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The response of freshwater plankton communities to temporal concurrence of agrochemical mixtures

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ABSTRACT

The response of freshwater plankton communities to temporal concurrence of agrochemical mixtures

Freshwater ecosystems regularly experience pulsed inputs of nutrients and other pollutants as a result of temporally variable applications of agrochemicals combined with runoff events. In this study, we evaluate how planktonic communities respond to repeated and pulsed insecticide disturbances and if the response depends on temporal concurrence with nutrient pulses. We conducted an experiment using mesocosms to assess the ecotoxicological effects of a commonly used insecticide (chlorpyrifos) and nutrients (nitrogen and phosphorus) on plankton communities. The mesocosms (300 L) were established outdoors for 10 weeks. The experiment consisted of 3 treatments: nutrient pulse of nitrogen and phosphorus every two weeks (N; considered as control), nutrients and insecticide pulsed simultaneously every two weeks (NI), and nutrients and insecticide pulsed in alternating weeks (N_I). Insecticide and nutrient pulses consisted of 2 µg/L of chlorpyrifos, 560 µg/L of nitrogen, and 39.9 µg/L of phosphorus. Zooplankton abundance, community structure, and diversity were used as structural indicators. Chlorophyll *a* and net production were used as functional indicators. We found no effect of the treatments on zooplankton abundance, while richness and Shannon diversity was lower in treatments with pulsed insecticide (NI and N_I) compared to control treatment (N). Phytoplankton biomass was higher in the treatments with insecticide than in the controls (N). Higher phytoplankton biomass could be explained by an indirect effect shift from a cladocera-dominated to a copepod-dominated community in response to the insecticide treatment. Overall, the insecticide disturbance had direct and indirect effects on the community and did not depend on whether insecticides were pulsed synchronously or asynchronously with nutrients.

Key words: agrochemicals, mixtures, frequency, assessment, risk, plankton

RESUMEN

La respuesta de comunidades de plancton de agua dulce a la concurrencia temporal de mezclas agroquímicas

Los ecosistemas de agua dulce experimentan regularmente entradas en forma de pulsos de nutrientes y otros contaminantes como resultado de distintas aplicaciones de productos agroquímicos combinadas con eventos de escorrentía. En este estudio, evaluamos cómo responden las comunidades planctónicas a las perturbaciones repetidas y pulsadas de insecticidas y si la respuesta depende de la concurrencia temporal con los pulsos de nutrientes. Realizamos un experimento con mesocosmos para evaluar los efectos ecotoxicológicos de un insecticida de uso común (clorpirifos) y los nutrientes (nitrógeno y fósforo) en las comunidades planctónicas. Los mesocosmos (300 L) se establecieron al aire libre durante 10 semanas. El experimento consistió en 3 tratamientos: pulso de nutrientes de nitrógeno y fósforo cada dos semanas (N; considerado como control), nutrientes e insecticida pulsados simultáneamente cada dos semanas (NI), y nutrientes e insecticida pulsados en semanas alternas (N_I). Los pulsos de insecticida y nutrientes consistieron en 2 µg/L de clorpirifos, 560 µg/L de nitrógeno y 39,9 µg/L de fósforo. Se utilizaron como indicadores estructurales la abundancia de zooplancton, la estructura de la comunidad y la diversidad. Como indicadores funcionales se utilizaron la clorofila a *y la producción del ecosistema. No se encontró ningún efecto de los*

tratamientos sobre la abundancia del zooplancton, mientras que la riqueza y la diversidad de Shannon fueron menores en los tratamientos con insecticida pulsado (NI y N_I) en comparación con el tratamiento de control (N). La biomasa de fitoplancton fue mayor en los tratamientos con insecticida que en los controles (N). Esta situación podría explicarse como un efecto indirecto del cambio de una comunidad dominada por cladóceros, a una dominada por copépodos y su capacidad de filtración. En general, la perturbación por insecticida tuvo efectos directos e indirectos en la comunidad; y, no dependió de si se pulsó de forma sincrónica o asincrónica con los nutrientes.

Palabras clave: *agroquímicos, mezclas, frecuencia, evaluación, riesgo, plancton*

INTRODUCTION

Anthropic pressures are affecting ecosystems resulting in biodiversity losses worldwide, and this fact is especially dramatic in freshwater ecosystems (Sala et al., 2000). One of the most significant pressures challenging the ecological integrity of freshwater ecosystems is intensive agriculture characterized by applications of pesticides and fertilizers (Parra et al., 2005; Stendera et al., 2012). As a result of repeated agrochemical application and run-off events, freshwater ecosystems often receive pulses of agrochemical mixtures at varying frequencies (i.e. synchronized vs. asynchronized) with consequences for aquatic ecosystem biodiversity, structure and function (Hillebrand & Kunze, 2020).

The pulse interval of stressors is important because it may cause sublethal effects and can allow or prevent recovery of the community between pulses, resulting in pulses that are either independent or cumulative in nature (Hillebrand & Kunze, 2020; Hoang et al., 2007). Additional work has shown that species and communities respond differently to single versus chronic pulses of contaminants (Tlili et al., 2011). Therefore, in order to improve our understanding of the consequences of agricultural disturbances on aquatic ecosystems, we need to study them as they occur in natural ecosystems. Agricultural disturbances are often complex and involve multiple chemicals that may be pulsed repeatedly, either singly or in various combinations (Borgert et al., 2004; LeBlanc et al., 2012). Despite the complexity of human-induced disturbances in natural ecosystems, most toxicological evaluations have studied the effects of single pulses of a toxic substance on individual species that are well-known bioindicators (Earl & Whiteman, 2009; García-Muñoz et al., 2011; Hoang et al., 2007).

Two common agrochemical applications that have particularly strong effects in aquatic ecosystems are fertilizers and insecticides. Fertilizers often enter aquatic ecosystems in repeated pulses, particularly in agricultural areas with diverse crops, complex agrochemical application timing and variable rainfall events (Haygarth et al., 2012; Smith et al., 2001). Fertilizer run-off generates an overall increase in nutrient input into freshwater ecosystems leading to many changes including enhanced productivity, a reduction in biodiversity (Guignard et al., 2017), altered planktonic structure (Hall et al., 2004), and disrupted consumer-resource interactions (Holt, 2008; Miracle et al., 2007; Scheffer et al., 2008).

Similar to fertilizers, insecticides often enter ecosystems in repeated pulses depending on rainfall events and application schedules designed to maximize control of pests and disease (Reinert et al., 2002). Insecticides typically cause high mortality in the zooplankton community, especially in the larger cladocerans species which can in turn alter community structure (Downing et al., 2008; Kreutzweiser et al., 2004). For instance, the reduction in zooplankton and the shift towards smaller zooplankton species such as rotifers and copepods has indirect effects on phytoplankton by reducing grazing pressure (Hanazato, 1998). In addition, insecticides can impose strong selection pressures that can shape community structure over relatively short time scales (Hendry & Kinnison, 1999; Jansen et al., 2015). For example, a strong decrease in population abundance due to an insecticide can favour a new genotype in the population that is better adapted to the new contaminated environment, allowing the population

to increase by evolutionary rescue mechanisms (Bell, 2013; Fugère et al., 2020).

The aim of this study was to evaluate how freshwater ecosystems respond to repeated and pulsed insecticide disturbances and if the response depends on if insecticide pulses occur synchronously or asynchronously with nutrient pulses. Specifically, we used chlorpyrifos insecticide and nitrogen and phosphorus for the pulses. Nutrients and chlorpyrifos, a broad-spectrum organophosphate pesticide extensively used for agricultural purposes worldwide, were chosen because they represent common agrochemical contaminants that are frequently applied either alone or in combination. They are often applied repeatedly over a growing season, which, in combination with run-off from sporadic rainfall events, can lead to both synchronous and asynchronous pulses. We conducted the study using outdoor aquatic mesocosms in order to explore community responses to realistic toxic scenarios. To do this, we assembled diverse plankton communities collected from nearby natural ponds and then applied repeated biweekly pulses of nutrients alone, biweekly pulses of nutrients and insecticides applied synchronously in the same week, and biweekly pulses of nutrients and insecticides applied asynchronously in alternate weeks. Pulses of chlorpyrifos are predicted to temporarily reduce zooplankton abundance and increase phytoplankton biomass through a release of grazing pressure, whereas pulses of nutrients are expected to temporarily increase abundance of phytoplankton. We hypothesize that the response of the plankton communities in terms of structural and functional features will differ when nutrient and insecticide pulses occur synchronously or asynchronously.

METHODS

Freshwater planktonic communities in outdoor mesocosms

The response of freshwater planktonic food webs to synchronously or asynchronously pulsed disturbances was explored experimentally using replicated pond ecosystems established in mesocosms. Mesocosms were maintained outdoors at Ohio Wesleyan University's Kraus Nature Preserve, Delaware, OH USA. Fifteen cylindrical mesocosms were established in plastic tanks of 87.6 cm diameter and 45 cm depth. They were filled with 270 liters of well water and covered with mesh lids to avoid immigration by larger organisms. The well water initial nutrient concentration was 547 μg N/L and 43 μg P/L. We added nutrients to bring the concentrations up to 800 μg N/L and 57 μg P/L which are the average concentrations of the local pond communities we used to obtain plankton communities for this experiment. Previous work has shown that nitrogen and phosphorus are lost in these experimental mesocosms at the rate of approximately 5 % per day (Downing et al., 2008). In order to maintain these target concentrations all tanks received nutrient inputs every two weeks over the experimental period to match the loss rate of 5 % per day.

Mesocosms were first inoculated in early May with a naturally diverse assemblage of phytoplankton collected from 10 local ponds in order to allow plankton communities to assemble from a diverse species pool representing local diversity. Phytoplankton was strained through a 30 μm net to remove large zooplankton and macroinvertebrates. Phytoplankton populations were allowed to grow from the initial inoculum for two weeks before a diverse assemblage of zooplankton was added to the mesocosms.

Zooplankton was collected from the same 10 ponds as phytoplankton using a 35 μm plankton net and was added to the mesocosms after macroinvertebrates were removed. The mesocosms were exposed to natural environmental variability with respect to temperature and rainfall. The experiment started 16 days after zooplankton inoculation to give time for the populations to grow and the community to stabilize (Downing et al., 2008, Hall et al. 2004).

Experimental treatments of nutrients and insecticide

The experiment consisted of 3 treatments: nutrient pulses of nitrogen and phosphorus every two weeks (N), nutrients and insecticide pulsed simultaneously every two weeks (NI), and nutrients and insecticide pulsed in alternating weeks (N_I). Each treatment had five replicates for a total of 15 tanks.

Insecticide (I) pulses were delivered as 2 µg/L of chlorpyrifos every two weeks, representing an environmentally realistic worst case scenario observed in water bodies given the fact that toxicant concentrations can rise up to several orders of magnitude after rainfall events (Poletika et al., 2002; Rabiet et al., 2010). Nutrient (N) pulses were delivered as 560 µg/L of nitrogen and 39.9 µg/L of phosphorus every two weeks. Nutrients were added in the form of Na_2HPO_4 and $NaNO_3$. Nutrients were diluted in water and delivered via pipette in 5 ml increments to the mesocosms. Analytical grade Chlorpyrifos (Sigma Aldrich, Chlorpyrifos PESTANAL®) was diluted in acetone. Two ml of the acetone/Chlorpyrifos mixture were added to the insecticide treatments (NI and N_I) and two ml of pure acetone were added to the N treatments to serve as a control for potential effects of acetone (applied at approximately a 0.0001 % concentration). All mesocosms were gently stirred immediately after application of nutrients or insecticide.

Mesocosm sampling

Three days after pulse treatments were established, mesocosm sampling began weekly for 10 weeks. Zooplankton was sampled every week using integrated water samples (16 L) taken from each tank, filtered through a plankton net of 35 µm, handpicked to remove unwanted particulates (e.g. clumps of detritus, sand, etc.) and preserved in the lab with Lugol solution. The filtered water was returned to the mesocosm. Zooplankton was identified and counted using microscopy to the following taxonomic levels: ostracods, cyclopoid copepods, calanoid copepods, nauplii, copepodite, *Bosmina* sp., *Scapholebris* sp., *Daphnia* sp., *Chydorus* sp., *Alona* sp., *Pleuroxus* sp., *Simocephalus* sp. and *Ceriodaphnia* sp. Zooplankton abundances per liter, species richness and Shannon index were estimated from zooplankton counts. Phytoplankton biomass was assessed by chlorophyll *a* concentration. Water samples (300 ml) were taken, cold stored and transported to the laboratory to perform chlorophyll extractions. Chlorophyll *a* (Chl *a*) concentration was measured via extraction (Welschmeyer, 1994) and a flurometer (Turner Designs 700) to estimate the response of phytoplankton. In addition, net production was approximated by diurnal oxygen fluctuations (net production as gross production minus the respiration of all organisms using a YSI 550 Oxygen Probe; Cole et al., 2000; Downing & Leibold, 2010; Lind, 1979). Oxygen levels can be affected by production, respiration and atmospheric exchange but because atmospheric exchange should be comparable across all mesocosms, oxygen differences between mesocosms will largely be driven by differences in production and respiration induced by the different treatments. Oxygen was measured thirty minutes immediately before sunrise when oxygen levels had been depleted to their lowest levels due to respiration of heterotrophic and autotrophic organisms. Oxygen was measured again thirty minutes before sunset when oxygen levels are at their highest due to photosynthesis minus respiration by autotrophic and heterotrophic organisms during the day. To approximate net production, we subtracted dawn oxygen levels from dusk oxygen levels to obtain the net increase in oxygen over daylight hours. Therefore this measurement serves as a proxy for net production during daylight hours (i.e. gross primary production minus the respiration of autotrophs and heterotrophs during the day) (Cole et al., 2000; Downing & Leibold, 2010; Lind, 1979).

Water samples were taken to quantify nutrient (total N and total P) and chlorpyrifos concentrations on the final sampling day. Nutrients were analysed using spectrophotometry for total nitrogen (TN) and total phosphorus (TP) (APHA, 1980). Water samples from replicates receiving insecticides were collected on the final sampling day, two weeks after the final insecticide pulse had occurred. Samples were frozen and sent to Pace Analytical to estimate chlorpyrifos concentration remaining by the end of the experiment.

Data analysis

Prior to statistical analysis, data were tested for normality and homoscedasticity. Zooplankton abundance, chlorophyll *a*, and production data were log transformed to meet normality. Biological response variables were compared among mesocosms using linear mixed model regression in the statistical program R (lmer in package lme4,

Table 1. Statistical results (degrees of freedom-df; F statistic-F; level of significance *p-p*) of the linear mixed models testing the effects of pulse treatment (Treat), time (week) and their interaction (Treat*week) on zooplankton and phytoplankton communities, and of the Principal Response Curve (PRC) testing the effects of pulse treatment (Treat) on the zooplankton community. Bold values indicate significant effects ($p < 0.05$). *Resultados estadísticos (grados de libertad-df; estadístico F; nivel de significación* -p*) de los modelos lineales mixtos que prueban los efectos del tratamiento del pulso (Treat), el tiempo (semana; week) y su interacción (Treat*week) sobre las comunidades de zooplancton y fitoplancton, y de la curva de respuesta principal (PRC) que prueba los efectos del tratamiento del pulso (Treat) sobre la comunidad de zooplancton. Los valores en negrita indican efectos significativos (*p < 0.05).

Factors	df	Zooplankton Abundance (individuals/L)		Zooplankton Richness		Zooplankton Shannon diversity		Phytoplankton biomass		Production (mg O_2/L/hr)		PRC	
		F	*p-p*	F	*p-p*	F	*p-p*	F	*p-p*	F	*p-p*	F	*p-p*
Treat	2,12	2.63	0.113	**37.41**	**<0.001**	**14.53**	**<0.001**	**8.17**	**0.006**	0.65	0.541	10.16	**0.018**
Week	9,10	**2.13**	**0.033**	**3.31**	**0.001**	1.875	0.063	**5.78**	**<0.001**	**3.74**	**<0.001**	-	-
Treat* week	18,10	1.12	0.348	1.55	0.087	0.93	0.545	**3.48**	**<0.001**	1.67	0.06	-	-

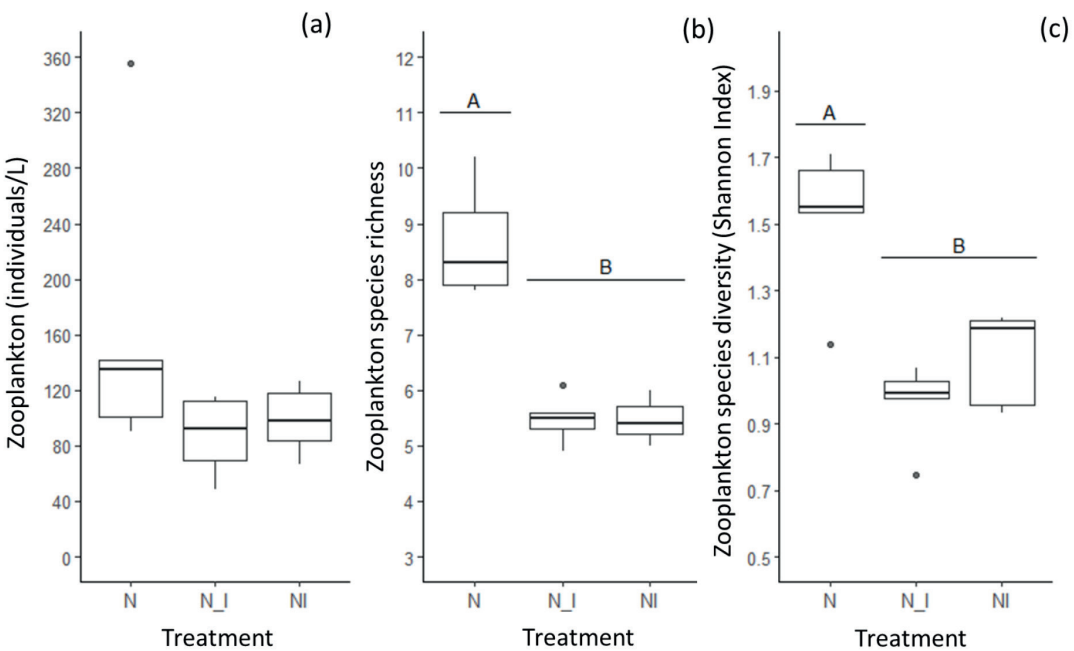

Figure 1. Zooplankton community response to pulse treatments averaged over time, expressed as (a) Zooplankton abundance, (b) Zooplankton richness and (c) Zooplankton diversity (Shannon's Index). Letters above bars indicate statistical difference between treatments as determined by Tukey *post hoc* tests ($p < 0.05$). Box plots show the median as the horizontal line, the boxes indicate the upper and lower quartiles around the median. *Respuesta de la comunidad de zooplancton a los tratamientos de pulso en promedio a lo largo del tiempo, expresada como (a) abundancia de zooplancton, (b) riqueza de zooplancton y (c) diversidad de zooplancton (índice de Shannon). Las letras encima de las barras indican la diferencia estadística entre los tratamientos según las pruebas* post hoc *de Tukey (*p < *0.05). Los diagramas de caja muestran la mediana como la línea horizontal, las cajas indican los cuartiles superior e inferior alrededor de la mediana.*

Bates et al. 2015) with treatment as a fixed effect and time as a random effect combined with *post hoc* Tukey tests to determine treatment differences. In addition, ordination analysis was performed to test the plankton community compositional responses. A Principal Response Curve (PRC) was done using R (Vegan package, Oksanen et al. 2020) software to analyze the zooplankton data set. PRC is a technique based on Redundancy Analysis (RDA) ordination techniques (Van den Brink & Ter Braak, 1999). The PRC analysis results in a diagram displaying the principal response of the community (left y-axis) for all sampling days (x-axis) by showing the deviations in time of the treatments compared to the controls. The species weights are presented in the right y-axis (1-D plot) which reveals the affinity of the different species with the principal community response. The species with a high positive weight are the most correlated to the main response showed by the PRC, while the species with a negative weight show the contrary trend to the main one reflected by the PRC. Species with weight close to zero means no response or are very dissimilar to the main response. Abundance data of zooplankton was ln (x + 1) transformed.

RESULTS

The zooplankton community was predominantly composed of cladocerans, copepods and ostracods. Nine different taxa were found: *Daphnia* sp., *Bosmina* sp., *Scapholebris* sp., *Chydorus* sp., *Simocephalus* sp., *Alona* sp., *Ceriodaphnia* sp., *Pleuroxus* sp., cyclopoid copepods, calanoid copepods and ostracods. Copepodite and nauplii within the copepods were counted as separate taxa and used for the ordination analysis.

After pulse treatments were established, zooplankton abundance did not vary significantly between treatments (Table 1, Fig. 1a), but did vary over time (Table 1, Fig. 2a). In contrast, zooplankton richness and Shannon diversity were both higher in the N treatment compared to the N_I and NI treatments (Table 1 Fig 2b-c). Zooplankton richness also varied similarly across time in

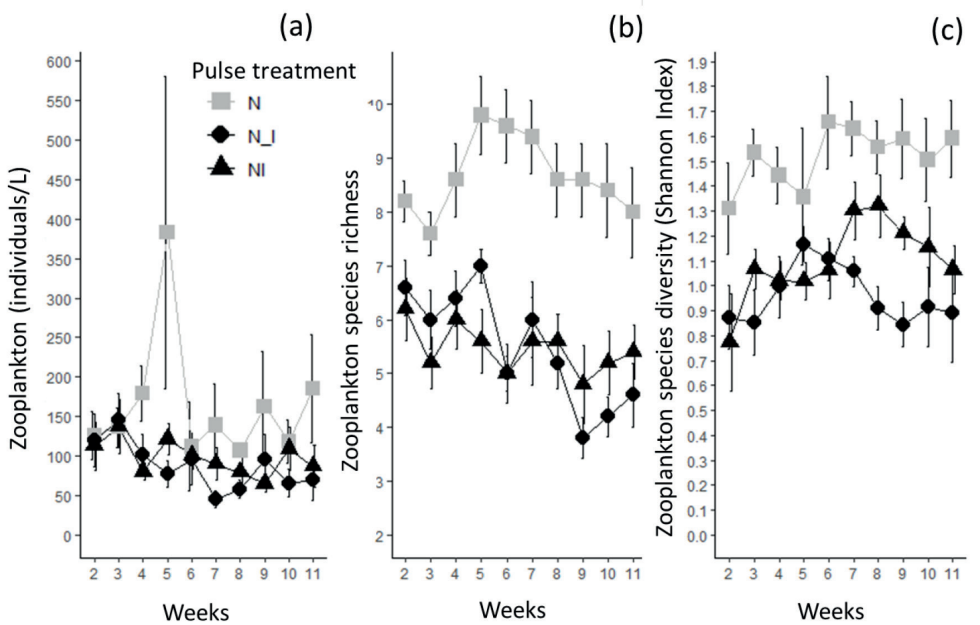

Figure 2. Temporal response of zooplankton communities to pulse treatments over 10 weeks, expressed as a) Zooplankton abundance, b) Zooplankton richness and c) Zooplankton diversity (Shannon index). *Respuesta temporal de las comunidades de zooplancton a los tratamientos de pulso durante 10 semanas, expresada como a) abundancia de zooplancton, b) riqueza de zooplancton y c) diversidad de zooplancton (índice de Shannon).*

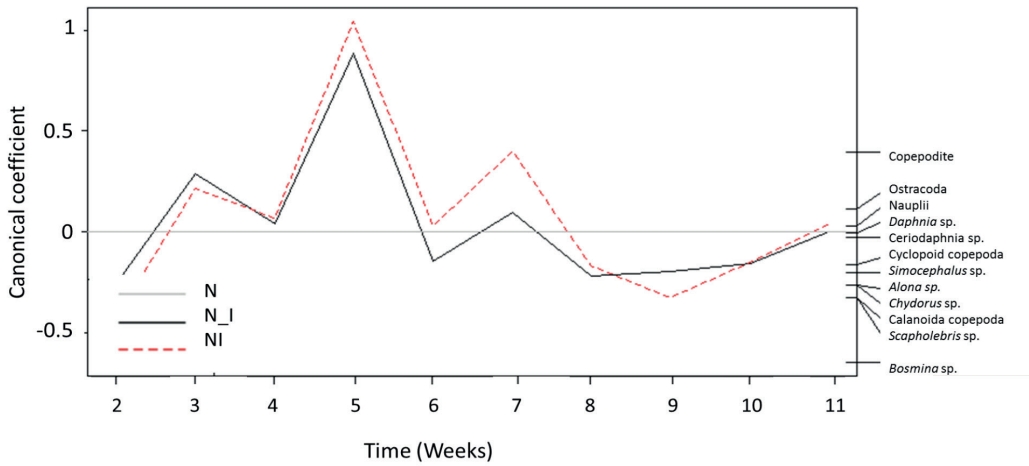

Figure 3. Principal Response Curve (PRC) showing the temporal responses of the zooplankton community to pulse treatments over 10 weeks. On the left y-axis, ordination method represents the main community response of the treatments (N_I and NI) over time with respect to the control (horizontal line corresponding to N treatment). The y-axis on the right summarizes the zooplankton community response based on its species composition; it represents the species weights expressed as the level of affinity that each taxa had with the main trend of the PRC. *Curva de respuesta principal (PRC por sus siglas en inglés) que muestra las respuestas temporales de la comunidad de zooplancton a los tratamientos de pulso durante 10 semanas. En el eje y de la izquierda, el método de ordenación representa la respuesta principal de la comunidad de los tratamientos (N_I y NI) a lo largo del tiempo con respecto al control (línea horizontal correspondiente al tratamiento N). El eje y de la derecha resume la respuesta de la comunidad de zooplancton en función de la composición de especies; representa los pesos de las especies expresados como el nivel de afinidad que cada taxón tuvo con la tendencia principal de la RPC.*

all treatments, with a slow decline in zooplankton richness after week 5 across all treatments (Table 1, Fig 2b). Shannon diversity did not vary significantly over time. (Table 1, Fig 2c). Zooplankton taxa composition was different between treatments as revealed PRC. The PRC was performed to characterize the degree of change and duration of the treatment effects upon the zooplankton community (Fig. 3). PRC was significant (Table 1), indicating that the disturbance treatments had detectable effects on the zooplankton community. The variance explained by the first axis which corresponds to the treatment was 37 % of the total data variability. Species weights (Fig. 3 right y-axis) indicate how different taxa are correlated to the main community response. It shows an increase in copepods in NI and N_I treatments and a decline in cladocerans.

Chl *a*, as an indicator of phytoplankton biomass, remained consistently low in the N treatment compared to Chl *a* in the N_I and NI treatments that oscillated between lower and higher concentrations (Table 1, Fig. 4a, Fig. 5a). Net production was not statistically different between treatments but did vary in the experiment over time (Table 1, Fig. 4 b, Fig. 5b).

TN and TP were measured at the end of the experiment and were similar across treatments (ANOVA, $F_{2,5} = 0.581$, $p > 0.05$). TN averaged 1130 µgN/L and TP averaged 82 µgP/L by the end of the experiment. Chlorpyrifos was detected at an average concentration of 0.1 µg/L (5 % of the pulse concentration) at the end of the experiment across 10 replicates sampled randomly from mesocosms with insecticide pulses, suggesting that chlorpyrifos was broken down quickly in the experimental mesocosms.

In summary, we found no differences in community response related to synchronous and asynchronous pulses. The insecticide pulses had negative effects on zooplankton species richness and diversity, no effects on zooplankton abundance, and positive effects on phytoplankton biomass. Our results show that insecticides change zooplankton community composition and reduce the zooplankton grazing pressure on phytoplankton.

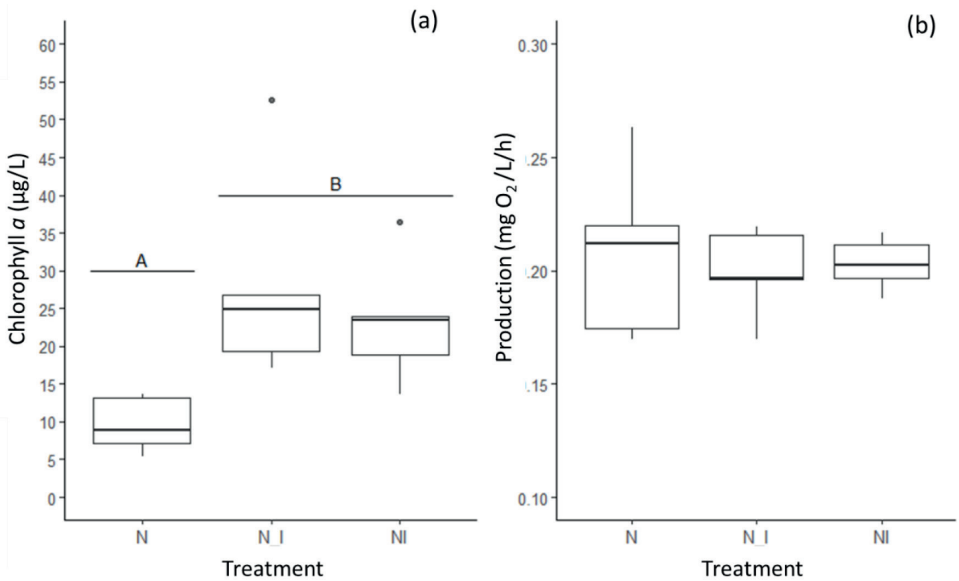

Figure 4. Phytoplankton community response to pulse treatments averaged over time, expressed as phytoplankton (a) and production (b). Letters above bars indicate statistical difference between treatments as determined by Tukey *post hoc* tests ($p < 0.05$). Box plots show the median as the horizontal line, the boxes indicate the upper and lower quartiles around the median. *Respuesta promedio de la comunidad de fitoplancton a los tratamientos de pulso a lo largo del tiempo, expresada como clorofila*-a *(a) y producción como concentración de oxígeno (b). Las letras sobre las barras indican la diferencia estadística entre los tratamientos según las pruebas* post hoc *de Tukey (*$p < 0.05$*). Los diagramas de caja muestran la mediana como la línea horizontal, las cajas indican los cuartiles superior e inferior alrededor de la mediana.*

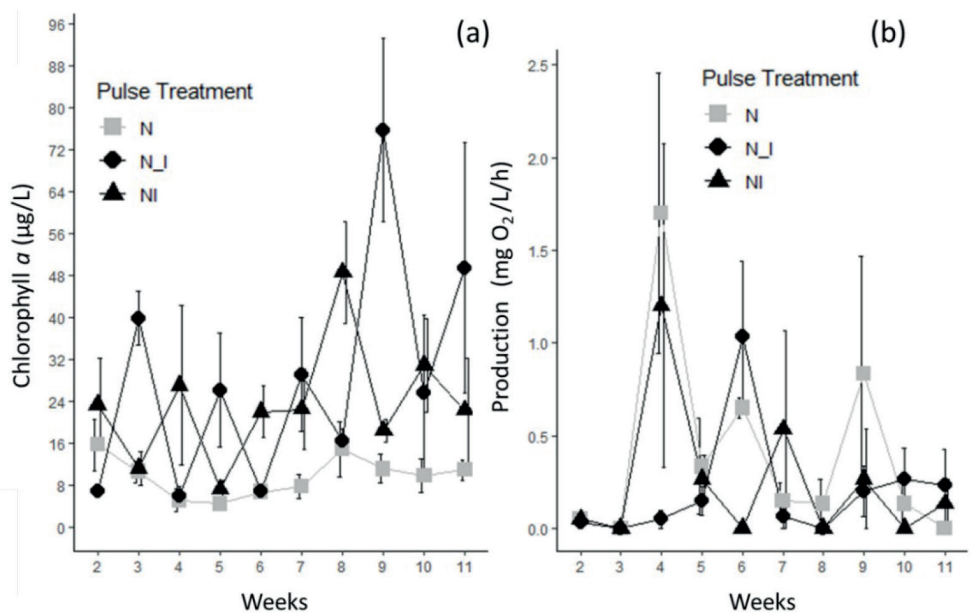

Figure 5. Temporal response of phytoplankton communities to pulse treatments over 10 weeks, expressed as a) Phytoplankton biomass, b) Production. *Respuesta temporal de las comunidades de fitoplancton a los tratamientos de pulso durante 10 semanas, expresada como clorofila*-a *(a) y producción como concentración de oxígeno (b).*

DISCUSSION

The mesocosm experiment was designed to study how freshwater ecosystems will respond to agrochemical disturbances (insecticides and nutrients) that are often pulsed (synchronously or asynchronously) in nutrient-enriched environments. We hypothesized that the synchronous vs. asynchronous pulses would differ in their impact on the community with synchronous pulses potentially showing stronger responses compared to asynchronous treatments.

On the contrary, we did not see significant differences in any response variable between NI and N_I, indicating that pulses of insecticide, whether pulsed synchronously or asynchronously with nutrients had a similar effect on pond communities.

The results obtained show that zooplankton abundance did not respond to the treatments, while richness and Shannon diversity were lower in both treatments with pulsed insecticide (NI and N_I) compared to the N treatment and did not show significant differences between the synchronous (NI) and asynchronous (N_I) treatments. This result agrees with studies that showed the effects of chlorpyrifos in nutrient-enriched systems appeared to be independent of its mixture with nutrients (Van Donk et al., 1995). The same conclusion was reached by Cuppen et al. (1995) where chlorpyrifos caused an adverse direct effect on the zooplankton community under a combined exposure of insecticide with nutrients. Previous studies report negative effects on zooplankton abundance under 1 µg/L chlorpyrifos exposure (van Wijngaarden et al., 2005), however, in our experiment we did not observe a negative impact on zooplankton abundance even under a higher chlorpyrifos concentration of 2 µg/L.

Unlike abundance which was not affected by the treatments, zooplankton species richness and diversity were both reduced in treatments receiving insecticides compared to N treatments, showing a significant and negative effect of insecticides on zooplankton community structure. In addition, PRC indicated that there was a community shift from a cladocera-dominated community in N treatments to a copepod-dominated community in treatments with insecticide (NI, N_I). This general shift towards copepod-dominated communities has also been found in other studies (Hanazato, 1998, 2001). Cladocera have been shown to be more sensitive to insecticides than copepods (Hanazato, 2001). Additionally, this result was also expected since a model cladoceran species *Daphnia magna* has a documented LC50 of 1 µg/L for chlorpyrifos (Moore et al., 1998). The initial insecticide treatments caused copepodites to become dominant, either due to decreases in competition from cladocerans which were more sensitive to the insecticide, or because they simply survived the insecticide resulting in them becoming more abundant than cladocerans. Then, over time, the copepodites matured into adult copepods and the PRC shows a shift towards more adult copepods by the end of the experiment.

The insecticide concentrations we used are within legal limits in order to explore realistic effects of chlorpyrifos on plankton communities and to allow for a chance for communities to recover between pulses. Brock et al. (2000) determined that signal of recovery from insecticides is only expected after two months from the last application if the exposure is lower than (0.1-1) x EC50 of the most sensitive standard test species. Our experiment did not last long enough after the last application to evaluate if recovery occurs. However, PRC show all communities become more similar by the end of the experiment. The observed changes in the communities exposed to insecticide seem to be mostly driven by the negative effects on cladoceran ecology (abundance, grazing pressures and diversity). However, it is known that cladoceran can rapidly develop tolerance to stressors (Brausch & Salice, 2011) which has the potential to impact further generations after both its application and degradation. Therefore, an increase in individual tolerance may explain the recovery trend by the end of the experiment, though differences between treatments are not significant. Increased tolerance could occur by acclimation, adaptation (genotype sorting, evolutionary rescue), or both as a result of the continuous selection pressure imposed by the insecticide pulses (Bickham, 2011). Such increases in tolerance could result in a population and/or community rescue (defined by Fugère et al., 2020 which defined community rescue as the

restoration of a population´s positive growth in a stressful environment), however, it might come with a cost which ultimately impacts populations and community (function, structure, and diversity) (Bell, 2013; López-Valcárcel et al., 2021). Ultimately, future studies will have to last long enough to allow for evolutionary processes such as genotype sorting in order to determine how important evolution can be for community recovery after insecticide disturbances.

Phytoplankton was higher in the treatments NI and N_I, suggesting a release from grazing pressure from zooplankton affected by the insecticide. Specifically, in our experiment, the difference in sensitivity to insecticides between cladocerans and copepods allowed copepods to dominate because copepods are less sensitive to insecticides and due to indirect effects of being released from food competition as cladocerans declined. The shift from cladocerans to copepods reduced grazing pressure and caused an increase in phytoplankton biomass. These indirect effects of reduced grazing pressures, detected through significant increases of phytoplankton biomass, are of extreme importance as a warning signal of potential eutrophication impacts (Schindler, 2006). In addition to insecticides reducing the grazing potential of the zooplankton community, insecticides could also affect phytoplankton growth (Chen et al., 2016), adding pressure to the already affected zooplankton taxa from the insecticide toxicity by altering food resources. In addition, the higher zooplankton richness and diversity in the N treatment is likely responsible for maintaining phytoplankton at lower and relatively constant levels across the experiment because a diverse zooplankton community containing cladocerans can more effectively maintain top-down control of the phytoplankton community. Production as measured by diurnal oxygen concentrations did not differ between treatments despite significant differences in phytoplankton biomass. This lack of treatment effect on production might be surprising given that phytoplankton should be a major determinant of production (oxygen) levels in these mesocosms. Net production as measured by diurnal oxygen fluxes in this experiment is influenced in large part by the phytoplankton community, but our measure of production also captures the influence of heterotrophic (e.g. microbial and zooplankton) communities on oxygen levels. In this study, we did not assess changes in microbial communities which are known to play a central role in production, and did not directly estimate respiration of autotrophic and heterotrophic organisms (Falkowski et al., 2008). We speculate that various unmeasured factors affected our measure of net production, for example microbial communities and environmental factors such as atmospheric exchange and temperature, may have had an effect on oxygen levels that resulted in similar net production levels across treatments in this study despite observed differences in phytoplankton biomass.

Our results suggest that the insecticide drives the response differences among the communities. However, we cannot disentangle how nutrients and pesticide interact (i.e. antagonistic, additive, or synergistic). For future experiments, understanding how nutrients and pesticide interact would provide relevant information for agrochemicals management and application plans (Allgeier et al., 2011; Villar-Argaiz et al., 2018). Specifically, identifying if synchronously agrochemical pulses results in synergistic effects would imply the need for regulating agrochemicals application with some delay between applications to prevent runoffs with both of them.

In summary, our results suggest that indirect effects can be as important as direct effects in determining the food web response to disturbances. While our results did not show a significant effect of pulse timing of multiple stressors, we encourage future work to incorporate ecologically realistic complexity such as studying the impact of multiple stressors that vary in pulse frequency and synchrony in order to better understand complex community responses to realistic disturbance scenarios.

CONCLUSIONS

This study was designed to gain a deeper understanding about how asynchronous and synchronous mixtures of stressors impact wetland communities. Our results show (i) that insecticides had a negative effect on zooplankton commu-

nities and did not depend on if nutrients pulses occurred synchronously or asynchronously with insecticide pulses; (ii) the importance of indirect effects caused through trophic interactions for understanding the impact of agrochemical stressors on freshwater communities; and, (iii) that multiple aspects of communities (richness, diversity, and abundance) should be monitored over time to fully capture a community's response to and recover from disturbances.

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