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Interactive effects of fish predation and sublethal insecticide concentrations on freshwater zooplankton communities

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ABSTRACT

Stresses imposed by insecticides and predators are possibly the most rigorous filters to which aquatic organisms are exposed in rivers and lakes associated with agricultural lands. However, their interactive effects on zooplankton communities are still unclear. This study elucidated the zooplankton community response to fish predation, the insecticide chlorpyrifos (CLP), and a combination of both factors, using a 30-day mesocosm experiment. The zooplankton assemblage was influenced by fish presence prior to CLP toxicity. Fish predation reduced microcrustacean density leading to a community dominated by microzooplankton (i.e.: rotifers and copepod nauplii). CLP decreased the species richness in treatments with and without fish, yielding an increase in the abundance of bdelloid rotifers, in the genera *Lepadella* and *Trichocerca*. The zooplankton:phytoplankton (< 20 μm) ratio decreased substantially when the two stressors, fish predation and insecticide toxicity, were combined. Although CLP dissipated relatively rapidly in the aqueous phase and accumulated in sediment and fish tissue, zooplankton richness was unable to recover. A possible explanation for this could be the inhibitory effect of CLP on resting stage hatchings in the sediment. Therefore, the combined effects of fish predation and CLP might influence zooplankton richness, leading to an assemblage dominated by rotifers that appeared to be resistant to both factors, with a limited capability to control phytoplankton growth. Thus, the effects of natural and anthropogenic stressors should be considered together when assessing community dynamics in aquatic ecosystems.

1. Introduction

The use of agrochemicals has increased dramatically from the middle of the 90 s decade (Etchegoyen et al., 2017). When applied to crop fields, these products can reach water bodies (e.g., through runoff), leading to changes in biotic communities (Bourguet and Guillemaud, 2016). However, the consequences of these disturbances can differ depending on the hydrological, physical and chemical variables of each system, as well as on the presence of other anthropogenic or natural stressors (Coors and De Meester, 2008; Relyea, 2018).

Natural stress imposed by predators and pesticide toxicity are possibly the most rigorous filters to which aquatic organisms are exposed in rivers and lakes associated with agricultural lands (Relyea, 2003). Sublethal concentrations of insecticides have the potential to alter predator-prey interactions, affecting prey and predator differentially (Coors and De Meester, 2008; Pestana et al., 2009; Rodrigues et al., 2018). In addition, predation pressure has the potential to enhance (Relyea, 2003; Hanazato and Hirokawa, 2004) or reduce the toxicity of an agrochemical (Janssens et al. 2018). Zooplankton comprise a particularly susceptible community to insecticides and predation pressure, the changes of which can have a strong impact on the ecosystem through either top-down and bottom-up processes (Day, 1989; Hanazato, 1998; Hua and Relyea, 2014; Bendis and Relyea. 2016). The effects of fish predation on zooplankton have been widely studied, as well as the impact of insecticides, with both factors individually having similar direct and indirect effects on community density, composition (species replacement), evenness, size and traits (Relyea and Hoverman, 2006; Laird et al., 2007; Iglesias et al., 2011; Smith et al., 2018).

Assessments of the combined effects of both stressors are still scarce, being mostly focused on the consequences for individual species

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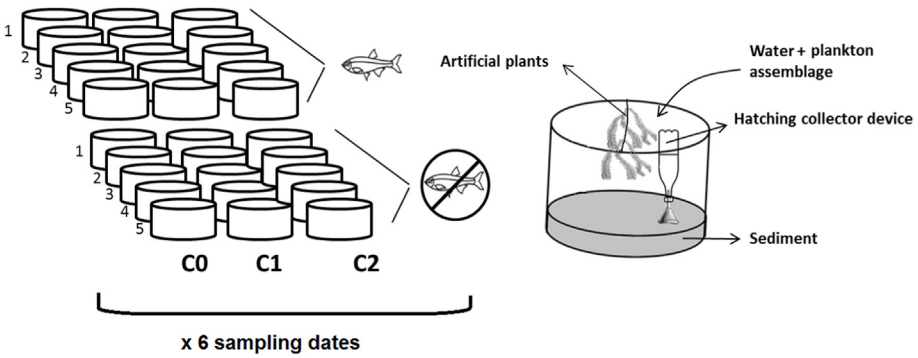

Fig. 1. Experimental design of the 30-days mesocosms experiment to test the individual and combined effects of the insecticide chlorpyrifos (CLP) and fish predation pressure on the zooplankton structure and dynamic. C0: Control; C1: 0.05 μg l^{-1} CLP; C2: 0.40 μg l^{-1} CLP.

(Hanazato, 1998; Chang et al., 2005; Campero et al., 2007; Lopez-Macisidor et al. 2008; Pestana et al., 2009; Geyer et al., 2016; Janssens et al. 2018). Therefore, there is still a gap in understanding of the relative impacts of these stressors at the community level.

The outcome of one stressor can mask, enhance or reduce the effect of the other. Thus, experimental assessments of the combined effects of both stressors becomes important for a better understanding of the magnitude of the impact and trajectories imposed on the species within a community (Mano and Tanaka, 2016).

This study evaluated the impact of fish predation pressure and an insecticide, as individual stressors and in combination, on zooplankton structure and dynamics, and their capacity to regulate phytoplankton. In addition, the capacity of zooplankton to recover from insecticide toxicity was also assessed. We hypothesized that: (1) fish predation and insecticide toxicity would influence different attributes of the zooplankton assemblages when acting individually or in combination, and that the dissipation of the insecticide in the aqueous phase would allow zooplankton assemblages to recover; and (2) the combined stress factors would act synergistically on zooplankton, reducing their capacity to regulate phytoplankton through predation.

2. Methods

2.1. Insecticide characteristics

The insecticide chosen was Clorpi® (Ciagro; Buenos Aires, Argentina), a commercial formulation based on chlorpyrifos (O,O-diethyl-O-3,5,6-trichloro-2-pyridylphosphorothioate; CLP), which is widely apply to corn, soybean and wheat crops worldwide. CLP is a hydrophobic broad-spectrum systemic phosphorothioate ester insecticide. It is ingested by organisms and also absorbed via dermic contact. It acts by phosphorylating acetyl cholinesterase both at neural synapses and in plasma (Sardar and Kole, 2005). Although the concentrations of this insecticide in natural streams can range from approximately 0.2 ng l^{-1} to 17 mg l^{-1} (Jergentz et al., 2005; Mugni et al., 2011; Bonansea et al., 2013), they could be even higher in periods of high agricultural activity and heavy rainfall. Even so, it has been demonstrated that much lower concentrations can affect zooplankton, with the calculated no observed effect concentration (NOEC) for *Daphnia magna* reported to be 0.013 μg l^{-1} (Van Wijngaarden et al., 2005). In a mesocosm experiment, CLP concentrations between 0.1 and 1.0 mg l^{-1} (applied as Chass®) decreased the abundance of different zooplankton taxa, including cladocerans (*Daphnia* group *galeata*), copepods (i.e.: cyclopoids and copepod nauplii) and rotifers (e.g.: *Keratella cochlearis*; López-Mancisidor et al., 2008).

2.2. Collection of experimental organisms

Plankton and fish were collected from a shallow lake of the Paraná River floodplain (31°37′ S, 60°41′W; Ecological Reserve of the University Campus, Santa Fe, Argentina), located in an area more than 30 km from agriculture land, so the presence of contaminants in detectable concentrations was unlikely. Zooplankton was collected with a 50 μm net and phytoplankton with a 10 μm net. They were placed in a 100 l tank with dechlorinated and continuously oxygenated water, and maintained under these laboratory conditions for one week prior to commencing the experiment.

Cnesterodon decemmaculatus (Jenyns), a small omnivorous-planktivorous fish was used as a top predator. The importance of this species as a predator of zooplankton was reported previously (Quintans et al., 2009). For acclimation purposes, collected fish (mean total length 4.3 ± 0.5 cm) were held for two weeks in containers of dechlorinated and continuously oxygenated tap water. Fish were fed once daily with zooplankton, until one day before the beginning of the experiment. Laboratory conditions were kept constant during the acclimation and experimental periods and were 20 ± 1 °C with a photoperiod of 18 h light, 6 h dark.

2.3. Experimental design

To test the individual and combined effects of fish predation pressure and the insecticide on the structure and dynamics of zooplankton, we used a completely randomized full factorial design (Fig. 1). Each experimental unit consisted of a cylindrical plastic container (38 cm diameter, 15 cm high) filled with 8 L of continuously oxygenated dechlorinated tap-water, a 1 cm thick natural sediment layer, and a phytoplankton and zooplankton assemblage. Before the distribution to each experimental unit, the phytoplankton and zooplankton assemblages were gently homogenized by transferring water from one container to another. In each container, a small plastic plant was included to provide refuge for zooplankton (Fig. 1). The sediment used for the experiment was obtained with a core (< 5 cm deep in the sediment) from the same lake used for collection of the organisms. An integrated sample of 20 sediment subsamples was obtained from the littoral and limnetic zones of the lake because the distribution of egg banks is patched in nature. This material was dried at 21 °C for 72 h in the laboratory and then stored in the dark at 4 °C for three months prior to the beginning of the experiment. This procedure was necessary to stimulate the hatching of zooplankton resting stages (Vandekerkhove et al., 2005). Finally, the stored and cooled sediments were homogeneously distributed to each experimental unit before the beginning of the experiment.

For the insecticide treatments, two CLP concentrations were used, based on concentrations observed in natural aquatic systems (Etchegoyen et al., 2017), 0.05 μg l^{-1} (C1), and 0.40 μg l^{-1} (C2), as well as controls without CLP addition (C0). For fish treatments, two concentrations were used, with fish (two individuals) and without fish. Fish density used in this experiment was similar to concentrations

observed in shallow lakes of the Middle Paraná River floodplain, including the lake from which organisms were collected (Scarabotti et al. 2011; Fleeger et al., 2003). Each treatment was replicated five times, thus a total of 30 experimental units were analysed. CLP and fish were introduced in the experiment on day 1, after the first phytoplankton and zooplankton sampling (see sampling procedure below).

Water for phytoplankton and zooplankton analyses were collected on days 1 (before fish and the insecticide addition), 2, 5, 8, 15 and 30 (n = 180 for each variable; Fig. 1). Phytoplankton samples were collected from the sub-superficial area using 50 ml bottles and immediately fixed with acidified Lugol's solution for preservation. Individual phytoplankton were counted using the Utermöhl (1958) method, and results are expressed as population density (ind. ml^{-1}). Organisms were separated into small (< 20 μm), medium (20–40 μm) and large taxa (> 40 μm) according to their maximum linear dimension (MLD; Lewis, 1976). Small group are consumable by zooplankton (Salmaso and Padisák, 2007; Kruk et al., 2016; Amorim et al., 2019).

Zooplankton samples were collected randomly from three points of each container using a tubular glass collector (30 cm long, 1.5 cm wide) and integrating the whole water column, using the method of Szlauer (1964), and preserving the volume obtained (50 ml) immediately with 10% formaldehyde and staining with erythrosine. A binocular microscope was used for quali-quantitative counting of zooplankton species. Rotifers and copepod nauplii were counted in a 1-cm^3 Sedgwick Rafter chamber due to their small size and higher abundances, and microcrustaceans in a 5-cm^3 Bogorov chamber. Taxonomic identification was conducted to the species level, whenever possible, using specific identification keys for zooplankton (Ringuelet, 1958; Koste, 1978; Pestana et al., 2009; Reid, 1985; Smirnov, 1992; Pestana et al., 2009).

To assess the number of animals contributed to the population from eggs in the sediment, we included a specific device in each container, consisting of a collector adapted from an inverted funnel placed over the sediment (Fig. 1). The design for this method was an adaptation of an *ex situ* emergence assessment method, previously used in other studies (García-Roger et al., 2005; García-Roger et al., 2008; Johansson et al, 1988). In the current study, the device in each container allowed for the monitoring of an area of about 25% of the sediment. Samples of hatched organisms were taken on days 2, 5, 8, 15 and 30, and were quantified by the same method used for the active zooplankton populations.

For nutrient (nitrate, nitrite, ammonium and soluble reactive phosphorus) analysis, 10 ml water samples were collected from the subsurface area on days 1 (before fish and insecticide addition), 5, 8 and 30. Samples were immediately filtered through 0.45 μm membrane filters, and were then kept frozen until analysed. Nitrate plus nitrite (NO_3^- + NO_2^-) levels were determined by reducing nitrate with hydrazine sulfate, and then determining the total nitrite of the solution by diazotization with sulfanilamide (Hilton and Rigg, 1983). Ammonium (NH_4^+) levels were quantified by the indophenol blue method and soluble reactive phosphorus (SRP) by the ascorbic acid method (APHA, 2005). The confidence limits of nutrient concentrations were ± 0.146 mg l^{-1} for NO_3^- + NO_2^-, ± 0.031 mg l^{-1} for NH_4^+, and ± 16 μg l^{-1} for SRP, considering a two-sided interval and 95% confidence. Water was monitored daily in all replicates over the 30 days for temperature with a standard thermometer, pH with a Hellige pH-meter, conductivity with a Hanna conductivity meter and dissolved oxygen with a Hanna oximeter.

In order to analyse the dynamics of CLP in the experimental system, water samples for CLP analyses were collected on days 1 (before fish and insecticide addition), 2, 5, 8, 15 and 30. The insecticide concentration was determined by solid phase extraction (SPE) and gas chromatography coupled to tandem mass spectrometry (GC-MS/MS). CLP was also quantified in sediment samples on days 1 (before fish and insecticide addition), 2, 5, 8, 15 and 30. The insecticide concentration in sediment was determined by solvent extraction, dispersive solid-phase extraction (dSPE) and GC-MS/MS. At the end of the experiment, fish were removed from test containers and sacrificed to evaluate the content of CLP in their muscle tissue. For this purpose, samples were purified by dSPE and measured by GC-MS/MS. All this analyses were developed in the central Laboratory of Analytical Services, Faculty of Chemical Engineering, Santa Fe, Argentina. All the methods were adapted and optimized from literature (Reference method: QuEChERS and related ones; EPA 8121, EPA 88141a). Quantification limits were 0.01 μg/l for water samples, 2 μg/kg for sediment and 10 μg/kg for fish tissue.

2.4. Statistical analysis

Zooplankton species richness, diversity and abundances were compared among all the experimental units on day 1 by one-way analysis of variance (ANOVA), to confirm that their initial values were similar at the beginning of the experiment. For the same reason, species composition was also checked using non-parametric multivariate analysis of variance (NPMANOVA).

To explore temporal and treatment effects on zooplankton species composition, non-metric multidimensional scaling (NMDS) was used, and was conducted using a Bray-Curtis similarity matrix on $\log_e$ (x) transformed relative abundances. Axe 1 and 2 in the ordination represented differences in zooplankton composition, thus, closer points were compositionally more alike than points farther away. To determine if zooplankton compositional differences observed graphically in the NMDS analysis were statistically significant, we conducted NPMANOVA. A similarity percentage procedure (SIMPER) with 9999 permutations was then performed on the Bray-Curtis triangular matrix to determine which species were potentially responsible for such differences.

Factorial repeated measure ANOVA (rm-ANOVA) was used to test the effect of fish treatment, CLP treatment, time and their interactions on 13 selected dependent variables including: zooplankton richness; Shannon-Wiener diversity; total abundance of each group (i.e.: Cladocera, Copepoda and Rotifera); proportion of small sized animals (including nauplii) with respect to total zooplankton (Microzoo:total zoo); relative abundance of the most common taxa (Daphniidae:total zoo, *Lepadella*:total zoo, *Trichocerca*:total zoo, *Lecane:*total zoo, *Brachionus:*total zoo and Bdelloidea:total zoo), and the zooplankton abundance to small phytoplankton (< 20 μm) abundance ratio (AbZoo:Abphyto). The small fraction of phytoplankton was used because it is considered the palatable group for zooplankton. This last ratio was used as an indicator of the ability of zooplankton to regulate phytoplankton population by predation. All data, except those of the initial sampling day (day 1), were used for the analyses. All statistical analyses were performed using the Past 1.76 (Hammer, Ø et al., 2001) and SPSS 18.0 software.

3. Results

3.1. Physico-chemical variables and CLP dynamics

The mean value of water pH, temperature, dissolved oxygen and conductivity remained relatively constant in all treatments during the experiment (rm-ANOVA: degrees of freedom, df = 4, p > 0.05 in all cases, Table 1). Nutrients varied through the exposure period but with a similar temporal trend in all treatments and controls (Appendix A: Fig. S1). Within the fishless group, concentrations of NH_4^+ were lower in treatments with insecticide, compared to the control (rm-ANOVA: F = 20.37, df = 4, p = 0.007; Appendix A: Fig. S1). Similarly, in the absence of fish, concentrations of SRP were lower in the CLP treatments than in the control (rm-ANOVA: F = 23.88, p = 0.01 and p = 0.005 for days 8 and 30, respectively; Appendix A: Fig. S1).

Fish accumulated 10 and 15 μg CLP kg^{-1} on average in treatments C1 and C2, respectively. The highest concentration of CLP in water was observed on day 2, while for sediment, the insecticide was detectable

Table 1
Mean values (± SD, n = 5) of pH, conductivity, water temperature and dissolved oxygen concentration (DO) in controls (C0) and CLP treatments (C1 and C2) during the experimental period.

	Without fish			With fish		
	C0	C1	C2	C0	C1	C2
pH	6.93 (0.05)	7.05 (0.04)	7.13 (0.02)	6.95 (0.09)	7.03 (0.04)	7.14 (0.02)
Conductivity (μs cm^{-1})	280.50 (11.20)	283.80 (9.40)	279.50 (5.50)	281 (8.70)	286.30 (7.27)	289.60 (10.60)
Temperature (°C)	22.50 (0.07)	22.20 (0.08)	22.30 (0.20)	22.40 (0.10)	22.30 (0.10)	22.30 (0.20)
DO (mg l^{-1})	7.47 (0.48)	6.94 (0.35)	6.93 (0.74)	7.38 (0.57)	7.54 (0.64)	6.79 (0.39)

from day 5 (Appendix B: Table S1). At the end of the experiment, CLP was only detectable in sediment and fish.

3.2. Initial plankton assemblages

At the beginning of the experiment (day 1), zooplankton species richness, diversity and abundance were similar among treatments (one-way ANOVA: F = 2.11, df = 5, p = 0.22; F = 1.6, df = 5, p = 0.27; F = 0.14, df = 5, p = 0.71; respectively). Accordingly, zooplankton composition was also the same among treatments (NPMANOVA: F = 1.21, p = 0.23). The mean zooplankton species richness was 9 (± 1) and the mean diversity index (Shannon-Wiener) was 1.7 (± 0.28) among all treatments. The mean abundances of cladocerans, rotifers and copepods were 476 (± 105), 141 (± 121) and 116 (± 96) ind. l^{-1}, respectively. The most abundant taxa characterizing the assemblage, based on mean density, were: *Ceriodaphnia cornuta* Sars, *Moina micrura* Kurz and *Alonella dadayi* Birgei for Cladocerans; *Lecane closterocerca* (Schmarda), *Lecane bulla* (Gosse) and *Lepadella patella* (Müller) for Rotifers; and nauplii larvae and Cyclopoidea copepodites for Copepods.

Phytoplankton abundance on day 1 was also similar among treatments, and averaged 14,978 (± 1300); 244 (± 120) and 394 (± 134) ind. ml^{-1} for the small green (< 20 μm), medium (20–40 μm) and large (> 40 μm) fractions, respectively (one-way ANOVA: F = 1.32, df = 5, p = 0.32; F = 3.22, df = 5, p = 0.06; F = 0.42, df = 5, p = 0.8, respectively). The whole assemblage was mainly represented by the small algae *Chlamydomonas prolifera* Skvortzov (small size class).

3.3. Effects of combined stressors on the zooplankton assemblages

Zooplankton species richness decreased in the presence of CLP, but not due to fish predation (Table 2, Fig. 2A). In fact, we found a significant positive effect of fish, but not of CLP, on Shannon-Wiener diversity (Table 2), with fish treatments producing, on average, greater diversity than fishless treatments (Fig. 2B).

Cladoceran and rotifer abundances, as well as the proportion of microzooplankton, changed due to fish presence, time, and the fish-time interaction (Table 2). Fish presence was correlated with an increase in the abundance of rotifers and microzooplankton, and a decrease in the abundance of Cladocera (Fig. 3A, B and D). Similarly, in the CLP treatments, the abundance of rotifers increased as well as the microzooplankton ratio, while cladocerans decreased by the end of the experimental period (Fig. 3A, B and D). The abundance of copepods varied with time and the interaction of CLP and fish (Fig. 3C, Table 2).

NMDS analysis indicated that each treatment triggered different species trajectories over time, depending on the presence or absence of fish and CLP (Fig. 4). However, in fishless treatments, the trajectories differed in the latter stages of the experiment, while in treatments with fish, they differed earlier (Fig. 4). These temporal differences were confirmed by NPMANOVA, which indicated that for fishless groups no significant differences in species composition were observed between CLP treatments until day 30 (F = 2.20, p = 0.0104; overall percentage of dissimilarity 62.3%). The species composition in the fish treatments showed statistically significant differences on day 8 (F = 2.23, p = 0.006; overall percentage of dissimilarity 35.18%) and on day 30 (F = 1.53, p = 0.04; overall percentage of dissimilarity 43.0%). SIMPER analysis showed that the differences between treatments with and without CLP in the presence of fish were represented by nauplii, *Lepadella ovalis* (Müller), *Testudinella patina* (Hermann), *Squatinella mutica* (Ehrenberg), *Lecane quadridentata* (Ehrenberg) and *Colurella obtusa* (Gosse), with a cumulative contribution of 84.0%. The main taxa responsible for the differences between treatments with and without CLP in the absence of fish were nauplii, Cyclopoidea, Harpacticoidea, *Ceriodaphnia cornuta*, *L. ovalis*, *T. patina* and *Mytilina mucronata* (Müller), with a cumulative contribution of 84%.

The most common zooplankton taxa were differentially affected by the treatments, time and their interactions. Fish presence and time alone, and in combination, were significantly correlated with a

Table 2
F and p values of the rm-ANOVAs comparing the effect of fish treatment, CLP treatment, time and their interactions on 13 selected traits (zooplankton indicators): zooplankton richness (Richness), Diversity index (Shannon-Wienner), proportion of small size animals including Copepoda nauplii (Microzoo:total zoo), abundances of each group (Cladocera, Copepoda, Rotifera), abundance of the most representative taxa (Daphniidae; *Lepadella*, *Trichocerca*, Bdelloidea; *Lecane*, *Brachionus*), and zooplankton to small phytoplankton ratios (AbZoo:AbPhyto).

	Fish		CLP		Fish xCLP		Time		Time x Fish		Time xCLP		Time x Fish xCLP	
	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
Richness	0.26	0.61	4.75	0.02	0.18	0.85	22.45	< 0.01	14.5	< 0.01	2.60	0.01	1.45	0.18
Diversity (Shannon)	4.40	0.05	0.16	0.85	0.17	0.84	22.15	< 0.01	3.22	0.01	2.09	0.03	2.80	< 0.01
Microzoo/total zoo	150.18	< 0.01	1.11	0.35	2.20	0.13	24.85	< 0.01	24.14	< 0.01	4.48	< 0.01	1.85	0.05
Cladocera	378.16	< 0.01	0.49	0.62	0.53	0.59	22.05	< 0.01	28.62	< 0.01	3.32	0.01	2.95	< 0.01
Copepoda	0.09	0.77	1.70	0.20	4.78	0.02	14.00	< 0.01	14.,00	< 0.01	30.00	0.01	30.00	0.02
Rotifera	682.57	< 0.01	3.11	0.07	1.72	0.20	71.26	< 0.01	37.22	< 0.01	3.30	0.01	4.19	< 0.01
Daphniidae	624.04	< 0.01	0.51	0.61	0.50	0.61	19.25	< 0.01	28.65	< 0.01	4.14	< 0.01	4.85	< 0.01
Lepadella	2.85	0.11	4.19	0.03	0.29	0.74	9.28	< 0.01	1.76	0.14	2.62	0.14	0.62	0.75
Trichocerca	1.20	0.32	5.53	0.04	2.51	0.11	2.23	0.07	3.71	< 0.01	2.08	0.93	1.78	0.09
Bdelloidea	15.10	0.01	4 14	0.05	0.19	0.82	9.66	< 0.01	10.72	< 0.01	2.01	< 0.01	2.43	< 0.01
Lecane	66.01	< 0.01	1.69	0.21	3.22	0.06	13.17	< 0.01	17.92	< 0.01	2.71	< 0.01	2.40	0.01
Brachionus	9.66	0.06	5.76	0.04	4.45	0.03	2.79	0.02	2.50	0.03	3.31	0.01	1.83	0.06
AbZoo:AbPhyto	13.94	0.03	12.89	< 0.01	7.87	0.01	7.03	< 0.01	12.5	< 0.01	8.37	< 0.01	10.05	< 0.01

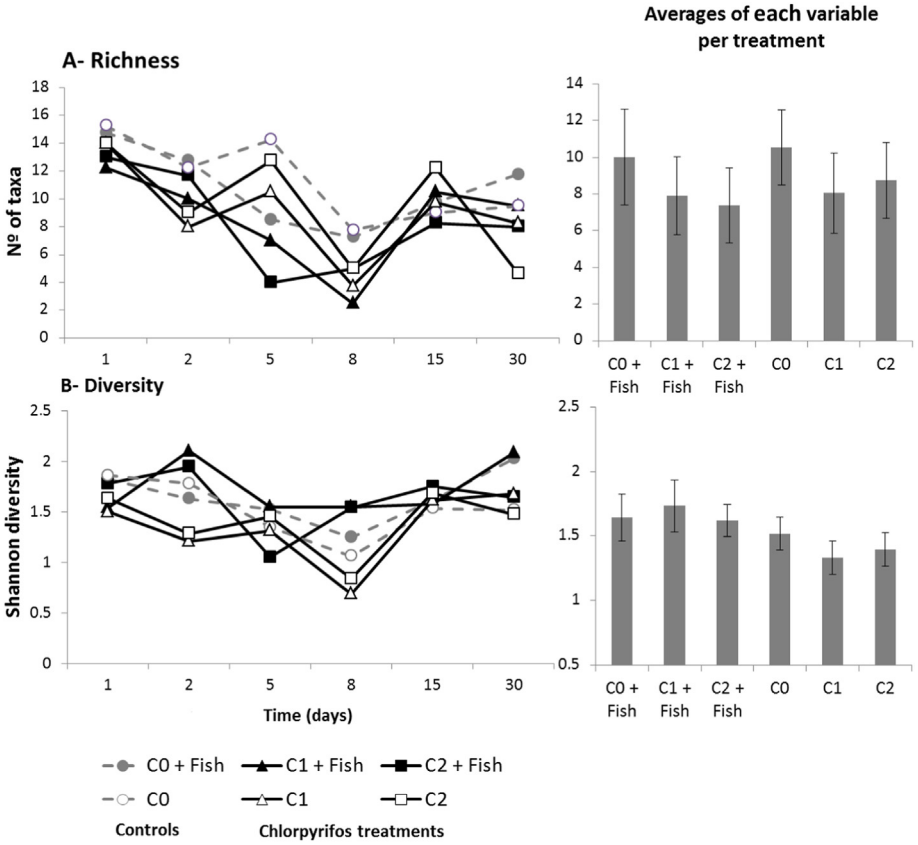

Fig. 2. Changes in the species richness (A) and diversity index (B) through the time (left panels) and their mean values at the end of the experiment (right panels). Vertical bars indicate standard deviation (± SD).

decrease in the relative abundance of cladocerans (mainly represented by *Ceriodaphnia cornuta*) and *Lecane* spp. (Table 2). CLP had an individual effect on *Lepadella* spp. *Trichocerca* spp. Bdelloidea and *Brachionus* spp. (Table 2). By contrast, the relative abundances of *Lepadella* sp. and *Trichocerca* sp. significantly increased in CLP treatments with respect to the controls, while fish presence demonstrated no significant effect (Table 2). Only bdelloid rotifers were significantly affected by fish, CLP and time, alone, and in combination (Table 2).

At the end of the experiment, only rotifers were determined to be hatching from sediment, (29 ± 14 individuals hatched in control, and 16 ± 6 individuals hatched in CLP treatments). The dominant rotifer taxa hatched were *Collurella* sp., *Lecane* spp. and *Lepadella* spp. Hatching in the control contributed significantly, with more species to the water column (21 ± 4), compared to hatching in CLP treatments (14 ± 3; one-way ANOVA: df = 5; $p < 0.05$). Finally, the zooplankton to phytoplankton ratios were reduced by the two stressors, fish and CLP, acting alone and in combination over the time (Table 2). By contrast, a significant decrease in the AbZoo:Abphyto ratio was only observed in treatments with the highest CLP concentration when fish were present (Fig. 5).

4. Discussion

Our hypothesis is that CLP and fish predation alone or in combination have differential effects on zooplankton community. Fish presence resulted in an early reduction of cladoceran abundance and an increase in the proportion of microzooplankton (rotifer and copepod nauplii) in relation to macrozooplankton. This result reflected the higher vulnerability of larger individuals (like cladocerans) to fish size-selective predation, and was consistent with previous studies which indicated that fish predation can cause shift in the size distribution of zooplankton toward small sized individuals (Brooks and Dodson, 1965; Scasso et al., 2001; Boveri and Quirós, 2007; Havens et al., 2009).

Although CLP at the concentrations evaluated did not appear to have an early effect on the abundance of Cladocera and Rotifera, as occurred with fish presence, it resulted in a general decrease in species richness in all treatments to the end of the experiment. This result was consistent with previous studies in which direct effects of insecticides were correlated with substantial species decline (Hanazato, 2001; Relyea and Hoverman, 2008). The reduced richness of the active zooplankton assemblage in the current study could be exacerbated by the lower richness found in hatchings from sediment. Previous studies have also documented a similar inhibitory effect on egg hatching due to insecticide pollution (Gutierrez et al., 2017), suggesting that the inhibition of egg banks deserves more attention when evaluating the effects of insecticide on active communities. Given that species richness is intimately related to ecosystem functions, such as primary production, energy flow and nutrient cycling, its decrease in nature might have direct consequences on ecosystem services (Clements and Rohr, 2009). Thus, species richness should be prioritized in evaluating the environmental integrity of aquatic systems.

CLP also correlated with an increase in microzooplankton and a reduction in cladocerans at the end of the experimental period. The decrease in cladocerans might have favoured the increase in rotifers, which were more tolerant to CLP, as previously demonstrated for other xenobiotics such as malathion and carbaryl (Hanazato and Yasuno, 1990; Relyea et al., 2005). Consistent with this, field studies found similar effects on species replacement after the application of CLP and other related insecticides (Kaushik et al., 1985; Chang et al., 2005; Smith et al., 2018).

Another key finding of the current study was that both stressors (i.e.: fish predation and toxicity) had differential effects on zooplankton composition. Compositional changes were observed earlier due to the presence of fish compared to CLP toxicity, which is not supported by the

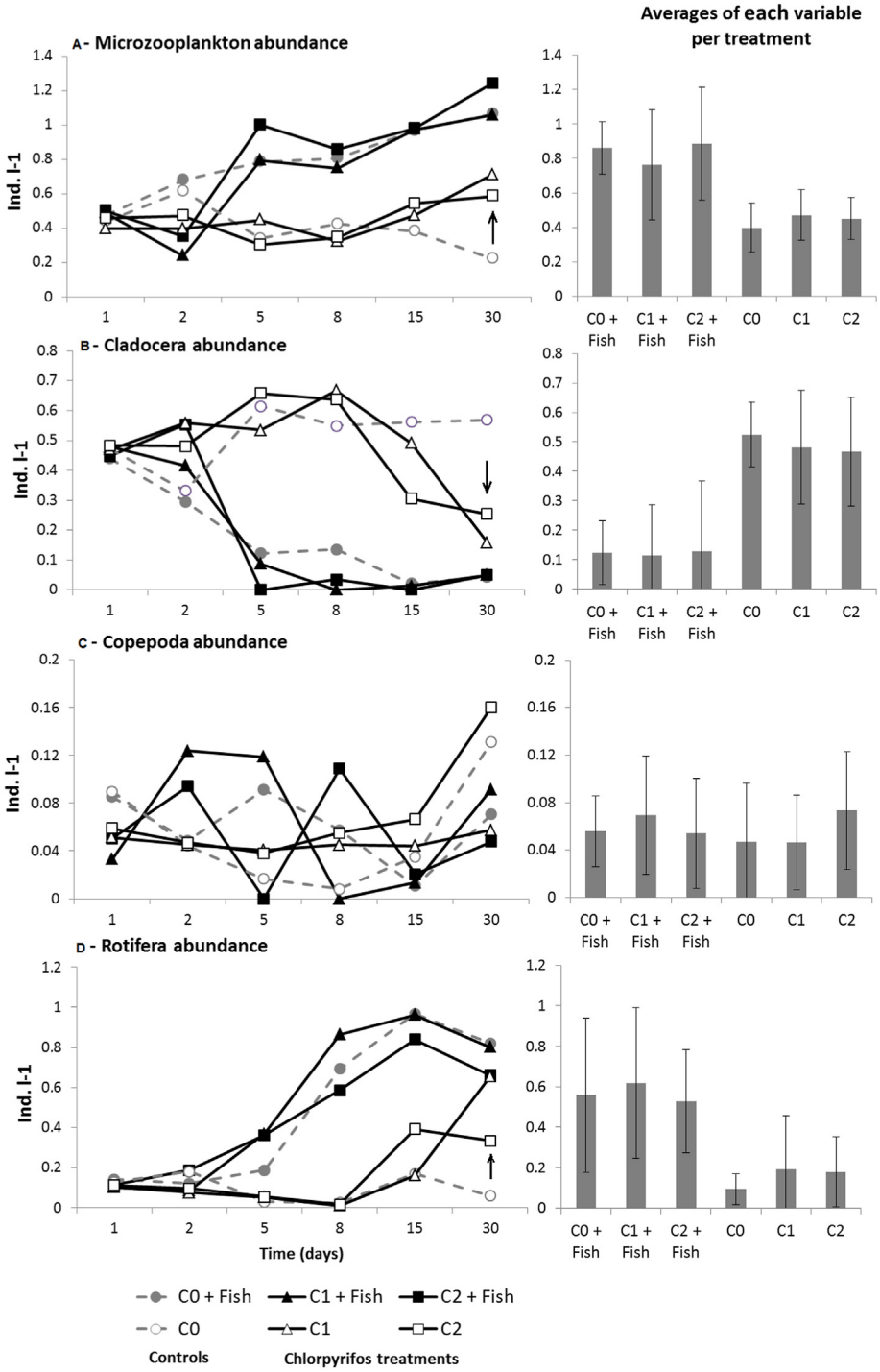

Fig. 3. Changes in the microzooplankton density (A), and absolute abundances of cladocerans (B), copepods (C) and rotifers (D) through the time (left panels) and their mean values at the end of the experiment (right panels). Arrows indicate significant differences of treatments with CLP (C1 and C2) from controls (C0) according to rm-ANOVA ($p < 0.05$). Vertical bars indicate standard deviation (± SD).

findings in other studies. For instance, Chang et al. (2005), who studied the effect of carbaryl on a zooplankton assemblage in a small-scale mesocosm, reported that the presence of predators affected the species succession that remained after insecticide application. In the current study, CLP toxicity was observed later than the effect of fish predation. In other words, fish predation quickly changed the initial zooplankton community to one dominated by rotifers and nauplii (due to size-selective consumption), while CLP caused a shift of this resulting microzooplankton assemblage.

By contrast, the fishless treatments had a relatively equal proportion of cladocerans, copepods and rotifers throughout the first two weeks of the experiment. CLP resulted in changes in the three groups from the third week, with cyclopoids, harpacticoids, *C. cornuta*, *L. ovalis*, *T. patina* and *M. mucronata* being the most affected taxa in terms of density reduction. At the end of the experiment with CLP treatment, cladoceran members were reduced, while Cyclopoid copepods and rotifers increased, possibly because of their higher tolerance to the insecticide compared to cladocerans (Gliwicz and Sieniawska, 1986; Hanazato, 1991; Relyea and Hoverman, 2008). These results were consistent with other micro- and mesocosm studies in which pesticides, including

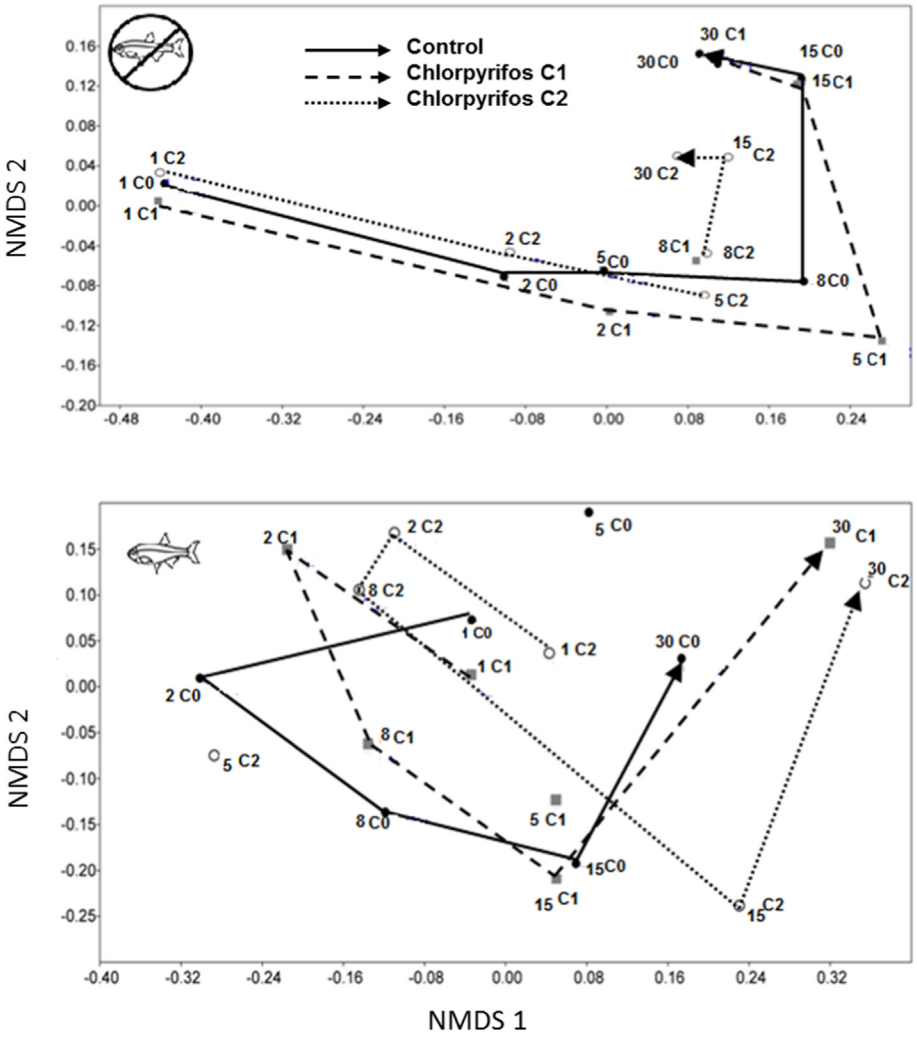

Fig. 4. Composition trajectory of the zooplankton in treatments without fish (upper panel) and in treatments with fish presence (lower panel) throughout the experiment. The inflection points represent the sampled days (e.g.: 1 C0: Day 1 for control; 1 C2: Day1 for treatment 2). For the fishless treatments (upper panel), the stress of the final NMDS analysis was 15.2%, and the percent variance explained by axes 1 and 2 is 60% and 16.4%, respectively. For treatment with fish (lower panel) the stress of the final NMDS analysis was 12.04%. The percent variance explained by axes 1 and 2 is 72.7% and 18.6%, respectively. Note that all three treatments clustered together on day 1, indicating that the treatments were compositionally similar at the starting point.

chlorpyrifos, resulted in the structure of zooplankton towards a community dominated by copepods and rotifers (Yasuno et al., 1988; Wendt-Rasch et al., 2003; Downing et al., 2008; López-Mancisidor et al., 2008).

The observed differential effects of fish predation, CLP toxicity and their combination on zooplankton composition were consistent with previous studies analyzing other combined stressors. These findings concurred with a growing body of research indicating that species exposed to combined stressors could be more sensitised and have less resilience than those exposed to single stressors (Hanazato and Yasuno 1989; Yan et al., 2004; Campero et al., 2007). The differential response of species to each stressor may be related to different physiological and behavioural defense mechanisms (Campero et al., 2007). Moreover, the interaction of stressors may produce outcomes not observed when stressors act individually. The decrease in zooplankton to small phytoplankton ratio (AbZoo:Abphyto), which was only observed in the group exposed to both fish and CLP, suggest that top-down effect with important consequences for ecosystem balance could emerge from the combination of both stressors.

The presence of CLP in the fish treatments resulted in an increase of most abundant taxa *Lepadella* spp., *Trichocerca* spp. and Bdelloidea spp. The increase of *Lepadella* in the water column was probably due to an increase in the emergence of resting eggs present in the sediment, given that this taxa accounted for the highest number of individuals emerging from the sediment. By contrast, the increase in the other two taxa, *Trichocerca* and Bdelloidea, might be related to other mechanisms, such as an increased reproduction rate favoured by an absence of competitors and a higher tolerance to CLP; however, this hypothesis requires more testing. On the other hand, the genera *Lecane* and *Ceriodaphnia* were not sensitive to the toxic effect of the insecticide, within the range of concentrations tested.

Despite the relatively rapid translocation of CLP from the water to the sediment and fish tissue, a full recovery of the zooplankton community was not observed. In fact, the zooplankton species composition changed over the time displaying different trajectories on the different treatment and failed to be homogeneous again (i.e.: similar in all experimental units). This result may indicate that the ecological effects of the insecticide may be persistent for at least one month. A possible explanation for this would be related to the presence of secondary metabolites in the system, such as TCP (3,5,6-trichloro-2-pyridinol). Although these metabolites were not measured in the current study, their presence might have indirectly affected the organisms present in the assemblage.

Finally, our results suggest that CLP could cause long-term effects by modifying the availability of nutrients. The lower NH_4^+ and SRP concentrations in the CLP fishless treatments would indicate a decrease in nutrient release from the bottom sediment to the water column due to the adverse effects of CLP on microorganisms (Ward, 1996; Das and Mukherjee, 2000). Although the reason that these effects were not observed in the CLP treatments in which fish were present was not determined, nutrient excretion by fish may have compensated for the CLP effects on N and P transformation. As the energy demanded by fish increases under toxic stress (Kumar et al. 2015), CLP could have increased fish metabolism and favoured the excretion of ammonia and

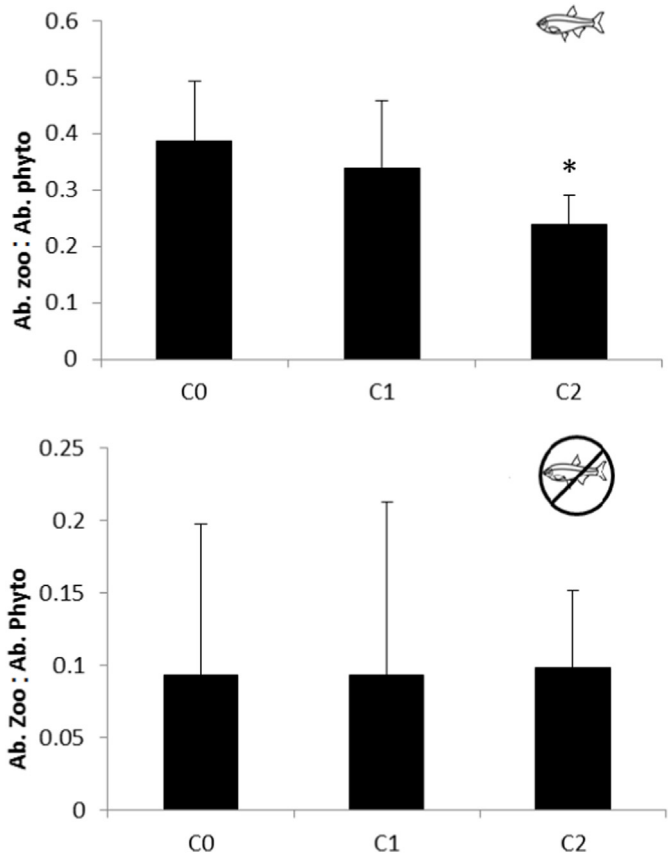

Fig. 5. Mean values of zooplankton to small phytoplankton ratio (AbZoo:Abphyto) in controls (C0) and CLP treatments (C1 and C2) in the presence (upper panel) and absence of fish (lower panel). Asterisks represent significant differences of C1 and C2 from C0 according to rm-ANOVA ($p < 0.05$). Vertical bars indicate standard deviation (± SD).

phosphate (Torres and Vanni, 2007).

In summary, we observed that fish predation, the insecticide CLP and a combination of both stress factors resulted in differential effects on zooplankton dynamics. In contrast to previous studies, the presence of fish influenced the zooplankton assemblage earlier than CLP toxicity. The mere presence of fish caused a rapid shift in zooplankton by reducing the abundance of large individuals, due to size-selective predation. CLP decreased species richness, possibly due to a selective toxic effect on sensitive species and through a general inhibitory effect on egg hatching from sediment. The combined effect of both stress factors resulted in a shift in zooplankton composition towards an increased proportion of microzooplankton, exerting a potentially synergistic effect on the consumption rate of small phytoplankton. Our study highlights the importance of considering multiple natural and anthropogenic stressors when evaluating the ecological dynamics of natural aquatic communities.

Credit author statements

María Florencia Gutierrez: provided the idea of the work, designed the experiment, processed zooplankton samples, made statistical analyses and wrote the manuscript. Florencia Rojas Molina: designed the experiment, processed zooplankton samples and contributed with the manuscript writting Diego Frau: processed the phytoplankton samples, contributed with statistical analyses and revised the manuscript. Gisela Mayora: processed the nutrient analyses and revised the manuscript. Yamila Battauz: processed the zooplankton hatchings from egg bank and revised the manuscript. All authors contributed with the experimental set up.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.110497.

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