

Periphyton responses to enrichment and nutrient dilution in two mesocosm experiments in a shallow hypereutrophic reservoir

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ABSTRACT

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Periphyton can contribute to maintaining oligotrophic conditions in restored shallow lakes and reservoirs. In two mesocosm experiments, we evaluated the periphyton responses to enrichment and nutrient dilution in a hypereutrophic reservoir. Effects of the isolated and combined N and P enrichment and dilution levels on periphyton biomass and structure on artificial substrate were investigated. For colonization of periphyton, glass slides were exposed for 14 days (enrichment experiment) and 21 days (dilution experiment) at 20 cm deep. We monitored the effects of N and P enrichment and dilution on the algal biomass, density, and species composition in the periphyton. P was a primary limiting factor for periphyton development. Periphyton algal density and biomass increased in response to combined and isolated P enrichment. Only the 75 % dilution had a positive and significant effect on biomass and algal density in the periphyton. In both mesocosm experiments, the periphyton structure changed, with *Nitzschia palea* (Kutz.) Smith responding positively to P enrichment and negatively to dilution. *Achnanthydium minutissimum* Complex becoming dominant following 50 % and 75 % eutrophic water dilution. Our results suggest that major environmental changes are necessary for a heterogeneous algal community structure in the periphyton in hypereutrophic conditions. We conclude that, after a restoration process that reduces P-competition with phytoplankton and shading, periphyton can become an important primary producer in a hypereutrophic reservoir.

Key words: algae species; artificial substrate; biomass; tropical shallow reservoir

RESUMEN

Respuestas del perifiton al enriquecimiento y dilución de nutrientes en dos experimentos de mesocosmos en un embalse hipereutrófico bajo

El perifiton puede contribuir a mantener las condiciones oligotróficas en lagos y embalses poco profundos restaurados. En dos experimentos usando mesocosmos, evaluamos las respuestas del perifiton al enriquecimiento y a la dilución de nutrientes en un embalse hipereutrófico. Se investigaron los efectos del enriquecimiento y dilución de N y P aislados y combinados sobre la biomasa del perifiton y la estructura del sustrato artificial. Para la colonización del perifiton, se colocaron portaobjetos de vidrio exponiéndolos durante 14 días (experimento de enriquecimiento) y 21 días (experimento de dilución) a 20 cm de profundidad. Monitorizamos los efectos del enriquecimiento y dilución de N y P en la biomasa de algas, la densidad y la composición de especies en el perifiton. El P fue el principal factor limitante para el desarrollo de perifiton. La densidad y la biomasa de algas aumentaron en respuesta al enriquecimiento combinado y aislado de P. Solo la dilución del 75 % tuvo un efecto positivo y significativo sobre la biomasa y la densidad de algas en el perifiton. En ambos experimentos de mesocosmos, la estructura del perifiton cambió, con *Nitzschia palea* (Kutz.) Smith respondiendo positivamente al enriquecimiento de P y negativamente a la dilución. *Achnanthydium minutissimum* Complex se vuelve dominante después de una dilución de agua eutrófica al 50 % y 75 %. Nuestros resultados sugieren que los cambios ambientales importantes son necesarios para una estructura heterogénea

de la comunidad de algas en el perifiton en condición hipereutrófica. Concluimos que, después de un proceso de restauración que reduce la competencia de P con el fitoplancton y el efecto sombra, el perifiton puede convertirse en un importante productor primario en un embalse hipereutrófico.

Palabras clave: *especies de algas; sustrato artificial; biomasa; embalse tropical poco profundo*

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INTRODUCTION

Periphyton is largely involved in the lake functioning, contributing to primary production and nutrient cycling (Vadeboncoeur & Steinman, 2002). However, in eutrophic ecosystems, periphyton development can be attenuated due to the strong phytoplankton-related competition for nutrients and shading, especially during the bloom periods (Romo et al., 2007, Borduqui & Ferragut, 2012). Studies have demonstrated that the periphytic communities can indicate environmental changes and act in bioremediation of impacted freshwater ecosystems (Jöbgen et al., 2004, Wu et al., 2010). Periphyton biomass, nutrient content and species composition have been used as a sensor/indicator of water quality changes, as eutrophic processes (Gaiser, 2009). Periphyton can adsorb, absorb and consume nutrients, which can contribute to improving the ecological quality of enriched lakes (Dodds, 2003, Li et al., 2015). Overall, the periphyton appears to be a promising tool for lake restoration and eutrophication control (Gaiser, 2009, Wu et al., 2017). On the other hand, studies have reported that high periphyton biomass can cause the decline of submerged macrophyte communities that function to maintain the clear-water phase in restored temperate lakes (Roberts et al., 2003, Hilt et al., 2018). Thus, considering the restoration of shallow lakes and reservoirs, periphyton can contribute to maintaining oligotrophic conditions or prevent the establishment of a clear phase. Therefore, the responses of periphyton to different restoration techniques need to be further investigated.

Various studies demonstrated that the nutrient stoichiometry, as well as interactive effects between light and nutrients, can influence the primary production, biomass, and structure of periphyton in freshwater ecosystems (Stelzer

& Lamberti, 2001, Hillebrand & Kahlert, 2001, Zhang et al., 2015). The changes in periphyton biomass and structure can be strongly related to the N:P stoichiometry in lakes and streams (Stelzer & Lamberti, 2001, Hillebrand & Kahlert, 2001). For algal growth, the C:N:P ratio is the most explored stoichiometric relationship given the importance of these elements. Despite advances in knowledge about the effects of changes in the N:P ratio on periphyton, there are still gaps, especially regarding the algal species composition. A greater understanding of the periphyton responses to changes in the N:P ratio can help in predicting the community's response to ecosystem changes. Several environmental processes can alter the N and P stoichiometry, including the reservoir and lake restoration techniques, as oligotrophication (Schindler et al., 2016, Penuelas et al., 2020). Oligotrophication consists of reducing the nutrient concentration in eutrophic lakes and reservoirs (Sommer et al., 1993, Anderson et al., 2005). The oligotrophication effects need to be better understood for the development of more efficient techniques for restoring eutrophic environments and for the maintenance of restored systems (Søndergaard et al., 2007, 2013).

In two mesocosm experiments, we experimentally evaluated the periphyton responses to enrichment and nutrient dilution in a hypereutrophic shallow reservoir. In both mesocosm experiments, we focus on the effects of N:P ratio changes on periphyton biomass and structure on artificial substrate. Most restoration processes imply in N:P ratio changes over time (Schindler et al., 2016) and, thus, a better understanding of periphyton responses to ratio changes can contribute to more effective predictions in shallow lakes and reservoirs. For the enrichment experiment, we take into consideration that periphyton growth is primarily P-limiting in local reservoirs (Ferragut

& Bicudo, 2010, Lambrecht et al., 2019). Thus, we hypothesized that periphyton is limited by P even in a hypereutrophic reservoir. For the dilution experiment, we consider that oligotrophication tends to reduce the N:P ratio and phytoplankton biomass (Schindler et al., 2016, Amaral et al., 2020), which can control the periphyton growth (Borduqui et al., 2012). For eutrophic conditions, our second hypothesis is that nutrient dilution can increase biomass and change the algal community structure in periphyton. In both mesocosm experiments, the periphyton results can contribute to a greater understanding of the effects of oligotrophication in shallow eutrophic reservoirs.

MATERIALS AND METHODS

Study Area

Two mesocosm experiments were carried out in Garças Lake ($23^{\circ} 38' 20.03''$ S; $46^{\circ} 37' 19.20''$ W, Fig. 1), a eutrophic reservoir located in the Parque Estadual das Fontes do Ipiranga, in the south-eastern part of the state of São Paulo, Brazil. The reservoir has an area of 88 156 m², a volume of 188 785 m³, an average depth of 2.1 meters and a maximum depth of 4.7 meters. Despite the improvement of reservoirs in water quality

in recent years, it is classified as hypereutrophic (Bicudo et al., 2020). Both experiments were carried out during the winter, which is characterized by mild temperatures and low rainfall. The first experiment was conducted from June 30 to July 20, 2017, and the air temperature varied from 10 to 19 °C (average 15.1 °C) and the total accumulated rainfall was 2.3 mm (average 0.1 mm). The second experiment was performed from August 4 to August 30, 2017, and the air temperature varied from 19.4 to 25.4 °C (average 22.6 °C) and the total accumulated rainfall was 74.4 mm (on average 3.7 mm). The climate data were obtained from Boletim Climatológico Anual da Estação Meteorológica do IAG/USP (<http://estacao.iag.usp.br/boletim.php>).

Experimental Designs

Consulting the bathymetry of the Garças Reservoir, we installed the mesocosms randomly in the littoral zone of the reservoir. The mesocosms were positioned according to the light availability, to avoid limitations by this resource. Once installed in the littoral zone, then the treatments were randomly (by draw) designed for each experimental unit. The enrichment experiment (EE) was conducted from June 30 to July 20, 2017, with water

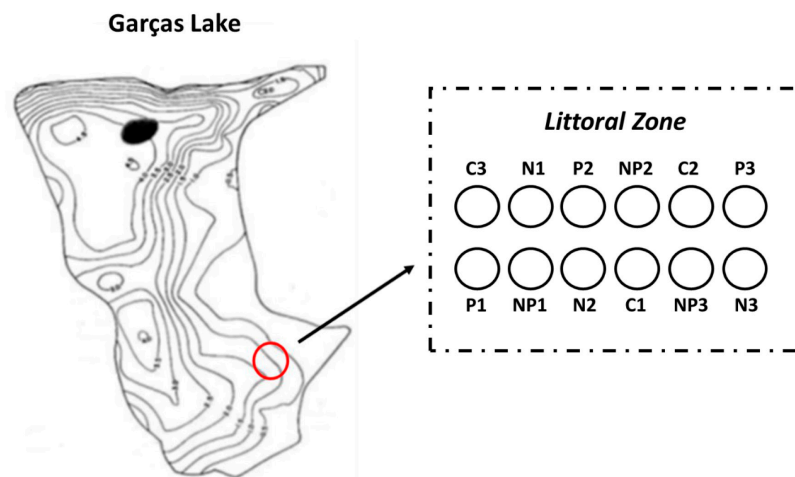


Figure 1. Bathymetric map of Garças Reservoir with location of experiment on the littoral zone and a schematic of the treatment distribution after randomization. Source: Bicudo et al. (2002). *Mapa batimétrico del lago Garças con la ubicación de las unidades experimentales en la zona litoral y esquema de distribución del tratamientos al azar.* Fuente: Bicudo et al. (2002).

and periphyton sampling on 14 experimental days (July 20). The second experiment was simulating oligotrophic conditions, which was conducted from August 9 to August 30, 2017, and water and periphyton sampling were performed on 21 experimental day (August 30). Aiming to create environmental conditions closer to natural conditions, both the enrichment and oligotrophic simulating dilutions were carried out in open-bottom mesocosms in littoral zone. The open-bottomed mesocosms were constructed of PVC cylinders (0.5 m in diameter, 120 cm in length) and had a maximum volume of 151 l. The depth of the water column was standardized at 80 cm. In both experiments, the mesocosms were acclimated for 4-5 days after installation.

To evaluate the effects of enrichment and dilution of nutrients on periphyton, we chose the inert substrate. The glass slides (76 mm × 25 mm) served as substrate for periphyton colonization. Considering that it is a consensus that natural substrates interfere in the periphyton structure, we used glass slides, which is inexpensive and easy to handle substrates. In the acrylic support, the substrates were positioned vertically to minimize the effect of the deposition of particulate matter. The acrylic support was fixed to 20-cm depth to avoid photoinhibition (Gil-Gil et al., 2011). The periphyton was removed from the artificial substrate by scraping using steel blades and distilled water jets.

The enrichment experiment (EE) consisted of four treatments in replicate ($n = 3$): Control, no nutrient addition; P+, isolated phosphorus addition, (N-limiting); N+, isolated nitrogen addition (P-limiting); and NP+, combined nitrogen and phosphorus addition (no-limitation). The mesocosms were positioned in the function of light availability, to avoid limitation resource. Once installed, the treatments were randomly designed to each experimental unit (Fig. 1). The initial concentration of the DIN and PO₄-P were 130 µg/l and 5 µg/l, respectively. In order to adjust the N:P molar ratios to 16:1 (NP+), < 16:1 (N+) and > 16:1 (P+) defined amounts of salts were added to the mesocosms. The N:P molar ratios were monitored on experimental days 1, 4, 8, and 11. The ammonium nitrate (NH₄NO₃ Merck) and monobasic potassium phosphate (KH₂PO₄

Merck) were used for enrichment. The first enrichment was performed on experimental day 6.

The nutrient dilution experiment (NDE) involved diluting the eutrophic reservoir water into mesocosms. The experiment consisted of four treatments in replicate ($n = 3$): Control undiluted reservoir water (C), reservoir water diluted by 25 % (D25), reservoir water diluted by 50 % (D50) and reservoir water diluted by 75 % (D75, which corresponds to oligotrophic conditions). Distilled water was used to dilute the eutrophic water and was added slowly to avoid resuspension of the sediment. More details can be found in Amaral et al. (2020).

Variables analysed

In the enrichment and dilution experiments, the temperature, electrical conductivity and pH were measured with a multiparameter probe (Horiba U-53, Kyoto, Japan). According to the methods described in the APHA (2005), dissolved oxygen (DO) (azide-modification method), alkalinity (titration method), free CO₂ (calculated from alkalinity and pH), nitrate (cadmium-reduction method), ammonium (phenol hypochlorite method), orthophosphate (ascorbic acid method) and total nitrogen (TN) and phosphorus (TP) (alkaline persulfate method) were determined. For determining the dissolved nutrient fraction, water samples were filtered through glass fiber filters (GF/F, Whatman, Maidstone, UK). The underwater radiation was measured using a quantum sensor (LI-250A, LI-COR; Lincoln, NB, USA). The light attenuation coefficient (k) was determined by the following equation:

$$k = \frac{\ln(I_1) - \ln(I_2)}{z}, \text{ equation 1}$$

where I_1 is the light in the upper layer, I_2 is the light in the below layer and z is the depth (0.2 m) (Kirk, 1994).

For determining periphyton chlorophyll-*a* concentrations (pheophytin-corrected) samples were filtered through glass fiber filters (GF/F, Whatman) using 90 % ethanol as the solvent (Sartory & Grobbelaar, 1984). The glass-fiber filters were pre-calcined, weighed, and later used to de-

termine the dry mass (DM) and ash-free dry mass (AFDM) according to APHA (2005). The periphyton samples were preserved in 4 % formalin for qualitative analysis, which was performed under an optical microscope (Zeiss Axioskop 2, with phase contrast) and the images were obtained with a high-resolution camera (AxioCam MRc Rev. 3). The periphyton samples were preserved in Lugol acetic solution for quantitative analysis, which was performed with an inverted microscope (Zeiss Axio Observer D1 with 2.5× optovar and image

capture), following the Utermöhl method. The algal density was expressed by area of substrate (ind/cm²). Permanent slides were made for the identification of diatoms (ECS, 2003). The algal biovolume was obtained according to Fonseca et al. (2014) or estimated as described by Hillebrand et al. (1999). The counting limit was determined by species rarefaction curve and at least 95 % of counting efficiency. The species that contributed ≥ 5 % of the total density of each algal community were considered descriptor species.

Table 1. One-way RM-ANOVA results, the mean and standard deviation of abiotic variables and phytoplankton chlorophyll-*a* ($n = 3$) in the isolate and combined N and P enrichment treatments. *Resultados del RM-ANOVA de una vía, la media y la desviación estándar de las variables abióticas y la concentración de clorofila-a del fitoplancton ($n = 3$) en los tratamientos de enriquecimiento de N y P combinados y aislados.*

Variables	Enrichment Experiment				one-way RM-ANOVA	
	Control	N+	P+	NP+	F	P
Conductivity (µS/cm)	243.0 ±18.0	251.3 ±4.0	216.0 ±23.6	244.3 ±5.5	-	ns
DO (mg/l)	5.9 ±0.0	6.7 ±1.3	6.0 ±2.2	6.9 ±0.4	1537	<0.001
Free CO ₂ (mg/l)	49.0 ±7.9	18.7 ±6.8	7.5 ±0.8	4.2 ±1.4	5.746	0.021
Light Attenuation (k)	2.6 ±0.2	3.6 ±1.0	4.3 ±0.3	4.6 ±0.4	9.03	0.012
DIN (µg/l)	651.5 ±243	1997 ±160	204.4 ±252	1127 ±111	82.68	<0.01
pH	6.2 ±0.4	6.8 ±0.1	7.1 ±0.0	7.4 ±0.2	-	ns
PO ₄ -P (µg/l)	5.0 ±0	5.0 ±0	104.9 ±37	133.2 ±21	31.36	<0.001
N:P molar ratio	288.5 107.6	884.2 71.0	1.4 0.6	19.0 2.8	130.64	<0.001
TP (µg/l)	92.4 ±23	227.4 ±20	383.5 ±113	432.9 ±73	7.34	0.011
TN (µg/l)	817.8 ±468	1901 ±157	632.5 ±381	1697 ±597	4.99	0.030
TDS (mg/l)	157.7 ±11.5	163.7 ±2.9	146.7 ±6.4	159.0 ±4.0	5.80	0.050
Temperature (°C)	14.5 ±0.4	14.9 ±0.1	15.1 ±0.1	15.4 ±0.1	-	ns
Phytoplankton chlorophyll- <i>a</i> (ug/L)	95.7 24.0	120.4 51.7	149.7 42.7	326.5 64.5	14.23	0.001

ns no significance

Data analysis

The significant difference between the average values of the abiotic and biotic variables between treatments was determined by the analysis of variance (one-way RM-ANOVA; $\alpha = 0.05$). The multiple mean comparison test was performed using the Tukey Test ($\alpha = 0.05$). Data were logarithmized (except pH) for data normality and homogeneity of variance. Statistical analyses were performed using SigmaPlot 12.0 (Systat Software, Inc.). The permutational multivariate analysis of variance (one-way PERMANOVA; $\alpha = 0.05$) was applied to detect significant differences in the periphyton taxonomic structure among treatments. This analysis was done using Bray-Curtis similarity and 4999 permutations using the PAST 4.04 statistical software (Hammer et al., 2001; <https://nhm.uio.no/english/research/infrastructure/past/>).

Principal Components Analysis (PCA) was used for the assessment of environmental data. PCA was performed based on covariance matrix with environmental data transformed by logarithm [$\log(x + 1)$] using PC-ORD 6.0 (McCune & Meford, 2011). The Pearson correlation coefficient (r) was also applied to measure the degree of the linear relationship between PCA scores of axes 1 and 2 and the density of the most abundant species.

RESULTS

Enrichment experiment results

The values of most variables analysed were significantly different among treatments in the enrichment experiment, except temperature, pH and conductivity (Table 1). When compared to control, the highest TP and PO₄-P concentrations were detected in the P+ and NP+ treatments,

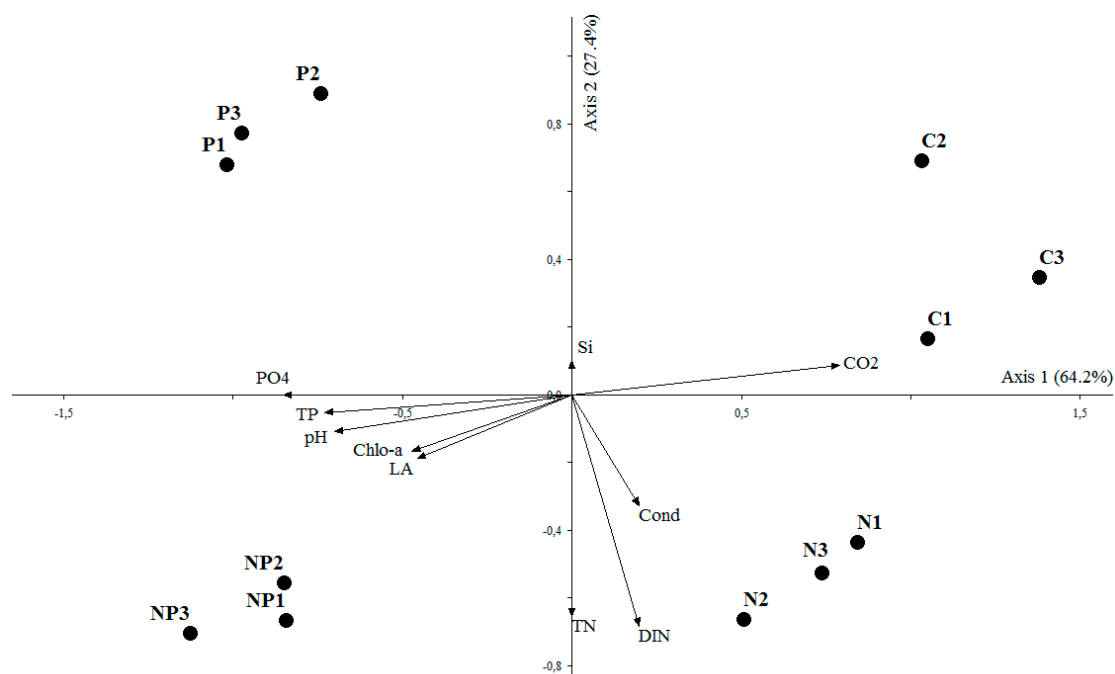


Figure 2. PCA of environmental variables in treatments in the enrichment experiment (C control, N N treatment, P P treatment, NP NP treatment). Vectors: CO₂ free CO₂, Cond conductivity, DO oxygen dissolved, pH pH, TN Total Nitrogen, DIN dissolved inorganic nitrogen, TP Total Phosphorus, LA Light Attenuation and Chlo-a Chlorophyll-a. *PCA de las variables ambientales en los tratamientos del experimento de enriquecimiento (C control, N tratamiento N, P tratamiento P, NP tratamiento NP). Vectores: CO₂ CO₂, conductividad cond, DO oxígeno disuelto, pH pH, TN nitrógeno total, DIN nitrógeno inorgánico disuelto, TP fósforo total, LA atenuación de luz y Chlo-a clorofila-a.*

while the highest DIN and TN concentrations were found in the N+ and NP+ treatments (Tukey: $p > 0.001$). There was a significant increase in light attenuation in the enriched treatments when compared to control. The N:P molar ratio was reduced in P+ and NP+ treatment compared to the control; significant differences between the control and P+ and NP+ treatments were found (Tukey: $p = 0.004$).

PCA ordered treatment C and N treatment on the positive side and P and NP treatments on the negative side of axis 1 (Fig. 2). Chlorophyll-*a*,

PO₄-P e TP concentrations and light attenuation were highly correlated with P and NP treatments scores (Pearson: $r = -0.73; -0.97; -0.90; -0.71$, respectively).

The highest periphyton chlorophyll-*a*, algal density, dry mass, and AFDM:Chlorophyll ratio were found in the P+ and NP+ treatments (Fig. 3A-D; Table 2). These attributes were significantly different among treatments. The lowest species dominance index was found in the N + treatment, while the highest species richness was detected in the P + treatment (Fig. 4A). The periphytic al-

Table 2. One-way RM-ANOVA results, the mean and standard deviation of abiotic variables and phytoplankton chlorophyll-*a* ($n = 3$) in the different dilution levels treatments. *Resultados del RM-ANOVA de una vía, la media y la desviación estándar de las variables abióticas y de la concentración de clorofila-a del fitoplancton ($n = 3$) en los tratamientos con diferentes niveles de dilución.*

Variables	Dilution Experiment				one-way RM-ANOVA	
	Control	T25	T50	T75	F	<i>p</i>
Conductivity (μS/cm)	485.0 ±46.1	409.0 ±24.5	336.0 ±42.6	284.7 ±29.7	12.47	0.002
DO (mg/l)	6.8 ±0.3	7.2 ±0.2	7.7 ±0.6	4.5 ±1.3	12.47	<0.01
Free CO ₂ (mg/l)	16.5 ±8.9	14.3 ±0.7	21.7 ±3.3	108.0 ±64.9	21.59	<0.001
Light Attenuation (k)	4.3 ±0.6	3.8 ±0.4	3.8 ±0.2	2.9 ±0.8	4.44	0.500
DIN (μg/l)	1024 17.0	664.5 103.0	453.9 91.0	431.9 58.0	40.00	<0.001
pH	6.9 ±0.2	6.8 ±0.1	6.6 ±0.1	5.8 ±0.3	20.13	<0.001
PO ₄ -P (μg/l)	>5.0 ±0.6	>5.0 ±0.3	>5.0 ±1.3	>5.0 ±1.4	-	-
N:P molar ratio	453.6 7.7	278.3 41.8	195.4 56.9	168.4 11.7	37.50	<0.001
TP (μg/l)	77.9 ±15	57.3 ±6	64.6 ±8	63.0 ±14	-	<i>ns</i>
TN (μg/l)	1865 ±210	1103 ±76	419.6 ±201	575.3 ±4	56.60	<0.001
TDS (mg/l)	151.7 ±9.5	127.3 ±5.1	97.3 ±11.4	78.3 ±5.5	38.80	<0.001
Temperature (°C)	19.6 ±0.2	19.6 ±0.1	19.0 ±0.1	18.9 ±0.1	-	<i>ns</i>
Phytoplankton chlorophyll- <i>a</i> (ug/l)	52.2 15.6	48.3 19.9	28.9 12.7	23.1 5.8	4.54	0.039

ns no significance

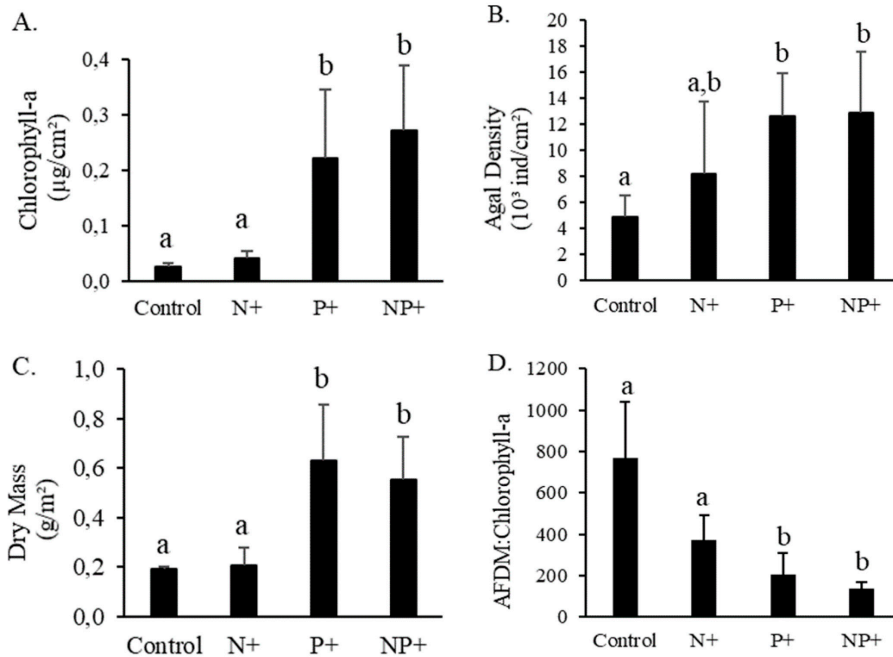


Figure 3. Chlorophyll-*a*, algal density, dry mass and AFDM:Chlorophyll-*a* ratio ($n = 3$; \pm SD) in the periphyton on artificial substrate at the enrichment experiment (A-C; Control; N+, isolate N addition; P+, isolate P addition; NP+, combined N and P addition). The same letters above bars indicate no significant difference between treatments (Tukey: $\alpha > 0.05$). *Clorofila-a*, densidad de algas, masa seca y relación AFDM: Clorofila-*a* ($n = 3$; \pm SD) en el perifiton sobre sustrato artificial en el experimento de enriquecimiento (AC; Control; N+, adición de N aislado; P+, adición de P aislado; NP+, adición combinada de N y P). Las mismas letras sobre las barras indican que no hay diferencia significativa entre tratamientos (Tukey: $\alpha > 0.05$).

gae community structure was significantly altered by isolated and combined by N and P enrichment (one-way PERMANOVA: $F = 1.8$; $p < 0.004$). The diatom *Nitzschia palea* (Kützing) W. Smith was the most abundant species in all treatments, representing 28 % to 48 % of the total density (Fig. 4B). Although it was the most abundant species in the treatments, *N. palea* density was significantly different between treatments (ANOVA one way: $F = 12.437$; $p = 0.002$). The *N. palea* density in the N treatment was different from the control, treatment P and NP (Tukey: $p < 0.031$). A negative correlation was observed for *N. palea* density and the PCA axis-1 scores ($r = -0.70$, $p = 0.011$). Overall, isolated N enrichment increased the density of *Microcystis aeruginosa* (Kützing) Kützing, while NP+ and P+ enrichment favoured the increase of *Fragilaria* spp. and *Coelastrum astroideum* (De Notaris) when compared to the control and N+ treatment.

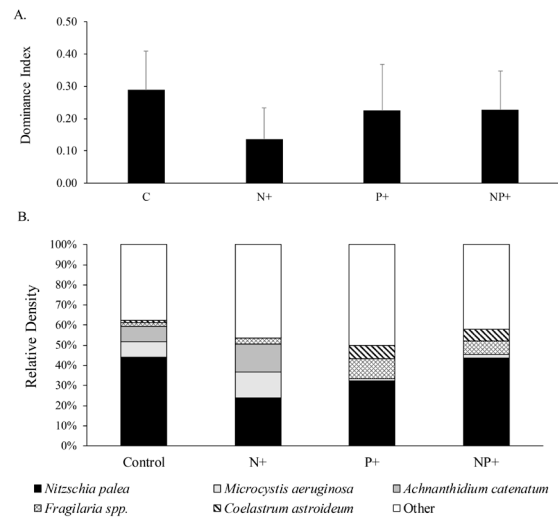


Figure 4. Dominance index (A) and descriptor species density (B) in the periphyton ($n = 3$) in the enrichment experiment. Índice de dominancia (A) y densidad de especies del descriptor (B) en el perifiton ($n = 3$) del experimento de enriquecimiento.

Nutrient dilution experiment results

The values of most environmental variables were significantly different among treatments in the dilution experiment, except temperature (Table 2). Significant differences in the TN and DIN concentrations between control and diluted treatments were found (Tukey: $p > 0.002$). The lowest DIN and TN concentrations were found in the diluted treatments. $\text{PO}_4\text{-P}$ concentration was below the limit of detection for the method ($< 5 \mu\text{g/l}$), whereas the TP concentration was significantly different among the treatments, particularly between the control and T75 (Tukey: $p = 0.029$). The N:P molar ratio was significantly reduced with dilution levels and were found significant differences between the control and diluted treatments (Tukey: $p = 0.004$).

PCA ordered D50 and D75 treatments on the positive side of axis 1 and Control and D25 treatment on the negative side (Fig. 5). In contrast with D50 and D75 treatments, the control and D25 treatment was highly correlated with conductivity, pH, DIN and TN concentrations (Pearson: $r = -0.88; -0.94; -0.83; -0.78$, respectively).

The periphyton chlorophyll-*a*, algal density, and AFDM:Chlorophyll ratio were significantly different among treatments (Fig. 6A-D, Table 4). Significant differences in the chlorophyll-*a* con-

centration and algal density were detected among T75 treatment and other treatments (Tukey: $p < 0.05$). The species dominance index was higher in the diluted treatments than in the control, while the richness decreased with the dilution (Fig. 7A). The periphytic algal community structure was significantly changed with dilution levels (one-way PERMANOVA: $F = 4.393$; $p < 0.003$). Notably, T50 and T75 treatments exhibited increased *Achnanthydium minutissimum* Complex, representing 93 % of the total density (Fig. 7B). However, the *A. minutissimum* density was significantly different between treatments (ANOVA one way: $F = 6.235$; $p = 0.017$). The *A. minutissimum* density in D75 treatment was different from the control and D25 treatment (Tukey: $p < 0.005$). A negative correlation was observed for *A. minutissimum* density in the PCA axis-1 scores ($r = -0.78$, $p = 0.0028$). Although *Nitzschia palea* density was progressively reduced with increasing dilution, it remained a descriptor species in the periphyton. *N. palea* density was significantly reduced by 15.6-fold and 13.1-fold in the T50 and T75 treatments, respectively. *N. palea* density was significantly different between treatments (ANOVA one way: $F = 11.33$; $p = 0.003$). The *N. palea* density in control was significantly different from the D50 and D75 treatments (Tukey: $p = 0.005$).

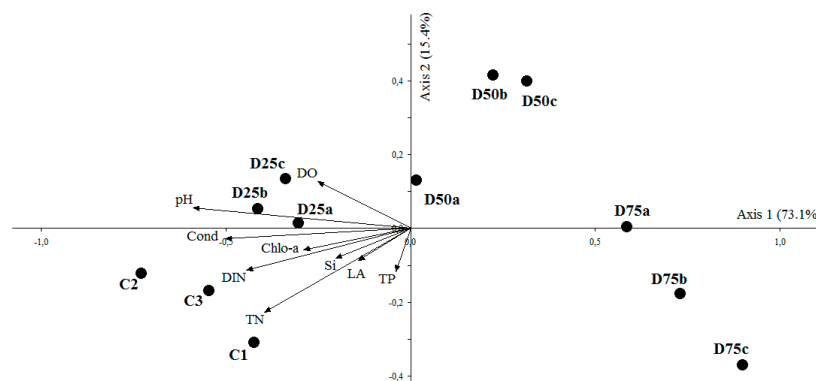


Figure 5. PCA of environmental variables in treatments in the dilution experiment (C control, D25 D25 treatment, D50 D50 treatment, D75 D75 treatment). Vectors: Cond conductivity, DO oxygen dissolved, pH pH, TN Total Nitrogen, DIN dissolved inorganic nitrogen, TP Total Phosphorus, Si Silicate, LA Light Attenuation and Chlo-*a* Chlorophyll-*a*. *PCA de las variables ambientales en los tratamientos del experimento de dilución (C control, D25 tratamiento D25, D50 tratamiento D50, D75 tratamiento D75). Vectores: Cond conductividad, DO oxígeno disuelto, pH pH, TN nitrógeno total, DIN nitrógeno inorgánico disuelto, TP fósforo total, Si Silicato, LA atenuación de luz y Chlo-a Clorofila-a.*

DISCUSSION

Enrichment experiment

We found the positive effect of isolated and combined P enrichment on algal biomass and density in the periphyton. AFDM:Chlorophyll ratio also indicated an increase in the algal component in the periphyton in high P availability. Thus, the P was a primary determining factor for algal growth in the periphyton, as evidenced in other local reservoirs (Ferragut & Bicudo, 2010, Santos & Ferragut, 2013, Lambrecht et al., 2019). Regarding environmental conditions, the isolated and combined P enrichment caused an increase in light attenuation and phytoplankton chlorophyll-*a*, while the N:P molar ratio was significantly reduced (N:P > 19). Meanwhile, the light availability had little or no effect on periphyton biomass and algal density since the increase in P availability increased the light attenuation. The phytoplankton is characterized by rapid nutrient uptake, while periphyton is typically characterized by nutrient storage (Hwang et al., 1998).

Therefore, the results evidenced that an imbalance between N and P (N:P < 100) and suggested the competition for P with phytoplankton in a shallow hypereutrophic reservoir.

Our findings showed that the algae community's dominance index in the periphyton was slightly reduced with increased dissolved N and P availability. However, the periphytic algal community structure remained simplified as in the control. In tropical reservoirs, eutrophication is one of the main drivers of diatom assemblage homogenization, showing a significant loss of species over time (Wengrat et al., 2018). Our findings showed that the periphytic algae community structure was characterized by the high density of *Nitzschia palea* in all treatments. However, the combined or isolated P addition increased the *N. palea* density when compared to N+ treatment and control. We found a positive response of *N. palea* even in the P+ treatment, where the condition was N-limiting (N:P = 1.4) and the DIN concentration was reduced (204.4 µg/l). *Nitzschia palea* has been described as an indicator of eutrophic and hypereutrophic conditions (Salomoni

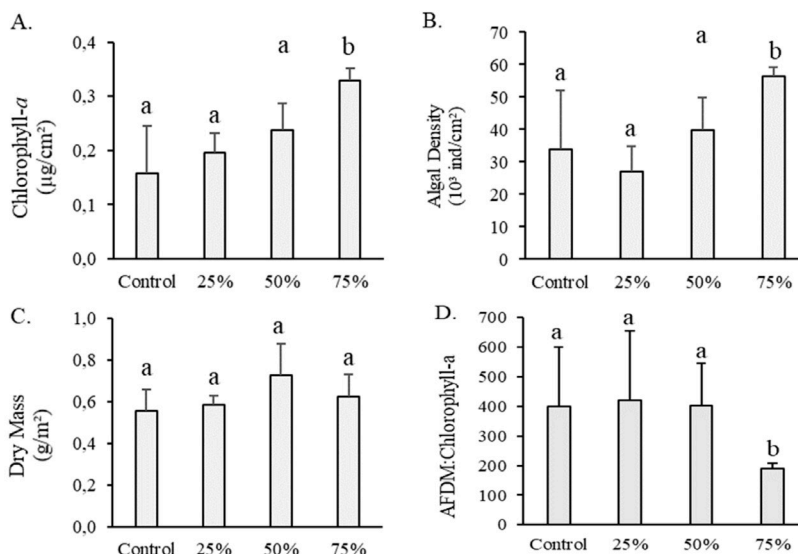


Figure 6. Chlorophyll-*a*, algal density, dry mass and AFDM:Chlorophyll-*a* ratio in the periphyton on artificial substrate ($n = 3; \pm SD$) in the dilution experiment (25 %, 50 % and 75 % dilution). The same letters above bars indicate no significant difference between treatments (Tukey: $\alpha > 0.05$). *Clorofila-a*, *densidad de algas*, *masa seca* y *relación AFDM:Clorofila-a* en el perifiton sobre sustrato artificial ($n = 3; \pm SD$) en el experimento de dilución (dilución al 25 %, 50 % y 75 %). Las mismas letras encima de las barras indican que no hay diferencias significativas entre los tratamientos (Tukey: $\alpha > 0.05$).

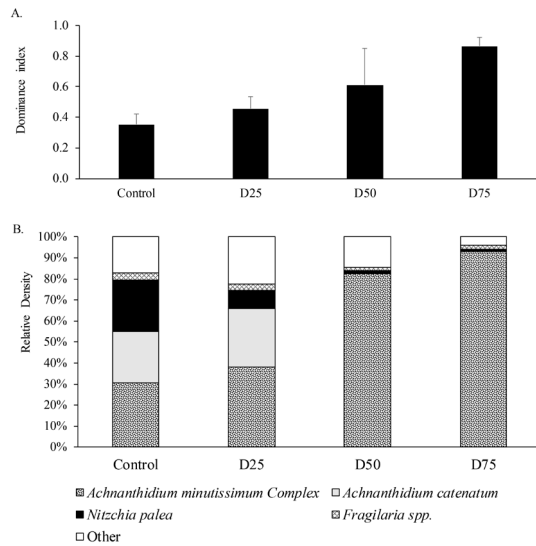


Figure 7. Dominance index and (A) and descriptor species density (B) in the periphyton ($n = 3$) on artificial substrate in the dilution experiments. Índice de dominancia y (A) y densidad de especies del descriptor (B) en el perifiton ($n = 3$) sobre sustrato artificial en los experimentos de dilución.

et al., 2006; Potapová & Charles, 2007, Trobajo et al., 2009) and has been associated with increased P concentrations (Ferragut & Bicudo, 2010). However, Machado et al. (2016) evaluated the metabolic perspective on ecological plasticity, demonstrating that *N. palea* can survive in a wide range of habitats (Machado et al., 2016). Additionally, isolated and combined P enrichment stimulated the growth of *Coelastrum asteroideum*. This species appears to respond positively to increase in nutrient availability (Amaral et al., 2020) and, still, it can grow optimally in urban wastewater (Úbeda et al., 2017). Our findings showed a significant change in the periphyton structure due to enrichment. However, species with adaptive strategies for the hypereutrophic conditions remained in the community during the short-term experiment.

Despite occur in all treatments, grazing on periphyton could influence the community structure. However, a previous experimental study has shown that zooplankton does not exert grazing pressure on the phytoplankton and epipelon (Amaral et al., 2020). In the eutrophic waters, the zooplankton grazing on phytoplankton is size-spe-

cific, which can explain the weak top-down control on the algal community (Wong et al., 2016). The classic control of phytoplankton by large zooplankton is not commonly observed in warm lakes (Jeppesen et al., 2007). Therefore, our results suggested that isolated and combined N and P enrichment changed the algal community structure in periphyton, mainly the algal biomass density.

Nutrient dilution experiment

Our findings showed that only the 75 % dilution had a positive and significant effect on biomass and algal density in the periphyton when compared to control. The dilution reduced the nutrient availability and improved the stoichiometric N and P balance, which was close to 16. In addition, the light attenuation was significantly reduced at different dilution levels. Therefore, in the treatment with 75 % dilution, the increase in light availability and the decrease in the P-limitation promoted the greater development of periphyton. According to Amaral et al. (2020), the dilution of eutrophic water resulted in a progressive reduction in the phytoplankton biomass, which decreases by > 50 % in the T75 treatment. In eutrophic lakes, periphyton development is severely hampered due to the strong phytoplankton-related competition for nutrients and shade (Romo et al., 2007, Borduqui & Ferragut, 2012). Therefore, besides reducing the competition for P, the decrease in phytoplankton biomass due to eutrophic water dilution increased the light availability for periphyton, promoting improved community development.

Regarding the periphyton structure, *Achnanthydium minutissimum* Complex was dominant with 50 % and 75 % de dilution, where it represented 82.5 % and 93.0 % of the total density. Thus, we found an increase in the dominance index with the nutrient dilution. In addition to the reduction in nutrients, there was the reduction of the N:P ratio, phytoplankton chlorophyll-*a*, light attenuation, which allowed the success of *A. minutissimum* complex. These species may respond to both increased and decreased nutrient availability and may have different ecological roles (Potapová & Charles, 2007, Potapová & Hamilton, 2007). However, *A. minutissimum* complex is R-strategists, which was characterized by small size and

high reproduction rates (Biggs et al., 1998, Potapová & Charles, 2007). Once the nutrient dilution acts as a disturbance in the eutrophic environment during the long term (Verbeek et al., 2018), the dilution process can increase the competition for a nutrient-limited among algae communities. These characteristics mentioned above consequently favoured the high density of *Achnantheidium minutissimum* Complex observed in the dilution treatments compared to Control. In contrast to the enrichment experiment, we observed a drastic reduction in the *Nitzschia palea* density with 50 % and 75 % de dilution. Like in the enrichment experiment, the periphyton structure had a significant change, however, nutrient dilution acted as a disturbance favoring r-strategist species.

Achnantheidium minutissimum Complex was dominant in treatments with 50 % and 75 % dilution and co-abundant with *Achnantheidium catenatum* in the control and D25 treatment. These species are commonly reported in floristic and ecological studies worldwide (Marquardt et al., 2017), including in the reservoirs of the study area (Borduqui & Ferragut, 2012, Fonseca, et al., 2014). Regarding the habitat, species of *A. minutissimum* complex are found globally in the periphyton, especially in the benthic environment (Potapová & Hamilton, 2007). In contrast, *A. catenatum* is more commonly found in plankton (Hlúbiková et al., 2011), however, the species may be abundant in phytobenthos in the river (e.g., Blanco et al., 2010) and reservoirs (e.g., Zorzal et al., 2017). Considering the fixation strategy to the periphytic matrix, algae are classified as firmly adhered and loosely adhered (Sládecková & Sládeček, 1964, 1977). *A. minutissimum* is an adnate nonmotile diatom and, therefore, it is firmly attached to the periphytic matrix. In contrast, *A. catenatum* must be loosely adhered to the matrix due to the absence of an adhesion structure. Thus, both species are in the periphyton, independently of the matrix adhesion strategy. In addition, studies indicate that the species of the *A. minutissimum* Complex prefer environments with low levels of phosphorus (e.g., McCabe & Cyr, 2006, Potapová & Hamilton, 2007). On the other hand, *A. catenatum* is predominantly associated with eutrophic environments (Marquardt et al., 2017, Zorzal et al., 2017). In the reservoir studied here, *A. catena-*

tum was found of a marked eutrophication phase in a paleolimnological study (Costa-Böddeker et al., 2012). Therefore, our results are supported by the regional and international literature, which reported *A. minutissimum* Complex is more associated with the oligo – mesotrophic condition and *A. catenatum* with eutrophic conditions.

In the studied reservoir, Amaral et al. (2020) observed that oligotrophication (50 % and 75 %) increased the zooplankton total density, but the community still did not exert a strong grazing pressure on the algal assemblage. Studies reported weakness of top-down control in phytoplankton by zooplankton reported in eutrophic waters (Jeppesen et al., 2007, Wong et al., 2016) and in the reservoir studied here (Amaral et al., 2020). Similarly, our results suggested that the herbivory was not decisive for periphyton responses. Thus, the dilution of eutrophic water caused the changes in nutrient and light availability which promoted differences in periphytic algae responses.

In mesocosm experiments, periphyton responses may sometimes not be representative of natural conditions, especially species-level responses. The responses of the algal community in the periphyton on artificial substrate may not be representative of the response of the natural community, especially on macrophytes (Vadeboncoeur et al., 2006). However, in the case of the reservoir studied here, previous observational studies have reported the dominance of Bacillariophyceae in the dry period, especially with high abundances of *Nitzschia palea* and *Achnantheidium minutissimum* Complex (Borduqui et al., 2008, Borduqui & Ferragut, 2012). Therefore, periphytic algal species abundant in treatments are commonly found in the studied reservoir.

Overall conclusions

Based on two mesocosm experiments, we found that reducing the N:P ratio by reducing P-limitation (P addition) and diluting eutrophic water improved algae growth in the periphyton. Our results suggest that major environmental changes are necessary for the development of a heterogeneous algal community structure in the periphyton, as observed in the mesotrophic reservoir in the study area (Santos et al., 2013, Pelegrini &

Ferragut, 2018). We concluded that the reduction of shading and P-competition are fundamental requirements for the establishment of periphyton in the studied hypereutrophic reservoir. After a restoration process that includes reducing competition for P with phytoplankton, the periphyton can become an important primary producer and contribute to biodiversity in eutrophic reservoirs. We highlight additional studies on nutrient enrichment and dilution effects on periphyton on natural substrates are needed to better support future reservoir restoration processes.

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