From molecules to monitoring: integrating genetic tools into freshwater quality assessments

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CHAPTER 5

Environmental DNA metabarcoding reveals comparable responses to agricultural stressors on different trophic levels of a freshwater community

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ABSTRACT

Freshwater habitats are under stress from agricultural land use, most notably the influx of neonicotinoid pesticides and increased nutrient pressure from fertilizer. Traditional studies investigating the effects of stressors on freshwater systems are often limited to a narrow range of taxa, depending heavily on morphological expertise. Additionally, disentanglement of multiple simultaneous stressors can be difficult in field studies, whereas controlled laboratory conditions do not accurately reflect natural conditions and food webs. To overcome these drawbacks, we investigated the impacts of two agricultural stressors (the neonicotinoid insecticide thiacloprid and fertilizer) in full-factorial design in a semi-natural research site, using environmental DNA sampling to study three different taxonomic groups representing three trophic levels: bacteria (decomposers), phytoplankton (primary producers), and chironomids (consumers).

The results show considerably impact of both stressors across trophic levels, with an additive effect of fertilizer and thiacloprid on community composition at all levels. These findings suggest that agricultural stressors affect the entire food web, either directly or through cascade reactions. They are also consistent with morphological assessments that were performed in the same study site, even at a lower number of replicates. The study presented shows that the use of multi-marker environmental DNA provides a more comprehensive assessment of stressor impacts across multiple trophic levels, at a higher taxonomic resolution than traditional surveys. Additionally, over a thousand putative novel bio-indicators for both agricultural stressors were discovered. We encourage further investigations into stressors impacts at different trophic levels, which will lead to more effective monitoring and management of freshwater systems.

5.1 INTRODUCTION

Freshwater ecosystems contain a rich diversity of both taxa and microhabitats, despite the fact that they cover less than one percent of the Earth's surface. They are disproportionately affected by anthropogenic impacts, and seem to be under greater threat than terrestrial and marine systems (Dudgeon et al. 2006, WWF 2014). Effective monitoring of biological quality of freshwater systems is essential for timely interventions, especially since freshwater is not only important for the management of aquatic flora and fauna, but also for the 'ecosystem services' that are essential to people's well-being and health (Corvalan et al. 2005).

One of the most important stressors to freshwater systems is agricultural land use as many freshwater habitats are directly connected to agricultural land. Next to the removal and fragmentation of habitat, pesticide and fertilizer use are the most prominent stressors here (Matson et al. 1997, Schreiner et al. 2016). While pesticides are used on agricultural land to prevent crop losses by pests, they may enter adjacent freshwater through spray drift, run-off, and seepage. The widespread use of neonicotinoid insecticides in agriculture has been subject of debate as they are found to impact non-target species, including many freshwater invertebrate species (Pisa et al. 2014, Morrissey et al. 2015, Raby et al. 2018), and have the potential to disrupt the entire food web (Yamamuro et al. 2019). Research has shown that neonicotinoid insecticides can negatively impact macroinvertebrate communities and have significant effects on food web structuring since invertebrates are critical in the transfer of nutrients from the primary producers to the consumers at the top of the food chain (Van Dijk et al. 2013, Chagnon et al. 2015, Schrama et al. 2017). The effects of neonicotinoids and the interaction with other common stressors such as increased influx of nutrients or fine sediments have been studied via morphological assessments in model systems (Barmentlo et al. 2019, Chará-Serna et al. 2019), showing alternative impacts of neonicotinoids to macroinvertebrate communities in combination with other stressors.

Traditional morphological surveys, such as employed in the above-mentioned studies, have several drawbacks which have implications on the quality and quantity of data that is collected. Morphological assessments of macroinvertebrate communities rely on skilled taxonomists, may be biased between assessors (Haase et al. 2010) and are labor-intensive and therefore often expensive (Jones 2008). The costs specifically affect decisions made on sampling frequency and intensity, and the time-consuming nature can cause delays that prevent timely interventions into impacted systems (Keeley et al. 2018). Additionally, traditional morphological surveys are limited in

accurately assessing many taxa that are likely to be affected by stressors, such as bacteria or planktonic organisms. Tools used to assess impact of pollutants on the aquatic ecosystem thus need to be refined (Schwarzenbach et al. 2006).

In the last decade, molecular tools, including environmental DNA metabarcoding, have become more common place for detecting and identifying taxa. Environmental DNA (eDNA) refers to any DNA collected from the environment without specifically collecting or isolating target specimens (Taberlet et al. 2012b). Community assessments using eDNA from soil have been standard practice for microbiologists for some time, but only more recently has this tool become one of the standard approaches for surveying freshwater biota, especially fish (e.g. Hänfling et al., 2016; Shaw et al., 2016). The use of eDNA has also found its way into environmental impact studies, such as studies on the impact of aquaculture on benthic sediments (Pochon et al. 2015, Stoeck et al. 2018). eDNA enables the detection of other, potentially more informative, organism groups than those studied in traditional impact studies (Macher et al. 2018). The use of eDNA allows for the defining of new indicators to stressors (e.g. Chariton et al., 2014; Li et al., 2018), and metabarcoding techniques can lead to the creation of new MOTU-based biotic indices (Apothéloz-Perret-Gentil et al. 2017). Despite their potential, most eDNA-based impact assessments still focus on one or few taxonomic groups, and only recently have multi-marker approaches been introduced to evaluate different taxonomic groups simultaneously (Andújar et al. 2018a, Keeley et al. 2018, Laroche et al. 2018, Li et al. 2018b, Cordier et al. 2019).

Impact assessments are often performed directly in the field, where the myriad of simultaneous stressors make it difficult to identify the impact of individual stressors (Piggott et al. 2015, Côté et al. 2016). Multi-trophic (eDNA) approaches have proven to provide stronger correlations with environmental variables than approaches that use a single guild (Keeley et al. 2018), but the possibility that different guilds respond differently to stressors make interpretation of novel multi-trophic eDNA approaches in natural settings difficult. Due to a lack of multi-trophic impact assessment studies where results gathered using eDNA and traditional approaches are combined, it remains unclear to what extent eDNA-based assessments can accurately detect the impacts in such complex environments.

In this study, we assess the impact of two main agricultural stressors on multiple trophic levels in naturally colonized freshwater communities in outdoor experimental ditches. In a full factorial setup, we use eDNA to assess the single and combined impacts of fertilizer and pesticide (the neonicotinoid thiacloprid) application on the richness, taxonomic composition and community dissimilarity of three trophic levels: bacteria, representing decomposers; phytoplankton, representing primary

producers; and chironomids, as representatives of the primary and secondary consumers, as well as a traditional indicator group for water quality. Using eDNA in this experimental impact assessment allows us to achieve the following aims: (1) to assess multi-trophic impacts on taxon groups that may be sensitive to stressors, but are not traditionally used in freshwater impact assessments due to their difficulty in identification, using novel multi-marker eDNA approaches; (2) to assess the impact of two agrochemicals on freshwater communities, while also being able to compare results with a concomitant traditional morphology-based impact study (Barmentlo et al. 2019); and (3) to pinpoint potential new bio-indicators for the health of freshwater ecosystems.

5.2 MATERIALS AND METHODS

5.2.1 Experimental setup

Environmental DNA sampling was performed in 20 experimental ditches located in the outdoor research facility the 'Living Lab' (see Barmentlo et al. (2019) for a detailed description of the site and treatments). Prior to the experiments, ditches were left connected to the adjacent reservoir for six months to allow for natural colonization of freshwater communities in the ditches. Before starting the experiment, ditches were hydrologically closed off using acrylic plates to avoid cross-contamination between treatments and to isolate the ditches from the reservoir. Subsequently, the ditches were exposed to two different agrochemical stressors in a full factorial design (five ditches per treatment): (1) control, with no added substances; (2) addition of the insecticide thiacloprid (Sigma-Aldrich, Zwijndrecht, The Netherlands) in two spikes (week 20 and 22) with a nominal time weighted average concentration for one month of 0.4 µg/l; (3) addition of nutrients in the form of three sachets with 75g of slow-releasing artificial fertilizer granulates ('Osmocote'; N:P:K = 15:9:11 combined with microelements) per ditch that were replaced every six weeks; and (4) a combination of thiacloprid and fertilizer in the same concentrations and application as described for the single-treatment ditches.

5.2.2 Sampling and DNA extraction

Environmental DNA sampling was performed in five replicate ditches for each treatment (20 in total) at four time points: two weeks prior to the start of the treatment (May 1st, 2017; week 18), and two weeks (May 31st, 2017; week 22), four weeks (June 13th, 2017; week 24) and seven weeks (July 6th, 2017; week 27) after the start of the treatments. Surface water samples were collected in the morning from the center of

each ditch using sterilized bottles and filtered within two hours in the laboratory. Filtration was performed using 0.2 µm polyethersulfone (PES) filter membranes (Sartorius, Göttingen, Germany) placed in sterilized Nalgene filter units (Thermo Fisher, Waltham, MA, USA) attached to a vacuum pump. Up to 300 ml of water was filtered for each of the 20 ditches. A modified CTAB extraction protocol adapted from Turner et al. (2014) was used for DNA extraction (Chapter 4).

5.2.3 DNA amplification and MiSeq sequencing

Three different markers for three different taxa groups were analyzed separately, using group-specific primers: a ±400 bp fragment of 18S rRNA V4 subregion for phytoplankton (Zimmermann et al. 2011), a 273 bp fragment of the 16S rRNA for bacteria (Klindworth et al. 2013) and a 235 bp fragment of COI for chironomids (Bista et al. 2017) (for primers, see Supplemental Table S5.1). For each of the PCRs, all of the 80 reactions for each marker (20 replicate ditches, 4 time points) were performed in duplicate. The chironomid PCR contained two samples of DNA extracted from two chironomid specimens unlikely to occur in the setup were used as a contamination control. This control was used to estimate cross-contamination between samples during the amplification and correct MOTU tables of all three markers accordingly, using a tool based on Larrson et al. (2018). Cross-contamination was assumed to be the same for all three markers.

Dual-indexed Illumina amplicon libraries were prepared using a two-step PCR protocol, in which the first PCR used primers with 5' Illumina tails. Initial PCRs were performed in 25 µl reactions containing 1x Phire Green Reaction Buffer, 0.5 mM dNTPs, 0.5 µl Phire Hot Start II DNA Polymerase (Thermo Fisher, Waltham, MA, US), 0.5 µM of each primer and 2.0 µl of template DNA. Initial denaturation was performed at 98°C for 30 seconds, followed by 35 cycles at 98°C for 5 seconds, 50°C for 5 seconds and 72°C for 15 seconds, followed by final elongation at 72°C for 5 minutes. PCR products were checked on E-Gel 96 pre-cast agarose gel (Thermo Fisher, Waltham, MA, USA) and cleaned with a one-sided size selection using NucleoMag NGS-Beads (Macherey-Nagel, Düren, Germany), in a 1:0.9 ratio. Dual-index PCRs were performed using 2.0 µl of PCR product from the first round in a 20 µl reaction containing 1x TaqMan Environmental Master Mix 2.0 (Thermo Fisher, Waltham, MA, USA) and 1.0 µM of each primer. Initial denaturation was performed at 95°C for 10 minutes, followed by 10 cycles at 95°C for 30 seconds, 55°C for 60 seconds and 72°C for 30 seconds, followed by final elongation at 72°C for 7 minutes. These PCR products were quantified on the QIAxcel (Qiagen, Venlo, the Netherlands) and each replicate of each marker was pooled equimolarly separately. Pools were cleaned with

a one-sided size selection using NucleoMag NGS-Beads, ratio 1:0.9, then quantified on the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) with the DNA High Sensitivity Kit. The pools for the bacteria and chironomids were combined equimolarly and sequenced on one run of Illumina MiSeq (v3 Kit, 2x300 paired-end), the pools for the phytoplankton were combined equimolarly and sequenced on a separate run, both at BaseClear BV (Leiden, the Netherlands).

5.2.4 Bioinformatics

Quality filtering and clustering of all data was performed in a custom pipeline on the OpenStack environment of Naturalis Biodiversity Center through a Galaxy instance (Afgan et al. 2018). Raw data was filtered with Sickle (Joshi & Fass 2011) and merged with FLASH v1.2.11 (Magoč & Salzberg 2011), non-merged reads were discarded. Primers were trimmed from both ends using Cutadapt v1.16 (Martin 2011) and any read without both primers present and anchored was discarded. PRINSEQ v0.20.4 (Schmieder & Edwards 2011) was used to filter reads based on length (390-420 bp for phytoplankton, 248-254 bp for bacteria, 230-250 bp for chironomids). Sequences were dereplicated and clustered into Molecular Operational Taxonomic Unit (MOTUs) using VSEARCH v2.10.3 (Rognes et al. 2016) with a cluster identity of 98% and a minimal accepted abundance of 2. MOTU tables were corrected using the occurrence of control chironomids in field samples (rate of spread 0.003, cutoff value 5 reads). PCR replicates were combined, including all MOTUs that were present in at least one replicate.

MOTU sequences were compared to custom reference databases using BLAST+ (Camacho et al. 2009). Phytoplankton MOTUs were compared to a dataset that included all 18S rRNA sequences from GenBank (Benson et al. 2005) (sequences downloaded 21 August 2018), bacteria were compared to Silva SSUParc 132 (Quast et al. 2013), chironomids were compared to a custom reference (Chapter 3) based on specimens collected in the Netherlands as part of a national DNA barcoding campaign (Beentjes et al. 2015), supplemented with sequences obtained from BOLD (Ratnasingham & Hebert 2007).

5.2.5 Taxonomic assignment and diversity analysis

A 98% cutoff was used for species-level identification, and a custom lowest common ancestor (LCA) script (Chapter 3) was used to identify MOTUs in those cases where no direct hits above 98% with the reference database were found. LCA was performed on the top 10% hits with bitscore >170, a minimum identity of 85% and a minimum coverage of 90% (90% identity and 100% coverage for the bacteria). The LCA was set to identify MOTUs no further than genus level. Normalized read abundances

were used in the analyses, based on the assumption that initial communities were all similar in terms of species composition and abundances. Differences in relative abundances in time, relative to the control samples, are assumed to be caused by the treatments (Beermann et al. 2018, Barmentlo et al. 2019).

5.2.6 Statistical analyses

Potential effects of the agrochemicals and time were assessed on the three different communities (bacteria, phytoplankton and chironomids). The effects of both fertilizer, thiacloprid, time, and all possible interactions were investigated on the normalized MOTU abundances using permutational analysis of variance (PERMANOVA, function adonis, R package VEGAN). Bray-Curtis was used as measure for dissimilarity, with 999 permutations. We accounted for the repeated measure design by including ditch number as a random variable. Differences in richness were analyzed with ANOVA (R package STATS). Potential effects on beta dispersion were investigated by using distance-based dispersion tests (function betadisper, R package VEGAN). Correlation between the distance matrices for the three communities analyzed in this study and the morphological assessment was investigated using a Mantel test (function mantel.rtest, R package ADE4, 999 permutations). Indicative MOTUs for each of the treatments independently were identified using the multipatt function (R package INDICSPECIES).

Morphological assessment in the original study by Barmentlo et al. (2019) was performed at three moments: before treatment, one month after treatment (June) and four months after treatment (September). The assessment in June was performed at the same time as the measurement four weeks after treatment start presented in this paper. Data from the morphological assessment in June was compared directly to eDNA results from the same week.

5.3 RESULTS

5.3.1 Sequence run statistics and taxonomic assignment

After merging, filtering and clustering, and with the correction for cross-contamination applied, the replicates combined and non-target MOTUs omitted, there was a total of 5,383 MOTUs for bacteria, 2,819 for phytoplankton and 692 for chironomids. The bacteria data contained 4,011 MOTUs (74.5% of total MOTUs) that could be identified at least at phylum level, with the largest groups being Gammaproteobacteria (30.1%) and Bacteroidetes (20.9%). In the phytoplankton data, 1,773 MOTUs (62.9% of total MOTUs) could be identified to at least phylum

level of relevant taxa, mostly Chlorophyta (45.4%) and Stramenopiles (34.5%). For the chironomid dataset, 368 MOTUs (53.2% of total MOTUs) could be identified as Chironomidae on genus or species level, representing 64 species from 35 genera; 207 MOTUs were only identified up to genus level. One sample (a sample from a ditch with a fertilizer treatment from week 18) did not contain any chironomid reads. The morphological study by Barmentlo et. al (2019) confirmed the presence of chironomids in this ditch, proving this a false negative; the sample was therefore omitted from the analyses presented here.

5.3.2 Effects on MOTU richness

Richness changed significantly over time irrespective of treatment for all three investigated communities, following a similar pattern for all three, with a peak in richness in week 22 (Figure 5.1). Looking at the different weeks separately, there was no significant effect of any treatment on the MOTU richness for phytoplankton or chironomids. For bacteria, the richness observed in ditches with combined treatment of fertilizer and thiacloprid was significantly higher than the richness observed with addition of only thiacloprid ($p = 0.003$) or the addition of only fertilizer ($p = 0.013$), but not higher than the richness observed in control ditches, and only in week 22 (two weeks after application of treatments). There was no significant difference in the number of reads between treatments for each week.

5.3.3 Effects on community dissimilarity

Before the application of any agrochemical, there were no statistically significant differences between community species compositions of the prospective treatments. After application of the agrochemicals, fertilizer and thiacloprid addition showed a significant interaction, irrespective of time, leading to dissimilar communities relative to the control for all three communities ($p = 0.001$ for all comparisons) (Table 5.1). The interaction between thiacloprid and fertilizer was most pronounced in the weeks directly after application of thiacloprid, where the dissimilarity between control ditches and ditches treated with both agrochemicals was higher than dissimilarities between control ditches and ditches treated with only thiacloprid or fertilizer (Figure 5.2). Two weeks after the start of the treatments, the impact of thiacloprid addition was more pronounced than the addition of fertilizer, with the former having a significant impact on the dissimilarity in all groups ($p = 0.001$, Table 5.2), while the impact of fertilizer was only significant for bacteria and phytoplankton ($p = 0.021$ and 0.001, respectively). Thiacloprid centroids were more distant from the control than the fertilizer centroids for all three groups in week 22 and 24 (two

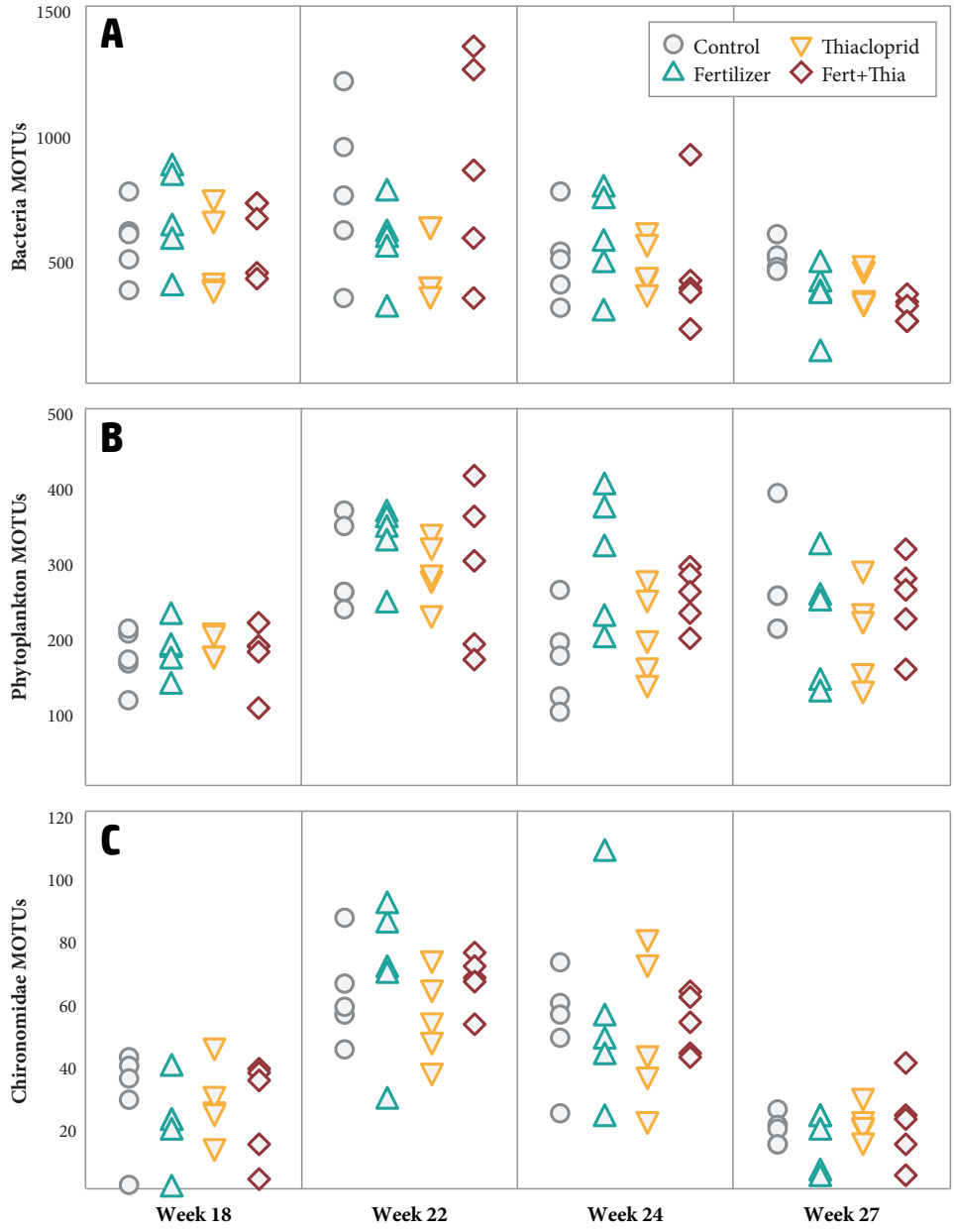


FIGURE 5.1. Observed number of MOTU for each of the taxonomic groups: (A) bacteria, (B) phytoplankton, and (C) chironomids, in control situation , and with added fertilizer, thiacloprid, and combined treatments.

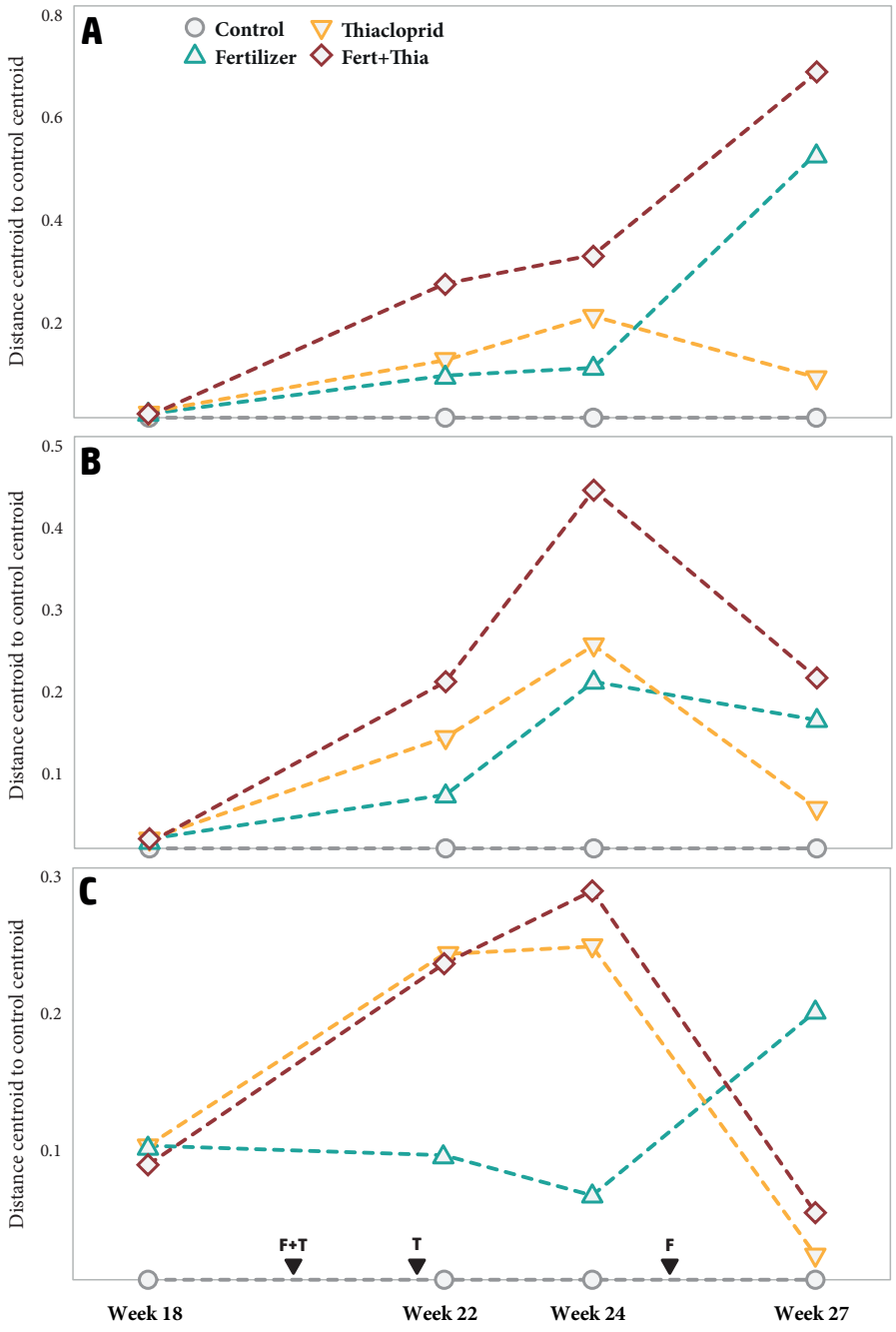


FIGURE 5.2. Average distance from centroid to the control centroid, for each of the taxonomic groups: (A) bacteria, (B) phytoplankton, and (C) chironomids. Moments of treatment application for thiacloprid (T) and fertilizer (F) are provided on the x-axis of panel C.

and four weeks after treatment), indicating that thiacloprid had a greater effect on the community composition in the short-term than fertilizer, albeit much more pronounced for chironomids. This reversed after the addition of fresh fertilizer pellets in week 25 as the effects of fertilizer became more pronounced compared to those of the thiacloprid addition (Figure 5.2). There was one sample in the control ditches prior to application of treatments, where we found only a single MOTU that was identified as a chironomid. This formed an outlier in the analysis of the chironomid data (Supplemental Figure S5.2C), and caused the centroid of the control samples in this measurement (week 18) to shift relative to the centroids of the other sets of ditches, explaining why the distances between centroids in week 18 were already elevated prior to start of treatment (Figure 5.2C).

The effect of time on dissimilarity was prominent, being larger than most effects of the agrochemicals, indicating that continued species turnover occurred. Two-way interactions of time with both fertilizer and thiacloprid were significant for all three communities studied (Table 5.1). There were no significant three-way interactions for any of the three groups, indicating that the interaction between the effects of fertilizer and thiacloprid occurred irrespective of the time point sampled. Studying the individual weeks separately, there was a significant effect of thiacloprid addition on community dissimilarity compared to control ditches in all three groups in week 22 and 24. Fertilizer had a significant effect on the composition of phytoplankton and bacteria in all three weeks after the start of the treatments (Table 5.2).

Beta-dispersion was significantly higher in treatments containing fertilizer for both bacteria and phytoplankton ($p < 0.001$ and $p = 0.005$, respectively), meaning that communities diverged when fertilizer was added to the system. Thiacloprid addition had a significant effect on chironomids, leading to convergence of the communities across the replicate ditches ($p = 0.002$) (Supplemental Figure S5.1). There were moderate, but significant correlations between all three Bray Curtis distance matrices of the three taxon groups. The correlation between bacteria and phytoplankton was stronger (Pearson $r = 0.820$, $p = 0.001$) than correlations of bacteria and phytoplankton with chironomid data ($r = 0.447$ and $r = 0.465$, respectively, $p = 0.001$). This indicates that community dissimilarities caused comparable patterns for both bacteria and phytoplankton (Supplemental Figure S5.2).

5.3.4 Effects on taxonomic composition

While the treatments had no apparent effect on the observed richness compared to control ditches, there were considerable shifts in the relative abundance of different taxa for all three communities analyzed in this study (Figure 5.3).

TABLE 5.1. PERMANOVA results (F-statistic, R2 and p-values) for the different treatments and the combined effects, including the three-way interaction with time, for data from all measurements combined. Significant p-values are marked with an asterisk (*).

	Bacteria			Phytoplankton			Chironomidae		
	F	R2	p-value	F	R2	p-value	F	R2	p-value
Thiacloprid	6.823	0.034	**0.001***	2.605	0.017	**0.001***	4.068	0.038	**0.001***
Fertilizer	12.329	0.061	**0.001***	6.751	0.044	**0.001***	1.192	0.011	**0.001***
Time	29.331	0.436	**0.001***	19.850	0.387	**0.001***	8.950	0.254	**0.001***
Fert:Thia	2.170	0.011	**0.001***	1.893	0.012	**0.001***	1.017	0.010	**0.001***
Thia:Time	3.100	0.046	**0.002***	2.115	0.041	**0.016***	1.531	0.043	**0.041***
Fert:Time	4.785	0.071	**0.001***	2.911	0.057	**0.002***	0.872	0.025	0.695
Fert:Thia:Time	1.543	0.023	0.195	1.287	0.025	0.269	0.846	0.024	0.755

TABLE 5.2. PERMANOVA results (F, R2 and p-values) for the different treatments for each of the time points evaluated separately. Significant p-values are marked with an asterisk (*).

		Bacteria			Phytoplankton			Chironomidae		
		F	R2	p-value	F	R2	p-value	F	R2	p-value
Week 18	**Thiacloprid**	0.675	0.036	0.809	0.502	0.028	0.711	1.032	0.060	0.352
	Fertilizer	1.329	0.071	0.201	1.190	0.066	0.287	0.316	0.018	0.990
	Fert:Thia	0.728	0.039	0.757	0.392	0.022	0.803	0.964	0.056	0.392
Week 22	**Thiacloprid**	6.428	0.234	**0.001***	3.520	0.144	**0.001***	4.087	0.181	**0.001***
	Fertilizer	3.044	0.111	**0.021***	3.553	0.145	**0.001***	1.460	0.064	0.152
	Fert:Thia	2.005	0.073	0.069	1.348	0.055	0.179	1.093	0.048	0.335
Week 24	**Thiacloprid**	4.318	0.167	**0.001***	3.007	0.117	**0.004***	3.006	0.145	**0.001***
	Fertilizer	3.806	0.147	**0.003***	4.210	0.164	**0.001***	0.857	0.041	0.662
	Fert:Thia	1.740	0.067	0.114	2.514	0.098	**0.010***	0.860	0.042	0.636
Week 27	**Thiacloprid**	2.027	0.061	0.070	0.892	0.041	0.550	1.269	0.066	0.173
	Fertilizer	13.598	0.410	**0.001***	4.119	0.189	**0.001***	1.121	0.059	0.311
	Fert:Thia	1.514	0.046	0.149	0.750	0.034	0.764	0.728	0.038	0.782

There were noticeable changes in the relative abundances of the phyla of bacteria within the different treatments (Figure 5.3A). Bacteriodetes were more abundant in ditches with added thiacloprid (representing 48.5% of the total reads versus 30.3% in the control ditches), mainly at the expense of Proteobacteria (21.4% versus 27.3%) and Verrucomicrobia (1.2% versus 7.1%). Actinobacteria represented a larger proportion of the reads in ditches without added fertilizer (33.4% of the total reads in control ditches versus 23.5% in the fertilizer ditches), a trend that continued into week 27, where the difference was 28.8% versus 3.6% on average. The relative abundances of Actinobacteria were also lower under the addition of thiacloprid to the point where they were nearly absent (0.8% of the total reads in combined agrochemical ditches compared to 32.2% in the control) in the combined treatment in week 27. In weeks 24 and 27, the relative composition at the phylum level seemed more affected by the addition of fertilizer than by thiacloprid, and both Bacteroidetes and Alphaproteobacteria became more abundant within the fertilizer treatment relative to the control (49.3% versus 29.5% and 12.1% versus 2.8%, respectively for both groups).

The phytoplankton community compositions changed considerably as well, and were significantly affected by both fertilizer and thiacloprid (Table 5.2). The read distribution (Figure 5.3B) reflected these changes as well. Effects were subtle in week 22 (two weeks after application of treatments), with the thiacloprid treatment showing higher proportions of chrysophyte reads (average of 51.3% versus 25.8% in control ditches), mostly at the cost of diatoms (3.7% versus 19.6%) and chlorophytes (35.3% versus 47.3%). In week 24, the composition changed considerably, showing a shift towards a system that was dominated by chlorophytes in ditches with added thiacloprid (76.3% versus 36.0% in control ditches), now at the expense of chrysophytes (5.5% versus 25.9%) and other stramenopiles (12.6% versus 20.3%). Cryptophytes were detected with much higher relative read abundances in ditches without added fertilizer (16.2% in control ditches versus 1.5% with fertilizer added), and went almost undetected in the ditches with a combined treatment, representing only 0.015% of the reads. The addition of fresh fertilizer pellets, in week 25, again changed the composition, bringing about a large shift in communities for ditches that received fertilizer. These were dominated by various groups of chlorophytes (60.6% versus 24.9% in control ditches), whereas control and thiacloprid-only ditches were dominated by the various stramenopile groups (60.0% versus 27.0% in ditches with fertilzer), most notably chrysophytes (29.0% versus 10.6%) and diatoms (17.0% versus 1.9%). Cryptophytes represented 11.9% of the reads in treatments without fertilizer, but went nearly undetected in the treatments with fertilizer (0.4% of the

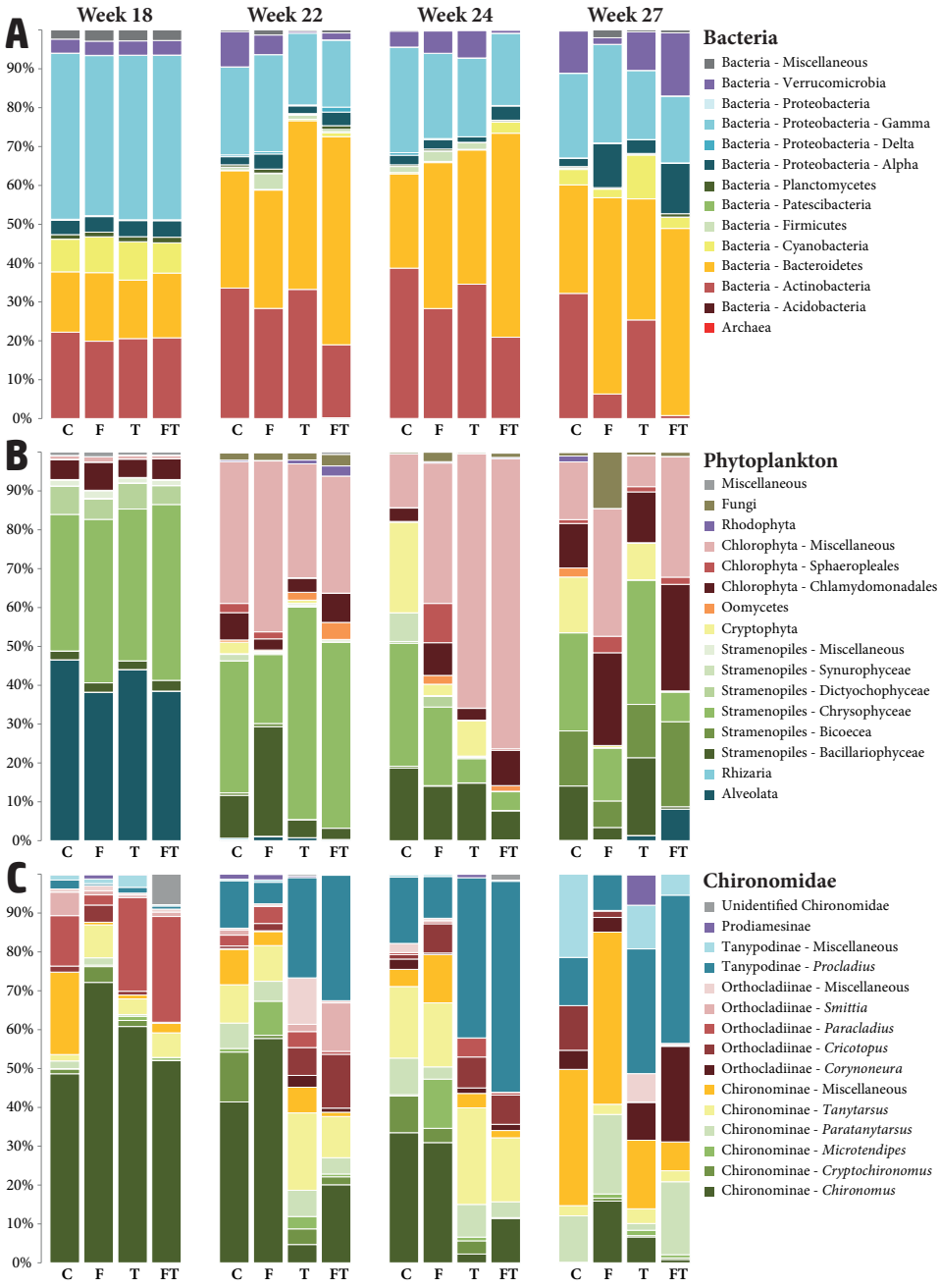


FIGURE 5.3. Read distributions observed for each of the different treatments and control both prior to (week 18) and after application of treatments (week 22-27) for each of the taxonomic groups: (A) bacteria, (B) phytoplankton and (C) chironomids, in control situation (C), and with added fertilizer (F), thiacloprid (T) and combined treatments (FT).

reads). At this point in time, thiacloprid no longer showed a significant effect on the community composition (Table 5.2).

For the chironomids, the most notable differences were observed between ditches with and without added thiacloprid (Figure 5.3C). With thiacloprid addition, the genus *Chironomus* was no longer the most abundant and declined strongly in read abundance (12.4% in ditches with thiacloprid versus 50.0% in control ditches), also compared to week 18 (before the start of treatments), where on average this genus represented 57.7% of the reads. Thiacloprid shifted the community composition towards genera outside of the subfamily Chironominae, such as *Procladius* (subfamily Tanypodinae) (29.0% versus 8.9% in control ditches) and *Cricotopus* (subfamily Orthocladiinae) (10.5% versus 1.3%). This shift continued in week 24, where thiacloprid ditches became dominated by *Procladius* (47.7% versus 13.9%), at the expense of *Chironomus* (6.8% versus 32.2%). In week 27, *Procladius* remained more abundant in the thiacloprid ditches, although not as pronounced as in week 24. The genus *Corynoneura* was also much more abundant in these ditches (17.2% versus 4.3%).

5.3.5 Indicator taxa

Indicator analysis on the three assessments after start of treatments separately identified 624 bacterial MOTUs, 470 phytoplankton MOTUs and 46 chironomid MOTUs that were indicative for either absence or presence of either of the two added agrochemicals in one or more of the three post-treatment measurements (Supplemental File S5.1, summarized in Table 5.3). With the observations of the three assessments combined (week 22, 24, 27), the indicator analysis identified 552 bacterial MOTUs, 76 of which acted as indicators for both agrochemicals. The

TABLE 5.3. Summarized indicator species analysis results, with the number of indicative MOTUs found for each of the three taxonomic groups: indicators for absence (F-) and presence (F+) of nutrients, and absence (T-) and presence (T+) of thiacloprid. Analysis was performed on data from each post-treatment measurement (week 22, 24 and 27), and combined data of the three measurements. An overview of all indicator MOTUs is provided in Supplemental File S5.1.

	Bacteria				Phytoplankton				Chironomidae			
	F-	F+	T-	T+	F-	F+	T-	T+	F-	F+	T-	T+
Week 22	52	54	128	50	60	63	39	56	1	8	13	10
Week 24	43	46	110	39	12	127	40	48	3	4	5	11
Week 27	194	109	25	4	90	91	9	2	3	0	0	3
Week 22-27	172	212	176	68	126	222	93	65	4	8	20	15

majority were indicators for the absence of both fertilizer and thiacloprid (51) or the presence of both (21), the remaining four were indicative for the presence of one agrochemical stressor and the absence of the other. For phytoplankton, we found 446 indicators in total, in which 60 acted as indicators for both agrochemicals, again mainly for absence (15) or presence (39) of both treatments. In the combined chironomid data there were 46 indicative MOTUs, with only a single MOTU that acted as indicator for both presence of thiacloprid and the presence of fertilizer. We did observe lower fidelity values for the combined measurements, due to the fact that indicative MOTUs for all three groups were not observed in the ditches in each of the three assessments after the introduction of agrochemicals.

5.3.6 Comparison to morphological assessment

Patterns observed in stressor responses as measured by distances between centroids in week 24 were similar for all three taxonomic groups assessed in this study, as well as the morphological assessment of macroinvertebrates assessed by Barmentlo et al. (2019) at the same sampling timepoint. The thiacloprid treatment showed more distance relative to the control than the fertilizer treatment, whereas the combined treatment showing the largest deviation for all four assessments (Figure 5.4), although the morphological assessment made use of nine replicates instead of the five replicates that were used for eDNA evaluation. The distances between control

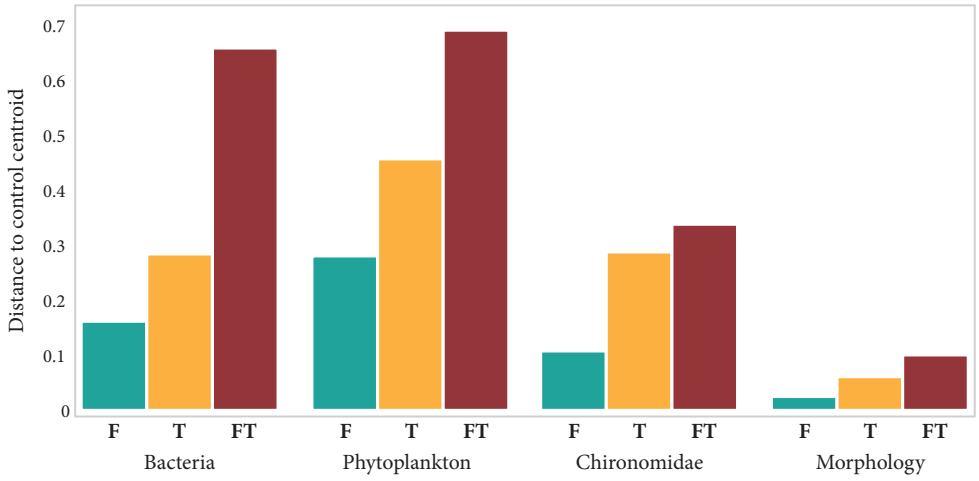


FIGURE 5.4. Centroid distance to the control centroid in week 24 (one month after application of the agrochemicals), for the bacteria, phytoplankton, and chironomids assessed with environmental DNA, as well as the macroinvertebrates assessed with morphological methods (see Barmentlo et al. 2019), exposed to fertilizer (F), thiacloprid (T), and combined agrochemical addition (FT).

centroids varied when using fewer replicates, but in all three eDNA assessments the pattern described above was observed with as little as three replicates (out of five) (Supplemental Figure S5.3B-D). For morphological data, at least seven (out of nine) replicates were needed to reveal this pattern (Supplemental Figure S5.3A). There were, however, no significant correlations with any of the eDNA-based distance matrices and the distance matrix of the morphological assessment. The morphological assessments also showed no significant treatment effect on richness nor abundance of macroinvertebrates (Barmentlo et al. 2019).

5.4 DISCUSSION

Our study shows that environmental DNA can be used to investigate the effects of agrochemical stressors on multiple trophic levels in a freshwater community. The factorial design of our semi-natural research site allowed us to separate the effects of the addition of fertilizer (nutrients) and the neonicotinoid thiacloprid on the taxonomic composition of freshwater organisms. Clear impacts of both agricultural stressors were observed for all three taxonomic groups. Moreover, the introduction of realistic levels of both agrochemicals in the ditches had strong additive effects on the three trophic levels analyzed. Our findings are in line with simultaneous morphological assessments of macroinvertebrates conducted during the same experiment (Barmentlo et al. 2019) and previously reported effects of neonicotinoids on macroinvertebrates and zooplankton (e.g. Yamamuro et al., 2019), and we show that eDNA-based impact assessments can provide useful insights into stressor responses in taxa that are usually not included in traditional assessments. The three groups evaluated in this study have been observed to contain numerous indicative taxa that have potential as novel bio-indicators for environmental stress.

The similar distributions of reads across the various taxa in the measurements before the application of the treatments confirms the assumption that the initial communities were all similar in terms of composition and abundances (Figure 5.3). Subsequent agrochemical addition strongly affected community composition of all three trophic levels investigated (Tables 5.1 and 5.2). The effect of time on dissimilarity was considerable, being larger than the single effects of the agrochemicals, indicating that natural species turnover occurred. Other studies have also found large fluctuations in macrofaunal community composition under normal conditions, even in relatively short periods of time (Bista et al. 2017, Chapter 4).Despite these fluctuations in community composition caused by species turnover, the experimental design of the present study still allowed for clear differentiation in those patterns

caused by seasonal turnover and those caused by external stressors.

Fertilizer addition caused significant changes in community composition of both bacteria and phytoplankton, with replicate ditches showing higher dissimilarity (divergence) compared to control ditches (Supplemental Figure S5.1). Community dissimilarities showed comparable patterns (Supplemental Figure S5.2) and strong correlations for bacteria and phytoplankton. This was expected as eutrophication has long been known to be associated with increased growth in phytoplankton (Heisler et al. 2008), and interactions such as nutrient cycling between phytoplankton and bacteria at the base of the food web (Seymour et al. 2017) render bacterial communities sensitive to changes in phytoplankton communities (and vice versa). Chironomids were also sensitive to the addition of fertilizer, although these fertilized communities were generally more similar to the control than to the thiacloprid treatment (Supplemental Figure S5.2). Nutrient pressure has been shown to have effects on freshwater macroinvertebrates in previous research (e.g. Donohue et al., 2009), since eutrophication can lead to oxygen depletion and changes in food availability.

The thiacloprid concentration used in this study (a nominal time weighted average of 0.4 μg/l) is considered an realistic concentration as it is based on surface water concentrations from the Netherlands, and earlier research has already shown that freshwater macroinvertebrates are affected by neonicotinoids at concentrations observed in surface water (e.g. Morrissey et al. 2015, Sánchez-Bayo et al. 2016). Indeed, thiacloprid addition had a much larger impact on the chironomid community structure than fertilizer addition and resulted in a significant convergence (Supplemental Figure S5.1). Even after thiacloprid had dissipated from the water column after only a few weeks due to its rapid adsorption to the sediment (DT90 = 11.1 days; Barmentlo et al. 2019), the legacy effect of thiacloprid was still larger than the effect of the fertilizer (Table 5.2). This suggests that even a single spike of thiacloprid can have a lasting impact on large parts of the macrofaunal community. There was an additive effect of both agrochemicals, as the impact of a combined treatment effect of fertilizer and thiacloprid was greater than that of each treatment separately, and communities under a combined treatment were more dissimilar relative to the control than communities exposed to a single agrochemical (Figure 5.2, Tables 5.1 and 5.2). Most two-way interactions between fertilizer and thiacloprid were not significant, however, suggesting the effect was additive, rather than synergistic (Table 5.2).

Addition of agrochemicals strongly affected the community compositions. Changes in composition where most notable for chironomids, for which most MOTUs could be identified at species or genus level. For instance, we observed

that thiacloprid treatment ditches (and combined effect ditches) had much lower ratios of the subfamily Chironominae, which were accompanied by higher ratios of species belonging to the subfamilies Tanypodinae and Orthocladiinae. These latter subfamilies were apparently less susceptible to the presence of thiacloprid, which is consistent with findings from previous studies that showed significant effects on Chironominae in response to neonicotinoid insecticides (Langer-Jaesrich et al. 2010, Williams & Sweetman 2019). Whilst the direct effects of fertilizer on bacterial and phytoplankton communities have been studied before (Carvalho et al. 2013), there is little research on the effects of neonicotinoid insecticides on those communities. One study suggests that algal blooms appear to increase in size under stress from the neonicotinoid imidacloprid (Sumon et al. 2018). The neonicotinoid insecticide thiacloprid, meant to target pest insects, also affected bacterial and algal community composition in the present study. Our data suggests that thiacloprid has an important impact on the structuring of the communities (Tables 5.1 and 5.2,Figure 5.3). It is likely that some of these effects on phytoplankton and bacteria communities have been caused by food web cascades, especially as many of the affected Chironominae are common feeders on these microbes. Indeed, previous research showed that even under stress from pesticide mixtures, biotic interactions played a major role in the structuring of plankton communities (Pereira et al. 2018). Similarly, responses to nutrient pressure by fertilizer in macroinvertebrates may also partly be caused by cascade reactions, such as the aforementioned changes in food availability. Processes like eutrophication can have a significant impact on total community composition and food web structure via trophic cascades (Davis et al. 2010, Suikkanen et al. 2013), and a recent study evaluating anthropogenic stressors on freshwater food webs showed that macroinvertebrates had different reactions to fertilizer, herbicide and insecticide, depending on their food source (Schrama et al. 2017). The authors also noted, however, that cascading effects in the food web were hard to explain, and found some suggestions of shifts in diet induced by stressors.

Results from the morphological assessment closely matched the presently observed patterns regarding dissimilarity relative to the control; there was an increase in effect size from fertilizer to thiacloprid to the mixture treatment for all communities investigated, although no significant effects were detected on the beta dispersion of the community in the morphological assessment (Barmentlo et al. 2019). In this study, however, we observed these stressor impact patterns at a lower number of replicates compared to the traditional assessment (Supplemental Figure S5.3). Data for the morphological assessment was log10(x+1) transformed, due to the uneven distributions in species, where zooplankton species often dominated

samples, and chironomids were only identified at family level (Barmentlo et al. 2019). This transformation resulted in considerably lower centroid distances than those observed within the present study, an effect which was amplified by the difference in taxonomic units observed (83 morphological taxa, versus 4,011, 1,773 and 368 MOTUs for the bacteria, phytoplankton and chironomids, respectively). Nevertheless, the morphological assessment of the macroinvertebrates reflected a similar pattern to the eDNA assessment, with the thiacloprid treatment showing more distance relative to the control than the fertilizer treatment and the combined treatment showing the largest deviation (Figure 5.4), irrespective of the biota that were sampled. This indicates how strongly interconnected the different trophic levels are and that potential cascading food web responses to stressors can occur even in non-target biota.

A full-factorial experimental setup such as the one used in this study allows for focused research into the individual and combined impacts of stressors on communities. However, most impact assessments are done in fully natural settings, where the interplay between multiple stressors is much harder, if not impossible, to disentangle (Piggott et al. 2015, Côté et al. 2016). Our semi-natural controlled experimental setup reflected key parts of the food web, and our study shows that using eDNA can successfully describe the effects of agricultural stressors to freshwater communities in a semi-realistic setting. This provides much needed confidence in the application of such an approach in impact studies in natural environments in which disentanglement of the impact of different (a)biotic stressors is even more difficult.

As observed in this study, MOTUs of different taxonomic groups present consistent patterns under the effects of stressors (Figures 5.2 and 5.4). Previous research has already shown that MOTU-based approaches can provide better resolution in impact assessments, such as with undescribed cryptic diversity demonstrating contrasting responses to stressors (Macher et al. 2016), or reference databases being unable to identify all the encountered molecular variation (Beermann et al. 2018). Several studies have shown that MOTU-based assessment methods can accurately predict stressor impact on water systems (e.g. Andújar et al. 2018a, Li et al. 2018b). However, the inability to identify all MOTUs to species or even genus level complicates the ecological interpretations of shifts in communities caused by external stressors. Taxonomic hiatuses in the reference database are large, especially for microorganisms such as the freshwater bacteria and phytoplankton studied in this paper. Accumulating MOTUs based on the higher-level taxonomic assignments could be possible, in order to assign some ecological value to such indicators. The MOTUs, however, may represent a wide variety of ecological groups, and accumulating them into a single entity would decrease the sensitivity of any such bioindicators (Jones 2008). While it

may be difficult to link ecological information to unidentified MOTUs, they can still be of use in comparative studies, such as impact assessments (Li et al. 2018b).

Our analyses revealed a large number of indicative MOTUs for all three trophic levels assessed (Table 5.3), suggesting that many potential new bioindicators are hidden in taxon groups that are either difficult to identify (e.g. chironomids) or are mostly neglected in traditional bioassessments of water impacted by anthropogenic stressors (e.g. bacteria and phytoplankton). Especially for bacteria and phytoplankton, many indicative MOTUs were found, for both stressors, but many MOTUs could not be resolved to species or genus level. Most of the chironomid MOTUs identified as positive indicators for the presence of thiacloprid could be assigned to the genus *Procladius* (Tanypodinae). These observations match the findings of a recent morphological study performed in the same experimental ditches where *Procladius choreus* was the most abundant remaining species under the stress of increasing levels of thiacloprid (Barmentlo et al. in prep).

One key limitation for assigning indicator taxa for freshwater communities is the large fluctuations in community composition over time. The large community turnover caused low fidelity scores for many indicator MOTUs observed in the indicator analysis on the combined data for the three post-treatment measurements, due to the fact that many MOTUs do not occur in all time points (Table S5.2). Moreover, indicator MOTUs might not only be specific to a certain time frame, but can also be spatially limited, as it was previously observed that indicator species for the impact of offshore oil and gas drilling were highly specific to site conditions (Laroche et al. 2018). Impact assessments based on novel indicators, or even based on MOTUs, should preferably be time- and location-independent, to make their application on a broader scale feasible. This could prove challenging, especially when looking at microorganisms such as bacteria or phytoplankton taxa observed in the current study, as these groups tend to have a large turnover in their community composition on a relatively small time scale (Chapter 4). However, the huge potential for these novel bio-indicators in large-scale impact assessments would make any efforts into a better understanding of their occurrence and behavior worthwhile.

5.5 CONCLUSIONS

We have shown that environmental DNA metabarcoding at multiple trophic levels provides insights into changes in freshwater communities under pressure of agricultural stressors. The full-factorial design of the mostly natural study site allowed us to observe the impact of single stressors. We found an additive (but not

synergistic) effect of artificial fertilizer and the insecticide thiacloprid on community composition at the level of decomposers, primary producers, and consumers. This effect of multiple stressors was consistent with observations reported in traditional morphological assessments of the same experimental setup. These effects were even detected with a lower number of replicates than the traditional morphological study, indicating the robustness of using environmental DNA metabarcoding in impact assessments. While both agrochemicals directly influenced different taxa at different trophic levels, the neonicotinoid insecticide thiacloprid, meant to target pest insects, also affected bacterial and algal community composition, be it directly or through cascade reaction through the food web. We encourage the use of multi-marker eDNA for impact assessment across trophic levels in freshwater ecosystems, as it (1) provides a more comprehensive assessment of impacts on the entire food web, (2) provides more information at a higher taxonomic resolution compared to traditional morphological surveys, even if MOTUs are not all assigned to species level, and (3) allows for discovery of novel indicator taxa. The incorporation of eDNA methodology contributes to ecosystem understanding and would allow for more effective monitoring and management of freshwater systems, and help safeguard the ecosystem services they contribute to humanity.

5.6 ACKNOWLEDGEMENTS

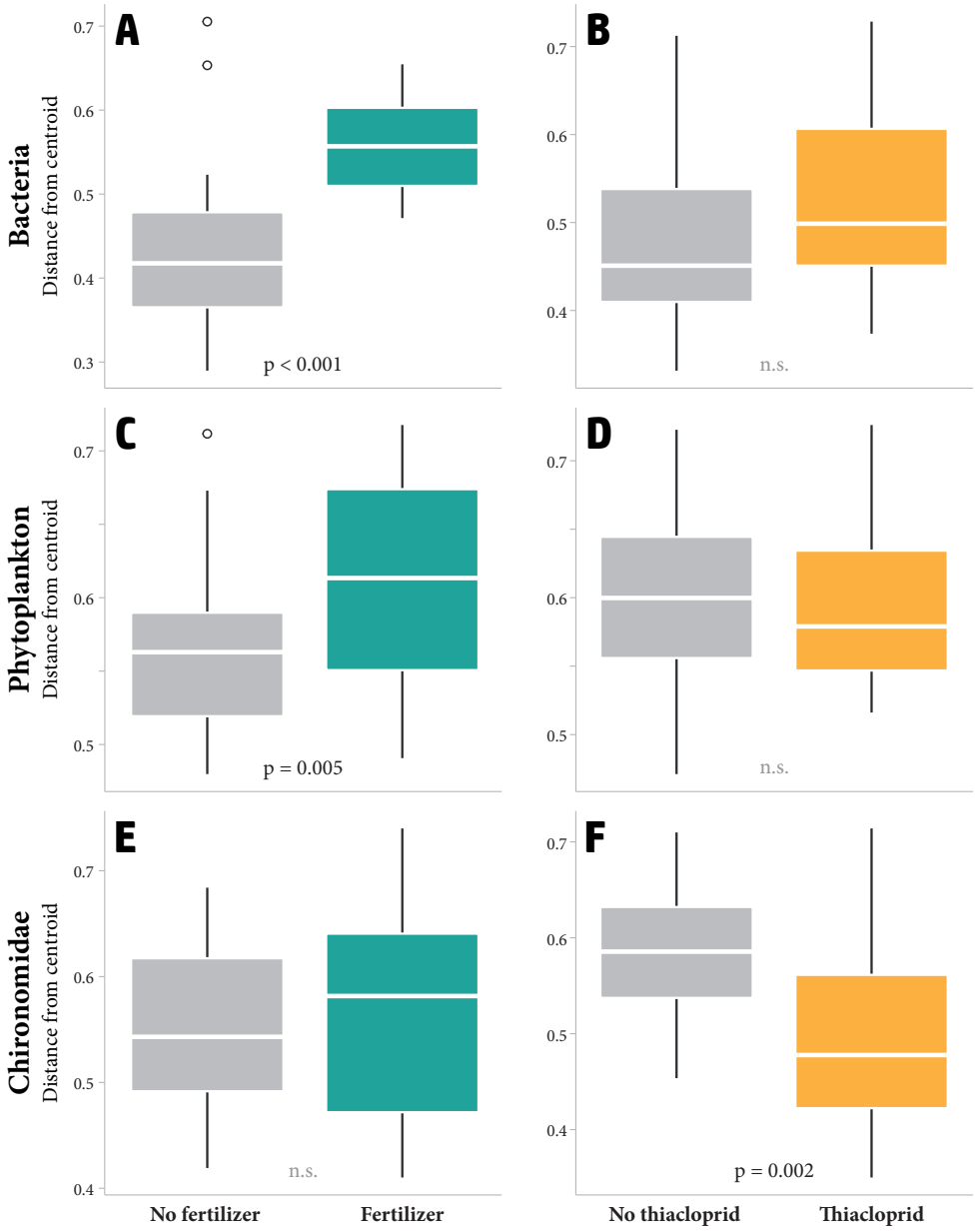
We thank Sam Boerlijst for his assistance with DNA extractions, and André van Nieuwenhuijzen for his critical look at the identifications of chironomids obtained from the eDNA metabarcoding. Experiments were performed in the outdoor experimental laboratory "Levend Lab" established by crowdfunding by Maarten Schrama and Martina Vijver at Leiden University.

5.8 SUPPLEMENTARY MATERIALS

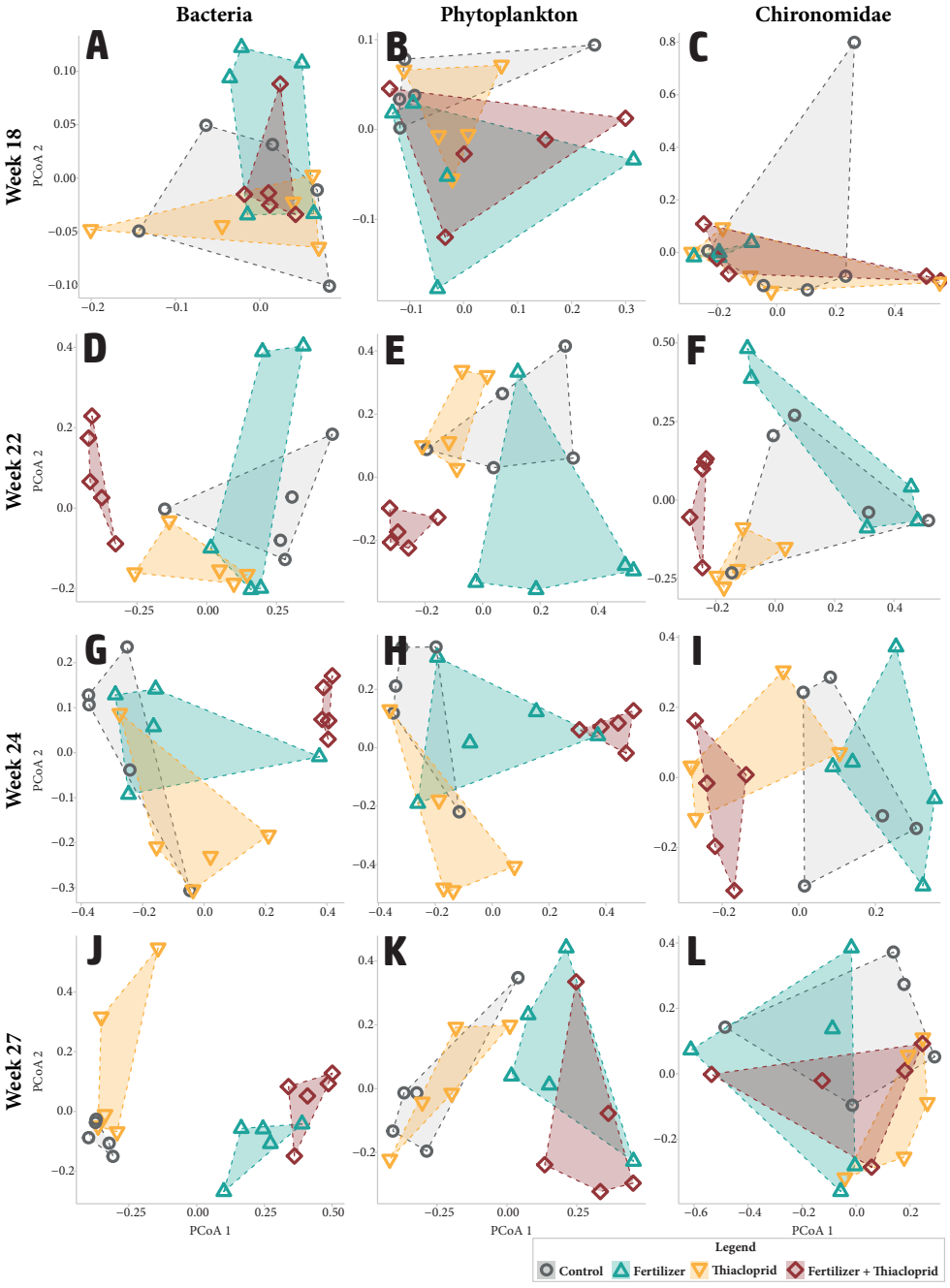
SUPPLEMENTARY FILE S5.1. Indicative MOTUs for bacteria, phytoplankton and Chironomidae for either absence or presence of either of the two added agrochemicals in one or more of the three post-treatment measurements. **https://doi.org/10.22541/au.159236833.30909538**

SUPPLEMENTARY TABLE S5.1. Sequences for primers used in the first and second round amplification.

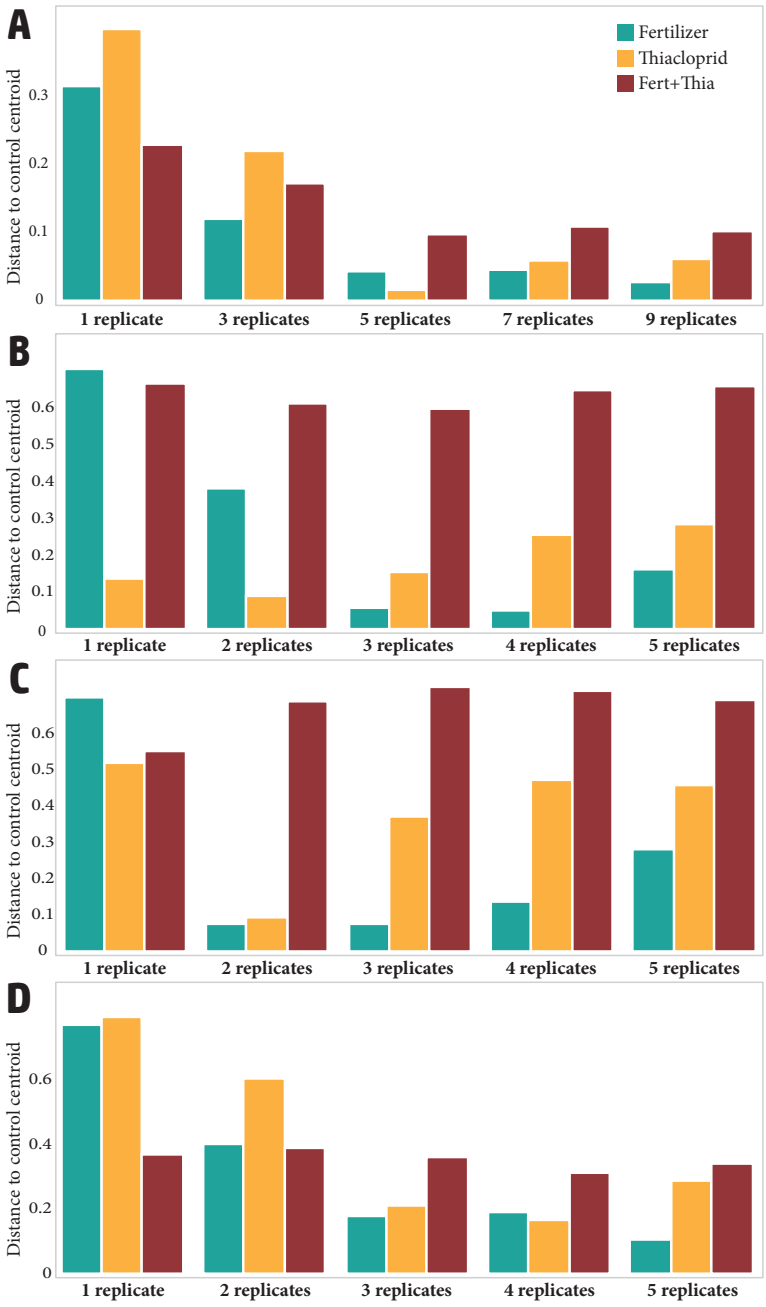
First Round	
Primer set	Sequence (Universal tail – template-specific primer)
Bacteria (Klindworth et al. 2013)	
S-D-Arch-0519-a-S-15	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CAGCMGCCGCGGTAA
S-D-Bact-0785-a-A-21	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TACNVGGGTATCTAATCC
Phytoplankton (Zimmerman et al. 2011)	
D512for	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG ATTCCAGCTCCAATAGCG
D978rev	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GACTACGATGGTATCTAATC
Chironomidae (Bista et al. 2017)	
LCO-1490	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGTCAACAAATCATAAAGATATTGG
COIA-R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CARAAWCTTATATTATTTATTCGDGG
Second Round	
Primer	Sequence (Illumina adapter – index – universal tail)
NEX-F	AATGATACGGCGACCACCGAGATCTACAC [i5 index] TCGTCGGCAGCGTC
NEX-R	CAAGCAGAAGACGGCATACGAGAT [i7 index] GTCTCGTGGGCTCGG



Supplemental Figure S5.1. Beta dispersion in weeks 22 to 27 under the two different treatments (tested independently) for bacteria exposed to fertilizer (A) and thiacloprid (B), phytoplankton exposed to fertilizer (C) and thiacloprid (D), and chironomids exposed to fertilizer (E) and thiacloprid (F). Fertilizer caused significant divergence in bacteria and phytoplankton communities (A and C), whereas thiacloprid caused significant convergence in chironomid communities (F). ANOVA p-values are provided in the panels.



Supplemental Figure S5.2. PCoA plots for each of the four measurements, both prior to (week 18, A-C) and after application of treatments (week 22-27, D-L) for of the three taxonomic groups: bacteria, phytoplankton and chironomids.



Supplemental Figure S5.3. Average distance from centroid to the control centroid in week 24 for the (A) macroinvertebrates assessed with morphological methods (Barmentlo et al. 2019), and (B) bacteria, (C) phytoplankton and (D) chironomids assessed with eDNA (this study), at different numbers of replicates.