Neonicotinoids and fertilizers jointly structure naturally assembled freshwater macroinvertebrate communities

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HIGHLIGHTS

- Aim: assessing combined effect of two model agrochemicals on aquatic communities.
- Experiments under natural conditions with a neonicotinoid and fertilizer.
- Taxa richness and total abundance were unaffected at realistic concentrations.
- Structure of the communities shifted markedly, with long-term consequences.
- Joint application exhibited the strongest effects on community structure.

GRAPHICAL ABSTRACT
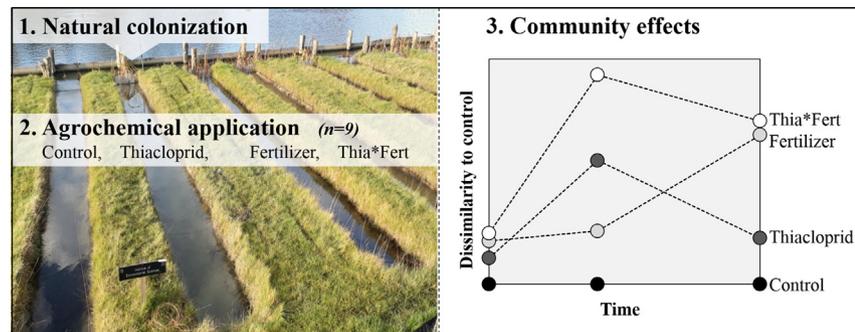
ABSTRACT

Although it is widely acknowledged that a decline of freshwater biodiversity jeopardizes the functioning of freshwater ecosystems, the large number of (human-induced) pressures jointly acting on these systems hampers managing its biodiversity. To disentangle the magnitude and the temporal effects of these single and interacting pressures, experiments are required that study how these pressures affect the structuring of natural communities.

We performed experiments with naturally assembled invertebrate communities in 36 experimental ditches to assess the single and joint effects of environmentally relevant concentrations of two commonly co-occurring stressors: fertilizer inputs and neonicotinoid insecticides, in this case thiacloprid. Specifically, we explored whether these agrochemicals result in sustained changes in community structure by inspecting divergence, convergence and short- /long-lived dissimilarity of communities, when compared to a control treatment.

Our results indicate strong impacts on the abundance of different taxa by exposure to the agrochemicals. However, we found no effect of any treatment on total abundance, taxon richness or convergence/divergence (measured as beta dispersion) of the communities. Moreover, we found contrasting responses when both joint stressors were present: when considering abundance of different taxa, we observed that fertilizer additions reduced some of the thiacloprid toxicity. But when assessing the community structure, we found that exposure to both stressors consistently resulted in a more dissimilar community compared to the control. This dissimilarity was persistent up to four months after applying the agrochemicals, even though there was a turnover in taxa explaining this dissimilarity. This turnover indicates that the persistent dissimilarity can potentially be attributed to a rippling effect in the community rather than continued toxicity. Such shifts in natural freshwater invertebrate

communities, months after the actual exposure, suggests that stressors may have important long-term repercussions for which may subsequently lead to changes in ecosystem functioning.

1. Introduction

Globally, we are witnessing a strong decline in freshwater aquatic biodiversity, which negatively affects the functioning of freshwater ecosystems (Dudgeon et al., 2006; Schwarzenbach et al., 2006). These declines have been widely attributed to anthropogenic pressures (Schwarzenbach et al., 2006; Rockström et al., 2009), but the large number of different pressures that are found jointly in freshwater ecosystems hampers the identification and prediction of their adverse effects to freshwater ecosystems. As biota drives ecosystem functioning, an understanding of how different anthropogenic pressures jointly affect and structure freshwater communities is thus essential to effectively manage and conserve these ecosystems.

Studying how anthropogenic stressors impact the structure of aquatic communities has proven notoriously difficult as natural communities are affected by a variety of pressures that generally operate at the same time (Côté et al., 2016). Two of the most prominent anthropological pressures on freshwater ecosystems result from the emission of agricultural fields: pesticides and fertilizers (Matson et al., 1997; Davis et al., 2010; Malaj et al., 2014), agrochemicals that are often applied and found together (Schreiner et al., 2016). Especially the effects of neonicotinoid insecticides have gained increasing attention (see e.g. Hallmann et al., 2014; Pisa et al., 2015) as they are among the most commonly used pesticides on the global market (Jeschke et al., 2011) and consequently show elevated concentrations in surface waters (Morrissey et al., 2015). Maximum observed concentrations are typically found around 0.1–1 μg/L (see Fig. 2b in Morrissey et al., 2015), but were also found to reach up to 320 μg/L for the neonicotinoid imidacloprid in Dutch surface waters (van Dijk et al., 2013).

Over the past decades, it has become apparent that, in addition to agricultural pest insects, non-target invertebrates including freshwater macrofauna experience high toxicity from neonicotinoids at concentrations that are found in surface waters across the world (Morrissey et al., 2015; Miles et al., 2017; Vijver et al., 2017). Several authors have provided evidence that such concentrations also affect invertebrate populations and communities (Beketov et al., 2008; Miles et al., 2017; Barmentlo et al., 2018b; Rico et al., 2018). However, recent data suggests that such effects can be reduced by increased nutrient levels or food quality as this may allow for compensatory feeding (Ieromina et al., 2014; Barmentlo et al., 2018a). Fertilizers (or nutrients) themselves also present an important stressor for aquatic communities (e.g. Davis et al., 2010). This entanglement of different types of processes thus explains why the study of the combined effects of different stressors, such as neonicotinoids and fertilizers, to natural communities has been proven to be so challenging (Alexander et al., 2013; Côté et al., 2016).

Obtaining understanding on how the single and joint effect of pesticides and fertilizers affect communities requires experiments that disentangle the single and interacting pressures that operate on natural freshwater communities. When comparing to a control situation, such data allow for the examining of changes in the structuring of communities. A powerful tool to analyze such changes is by identifying whether the stressors alter the state of the communities, distinguishing two important changes in community state: 1) convergence: a perturbation causes all communities to become reduced to one state and 2) divergence: a perturbation results in communities to evolve to multiple states or no clear state (Houseman et al., 2008). A lower degree of variation between communities (lower beta dispersion) after a disturbance indicates convergence and can possibly be a result of ecological filtering, for example, due to

neonicotinoids that harm specific species (Fig. 1A, scenario i). On the other hand, a higher degree of variation after a disturbance (higher beta dispersion) would indicate divergence, which would for example be due to fertilizer inputs that broaden or add ecological niches by increasing productivity; Fig. 1A, scenario iii). In addition, communities can become more or less dissimilar compared to a control state (Fig. 1B) irrespective of the degree of variation between communities.

To move towards an integral understanding of the joint effects of fertilizers and neonicotinoids under natural conditions, we distinguish three possible outcomes of these agrochemicals on naturally assembled freshwater invertebrate communities. Assuming that environmentally relevant concentrations of both agrochemicals indeed affect the communities, the joint effect can result in i) a qualitatively different shift in community composition, but one of the pressures masks or reduces the effect of the other ii) an additive effect of both agrochemicals, or iii) a combined effect of both agrochemicals that is stronger than the single effects. This study aims to evaluate the single and joint effect of agrochemical stressors on taxa abundance and structuring of communities, both in terms of beta dispersion (Fig. 1A) and dissimilarity (Fig. 1B). Moreover, we test whether shifts in community composition are short-lived or relatively long-lived, i.e. whether the changes persist up to a four-month period (Fig. 1B). To this end, we assessed the impact of environmentally relevant concentrations of a model neonicotinoid, thiacloprid, and fertilizer in experimental ditches on naturally assembled freshwater communities at three different time points: one month before treatments (assuming no effects and similar communities), one month after and four months after application of the agrochemicals.

2. Methods

2.1. Experimental site

The experiment took place in the outdoor facility 'Living Lab' of Leiden University (the Netherlands; Fig. 2). In November 2016, 36 adjacent ditches with a length of 10 m, a width of 0.8 m at the surface level and 0.4 m at the sediment level on a depth of 0.3 m were dug adjoining to an existing water level compensation reservoir in Oegstgeest, The Netherlands (Fig. 2). The reservoir has an average depth of 1.5 m and is connected to the Old-Rhine watershed through ditches and canals. The reservoir is located next to uncultivated grassland (for the past ~30 years, owned by Leiden University) and characterized by low levels of soluble nutrients and absence of any pesticide residues as there are no agricultural (or agrochemical maintenance) practices in close proximity. A longstanding undisturbed ecological community of invertebrates, fish, birds and plants is present in the reservoir, yielding full ecosystem complexity. The ditches are protected from waves from the reservoir by a wooden barrier, thus more naturally mimicking actual drainage ditches and protecting the testing facility from erosion. All ditch banks are covered by organically grown grass. Furthermore, between the wooden barrier and the ditch entrance, a row of *Typha latipholia* was planted in order to attract invertebrates that would colonize the ditches. Fish were retained from accessing the ditches by placing 5 mm netting (dug into the sediment and reaching 10 cm above the water surface level) between the wooden barrier and the ditch entrance. Birds were effectively repelled from the ditches by a brightly colored orange wire hanging 5 cm above the water surface level over the length of each ditch.
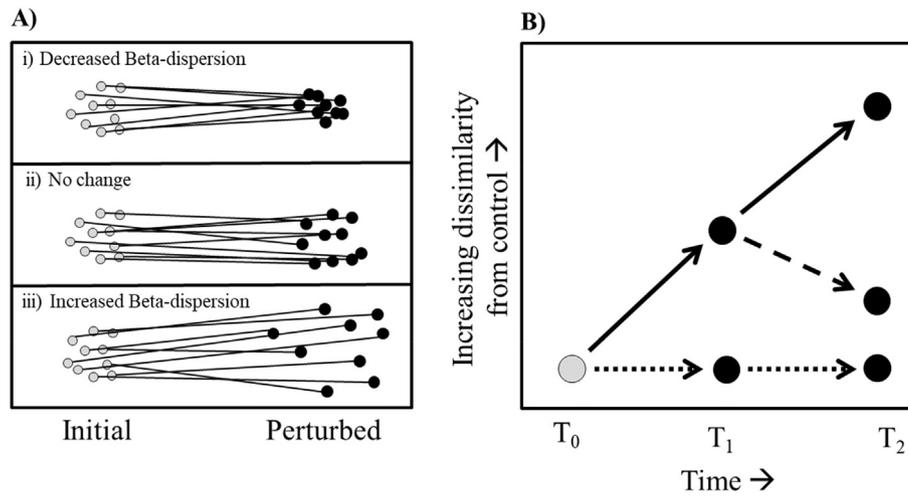
Fig. 1. (A) Effects on beta dispersion; communities can either gain niche width between the initial and perturbed state (top panel; divergence), do not change (middle panel), or lose niche width (lower panel; convergence). (B) Effects on community similarity; pressures can cause the communities to become more dissimilar (straight arrows), veer back to the control (dashed arrow) or stay similar (finely dashed arrow) to the control. Circles denote the individual communities.

2.2. Experimental setup

The 36 experimental ditches had been dug by removing 1.8 m of top-soil and were left connected to the reservoir for six months (November – April) to form naturally colonized communities of invertebrates and plants. As we dug nearly two meters down, the ditches were initially characterized by clay with low levels of organic matter. Therefore, we transplanted as little as 10 L of organically rich sediment from the adjoining reservoir into each experimental ditch in February 2017. In April 2017, we sampled the biota residing in the ditches (see 2.3 for the sampling procedure) in order to establish if natural communities have formed comparably between the ditches. Then, in order to avoid cross-contamination of agrochemicals between treatments, we ensured that all ditches became hydrologically isolated from the reservoir using 1000*500*2 mm acrylic plates that were hammered firmly into the ditch banks and 15 cm deep into the sediment. After this, the total ditch length was 9 m. Hereafter, we started application of the different agrochemicals. The experiment entailed a full factorial design of four different treatments with nine replicates per treatment: i) control (no added substances, 'C'), ii) thiacloprid addition ('T'), iii) fertilizer addition ('F') and iv) thiacloprid and fertilizer addition ('TF') (See Appendix figure A1 for a schematic overview of the block design). Substances were added to the ditches following a block design in order to account for the effects of possible naturally occurring gradients. We use thiacloprid as a model neonicotinoid as it is among the most toxic of neonicotinoids to non-target organisms and like most neonicotinoids abundantly present in surface waters (Morrissey et al., 2015; Leiden University and Rijkswaterstaat-WVL, 2017).

Starting in April 2017 (after hydrologically isolating the ditches), we enriched half of the ditches continuously with fertilizer by hanging three sachets filled with 75 g of slow-releasing granulates (Osmocote; N:P:K = 15:9:11 combined with microelements, Dordrecht, The Netherlands) in each ditch. These sachets were replaced with fresh granulates every six weeks. Fertilizer addition was aimed to approach nutrient levels that have been experimentally showed to stimulate freshwater algal growth (see Ieromina et al., 2014). In May 2017, we started two biweekly applications of thiacloprid (99.9% purity; Sigma-Aldrich, Zwijndrecht, The Netherlands; see Appendix for details on the application procedure). We aimed to maintain a nominal time weighed average concentration (TWA) of 0.4 μg/L for the duration of one month. This environmentally relevant concentration of thiacloprid was determined from data retrieved from the Leiden University and Rijkswaterstaat-WVL dataset (2017) that shows grab samples of surface water concentrations of thiacloprid in the Netherlands in the period 2011–2015 (discussed and shown in Barmentlo et al., 2018a). The experimental concentration (0.4 μg/L) falls in the top 10% of detect concentrations measured in the month May. However, note that thiacloprid is highly likely to quickly adsorb to sediment (given its log K_{ow} of 1.26; USEPA, 2003) and consequently the chance to detect the maximal surface water concentration via grab sampling is low. In addition, the smaller waters like we simulate here are often not monitored and are generally the first recipient of agrochemical loadings, meaning that they likely receive higher neonicotinoid concentrations. Our experimental concentration is not exclusive to the Netherlands but also fits the range for other countries and neonicotinoids (see Fig. 1B in Morrissey et al., 2015) To investigate the effectiveness of our nutrient enrichments and thiacloprid addition, we monitored dissolved nitrate (NO_3^-) and phosphate (PO_4^{3-}) concentrations using a NOVA 60 Spectroquant® photometer (Merck). These analyses were performed with water samples that were retrieved from the middle of each ditch, 5 cm below the surface. From these water samples, we also determined the thiacloprid concentration one hour after application of a spike. Subsequently, we measured randomly selected blocks within the block design daily for a week after the first thiacloprid application, then biweekly and finally monthly over the summer period. Thiacloprid concentrations were determined using liquid chromatography tandem mass spectrometry (Agilent Technologies; see Roessink et al., 2013 for the detailed procedure). We also monitored the possible effects of the agrochemicals on several physicochemical parameters including, water temperature, pH, dissolved oxygen (DO) and conductivity. See

Fig. 2. Photographic overview of the experimental site 'the Living Lab'. Top to bottom: adjoining reservoir, wave barrier, macrophytes, netting, experimental ditches.

the Appendix for a detailed description on the methods and results for thiacloprid and nutrient concentration determination and for the physicochemical responses.

2.3. Community sampling

One month before (April), one month after (June) and four months after (September) the first application of thiacloprid (May), we sampled the macroinvertebrate communities residing in each experimental ditch. Sampling was conducted by quickly and simultaneously placing two acrylic plates with a width of 1 m into a ditch, thus isolating 1/9 of its length. By sampling all macroinvertebrates in this meter of ditch, we were able to standardize the number of taxa and number of animals caught to a fixed volume of water and sediment rather than the number of sweeps with a dipping net over a fixed length (as is the common practice, for example Ieromina et al., 2015). Within this 1 m of ditch, macroinvertebrates were sampled by sweeping with a 25 * 25 cm dipping net (150 μm). All plants within the compartment were carefully washed by hand to retrieve all macroinvertebrates. In order to catch the benthic macroinvertebrates, we sampled the top 3-5 cm of the sediment and ditch banks and sieved out the invertebrates using a 500 μm sieve. We stopped sampling when subsequent nets remained empty. All invertebrates were carefully collected in large white trays, sorted on species groups with the naked eye (e.g. leeches, beetles) and then immediately identified to the lowest possible level using a stereomicroscope (magnification: 20-40×) at the on-site laboratory. In order to determine the species composition in all 36 communities with minimum disturbance to the community within the shortest possible time span (i.e. within one week to minimalize time-dependent effects), we estimated the abundances of highly dominant taxa (such as Branchiopoda and Maxillopoda) by sieving these animals over a fine sieve (106 μm) and subsequently placing them in 500 mL of water. A subsample of this fixed volume was analyzed until at least 50 individuals were counted or 25% of the total sample volume was counted to ensure a representative sample of the community. The number of animals within the subsample was then multiplied to fit the total filtrate volume. Directly after identification, macroinvertebrates were released back into the ditch compartment they originated from in order to avoid a decline in communities in the ditches. The whole procedure from sampling to release took 1–2 h per ditch.

2.4. Statistical analyses

In order to detect the formation of multiple community states as a result of either thiacloprid addition ('added' or 'not added') or fertilizer addition ('added' or 'not added') and their possible interaction, we tested for dissimilarity of the communities using Permutational multivariate analysis of variance (PERMANOVA, function 'adonis', R package 'Vegan') with 999 permutations. We log10(x + 1) transformed all count data and used Bray-Curtis as our measure of dissimilarity as recommend by Tebby et al. (2017) for data 1) with large differences in abundances between species and 2) where the less abundant species may be affected. This was evaluated by examining the histograms per species. Our full model investigated the effects of fertilizer, thiacloprid, time (April, June, September, included numerically) and all possible interactions while including the respective ditch measured as a random variable to account for potential effects of the ditch. As freshwater communities are highly variable between seasons (because of the high turnover in abundances and species), we investigated the relative effects of both fertilizer and thiacloprid per month as well to gain more insight on the specific community responses. In these monthly models, we included ditch number as a fixed effect (ranging from 1 to 36, aligned from left to right at the experimental site) and all possible interactions with any of the treatments in order to evaluate the effects of possible natural gradients within the experimental setup. To test whether the dispersion of the data was homogeneous across the different

treatments, we used permutated (999) distance-based dispersion tests (function 'betadisper', R package 'Vegan'). Deviations from such homogeneity can point to convergence (decreased distances) or divergence (increased distances) within a treatment compared to the control. Finally, when treatments indicated a significant effect on the community centroids, we calculated the average contribution of individual taxa to the overall Bray-Curtis dissimilarity using SIMPER (similarity percentage, R package 'Vegan').

We also investigated for possible effects of thiacloprid ('added' or 'not added'), nutrients ('added' or 'not added'), time and all possible interactions on total taxon richness, total abundance and average abundance per taxon using linear mixed effect modelling (function 'lme') while accounting for the repeated measure design by including the ditch as a random effect. In order to more closely examine the effects of both agrochemicals and their possible interaction on the community structure, we investigated their potential effect on the taxonomic class abundance per month using factorial ANOVA (function 'lm') We tested for these differences to identify whether group-specific effects of the agrochemicals occurred: for example lower insect abundance because of thiacloprid (an insecticide) addition. We tested for normal distribution of the model residuals using Quantile Quantile-plots and homogeneity of variances using Levene's. If either assumption was not met (i.e., for the models on Insecta and Maxillopoda in June and for Malacostraca and Gastropoda in September), we log transformed the data accordingly. Prior to the analyses, we omitted one value in the thiacloprid treatment in September due to the extremely high number of Daphniidae observed (18,400 individuals), which heavily skewed the data as the treatment average was 571 individuals in total. This was due to an unexplained and unusual high concentration of phosphate (>1 mg/L) in this ditch in September. Statistical significance was considered at $p < 0.05$ and marginal significance is reported at $0.05 < p < 0.10$. All statistical analyses were performed with R (version 3.5.0; R Core Team, 2018).

3. Results

3.1. Effectiveness of the treatments

The actual TWA concentration of thiacloprid during the month after the first spike was 0.46 μg/L (see Appendix, Table A1). Thiacloprid concentrations in the water declined rapidly with an average DT50 of 3.3 days (SD 0.1) and DT90 of 11.1 days (SD 0.4) (Appendix Fig. A2). This was expected due to the high log octanol-water partition coefficient of thiacloprid (log K_{ow} = 1.26; USEPA, 2003), thus thiacloprid was likely to adsorb to the sediment. There was no effect of fertilizer addition on the thiacloprid concentration at any given time ($p > 0.05$), meaning that thiacloprid did not degrade more rapidly because of e.g. increased bacterial degradation. Fertilizer addition significantly increased the TWA concentrations of both nitrate and phosphate ($F_{1,32}$ = 4.7, $p = 0.037$; $F_{1,31}$ = 12.0, $p = 0.002$) with a factor of 1.3 and 1.4 respectively (see Appendix, table A1 for the actual concentrations).

3.2. Colonization, taxon richness and total abundances

The broad range of observed taxa from different classes of macroinvertebrates illustrate that the experimental ditches were effectively colonized (Figs. 3 and 4A,B,C) with a total of 59, 83 and 97 identified taxa in the months April, June and September, respectively (see Appendix for the full lists of taxa per month). Average taxon richness per meter ditch in the controls increased significantly over time from 18.0 taxa in April to 20.9 taxa in June and 27.2 in September ($F_{1,68}$ = 127.9, $p <$ 0.001; Fig. 3A). However, note that the abundance per taxon significantly decreased in September (5.8) compared to July (25.8, $F_{1,8}$ = 39.6, p < 0.001). There were no effects of the treatments on the total taxon richness at any given month (thiacloprid: $F_{1,65}$ = 1.5, $p > 0.05$ and fertilizer: $F_{1,65}$ = 1.2, p > 0.05). There were also no observed
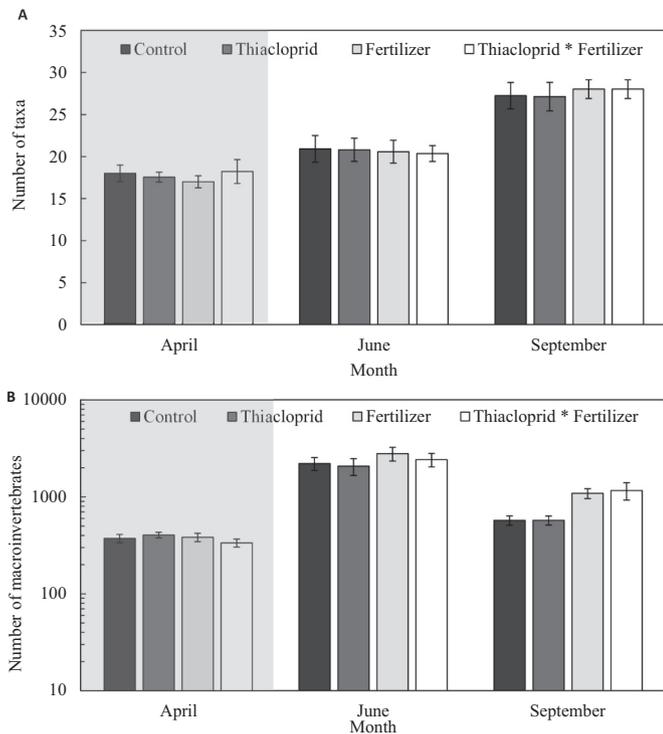
Fig. 3. Average ($n = 9$) A) macroinvertebrate taxon richness ($\pm$SE, total number of identified taxa) and B) total macroinvertebrate abundance ($\pm$SE) per treatment in 1 m of ditch per month (spiking was conducted in May 2017). Grey shading shows data before the addition of the agrochemicals.
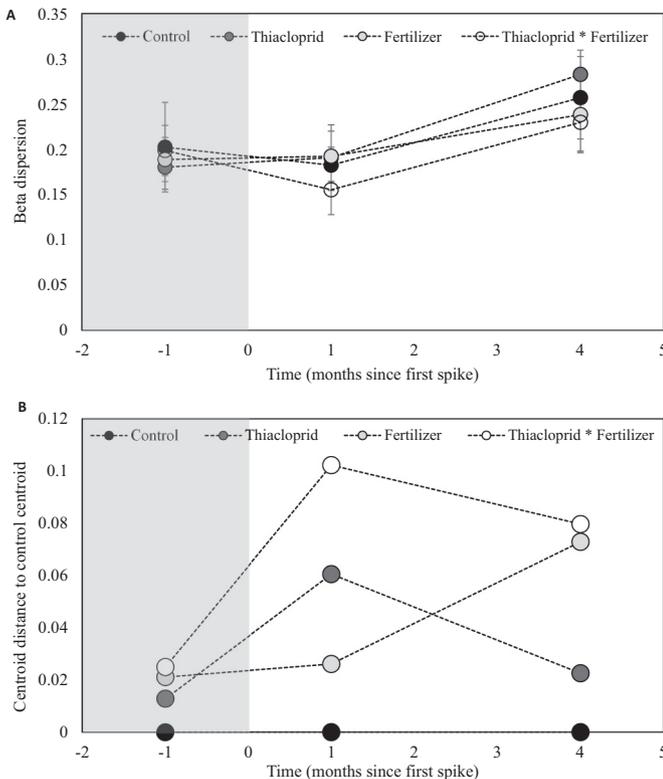

Fig. 4. A) Average beta dispersion (distance to centroid, $\pm$SD) per treatment and B) Centroid distance (Principal Coordinates Axes 1 and 2) to the control centroid per month. Treatments were applied at $t = 0$ (May). Grey shading shows data before the addition of the agrochemicals.

significant effects of the treatments on the total number of animals in either April or June (thiacloprid: $F_{1,32} = 0.3$, p > 0.05 and fertilizer: $F_{1,32} = 1.4$, p > 0.05). However, fertilizer addition increased the total number of animals four months after the first application ($F_{1,32} = 18.5$, $p < 0.001$; Fig. 3B) by 100% compared to the control.

3.3. Temporal dynamics in dissimilarity and dispersion

Both thiacloprid and fertilizer induced significant dissimilarity interacting with time relative to the control ($F_{1,100} = 12.5$, $R^2 = 0.06$, p < 0.001 and $F_{1,100} = 6.2$, $R^2 = 0.03$, p < 0.001 respectively); the thiacloprid treatment more strongly affected the community centroid in June, while the effect of fertilizer application was stronger in September (Fig. 4B). Time, fertilizer and thiacloprid also showed a significant three-way interaction resulting in communities that were most dissimilar from the control in both June and September compared to the single treatments ($F_{1,100} = 3.4$, $R^2 = 0.02$, p < 0.001; Fig. 4B). The effect of time on the community dissimilarity was strong ($F_{1,100} = 59.7$, $R^2 = 0.27$, p < 0.001), showing that the communities were highly dynamic in time. When investigating the communities more closely per month, we found no difference between community centroids in the prospective treatments before the addition of the agrochemicals (April; PERMANOVA: $p > 0.05$, Fig. 4A). Community centroid shifted significantly with ditch number, which is a (fixed effect) spatial variable in these analyses ($F_{1,28} = 5.8$, $R^2 = 0.15$, p < 0.001), indicating a possible underlying spatial gradient affecting macroinvertebrate communities. This spatial impact on the communities was also present, but weaker over time, in both June and September ($F_{1,28} = 5.7$, $R^2 = 0.13$, p < 0.001 and $F_{1,28} = 2.6$, $R^2 = 0.07$, p < 0.001 respectively), but never interacted with any treatment effect (p > 0.05 for all possible interactions).

We found no significant effect on the distances to the centroids (beta dispersion) in the prospective treatments before the addition of the agrochemicals (April; beta dispersion tests, thiacloprid: $F_{1,34} = 0.4$, p > 0.05 and fertilizer: $F_{1,34} = 0.1$, p > 0.05, Fig. 4A). Moreover, there was no observed difference in the distances to the centroid (beta dispersion) for both thiacloprid and fertilizer addition in June ($F_{1,34} = 1.6$, p > 0.05 and $F_{1,34} = 1.7$, p > 0.05 respectively; Fig. 4A). Fertilizer addition did appear to reduce the average distance to the centroid in September ($F_{1,34} = 4.7$, $p = 0.037$; Fig. 4A), but only in the thiacloprid – thiacloprid*fertilizer comparison. We also evaluated this effect of fertilizer using raw data (as recommended for data with large differences in abundancies, see Tebby et al., 2017) and observed no deviation from homogeneity ($F_{1,34} = 0.2$, p > 0.05). Because of the large differences in total abundancies (Fig. 3C) and the incoherency with the raw data, we consider that the slight deviation from homogeneity of the $\log(x + 1)$ transformed model, if at all present, is negligible.

3.4. Monthly taxonomic class-specific responses to the agrochemicals

While there was no effect of the treatments on total abundance in June, the abundances of the different taxonomic classes were affected in this month (Fig. 5B). We observed that the addition of thiacloprid resulted 51% lower abundances of individuals belonging to the class Insecta compared to the control treatment in June, one month after application ($F_{1,32} = 5.0$, $p = 0.032$; Fig. 5B). In June, the Insecta class was dominated by the family Chironomidae which contributed, on average, 83% to the total abundance within the control treatment. This explained most of the observed lower insect abundance, but there were clear effects in other insect taxa as well (see Table 1), most notably a 97–100% lower abundance of the alderfly *Sialis lutaria*. As a result of fertilizer addition, we observed marginally significant higher abundances for Insecta (35%) and Maxillopoda (29%) in June relative to the control ($F_{1,32} = 3.8$, $p = 0.060$ and $F_{1,32} = 4.0$, $p = 0.055$ respectively; Fig. 5B). The class Malacostraca showed significantly higher abundances in the fertilization treatment (93%) in June ($F_{1,32} = 4.2$, $p = 0.048$;
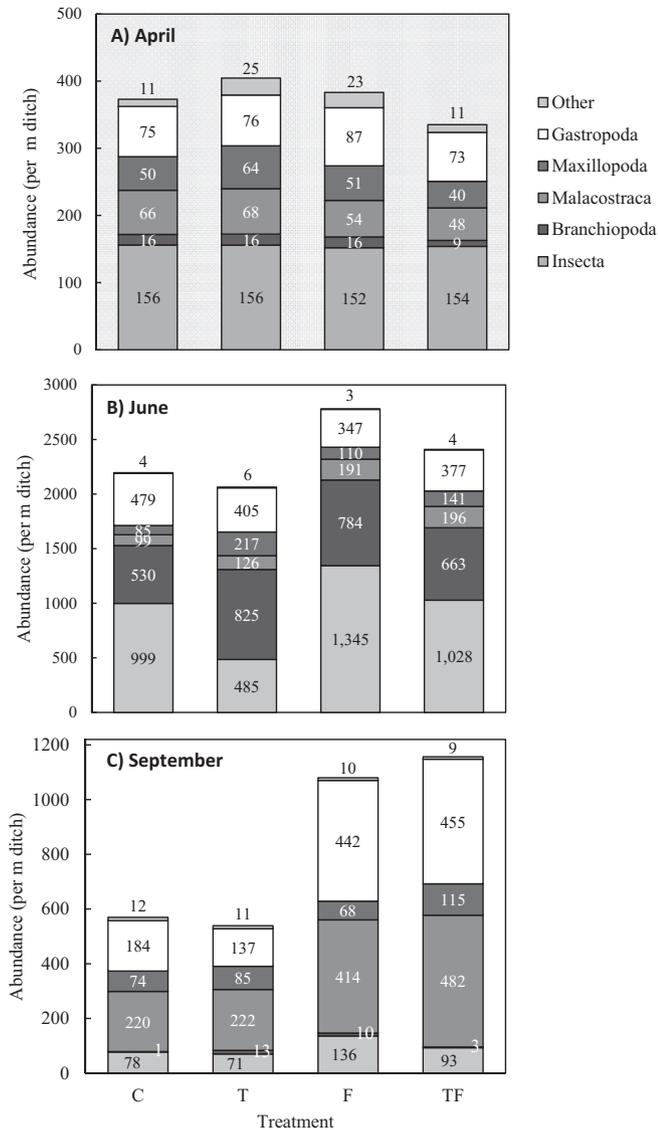
Fig. 5. Average macroinvertebrate class abundance (n = 9) of the most abundant classes one month before (A), one month after (B) C) and four months after the first thiacloprid application. C = control, T = thiacloprid, F = fertilizer and TF = thiacloprid and fertilizer. 'Other' is the sum of all other identified classes. Grey shading shows data before the addition of the agrochemicals.

Fig. 5B) relative to the control. In September, the number of Malacostraca again significantly higher by fertilizer addition ($F_{1,32} =$ 10.2, $p = 0.003$; Fig. 5C) compared to the control. The number of Gastropoda in September was also significantly higher due to fertilizer addition compared to the control (104%, $F_{1,32} = 16.9$, $p < 0.001$, Fig. 5C). For Insecta, we observed a marginal higher number by fertilizer addition, similar to June (75%, $F_{1,32} = 3.9$, $p = 0.058$).

3.5. Taxon specific responses to the agrochemicals

Both in June and September, thiacloprid significantly shifted the community centroids ($F_{1,28} = 3.7$, $R^2 = 0.09$, p < 0.001 and $F_{1,28} =$ 2.1, $R^2 = 0.05$, $p = 0.002$ respectively; Fig. 4B). The taxa that contributed most on average to the observed dissimilarity between the 'C' and the 'T' treatments in June are *S. lutaria*, Daphniidae, *Chaoborus* sp. and *Notonecta viridis* (average contribution to the dissimilarity: 6.93, 6.50, 4.62 and 4.30%, respectively; Table 1). This was partially comparable to those taxa contributing most to the dissimilarity between the 'C and 'TF' treatments: *S. lutaria,* Daphniidae, *Helophorus minutus* and

Helophorus aequalis (average contribution to the dissimilarity: 7.27, 5.52, 5.05 and 4.69%, respectively; Table 1). For *S. lutaria*, we observed 0.8 and 0.1 individuals on average in the 'T' and 'TF' treatments versus 25.0 in the control. Four months after application, in September, the taxa contributing most to dissimilarities between the 'C' and 'T' treatments shifted to *Proasellus coxalis*, Daphniidae and *Ortheterum cancellatum* and *Cymatia coleoptratra* (average contribution to the dissimilarity: 5.29, 3.71, 3.38 and 3.15%, respectively). This was partially similar to the C-TF comparison where *P. coxalis*, *Hippeutis complanatus*, *O. cancellatum* and *Sigara lateralis* contributed most (average contribution to the dissimilarity: 4.48, 4.00, 3.61 and 3.60%, respectively). Solely fertilizer addition also significantly shifted the community centroid in September ($F_{1,28} = 2.6$, $R^2 = 0.07$, p < 0.001; Fig. 4). Contributing most to this observed dissimilarity between the 'C and the 'F' treatment were *P. coxalis*, *C. coleoptrata*, *H. complanatus* and Coenagrionidae (average contribution to the dissimilarity: 5.74, 4.58, 3.68 and 3.28% respectively, Table 1).

4. Discussion

Using a well-replicated experimental design with a semi-natural setup, our results show that at environmentally relevant concentrations fertilizers and neonicotinoid insecticides jointly structure aquatic macroinvertebrate communities. One of our expected outcomes was that fertilizer input could 'mask' or reduce the effects of neonicotinoids. While we found such effects at the species or family level, our results suggest the opposite effect for communities: the magnitude of effects on community structure was greater for the joint application when compared to the effects of the single stressors. Moreover, our results indicate long-term legacy effects of the stressors; community structure was still altered four months after the neonicotinoid was applied. Our observations with natural aquatic community assemblages add to previous laboratory and mesocosm studies which show isolated effects on individual sensitive species (e.g. Beketov et al., 2008; Roessink et al., 2013), as we show that effects can feed through to higher levels of organization in aquatic ecosystems and that species turnover strongly affect community composition in response to toxic stress.

This is, to our knowledge, the first study to investigate the joint effect of agricultural pressures to fully naturally (re-)assembled aquatic invertebrate communities of macroinvertebrates. Previous studies have addressed effects of neonicotinoids on communities by focusing on either recovery time of sensitive species (e.g. univoltine species, see Beketov et al., 2008), species with sensitive traits to chemical pollution (SPEAR, see Liess and Beketov, 2011), predator-prey interactions (Alexander et al., 2016), or food-web structure (Schrama et al., 2017). Most of these studies report that application of neonicotinoids result in a decline in total abundance and/or richness (for example: Beketov et al., 2008; Alexander et al., 2013; Rico et al., 2018). Using our study system, experimental ditches that are open to natural (re)colonization, we find that none of these relatively simple metrics were affected by the treatments: total abundance, taxon richness and beta dispersion (variation) between communities all remained remarkably similar between treatments. However, our results do show pronounced treatment-induced changes in community composition, where single and combined stressors result in different communities compared to a situation not exposed to stressors. For example, thiacloprid application resulted in 51% lower insect abundance, especially pronounced in Chironomidae (50% lower) and larger predator species (*S. lutaria*, *Notonecta viridis* and *Gerris thoracicus;* combined lower abundance of 96%). In contrast, we observed a strong increase in the abundance of multivoltine taxa such as Daphniidae and Cyclopidae, thus explaining the lack of effects on total abundance. While chironomids have previously been identified as a family of insects that is particularly sensitive to neonicotinoids (Langer-Jaesrich et al., 2010), the lower abundances in large predators was not expected as toxicity values for these species generally are several-fold higher than our concentrations (see Roessink et al., 2013

Table 1
Average contribution ('contr', %, highest to lowest, SIMPER analysis) to the observed dissimilarity per taxon (log10[x + 1] transformed). Shown are taxa (species groups and *species*) that contribute for >2.5% to the observed dissimilarity. 'NS' means no significant difference (PERMANOVA). Yellow colors indicate average lower and blue colors higher abundances compared to the control.

| Month | Comparison | | | | | |
| | Control - Thiacloprid | | Control - Fertilizer | | Control – Thia*Fert | |
	Taxon	Contr. (%)	Taxon	Contr. (%)	Taxon	Contr. (%)
June	*S. lutaria*	6.93	NS	NS	*S. lutaria*	7.27
	Daphniidae	6.50			Daphniidae	5.52
	Chaoborus sp.	4.62			*H. minutus*	5.05
	N. viridus	4.30			*H. aequalis*	4.69
	Cyclopidae	3.88			Chironomidae	4.17
	G. thoracicus	3.81			*V. piscinalis*	4.15
	C. coleoptrata	3.78			*N. viridis*	4.11
	H. aequalis	3.76			*G. thoracicus*	3.70
	V. piscinalis	3.63			*H. pusillus*	3.33
	Chironomidae	3.62			Cyclopidae	3.06
	P. antipodarum	3.09			*P. coxalis*	2.94
	H. minutus	2.93			*H. complanatus*	2.74
	G. tigrinus	2.59			*G. tigrinus*	2.66
September	*P. coxalis*	5.29	*P. coxalis*	5.74	*P. coxalis*	4.48
	Daphniidae	3.71	*C. coleoptrata*	4.58	*H. complanatus*	4.00
	O. cancellatum	3.38	*H. complanatus*	3.68	*O. cancellatum*	3.61
	C. coleoptrata	3.15	Coenagrionidae	3.28	*S. lateralis*	3.60
	A. aquaticus	2.81	Daphniidae	3.00	*C. coleoptrata*	3.12
	Cyclopidae	2.74	*A. aquaticus*	2.72	*A. aquaticus*	2.91
	Sigara sp.	2.73	*Sigara* sp.	2.69	*E. octoculata*	2.84
	L. hyalinus	2.64			Coenagrionidae	2.83
	Coenagrionidae	2.60			Cyclopidae	2.80
	Chironomidae	2.60			*G. tigrinus*	2.61
					Tubifex sp.	2.52

and Morrissey et al., 2015). Several of these species that showed lower abundances fulfill important ecosystem process within as well as outside of the aquatic ecosystem as they are, for example, common food sources for terrestrial predators such as birds and bats (Hallmann et al., 2014). Not only do these results suggest a potential impact on the (aquatic) predator trophic level by neonicotinoids, they also suggest that studying metrics such as diversity and abundance under strongly controlled conditions may paint a too simple story. As such, the inclusion of natural (re)colonizing communities in the experimental setup (to allow for species turnover) opens up unique research possibilities to evaluate effects of single and multiple co-occurring stressors on natural communities and its concomitant impacts on ecosystem functioning. The current results already point towards changes in ecosystem

functioning as the several species that showed relative higher or lower abundances are important players for several ecosystem processes (such as emergent taxa as a food source for birds and bats, chironomids that might be important for phytoplankton abundance and OM degradation or planktonic species that might be important for phytoplankton abundance).

Results from our study show that joint stressors have a qualitatively different effect from the effect of the single stressors, both at the individual species and at the community level. The sole addition of thiacloprid resulted in a community structure that was dissimilar from the control. These results are in line with Beketov et al. (2008) who already showed that inoculated macroinvertebrate communities in artificial stream mesocosms were affected by thiacloprid at a nominal concentration of

3.2 μg/L. We found different communities at over six times lower TWA concentration (0.5 μg/L), that is representative for concentrations observed in surface waters (Morrissey et al., 2015). Like thiacloprid, ditches with fertilizer were also statistically significantly dissimilar to the control, but this was found only four months after the introduction of the treatment. These effects were most clear when considering total abundance, which increased by 100%. Such increases in abundance by additional nutrients are well-known (for example Davis et al., 2010) and were anticipated. When studying both stressors in concert we found that fertilizer caused a marked decrease in toxicity of the neonicotinoid to some taxa; Chironomidae abundance was 50% lower after thiacloprid application, but the addition of fertilizer nearly nullified this response. This is in accordance with previous studies that found reduced effects on nutrients on species-specific neonicotinoid toxicity (Alexander et al., 2013; Ieromina et al., 2014; Barmentlo et al., 2018a, 2018b). However, fertilizer addition did not reduce the effect of thiacloprid on community structure as a whole. In fact, the joint application of both agrochemicals resulted in a community composition that was most dissimilar from the control. This is likely caused by a combination of two effects. On the one hand, taxa that disappeared after thiacloprid application were also absent from the mixture treatment as exemplified by the alderfly S. lutaria (97 and 100% lower abundances, respectively). On the other hand, some taxa that reacted strongly and positively to fertilizer increased even stronger in the mixture treatment. For example, abundance of beetles of the genus Helophorus showed 49% higher abundances in the ditches with solely fertilizer addition compared to the control, whereas they had 265% higher abundances in the mixture. This could potentially be explained by an initial filling of a (wider) opened ecological niche (a double indirect effect, see Gessner and Tlili, 2016) and, subsequently, dissimilarity remained as the community state did not return to the control state. Overall, such observed shifts in community structure suggest that neonicotinoids can cause a rippling effect in the community that can even be amplified by the presence of nutrients.

Our results indicate that stressor-induced ecological differentiation can persist onto the next season. Particularly for the treatments containing neonicotinoids this is a remarkable effect because thiacloprid was removed rapidly from the water column (>95% concentration decrease within two weeks). Dispersal limitation cannot explain this phenomenon as 'polluted' ditches were directly next to 'unpolluted' ditches and the adjacent lake. We speculate that there are three important explanations for the lack of, or slow, recovery. First, the slow recovery of uni- and semivoltine species (as suggested and found earlier by Beketov et al., 2008 and Rico and Van den Brink, 2015) may be underlying the lower abundances in both June and September, as shown by for example S. lutaria (explaining 2.48% of the dissimilarity in September, not shown in Table 1) and the water boatmen Cymatia coleoptrata (uni/bivoltine species, Table 1). Second, neonicotinoids form a nearly irreversible bond to the post-synaptic nicotinergic acetylcholine receptors (nAChR) in insect central nervous systems (Tennekes, 2010), likely continuing to hamper the performance of the toxicologically sensitive species that were developing over these months. Third, effects in spring could lead to cascading effects later in the season. This idea is supported by the observation that taxa that explained most of the dissimilarity between the control and thiacloprid treatment in September were not necessarily the same taxa as in June (see Table 1). The most likely ecological explanation for this is that thiacloprid induced not only a short-term toxicity effect (as discussed in the previous paragraph) but also a long-term rippling effect in the aquatic communities. As we found no differences in beta dispersion (for all months and treatments), we found no indications for divergence, convergence or unidentified community states. This means that the observed agrochemical-induced (rippling) effects on the community structure had a similar pattern within treatments and were thus consistent. Such consistent patterns of structuring of aquatic communities may help future studies and managers to help predict the effect of fertilizers and neonicotinoids in the natural aquatic environment.

5. Conclusions

We identify fertilizer and neonicotinoids as important single and joint drivers of freshwater macroinvertebrate communities. The application of both agrochemicals resulted in consistently altered macroinvertebrate communities. The neonicotinoid thiacloprid induced persistent changes in the communities without affecting the magnitude of the variation between the communities (i.e., no changes in beta dispersion). Given the key role of biota in maintaining ecosystem processes and ultimately the functioning of the ecosystem (Hooper et al., 2005), our results strongly suggest that a variety of ecosystem processes may be affected. These impacts go beyond the period that these substances can be retrieved from the water column and even beyond aquatic ecosystems per se. Our findings illustrate how agricultural stressors can propagate through aquatic ecosystems with inherent risks for their functioning and the services they provide.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.07.110.

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