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REMOVAL OF NITROGEN AND PHOSPHORUS BY MICROBIAL STIMULATION IN A MESOCOSM OF EUTROPHICATED WATER

Remoción de nitrógeno y fósforo por estimulación microbiana en un mesocosmos de agua eutrofizada

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ABSTRACT

With the accelerated industrialization and expanding urbanization of today, healthy water resources are becoming increasingly important. However, changes in environmental conditions and diverse anthropogenic activities lead to water contamination, which is a global concern. The use of water reservoirs to sustain domestic, agricultural, and industrial activities alters the water cycle, while non-treated effluents and the lack of hydric intake into reservoirs increase the concentration of macronutrients in these systems, such as nitrogen (N), phosphorus (P), carbon (C), calcium (Ca), and sulfates, thereby raising the probability of eutrophication problems. This leads to the rapid multiplication of plankton, resulting in the variation in dissolved oxygen (DO), the deterioration of water quality, and the death of organisms inhabiting these waters, further affecting the utility value of their resources. In this study, the removal of N and P was evaluated in a sample of eutrophicated water from a dam through the bio-stimulation of the native population in a mesocosm with controlled aeration for 25 days, achieving removals of 99 and 95% of the total N and P, respectively. These results highlight the importance of stimulating native microorganisms by increasing the OD, to improve biological treatments for nutrient removal; additionally, the importance of using systems that simulate the environmental conditions of water bodies to study their biotreatability is shown.

Palabras clave: microorganismos nativos, bioestimulación, macronutrientes, bio-tratabilidad, oxígeno disuelto.

RESUMEN

Con la acelerada industrialización y la creciente urbanización, los recursos hídricos sanos son cada vez más importantes. Sin embargo, los cambios en las condiciones ambientales y las actividades antrópicas generan contaminación del agua, lo cual genera preocupación a nivel global. El uso de reservas de agua para sostener actividades domésticas, agrícolas e industriales altera el ciclo de este recurso, mientras que las descargas no tratadas y la falta de captación hídrica en los embalses aumenta la concentración de macronutrientes, como nitrógeno (N), fósforo (P), carbono (C), calcio (Ca) y sulfatos. Esto incrementa la probabilidad de que se presenten problemas de eutrofización, provocando que el plancton se multiplique rápidamente y cause variación del oxígeno disuelto (OD), deterioro de la calidad del agua y muerte de los organismos que ahí

habitan, afectando además el valor de uso de estos recursos hídricos. En el presente trabajo se evaluó la remoción de N y P en una muestra de agua eutrofizada proveniente de una presa, mediante la bioestimulación de la población nativa, en un mesocosmos con aireación controlada por un periodo de 25 días, logrando una remoción del 99 y el 95 % del N y P, respectivamente. Estos resultados destacan la importancia de estimular microorganismos nativos a partir del aumento en el OD, con el fin de mejorar los tratamientos biológicos relacionados con la remoción de nutrientes. Adicionalmente, se muestra la importancia de utilizar sistemas que simulen las condiciones ambientales de los cuerpos de agua para estudiar su biotratabilidad.

INTRODUCTION

Water is an essential resource for all living organisms and is utilized for a wide range of socioeconomic activities (Bhattacharya et al., 2018). However, water resources have been threatened by increasing population growth and industrial activities, affecting the availability of clean water for various human uses, posing environmental and health hazards, and incurring a financial burden for the community and the government (Nelliyat 2016). Within water pollution problematics, eutrophication is the enrichment of nutrients, especially phosphorus (P) and nitrogen (N), in slow-flowing waters like lakes, rivers, and dams; it is characterized by the excessive growth of phytoplankton, resulting in the decrease of water quality and the dissolved oxygen (DO) variation (Russel et al. 2020, Astuti et al. 2022). N and P are not the only nutrients associated with eutrophication, but they have shown the highest impact on water quality; the concentrations of carbon (C), calcium (Ca), and sulfates can also influence this phenomenon (Russel et al. 2020).

While the underlying causes of eutrophication are well understood, mitigating its effects remains challenging because of the complex pollution present in water bodies and the intricate interplay of numerous factors therein caused by the complex reactions involved, as different physical, chemical, and microbial processes interact simultaneously (Dodds and Whiles 2020). One of the factors that significantly affects eutrophication is aeration, as it directly influences the levels of DO in the water and the activity of microbial communities involved in nutrient cycling (Smolders et al. 2006).

Although various physical and chemical technologies have been established for water treatment, they present limitations, including their complex nature and relatively slow implementation pace, the generation of significant volumes of potentially harmful byproducts, and considerable expenses. Consequently, there has been a notable trend in surface water remediation towards decentralized and sustainable approaches. These latter alternatives can offer economically viable solutions with reduced maintenance costs, along with their remarkable renewal ability (Nelliyat 2016).

Microbial communities play a crucial role in nutrient removal within eutrophicated systems, with C/N and P/N ratios being critical for regulating microbial activity in these environments. A high C/N ratio promotes catabolic processes, as N can become the limiting nutrient, leading to growth restriction and the increment of catabolism to fill metabolic needs. In contrast, when the C/N ratio is low and close to cellular requirements, microorganisms can perform anabolic processes through the assimilation of C and N, thereby fostering microbial growth (Guo et al. 2020, Khan et al. 2024). Regarding the N/P ratio, it is also crucial for microbial growth (Wierzchowska et al., 2021), influencing the composition of microbial communities and their functional capabilities (Feng et al., 2023). Specifically, P-accumulating organisms (PAO) can store P intracellularly in excessive amounts and release it under anoxic conditions (Boyd 2020, Dai et al. 2022). In parallel, denitrifying bacteria contribute to N removal through a series of metabolic processes that convert nitrate (NO_3^-) into N gas (N_2), effectively removing bioavailable N from the ecosystems (Smolders et al. 2006). The combined action of these two microbial groups can mitigate nutrient overloads, promoting ecological balance and improving water quality in eutrophicated systems (Dai et al. 2022).

The nutrient removal capability of a stimulated native population from an eutrophicated water was evaluated by modulating DO in a mesocosm to simulate natural conditions while isolating the system from external disturbances (Ren et al. 2017). The results obtained highlight the importance of using a mesocosm approach, combined with the stimulation of a native inter-domain microbial population,

to achieve efficient N and P removal yields. Unlike traditional methods that mainly focus on single-domain systems such as microalgae, this study demonstrates that nutrient removal can be enhanced by integrating diverse microbial domains. Additionally, it is shown that a mesocosm approach facilitated the examination of the microbial processes involved in nutrient uptake, providing a realistic yet controlled environment for a better understanding of nutrient bio-removal mechanisms.

MATERIALS AND METHODS

Water sample and general experimental procedure

The general workflow of the current study is presented in **figure 1a**. The water sample was collected from the visibly eutrophicated water of Santa Catarina Dam in Querétaro, Mexico (20º 47' 17.5" N, 100º 27' 10.4" W), presumably impacted by domestic and agricultural activities (**Fig. 1b**). The sample was stored under the conditions specified by Mexican Standard NMX-AA-003-2019 (SCFI 2019) until further analysis. Experimentation began with an initial physical and chemical characterization of the water (n = 3), for which pH and conductivity measurements were performed using potentiometric methods, following Mexican Standards NMX-AA-008-SCFI-2016 (SCFI 2016) and NMX-AA-093-SCFI-2018 (SCFI 2018), respectively. These physical and chemical determinations also included total N (TN), total P (TP), DO, organic matter, biological oxygen demand (BOD_5), and chemical oxygen demand (COD); besides, a water biotoxicity test was conducted. Additionally, the presence of P-accumulating organisms (PAO) and denitrifying bacteria within the water microbial community was determined. Afterward, an aerated mesocosm was set under natural light and environmental temperature conditions, where the aeration was controlled at 12 L/min, and TN and TP removals were periodically monitored for 25 days.

Determination of the presence of PAO and denitrifying organisms

Mineral acetate medium (MMA) (g/L: $CH_3COONa \cdot 3H_2O$, 3.68; $Na_2HPO_4 \cdot 2H_2O$, 0.029; NH_4Cl, 0.057; $MgSO_4 \cdot 7H_2O$, 0.132; K_2SO4, 0.027; $CaCl_2 \cdot 2H_2O$, 0.017; HEPES buffer, 12; agar, 15) was used to prepare solid agar plates to quantify PAO. MMA was adjusted to pH 7 with 1 M NaOH and supplemented with 2 mL of trace solution (g/L: EDTA, 50; $FeSO_4 \cdot 7H_2O$, 5; $CuSO_4 \cdot 5H_2O$, 1.6; $MnCl_2 \cdot 4H_2O$, 5; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.1; H_3BO_3, 0.05; KI, 0.01; $CoCl_2 \cdot 6H_2O$, 0.05). Serial dilutions were prepared with 0.9% NaCl (w/v) from 10^{-1} to 10^{-5} and plated using the spreading plate technique, with the plates incubated at 30 ºC. After three days, colonies were picked and re-plated in MMA using the stab culture method. This process was repeated once more, and PAO were considered the ones that survived the first plating and the two subsequent transfers (Bao et al. 2007). In contrast, the Most Probable Number (MPN) technique was used for denitrifying

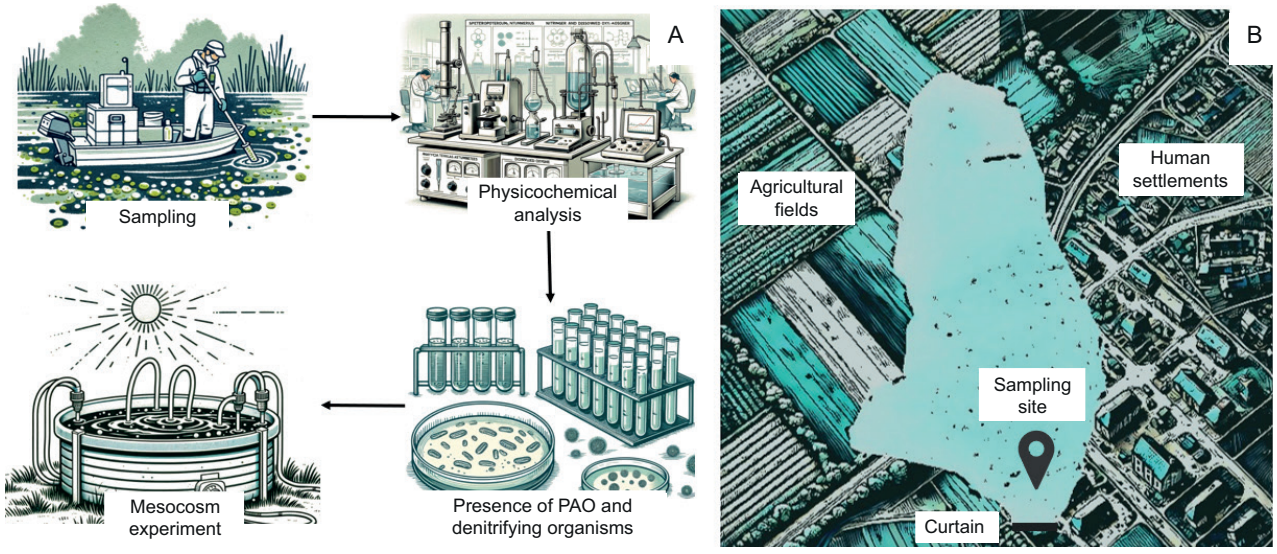

Fig. 1. (A) General workflow of the present study and (B) representation of the sampling site and its surroundings.

bacteria. To do so, 0-, 10-, and 100-times water dilutions were prepared by using 0, 9.0, and 9.9 mL of nutrient broth with 1.5 g/L of KNO_3 and 10.0, 1.0, and 0.1 mL of the water sample, respectively. Durham tubes were added, and samples were incubated at 28 ºC for 48 h. The presence of denitrifying bacteria was then determined by gas production using the MPN tables (Böllmann and Martienssen 2020).

Physical and chemical characterization of water

Dissolved oxygen (DO)

DO was assessed following the Mexican Standard NMX-AA-012-SCFI-2001 (SCFI 2001). To this end, oxygen was fixed by adding 0.33 mL of 3 M $MnSO_4$ to 50 mL water samples in Winkler bottles, followed by the addition of 0.33 mL of alkaline iodide-azide solution (g/L: NaOH, 500; KOH, 700; KI, 150; with the addition of 10 g/L of NaN_3 previously dissolved in 40 mL of distilled water). The bottles were vigorously shaken and allowed to settle. Subsequently, 0.33 mL of concentrated H_2SO_4 was added and mixed thoroughly. Afterward, the samples, with an initial amber color, were titrated with 0.025 M $Na_2S_2O_3$ until they changed to pale yellow; two drops of 20 g/L $C_6H_{10}O_5$ indicator were then used to produce a blue color, continuing the titration with 0.025 M $Na_2S_2O_3$ until the color disappeared.

Total nitrogen

TN was determined by the micro Kjeldahl method, as described in the Mexican Standard NMX-AA-026-SCFI-2010 (SCFI 2010). First, 25 mL samples in 125 mL flasks were diluted by half with distilled water, neutralized to pH 7, and treated with 10 mL of digestion reagent (g/L: K_2SO_4, 134; $CuSO_4$, 7.3; and mL/L: H_2SO_4, 134) by heating them to boiling point (containing boiling beads) until a light green color was achieved. Samples were then distilled with 10 mL of thiosulfate hydroxide solution (g/L: NaOH, 500; $Na_2S_2O_3 \times 5H_2O$, 25), and the condensates were collected in 10 mL of boric acid indicator solution (20 g/L of H_3BO_3 with 10 mL/L of indicator mix: 2 g/L of red methyl and 1 g/L of methylene blue previously dissolved in CH_3CH_2OH) until they turned emerald-green. Finally, samples were titrated with 0.006M H_2SO_4 until a violet turn.

Total phosphorus

TP was determined using a modified protocol from the Mexican Standard NMX-AA-029-SCFI-2001 (SCFI 2001). Briefly, 25 mL samples were mixed with 0.5 mL of a strong acid solution (mL/L: H_2SO_4, 300; HNO_3, 4) plus 0.2 g of $(NH_4)_2S_2O_8$. The samples were subsequently heated until the volume was reduced to 5 mL, then diluted to 15 mL with distilled water, and neutralized with 1N NaOH using a drop of phenolphthalein 1% (w/v) as an indicator. Two milliliters of ammonium molybdate solution (g/L: $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 25 g; and mL/L: H_2SO_4, 280) and five drops of stannous chloride solution (2.5 g $SnCl_2 \times 2H_2O$ in 100 mL of $C_3H_8O_3$) were added and vigorously mixed. The absorbance was finally read in a spectrophotometer (SP-UV1100 DLAB) at 690 nm after 10 min of the addition of stannous chloride.

Organic matter

Organic matter was determined by the Walkley and Black method, according to the Mexican Official Standard NOM-021-RECNAT-2000 (SEMARNAT 2002). To do so, 10 mL of each sample were digested with 10 mL of 1N $K_2Cr_2O_7$ and 20 mL of concentrated H_2SO_4 and diluted in 200 mL of distilled water; then, 5 mL of concentrated H_3PO_4 was added. Each sample was colored with five drops of diphenylamine indicator (0.5 g of $C_{12}H_{11}N$ dissolved in 20 mL of water and then mixed with 100 mL of concentrated H_2SO_4) and titrated with $FeSO_4$ 1 M until it turned to a green-brown color.

Chemical oxygen demand (COD)

COD determination followed the Mexican Standard NMX-AA-030/2-SCFI-2011 (SCFI 2011). Thus, 2 mL of the sample were placed in a digestion tube, mixed by inversion, and heated to reflux at 150 ºC for 2 h. The sample was then cooled to 60 ºC, mixed again, and cooled at room temperature. Finally, the absorbance was measured at 600 nm using a spectrophotometer (SP-UV1100 DLAB).

Biochemical oxygen demand (BOD_5)

BOD_5 was analyzed according to the Mexican Standard NMX-AA-028-SCFI-2001 (SCFI 2001). Water samples were neutralized to pH 7, and DO concentration was measured as previously described. Then, the samples were incubated at 20 ºC for five days in sealed bottles, and DO concentration was measured again after this incubation period.

Biotoxicity test

The biotoxicity test of the water was conducted using *Lactuca sativa* seeds, placing 10 seeds in a filter paper (pore size: 1 µm) inside plastic Petri dishes, which were hydrated with 3 mL of distilled water for the control or with 25, 50, 75, and 100% water sample dilutions with distilled water. The plates were sealed

with parafilm and incubated in darkness for seven days. Then, germination percentages were obtained, and root lengths were measured (Priac et al 2017). Results were analyzed using the Normalized Residual Germination (NRG) and Normalized Residual Root Elongation (NRE), proposed by Joseph et al. (2014):

$$IGN = \frac{Germ_x - Germ_{control}}{Germ_{control}} \quad (1)$$

$$IER = \frac{Elong_x - Elong_{control}}{Elong_{control}} \quad (2)$$

where $Germ_x$ and $Elong_x$ are the average germination percentage and average root elongation in the water sample, respectively, while $Germ_{control}$ and $Elong_{control}$ are the average germination percentage and average root elongation in the control, respectively. Based on these calculations, water toxicity can be classified as low (0 to –0.25), moderate (–0.25 to –0.5), toxic (–0.5 to –0.75), or highly toxic (–0.75 to –1).

Additionally, the median lethal concentration (LC_{50}) was calculated using the following formula:

$$CL_{50} = C1 + \left(\frac{C1 - G1}{G2 - G1}\right) * (C2 - C1) \quad (3)$$

where C1 is the water dilution concentration with the highest germination percentage; G1 is the germination percentage at C1; C2 is the concentration at which there is a significant decrease in germination compared to the previous concentration (>10%), and G2 is the germination percentage at C2.

Mesocosm experiment

A water sample of 40 L, collected directly from the reservoir, was placed in a circular tank of 82 L capacity immediately after sampling. Aeration at 12 L/min was continuously supplied and controlled, provided through plastic pipes with perforations every 0.5 ± 0.2 cm to ensure small bubble sizes. The mesocosm was set inside a greenhouse with controlled environmental illumination and temperature for 25 days, without replenishing the water lost through evaporation. During this period, the daily illumination average in Querétaro City was 100 000 lux, with daylight hours typically between 7:00 and 18:46 LT (photoperiod 11/13 h). The temperature in the greenhouse was around 30 ºC during the day and 10 ºC at night. Samples were taken every seven days, filling 250 mL flasks with 200 mL of water for further determinations (Guy-Haim et al. 2017). Additionally, the total biomass was determined by the dry weight method at the beginning and the end of the experimentation, centrifuging 40 mL of water at 5000 × g for 10 min and placing the pellets in aluminum trays at a constant weight, letting them dry in an oven at 105 ºC for 24 h; differences in weights were considered to calculate biomass accumulation (Lu et al. 2017). N and P removals in water were assessed after withdrawing biomass by centrifugation; in contrast, C/N initial ratio in the system and C uptake were calculated considering biomass contribution, where C was determined from organic matter concentration, following the Mexican Official Standard NOM-021-RECNAT-2000 (SEMARNAT 2002):

$$\text{Organic matter} = \text{Organic C} \times 1.724 \quad (4)$$

Mathematical model of nutrient consumption

The N and P removal data experimentally obtained was analyzed using the model reported by Haro and Perales (2015), which may provide a predictive framework for nutrient consumption by microorganisms, using the equation:

$$S = \frac{\left(\frac{X_0}{Y_0} + S_0\right)(S_0 - S_{na}) - S_{na}\left(S_0 - \left(\frac{X_0}{Y_0} + S_0\right)\right) e^{\mu t}}{(S_0 - S_{na}) - \left(S_0 - \left(\frac{X_0}{Y_0} + S_0\right)\right) e^{\mu t}} \quad (5)$$

where S represents the N or P uptake at a specific time; S_0 is the initial nutrient concentration in water (excluding the biomass content); S_{na} is the non-assimilable substrate concentration determined with the final concentration in the water; X_0/Y_0 is the ratio of biomass concentration to nutrient concentration or the nutrients in the biomass at t_0; μ is the maximum nutrient uptake rate, and t is time in days. The term μ was calculated as follows:

$$\mu = \frac{ln\left(\frac{S_{t0}}{S_\mu}\right)}{t_\mu - t_0} \quad (6)$$

And the term X_0/Y_0 can be calculated by:

$$S_{t0} = \frac{X_0}{Y_0} + S_0 \quad (7)$$

where S_{t0} is the total initial concentration of the nutrient in the medium (considering the nutrients dissolved in the water and within the biomass), S_μ is the concentration of the nutrient where the highest removal was observed, S_0 is the initial concentration of the nutrient in water (excluding the biomass content), t_μ is the time where the highest removal was observed, and t_0 is the initial time.

Statistical analysis

Minitab 19 was used for the statistical analyses presented in this work. Different letters represent data groups that are significantly different ($p \leq 0.05$).

RESULTS AND DISCUSSION

Initial water characterization

The initial characterization of the water sample from the reservoir is presented in **table I**, which may be considered crucial information to understand its quality status and potential biotreatability. The recorded pH (9.25) indicates an alkaline condition, whereas previously it has been reported that pH values above 9 can harm most aquatic organisms, affecting their metabolism and reproduction (Dodds and Whiles 2020). Additionally, high pH values can influence the solubility of nutrients and heavy metals, increasing their availability and potential toxicity. The measured conductivity (1002 µS/cm) suggests a high presence of dissolved ions, which may indicate pollution from anthropogenic activities, like non-treated discharges or runoffs carrying dissolved salts, minerals, and pollutants from the surrounding environment into the reservoir (Dodds and Whiles 2020). Conductivity can also be correlated with nutrient concentrations, such as nitrates and phosphates, which are indicators of organic contamination that can exacerbate eutrophication (Boyd 2020).

The DO (14.04 mg/L) is at the upper limit of established water quality guidelines. According to EPA (2023), DO levels in natural surface waters generally range between 5 and 14 mg/L, indicating good aeration and a high autotrophic capability, primarily due to the photosynthetic activity of the microalgae and cyanobacteria present. However, high DO levels can also indicate eutrophic conditions, which may fluctuate rapidly and affect the stability of ecosystems. Furthermore, a high DO content may indicate water movement, such as when a dam gate is opened, leading to increased aeration and mixing of the water column and the sediment. This water movement can enhance oxygen diffusion and distribution throughout the reservoir, contributing to elevated DO levels (Nguyen et al. 2016). Direct water quality indicators, such as BOD_5 and COD, reflect the level of organic contamination in the water (Bhattacharya et al. 2018, Boyd 2020). The BOD_5 was 86.40 mg/L, exceeding typical surface water regulatory limits, which range from 3 to 6 mg/L (EPA 2023). The BOD_5 elevated value suggests a high oxygen demand for microbial organic decomposition. High levels of BOD_5 can lead to hypoxia, as the decomposition of organic matter depletes oxygen, adversely affecting aquatic life (Ansari and Gill 2014).

COD, on the other hand, measures the total amount of substances that can be chemically oxidized, providing an overall indication of the oxidative potential of water. It has been reported that good-quality surface waters typically have COD values below 50 mg/L, with values exceeding 100 mg/L often indicating significant pollution from anthropogenic discharges (Ansari and Gill 2014, Boyd 2020). Contrastingly, the Official Mexican Standard NOM-001-SEMARNAT-2021 (SEMARNAT 2022) sets a maximum permissible COD limit of 140 mg/L for discharges into reservoirs. Thus, the COD value obtained (182.30 mg/L) indicates the existence of complex organic compounds that require significant oxidation to decompose (Sharma and Walia 2015). In addition, the total organic matter load (2726.29 mg/L) reaffirms the considerable organic pollution, probably from various sources, such as discharges of untreated wastewater, besides detritus or algal exudates that may contribute to this

TABLE I. INITIAL CHARACTERIZATION OF WATER.

Parameter	Results	Units
pH	9.25	—
Conductivity	1002.00	µS
Dissolved oxygen	14.04 ± 3.02	mg/L
Biochemical oxygen demand$_5$	86.40 ± 1.21	mg/L
Chemical oxygen demand	182.30 ± 27.34	mg/L
Organic matter	2716.29 ± 419.35	mg/L
Total nitrogen	88.34 ± 22.60	mg/L
Total phosphorus	1.44 ± 0.01	mg/L
Biotoxicity	86.36 ± 0.02	% CL_{50}
Denitrifying organisms	140 ± 7	Most probable number for 100 mL
Polyphosphates accumulation organism	36.66 ± 5.13	Colony forming units/mL

high organic matter content (Boyd 2020, Dodds and Whiles 2020).

The concentration of TN (88.34 mg/L) indicates a strong N contamination, being approximately three times higher than what is indicated in the Official Mexican Standard NOM-001-SEMARNAT-2021 (SEMARNAT 2022). TP (1.44 mg/L), although within the regulatory limits established in the standard, is still considerably high and can significantly contribute to eutrophication if compared to international regulations, where it is stated that TP concentrations exceeding 0.1 mg/L indicate an eutrophicated system (EEA 2018). High N levels can cause eutrophication, promoting excessive algal growth and affecting water quality and the health of the ecosystems (Russel et al. 2020). P is considered a critical limiting nutrient in aquatic ecosystems, and its excess can also trigger algal blooms (Dodds 2006). The toxicity indices NRG (equation 1) and NRE (equation 2) were found to be –0.40 and –0.51, respectively, when using the water sample at 100% concentration, placing the water quality in the moderately toxic and toxic categories, respectively. In addition, this toxicity test yielded an LC_{50} of 86.36% (equation 3), suggesting that the water represents a potential risk to organisms, like sublethal effects that may include reduced growth rates, impaired reproduction, and behavioral changes, which can disrupt aquatic ecosystems and affect the health of higher trophic levels (Guy-Haim et al. 2017, Hope et al. 2020, Srivastava et al. 2022). Unlike the NRG, which only measures germination in binary terms (i.e., whether it occurs or does not) (Joseph et al. 2014, Haro and Perales 2015), NRE (the elongation metric) may be considered a more sensitive indicator of environmental toxic stress, as it reflects the extent of root growth inhibition, providing a nuanced measure of sub-lethal effects. Thus, the system is indeed experiencing significant toxicological impacts (Joseph et al. 2014) probably due to nutrient and organic pollution stress.

Regarding microbial diversity, consortia may include microalgae, archaebacteria, bacteria, and fungi, whose proliferation may be linked to nutrient availability and water toxicity. Specifically, selective culture media tests confirmed the presence of relevant microorganisms for the removal of N and P. The presence of denitrifying organisms (140 MPN/100 mL) indicates potential denitrifying activity that is essential for reducing nitrates to gaseous forms like N_2 in the water (Böllmann and Martienssen 2020). Additionally, the presence of PAO (36.6 CFU/mL) in the system suggests the presence of biological P removal processes; that is, the microbial community includes organisms capable of sequestering P. The presence of both types of microorganisms has the potential to mitigate the impact of eutrophication by reducing N and P, as has been reported by different authors (Zhang et al. 2013, Böllmann and Martienssen 2020, Srivastava et al. 2022).

Analysis of the mesocosm

The visual transformation within the experimental mesocosm over the 25 days assessed is illustrated in **figure 2**. Initially, on day 0 (**Fig. 2a**), the water exhibited high turbidity, characterized by the significant presence of suspended particles and a green hue that can be attributed to the presence of microalgae and cyanobacteria (Ansari and Gill 2014). A notable reduction in turbidity was observed throughout the experiment, as on days 7 (**Fig. 2b**) and 14 (**Fig. 2c**)

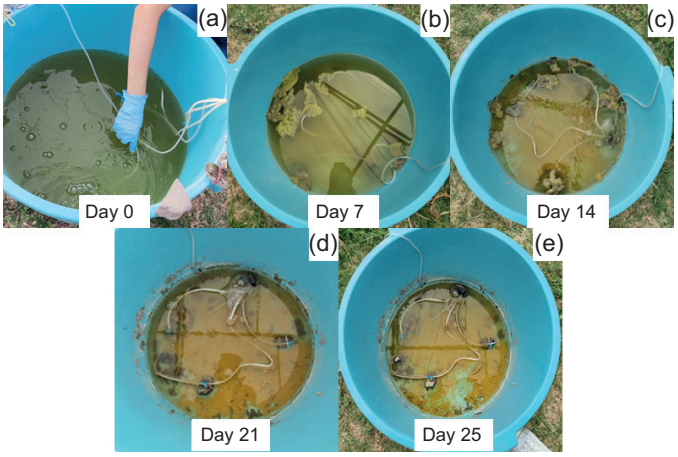

Fig. 2. (a-e) Visual evolution of the experimental mesocosm from day 0 to day 25.

the turbidity significantly decreased. By days 21 and 25 (**Fig. 2d**, **e**), the water became clearer, indicating a steady decline in turbidity along the experiment. This reduction in turbidity likely points to particle sedimentation processes (Hope et al. 2020). Besides, turbidity reduction serves as a critical indicator of water quality improvement, as the decline in particulate matter and increased water clarity are often linked to microbiological activity and the sedimentation of organic detritus (Bhattacharya et al. 2018, Hope et al. 2020).

In addition, by day 7 (**Fig. 2b**), the formation of a biofilm on the surface was evident. By day 14 (**Fig. 2c**), biofilms developed on both the upper and lower parts of the container, with a more pronounced accumulation at the bottom. This trend continued, and by days 21 (**Fig. 2d**) and 25 (**Fig. 2e**), biofilm formation was observed only at the bottom. The biofilm may have consisted of a complex microbial community, potentially comprising diverse microorganisms such as microalgae, bacteria, fungi, and protozoa; these inter-domain interactions create gradients of energy sources and chemicals, influencing the functionality of the biofilm, as observed in similar studies (Saini et al. 2023, Zhang et al. 2023). Biofilms play a crucial role in water treatment processes by adsorbing and degrading pollutants, contributing to nutrient cycling and water quality improvement through the transformation of contaminants (Dai et al. 2022, Aguilar-Aguilar et al. 2023). Additionally, they provide a protective environment for microbial communities, facilitating higher efficiencies in the removal of nutrients such as N and P (Mkpuma et al. 2024).

Since the mesocosm was exposed to the atmosphere without water inflow, the contained volume decreased over days. This reduction in volume could have affected the concentration of nutrients in the water. Therefore, N and P removals were calculated in absolute units (mg) rather than concentration (mg/L) to assess the changes in TN and TP levels accurately. This approach considered the changing volume of water in the mesocosm at each sampling time, by normalizing TN and TP to the actual water volume at the time of sampling; in this way, the registered changes can be attributed to biological and chemical processes within the mesocosm, rather than merely reflecting concentration changes caused by volume fluctuations. The system showed a significant reduction in TP and TN loads in water, as shown in **figure 3a** and **b**, respectively. Initial TP (32.9 mg) decreased 95% (to 1.70 mg) by the end of

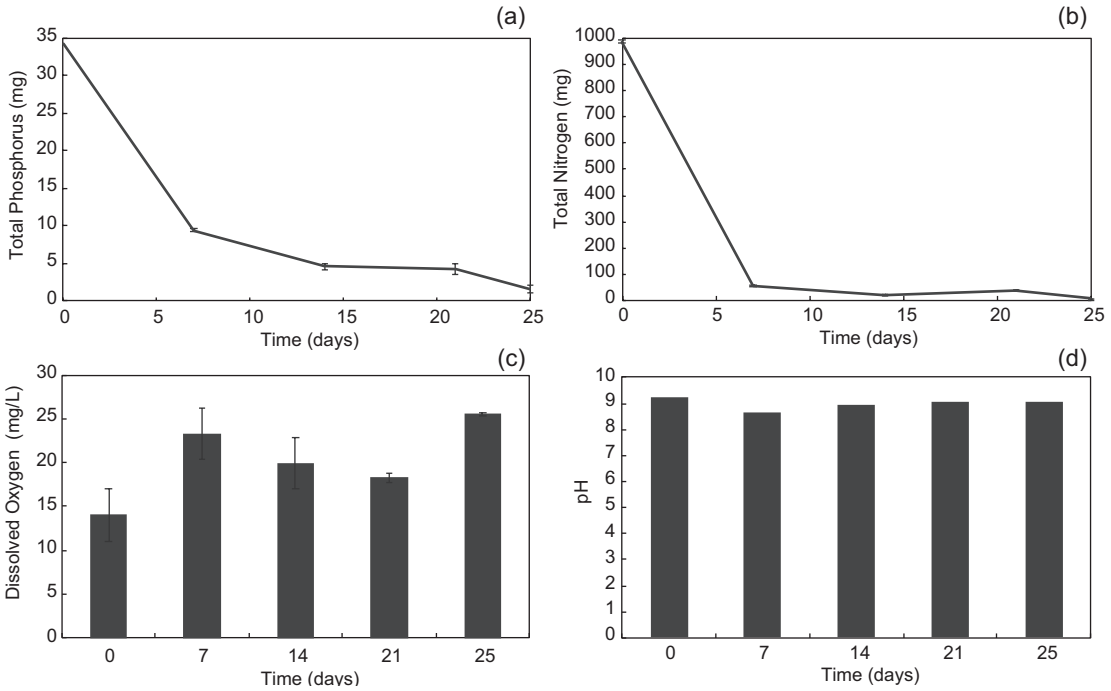

Fig. 3. (a) Kinetics of total phosphorus, (b) total nitrogen removals, (c) fluctuations of dissolved oxygen, and (e) pH in the experimental mesocosm through 25 days. The data are presented as means with their corresponding standard deviations. Groups of data that differ significantly ($p < 0.05$) are indicated by uppercase letters.

the experiment. The accelerated decrease of TP in the first seven days suggests a high P uptake rate by the microbial community, presumably by the biofilm and suspended microorganisms that were not considered to calculate nutrient removals. Similarly, the TN load dropped from an initial 943 mg to less than 60 mg within the first seven days, reaching 7 mg on day 25, indicating an effective TN removal process of 99%, likely through biomass consumption for metabolic processes like growth or nitrification followed by denitrification (Dai et al. 2022).

Nutrient uptake rates in aquatic systems can vary significantly depending on environmental conditions, nutrient availability, and the specific types of microorganisms present. Although the determination of C/N uptake can be difficult in complex systems due to the presence of diverse compounds differentially available for microorganisms (Seitzinger and Sanders 1997), it has been reported to be around 4:1 for phytoplankton (Kim et al. 2020), being also shown that this ratio reaches 10:1 under stressful environments (Kim et al. 2022) like eutrophication conditions (Chen et al. 2017). The initial C/N ratio in the system was 18:1 (with biomass), where C (equation 4) and N initial loads were 63 022.97 and 3533.60 mg, respectively. Only 0.52% of N was present at the end of experimentation, and, considering the reported C/N uptake ratio of 10:1 in eutrophicated waters, 55.77% of C (27 871.47 mg) might have been consumed (that is, approximately 44.22% of C remained in the system).

The DO level, influenced by the 12 L/min aeration, initially increased from 14.04 to 23.24 mg/L by day 7 (**Fig. 3c**), and a decreasing trend was observed on days 14 and 21, before another increase on day 25. It can be observed that the DO level seemed to stabilize around 20 mg/L, suggesting a balance between oxygen supply and consumption, which may indicate a stable microbial activity within the system (Bhattacharya et al. 2018, Saini et al. 2023, Zhang et al. 2023). High DO levels, above 2 mg/L, are essential for aerobic microbial processes, and levels between 4 and 8 mg/L are necessary for organic matter oxidation and nitrification (Chen et al. 2014). The pH remained relatively unchanged throughout the experiment (**Fig. 3d**), indicating a buffered system. Maintaining a stable pH is critical for the optimal activity of various microbial communities involved in nutrient cycling (Shirdashtzadeh et al. 2022).

Furthermore, the high DO levels with substantial reductions in TP and TN, suggest that the aerated mesocosm improved water quality by promoting microbial activity, such as the growth and activity of PAO and denitrifying microbial members (Aguilar-Aguilar et al. 2023). These observations suggest a beneficial synergy among the members of the biofilm, which may have facilitated nutrient cycling. Biofilms likely form microenvironments with oxygen gradients, allowing both aerobic and anaerobic processes to occur. These heterogeneous conditions support the breakdown of nutrients, pollution removal, and stabilization of water quality parameters, with a previously demonstrated effectiveness of biofilms in nutrient removal and water quality improvement (Dai et al. 2022, Saini et al. 2023).

Additionally, the total biomass at time 0 and the end of the experimental process was determined (**Fig 4**). The initial biomass was 541.67 mg/L, which increased 34% to 727.33 mg/L after 25 days. This significant increment corroborated an active microbial growth in the system (Dai et al. 2022, Aguilar-Aguilar et al. 2023), which may have influenced the DO levels and the pH (**Fig. 3**), as it is known that the photosynthesis performed by photoautotrophic microorganisms affects DO and can regulate pH (Hope et al. 2020, Srivastava et al. 2022). Biomass growth can be attributed to various factors, including nutrient availability and the environmental conditions during the experiment. That is, the constant aeration rate of 12 L/min likely improved the oxygenation of the system, favoring both the metabolic activity of aerobic microorganisms and the photosynthesis of photoautotrophs (Boyd 2020). PAOs can accumulate P under aerobic conditions, whereas denitrification is also promoted. Besides, the significant increase in biomass suggests a high rate of nutrient conversion to microbial biomass with the possible enrichment of

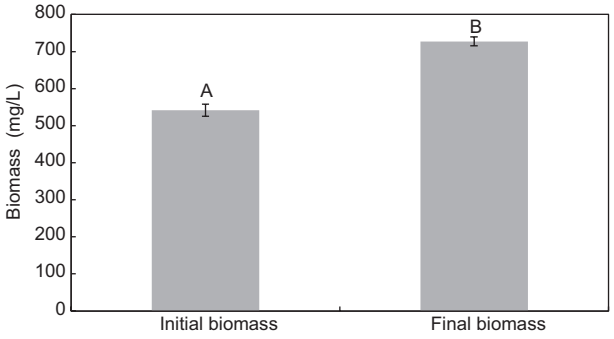

Fig. 4. Biomass quantification at the beginning and the end of experimentation. The data are presented as means with their corresponding standard deviations. Groups of data that differ significantly ($p < 0.05$) are indicated by uppercase letters.

PAOs and denitrifiers, which could have contributed to the reduction of dissolved nutrient concentrations in the water (Ansari and Gill 2014, Dai et al. 2022).

Predictive model of nutrient removal

Predictive models are crucial for understanding the dynamics of ecological systems, as they enable the simulation of processes related to nutrient cycling and removal. These models offer valuable insights into how systems respond to various factors, enabling the analysis of different scenarios related to environmental changes (Chen et al. 2014). Nutrient removal depends on microbial adaptability to ensure that these processes remain effective despite fluctuations in environmental factors (Shirdashtzadeh et al. 2022, Niu et al. 2024). Furthermore, continuous validation against experimental data and improvement of these models enhance the accuracy and reliability of the theoretical frameworks underlying nutrient removal processes (Haro and Perales 2015, Liu et al. 2023, Wang et al. 2024). One of these models, the phototreatment model (PhBT), was proposed by Haro and Perales (2015) to predict nutrient removal in discontinuous experiments by considering biomass growth and nutrient assimilation. PhBT was used in the present study to predict the removals of TN and TP (equation 5) by the native consortia in the mesocosm over 50 days.

In the theoretical model (**Fig. 5**), TP exhibited an exponential decrease of 57%, from 35 to approximately 15 mg over 25 days. This behavior indicates a rapid initial absorption, followed by a de-accelerated uptake phase as P concentration decreases and reaches a constant residual value. The experimental data also showed a rapid decrease in TP during the initial days; however, this reduction stabilized earlier (**Fig. 3a**) than predicted theoretically (**Fig. 5**). Notably, the experimental removal of TP was 2 times higher than that predicted by the model, indicating an enhanced efficiency of TP removal in the mesocosm.

In the case of TN, the removal efficiency was 5% higher in the experimental process (99%) compared to the theoretical prediction (94%). Similar to TP removal, the experimental results showed a more pronounced initial decrease in TN concentration, stabilizing at lower values earlier than theoretically predicted. This could indicate an initial phase of high denitrifying microbial activity followed by a limited availability of organic N and the emergence of other limiting factors, like micronutrients (Haro and Perales 2015). Under the predictive model, the trends for TN and TP removal remained asymptotic from day 25 to day 50.

It is important to highlight that the PhBT model has primarily been used for microalgal removal kinetics, whereas this study employed a native interdomain consortium. Thus, the accelerated experimental removal of nutrients (TN and TP) compared to the theoretical prediction may be due to the simultaneous use of different microorganisms (Dodds 2006, Bhattacharya et al. 2018). In microalgal systems, nutrient removal primarily depends on the photosynthetic activity and growth rates of these microorganisms, which typically follow a more gradual kinetic pattern (Ansari and Gill 2014). However, the relationship between the consortium members used in this study may have enhanced nutrient removal. Interactions among various microbial domains can create more dynamic and efficient nutrient cycling and removal systems. For example, bacteria can support the growth of microalgae by providing essential nutrients and growth factors, such as carbon dioxide and

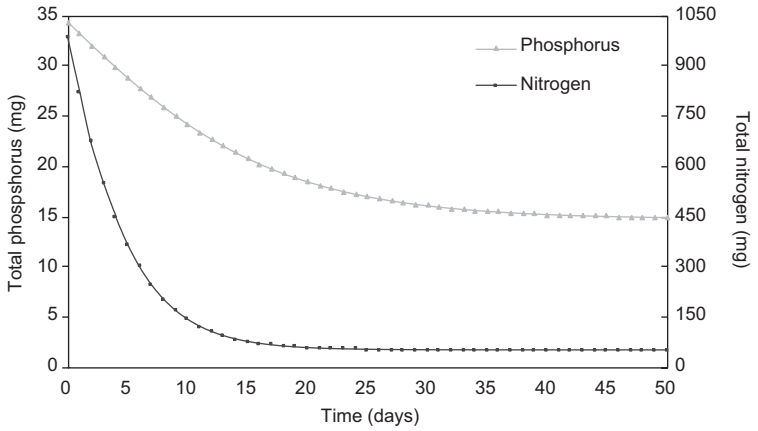

Fig. 5. Theoretical kinetics of nitrogen and phosphorus removal.

vitamins, thereby promoting the overall efficiency of photosynthesis and nutrient uptake (Ansari and Gill 2014, Boyd 2020). These relationships also enhance the resilience of the microbial community, enabling more effective adaptation to environmental changes and thereby maintaining stable and efficient nutrient removal rates (Bhattacharya et al. 2018). The presence of diverse microbial members also facilitates the production of extracellular polymeric substances (EPS), which contribute to biofilm formation and nutrient retention, providing a synergic effect that improves the general performance of the removal system (Dodds 2006, Aguilar-Aguilar et al. 2023).

Overall, the results obtained from the mesocosm study highlight the potential of using controlled environments to simulate natural systems for studying nutrient removal and improving water quality. While this study focused on nutrient removal under eutrophic conditions, specifically targeting N and P, the complexity of natural water systems could significantly impact these processes. In real-world environments, complex organic pollutants, such as hydrocarbons, pose additional challenges to nutrient cycling, as they often exhibit low solubility and require specific microbial communities for their effective degradation. Thus, these pollutants can alter microbial dynamics, potentially inhibiting or diverting the activity of the microorganisms involved in nutrient removal. Consequently, the coexistence of diverse contaminants may reduce the efficiency of bioremediation processes by introducing competing metabolic demands, complicating nutrient cycling, and posing additional barriers to water quality restoration efforts (Lizardi-Jiménez et al. 2012, Narciso-Ortiz et al. 2020). Biotechnological developments using inter-domain processes, rather than single strains or intra-domain interactions, may represent promising alternatives to enhance sustainable water treatment procedures. In addition, the generation of theoretical models to predict nutrient removal by microbial consortia seems like an imminent need to optimize these processes based on the knowledge of complex biological interactions (Haro and Perales 2015, Wang et al. 2024). In addition, characterizing the microbial consortia involved in TP and TN removals could lead to more reliable predictions and a better understanding of nutrient removal processes.

CONCLUSIONS

A controlled mesocosm environment, designed to balance ecological realism and experimental control, facilitated DO manipulation through aeration and promoted microbial nutrient removal activity (TN and TP) in eutrophicated water, achieving up to 99% TN and 95% TP removals. The enhanced nutrient reduction compared to a predictive theoretical model can be attributed to the use of a native inter-domain consortium stimulated in the system, which may significantly improve nutrient removal processes for water treatment. Future research should focus on optimizing the balance between aeration and microbial growth of keystone species to maximize efficiency.

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