinoid concentrations, respectively ($F_{1,34} = 15.1$, $P < 0.001$). Both Trichoptera and Odonata appeared to experience a similar decrease in relative contribution (Fig. 3), but these were not confirmed statistically ($P > 0.05$ for both comparisons).

Nevertheless, as reflected by their abundances, the overall biomass of all orders decreased substantially with increasing thiacloprid concentrations.

Effects of Neonicotinoid Exposure on Diversity. Effects of neonicotinoid exposure on species diversity were investigated for the most species-rich family of insects, the Chironomidae (order: Diptera). One month after the first application of the neonicotinoid (i.e., after >95% neonicotinoid removal from the aqueous phase), we identified 477 male individuals belonging to 29 different species of three different subfamilies of Chironomidae (Chironominae, Orthocladiinae, and Tanytopodinae; see *SI Appendix*, Table S1 for the full list of species). Neonicotinoid exposure induced a strong dose–response driven decline in species diversity. We calculated a 50% reduction (or EC_{50}) of Shannon H -diversity to occur at a spike concentration of 1.11 $\mu\text{g/L}$ (SE 0.41). Mean Shannon diversity (H) was 1.60 and 1.67 for the concentrations 0 and 0.1 $\mu\text{g/L}$ thiacloprid and decreased to 0.95 and 0.16 at 1.0 and 10 $\mu\text{g/L}$ thiacloprid, respectively (Fig. 4). A similar decline was observed in Shannon evenness (J): 0.88 to 0.87, 0.60, and 0.33 with increasing concentrations ($EC_{50} = 3.24 \mu\text{g/L}$, SE 1.81). These effects on evenness represent an underestimation as species richness was reduced to

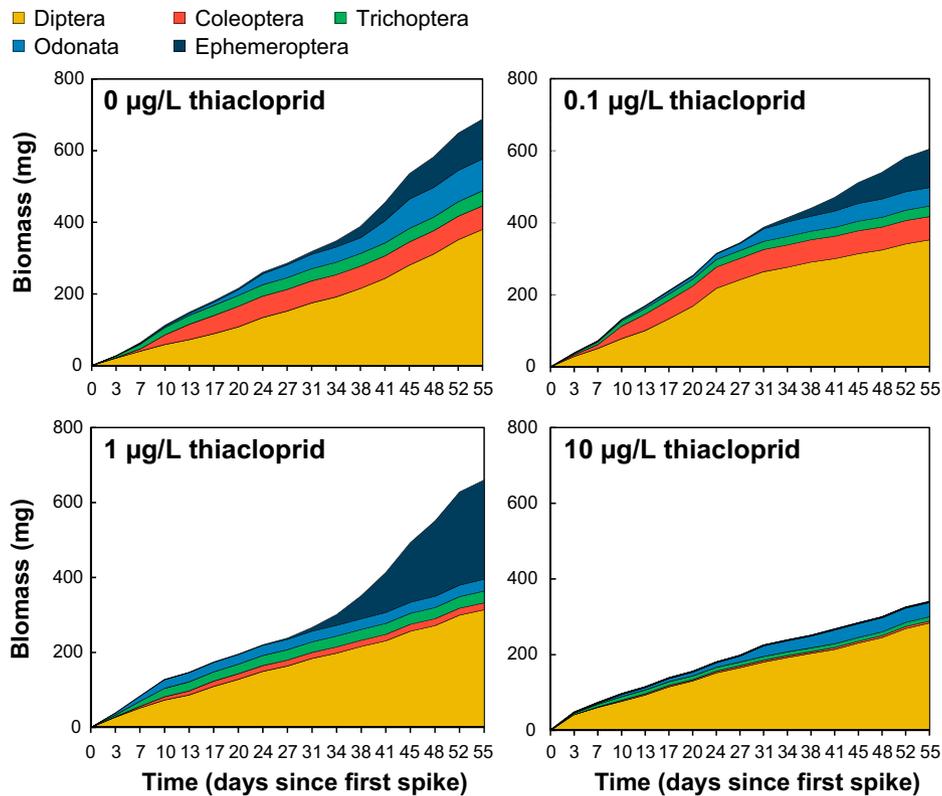


Fig. 3. Thiachloprid-induced changes in average insect biomass of the different taxonomic orders over time per nominal spike concentration of neonicotinoid. The neonicotinoid thiachloprid was applied at $t = 0$ and $t = 15$ d.

only one species, *Procladius choreus*, in 60% of the samples of the highest concentration which prevented an evenness calculation (SI Appendix, Fig. S3). In contrast to all other Chironomidae, this species actually strongly increased in abundance by 301 and 310% on average at the two highest test concentrations relative to the control (Dunnett’s post hoc test: $T = 2.5$, $P = 0.024$ and $T = 2.4$, $P = 0.030$, respectively). This strong increase also explains for the most part the lack of large effects on total and Diptera emergence (see Fig. 2 “Total” and “Diptera”), as *P. choreus* made up on average 77% of the total emergence (assuming that thiachloprid had equal effects on females since we only identified males) at this point in time at the highest test concentration compared to 17% for the control (SI Appendix, Fig. S4).

Discussion

The debate on the insect decline within the scientific community (4, 6), among policy makers (26), and in the media (including news

articles, e.g., refs. 42 and 43), often concentrates on the role of insecticides and their supposed negative impacts on insect populations. Despite this awareness, the actual risks to natural insect populations and ecosystems are largely unknown due to a lack of well-controlled, field-realistic experiments (39, 40). Our results show that field-realistic levels of neonicotinoids in surface water negatively affect abundance and biomass of orders of major emerging insect orders. We provide evidence that more common neonicotinoid surface water concentrations (41) halve the species diversity of the most species-rich freshwater insect family, the Chironomidae. At the highest test concentration, Chironomidae diversity mostly dropped to a single species, while emergence of three other insect orders (Coleoptera, Ephemeroptera, and Trichoptera) was close to nonexistent.

The observed impacts of insecticide application were not as apparent in the total cumulative abundance, as this metric was largely distorted by the opposite response of a single Chironomidae species. This observation nicely illustrates that identification to

Table 1. Cumulative biomass (in mg/60 cm² surface water \pm SE) per insect taxonomic order on the final day of collection ($t = 55$) since the first neonicotinoid application ($t = 0$)

| Biomass per taxonomic order | Concentration spiked thiachloprid ($\mu\text{g/L}$) | | | |
|-----------------------------|---|----------------------|-----------------------|----------------------|
| | 0 (control) | 0.1 | 1 | 10 |
| Total | 690.1 (± 96.0) | 612.7 (± 73.2) | 662.4 (± 112.2) | 344.6 (± 27.2) |
| Coleoptera | 65.8 (± 15.1) | 64.7 (± 26.5) | 18.6 (± 3.3) | 5.4 (± 1.6) |
| Diptera | 379.8 (± 86.0) | 352.3 (± 38.3) | 313.8 (± 28.6) | 283.3 (± 29.5) |
| Ephemeroptera | 111.1 (± 31.1) | 106.6 (± 27.5) | 264.7 (± 92.1) | 1.7 (± 0.6) |
| Trichoptera | 42.2 (± 9.0) | 29.2 (± 7.7) | 31.5 (± 7.8) | 11.0 (± 3.3) |
| Odonata | 88.9 (± 17.9) | 51.6 (± 8.7) | 33.1 (± 9.0) | 38.7 (± 16.0) |
| Hemiptera + hymenoptera | 2.3 (± 1.3) | 8.2 (± 3.9) | 0.7 (± 0.6) | 4.5 (± 2.3) |

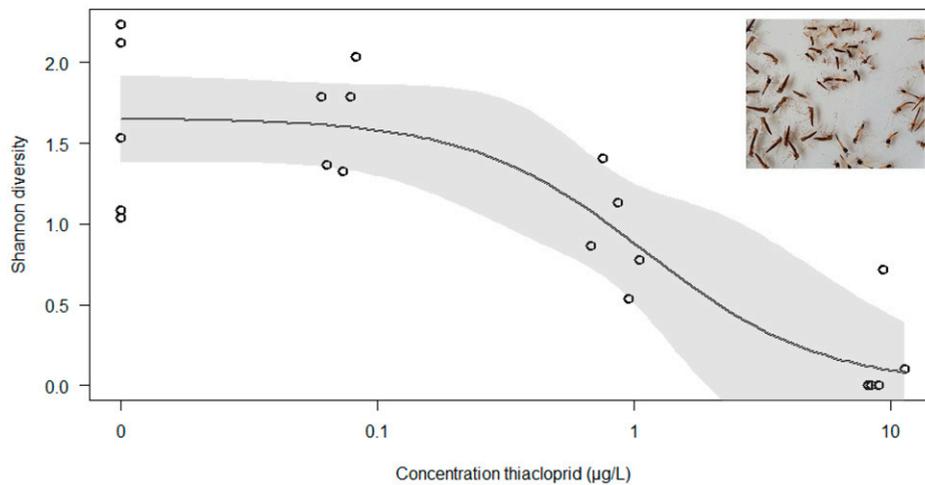


Fig. 4. Dose–response model ($\pm 95\%$ confidence intervals in gray shading) of male Chironomidae (photo inset shows an impression of control diversity) (H , $n = 5$) per spike concentration of the neonicotinoid thiachloprid 1 mo after the first spike. The x-axis is log transformed for presentation purposes.

species level is sometimes essential to understand insecticide-induced impacts to natural communities. These results are largely in line with a previous stream mesocosm study where significant reductions in emerging insects were observed at concentrations of $3.2 \mu\text{g/L}$ thiachloprid and higher (37). However, this previous research found that after 8 wk, thiachloprid no longer exhibited an effect on either abundance or diversity, which was explained by the strong dominance of multivoltine taxa that filled the vacant niche space. In contrast, we found that there was still a near full stop in emergence for four out of five major taxonomic orders after 8 wk while a strong dominance of multivoltine taxa was also present. The responses of Coleoptera, Ephemeroptera, Trichoptera, and Odonata were all relatively straightforward: field-realistic neonicotinoid surface water concentrations reduced their abundance as well as their relative contribution to total emerged biomass. These results for the order of Odonata match the growing body of literature that show the negative effects of field-realistic neonicotinoid concentrations on Odonata species (22, 41, 44, 45). Emergence of Coleoptera, Ephemeroptera, and Trichoptera were near absent at our highest test concentration, which is largely in line with results for thiachloprid of single species bioassays within these orders (30, 46, 47) and results from mesocosm experiments that assessed abundances of insect aquatic life stages (37, 48). Nevertheless, we observed that Ephemeroptera were thriving at nominal spike concentrations that are considered toxic ($1.0 \mu\text{g/L}$) (46), thus highlighting the importance of field-realistic experiments. As our ditches are continuously (re)colonized, we hypothesize that Ephemeroptera egg deposits occurred after removal of most of the neonicotinoid from the aqueous phase. This explanation coincides with the observed very rapid removal of thiachloprid after application ($\text{DT}_{50} = 3.6 \text{ d}$, see *Neonicotinoid Application*) and the fast development of commonly occurring Ephemeroptera species in these systems [generally a few weeks in this season and habitat type (49)]. At the lowest test concentration, shortly after neonicotinoid application, we observed a temporary increase in abundance and a coinciding decrease in biomass of Diptera, indicating that Diptera diversity shifted toward species with smaller and/or lighter imagoes, which often have shorter life cycles (50) (such as *P. choreus*; see later in this section). This finding may also apply to the results found for Ephemeroptera. Species with a short aquatic lifespan might have avoided neonicotinoid exposure or even benefited from vacant niche space. Despite these exceptions, the overall picture emerging is that total cumulative abundance of imagoes of all orders of insects was negatively associated with increasing neonicotinoid concentration.

While the total abundance of Chironomidae did not decrease as much as in other groups, its species diversity exhibited a clear response to increasing thiachloprid concentrations. Species diversity was halved at a spike concentration of $1.0 \mu\text{g/L}$ thiachloprid, which averages out to a time-weighted average concentration of $0.3 \mu\text{g/L}$ during the first month after spiking (after which these Chironomidae identifications were performed). These spike and time-weighted average concentrations approximate the neonicotinoid geometric mean for peak and average surface water concentrations of 0.63 and $0.13 \mu\text{g/L}$ based on 27 studies (30). However, data from these studies and most other surveys rely on grab samples, which are shown to underestimate average concentrations by 50% and peak surface water concentrations by a magnitude of 1 to 3 orders (51), and neonicotinoid concentrations up to $320 \mu\text{g/L}$ have been observed (29). Surveys that include thiachloprid surface water concentrations, however, are relatively scarce (29). For the Netherlands, survey data on thiachloprid concentrations from 2013 to 2017 revealed that, when thiachloprid was detected in surface water, 8% of all samples fell in the concentration range of 0.1 to $1 \mu\text{g/L}$ (41). The nominal spike concentration of $1.0 \mu\text{g/L}$ also approximates the concentration where 50% mortality of the common test species *Chironomus riparius* was previously observed in laboratory experiments ($1.6 \mu\text{g/L}$) (52). Indeed, in surface water mesocosm or field experiments, a reduction in Chironomidae larvae diversity and/or abundance due to exposure to the neonicotinoid imidacloprid have also been observed, however, typically at test concentrations that are a factor 2 to 100 higher than presented here (38, 53). For thiachloprid, in our experimental ditch system, large reductions in Chironomidae larvae as well as in other mesocosm setups have also been observed at concentrations that fall within our concentration range (37, 48, 54). As removal of species can be considered more critical than reduced survival, our results show that toxicity in the field can be far more severe, as has also been observed before for thiachloprid at the experimental site (55). We further stress these strong results of decreasing species diversity with increasing thiachloprid concentration, as Shannon diversity indices are generally considered relatively insensitive endpoints in ecotoxicological mesocosm assessments (56). For example, an earlier wetland study on the effects of the neonicotinoids imidacloprid, clothianidin, and thiamethoxam only showed subtle effects of these substances on the timing of insect emergence but no strong, negative effects on diversity or abundances (57). Such differences in results for Chironomidae or the other insects compared to previous studies can potentially be attributed

to our experimental setup with high statistical power ($n = 9$) and the fact that we tested four different concentrations, whereas many mesocosm studies are limited to two or three concentrations (including the control).

We found that one species of Chironomidae, *P. choreus*, showed a contrasting response and increased strongly in abundance with increasing neonicotinoid concentration, explaining the weaker decline in total emergence and in Diptera emergence compared to the other orders. This illustrates the importance of subtle interactions in an ecosystem that consequently lead to contrasting responses, which underscores the importance of performing field-realistic experiments. We hypothesize that the mostly predaceous *P. choreus* larvae moved into the vacant niche of aquatic predators as is reflected by the strong declines in the predaceous Odonata. Furthermore, earlier experiments also showed observed losses in other invertebrate predator taxa (48) and predator activity (41) due to thiacloprid exposure in the same test system. Chironomidae often make up the vast majority of aquatic emergent insect abundance in freshwaters (58) and can be considered useful indicators for water quality (59) due to their abundance and wide range of species with specific habitat preferences. Results of our study show that neonicotinoids can enact changes in Chironomidae diversity, and, as such, temporal shifts in their diversity could potentially be a readily applicable indicator for sites that are suspected of neonicotinoid pollution.

In addition to affecting insects directly, neonicotinoid usage and surface water concentrations have been associated with a decrease in insectivorous bird species (60, 61). One hypothesis is that such decreases in insectivorous birds could be due to a food source (insects) depletion as a result of the use of neonicotinoids (60–62). A comprehensive study on an assemblage of 10 insectivorous bird species showed that aquatic insects compromised 10 to 40% of the annual energy budget and in spring and autumn and, depending on species, this increased up to 90% (63). The nutritional value of aquatic insects for insectivorous birds has been observed to be higher compared to terrestrial insects due to the higher concentration of highly unsaturated omega-3 fatty acids (64), which further highlights the importance of aquatic insects in insectivorous bird diets. Results from our experimental study confirm the hypothesis that neonicotinoids can have a detrimental effect on food abundance to insectivorous birds: the biomass of emerging insects decreased across five major orders, together comprising >99% of the total biomass. What is more, the present study was carried out when insectivorous birds in Western Europe forage for insects to feed their young (65), which matches the timeline of increasing neonicotinoid surface water concentrations in the Netherlands (55). In this sense, our results might even underestimate the magnitude of the effects, considering that we only applied two spikes and aqueous neonicotinoid concentrations were removed in a matter of days.

Our well-replicated experiment with naturally assembled insect communities allowed us to identify a causal relationship between increasing levels of neonicotinoids and a steady decline in the emergence of aquatic insects. Based on these results, we conclude that this realistic range of neonicotinoid insecticides in surface waters has a range of distinct negative effects on abundance, biomass, and diversity of emerging aquatic insects. Although our results come from a specific freshwater type under temperate climate conditions, they are likely a conservative estimation because we only tested a single neonicotinoid that was rapidly removed from the system. In most field situations, multiple pesticides are found across much longer time spans (66), likely with additive effects and possibly multiplicative effects as well. Likewise, interactions with adjuvants or environmental factors might also add to or alter the exhibited neonicotinoid toxicity (55, 67–69), which further

complicates predictions on the actual magnitude and contribution of neonicotinoids in the observed insect decline. Nevertheless, given the urgency of the insect decline, our results highlight the need to reconsider the mass usage of neonicotinoids to preserve freshwater insects as well as the life and services depending on them.

Methods

Test Location. The experiment was carried out in 36 experimental freshwater ditch-ecosystems at the outdoor “Living Lab” facility of Leiden University (Leiden, The Netherlands). Each ditch-ecosystem (length-width-depth: 10-0.8-0.3 m; volume: 1,750 L) was placed side-by-side and adjoined to a small lake (Fig. 1). This lake provides a natural source of plants, microbes, and invertebrates that (re)colonize the ditches, leading to similar communities (48). The ditches are colonized by large numbers of invertebrates ranging across 31 different taxonomic orders, among which are 9 insect orders. For more details on the experimental site, see ref. 48 and <http://mesocosm.org/>. After an initial colonization period (November 2017 to April 2018), each ditch was hydrologically isolated from the adjoining lake with 1,000 × 500 × 2 mm acrylic plates that were placed at the end of each ditch. We specifically mimic ditches as these systems are generally the first surface water bodies to receive agricultural contaminants (due to processes such as runoff from agricultural fields) and consequently the highest concentrations of pesticides are found here.

Sampling of Emergent Insects. Each experimental ditch-ecosystem was equipped with a single emergence trap at the center of the ditch to continuously trap and sample emerging insects from mid-April to mid-July. Each trap consisted of a pyramid of stainless-steel rods (length-width-height: 60-60-74 cm) that was fitted with No-See-Um netting (300 holes/cm²). All traps were installed 3 cm below the water surface level (connected with tie-raps to bamboo sticks that were tightly stuck in the ditch banks) to prevent the escape of emerging insects. Furthermore, each trap was equipped with the insect collection system according to ref. 70 consisting of two 500-mL polyethylene bottles connected to each other via cutout caps (Ø 3.5 cm) that are glued together (Fig. 1). The upper (capture) bottle is connected to the trap via a 6.5 by 4.5 cm opening in both the netting and bottle. The lower (collection) bottle is filled with 100 mL of 80% ethanol (20% tap water) as a capture and preservative medium for the insects. These lower bottles were replaced twice a week (every Monday and Thursday) for bottles with fresh preservative. The collected insects were then transferred to 25-mL glass Falcon snap caps with freshly prepared 80% ethanol.

Neonicotinoid Application. To evaluate whether experimental communities were similar prior to the experimental treatments, we assessed insect emergence in the month before applying the experimental treatments. After this 1-mo period, we started application of thiacloprid in mid-May and continued to follow emergence for two more months. We applied spikes of thiacloprid (CAS no. 111988-49-9, purchased from Sigma-Aldrich) in four different concentrations: 0, 0.1, 1, and 10 µg/L thiacloprid with nine replicate ditches per concentration. Two weeks after this initial spike, we again applied these spikes in the same concentrations in order to maintain concentrations for 1 mo as could be predicted from earlier observations on thiacloprid removal from the aqueous phase (55). Before application, no detectable thiacloprid was present in the water and sediment based on analytical measurements that were below the detection limit measurements in the control and in ref. 51 as well as the fact that there are no agrochemical maintenance practices present in the close proximity of the test system. The 0 to 10 µg/L concentration range represents most of the range of neonicotinoid concentrations observed in surface waters across the globe (29, 31, 32, 71–73). We chose to solely administer thiacloprid without adjuvants as we aimed to investigate field-realistic surface water concentrations of thiacloprid, meaning that a multitude of adjuvants could be considered relevant because of different application methods and testing of these different (concentration) combinations would become unfeasible. Stock solutions of thiacloprid were prepared in 1 L of demineralized water in glass bottles. These stock solutions were diluted in a 10 L glass bottle with ditch water of the ditch the treatment was imposed on. This solution was then evenly poured over the entire length of the ditch. We monitored the thiacloprid concentrations using LC-MS/MS analysis (see ref. 55 for the sampling procedure and ref. 46 for chemical analysis). Direct measurements after application of the two neonicotinoid spikes showed that our actual spike concentrations were 0 (control), 0.08 (nominal 0.1), 0.85 (nominal 1.0), and 9.86 µg/L (nominal 10 µg/L). Thiacloprid dissipated rapidly with DT50 (half-life) of 3.6 d and a DT90 of 12.0 d (41).

Insect Identification. All trapped insects were first identified to the order level. Larger specimens of other taxonomic orders, such as the dragonfly *Orthetrum cancellatum*, were identified to species level. Apparent dominant taxa, such as the beetle genus *Helophorus*, were also determined beyond the order level in order to correct for their dominance during the biomass assessment (see *Insect Biomass*). For the order of Diptera, we identified all individuals to family level using a stereo microscope and the European Diptera key (74). All these identifications were performed by a single observer. During identification, it became apparent based on morpho species that there were strong effects of thiacloprid on Chironomidae diversity. Therefore, because Chironomidae are among the most sensitive species to neonicotinoids (30, 54), we identified all individual Chironomidae to the species level for a subset of the experimental ecosystems. As this is a labor-intensive process, we selected five replicates per concentration (the central 20 ditches within the block design) 1 mo after the initial application, being the middle of the experimental period. Only male specimens were selected, as these are much easier to identify than females. After separating the males from the female Chironomidae using a stereomicroscope, all specimens were dissected following the protocol by ref. 75, embedded in Euparal and finally identified using a compound microscope and refs. 75–77 as guidelines for determination. These identifications for specifically the Chironomidae were performed by a second observer.

Insect Biomass. We determined the relative contribution of the different insect groups to total biomass by weighing randomly selected specimens per taxonomic order and per Diptera family from different treatments and dates collected. With this method, potential intraspecific variation due to neonicotinoid exposure is overlooked, but the bulk of the sample is preserved for future reference and analysis. As differences in body size can skew biomass estimates, we had different protocols for biomass determination for samples of the orders of Odonata, Coleoptera, and Hymenoptera, depending on body size. For larger taxonomic orders (such as Odonata), one to a few insects were dried for 3 d at 30 °C per sample to determine the biomass per individual. For Coleoptera, two dominant size classes were apparent (>4.5 mm and <4 mm), mostly belonging to the genus *Helophorus*, and therefore these were dried and weighed separately. Differences in size within the order of Ephemeroptera and Trichoptera were relatively small, and we therefore considered that random selection of individuals captured any relative spread in biomass. For smaller taxonomic orders (such as Diptera, often <1 mg dry weight per insect), about 5 to 20 insects were dried and weighed per sample using a

microbalance. To further avoid large skews in biomass, we took a large number of samples (in total 91, roughly 30 per month) consisting of multiple specimens per sample for the species-rich family of Chironomidae in order to capture the full range of differences in dry weight. In some cases, specimens of a certain group (such as Hymenoptera) were both small and very rare. In these instances, we used the dry weight of a comparable size class. All weights were divided by the number of specimens per sample in order to obtain the biomass per individual. This was repeated, if the number of specimens were sufficient, 20 times to determine an average biomass per taxon.

Statistical Analyses. All collected insect numbers and biomass data were first transformed to cumulative data. We then assessed the effects of neonicotinoid concentration on total emergence and on taxon emergence both in abundance and biomass. For this, we used generalized additive models (function gam, package “mgcv”) with a Poisson distribution and nominal spike concentrations, time (in days), and their interaction as explanatory variables. As thiacloprid concentrations declined rapidly (see *Neonicotinoid Application*), we used the nominal spike concentration rather than the actual concentration per sampling date. We smoothed the terms for nominal spike concentration and its interaction with time using factor smooths with 24 kn, which is a knot for every point in time that insects were collected from the trap. Finally, we accounted for the repeated measures design by including ditch ID as a random factor. Effects of actual neonicotinoid spike concentrations on Chironomidae diversity were investigated by dose–response modeling using Shannon diversity (*H*) and Shannon evenness (*J*) metrics as response variables (function drm, package “drc”). All statistical analyses were performed using R [version 3.5.1 (78)].

Data Availability. Excel files data have been deposited in Dryad repository (79).

ACKNOWLEDGMENTS. We are grateful to our students Janneke van der Horst, Justin Knetsch, and Mitchel van 't Riet for their valuable contribution to the experimental work. We are also grateful to Pasquale Ciliberti for his assistance with insect identification and to Edwin Giesbers for photographs presented. The funding for the “Living Lab” facility largely came from crowdfunding. NWO-VIDI number 864.13.010 granted to M.G.V. was used to support S.H.B.

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