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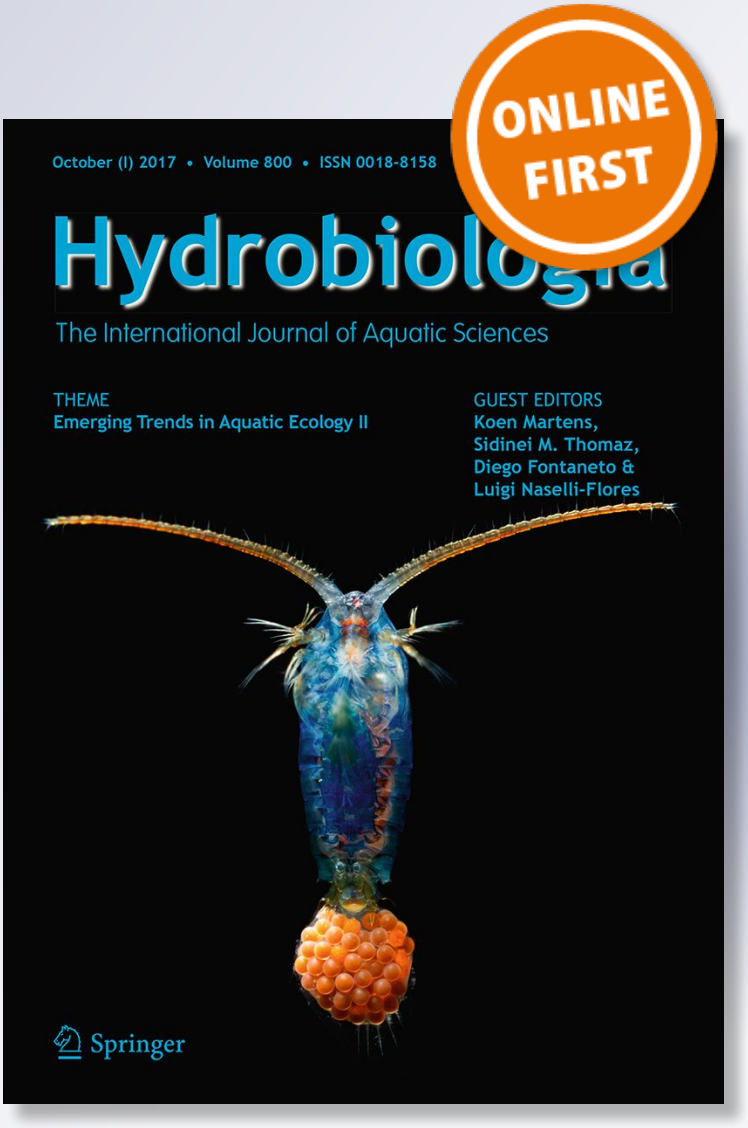

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PRIMARY RESEARCH PAPER

Approaches for phosphorus removal with calcium hydroxide and floating macrophytes in a mesocosm experiment: impacts on plankton structure

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Abstract Cultural eutrophication has promoted the application of several mitigation strategies in the last 50 years. In this study we tested the combined effects of two techniques: calcium hydroxide [($Ca(OH)_2$), lime] and a free-floating macrophyte (*Salvinia rotundifolia* Willd) to examine the soluble reactive phosphorus removal capability and the effects on plankton (phytoplankton and zooplankton) structure in a in situ lake mesocosms experiment. The experiment lasted 10 days (n = 12, 500 l each) with a control and three treatments (lime (CH), plants (FM), and the combination of both (CH + FM)). Samples of several physical and chemical variables (including nutrients) and phytoplankton were taken at the beginning, 2 days after, 4 days, and 10 days (end of the experiment). Zooplankton was sampled at the beginning and at the end. The highest depletion effect of soluble reactive phosphorus (SRP) was observed in presence of lime. Phytoplankton biovolume was highly and negatively affected in lime treatments (CH and CH + FM). Zooplankton changed from Rotifera to Cladocera and Copepoda in presence of macrophytes. We conclude that lime + plants reduces more effectively SRP, phytoplankton biovolume and promotes herbivorous zooplankton development; becoming by this way, in a suitable mitigation strategy to be explored in future field manipulation studies.

Keywords Cyanobacteria · Eutrophication · Mitigation strategies · Floating macrophytes · Calcium hydroxide

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Introduction

As Cyanobacterial Harmful Algal Blooms (Cyano-HABs) proliferation is among the most threatening consequences of freshwater eutrophication (Rastogi et al., 2015), a large amount of physical, chemical and biotic approaches has been proposed, implemented

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and evaluated in the last 50 years to reduce eutrophication and CyanoHABs events (Ibelings et al., 2016; Paerl et al., 2016). In comparison with well-documented research in temperate lakes, fewer studies have been done to explore the potential of using biotic and abiotic methods in eutrophic subtropical/tropical lakes (Zhang et al., 2001; Huser et al., 2016; Liu et al., 2018); suggesting evidence that this kind of environments may respond differently to the same treatments proposed in temperate systems by differences in environmental conditions (Jeppesen et al., 2005).

Nowadays, the prevailing technologies used to remove phosphorus in water systems may be too costly and sometimes non-ecofriendly. Therefore, research is oriented towards developing and proving low-cost and sustainable technology for nutrient removal (Dhote & Dixit, 2009). In this study, two main mitigation strategies for eutrophication are discussed: the use of macrophytes (Dhote & Dixit, 2009; Bakker et al., 2013) and the use of flocculants and modified clays (Jančula & Maršálek, 2011; Copetti et al., 2016); both tested alone and in combination to remove phosphorus from the water column and make it inaccessible for phytoplankton growth.

Macrophytes have a highly positive influence on eutrophic aquatic systems by capturing nutrients, constituting a refuge for zooplankton, competing for light with microalgae, and producing allelopathic substances (Meerhoff et al., 2003). Several experiments have tested the use of macrophytes for restoring eutrophic waters (Gulati et al., 2008), yet its effects on biotic compartments, such as zooplankton or non-toxic phytoplankton, have not been deeply studied (Zhang et al., 2001). On the other hand, the use of modified clays or flocculants is based on their capability to react preferentially with free phosphate compounds in water, and rapidly form phosphate minerals which get trapped in the sediments. These reactions are frequently redox dependent (making them reversible in anoxic conditions), or are sensitive to pH changes, hence affecting their removal efficiency (e.g., Copetti et al., 2016). Moreover, the effects of clays or flocculants on biotic compartments are not frequently considered (e.g., Luengo et al., 2017; Wang et al., 2017) leading to uncertainties about their potential effects on living organisms. In this study, we aimed to test two restoration techniques (calcium hydroxide and floating macrophytes) to examine the soluble reactive phosphorus removal capability, analyzing also their effects on plankton (phytoplankton and zooplankton) structure in a in situ lake mesocosms experiment.

Materials and methods

Experimental set-up

A field experiment was performed during the summer season of 2015 (from November 30th to December 10th) in 12 mesocosms of 500 l in a eutrophic shallow urban lake (the Quillá Lake, Santa Fe, Argentina). Each mesocosm consisted of a polyethylene (150 µm thick) bag cylinder of 80 cm in diameter and 100 cm height, attached to a circular floating ring. All mesocosms were filled with 1 kg of the lake's natural sediment, being added four individuals of *Jenynsia lineata* Jenyns (the most abundant planktivorous fish in the lake) on each vessel. The fish density used (4 individuals per treatment) was in accordance with density values observed in the field (Frau et al., unpublished data). A control (with only fish and sediments) and three treatments with three replicas each were assigned ($n = 12$): calcium hydroxide (lime) (CH) with 200 g m^{-2} of $Ca(OH)_2$ per treatment, floating macrophytes (*Salvinia rotundifolia* Willd) (FM) with 25% of coverage at the beginning of the experiment and lime + plants (CH + FM) (Fig. 1).

Samplings and laboratory analyses

Samples of phytoplankton and physical–chemical variables were done at T_0 (the beginning of the experiment), T_2 (2 days after), T_4 (4 days), and T_{10} (10 days). Zooplankton sampling was done at the beginning (T_0) and at the end of the experiment (T_{10}). We chose this sampling frequency due to the large volume of water needed for sampling (30 l per sampling). Nutrients measured in the study were ammonium, nitrites + nitrates and soluble reactive phosphorus (SRP) and were taken using 100 ml bottles for each one. The quantification was done in the laboratory following the methods indicated in APHA (2005). Nutrient concentrations were expressed as µg l^{-1}. Nitrite + nitrate + ammonium concentration was considered as dissolved inorganic

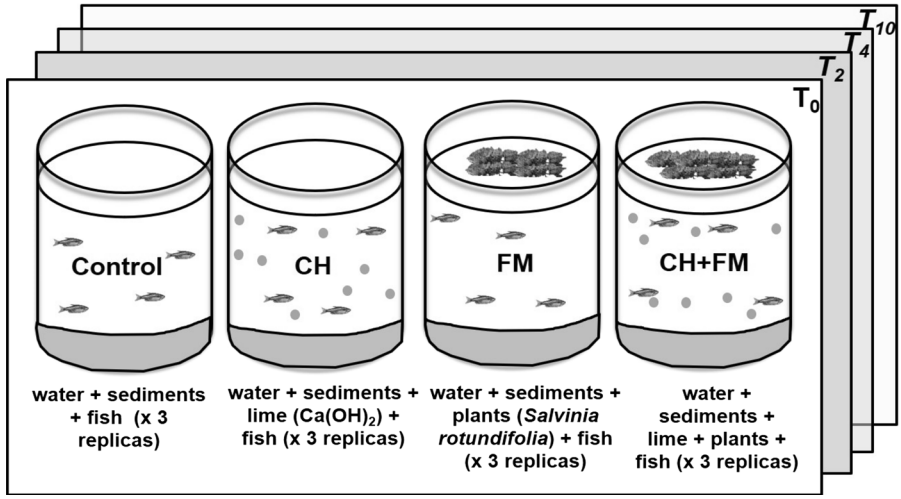

Fig. 1 Experimental design: Control, calcium hydroxide (CH), floating macrophytes (FM), and calcium hydroxide plus floating macrophytes (CH + FM) at sampling T_0 (beginning of the experiment), T_2 (2 days after), T_4 (4 days after), and T_{10} (10 days after)

nitrogen (DIN) for the rest of analyses. Several physical–chemical variables were taken in situ: water temperature (°C), pH, conductivity (mS cm^{-1}), and dissolved oxygen (mg l^{-1}) using HANNA multiparametric probes, depth (to control the volume of water on the mesocosms) with an ultrasonic prove (m), and transparency with a Secchi disk (m). Phytoplankton samples were taken from the subsurface and immediately fixed with 1% acidified Lugol solution. Samples were counted according to Utermöhl (1958) and density was expressed as ind ml^{-1}. Phytoplankton biovolume was estimated measuring at least 10 individuals of each taxon and following the formulas and criteria proposed by Hillebrand et al. (1999). The values obtained were expressed as mm^3 l^{-1}. Taxonomic classification was made according to Lee (2008) following keys and specific bibliography of each algal group, such as Komárek & Fott (1983), Tell & Conforti (1986), Krammer & Lange-Bertalot (1991), Zalocar de Domitrovic & Maidana (1997), and Komárek & Anagnostidis (1999, 2005), among other authors and recent revisions. Finally, all phytoplankton taxa were classified according to the morpho-functional classification of Salmaso & Padisák (2007) (MFGs). This classification is based on Weithoff (2003) who discriminates groups by: motility, potential capacity to obtain carbon and nutrients by mixotrophy, specific nutrient requirements, size, shape, and presence of gelatinous envelopes. Analyses were performed with those MFGs which represented ≥ 2% of total phytoplankton biovolume at the beginning of the experiment. For zooplankton density estimation 30 l of water were filtered through a net of a 55 µm pore. Samples were immediately fixed with 10% formalin + erythrosine solution. Microzooplankton (Rotifera and Copepoda nauplii) counting was carried out with an optical microscope in a Sedgwick Rafter chamber of 1 ml. The counts of the macrozooplankton (Cladocera and Copepoda) were done in a Bogorov chamber (5 ml). A minimum of 100 individuals were counted in each sample (José de Paggi & Paggi, 1995). Zooplankton taxonomic identifications were based on Korovchinski (1992), Alekseev (2002) and Korínek (2002), among others.

Statistical analyses

A two-way repeated measures (RM) ANOVA was applied for assessing the effect of the different mitigation techniques (inter-subjects factor) and time (intra-subjects factor) on nutrients concentration (SRP and DIN) and phytoplankton total and morpho-functional groups. When the RM ANOVA results were significant ($P < 0.05$), post hoc analyses were performed. Tukey test was applied to make comparisons among control and treatments (CH, FM, and CH + FM), while Bonferroni test was used to compare sampling dates (T_0, T_2, T_4, and T_{10}). Environmental variables were analyzed by using a randomized complete block designs ANOVA (RCB ANOVA)

where experimental units were grouped into blocks (sampling dates) according to known or suspected variation which is isolated by the blocks. Zooplankton was analyzed using a one-way ANOVA comparing control and each treatment at the beginning (T_0) and at the end of the experiment (T_{10}) by using Tukey test for post hoc comparisons. In all cases, the data were $\log_{10}(x + 1)$ transformed to guarantee the application of parametric tests (Zar, 2010). Normality and homoscedasticity of data were previously tested for all data sets analyzed. Changes in species composition (phytoplankton and zooplankton) were considered at the beginning (T_0) and at the end of the experiment (T_{10}) using the Shannon test and the equitability test. All statistical analyses were performed with PAST software v. 3.1 (Hammer et al., 2001).

Results

Environmental variables

Throughout the experiment, pH showed slightly higher values in treatments with lime (pH > 9, but less than 10), the dissolved oxygen concentration showed lower values in the treatment with plants (DO < 8, but higher than 6 mg l^{-1}) and the Secchi disk was higher in the treatment with lime (> 15% than the rest of treatments) (Table 1). These differences were confirmed by the RCB ANOVA for pH ($F = 24,\ 1\ P < 0.001$) between control and lime treatments (CH and CH + FM) ($P < 0.001$ for both, control < CH and control < CH + FM). For dissolved oxygen concentration ($F = 1.73\ P < 0.001$) for control and plants (FM) ($P = 0.04$, C > FM), and Secchi disk ($F = 8.47\ P < 0.001$) between control and lime treatment (CH) ($P < 0.001$, C < CH). Non-differences were found for temperature ($F = 2.01$ $P = 0.11$), depth ($F = 2.53$ $P = 0.07$) and conductivity ($F = 1.73$ $P = 0.17$).

Nutrient concentration variations

The one-way ANOVA performed at the beginning of the experiment showed similar concentrations of soluble reactive phosphorus (SRP) and dissolved inorganic nitrogen (DIN) among control and treatments ($F = 1.05$ $P = 0.45$ for SRP and $F = 1.14$ $P = 0.753$ for DIN). The soluble reactive phosphorus (SRP) at the beginning of the experiment was of 259.4 ± 56.1 μg l^{-1}, but markedly dropped at T_2 in all treatments. However, this drop was more evident at T_2 in those treatments with lime with percentage values reductions of 43% in CH and 66% in CH + FM in comparison with control. In the treatment with macrophytes, a 37% SRP drop compared to control was observed. The most noticeable SRP drop was observed at T_4 when concentrations dropped in the lime treatments (CH: 62% and CH + FM: 96%) compared with control. In the FM treatment an increment of 45% in comparison with control was observed. At the end of the experiment (T_{10}), SRP concentrations in the treatments with lime (CH and CH + FM treatments) were lower than in control. Drops of 70% respect to control in CH treatment, 16% in FM treatment and 66% in CH + FM treatment were observed (Fig. 2a).

The RM ANOVA excluding T_0 data (due to the high variation found between T_0 and T_2) showed differences in SRP for the effect of time ($F = 51.83$

Table 1 Mean environmental (± standard deviation) values throughout the experiment in the control and the three treatments: calcium hydroxide (CH), floating macrophytes (FM), and calcium hydroxide plus floating macrophytes (CH + FM)

	Control	CH	FM	CH + FM	Total exp.
Temperature (°C)	26.4 ± 0.3	26.6 ± 0.0	26.5 ± 0.1	26.6 ± 0.2	26.7 ± 0.7
pH	8.1 ± 0.1	9.6 ± 0.2	8.2 ± 0.0	9.1 ± 0.1	8.9 ± 0.9
Conductivity (mS cm^{-1})	2.6 ± 0.0	2.5 ± 0.0	2.5 ± 0.0	2.5 ± 0.1	2.8 ± 0.4
DO (mg l^{-1})	9.0 ± 1.3	9.7 ± 0.6	7.8 ± 0.3	8.7 ± 0.5	9.8 ± 2.7
Depth (cm)	75.7 ± 16.9	85.2 ± 2.6	80.2 ± 4.4	81.6 ± 2.0	73.1 ± 14.5
Secchi Depth (cm)	47.7 ± 0.9	69.1 ± 2.3	50.7 ± 1.6	61.0 ± 13.2	53.5 ± 18.7

It is also shown the total mean and standard deviation for each variable during the experiment

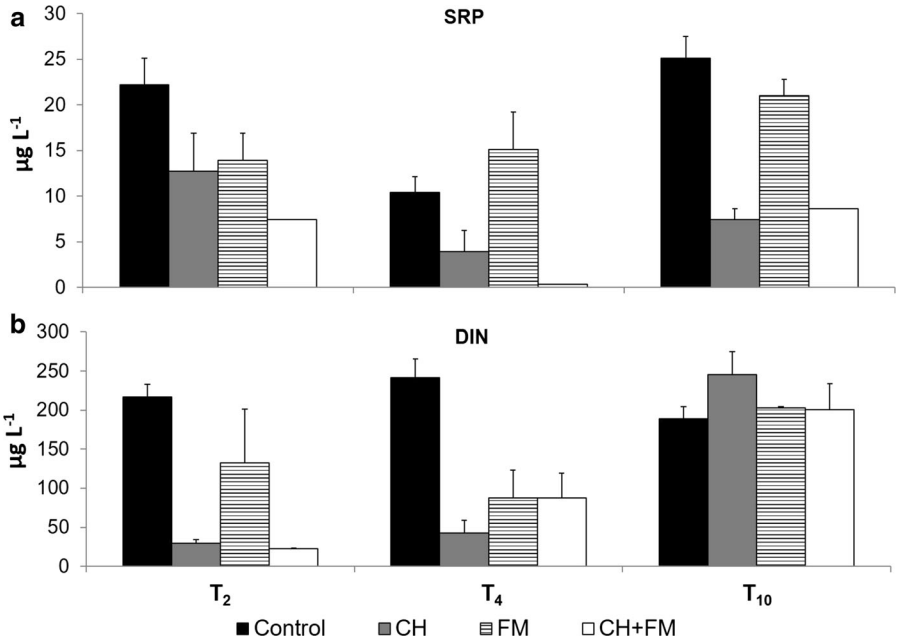

Fig. 2 Soluble Reactive Phosphorus (SRP) (**a**) and Dissolved Inorganic Nitrogen (DIN) (**b**) concentrations registered in the Control (C) and the rest of treatments: calcium hydroxide (CH), floating macrophytes (FM), and calcium hydroxide plus floating macrophytes (CH + FM) at T_2 (2 days after), T_4 (4 days after), and T_{10} (10 days after). Nutrients concentration at T_0 (beginning of the experiment) were: 259.4 ± 56.1 μg l^{-1} for SRP and 345.8 ± 68 μg l^{-1} for DIN

$P < 0.01$), the effect of treatments ($F = 322.29$ $P < 0.01$) and the combination of both factors ($F = 9.69$ $P < 0.004$). Tukey test found differences among control and lime treatment ($P < 0.01$, control > CH) and control versus CH + FM ($P < 0.01$, control > CH + FM). Bonferroni test showed differences among T_2 and T_4 ($P = 0.001$, $T_2 > T_4$) and T_4 versus T_{10} ($P = 0.002$, $T_4 > T_{10}$).

DIN concentration also demonstrated high concentrations at the beginning of the experiment (345.8 ± 68.0 μg l^{-1}). Drop in the control and all treatments at sampling T_2 and T_4 were observed; being more remarkable in the treatments (CH, FM and CH + FM). The trend at the end of the experiment (T_{10}) was of a homogenization in DIN concentrations in the three treatments (CH, FM and CH + FM) with values registered in control (Fig. 2b). Statistical differences among DIN concentrations (excluding T_0 data) were found for the effect of time ($F = 22.84$ $P = 0.001$), the effect of treatments ($F = 26.31$ $P < 0.01$), and the combination of both ($F = 5.52$ $P < 0.005$). Tukey test showed differences among control with the rest of treatments (CH, FM and CH + FM) ($P < 0.01$, control > CH, FM and CH + FM). The Bonferroni test showed differences between T_2 and T_{10} ($P < 0.01$, $T_2 < T_{10}$) and T_4 versus T_{10} ($P < 0.01$, $T_4 < T_{10}$).

Plankton response

At the beginning of the experiment we identified a total of 58 species of phytoplankton corresponding 26 to Chlorophyceae, 15 to Cyanobacteria, 7 to Euglenophyceae, 7 to Bacillariophyceae, 2 to Dinophyceae, and 1 to Haptophyceae. The dominant species during the experiment were mainly Cyanobacteria species (*Raphidiopsis mediterranea* Skuja, *R. curvata* Fritsch & Rich, *Glaucospira laxissima* (West) Simic, Komárek & Dordevic, and *Oscillatoria* sp.), followed by Bacillariophyceae (*Cyclotella meneghiniana* Hustdt), Dinophyceae (*Peridinium* sp.), and Euglenophyceae (*Trachelomonas volvocina* (Ehrenberg) Ehrenberg). Sixteen morpho-functional groups (MFGs) were identified, but only 6 which represented ≥ 2% of total phytoplankton biovolume were used in the statistical analyses. They were: large Chrysophyceae (1a-LargeChry), large Dinophyceae (1d-LargeDino), small Euglenophyceae (2c-SmallEugle), Cyanobacteria order Oscillatoriales (5a-FilaCyano), order Nostocales (5e-Nostocales), and large centric Bacillariophyceae (6a-LargeCent).

The one-way ANOVA comparing control and treatments (CH, FM and CH + FM) performed at the beginning of the experiment (T_0) for the total phytoplankton biovolume and MFGs showed lack of significance for all of them: total phytoplankton

biovolume ($F = 0.5$ $P = 0.6$), 1a-LargeChry ($F = 0.53$ $P = 0.67$), 1d-LargeDino ($F = 0.8$ $P = 0.48$), 2c-SmallEugle ($F = 2.03$ $P = 0.18$), 6a-LargeCent ($F = 1.21$ $P = 0.36$), 5a-FilaCyano ($F = 2.46$ $P = 0.13$), and 5e-Nostocales ($F = 0.14$ $P = 0.93$).

A strong drop in total biovolume (> 80%) was observed at T_2 in the treatments with lime (CH and CH + FM treatments), compared to the control and plants (FM treatment) which showed similar and higher values of biovolume (mean values between 60 and 80 $mm^3\ l^{-1}$). The drop was not consistent during samplings, showing a recovery at T_4 especially in lime (CH treatments). Finally, a drop at the end of the experiment (T_{10}) with values < 20 $mm^3\ l^{-1}$ was observed in all treatments in comparison with control (52 $mm^3\ l^{-1}$). The effect of plants (FM treatment) was only noticeable at T_{10} (Fig. 3). The RM ANOVA confirmed these patterns showing differences by the effect of time ($F = 46.36$ $P < 0.01$), by the effect of treatments ($F = 18.17$ $P = 0.001$) and by the combination of both factors ($F = 4.6$ $P = 0.015$). The Tukey test showed differences among control and lime treatments (CH and CH + FM) ($P < 0.01$, control > CH, CH + FM for both); except control vs. FM ($P = 0.089$). The Bonferroni test revealed differences among T_0 and the rest of the sampling days (T_2, T_4 and T_{10}) ($P < 0.01$ for all, $T_0 > T_2$, T_4, T_{10}). Differences ($P < 0.05$) were also found between T_2 versus T_{10} ($T_2 > T_{10}$), T_4 versus T_{10} ($T_4 > T_{10}$).

An increase in the percentage relative contribution to total biovolume of 5a-FilaCyano and 5e-Nostocales (both cyanobacteria groups) was observed at T_{10} (end of the experiment) compared to the values registered at the beginning (Fig. 4a, b). This increment was more noticeable in control and those treatments with presence of plants (MF and CH + MF treatments). In the CH treatment 5a-FilaCyano and 1d-LargeDino groups were the dominant groups (38% and 40%, respectively) (Fig. 4b). Alpha diversity estimators (Shannon diversity and equitability) showed similar values at the beginning and at the end of the experiment. Values were between 3.01 and 3.17 bits ind^{-1} Shannon diversity and 0.98–0.99 of equitability at T_0 and 2.67–2.97 bits ind^{-1} of Shannon and 0.97–0.99 for equitability at T_{10}.

Concerning to the different MFGs, all of them showed a drop in biovolume in presence of lime (CH and CH + FM) and compared with control at T_2 (Fig. 5a–f). A recovery with respect to T_2 was observed for 1d-LargeDino, 5a-FilaCyano and 5e-Nostocales at T_4 in CH, but not in CH + FM (Fig. 5a, b, e, f). In presence of plants (FM) none of the groups experienced a remarkable effect of biovolume decrease in T_2, except 6a-LargeCent. This pattern was maintained at T_4, being the drop only noticeable at T_{10}. At the end of the experiment (T_{10}) only FilaCyano and 5e-Nostocales maintained higher values of biovolume in the control, and a recovery in their biovolumen was observed in the CH + FM treatment.

The RM ANOVAs found differences for the 5e-Nostocales, 1a-LargeChry, 1d-LargeDino, 2c-SmallEugle, and 6a-LargeCent for the effect of time ($P < 0.01$), the effect of treatments ($P < 0.01$) and the combination of both factors ($P < 0.01$). Tukey test showed differences among control with CH and

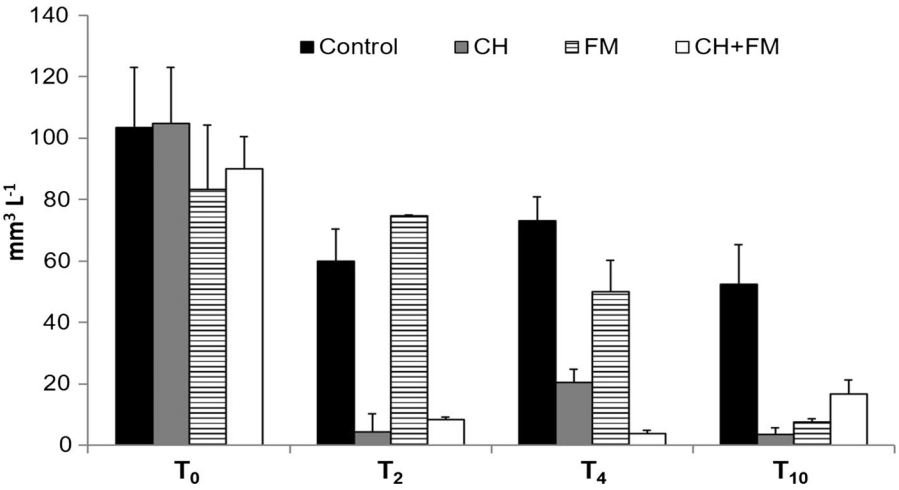

Fig. 3 Total phytoplankton biovolume among control and the rest of treatments: calcium hydroxide (CH), floating macrophytes (FM), and calcium hydroxide plus floating macrophytes (CH + FM) at sampling T_0 (beginning of the experiment), T_2 (2 days after), T_4 (4 days after), and T_{10} (10 days after)

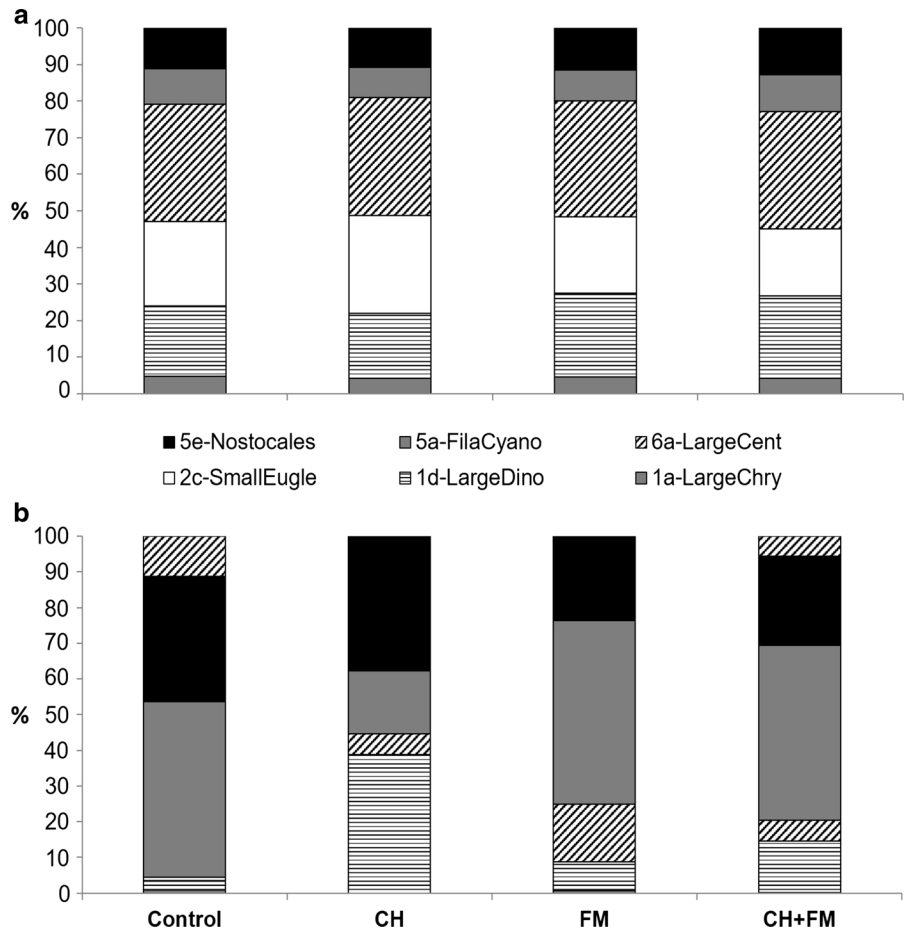

Fig. 4 Percentage contribution of the different morpho-functional groups (MFGs) of phytoplankton to the total biovolume at the beginning of the experiment (T_0) (**a**) and at the end (T_{10}) (**b**)

CH + FM treatments ($P < 0.01$, control > CH, control > CH + FM), but not for control versus plants (FM treatment) in almost any case ($P > 0.05$). The exception was 1a-LargeChry which also showed differences with plants (FM) ($P < 0.001$, C > FM). Bonferroni test showed differences among T_0 and the rest of the sampling days ($P < 0.01$, $T_0 > T_2, T_4, T_{10}$). Differences ($P < 0.05$) were also found for 1a-LargeChry, 1d-LargeDino and 6a LargeCent between T_2 versus T_{10} ($T_2 > T_{10}$), T_4 versus T_{10} ($T_4 > T_{10}$). The exception was 2c-SmallEugle for T_4 versus T_{10} ($P = 0.789$) and 5e-Nostocales which only showed differences for T_0 versus T_{10} ($P = 0.005$, $T_0 > T_{10}$) and T_{10} versus T_2 and T_4 ($P = 0.020$ and $P = 0.015$, respectively). The 5a-FilaCyano group demonstrated differences only by the effect of treatment ($F = 29.07$ $P < 0.01$), showing the Tukey test differences among control and the three treatments ($P < 0.05$, control > CH, FM, CH + FM).

Concerning zooplankton, we identified throughout the experiment a total of 30 species corresponding mainly to Rotifera (26 species); the dominant species were *Brachionus budapestinensis* Trusted and *B. caudatus* Pallas. We also identified 3 species of Cladocera with *Alona glabra* Pallas as dominant and 1 Copepoda with *Euclyclops* sp. as dominant. No Cladocera species were found at the beginning of the experiment. At the beginning of the experiment, the one-way ANOVA showed lack of significance for all groups considered: total zooplankton ($F = 1.26$ $P = 0.35$), Rotifera ($F = 1.26$ $P = 0.35$) and Copepoda ($F = 1$ $P = 0.99$).

At T_0 the assemblage was entirely dominated by Rotifera with density values higher than 800 ind l^{-1} in all treatments (Fig. 6a) and a zooplankton structure completely dominated by Rotifera (Fig. 6c). At the end of the experiment (T_{10}), a drop in density of Rotifera in control and the three treatments (higher

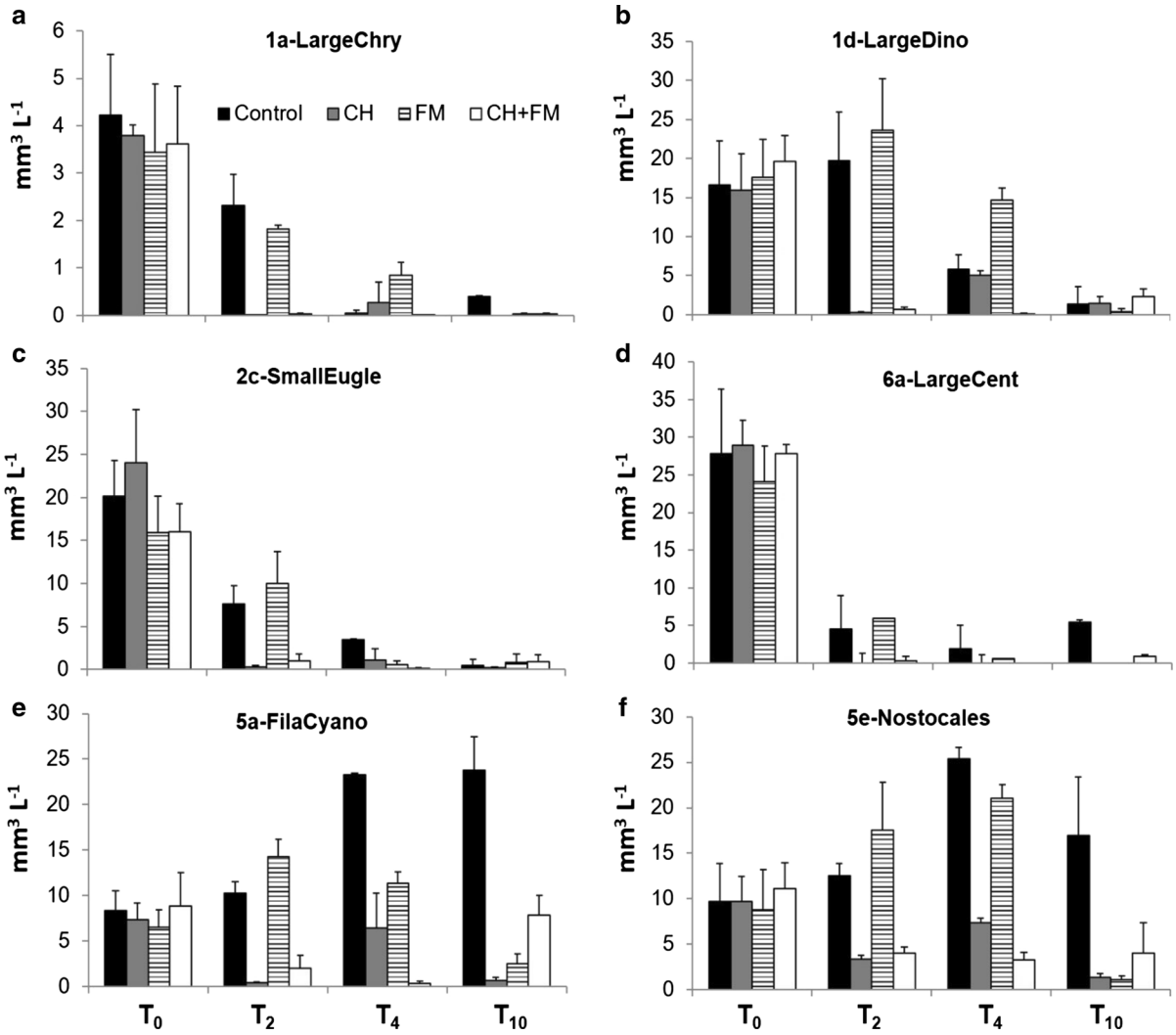

Fig. 5 Biovolume of the different morpho-functional groups (MFGs) of phytoplankton in control and the rest of treatments: calcium hydroxide (CH), floating macrophytes (FM), and calcium hydroxide plus floating macrophytes (CH + FM) at sampling T_0 (beginning of the experiment), T_2 (2 days after), T_4 (4 days after), and T_{10} (10 days after). Key: large Chrysophyceae (1a-LargeChry), large Dinophyceae (1d-LargeDino), small Euglenophyceae (2c-SmallEugle), Cyanobacteria order Oscillatoriales (5a-FilaCyano), Cyanobacteria order Nostocales (5e-Nostocales), and large centric Bacillariophyceae (6a-LargeCent)

than 90%) was observed (Fig. 6b). This decrease in Rotifera density was accompanied by an increase in Copepoda (98% compared with the beginning) in those treatments with plants (73 ± 40 ind l^{-1} in FM and 166 ± 80 ind l^{-1} in CH + FM treatment), being mainly *nauplii* larvae and adults of *Eucyclops* sp. Cladocera showed at the end of the experiment an increment of 100% in those treatments with plants (8 ± 5 ind l^{-1} in FM and 16 ± 11 ind l^{-1} in CH + FM) (Fig. 6d). No individuals where registered in control and CH treatment. A small increment was observed in the Shannon diversity and equitability when species composition was compared. Values were between 1.09 and 1.15 bits ind^{-1} of Shannon and 0.44–0.52 of equitability at T_0 and 0.89–2.03 bits ind^{-1} of Shannon and 0.39–0.69 for equitability at T_{10}. The one-way ANOVA at T_{10} (end of the experiment) showed lack of significance when treatments were compared for total zooplankton ($F = 1.99$ $P = 0.94$), Rotifera ($F = 3.31$ $P = 0.07$) and

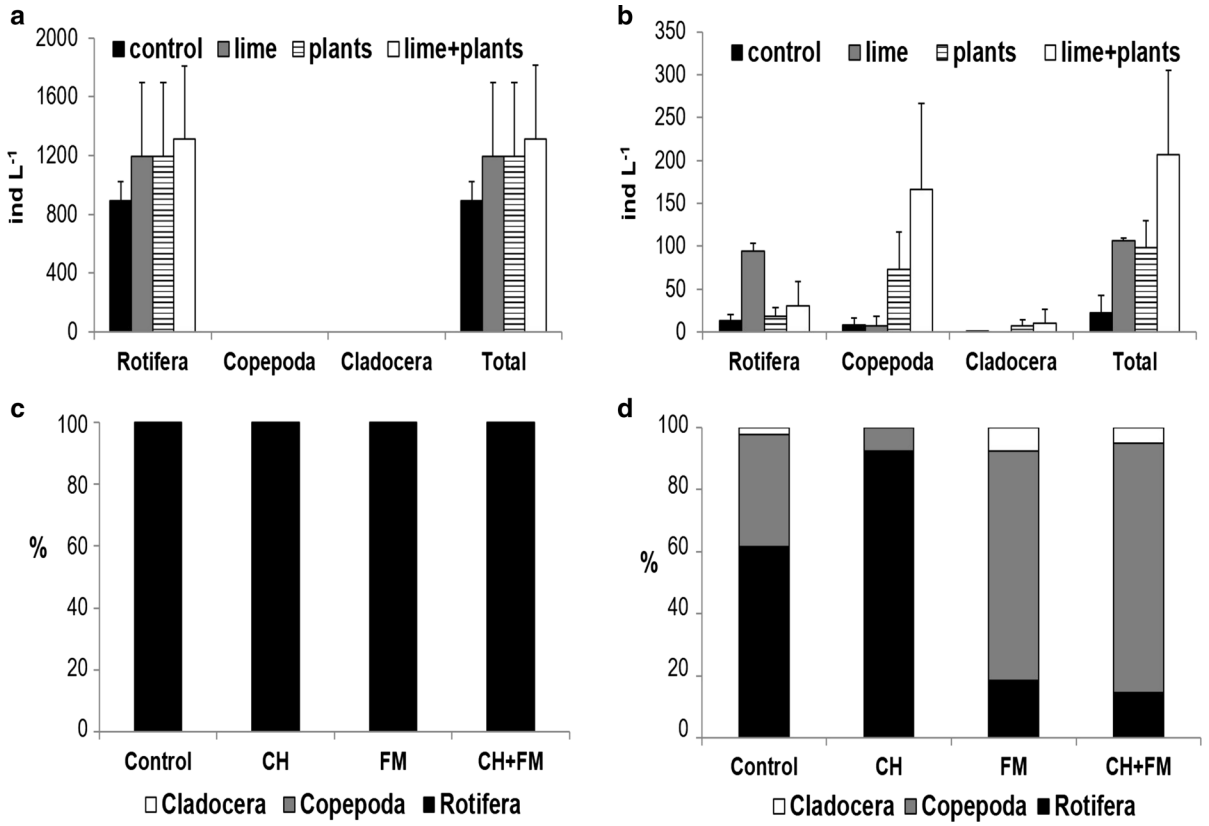

Fig. 6 Zooplankton density at the beginning (T_0) (**a**) and at the end (T_{10}) (**b**) of the experiment, being also shown the percentage contribution of the different groups to the total zooplankton density at the beginning (T_0) (**c**) and at the end of the experiment (T_{10}) (**d**) in the control and the different treatments: calcium hydroxide (CH), floating macrophytes (FM), and calcium hydroxide plus floating macrophytes (CH + FM)

Cladocera ($F = 2.22$ $P = 0.16$) but showed differences for Copepoda ($F = 3.31$ $P = 0.027$). The Tukey test found differences between control and treatments with plants ($P = 0.03$ for FM and $P = 0.02$ for CH + FM, control < FM, control < CH + FM).

Discussion

Phosphorus removal efficiency

The use of macrophytes in the mitigation of eutrophication is a technique which has shown to reduce external phosphorus inputs and water turbidity in experimental and field studies of temperate (e.g., Immers et al., 2013; Zeng et al., 2017) and subtropical areas (e.g., Tripathi et al., 1991; Maine, 2007, 2009). In this study, we tested a free-floating macrophyte (*Salvinia rotundifolia*), a small sized species with 2 or 3 leaves and a dense system of submerged tubular hairy modified leaves, analogous to roots in function. Native from South America, some species have been reported as invasive (e.g., *Salvinia molesta* Mitch) in the U.S, Australia and several countries of Europe (e.g., McFarland et al., 2004; Owens & Smart, 2010). Nonetheless, our results suggest that this genus may be used in mitigation of eutrophication of subtropical shallow lakes considering its affinity to stagnant waters, high tolerance to elevated conductivity, and high vegetative reproduction rate, being necessary however more field studies to confirm our findings.

In our experiment, *S. rotundifolia* increased its coverage area in a 75% at the end of the experiment. Despite this increment of biomass, results showed that this plant alone was a poor phosphorus removal organism. Debusk & Reddy (1987) proved the high capability of *S. rotundifolia* to up-take phosphorus (they reported 26.5 mg m^{-2} day^{-1}). However, other

studies have reported that *Salvinia* species optimum SRP up-take is done to low pH values (5 to 6) and low temperatures ($\sim$ 20°C), being ammonium the preferred form of nitrogen (Gaudet & Koh, 1968; Debusk & Reddy, 1987; McFarland et al., 2004; Owens et al., 2005; Owens & Smart, 2010). In this experiment, the ability of *S. rotundifolia* to remove high quantities of phosphorus may be reduced because pH was always near 8 in those treatments with plants, nitrite-nitrate was the main form of DIN and the temperature was about 26°C during samplings. Nonetheless, our results showed that *Salvinia* effect was more important when it was combined with lime. The highest reduction of SRP was observed 4 days after beginning (T_4) in CH + FM treatment, being phosphorus removal even better than in the lime treatment (CH) alone. Moreover, as we will discuss forward, the presence of plants could be an important plankton structure driver that could improve eutrophication mitigation strategies.

Lime addition was an effective technique for reduction of SRP concentration, either when was used alone (CH treatment), or in combination with plants (CH + MF). Different forms of lime [$CaCO_3$ and $Ca(OH)_2$] have been widely tested in temperate stratified deep lakes in the past (e.g., Murphy & Prepas, 1990; Prepas et al., 1990; Dittrich & Koschel, 2002), but in shallow subtropical lakes the information is scarce. Calcium hydroxide [$Ca(OH)_2$] may dissociate in water and form calcium carbonate ($CaCO_3$) which react with SRP and precipitate (Babin et al., 1989). It can also interact with available forms of SRP at pH near 10 and form hydroxyapatite [$Ca_5(PO_4)_3(OH)$], an insoluble water compound which also precipitate (Prepas et al., 1990). Both processes would make phosphorus inaccessible for phytoplankton. Once in sediments, these forms of calcium + phosphorus may continue to be absorbed preventing the release of phosphate into the overlying water, and even creating a superficial layer which prevents phosphorus liberation from deeper sediment layers.

In comparison with other metallic compounds commonly used in restoring eutrophic waters, calcium hydroxide presents some advantages. It does not have toxic effects -as copper or aluminum compounds widely used in other mitigation strategies- and it may produce products like hydroxyapatite which is not a redox dependent reaction (Babin et al., 1989; Prepas et al., 1990). Moreover, in comparison with other substances widely applied as lanthanum-modified clays, which can cost thousands of dollars per ton (Copetti et al., 2016), calcium hydroxide could be a low-cost agent (between 20 and 100 dollars per ton) and may have similar effects.

In our experiment, lime showed the highest removal efficiency 4 days after beginning (T_4), being this effectiveness decreased at the end of the experiment (T_{10}) in both CH and CH + FM treatment. These effects may be related first with the method of application, since lime was added to the surface, and dissolution and further interaction with the whole water column may take time. Second, phosphorus liberation from organic matter produced by vegetation, by death of planktonic organisms, or even from portions of sediments which were not in contact with lime, could contribute to increase phosphorus concentration in the water column at the end of the experiment.

The lime concentration used exerted a minor effect on the pH of water (slightly increments where observed in those treatments with lime). This is a highlight since the rapid increment of pH could be toxic for living organisms like fish (Dittrich et al., 2002). Zhang et al. (2001) suggest that small additions could be more effective in the removal of SRP without affecting pH in hard water lakes; being this a strategy which should be proven in further field studies in subtropical eutrophic systems.

Despite dissolved inorganic nitrogen (DIN) was not the target of the tested restoring techniques applied, is an important nutrient for phytoplankton and particularly for CyanoHABs development. The primary drop effect observed in DIN concentration in treatments may be related with the flocculation effect of lime (and subsequent deposition in the sediments), and the nutrients up-take of plants in the CH and FM treatments, respectively. The similar concentrations registered at the end of the experiment in the three treatments (CH, FM and CH + FM) and the control however, may be relate with organic matter released during the experiment or portions of sediments not covered with lime.

Plankton assemblage response

A consistent effect of decrease on total phytoplankton biovolume was observed in the treatments with lime addition (CH) and particularly in the treatment with lime and plants (CH + FM treatment). This pattern

was also consistent for almost all groups of phytoplankton identified; demonstrating the high power of flocculation which has lime compounds (Zhang & Prepas, 1996; Prepas et al. 2001). An effect probably improved at T_{10} by removal of SRP near limiting conditions for phytoplankton development (< 5 µg l^{-1}, Reynolds, 2006). In CH + FM treatment the presence of vegetation could also control phytoplankton biovolume by competing for nutrients, light and/or producing allelopathic substances. Phytoplankton control by free-floating plants is a phenomenon widely proven in several studies when macrophyte surface cover is high (Meerhoff et al., 2003; Bicudo et al., 2007). Kuiper et al. (2017) suggest carrying out harvests or mowing submerged macrophytes, thus allowing the phosphorus present in the plant biomass be removed from the system.

Biovolume recuperation in CH and CH + FM treatment was observed especially for 5a-FilaCyano dominated by *Glaucospira laxissima* (G.S.West) Simic, Komárek & Dordevic and 5e-Nostocales dominated by *Anabaenopsis arnoldii* Aptekar. Both species could be considered as CyanoHABs, and this is a pattern reported by Zhang et al. (2001) in a large field experiment made in several temperate lakes in Canada. This resistance may be favored by their buoyancy capability and by their elongated form which prevent sinking. Moreover, Cyanobacteria can store nutrients, resist changes in pH conditions (Carey et al., 2012), some of them show affinity to high conductivities indeed, both species found here are good examples (Santos & Sant'Anna, 2010; Malone et al., 2012). Moreover, depending on species, Cyanobacteria can resist changes in light penetration under floating macrophytes covers (Sinistro, 2006).

From a compositional perspective, at the end of the experiment those treatments treated with lime (CH and CH + FM) presented an increment of dominance in 1d-LargeDino and Cyanobacteria MFGs. Dinophyceae species may be benefited in this treatment by the high transparency levels observed -product of flocculation- resisting the low nutrient availability with phagotrophy (Modenutti, 2014). Shannon diversity and Equitability showed lack of relevant changes in all treatments indicating that the effects of lime or vegetation were similar in a diversity level.

For zooplankton, a slightly increment of diversity and equitability at the end of the experiment suggest a positive effect of vegetation on zooplankton. This change was also noticeable with an increment of the number of individuals of Copepoda (mainly nauplli larvae and adults of *Eucyclops* sp.) and Cladocera (3 species emerged during the experiment, mainly *Alona glabra*) in those treatments with plants (FM and CH + FM). Meerhoff et al. (2003) have already proved the effect of free-floating macrophytes as refuge for zooplankton in subtropical lakes since this type of plants is not commonly visited by small planktivorous fish. Floating macrophytes may have a better development of biofilms (food items available for zooplankton) in their roots, making them more desirable and contributing with the habitat heterogeneity in the littoral zone. The observed decrease in density (and high standard deviation), especially for Cladocera could be explained by the short time of the experiment (10 days) since Cladocera species may require more time to be developed. Despite their low nutritional value, zooplankton may feed on Cyanobacteria effectively (Kozlowsky-Suzuki et al., 2003; Panosso et al., 2003), becoming a potential complementary control of CyanoHABs in these kinds of environments.

Conclusions

The treatment of lime plus plants shows to be a good strategy for the treatments of cultural eutrophicated waters in experimental conditions. The evidence compiled suggests that lime addition may be an effective flocculant of phytoplankton, reducing also SRP availability; being vegetation important as phytoplankton competitor and as zooplankton refuge. However, it is remarkable the capability of filamentous cyanobacteria to resist the mitigation effects tested. Further field manipulation studies will allow us to improve these techniques and propose more feasible and eco-friendly approaches to mitigate cultural eutrophication in subtropical lakes.

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