Ann. Limnol. - Int. J. Lim. 2019, 55, 5
© EDP Sciences, 2019
https://doi.org/10.1051/limn/2019004

Available online at:
www.limnology-journal.org

RESEARCH ARTICLE

Plankton community responses to environmentally-relevant agrochemical mixtures

Ana del Arco*, Francisco Guerrero, Francisco Jiménez-Gómez and Gema Parra

Departamento de Biología Animal, Biología Vegetal y. Ecología. Centro de Estudios Avanzados en Ciencias de la Tierra (CEACTierra), Universidad de Jaén, Campus de las Lagunillas, s/n., 23071 Jaén, Spain

Received: 7 September 2018; Accepted: 18 February 2019

Abstract – Chemicals do not occur alone in the environment but most ecotoxicological assessments target the effect of single chemicals on aquatic communities and the establishment of legal limits is based on them. The present study assesses how plankton communities respond to single and mixture treatments of copper sulphate and ammonium nitrate where both agrochemical concentrations are below legal limits. Twenty-five microcosms were used to assess the effects of four treatments ($n = 5$): (1) low nitrate (L) of 25 mg L^{-1}; (2) high nitrate (H) of 50 mg L^{-1}; (3) copper treatment (CU) of 0.04 mg L^{-1} of copper; and (4) interaction treatment (I) of 50 mg L^{-1} of nitrate applied together with 0.04 mg L^{-1} of copper, and the controls (C). Plankton abundance, phytoplankton biovolume and zooplankton community structure (changes in the diversity and richness) were used as structural endpoints, and oxygen production and litter decomposition as functional indicators. Overall, results show no effect on the plankton community exposed to agrochemical under legal limits in single neither in mixture treatments. Only by the end of the experiment, total zooplankton abundance shows differences between interaction treatment (I) and the rest of the treatments and controls. Concretely, the interaction treatment suggests how a nutrient enhancement from ammonium nitrate addition may counterbalance the toxic effect of copper sulphate on zooplankton, most likely as a result of higher phytoplankton availability that positively influences zooplankter survival. Both drastic and subtle effects on communities are relevant for disentangling how chemicals interact under European current legal limits.

Keywords: agrochemical / mixtures / legal limits / natural assemblages / plankton

1 Introduction

Intensive agricultural practices are characterized by multiple applications of agrochemicals, mixtures of which enter water bodies by spray drift, runoff or leaching, potentially causing adverse effects on freshwater ecosystems (Parra *et al.*, 2005; Van Wijngaarden *et al.*, 2005a).

Consumption of manufactured fertilizers based on nitrogen in Europe is translated into 10.4 million tons of nitrogen (N) in 2011/2012 (Eurostat, 2017a). At the same time, fungicides, together with bactericides, account for 38.4 thousand tons (Eurostat, 2017b). Ammonium nitrate is a broad-spectrum fertilizer used worldwide. Ammonium quickly transforms into nitrates (NO_3^-) by nitrification processes, even though nitrate naturally occurs in aquatic systems, its concentrations have drastically increased owing to agricultural runoff (Camargo and Ward, 1995). A major consequence of nutrient increase is eutrophication, which triggers phytoplankton blooms due to nutrient availability or species interactions changes (*i.e.* disruption to grazing pressures from zooplankton) effects leading to water quality degradation (Schindler, 1977; Schindler, 2006; Dokulil and Teubner, 2011). In addition, many studies warn of the effect of nitrates on freshwater vertebrates and invertebrates (*e.g.* Camargo and Ward, 1995; García-Muñoz *et al.*, 2011). Now focusing on the fungicide, copper sulphate is used as a fungicide, herbicide and algaecide worldwide (Kungolos *et al.*, 2009), and may therefore reach aquatic systems both by direct application or runoff. Apart from its persistence and negative environmental effects, copper, as a heavy metal, is a public concern due to its impact on human health (Duruibe *et al.*, 2007). Copper induces disruptive effects in aquatic systems, such as changes in community structure, carbon transport across the food web, bioavailability and ecological interactions (Havens, 1994; Mastin and Rodgers, 2000; del Arco *et al.*, 2014). The importance of unbalanced food web changes for the whole community cannot be overlooked. For instance, a microcosm

*Corresponding author: aarco@ujaen.es; anadelarco@ugr.es

experiment assessing the adverse effects of an insecticide (Chlorpyrifos) in plankton communities showed that changes in grazing pressures owing to a decrease in microcrustacean populations resulted in signs of eutrophication (increases in chlorophyll *a* and algae abundances *versus* decreases of dissolved oxygen and pH changes) which will have adverse effects on both the biota and water quality parameters (Van Wijngaarden *et al.*, 2005a). Furthermore, looking at zooplankton under Cu exposure, Parra *et al.* (2005) found adverse effects on hatching rates and nauplii survival in copepods (*Arctodiaptomus salinus*) under copper concentrations lower than expected field values. Although both agrochemicals have different lifespans, grades of reactivity and persistence, seems realistic that fertilizers and fungicides co-occur in the environment, especially in wetland watersheds surrounded by intensive agriculture. By this reason, ammonium nitrate as a fertilizer and copper sulphate as a fungicide have been the selected agrochemicals for the microcosm experiment presented here.

Under the multi-stressors framework to seek the means to identify, diagnose and tackle those stressors effects is key to identify and priorities water management strategies (Ormerod *et al.*, 2010). Pointing out this concern, legal limits are mostly established based on single test species considering only single chemical exposure and standard species. Even though safety factors are applied to counterbalance the limitations of single species tests, it may not be enough to prevent the environmental risks of stressor mixtures and to understand the consequences in the ecosystem. However, despite efforts towards more complex scenarios (*e.g.* microcosms experiments), there is a lack of environmentally realistic exposure because most of the studies disregard chemical mixtures that unavoidably occur in natural water bodies. It is well-known that chemicals co-occur, so then, potentially they can interact resulting in additive (sum of the individual effects), antagonistic (less than additive) and synergistic (more than additive) effects (Lydy *et al.*, 2004; Jonker *et al.*, 2005). In this sense, there is a lack of studies for routinely-found chemical concentrations and their cocktail effects on ecosystems (Laskowski *et al.*, 2010; De Laender and Janssen, 2013; Van den Brink, 2013) this brings up the question whether current legal limits are safe enough. Our study presented here seeks to contribute to the claim of the need for more complex multispecies experiments exposed to mixtures targeting community responses (De Laender and Janssen, 2013).

Therefore, the aim of our study was to evaluate how treatments with agrochemical mixtures within legal limits affect freshwater ecosystems, focusing on plankton community. Our hypothesis is that no effects of exposure to a single toxicant on the community are expected because the doses are under legal limits but treatments with mixtures will have effects on the community because of toxicants' co-occurrence resulting on potential chemicals effect interactions.

2 Materials and methods

2.1 Microcosm set-up

The effects of agrochemical mixtures on plankton communities were assessed using microcosms with naturally-occurring plankton communities from a local pond [Casillas wetland, UTM 30SVG1084 with a surface area of 2.7 ha (Ortega *et al.*, 2006)]. Microcosms (circular plastic buckets with a volume of 50 L) were filled with 45 L of commercial mineralized water, covered with mesh lids to avoid immigration by larger organisms and/or spores and randomly located outdoors in an experimental wetland infrastructure at the University of Jaén. Hauls with a plankton net (53 μm) were done in a local pond (Casillas wetland) to collect plankton samples which were homogenized and equally distributed among the microcosms. At the same time, superficial sediment layers of the pond were extracted using a shovel to set a 5-cm layer in all microcosms after their homogenization. A diverse zooplankton community was developed from all the inoculated pond plankton samples up to hatching from the sediment resting eggs, with the presence of rotifers, cladocerans, copepods and ostracods species. The phytoplankton community developed from the inoculated pond water coming from the plankton and sediment samples.

The plankton community and sediment were inoculated in March 2013 to allow the development of a plankton community in the microcosms before the start of the experiment a month later in April 2013. The exposure experiment lasted 49 days (from D0 until D49). The agrochemical mixtures were spiked with a single treatment on D0 after plankton samples were taken to capture initial community conditions. The treatments consisted of four different agrochemical perturbations and the controls (C and single or interaction treatments, $n = 5$). The agrochemical perturbation treatments included two single treatments of nitrate of 25 mg L^{-1} (low nitrate treatment, L) and 50 mg L^{-1} (high nitrate treatment, H), one single treatment of copper of 0.04 mg L^{-1} (copper treatment, CU), and an interaction treatment with 50 mg L^{-1} of nitrate applied together with 0.04 mg L^{-1} of copper (interaction treatment, I). The concentrations of nitrate and copper employed in the treatments were selected based on the legal limits established by Council Directive 80/778/EEC (revised as Council Directive 98/83/EC) for nitrates (50 mg L^{-1}; while 25 mg L^{-1} was selected to explore a lower concentration within legal limits), Council Directive 91/676/EEC for nitrates and Boletín Oficial del Estado (BOE, 2011) for copper (0.04 mg L^{-1}) following the Water Framework Directive (WFD) (Directive 2000/60/EC).

2.2 Physical and chemical variables

The physical and chemical conditions of the microcosms were assessed weekly in the morning (8 a.m.). The environmental conditions measured *in situ*, using a field probe (YSI-556 MPS), were temperature, pH, dissolved oxygen (DO), and conductivity (μS cm^{-1}). Alkalinity was measured in the laboratory, water samples (100 mL) were taken and transported in cold and darkness conditions for analysis using an 848 Titrino Plus device.

Nitrate and copper stock solutions were prepared using ammonium nitrate (NH_4NO_3) and copper sulphate ($CuSO_4$), respectively. A single agrochemical treatment (aliquots of the stock solutions) was spiked with its corresponding treatment on D0 on the surface of the water in the microcosm after the first physical, chemical and biological sampling. The micro-

cosms were gently stirred to ensure homogeneous agrochemical distribution along the water column. After the treatment, a sample of 50 mL of water was taken to corroborate whether nominal concentrations were reached. A standard laboratory protocol was used to analyze nitrate concentrations (APHA, 1995). The ammonia concentration was measured by photometric water analysis using a NANOCOLOR kit (DIN 38406-5, German Institute for Norms, Photometric determination as indophenol, range from 0.05–3.00 mg $NH_4^+ L^{-1}$); and copper was analyzed by inductively coupled plasma mass spectrometry (ICP-MS). Average exposure concentrations (AEC) were calculated in controls and treatments as the average concentration of agrochemicals throughout the experiment.

2.3 Biological endpoints

The phytoplankton community response to the toxicants based on abundance and the community size structure endpoint was evaluated weekly by chlorophyll *a* (Chl *a*) and flow cytometry measurements, respectively. The endpoints were a proxy of phytoplankton abundance (cellular densities measured by cytometry and Chl *a* concentration), biovolume and community size structure (pico-, ultra-, nano- and microphytoplankton biovolume size classes). Chl *a* concentration was determined by fluorometry (AquaFluor from Turner Design). A calibration curve was calculated based on samples that were filtered through Whatman GF/C glass microfiber filters, and extracted in 90% acetone for 24 h at 4 °C (Strickland and Parsons, 1968). Cytometry analysis was performed on water samples preserved in glutaraldehyde (2% f.c.), frozen in liquid nitrogen and stored at 80 °C until analysis with a BD LSRFortessa flow cytometer. Calibration spheres were used to obtain a cell-size calibration curve. Four cell-size groups were studied: picophytoplankton (0.4–2 μm^3), ultraphytoplankton (2–8 μm^3), nanophytoplankton (8–20 μm^3) and microphytoplankton (> 20 μm^3). An acquisition time of 180 s at a rate of 60 $\mu L\ min^{-1}$ was a set parameter to measure the abundance of population cells. These data were analyzed with FACSDIVA software.

The zooplankton was identified, counted and grouped to assess community response to the toxicants weekly by abundance, community structure and diversity based on the lowest taxonomic practical levels (TPL) (Van Wijngaarden *et al.*, 2005b; del Arco *et al.*, 2016). The endpoints assessed were abundance ($ind\ L^{-1}$), community structure (PRC), richness and diversity (Shannon-Wiener diversity index, using a natural logarithm). Integrated water samples with zooplankton (0.5 L, plankton net of 60 μm) were taken from each microcosm and preserved in formaldehyde (4% f.c.). The filtered water was returned to the microcosm. Zooplankton was counted, identified and grouped into the following eight taxonomic practical levels (TPL): Ostracods, calanoid copepods (*Neolovenulla alluaudi*), cyclopoid copepods (*Acanthocyclops* sp. plus *Metacyclops* sp.), nauplii (calanoida plus cyclopoida), *Ceriodaphnia reticulata*, *Alona* sp., *Macrothrix hirsuticornis* and rotifers.

Oxygen production was estimated by diurnal oxygen fluctuations as a proxy of ecosystem productivity (Cole *et al.*, 2000; Downing and Leibold, 2010). It was measured weekly at the start (8 a.m.) and at the end (8 p.m.) of the day, with a spring-summer photoperiod (12:12), using a field probe (YSI-556 MPS).

Litter decomposition was assessed by incubating alder leaves (*Alnus glutinosa*) in order to compare the percentage of ash free dry mass (AFDM) between controls and treatments at the end of the experiment following the protocol of Gessner and Chauvet (1994). Litter decomposition aimed at obtain information of microbial activity in our controlled experimental microcosms, although in natural systems other influencing physical and biological factors (*i.e.* physical abrasion, nutrient enrichment) should be considered (Castela *et al.*, 2008; Pérez *et al.*, 2011; del Arco *et al.*, 2012).

2.4 Statistical analysis

The effects of single agrochemical treatments and mixtures in the planktonic community at both structural and functional levels were assessed through analysis of variance by linear mixed effects (LME) models (Pinheiro *et al.*, 2017) followed by a *post hoc* analysis (least squares means) when significant differences due to treatment, time or their interaction were detected (Lenth, 2016). The model considered the treatment, time and their interaction as fixed effects and the microcosms as the random effects. In addition, the zooplankton community structure was analyzed by principal response curves (PRC, van den Brink and ter Braak, 1999). PRC is an ordination analysis based on redundancy analysis ordination (RDA) that allows a graphic observation of the overall community response to the treatments during the experiment compared to the controls (van den Brink and ter Braak, 1999; Roessink *et al.*, 2005; Zafar *et al.*, 2012). In addition, the species weights are represented at the side of the graph, which inform about the affinity of the different species with the overall response showed by the PRC. Species can have a positive, negative or null value, meaning that the species changes are directly, indirectly or not correlated to the main response trend respectively. Differences between the curves of the different treatments respect to the controls indicate changes in communities due to the agrochemical exposure.

3 Results and discussion

3.1 Physical and chemical variables

Nitrate and copper measurements were taken to corroborate intended nominal concentrations and average exposure concentrations (AEC) calculated in each treatment (Tab. 1). During the experiment, the average percentages of nitrate concentrations were 51.6 ± 33.9% (L treatment), 55.3 ± 30.4% (H treatment) and 47.4 ± 34.5% (I treatment) of the target nominal concentrations in each treatment. The percentages of nominal copper concentrations were 38.9 ± 11.0% (CU treatment) and 46.0 ± 10.5% (I treatment). Although nitrate was the study target, levels of ammonium were assessed to account for its toxicity because of its ecological importance. Ammonium concentrations were lower than 0.05 mg $NH_4^+ L^{-1}$ in all treatments.

All experimental microcosms experienced similar physical and chemical conditions (aside from the agrochemical treatments)

Table 1. Nominal target concentrations, concentrations measured right after adding the treatments (D0) and average exposure concentration (AEC) in mg L^{-1} during the experimental period in each treatment (mean ± SD, $n = 5$).

Treatments	Toxicant	Nominal target concentration (mg L^{-1})	D0 ± SD	AEC (mg L^{-1})
L	NO_3^-	25	30.8 ± 11.1	14.0 ± 5.5
H	NO_3^-	50	41.0 ± 24.4	24.1 ± 14.4
CU	Cu	0.04	0.051 ± 0.001	0.015 ± 0.044
I	Cu	0.04	0.058 ± 0.001	0.018 ± 0.042
	NO_3^-	50	46.2 ± 28.9	14.0 ± 5.5

Table 2. Results of the linear mixed effects (LME) analysis showing the effects of treatment, time and theirs interaction on physical-chemical, functional and structural endpoints along the experiment. Litter decomposition was analyzed only at the end of the experiment (D49). Bold values indicate significant effects ($p < 0.05$).

Endpoint	Treatment	Time	Treatment × time
Physical-chemical			
Temperature (C)	$p = 0.845$	$\mathbf{p < 0.001}$	$p = 0.993$
pH	$p = 0.547$	$\mathbf{p < 0.001}$	$p = 0.172$
Conductivity (µS/cm)	$p = 0.090$	$\mathbf{p < 0.001}$	$p = 0.070$
% DO	$p = 0.749$	$\mathbf{p < 0.001}$	$p = 0.070$
O_2 (mg L^{-1})	$p = 0.584$	$\mathbf{p < 0.001}$	$p = 0.437$
Alkalinity	$p = 0.468$	$\mathbf{p < 0.001}$	$p = 0.177$
Functional			
Oxygen production (mg O_2 h^{-1})	$p = 0.412$	$\mathbf{p < 0.001}$	$p = 0.143$
Litter Decomposition	$p = 0.466$	–	–
Structural – phytoplankton			
Total phytoplankton abundance (cell L^{-1})	$p = 0.439$	$p = 0.031$	$p = 0.374$
Total phytoplankton biovolumen (µm^3)	$p = 0.186$	$\mathbf{p = 0.001}$	$p = 0.680$
Picophytoplankton (cell L^{-1})	$p = 0.175$	$\mathbf{p < 0.001}$	$p = 0.637$
Ultraphytoplankton (cell L^{-1})	$p = 0.972$	$p = 0.006$	$p = 0.882$
Nanophytoplankton (cell L^{-1})	$p = 0.210$	$p = 0.007$	$p = 0.457$
Microphytoplankton (cell L^{-1})	$p = 0.268$	$p = 0.012$	$p = 0.393$
Chl-a (µg L^{-1})	$\mathbf{p = 0.016}$	$\mathbf{p < 0.001}$	$p = 0.196$
Structural – zooplankton			
Total Zooplankton (ind L^{-1})	$\mathbf{p = 0.013}$	$\mathbf{p < 0.001}$	$\mathbf{p < 0.001}$
Rotifera (ind L^{-1})	$p = 0.066$	$\mathbf{p < 0.001}$	$\mathbf{p < 0.001}$
Cyclopoida copepods (ind L^{-1})	$p = 0.172$	$\mathbf{p < 0.001}$	$p = 0.546$
Calanoida copepods (ind L^{-1})	$p = 0.498$	$\mathbf{p < 0.001}$	$p = 0.271$
Nauplii (ind L^{-1})	$p = 0.461$	$\mathbf{p < 0.001}$	$p = 0.086$
Ceriodaphnia reticulata (ind L^{-1})	$p = 0.545$	$p = 0.002$	$p = 0.766$
Macrothrix hirsuticornis (ind L^{-1})	$p = 0.335$	$\mathbf{p < 0.001}$	$p = 0.275$
Alona sp. (ind L^{-1})	$p = 0.071$	$\mathbf{p < 0.001}$	$\mathbf{p = 0.017}$
Ostracoda (ind L^{-1})	$p = 0.598$	$\mathbf{p < 0.001}$	$p = 0.422$

as indicated by the lack of any statistically significant differences in temperature, pH, alkalinity, percentage of dissolved oxygen and conductivity (Tab. 2). The average measurements during the experiment were 16.6 ± 3.0 °C; 8.0 ± 0.1 pH; 87.3 ± 45.1 bicarbonate mg L^{-1}; 71.7 ± 13.7% DO and 317.0 ± 77.3 µS/cm respectively. Taking this into account, the responses presented in the results will be linked to the experimental treatments because nominal concentrations of individual agrochemicals and mixtures were achieved and no physical and chemical differences between microcosms were detected.

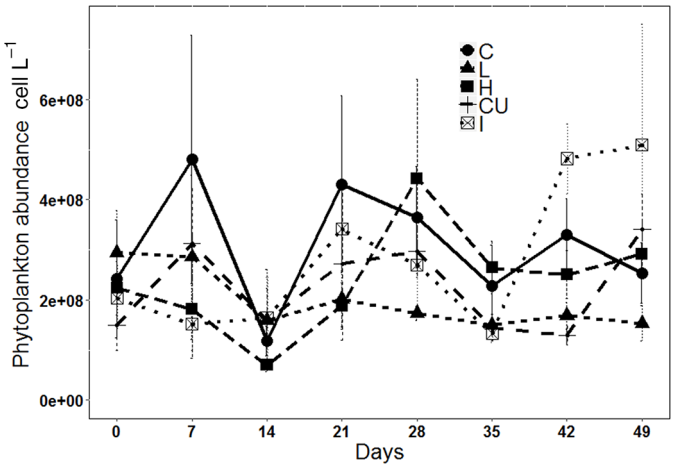

Fig. 1. Phytoplankton total abundance (cell L^{-1}) values in each treatment. C, L, H, CU and I stand for controls, low nitrate, high nitrate, copper and interaction of nitrate-copper mixture respectively (mean ± SD, $n = 5$).

3.2 Biological endpoints

No statistical differences in total phytoplankton abundance (Fig. 1), total biovolume and the biovolume of the cell-size classes between controls and treatments were found for the interaction of treatment × time (Tab. 2). Neither immediate nor delayed consequences (influenced by life cycle generations or disruption in trophic changes that may take longer to show effects different from mortality) were detected in terms of cell-size class composition of phytoplankton communities based on the absence of differences between controls and treatments (Tab. 2, Fig. 2). Changes in phytoplankton cell size was used instead of species identification as it could be an appropriated fast endpoint to complement the explanation of the changes registered in zooplankton abundance and community shifts (Quiñones, 1994; Kasai and Hanazato, 1995). For instance, these include warnings about species changes, which may differ in grazing capacity and will result in changes to phytoplankton cell-size abundance. It is well-known that edibility of phytoplankton modulates zooplankton grazing capacity (Miracle *et al.*, 2007; Holt, 2008; Scheffer *et al.*, 2008; Cumming *et al.*, 2013) which could influence zooplankton fitness and consequently its response to the toxicants. However, the absence of differences between treatments in terms of phytoplankton cell size pointed out the necessity for including other flow cytometry identifiers as pigments or cellular features to increase the usefulness of this tool.

Focusing on a more common proxy for phytoplankton, such as Chl *a*, no differences were shown related to the treatment × time (Tab. 2) but differences were denoted between the control and I treatment (Tab. 2, *post hoc* = 0.009). The Chl *a* graph (Fig. 3) suggests that such a specific difference would most likely be related to the variance of I treatment on the last sampling day (D49), probably related with the smoother tendency to increase Chl *a* values than the other treatments and controls after D28. This could be related to the availability of nitrogen, leading to an increase in phytoplankton abundance by the end of the experiment (Figs. 1 and 3) together with a decrease in grazing pressure from zooplankton exposed to copper based on the known detrimental effects of Cu on zooplankton (Parra *et al.*, 2005; Gama-Flores *et al.*, 2007). No negative effect of CU treatments on phytoplankton was observed as our treatment (0.04 mg Cu L^{-1}) is an order of magnitude lower than an effective concentration (EC_{10}) of 0.265 mg Cu L^{-1} for Chl *a* reported in the literature (Pérez *et al.*, 2010). Both the mild phytoplankton responses to low toxicants exposure and the complexity of the potential counterbalance effects on the different trophic levels make it impossible to disentangle the contribution of each agrochemical effect in the interaction treatment, more sampling days would be necessary. Therefore, based on our results and on the endpoints selected, phytoplankton was not affected by the treatments despite the crucial role of nitrogen availability in shaping both phytoplankton abundance and community structure (Conley *et al.*, 2002; Carpenter, 2008; Paerl *et al.*, 2010). Moreover, the functional indicators selected, oxygen production and litter decomposition did not show statistical differences between treatments and controls throughout the experiment (Tab. 2). Consequently, we corroborate the hypothesis of unexpected effects on the community with single exposure since chemicals were under legal limits. On the contrary, the second part of our hypothesis, co-occurring chemical having interacting effects, must be rejected since the phytoplankton community in I treatment was not different compared to the one in the controls.

In relation to zooplankton, richness (5.0 ± 0.7; 5.6 ± 0.2; 5.4 ± 0.5; 4.8 ± 0.9 and 5.0 ± 0 in the C, L, H, CU and I treatments, respectively) and the Shannon-Wiener diversity index (1.69 ± 0.25; 1.32 ± 0.63; 1.27 ± 0.60; 1.22 ± 0.60 and 1.82 ± 0.33 in the C, L, H, CU and I treatments, respectively) based on TPL at the end of the experiment suggested that zooplankton communities were highly similar despite the treatments. However, statistical analysis detected differences in total abundance and in the abundance of some taxa (rotifers and *Alona* sp., Tab. 2) between controls and treatments and among treatments. There is a broadly increasing rule of sensitivity from copepods to cladocerans in the literature (Hanazato, 1998). In accordance with this, in our experiment, copepods did not respond to the treatments, possibly because the copper concentration tested in this study (0.04 mg L^{-1}) is lower than the published LC_{50} for copepods (0.247 mg L^{-1}, Lalande and Pinel-Alloul, 1986) while *Alona* sp., as a cladoceran expected to be more sensitive, showed differences, with abundance being lower in CU and I treatments. Now, looking at the rotifers, attending to the data in the literature (Gama-Flores *et al.*, 2007), an effect was expected on rotifers based on a reported decrease in population abundances up to 41% in *Brachionus calyciflorus* when it was exposed to 0.0375 mg L^{-1}, 0.075 mg L^{-1}, 0.15 mg L^{-1} of copper sulphate for acute and chronic tests (Gama-Flores *et al.*, 2007) with the first two concentrations being similar to the copper concentrations of this study. PRC results were consistent with this result ($p < 0.001$, Fig. 4), showing how the response of rotifers dominates changes in zooplankton. Looking closer at the differences in rotifers and *Alona* sp. *post hoc* denoted *p*-values > 0.05 for rotifers and only found marginal differences for *Alona* sp. between CU and H treatments ($p = 0.065$). In addition, changes in the abundance of the rotifer population mimic the dynamic of total zooplankton abundance, so the

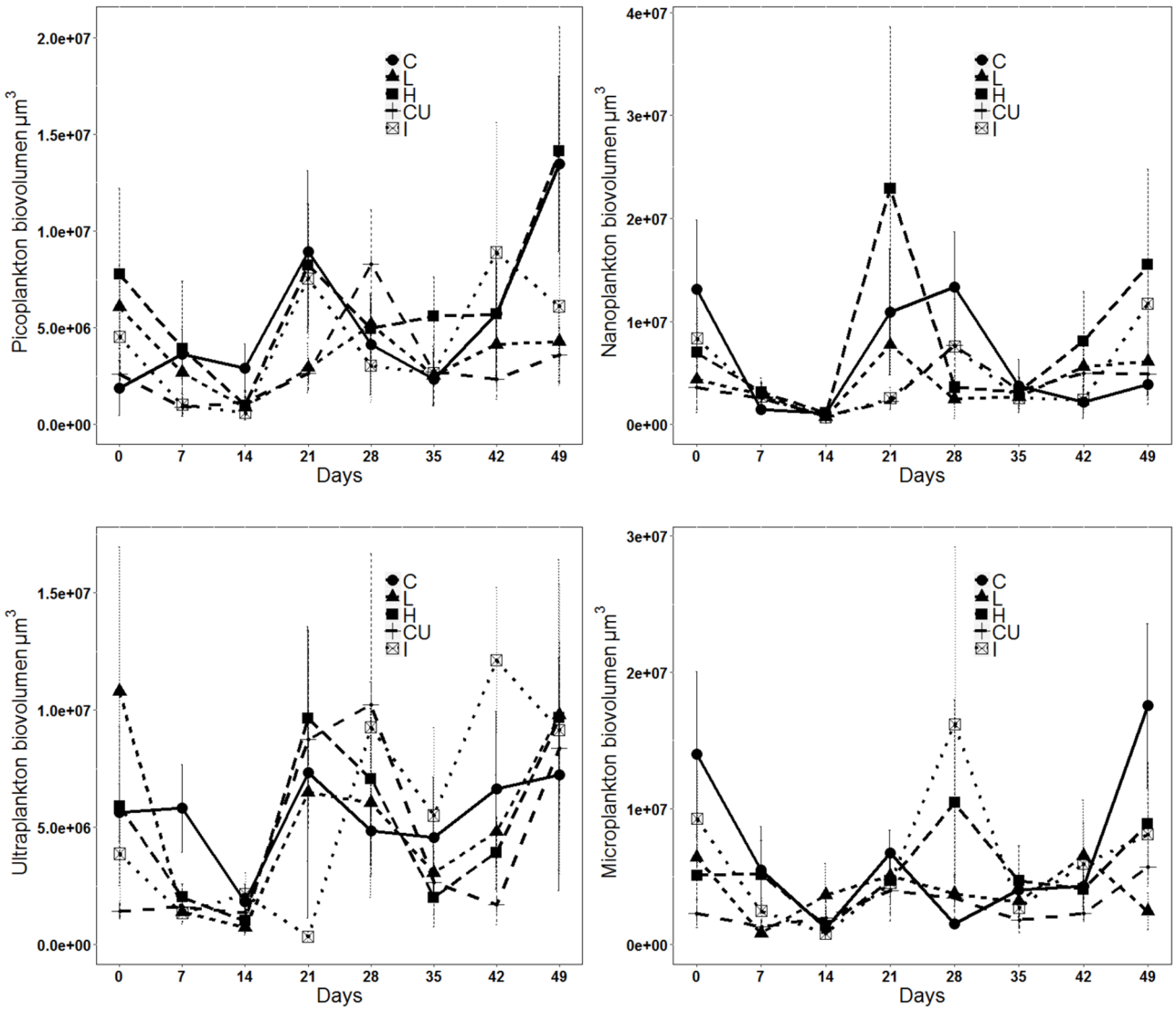

Fig. 2. Phytoplankton community structure represented by cell size classes' biovolume (μm^3) values in each treatment: C, L, H, CU and I stand for controls, low nitrate, high nitrate, copper and interaction of nitrate-copper mixture respectively (mean ± SD, $n = 5$).

differences found were dominated by rotifers, which had a high variability by the end of the experiment as total zooplankton. The grazing capacity of rotifers has been reported to be low to control phytoplankton development comparing with macro-zooplankton (Miracle *et al.,* 2007). Nevertheless, its importance in the aquatic community cannot be neglected, and there is a review that highlights their relevance as predators on bacteria, flagellates and even small ciliates (Arndt, 1993). To sum up, as in the phytoplankton community, the zooplankton community did not show any effect caused by exposure to single toxicants. However, the interaction treatment (I) by the end of the experiment suggests a counterbalance effect between the chemicals because of their mixture in the microcosms water column. Although we have stated that differences in zooplankton were not strong enough to denote negative treatment effects, nonetheless, we would like to take a closer look at D49. On D49, differences in zooplankton suggested that nitrate addition (higher zooplankton abundance in L, H and I treatments) could counterbalance the negative toxic effect of copper (CU treatment showing the lower zooplankton abundance on D49 with respect to C and the other treatments). This result could be explained by a compensation of the negative effects of copper as a consequence of major food availability due to the addition of nutrients favoring phytoplankton growth in I treatment similar to L and H treatments by the end of the experiment (D49, Figs. 1 and 3). Caramujo and Boavida (1999) stated the importance of food quality for reproductive cycles and development stages of zooplankton taxa. In the same line, Chandini (1988) reported the negative influence of food availability on the survivorship, growth and reproduction of *Echiniscatri serialis* when exposed to sublethal concentrations of cadmium. Focusing in rotifers, that within this community could be the most sensitive individuals to copper, their populations decreased under CU treatments on D49, while the decrease is softer in the I treatment owing to the addition of nitrate that could lead to an increase in phytoplankton, resulting in more food availability for rotifers that compensates the toxic effect of copper. Copper

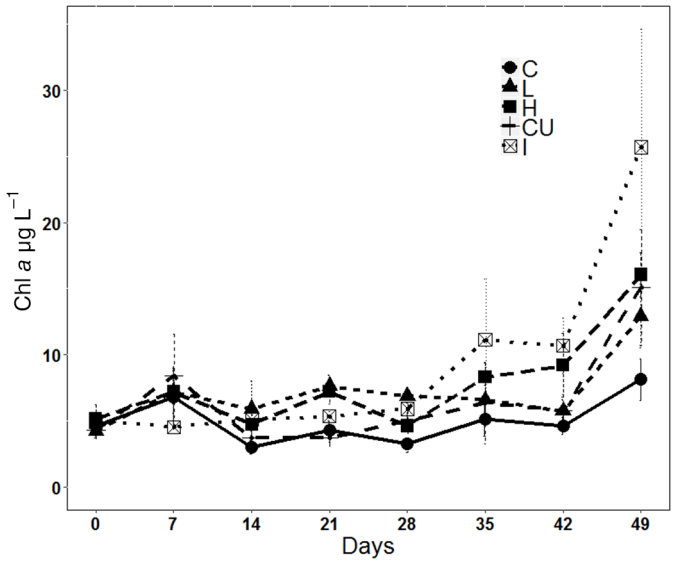

Fig. 3. Chlorophyll-*a* mean values in each treatment: C, L, H, CU and I stand for controls, low nitrate, high nitrate, copper and interaction of nitrate-copper mixture respectively (mean ± SD, $n = 5$).

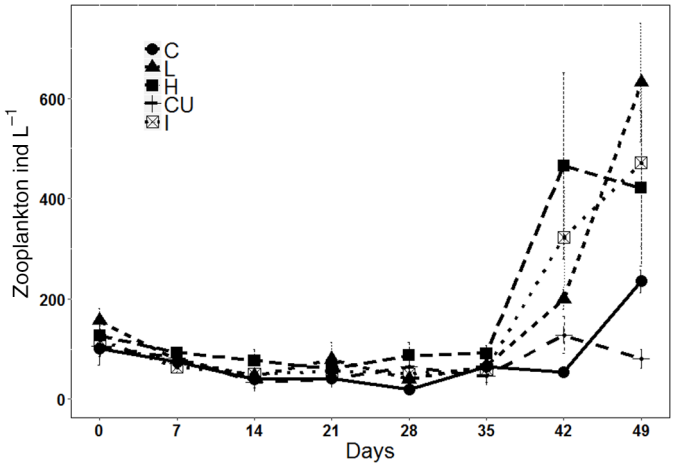

Fig. 4. Zooplankton total abundance (ind L^{-1}) values in each treatment: C, L, H, CU and I stand for controls, low nitrate, high nitrate, copper and interaction of nitrate-copper mixture respectively (mean ± SD, $n = 5$).

exposure was within legal limits (0.04 mg L^{-1}) no effect on rotifers would have been previewed; working so close to the limit of sensitivity may explain the subtle response of rotifers detected at the end of the experiment. Other studies have reported acute testing (48-h exposure) of rotifer species neonates (*Lecane hamate* and *L. quadridentata)* resulting in LC_{50} values of 0.06–0.33 mg L^{-1} (Pérez-Legaspil and Rico-Martínez, 2001). However, another study has described a lower value of LC_{50} (24-h exposure) for *Brachionus calyciflorus* of 0.02 mg L^{-1} for copper sulphate (Snell and Persoone, 1989). Additionally, the most relevant fact is that mixtures of agrochemicals may modulate the response of rotifers. Even subtle changes that may be overlooked could

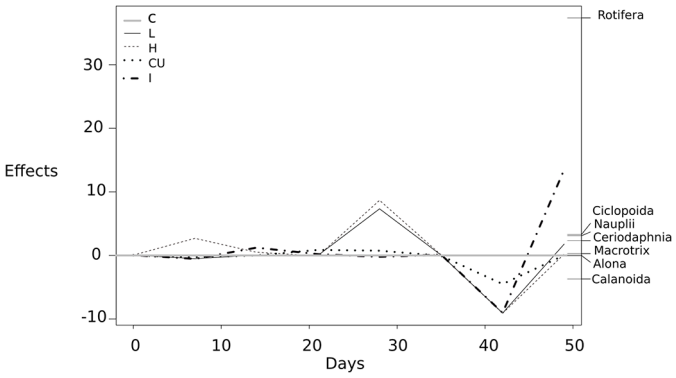

Fig. 5. Principal Response Curve (PRC): ordination method representing the main community response (y-axis, effect) to the treatment effect over time (x-axis, days) with respect to the controls (continuous line in the middle of the graph represent the C and the lines in black, red, green and blue represent L, H, CU and I treatments). The axis on the right summarizes the zooplankton community response based on its more influent taxa; it represents the species weights expressed as the level of affinity that each taxa had with the main trend of the PRC ($n = 5$).

trigger a community shift or changes in process rates in the long term, leading to impacts of higher magnitude (Scheffer *et al.*, 2008). In this respect, Zagarese (1991) and Hanazato (1998) described the consequences of zooplankton abundance and taxa shifts on the whole community structure, influencing the spring clear-water phase in lakes and fish larvae development. In addition, this community response capacity would not mean the absence of a negative effect with respect to unexposed communities, but it showed that the community response is more highly complex than expected under a mixture of chemicals because the magnitude of the effects is highly dependent on which species are changing and theirs role in the community (Fig. 5).

4 Conclusions

To sum up our general hypothesis, as expected no single toxicant effects were detected after the application of treatments because doses were within legal limits. Regarding the second part of the hypothesis, that mixture treatments would have effects on the community, no effects were detected apart from the differences between single and mixture treatments on D49. It can be argued whether 49 days are sufficient to monitor the medium-term effects of sublethal concentrations at community levels and the importance of toxicant prints at lower analytical endpoints (*i.e.* individual fitness, genetic changes) when working under these low concentrations because, independently of their concentration (low in this research), they are anthrophic-related. This experiment was motivated by the claim of using the current legal limits which are based on single species testing, and even if security factors are applied, the root of the studies may lack complexity to capture community responses (Van den Brink, 2013; De Laender and Janssen, 2013). Despite of the non-significant effects found, we support the relevance of moving

towards more complex experiments considering toxicant mixtures. Both drastic and subtle effects on communities are crucial to understand ecological consequences and for more accurate ecological risk assessment of our current legal limits.

Acknowledgement. The authors would like to thank Luciana Shigihara and Renan Santos for their support in field and laboratory work, Veronica Ferreira for the material provided for functional indicators tests and Juan Francisco García Reyes for his help with chemical analysis. We also thank the Consejería de Medio Ambiente (Junta de Andalucía) for permission to take plankton samples in Casillas pond. This research has been partly supported by a grant from the University of Jaén (Spain) to Ana Isabel Del Arco Ochoa and the research group of Ecología y Biodiversidad de Sistemas Acuáticos (RNM–300), Spain.

References

APHA. 1995. Standard methods. 19th Edition. Washington, DC: American Public Health Association.

Arndt H. 1993. Rotifers as predators on components of the microbial web (bacteria, heterotrophic flagellates, ciliates) – A review. *Hydrobiologia* 255–265: 231–246.

Boletín Oficial del Estado. 2011. Available from http://www.boe.es/boe/dias/2011/01/22/pdfs/BOE-A-2011-1139.pdf.

Camargo JA, Ward JV. 1995. Nitrate (NO_3-N) toxicity to aquatic life: A proposal of safe concentrations for two species of nearctic freshwater invertebrates. *Chemosphere* 31: 3211–3216.

Caramujo MJ, Boavida MJ. 1999. Characteristics of the reproductive cycles and development times of *Copidodiaptomus numidicus* (Copepoda: Calanoida) and *Acanthocyclops robustus* (Copepoda: Cyclopoida). *J Plankton Res* 21: 1765–1778.

Carpenter SR. 2008. Phosphorus control is critical to mitigating eutrophication. *PNAS* 105: 11039–11040.

Castela J, Ferreira V, Graça MAS. 2008. Evaluation of stream ecological integrity using litter decomposition and benthic invertebrates. *Environ Pollut* 153: 440–449.

Chandini T. 1988. Effects of different food (*Chlorella*) concentrations on the chronic toxicity of cadmium to survivorship, growth and reproduction of *Echinisca triserialis* (Crustacea: Cladocera). *Environ Pollut* 54: 139–154.

Cole JJ, Pace ML, Carpenter SR, Kitchell JF. 2000. Persistence of net heterotrophy in lakes during nutrient addition and food web manipulations. *Limnol Oceanogr* 45: 1718–1730.

Conley DJ, Markager S, Andersen J, Ellermann T, Lars MS. 2002. Coastal eutrophication and the Danish National Aquatic Monitoring and Assessment Program. *Estuaries* 25: 848–861.

Council Directive. 1980. Council Directive 80/778/EEC of 15 July 1980 relating to the quality of water intended for human consumption. [1980] L229/11.

Council Directive. 1998. Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. [1998] L330/32.

Cumming GS, Ndlovu M, Mutumi GL, Hockey PAR. 2013. Responses of an African wading bird community to resource pulses are related to foraging guild and food-web position. *Freshw Biol* 58: 79–87.

De Laender F, Janssen CR. 2013. Brief communication: The ecosystem perspective in ecotoxicology as a way forward for the ecological risk assessment of chemicals. *Integr Environ Assess Manag* 9: 34–38.

del Arco A, Ferreira V, Graça MAS. 2012. The performance of biological indicators in assessing the ecological state of streams with varying catchment urbanisation levels in Coimbra, *Portugal. Limnetica* 31: 141–154.

del Arco AI, Guerrero F, Jiménez-Gómez F, Parra G. 2014. Shifts across trophic levels as early warning signals of copper sulfate impacts in plankton communities. *Appl Ecol Environ Res* 12: 493–503.

del Arco AI, Jiménez-Gómez F, Guerrero F, Parra G. 2016. Can a copper sulphate pulse below toxic threshold change plankton communities?. *Aquat Ecosyst Health Manag* 19: 64–73.

Dokulil MT, Teubner K. 2011. Eutrophication and climate change: Present situation and future scenarios. In: Ansari AA, Gill SS, Lanza GR and Rast W (eds.), Eutrophication: Causes, consequences and control. London: Springer, pp. 1–16.

Downing AL, Leibold MA. 2010. Species richness facilitates ecosystem resilience in aquatic food webs. *Freshw Biol* 55: 2123–2137.

Duruibe JO, Ogwuegbu MOC, Egwurugwu JN. 2007. Heavy metal pollution and human biotoxic effects. *Int J Phys Sci* 2: 112–118.

Eurostat. 2017a. Agri – environmental indicator – mineral fertiliser consumption.

Eurostat. 2017b. Pesticide sales statistics.

Gama-Flores JL, Castellanos-Paez ME, Sarma SSS, Nandini S. 2007. Effect of pulsed exposure to heavy metals (copper and cadmium) on some population variables of *Brachionus calyciflorus* Pallas (Rotifera: Brachionidae: Monogononta). *Hydrobiologia* 593: 201–208.

García-Muñoz E, Guerrero F, Bicho RC, Parra G. 2011. Effects of ammonium nitrate on larval survival and growth of four Iberian amphibians. *Bull Environ Contam Toxicol* 87: 16–20.

Gessner MO, Chauvet E. 1994. Importance of stream microfungi in controlling breakdown rates of leaf litter. *Ecology* 75: 1807–1817.

Hanazato T. 1998. Response of a zooplankton community to insecticide application in experimental ponds: A review and the implications of the effects of chemicals on the structure and functioning of freshwater communities. *Environ Pollut* 101: 361–373.

Havens KE. 1994. Structural and functional responses of a freshwater plankton community to acute copper stress. *Environ Pollut* 86: 259–266.

Holt RD. 2008. Theoretical perspectives on resource pulses. *Ecology* 89: 671–681.

Jonker MJ, Svendsen C, Bedaux JJM, Bongers M, Kammenga JE. 2005. Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose-response analysis. *Environ Toxicol Chem* 24: 2701–2713.

Kasai F, Hanazato T. 1995. Effects of the triazine herbicide, simetryn, on freshwater plankton communities in experimental ponds. *Environ Pollut* 89: 197–202.

Kungolos A, Emmanouil C, Tsiridis V, Tsiropoulos N. 2009. Evaluation of toxic and interactive toxic effects of three agrochemicals and copper using a battery of microbiotests. *Sci Total Environ* 407: 4610–4615.

Lalande M, Pinel-Alloul B. 1986. Acute toxicity of cadmium, copper, mercury and zinc to *Tropocyclops prasinus mexicanus* (Cyclopoida, copepoda) from three Quebec lakes. *Environ Toxicol Chem* 5: 95–102.

Laskowski R, Bednarska AJ, Kramarz PE, Loureiro S, Scheil V, Kudłek J, Holmstrup M. 2010. Interactions between toxic chemicals and natural environmental factors – A meta-analysis and case studies. *Sci Total Environ* 408: 3763–3774.

Lenth RV. 2016. Least-Squares Means: The R Package lsmeans. *J Stat Softw* 69: 1–33.

Lydy M, Belden J, Wheelock C, Hammock B, Denton D. 2004. Challenges in regulating pesticide mixtures. *Ecol Soc* 9: 1.

Mastin BJ, Rodgers JH. 2000. Toxicity and bioavailability of copper herbicides (clearigate, cutrine-plus, and copper sulfate) to freshwater animals. *Arch Environ Contam Toxicol* 39: 445–451.

Miracle MR, Alfonso MT, Vicente E. 2007. Fish and nutrient enrichment effects on rotifers in a Mediterranean shallow lake: A mesocosm experiment. *Hydrobiologia* 593: 77–94.

Ormerod SJ, Dobson M, Hildrew AG, Townsend CR. 2010. Multiple stressors in freshwater ecosystems. *Freshw Biol* 55: 1–4.

Ortega F, Parra G, Guerrero F. 2006. Usos del suelo en las cuencas hidrográficas de los humedales del Alto Guadalquivir: Importancia de una adecuada gestión. *Limnetica* 25: 723–732.

Paerl HW, Xu H, Mccarthy MJ, Zhu G, Qin B, Li Y, Gardner WS. 2010. Controlling harmful cyanobacterial blooms in a hyper-eutrophic lake (Lake Taihu, China): The need for a dual nutrient (N & P) management strategy. *Water Res* 45: 1973–1983.

Parra G, Jiménez-Melero R, Guerrero F. 2005. Agricultural impacts on Mediterranean wetlands: The effect of pesticides on survival and hatching rates in copepods. *Ann Limnol – Int J Limnol* 41: 161–167.

Pérez J, Menéndez M, Larrañaga S, Pozo J. 2011. Inter- and intra-regional variability of leaf litter breakdown in reference headwater streams of Northern Spain: Atlantic *versus* Mediterranean Streams. *Int Rev Hydrobiol* 96: 105–117.

Pérez P, Beiras R, Fernández E. 2010. Monitoring copper toxicity in natural phytoplankton assemblages: Application of fast repetition rate fluorometry. *Ecotoxicol Environ Saf* 73: 1292–1303.

Pérez-LegaspiI A, Rico-Martínez R. 2001. Acute toxicity test on three species of the genus *Lecane* (Rotifera: Monogononta). *Hydrobiologia* 446/447: 375–381.

Pinheiro J, Bates D, DebRoy S, Sarkar D, Team RC. 2017. nlme: Linear and nonlinear mixed effects models. *R Package Version 3.1-131* 69: 18637.

Quiñones RA. 1994. A comment on the use of allometry in the study of pelagic ecosystem processes. *Scientia Marina* 58: 11–16.

Roessink I, Arts GHP, Belgers JDM, Bransen F, Maund SJ, Brock TCM. 2005. Effects of lambda-cyhalothrin in two ditch microcosm systems of different trophic status. *Environ Toxicol Chem* 24: 1684–1696.

Scheffer M, Van Nes EH, Holmgren, M, Hughes T. 2008. Pulse-driven loss of top-down control: The critical-rate hypothesis. *Ecosystems* 11: 226–237.

Schindler DW. 1977. Evolution of phosphorus limitation in lakes. *Science* 195: 260–263.

Schindler D.W. 2006. Recent advances in the understanding and management of eutrophication. *Limnol Oceanogr* 51: 356–363.

Snell TW, Persoone G. 1989. Acute toxicity bioassays using rotifers. I. A test for brackish and marine environments with *Brachionus plicatilis*. *Aquat Toxicol* 14: 65–80.

Strickland JDH, Parsons TR. 1968. A practical handbook of seawater analysis. Ottawa: Fisheries Research Board of Canada, Bulletin 167, 293 p.

Van den Brink PJ. 2013. Assessing aquatic population and community-level risks of pesticides. *Environ Toxicol Chem* 32: 972–973.

van den Brink PJ, ter Braak CJF. 1999. Principal response curves: Analysis of time-dependent multivariate responses of biological community to stress. *Environ Toxicol Chem 18:* 138, 148.

Van Wijngaarden RPA, Broc TCM, Van Den Brink PJ. 2005a. Threshold levels for effects of insecticides in freshwater ecosystems: A review. *Ecotoxicology* 14: 355–380.

Van Wijngaarden RP, Brock TCM, Douglas MT. 2005b. Effects of chlorpyrifos in freshwater model ecosystems: The influence of experimental conditions on ecotoxicological thresholds. *Pest Manag Sci* 61: 923–935.

Water Framework Directive. 2000. Water Framework Directive of the European Parliament and the Council, of 23 October 2000, establishing a framework for Community action in the field of water policy. *Off J Eur Communities* 327: 1–72.

Zafar MI, Belgers JDM, Van Wijngaarden RPA, Matser A, Van Den Brink PJ. 2012. Ecological impacts of time-variable exposure regimes to the fungicide azoxystrobin on freshwater communities in outdoor microcosms. *Ecotoxicology* 21: 1024–1038.

Zagarese HE. 1991. Planktivory by *Odontesthes bonariensis* (Atherinidae: Pisces) larvae and its effects on zooplankton community structure. *J Plankton Res* 13: 549–560.

Cite this article as: del Arco A, Guerrero F, Jiménez-Gómez F, Parra G. 2019. Plankton community responses to environmentally-relevant agrochemical mixtures. *Ann. Limnol. - Int. J. Lim.* 55: 5