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In vitro assays with *Kappaphycus alvarezii* for the bioremediation of coastal eutrophic systems in Southeast Brazil

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Abstract: The alga *Kappaphycus alvarezii* (Doty) L.M.Liao 1996 may constitute a good bioremediator for eutrophicated coastal environments. In the present research, the ability of *K. alvarezii* to remove nutrients from the water was tested in vitro, under various conditions. The algae were obtained from a commercial aquaculture in Angra dos Reis, Brazil, and large water volumes were sampled from the same area and Rodrigo de Freitas Lagoon, Rio de Janeiro, Brazil. Eight experimental sets were prepared with variable concentrations of nutrients and preweighed pieces of algae. Water samples were periodically analyzed for ammonium, nitrite, nitrate, and phosphate. Algal samples were collected at the beginning and the end of the experiment to quantify biomass growth/decay, nitrogen, and phosphorus tissue contents. The ammonium concentration in the water decreased due to preferential consumption of this nutrient by the algae in the lagoon experiment (95%), as well as oxidation. However, in the fertilized experiments, concentrations are so high that algae cannot consume measurable ammonium concentrations. The algae rapidly incorporated nutrients in their tissues 0.007 to O.158 mg P g^{-1} and 0.052 to 0.330 mg N g^{-1}); however, they reduced their concentrations to values close to natural by the end of the experiment. Phosphorus removal rates were elevated at the beginning of the lagoon microcosm (52%), but no removal was observed in the other microcosms. Nitrogen removal rates were elevated at the end of all microcosms (up to 46%), except in the control. It was concluded that the use of *K. alvarezii* as a bioremediator in coastal lagoons is possible.

Key words: Coastal environments, Eutrophication, Microcosms, Nutrient absorption rate, Green Algae.

Experimentos in vitro com *Kappaphycus alvarezii* para a biorremediação de sistemas costeiros do Sudeste do Brasil. Resumo: A alga *Kappaphycus alvarezii* (Doty) L.M.Liao 1996 pode ser considerada um bom biorremediador para ambientes costeiros eutroficados. Na presente pesquisa, a capacidade de *K. alvarezii* em remover nutrientes da água foi testada in vitro, sob variadas condições. As algas foram coletadas em cultivos comerciais em Angra dos Reis, Brasil e foi amostrados grandes volumes de água do próprio local e da Lagoa Rodrigo de Freitas, Rio de Janeiro, Brasil. Oito experimentos foram preparados com variadas concentrações de nutrientes, e pedaços de algas pré-pesadas. Amostras de água foram periodicamente

analisadas para amônio, nitrito, nitrato e fosfato. Amostras de alga foram coletadas no início e no fim dos experimentos para quantificar o crescimento ou decaimento de biomassa, a concentração de nitrogênio e fósforo dos tecidos. No experimento da laguna, a concentração de amônio na água caiu devido ao consumo preferencial deste nutriente pelas algas (95%), assim como devido à oxidação. Não obstante, nos experimentos fertilizados, as concentrações são tão elevadas que as algas não foram capazes de consumir concentrações mensuráveis de amônio. As algas foram capazes de incorporar rapidamente nutrientes nos seus tecidos (de 0.007 a O.158 mg P g^{-1} e de 0.052 a 0.330 mg N g^{-1}), contudo suas concentrações reduziram a valores próximos aos naturais ao final do experimento. As taxas de remoção de fósforo foram elevadas no início do experimento da Lagoa Rodrigo de Freitas (52%), mas não foi observada remoção nos outros microcosmos. As taxas de remoção de nitrogênio foram elevadas no final de todos os microcosmos (até 46%), exceto no controle. Conclui-se que o uso da *K. alvarezii* como biorremediador em ambientes costeiros é possível.

Palavras-chave: Ambientes costeiros, Eutroficação, Microcosmos, Taxa de absorção de nutrientes, Algas verdes

Introduction

Marine macroalgae, especially red algae, are considered important renewable marine resources for the production of various natural products, such as food, animal feed, biostimulants, and hydrocolloids (Rakhasiya *et al.* 2023). *Eucheuma spp.*, *Kappaphycus alvarezii,* and *Gracilaria spp* are among the most cultivated seaweeds for the production of carrageenans, and agar (Debbarma *et al.* 2022). The production of *K. alvarezii* has consistently increased since the beginning of its commercial cultivation due to growing demand (Narvarte *et al.* 2022). In addition to their use in the food, cosmetic, and pharmaceutical industries, macroalgae can be applied for bioremediation of aquatic environments and can be a natural fertilizer in agriculture. The relevance of algae as a source of renewable energy in the production of biofuels can also be emphasized (Biris-Dorhoi *et al.* 2020).

According to FAO (2022), in the year 2020, the total production of aquiculture was 87.5 million tons of aquatic animals and 35.1 million tons of algae for both food and non-food products, representing a 1.4% increase from 2019. In 2020, production increased in countries such as China and Japan, while cultivation decreased in Southeast Asia and the Republic of Korea. Asian countries were the largest producers, accounting for 97% of the total algae production in 2020. China contributed 58% of the total, becoming the leading producer, followed by Indonesia with 27% and the Republic of Korea with 5%. *K. alvarezii* is a native alga from the Philippines, and one of the most well-known and economically valuable organisms for carrageenan production which led to its introduction in various countries (Das *et al.* 2021). In Brazil, specifically in Santa Catarina, the production of *K. alvarezii* between 2022 and 2023 reached 300.35 tons, increasing 194% compared with 2021/2022 (102.3 tons). A total of 31.3 tons per hectare were produced in a rather small area of 9.59 hectares. On average, each producer grew 13.65 tons in areas with an average size of 0.44 hectares (Santos 2023).

The chemical composition of the algae can vary significantly due to environmental conditions (light, temperature, habitat, salinity) and genetic differences (Biris-Dorhoi *et al.* 2020). Light and temperature impact the nutrient uptake capacity of the algae, thereby influencing their growth and productivity (Roleda & Hurd 2019). Red macroalgae contain polysaccharides (carrageenan or agar) that make up 40% to 50% of their dry matter (Carpena *et al.* 2022). In addition, they contain proteins, amino acids, sterols, carotenoids, bromophenols, and other natural bioactive compounds.

Besides being an important commercial product, macroalgae are part of the food web in coastal marine environments and are increasingly cultivated for bioremediation in the aquaculture sector, helping to mitigate environmental impacts (Guillén *et al.* 2022). Several studies have assessed the behavior of the macroalga *Ulva lactuca* in the presence of various pollutants in aquatic environments (Areco *et al.* 2021). This species is resistant to salinity variations, exhibits a high growth rate, and displays strong rates of nutrient absorption, particularly ammonium (NH_4^+), showing good development in eutrophic environments (Nielsen *et al.* 2012, Bonanno *et al.* 2020). The alga *Gracilaria gracilis* has also been shown to be effective at reducing dissolved nutrients such as nitrogen and phosphorus. Its cultivation tends to contribute significantly to both

eutrophication control and biotechnology due to its use for antibiotic and agar production (Spanò *et al.* 2022).

K. alvarezii has been studied for its potential use as a bioremediator. In some studies, it has been observed that its cultivation enhances carbon dioxide sequestration, and it acts as a nutrient sink when it is grown with other organisms under various salinities, improving water quality (Hayashi *et al.* 2010, Reis *et al.* 2011). The potential for removing nitrogen and phosphorus compounds from seawater has been shown to reduce 24% of nitrite and nitrate concentrations and 6% in phosphate concentration (Doty *et al.* 1987).

In this context, algae cultivation can improve water quality due to its potential for nutrient removal (Hayashi *et al.* 2008) and provide shelter for various species of fish, crustaceans, and other aquatic organisms (Paula *et al.* 1998). This study aimed to assess, with *in vitro* assays, the nutrient removal rate following the cultivation of the macroalga *Kappaphycus alvarezii* (Doty) L.M.Liao 1996 in environments with the addition of fertilizer, in natural conditions, and eutrophicated by anthropogenic activities.

Materials and Methods

The assay was carried out in the laboratory of the Institute of Radioprotection and Dosimetry (IRD/CNEN), where microcosms were installed. The samples collected during the assay were transported to the Institute of Geosciences at the University Federal Fluminense (UFF) for the determination of dissolved nutrients (ammonium, nitrite, nitrate, and phosphate), total nitrogen and total phosphorus (in algae tissue). The analyses were carried out at the Natural Water Analysis Laboratory (LAAN) of the UFF Environment and Sustainable Development Network (REMADS-UFF).

The first water collection was carried out near the shore of Conceição de Jacareí Beach at coordinates 23°01'59.81" S and 44°09'42.89" W (Fig. 1A). The algae collection site was close to Sororoca Island, with coordinates 23°02'16.69" S and 44°09'40.38" W (Fig. 1A), where unfertilized *K. alvarezii* were planted a few weeks before collection in floating rafts. Although no genetic evaluation is available, the main strain cultivated in Brazil is green, but the age of the algae is difficult to determine because the growth is vegetative. The second water collection site was Rodrigo de Freitas Lagoon at coordinates 22°58'20.50" S and 43°12'56.97" W (Fig. 1B), a choked coastal system that receives large quantities of untreated sewage from the drainage basin (de Souza & Azevedo 2020).

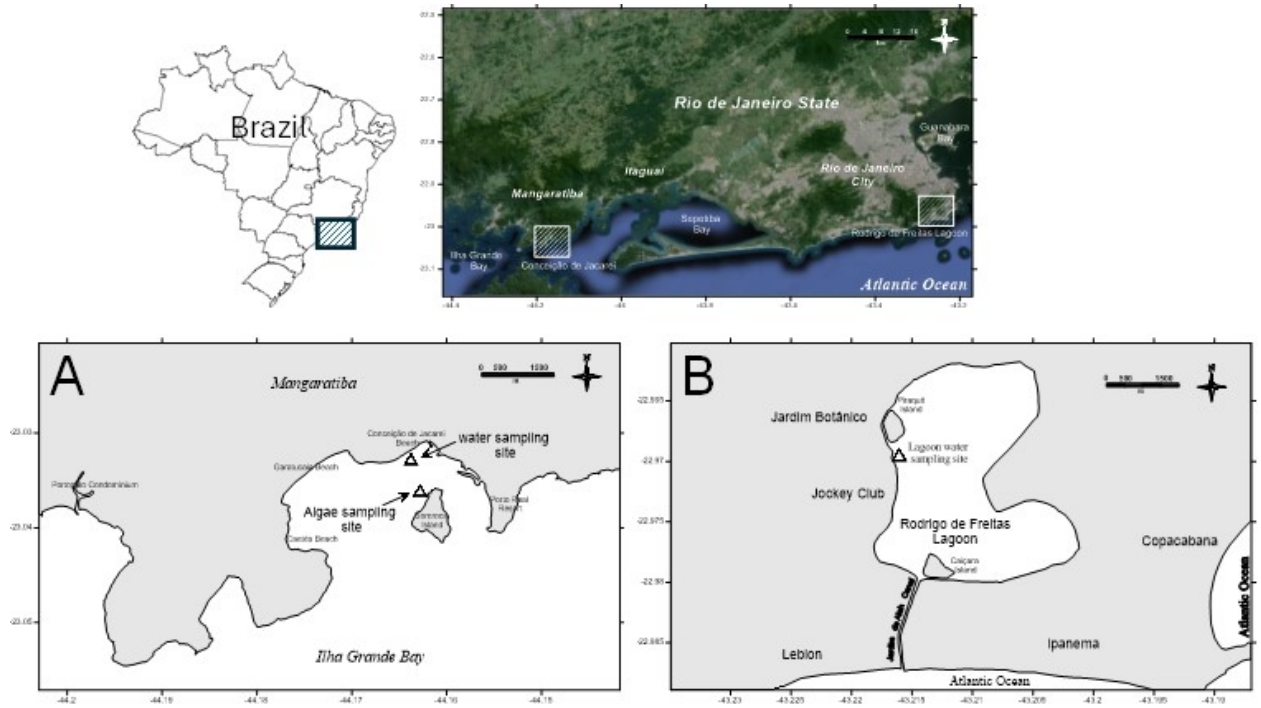

Figure 1- Location of the sampling sites: A) Conceição de Jacareí in the Ilha Grande Bay; B) Rodrigo de Freitas Lagoon, city of Rio de Janeiro.

Eight microcosms with a recirculating water system were used for this assay according to Guimarães *et al.* (2021) (Fig. 2). The setup of the experiments is summarized in Table I. A commercial fertilizer (inorganic fertilizer applied in hydroponics) was added to six of the microcosms (the composition is described in Table II) to test the effect of different concentrations on the growth of *K. alvarezii*. The hydroponics fertilizer was selected because it presents a composition that is adapted to aquatic systems and does not present any additives that promote adsorption into particles, being readily available for the plants. To assess the behavior of the algae in different environments, microcosms 1 (lagoon) and 2 (control) did not receive fertilizer, as they were filled with water from Rodrigo de Freitas Lagoon (naturally contaminated; Fig. 1B) and Conceição de Jacareí (Mangaratiba; Fig. 1A) uncontaminated seawater (Gomes *et al.* 2009), respectively.

Reservoirs 1 in all microcosms (Fig. 2) were carefully filled with seawater or lagoon water to a height of 15 cm. Within each microcosm, two 40 cm long ropes (10 mm thick) were placed (as shown in Figures 2 and 3), supporting algae pieces fixed with twine. Each rope held 6 pieces of approximately 3 cm of *K. alvarezii*, totaling 12 algae pieces in each microcosm. To enhance algae growth, besides adding fertilizer to the water, artificial lighting was provided by commercial LED SMD2835 lamps (red and blue), Grow Power brand panels, with 40 W (real) and 9500 lm, which were emitted in the wavelength range between 400-700 nm, featuring 81 light points and measuring 180x180x20 mm. The illumination system was connected to a 12-hour timer to simulate daylight/night dark periods (Fig. 3). A similar, but less efficient system was used by Castelar *et al.* (2014). The temperature was continuously monitored in the microcosms with an Onset Hobo temperature datalogger.

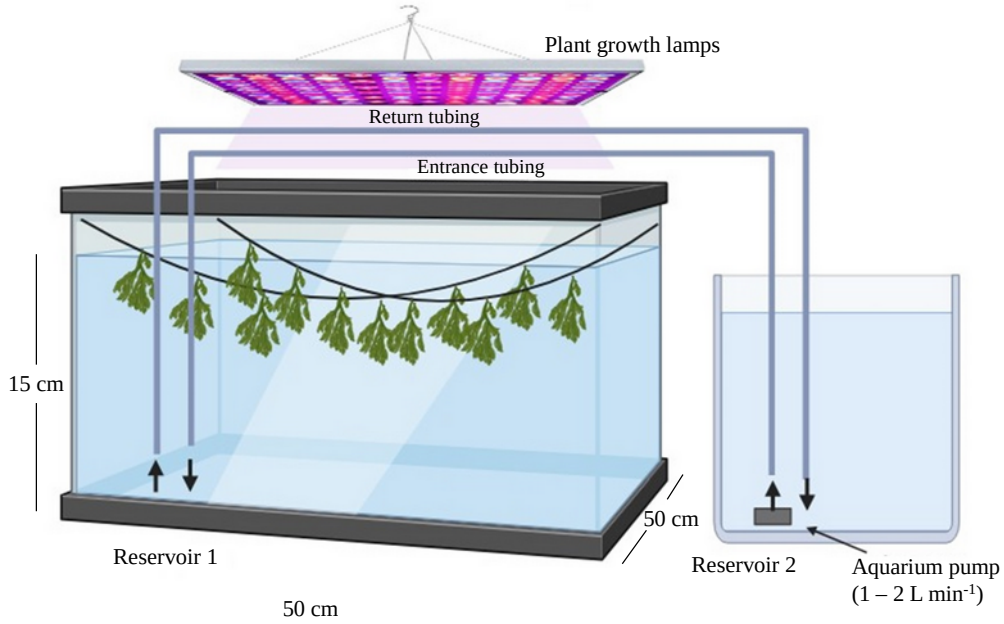

Figure 2 – Scheme of the microcosm system used for the *in vitro* assays.

Table I – Setup of the microcosm experiments in the present research.

Microcosm	Environmental setup	Fertilization
Microcosm 1	*K alvarezii* cultivation in Rodrigo de Freitas lagoon water	Natural
Microcosm 2	*K alvarezii* cultivation in seawater	No
Microcosms 3 & 4	*K alvarezii* cultivation in seawater – Microcosm A	40 g fertilizer in 40.5 L seawater
Microcosms 5 & 6	*K alvarezii* cultivation in seawater – Microcosm B	30 g fertilizer in 40.5 L seawater
Microcosms 7 & 8	K alvarezii cultivation in seawater – Microcosm C	20 g fertilizer in 40.5 L seawater

Figure 3 - On the left, the algae installed in the microcosms. On the right, the microcosms and their water exchange compartments are shown. Microcosms are colored by plant growth lamps.

Table II - Fertilization applied in each of the microcosms A, B and C. The values were calculated based on the recipe enclosed in the commercial product MLB-1008787979 (hydroponics fertilizer). The values in grams for each compound correspond to the value added to each of the 40.5 L microcosms.

Compounds	%	Microcosm A (3 and 4) in g	Microcosm B (5 and 6) in g	Microcosm C (7 and 8) in g
NO_3	7.8	3.104	2.328	1.552
NH_4	0.6	0.242	0.182	0.121
P_2O_5	3.0	1.200	0.900	0.600
K_2O	9.3	3.733	2.800	1.867
Mg	1.1	0.451	0.338	0.225
S	1.3	0.533	0.400	0.267
B	0.02	0.008	0.006	0.004
Cu	0.003	0.0013	0.0010	0.0007
Mn	0.02	0.007	0.005	0.003
Mo	0.02	0.010	0.007	0.005
Zn	0.01	0.003	0.002	0.001
Fe	2.0	0.800	0.600	0.400
CaO	8.8	3.533	2.650	1.767
Insoluble Minerals and organic compounds	65.9	26.375	19.781	13.187

At the beginning of the assays, seawater, lagoon water, and macroalgae were characterized in terms of their physicochemical parameters and nutrient concentrations, and the initial values of the microcosm experiments were recorded. Water and algae sampling, preparation, and the initiation of microcosm experiments took place on July 10th, 2022, with microcosm sampling measurements starting the following day (Fig. 4). The *in vitro* assays were conducted under uncontrolled laboratory temperature conditions. Water samples collected during the assays were stored in capped centrifuge tubes (50 ml)

and frozen for subsequent analyses. Water samples were collected at T0, T1 (2 days), T2 (9 days), and T3 (15 days), as described in Figure 4. The sampled macroalgae were transported to the laboratory in plastic bags, briefly rinsed with tap water to remove epiphytes and sediments, and introduced into the microcosms. Aliquots of the plant material were placed in an oven for evaluation of the water contents, which were determined after drying at 50°C until constant weight (two or three days drying period).

Water column measurements were carried out according to Figure 4, and after the assemblage, macroalgae biomass sampling was carried out on the eighth and the sixteenth days in the microcosms. To check the pH and Eh, a Hanna HI8424 portable meter was used, with specific electrodes for each parameter. Dissolved oxygen and temperature were measured with a Mettler-Toledo Seven 2Go-Pro optical oximeter. Water samples were collected, filtered through pre-weighed Whatman GF/C filters, and poured into 50 mL Falcon tubes (for nutrient determination). The concentrations of dissolved inorganic nitrogen (sum of ammonium, nitrite, and nitrate concentrations) and dissolved inorganic phosphorus (orthophosphate) were determined in the filtered samples. Analyses of ammonium, nitrite, and phosphate were performed according to the procedures described by Grasshoff *et al.* (1983). Nitrate analyses were performed according to Zhang & Fischer (2006). The detection and quantification limits of the analytical procedures were evaluated with blank assays and the results are presented in Table III.

Based on the results of the nutrient analyses, the removal rates (*RRs*) were calculated to verify the biofilter potential of *K. alvarezii* with the equation developed by Hayashi *et al.* (2008) (Equation 1).

$$RR(\%) = [(Ri - Rf)/(Ri)]\,x\,100 \quad \text{Eq 1}$$

where RR = Removal Rate; *Ri* = Initial nutrient concentration (element µM); *Rf* = final concentration relative to the days of cultivation (element µM).

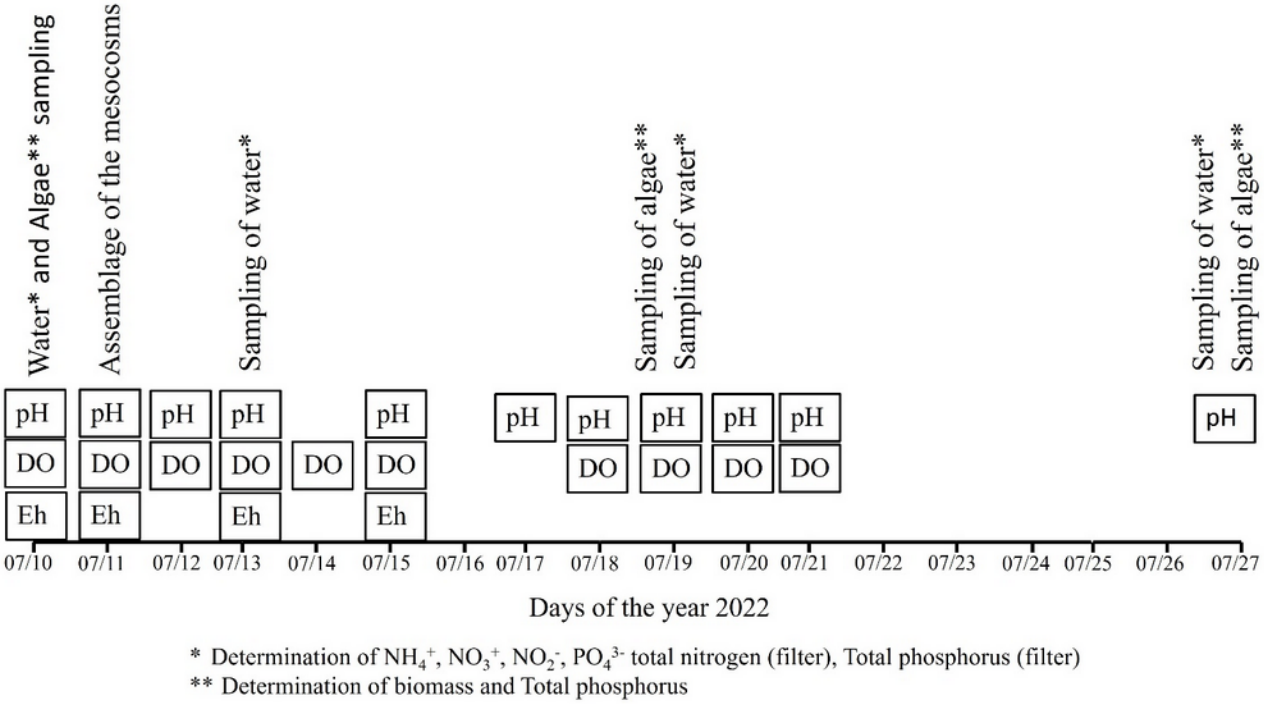

Figure 4 - Indications of the parameters measured in the water in the 16 days of assays in the microcosms.

Table III- Detection and quantification limits determined with blank assays.

	NH_4^+ (mg L^{-1})	NO_2^- (mg L^{-1})	NO_3^- (mg L^{-1})	PO_4^{3-} (mg L^{-1})	TN (mg L^{-1})	TP (mg L^{-1})
Detection limits	0.006	0.002	0.080	0.008	0.003	0.003
Quantification limits	0.010	0.003	0.130	0.013	0.005	0.005

RR values were calculated only for total nitrogen because we understand that conversions among chemical forms of nitrogen (nitrate-nitrite-ammonium and vice-versa) may occur due to biochemical processes not linked to algae removal.

Total nitrogen and phosphorus concentrations in the plant tissues were done in dried material, applying extraction with persulphate solution in an autoclave. This extraction is an adaptation of the procedure developed for the analysis of suspended material (Grasshoff et al. 1983). The extraction converted nitrogen and phosphorus into nitrate and phosphate which were analyzed by the same procedure described above for water samples.

Results

The results of the present research are presented in Table IV and Figures 5 – 9. Physico-chemical parameters measured during the assays and the raw results of nutrient concentrations were presented in Supplementary Materials 1 and 2.

Concentrations in the microcosms water: Seawater and lagoon water were quite different, with salinities of 34.09 PSU (marine condition) and 12.83 PSU (brackish) respectively, and pH values of 8.37 (marine) and 7.16 (lagoon), respectively. The introduction of fertilizers likely affected the physicochemical parameters, with a significant drop in pH (ranging between 5.5 and 6.5 in all microcosms; the raw data are presented in Supplementary Materials 1). The dissolved oxygen concentration was relatively stable throughout the assays at approximately 6 mg L-1, indicating that the water circulation in the system ensures aeration (Supplementary Materials 1). These microcosm assays were carried out at room temperature, but measurements (Onset Hobo temperature logger) showed slight fluctuations between 24°C and 26°C throughout the assays (Supplementary Materials 1).

Table IV shows significant variations in nutrient concentrations of the water during *K. alvarezii* cultivation (raw data are presented in Supplementary Materials 1). It is possible to observe that concentrations of nitrogen forms considerably oscillated, which may be attributed to interconversions of nitrogen forms and the effect of the algae removal or release to the other column. As discussed later, because the water was not aged (to remove phytoplankton) microalgae may also have a role in the removal or release of nutrients.

Figures 5 and 6 show the *removal rates (RRs*) in each experimental microcosm for dissolved inorganic nitrogen (molar summation of dissolved nitrogen forms) and dissolved inorganic phosphate, respectively. As shown in Figure 5, microcosm 2 (only seawater and algae) showed a small release in the first 9 days, then a strong release in the following period, which promoted a strong overall (T1-T3) release of nitrogen. The fertilization of the water (microcosms A, B, and C) did not yield stronger removal of nitrogen in the first experimental week (Figure 5). A greater removal efficiency was observed in the second period in these enriched microcosms, which barely compensated for releases of microcosms A and C in the first week. In low-salinity,

Table IV - Ammonium, nitrite, nitrate, and phosphate concentrations obtained throughout the assays.

	Assays	NH_4^+ (mg L^{-1})	NO_2^- (mg L^{-1})	NO_3^- (mg L^{-1})	PO_4^{3-} (mg L^{-1})
T0	Lagoon	0.20	0.03	0.58	0.16
	Sea	<0.01	0.03	1.13	0.10
T1 (2 days)	Microcosm 1 (lagoon)	0.39	0.02	0.60	0.67
	Microcosm 2 (sea)	<0.01	0.02	0.40	0.04
	Microcosm A	0.95	0.13	14.26	7.74
	Microcosm B	0.79	0.23	14.53	7.81
	Microcosm C	0.57	0.67	7.36	8.31
T2 (9 days)	Microcosm 1 (lagoon)	0.51	0.91	1.33	0.32
	Microcosm 2 (sea)	<0.01	0.01	0.40	0.05
	Microcosm A	0.93	1.60	16.94	9.72
	Microcosm B	0.74	1.51	12.58	10.04
	Microcosm C	0.54	1.34	8.06	10.58
T3 (15 days)	Microcosm 1 (lagoon)	0.01	0.67	1.07	0.14
	Microcosm 2 (sea)	0.01	0.02	0.75	0.05
	Microcosm A	0.96	1.39	15.22	10.87
	Microcosm B	0.74	1.38	11.96	10.97
	Microcosm C	0.61	1.39	6.40	11.58

naturally eutrophicated water (microcosm 1), a strong release of nutrients in the first week is compensated for removal in the second period but yields an overall release of nitrogen.

Figure 6 shows that in microcosm 1 algae promoted high removal rates throughout the entire experimental period. In the seawater (microcosm 2), phosphate concentrations are low (0.10 mg L-1), and regardless of plant growth, the algae did not seem to remove phosphate. Although the *RR* was very negative (-25%), the concentration in the water increased by only 0.01 mg L^{-1}. Microcosms A, B, and C presented very similar behaviors, with increasing concentrations in the water.

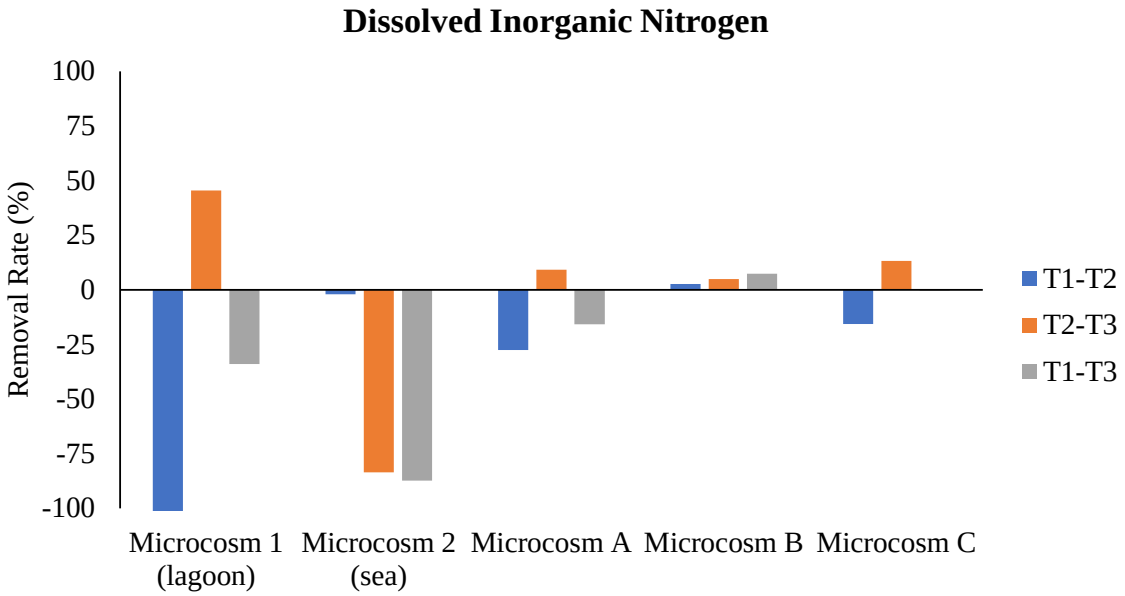

Figure 5- Removal Rates of DIN in microcosms, calculated according to the formula of Hayashi *et al.* (2010). Positive values indicate removal, while negative values indicate the release of nutrients. T1-T2 represents values from the beginning until day 9, T2-T3 indicates the values between the ninth and fifteenth days, and T1-T3 is the overall *RRs* of the assays.

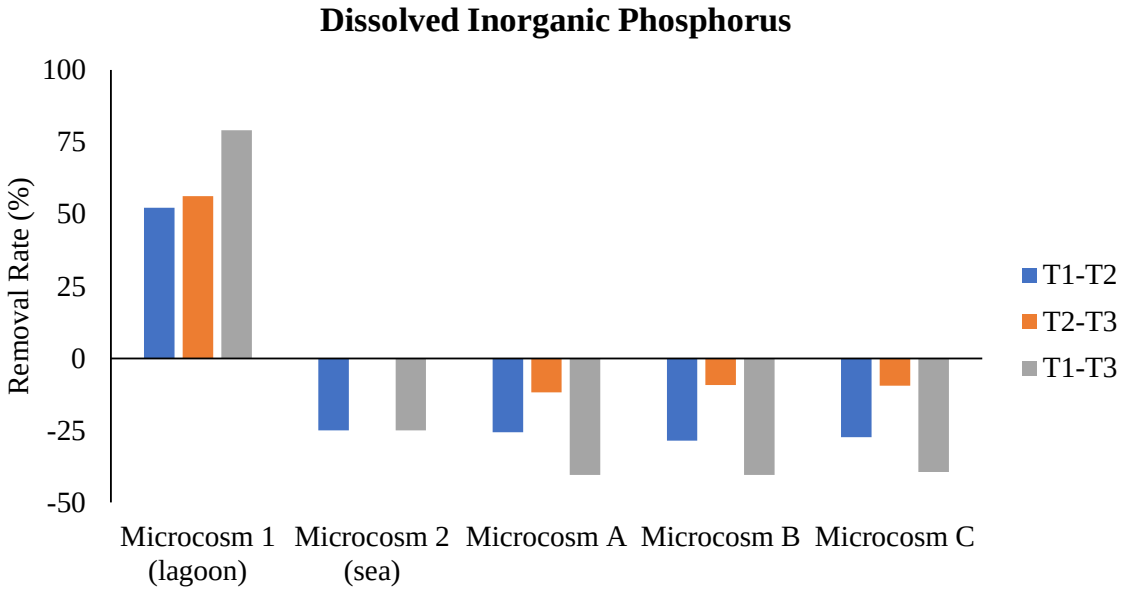

Figure 6- Removal Rates of DIP in microcosms, calculated according to the formula of Hayashi *et al.* (2010). Positive values indicate removal, while negative values indicate the release of nutrients. T1-T2 represents values from the beginning until day 9, T2-T3 indicates the values between the ninth and fifteenth days, and T1-T3 is the overall *RR* of the assays.

Biomass, nitrogen, and phosphorus in algae tissues: Figure 7 shows that the algal biomass tended to decrease toward the end of the assays, indicating senescence and decay of the organic matter. Measurements of biomass were performed at the beginning (the wet weight of algae introduced in each microcosm) and at the end of the experiments (the remaining algae were weighed); therefore, it was not possible to verify oscillations during cultivation.

Even though biomass generally decayed during the assays (Figure 7), total nitrogen and total phosphorus in the algal tissues tended to get enriched at the very beginning of the experiments (T1, 2 days), decreasing from sampling 1 to sampling 2 (Figures 7 and 8; Figure 4 indicates the sampling periods).

It is noteworthy that nitrogen and phosphorus concentrations in the natural environment collected algae (T0) were always lower than concentrations in the assays, even in the end, after a strong decrease. Although we cannot claim that this enrichment is attributed to the removal of nutrients, at least the rapid increase in T1 may indicate the removal of nutrients from the water column. We didn't calculate a balance of nutrients in the water because there are many parameters affecting nutrients that were not followed, like consumption by phytoplankton and bacteria, denitrification, and nitrification (chemical and biological).

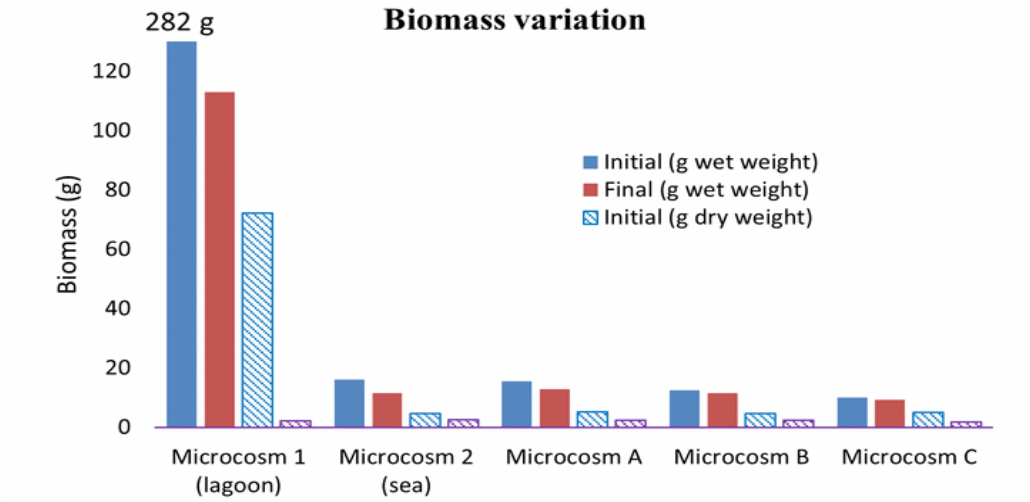

Figure 7 - Algal biomass at the beginning and end of the microcosm assays.

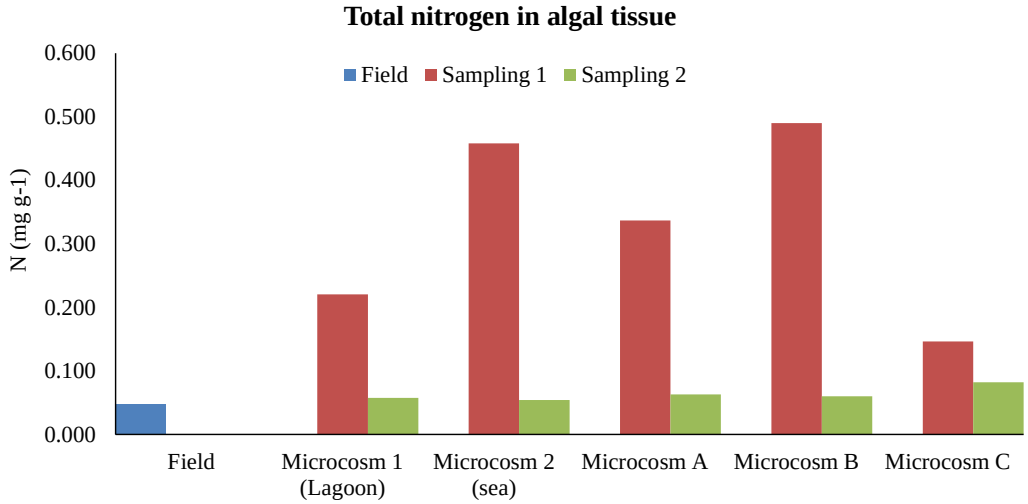

Figure 8 - Total nitrogen concentration in algae tissues. "Field" refers to freshly collected natural algae. Sampling 1 and 2 refer to macroalgae biomass measured on the eighth and the sixteenth days after the assemblage.

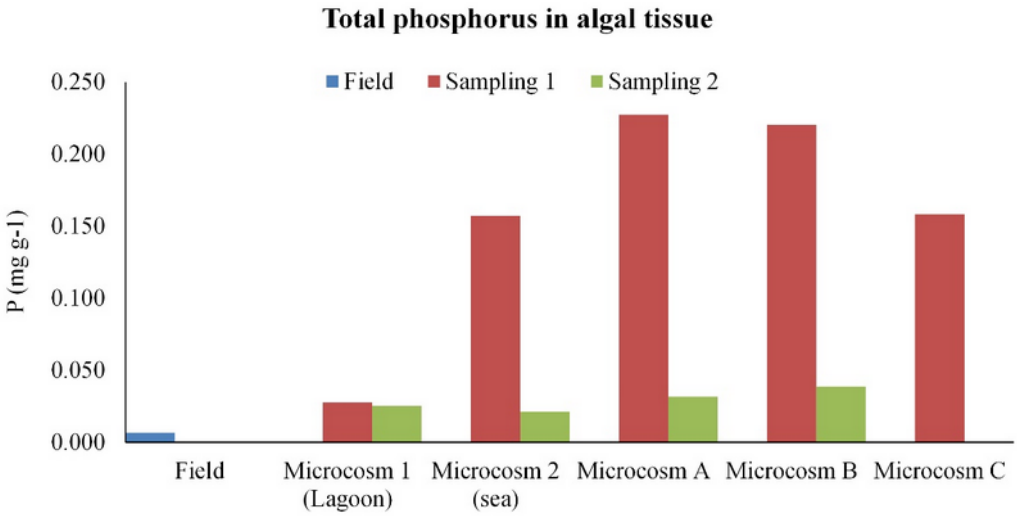

Figure 9 - Total phosphorus concentration in algae tissues. "Field" refers to freshly collected natural algae. Sampling 1 and 2 refer to macroalgae biomass measured on the eighth and the sixteenth days after the assemblage.

Discussion

Regardless of the variations of the physical-chemical parameters in the water column in the different experimental microcosms, the development of algae in saline and brackish water systems was similar. This is different from what was observed by Reis *et al.* (2011), who verified a twofold growth performance in seawater compared with brackish water (15 PSU) with the brown variant of *K. Alvarezii*. With other variants (green and red), these authors observed worse performances in lower salinities. It was expected that values of pH, temperature, and dissolved oxygen wouldn't oscillate, but it was verified that adding fertilizers promoted acidification of the systems. Even primary production (normally an alkalinizing process; Cunha & Wasserman (2003)) was not able to increase pH.

Variations in chemical forms of nitrogen (Table IV and Supplementary Materials 2) can be associated with a few factors that control dissolved nutrients, including oxidation, which converts ammonium into nitrite and nitrate, and macroalgae consumption and excretion. Phytoplankton which is present in the microcosms may also play a role in the removal or excretion of nutrients (Herbert 1999). For dissolved phosphate, primary producers (including algae and phytoplankton) significantly affected its concentrations (Williamson *et al.* 2018). It cannot be excluded that part of the added fertilizers could have remained undissolved in the marine waters of the assays due to association with iron (Yuan *et al.* 2020) or calcium (Anschutz *et al.* 2007). This can be a strategy of the fertilizer conception to have a constant release of dissolved phosphorus into the water. That may be valid for slower-growing plants, but not for algae.

The removal rates of dissolved inorganic nitrogen (Fig. 5) show strong release (negative values) for nitrogen at the beginning of all assays (T1 – T2) and a mild removal in the second period (T2 – T3). The behavior of phosphorus was distinct (Fig. 6), showing that algae are strongly affected by the availability of nutrients, and scarce or excess nutrients may change their metabolic state and therefore their removal rates from the water column (Pedersen 1994, Beardall & Raven 2021). Dissolved inorganic phosphorus in mesocosm 1 (Fig. 6) is an example where the availability of natural phosphate (unfertilized) promoted a more intense removal during all phases of the assay. Lenzi *et al.* (2003) showed that the provision of nutrients (in their case, from the sediment) in an eutrophicated system may promote an increase in algal biomass. These authors state that the regeneration of phosphate from the sediment was constant, and the algal biomass had to be managed; otherwise, its decomposition would release nutrients back into the sediment. In our case, considering that *K. alvarezii* is a commercially important plant, its production in sediment-contaminated areas is interesting because it is intended to be removed after growth.

In microcosms A, B, and C, significant removal or release of nutrients by plants could be discarded because the amount of dissolved phosphorus in the water is too high (due to the addition of fertilizer) to be affected by these inputs/outputs. On the other hand, the absorption of nutrients by algae in these enriched microcosms may not be noticeable

from water concentrations (Fig. 6). Dissolved inorganic phosphorus concentrations in the mesocosm 2 (natural seawater) were very low and could not be significantly affected by removal, nor by release (just a small release was observed).

From our results, it cannot be asserted that algae can remove ammonium to supply their nitrogen needs, however, the literature states that this is the preferred chemical form of nitrogen (Kim *et al.* 2007). Although Hayashi *et al.* (2008) reported an ammonium removal rate of 70% in integrated algae/fish farming, in our work this evaluation would be conjectural because oxidation due to aeration and bacterial nitrification/denitrification were not accountable (Kuenen & Robertson 1994).

The observed increase in DIN in microcosm 1 in the first 9 days (Fig. 5) may be attributed to the decomposition of organic matter in the water, considering that during collection the water was observed to be very turbid. However, within a few days, the water recirculation system in the microcosms seemed to have degraded the organic matter (probably BOD), and the water then became transparent (such as in a sewage treatment plant). This degradation of organic matter likely released some soluble nitrogen into the water column. However, at the end of the assay, it appears that the algae were able to remove much of this nitrogen from the water column.

For phosphate concentrations (corresponding to DIP) there was a removal of 79% in microcosm 1 (lagoon water) between T1 and T3, and 48% in microcosm 2 (control/seawater) that were far better than reported by Doty *et al.* (1987) who verified reductions of 6% in phosphate and 24% in nitrite and nitrate concentrations in experiments with *K. alvarezii* cultivated in the Philippines.

The cultivation of seaweed in microcosms is a consistent way of testing biological responses to strictly controlled environmental conditions; however, many studies have shown that these assays should not last very long (Hayashi *et al.* 2011, Peng *et al.* 2022). For many reasons, algae tend to decay after a few weeks, and in many cases, some authors have observed the incidence of ice-ice disease (Pedra *et al.* 2017). In our experiments, the decay of the algae started in the mean course, and measurements were only carried out in the end, so we could not follow any relevant growth in the first few days. Pires *et al.* (2021), in a 70-day growth assay with *K. alvarezii*, measured biomass every 7 days, and the growth rates increased and decreased alternately until the end of the assay. This should also be occurring in our work; however, we could not depict these alternations that should promote growth (and more intense nutrient removal) in the beginning and then, senescence in the end.

Although the biomass measurements indicate only the decay in the end, the analyses of the algal tissue nitrogen allowed to verify whether the absorption rates varied in relation to concentrations in the water and to better understand the removal process. For nitrogen and phosphorus, there is enrichment of the tissues at the beginning of the assay (Figs. 8 and 9), probably due to the strong change in environmental conditions. Luxury absorption of nutrients (Hurd *et al.* 2014) was reported in the Antarctic algae *Himantothallus grandifolius* and *Laminaria solidungula* subject to strong environmental changes and oscillations in nutrient availability (Korb & Gerard 2000). This behavior has been reported for *K. alvarezii* by Kambey *et al.* (2020) in experiments carried out in fish floating net cages in Indonesia. At the end of the assay, after 15 days of incubation the nitrogen and phosphorus were released back into the water column, but as shown in Figures 5 and 6, no release of nutrients was detected in the water column, probably because they were consumed by phytoplankton or bacteria.

Conclusion

The follow-up of the physical-chemical parameters in the assays indicates that it was possible to reproduce environmental conditions. Although the incorporation of fertilizers promoted a severe reduction in the pH, this parameter remained constant throughout the experiment, and apparently, the growth rates of the algae were not impacted by this parameter. As we observed, the algae could not survive for more than 15 days in the assay's environmental conditions.

In the first period of the experiment, *K. alvarezii* was able to grow in all experimental microcosms (even in lower salinities); however, the response in terms of nutrient removal is not clear, because there were several processes, including oxidation, consumption by phytoplankton that interfered in the evaluation. The oxidation process that was forced in the microcosms did not allow to distinguish removal of the different nitrogen forms, including ammonium which is reputed to be the preferential form consumed by algae. The analysis of removal/release rates (*RR*) of total nitrogen facilitated interpretations.

It was observed that the behavior of *K. alvarezii* in naturally eutrophicated and fertilized wa-

ters was quite distinct. A strong removal of phosphorus was observed in the naturally eutrophicated assay, while in the fertilized water this behavior was not observed, indicating that the response of algae to quality/quantity of fertilization is not direct, but it depends on various metabolic parameters. This can be confirmed by the strong enrichment in algal tissue nutrient content at the beginning of all assays, which should be interpreted as a luxurious accumulation.

The removal/release rates (*RR*) indicate that many processes take place simultaneously, including the release of nutrients through the decomposition of organic matter, removal, and release by macroalgae, and removal and release by microalgae. Therefore, the expected removal of nutrients from the water column as algae grow was not clearly observed. It would be interesting to remove organic matter and phytoplankton from the assays by previous filtrations and oxidations, but then, microcosms would not accurately reproduce environmental conditions. In future research, control of these parameters by measuring chlorophyll a, and total nitrogen, phosphorus, and carbon in filtered samples is a good idea to better understand the algal removal/release rates of nutrients in water assays. On the other hand, larger experiments with higher water circulation rates may constitute better environmental conditions for the growth of *K. alvarezii*, providing higher removal rates. Also, *in situ*, large-scale experiments should be carried out to better understand the use of these algae as bioremediators in the coastal environment.

Ethical statement

The present investigation did not involve regulated animals and did not require approval by an Ethical Committee

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SUPPLEMENTARY MATERIALS

In vitro assays with *Kappaphycus alvarezii* for the bioremediation of coastal eutrophic systems in Southeast Brazil

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Supplementary Material 1: Physical-chemical parameters measured daily in the microcosms: Temperature, pH, and dissolved oxygen (O_2)

	Temperature (°C)							
Date	Microcosm 1	Microcosm 2	Microcosm 3	Microcosm 4	Microcosm 5	Microcosm 6	Microcosm 7	Microcosm 8
7/11/2022	24.8	24.7	24.9	25.0	24.8	24.7	24.2	24.2
7/12/2022	24.3	24.2	24.8	24.7	24.2	24.4	24.3	24.9
7/13/2022	26.0	25.7	25.6	25.6	25.5	25.4	25.3	25.4
7/14/2022	25.3	24.9	24.9	25.0	25.0	25.0	25.3	25.2
7/15/2022	25.5	25.3	25.4	25.4	25.1	25.3	25.4	25.1
7/18/2022	26.0	25.8	25.8	25.8	25.7	25.7	25.8	25.8
7/19/2022	24.8	24.7	24.9	24.8	24.9	24.8	24.9	24.9
7/20/2022	26.2	26.0	25.9	25.9	25.8	25.8	25.8	25.8
7/21/2022	26.6	26.3	26.2	26.2	26.0	25.9	25.9	25.9
7/27/2022	-	-	-	-	-	-	-	-
	pH							
Date	Microcosm 1	Microcosm 2	Microcosm 3	Microcosm 4	Microcosm 5	Microcosm 6	Microcosm 7	Microcosm 8

7/11/2022	6.0	7.9	6.9	6.9	6.9	6.9	6.9	6.8
7/12/2022	6.4	8.3	6.7	6.8	6.8	7.0	6.9	7.0
7/13/2022	7.2	8.5	7.4	7.3	7.3	7.4	7.4	7.4
7/14/2022	-	-	-	-	-	-	-	-
7/15/2022	5.4	6.6	5.3	5.3	5.2	5.5	5.3	5.3
7/18/2022	6.0	7.0	6.0	6.0	7.0	6.0	7.5	6.0
7/19/2022	6.0	7.0	7.5	6.0	6.0	7.0	5.5	6.0
7/20/2022	5.5	7.0	6.0	6.0	6.0	5.5	5.5	6.0
7/21/2022	5.5	7.0	6.0	7.0	4.0	7.0	7.0	7.0
7/27/2022	6.0	7.0	6.0	6.0	6.0	6.0	6.0	6.0

	O_2 (mg L^{-1})							
Date	Microcosm 1	Microcosm 2	Microcosm 3	Microcosm 4	Microcosm 5	Microcosm 6	Microcosm 7	Microcosm 8
7/11/2022	-	8.5	8.7	9.0	8.8	8.8	8.6	8.4
7/12/2022	-	7.8	8.1	8.0	7.3	8.5	6.7	6.6
7/13/2022	-	5.4	6.6	6.3	4.2	6.4	5.1	4.8
7/14/2022	5.1	6.5	5.3	6.3	4.2	6.8	4.2	5.2
7/15/2022	5.1	6.7	5.7	5.8	4.5	6.9	4.7	5.2
7/18/2022	4.8	6.6	6.2	5.9	5.3	7.0	4.7	5.8
7/19/2022	5.4	6.9	6.6	6.2	5.8	7.0	5.3	5.5

7/20/2022	4.6	6.5	6.2	5.9	5.4	6.8	4.9	5.2
7/21/2022	3.7	5.9	6.1	5.6	5.5	6.8	6.0	5.2
7/27/2022	-	-	-	-	-	-	-	-

Supplementary Material 2: Concentrations and biomass in the microcosms (raw data).

		NH_4^+ (mg L^{-1})	NO_2^- (mg L^{-1})	NO_3^- (mg L^{-1})	PO_4^{3-} (mg L^{-1})	NT (Algae) (mg g^{-1})	TP (Algae) (mg g^{-1})	Biomass g ww	g dw
	Seawater	<0.01	0.03	1.13	0.10	0.05	0.01		
	Lagoon water	0.20	0.03	0.58	0.16				
July 13th, 2022									
	Microcosm 1 (lagoon)	0.39	0.02	0.60	0.67	0.22	0.03	16.00	4.65
	Microcosm 2 (control)	<0.01	0.02	0.40	0.05	0.46	0.16	282.00	72.15
Microcosm A	Microcosm 3	0.93	0.10	13.89	7.54	0.60	0.24	18.00	4.61
	Microcosm 4	0.98	0.15	14.64	7.94	0.07	0.22	13.00	5.65
Microcosm B	Microcosm 5	0.77	0.29	13.69	7.72	0.53	0.18	11.00	2.81
	Microcosm 6	0.80	0.18	15.36	7.90	0.45	0.27	14.00	6.65
Microcosm C	Microcosm 7	0.60	0.49	7.17	8.25	0.13	0.17	10.00	2.56
	Microcosm 8	0.55	0.85	7.55	8.37	0.16	0.14	10.00	7.65
July 19th, 2022									
	Microcosm 1 (lagoon)	0.51	0.91	1.34	0.31				
	Microcosm 2 (control)	<0.01	0.01	0.40	0.05				
Microcosm A	Microcosm 3	0.95	1.54	20.59	9.59				
	Microcosm 4	0.92	1.66	13.29	9.85				
Microcosm B	Microcosm 5	0.70	1.39	11.89	10.12				
	Microcosm 6	0.79	1.63	13.28	9.97				
Microcosm C	Microcosm 7	0.49	1.32	6.85	10.37				
	Microcosm 8	0.60	1.35	9.27	10.78				
									July 27th, 2022
	Microcosm 1 (lagoon)	0.01	0.67	1.07	0.14	0.06	0.03	113.00	2.17
	Microcosm 2 (control)	0.01	0.02	0.75	0.05	0.05	0.02	11.62	2.62
Microcosm A	Microcosm 3	0.83	1.46	15.49	10.65	0.06	0.03	14.70	2.64
	Microcosm 4	1.10	1.33	14.96	11.08	0.06	0.03	11.14	2.27

Microcosm B	Microcosm 5	0.70	1.36	12.08	11.09	0.06	0.03	11.64	2.47
	Microcosm 6	0.79	1.40	11.84	10.84	0.06	0.04	11.56	2.10
Microcosm C	Microcosm 7	0.56	1.44	6.41	11.29	0.08	0.05	7.71	1.58
	Microcosm 8	0.66	1.34	6.40	11.88	0.09	0.04	10.97	1.89

ww – wet weight; dw – dry weight