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RESEARCH

Advancing *Porphyra linearis* (Rhodophyta, Bangiales) culture: low cost artificial seawater, nitrate supply, photosynthetic activity and energy dissipation

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Abstract
Fertilizer use in agriculture and aquaculture significantly contributes to nitrate-rich effluent discharge into aquatic environments. *Porphyra*'s high surface area/volume enables efficient nutrient assimilation. This study aimed to identify a cost-effective, efficient artificial seawater medium for *Porphyra linearis* cultivation and determine the optimal nitrate concentration to enhance photosynthetic activity. *Porphyra linearis* was grown in three different salt media, with photosynthetic and biochemical parameters assessed, showing no differences. The nitrate experiment (7 days) using low-cost salt and varied concentration (0 to 6.5 mM) revealed optimal nitrate uptake at 3 and 5 mM, while 6.5 mM indicated saturation/toxicity. The phycobiliproteins contents did not increase compared to the 0 mM, but exhibited greater functionality, as evidenced by the enhanced photosynthetic parameters. Chlorophyll *a* peaked in 3 mM, whereas lutein and β-carotene peaked in 0 and 3 mM. The thalli turned greenish and appeared to have degraded branches under 0 mM. Growth rate was the same under all nitrate concentration and higher than under 0 mM. The presence of nitrate increased $ETR_{in\ situ}$ and $ETR_{max,}$ whereas the absence decreased the range between optimal irradiance for photoinhibition ($Eopt_{ETR}$) and saturated irradiance for photosynthesis (Ek_{ETR}) and between saturated irradiance for non-photochemical quenching (Ek_{NPQ}) and Ek_{ETR}, suggesting that under more nitrate available the algae dissipate less energy. *P. linearis* showed a wide range of nitrate use without variation in pigment composition in contrast to photosynthetic capacity. The 1.5 and 3 mM in cultivation significantly enhance the photosynthetic response of *P. linearis*, supporting their potential application in IMTA and bioremediation.

Keywords Nitrate assimilation · Bioremediation · Integrated multi-trophic aquaculture · Photosynthetic antennae · Pigments

Introduction

Practices such as using fertilizers for agriculture and the production of shellfish/fish are the main sources that discharge nitrate-rich effluents into the environment, leading to nitrate water pollution (Hooda et al. 2000). This influx of excess nutrients into surface waters can disrupt the ecological balance (Erratt et al. 2018). In response, there is an increasing search for biological methods for wastewater treatment, which are significantly more environmentally friendly compared to physico-chemical decontamination methods (Rani and Maróti 2022).

In the pursuit of cleaner wastewater treatment methods, bioremediation stands out as a noteworthy process. This approach employs plants and algae to absorb or neutralize environmental pollutants, thereby reducing their detrimental effects (Cunningham and Berti 1993). Additionally, Integrated Multi-Trophic Aquaculture (IMTA) represents an advanced technological approach in aquaculture, where nitrogen is identified as one of the main effluents released (Pereira et al. 2008). IMTA mitigates the dependency on chemical nitrogen fertilizers, which are widely used in conventional terrestrial agriculture, thereby curbing both the overall production costs and the risk of environmental pollution. Moreover, IMTA offers a range of benefits, such as the reliable availability of biomass, enhancements in its quality, and a stable chemical composition (Holdt and Edwards

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2014; Pliego-Cortés et al. 2019), which contributes to more sustainable aquaculture practices.

Porphyra sensu lato, a red macroalga, is economically significant in the food and pharmaceutical sectors, owing to its nutritional content and physiological effects resulting from its bioactive compounds (BACs) (Blouin et al. 2011; Zhou et al. 2012; Guedes et al. 2013; Mercurio et al. 2015; Morais et al. 2021). The notably high surface area relative to its volume of *Porphyra* enables efficient nutrient assimilation, which promotes their accelerated growth rates (Liu and Zou 2015), making it an excellent choice for use in bioremediation or/and IMTA. Research has shown that *Porphyra* is capable of absorbing dissolved nitrogen (Kraemer et al. 2004; He et al. 2008; Pereira et al. 2008; Wu et al. 2015), however these investigations generally examine scenarios with low nitrogen levels.

Despite the numerous benefits offered by *Porphyra*, its predominant cultivation is in Asian aquaculture farms, either offshore or in tanks. The central objective at these sites is to harvest biomass for culinary applications, without evaluating and understanding the photosynthetic activity, which could further enhance the production of this alga. Fortune Business Insights reports that the global market for commercial seaweed stood at US$14.11 billion in 2020 and is projected to grow to US$24.92 billion, reflecting a Compound Annual Growth Rate (CAGR) of 7.51%, by 2028. To understand the capacity of the nitrate use and growth rate it is necessary to know the effects of nitrate supply on photosynthesis and specifically the energy produced in the electronic transport chain and the energy dissipated. Nitrate is substrate of photosynthesis as is CO_2. The assimilation of nitrate and the conversion to proteins demands 1 ATP/4 NADPH. The effects of nitrate supply on photosynthetic efficiency, photosynthetic capacity and on energy dissipation as mechanism of photoprotection are evaluated by using *in vivo* chlorophyll *a* fluorescence technique. Therefore, this work was conducted with the objective of the selection of an optimal artificial seawater for the efficient and cost-effective cultivation of *Porphyra linearis* Greville and to determine the level of nitrate to be added to the water to achieve significant photosynthetic response, as a previous step for the scale up production of this important macroalgae scale in the IMTA systems and for bioremediation.

Materials & methods

Biological material

The gametophytes of *Porphyra linearis* were collected on the rocky shore of Santa Cristina Beach (43°61'N and 8°18'W), Galicia, Spain, in May 2023, by the 'Porto Muiños' company. Algal thalli were transported to the University Institute of Blue Biotechnology and Development (IBYDA) of the University of Malaga (Spain) in plastic containers containing seawater inside a thermal box. In the laboratory the thalli were washed with diluted artificial seawater ('Sea Salt') and healthy portions were selected and followed for acclimation with artificial seawater (32 psu) without nutrients for 14 days under controlled conditions: photosynthetic active radiation (PAR, λ= 400-700 nm) of 120 µmol photons m^{-2} s^{-1} (White LED light, 5000K, 54W, NU-8416, Nuovo), temperature of 15 ± 2 °C, photoperiod of 12 h L:12 h D, and continuous aeration.

Conditions of cultivation

Before starting the experiment with different nitrate concentrations an experiment was conducted with different types of salts (Instant Ocean, sea salt from a salina in Malaga, and a supplemented sea salt with KCl, $MgSO_4$, $MgCl_2$, KNO_3, and KH_2PO_4 to achieve ionic levels similar to Instant Ocean). The quantification of ions presents in the water prepared with each type of salt was evaluated using ion chromatography (930 Compact IC Flex - Metrohm) with a conductivity detector and an anion column and guard column Metrosep A Supp 7 – 250/4.0 (250 mm length and 4 mm diameter; Metrohm, Switzerland) in the Research Support Central Services (SCAI, University of Malaga, Spain). The analyses were performed in triplicate, and the results were expressed in mg L^{-1} (Suppl. Table 1).

The optimal or maximal quantum yield (F_v/F_m), effective quantum yield (Y(II)) (Supp. Fig. 1), electron transport rate (ETR) and non-photochemical quenching (NPQ) by rapid light curves (RLC) were determined (Suppl. Fig. 2). Growth rate, content of phycobiliproteins (PC: phycocyanin and PE: phycoerythrin), phenolic compounds, and antioxidant activity (Suppl. Table 2) were quantified at 7 and 14 days of cultivation, under the same conditions mentioned above, with the different types of salts, as well as the internal content of carbon (C), nitrogen (N) and sulfur (S), carbon and nitrogen ratio (C:N), carbon and sulfur ratio (C:S), and nitrogen and sulfur ratio (N:S) (Suppl. Table 2). After analyzing all the results, the 'Sea Salt' was chosen as it is the low-cost option with good photosynthetic and biochemical responses.

For the nitrate experiment the culture room conditions were the same as those in the acclimatization period. Following acclimatization portions of *P. linearis* (3.0 ± 0.05 g) were cultured in UV transparent polyvinylmethacrylate (Plexiglass XT-29080) cylindrical vessels filled with 750 mL artificial seawater (32 psu), with the addition of nitrate in different concentrations (0, 0.15, 0.5, 1.5, 3, 5 and 6.5 mM of KNO_3) (3 cylinders per each nitrate concentration) and 24 µM of PO_4 (the same for all treatments). The experiment lasted 7 days.

On the seventh day of the experiment photosynthesis measurements were performed in samples of all replicas, and then the samples were frozen at -80 ºC for extraction of the metabolites. As no statistical differences were observed between the results of the samples after the acclimation period and the samples exposed to 0 mM, serving as a negative control.

Nitrate quantification: Nitrate uptake efficiency (NUE) and nitrate uptake rate (NUR)

The nitrate quantification in water was carried out on the seventh day of the experiment. 1 mL of sample was added with 50 µL of Griess-reagent (sulphanilamide in 1.3N HCl (1% w/v) and N-(1-naphthyl)-ethylenediamine dihydrochloride (NED) in MiliQ water 0.005%, 1:1) and 100 µL VCl_3-reagent (Vanadium (III) chloride in 6N HCl (2 % w/v)) and gently mixed and incubated in a temperature controlled dry-bath at 60 °C for 25 min. Then the samples were cooled to room temperature in a water-bath and the absorbance was measured at 540 nm (García-Robledo et al. 2014). The quantification of total nitrate was determined using a standard curve of NO_3^- (1 to 25 µM - $R^2 = 0.99$; $y = 0.031567x$).

Relative growth rate (RGR)

The effect of salt type and of nitrate concentration on the growth of *P. linearis* was evaluated by the difference between fresh matter mass at the beginning and at the end of treatments (n = 3). Relative growth rates (RGR) were presented as percentage of daily growth and calculated according to the formula by Yong et al. (2013): RGR % $day^{-1} = [(W_f/W_0)^{t-1}] \times 100$, where RGR is relative growth rate (% day^{-1}), W_f is final fresh weight in gram, W_0 is initial fresh weight in gram, and time (t) in days.

Light microscopy

On the seventh day of the experiment fresh material of all replicates was extended between a slide and coverslip and observed under a light microscope (Leica DME, Germany) equipped with Leica ICC50 W camera. The software LAS-EZ (Leica) was used for the final image processing. The protoplast length measurements were performed using ImageJ software (version 1.53) with images obtained through light microscopy. Fifty cells from each treatment were measured, with one measurement per cell.

Lipophilic pigment quantification

The extractions, after 7 days of experiment, of lipophilic pigments (chlorophyll *a* and carotenoids) were performed with 100 mg fresh algae and 1.5 mL extraction solvent (methanol 100 %). After combining the algae with the extraction solvent they were macerated by 30 s using an UltraTurrax (T25, IKA, Germany) (18000 rpm) and then extracted at 4 ºC for 3 h. Following extraction, the samples were centrifuged at 2721.6 × *g* and the supernatant was collected.

The determination of pigments concentrations was carried out according to Gheysen et al. (2019). 500 µL of the algal extract was filtered through a 0.2 µm membrane before chromatographic analysis using a HPLC system (1260 Agilent InfinityLab Series, USA) with a diode-array detection (DAD) detector. The pigments separation was performed injecting 80 µL of extract algal into a Zorbax SB-C8 column (5 µm; 150 mm length and 4.6 mm diameter - Agilent), was kept at 30 °C and the samples at 5 °C. The mobile phases were methanol:ammonium acetate buffer (80:20, pH 7.2, 0.5 M), acetonitrile:ultra-pure water (90:10), and ethyl acetate, with a flow rate of 0.7 mL min^{-1}, and each run took 33 min. Pigments were detected at 444, 450 and 664 nm. Purified pigments were used as standards. The quantification was performed using a standard curve with pigments standards from DHI, Denmark (Chlorophyll *a*: 0.25 to 1.5 mg L^{-1} - $R^2 = 0.99$; $y = 178.52x - 9.97$; Lutein: 0.13 to 1 mg L^{-1} - $R^2 = 0.97$; $y = 797.1x - 50.372$; β-carotene: 0.19 to 1.5 mg L^{-1} - $R^2 = 0.99$; $y = 623.36x + 43.041$; where *y* represents the absorbance and *x* represents the concentration of pigment), and were expressed in mg per g of DW.

Bioactive compounds (BACS) extraction

The extractions of BACs were conducted in 500 mg of fresh algae and 5 mL of extraction solvent (distilled water with 2.5% sodium carbonate (SC)), performing alkaline hydrolysis. After combining the algae with the extraction solvent, they were macerated for 30 s using an UltraTurrax (18000 rpm) and then extracted at 80 ºC for 1.5 h. Following extraction the samples were centrifuged at 2721.6 × *g*, the supernatant was collected, and the pH was adjusted to pH 7.0 using lactic acid (pH meter Horiba LaquaTwin.). the algal extracts were used for the measurement of concentrations of BACs (phycobiliproteins for nitrate experiment and phycobiliproteins, phenols, and antioxidant activity for salt experiment).

Total soluble phycobiliproteins. Phycobiliproteins [phycocyanin (PC), and phycoerythrin (PE)] concentrations were determined using a UV–visible spectrophotometer (Shimadzu UV-2600, λ = 498, 615, and 651 nm), following the formulas described by Kursar et al. (1983). The analyses were conducted in triplicates and the results expressed in mg of pigment per g of dry weight (DW).

Total soluble phenols. The analyses of phenolic compounds were carried out using the spectrophotometric Folin-Ciocalteu method based on Folin and Ciocalteau (1927). Aliquots of 100 µL from the algal extracts were added to

700 µL distilled water, 50 µL of the Folin-Ciocalteu reagent (Sigma-Aldrich), and 150 µL of 20 % sodium carbonate, and incubated for 2 h at 4 ºC. Subsequently, readings were taken at 760 nm. The quantification of total phenolic compounds was determined using a standard curve of phloroglucinol (1 to 20 µg mL^{-1} - $R^2 = 0.99$; y = 0.0586x; where *y* represents the absorbance and *x* represents the concentration of phenols). The analyses were performed in triplicate and the results expressed in mg of phloroglucinol per g DW.

Antioxidant activity. Antioxidant capacity was determined by the ABTS radical scavenging assay. First the radical cation $ABTS^{+\bullet}$ was prepared by mixing 7 mM of ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid, 7 mM)) (Sigma-Aldrich) and 2.45 mM of potassium persulfate ($K_2S_2O_8$) in a sodium phosphate buffer solution (0.1 M, pH 6.5). The mixture was incubated in darkness at room temperature for 16 h for the complete formation of the radical. For the reaction, the $ABTS^{+\bullet}$ was diluted with phosphate buffet until absorbance at 727 nm was 0.75 ± 0.05. The assay was done by adding 950 µL of the diluted $ABTS^{+\bullet}$ solution and 50 µL of algal extract according to Re et al. (1999). The samples were shaken and absorbance was recorded at 727 nm at the beginning of the reaction (DO_i) and after 8 min of incubation (DO_f). The percentage of antioxidant activity (AA%) was calculated according to the formula:

$$AA\% = \left[\left(absDO_i - absDOf\right)/absDOi\right] * 100$$

The antioxidant compounds concentration was calculated using a standard curve of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma-Aldrich) (20 to 100 µg mL^{-1} - $R^2 = 0.99$; y = 15.8928x; where *y* represents the absorbance and *x* represents the concentration of Trolox), and the results expressed in µmol of Trolox equivalent antioxidant capacity (TEAC) per g of DW.

Total carbon, nitrogen, and sulfur

Total carbon (C), nitrogen (N), and sulfur (S) contents of the dry algal biomass were evaluated using a LECO-932 CNHS elemental analyzer in the SCAI, University of Malaga, Spain. The analyses were performed in triplicate, and the results were expressed in %.

Photosynthesis and energy dissipation as *in vivo* chlorophyll *a* fluorescence

In vivo variable chlorophyll *a* fluorescence was measured *in situ* directly under the cultivation conditions in the culture chambers. To achieve this, saturating pulses (>3000 µmol photons m^{-2} s^{-1}) were applied to algal thalli (five replicates per cylinder) during the dark phase of the daily photoperiod. This approach aimed to quantify three distinct fluorescence parameters: the basal fluorescence (F_o), the steady-state fluorescence (F^t), and the maximal fluorescence (F_m), which is measured in dark-adapted samples or F_m' in light-acclimated samples) using a portable pulse amplitude modulated fluorometer (875)). The optimal quantum yield (F_v/F_m) and the effective quantum yield (Y(II)) were assessed on the seventh day. Calculations for F_v/F_m and Y(II) were conducted using their respective standard formulas:

$$F_v/F_m = (F_m - F_o)/F_m$$
$$Y(II) = F_m\prime - F_t)/F_m\prime$$

The electron transport rate (ETR) was determined through rapid light curves (RLCs) by exposing samples to thirteen ascending irradiances, ranging from 0 to 1500 µmol photons m^{-2} s^{-1}, of actinic red light from Diving-PAM II. Firstly, algae were incubated in darkness for 15 min (Figueroa et al. 2003, 2014) to oxidize all the reaction centers before the beginning of the curves. After that, algae were exposed for 30 s of incubation at each irradiance level and a saturating pulse was applied to the samples. At each irradiance level of the RLCs, different parameters were calculated including the Y(II), ETR, and two different types of yield losses: the yield for non-photochemical quenching (Y(NPQ)) calculated as (F_t/F_m') - (F_t/F_m) and the yield for non-regulated energy dissipation (Y(NO)) calculated as F_t/F_m. Additionally, nonphotochemical quenching (NPQ) was calculated using the formula Y(NPQ)/Y(NO). The energy dissipation rate (EDR) was also evaluated at 124 µmol photons m^{-2} s^{-1} (intensity closest to that used in cultivation), at 631 µmol photons m^{-2} s^{-1} (median intensity of the RLC), and at 1509 µmol photons m^{-2} s^{-1} (maximum intensity of the RLC). The ETR (Schreiber et al. 1995) and EDR (Vega et al. 2024) was calculated as follows:

$$ETR\left(\mu mol\ e^{-}m^{-2}s^{-1}\right) = Y(II) \times E_{PAR} \times A \times F_{II}$$
$$EDR\left(\mu mol\ photons\ m^{-2}s^{-1}\right) = (Y(NO) + Y(NPQ)) \times E_{PAR} \times A \times F_{II}$$

where *Y(II)* is the effective quantum yield, *Y(NO)* is the yield loss related to passive thermal dissipation, and *Y(NPQ)* is the yield loss related to photoregulated process; E_{PAR} is the incident PAR irradiance expressed in µmol photons m^{-2} s^{-1}, *A* is the absorptance that was calculated as $A=1-(E_t/E_0)$ (E_t is the irradiance that passes through the thallus and E_0 is the emitted irradiance), and F_{II} is the fraction of chlorophyll *a* associated to PSII, being 0.15 in red macroalgae (Figueroa et al. 2003, 2014). The obtained ETR and NPQ vs. irradiance curves were fitted according to Eilers and Peeters (1988) and other parameters were obtained: photosynthetic efficiency of ETR (α_{ETR}), maximal ETR (ETR_{max}), saturated irradiance of ETR (Ek_{ETR}) and NPQ (Ek_{NPQ}) and optimal irradiance ($Eopt_{ETR}$), respectively). The Ek_{ETR} was subtracted from the Eopt values to detect the range of supported light intensities, without leading to photoinhibition,

and Ek_{NPQ} was subtracted from the Ek_{ETR} to know the range of light intensity in which the photosynthesis is maintained in steady state.

Statistical analysis

The data passed the Shapiro-Wilk normality test and the Bartlett test for homogeneity of variances, and all samples were within the normal range and exhibited homoscedasticity. Afterwards, the data were analyzed by unifactorial analysis of variance (ANOVA) (considering nitrate concentrations) followed by the Tukey's a posteriori test ($p \leq 0.05$). Statistical analyses were performed using the Statistica software package (Release 10.0). Data were also tested by principal component analysis (PCA). The graphical design was carried out using scripts written in Python language through the software Spyder to determine similarities of physiological variables that were analyzed in the present work.

Results

Different types of salt experiment

Growth Rate (GR) and Bioactive Compounds (BACs) In the salt experiment no statistical differences were observed at either seven or fourteen days in the growth rate, phycocyanin, phycoerythrin, and phenolics levels. No statistical differences were observed in antioxidant activity levels during seven days; however, after fourteen days, the samples exposed to Instant Ocean showed the lowest antioxidant activity, whereas those exposed to 'Sea Salt' and 'Supplemented Sea Salt' demonstrated the highest activity (Supp. Table 2).

Total internal carbon, nitrogen and sulfur In the salt experiment, no statistical differences were observed in internal carbon and sulfur levels during seven and fourteen days, nor in nitrogen levels after fourteen days. However, after seven days, the samples exposed to Instant Ocean showed the lowest nitrogen content, whereas those exposed to 'Sea Salt' and 'Supplemented Sea Salt' demonstrated the highest internal nitrogen content (Supp. Table 3).

Photosynthetic responses For the salt type experiment, the optimal or maximal quantum yield (F_v/F_m) did not show significant differences among the three salt types across all days analyzed (days 4, 7, 11, and 14) (Supp. Fig. 1a). Considering the effective quantum yield (Y(II)), after seven days of experimentation, the samples exposed to 'Sea Salt' and 'Supplemented Sea Salt' demonstrated the lowest yield; however, after eleven days, these samples exhibited the highest yield levels. For the other days, no statistical differences were observed (Suppl. Fig. 1b).

Exposure to three different salt types for seven days did not show a statistical difference in the electron transport rate (ETR) and non-photochemical quenching (NPQ) (Supp. Fig. 2a, b). On the other hand, after fourteen days of exposure, at certain irradiance points, the highest ETR was observed in samples exposed to 'Supplemented Sea Salt'; however, no statistical differences were observed in the NPQ (Suppl. Fig. 2c, d).

Table 1 Nitrate uptake efficiency (NUE, %) and in nitrate uptake rate (NUR, mmol of NO_3^- g^{-1} DW day^{-1}) in *P. linearis* after exposure to different nitrate concentrations for seven days ($n = 3$; mean ± SD). Different letters indicate significant differences according to the one-way analysis of variance (nitrate concentrations), followed by Tukey's post-hoc test ($p \leq 0.05$)

Nitrate concentration	NUE (% -7 days)	NUR (mmol NO_3^- g^{-1} DW day^{-1} - 7 days)
0 mM	-	-
0.15 mM	95.36 ± 4.40^{a}	0.12 ± 0.01^{d}
0.5 mM	99.51 ± 0.16^{a}	0.43 ± 0.15^{c}
1.5 mM	81.68 ± 7.07^{b}	1.02 ± 0.13^{ab}
3 mM	53.99 ± 3.74^{c}	1.26 ± 0.05^{a}
5 mM	27.66 ± 1.67^{d}	1.21 ± 0.06^{a}
6.5 mM	16.09 ± 2.13^{e}	0.92 ± 0.13^{b}

Nitrate concentration experiment

Nitrate quantification: Nitrate uptake efficiency (NUE) and Nitrate uptake rate (NUR) Considering the nitrate uptake efficiency after seven days of exposure, samples exposed to 0.15 and 0.5 mM demonstrated the highest nitrate absorption whereas samples exposed to 6.5 mM exhibited the lowest percentage of absorption, with higher concentrations showing reduced absorption. In terms of the daily uptake rate, for seven days of exposure, the daily absorption rate was highest

Table 2 Relative growth rate (% day^{-1}) of *Porphyra linearis* exposed to different nitrate concentrations after seven days (n = 3; mean ± SD). Different letters indicate significant differences according to unifactorial ANOVA and Tukey's test

Nitrate concentration	RGR (% day^{-1})
0 mM	-1.36 ± 0.71^{b}
0.15 mM	0.94 ± 0.43^{a}
0.5 mM	0.84 ± 0.35^{a}
1.5 mM	1.63 ± 0.91^{a}
3 mM	2.40 ± 0.96^{a}
5 mM	2.20 ± 1.17^{a}
6.5 mM	2.28 ± 0.11^{a}

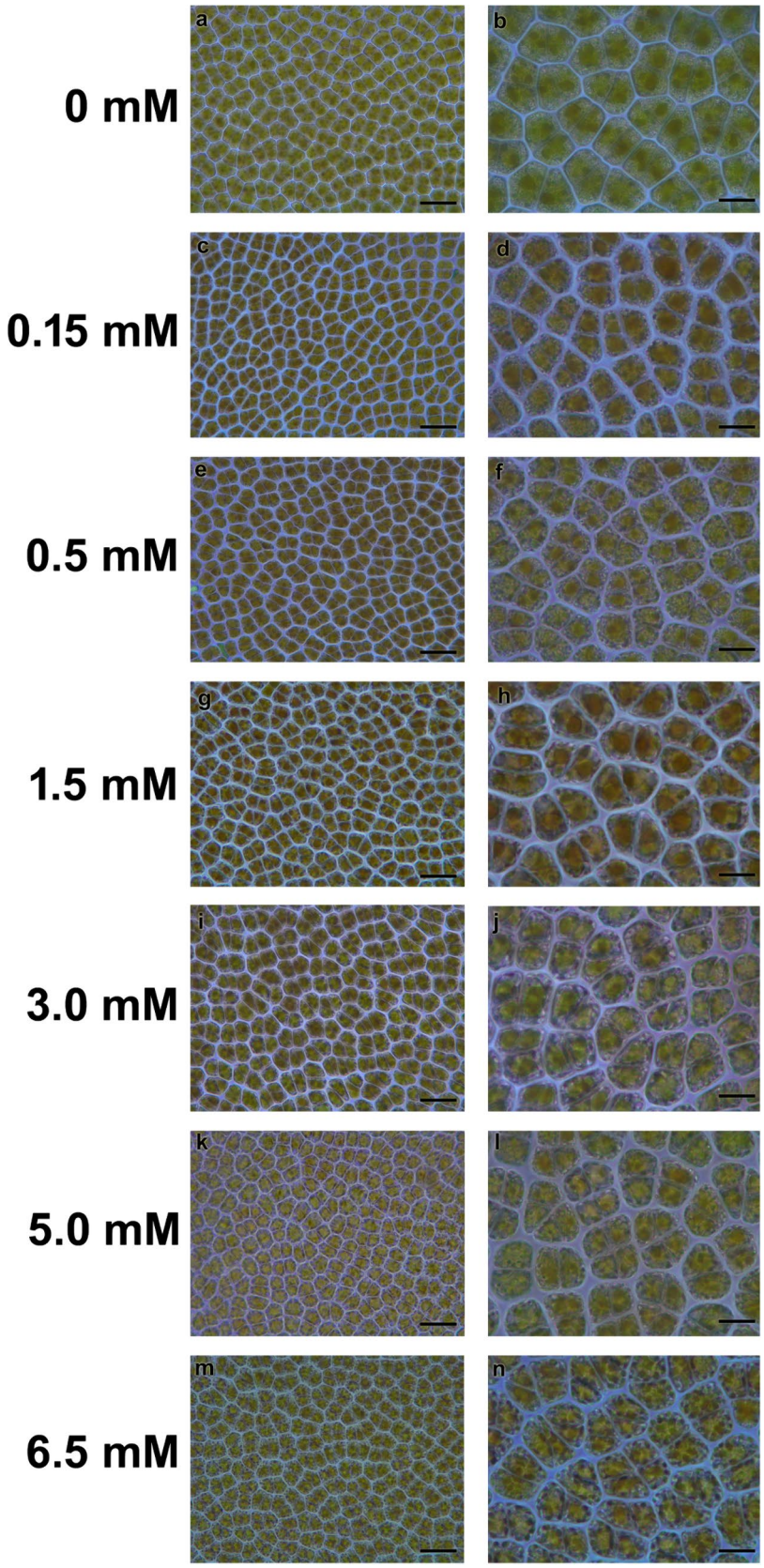

Fig. 1 Thalli of *P. linearis* exposed to different nitrate concentrations observed under light microscopy (scale bars in the first column = 50 µm, and in the second column = 20 µm)

in samples from 1.5, 3, and 5 mM, followed in a descending order with statistical differences from 6.5, 0.5, and 0.15 mM (Table 1). A positive and strong Pearson correlation between nitrate quantity and nitrate uptake rate was observed at seven days of exposure ($r = 0.844$, $p = 0.001$).

Relative growth rate (RGR) After 7 days of experimental treatment with different nitrate concentrations the RGRs of *P. linearis* were analyzed using one-way ANOVA. It was observed that the absence of nitrate results in a negative growth rate, while all nitrate concentrations analyzed demonstrated a positive growth rate. A positive and strong Pearson correlation between nitrate concentration and RGR was detected ($r = 0.655$, $p = 0.001$); however, no statistical differences were found among them (Table 2).

Effects of nitrate concentrations on microscopic morphology After seven days exposed to 0 mM of nitrate the chloroplasts showed depigmentation, turning greenish and appearing to have degraded branches (Fig. 1a, b). On the other hand, samples exposed to all nitrate concentrations maintained typical branched and brownish chloroplasts (Figure 1c-n).

Taking into account the protoplast length at the end of the experiment, samples exposed to 1.5, 3, 5, and 6.5 mM exhibited the longest lengths, while samples exposed to 0.5 mM showed the shortest. Samples exposed to 0 and 0.15 mM displayed a protoplast length statistically similar to both the longest and intermediate measurements (Table 3). A positive Pearson correlation was detected between the protoplast length and nitrate quantity ($r = 0.568$, $p = 0.007$).

Effects of nitrate concentrations on photosynthetic lipophilic pigments: Chlorophyll *a* and carotenoids Regarding chlorophyll *a*, the highest concentration was observed in the sample exposed to 3 mM after seven days of exposure. Samples exposed to 0, and 0.15 mM nitrate also displayed high levels of chlorophyll *a*, showing statistical similarities to the best response. In contrast, all other treatments exhibited the lowest quantities of this pigment (Fig 2).

The HPLC analysis detected only two carotenoids: lutein and β-carotene. The production of these carotenoids was most significant in the samples treated with 0 and 3 mM, followed by those with 0.15 mM. Conversely, samples treated with 1.5 and 6.5 mM exhibited the lowest quantities (Fig. 2).

No correlations between nitrate quantity and chlorophyll *a*, lutein, or β-carotene concentration were evident.

Effects of nitrate concentrations on photosynthetic hydrophilic pigments: Phycobiliproteins After seven days of culture, samples treated with 3 mM nitrate demonstrated the highest statistically significant phycoerythrin (PE) content, showing statistical similarities with the 0, 0.5, 5, and 6.5 mM samples. In contrast, the 0.15 mM nitrate samples exhibited the least favorable response (Fig. 3a). Regarding phycocyanin (PC) content, the 5 mM nitrate samples had the highest levels, with statistical similarities observed with the 0.5 and 6.5 mM samples, whereas 0.15 mM presented the lowest values (Fig. 3a).

Table 3 Protoplast length (μm) in *P. linearis* exposed to different nitrate concentrations ((n = 50; mean ± SD). Different letters indicate significant differences according to the one-way analysis of variance and Tukey's test ($p \leq 0.05$)

Nitrate concentration	Protoplast length (μm)
0 mM	17.72 ± 2.59^{ab}
0.15 mM	16.42 ± 3.97^{ab}
0.5 mM	15.17 ± 3.09^{b}
1.5 mM	19.40 ± 3.86^{a}
3 mM	19.24 ± 3.35^{a}
5 mM	19.72 ± 3.79^{a}
6.5 mM	19.23 ± 4.36^{a}

The PE:PC ratio was highest in samples treated with 3 mM nitrate and lowest in 5 mM (Fig. 3b).

A positive Pearson correlation between nitrate quantity and PC content was observed ($r = 0.528$, $p = 0.014$), but no correlation between nitrate quantity and PE concentration was evident.

Photosynthetic responses After seven days of exposure under different nitrate concentrations, the ETR exhibited statistical differences at seven specific irradiances, as denoted in the statistical table within the graph. At all irradiances where statistical differences were observed, samples exposed to 1.5, 5 and 6.5 mM nitrate concentrations displayed the highest ETRs, whereas the 0 mM nitrate sample consistently showed the lowest ETR (Suppl. Fig. 3a). At the end of the experiment the NPQ appears to be highest in samples exposed to 3 mM of nitrate and lowest in samples with 0 mM nitrate, with statistical differences only in the last four irradiances (Suppl. Fig. 3b).

After seven days, the lowest F_v/F_m was detected in the 0 mM nitrate treatment, while all other concentrations demonstrated the highest responses, with no significant statistical variance among them, but with a positive Pearson correlation between nitrate quantity and F_v/F_m ($r = 0.473$, $p = 0.03$). For the photosynthetic electron transport rate efficiency (α_{ETR}) and ETR saturated irradiance (Ek_{ETR}), no statistical differences were observed across all treatments. However,

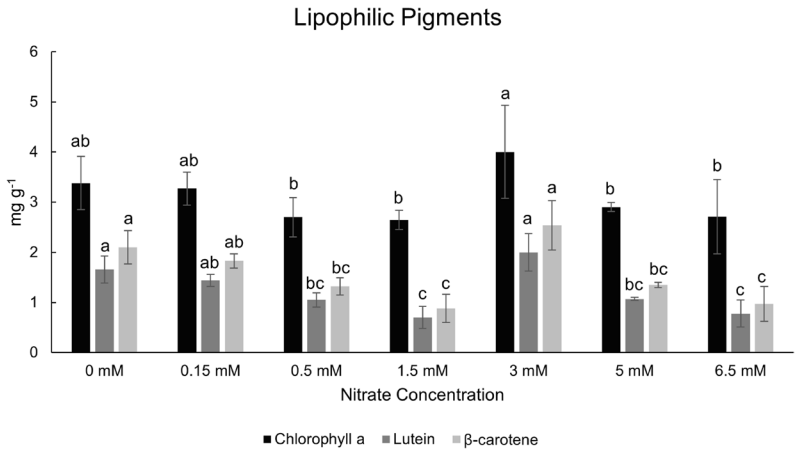

Fig. 2 Concentrations of lipophilic pigments (chlorophyll *a*, lutein and β-carotene) (mg g^{-1} dry weight) in *P. linearis* after exposure to different nitrate concentrations for seven days (n = 3; mean ± SD). Different letters indicate significant differences according to the one-way analysis of variance and Tukey's test ($p \leq 0.05$)

when considering the maximal ETR (ETR_{max}), the highest response was observed in the sample exposed to 6.5 mM of nitrate, whereas the least favorable response was identified in the 0 mM nitrate sample. Ek_{ETR} and ETR_{max} exhibited positive Pearson correlation with the nitrate concentration ($r = 0.439$, $p = 0.046$ and $r = 0.540$, $p = 0.012$, respectively), whereas α_{ETR} did not demonstrate any correlation.

For the optimal irradiance for ETR ($Eopt_{ETR}$), the best response was noted in the sample exposed to 1.5 mM of nitrate, with the least favorable response occurring in the 0 mM sample. Regarding the NPQ saturated irradiance (Ek_{NPQ}), the lowest intensity was found in the 0 mM sample, while the highest intensities were observed at nitrate concentrations of 1.5, 3, 5, and 6.5 mM. Considering the difference between $Eopt_{ETR}$ and Ek_{ETR}, the greatest range of intensity

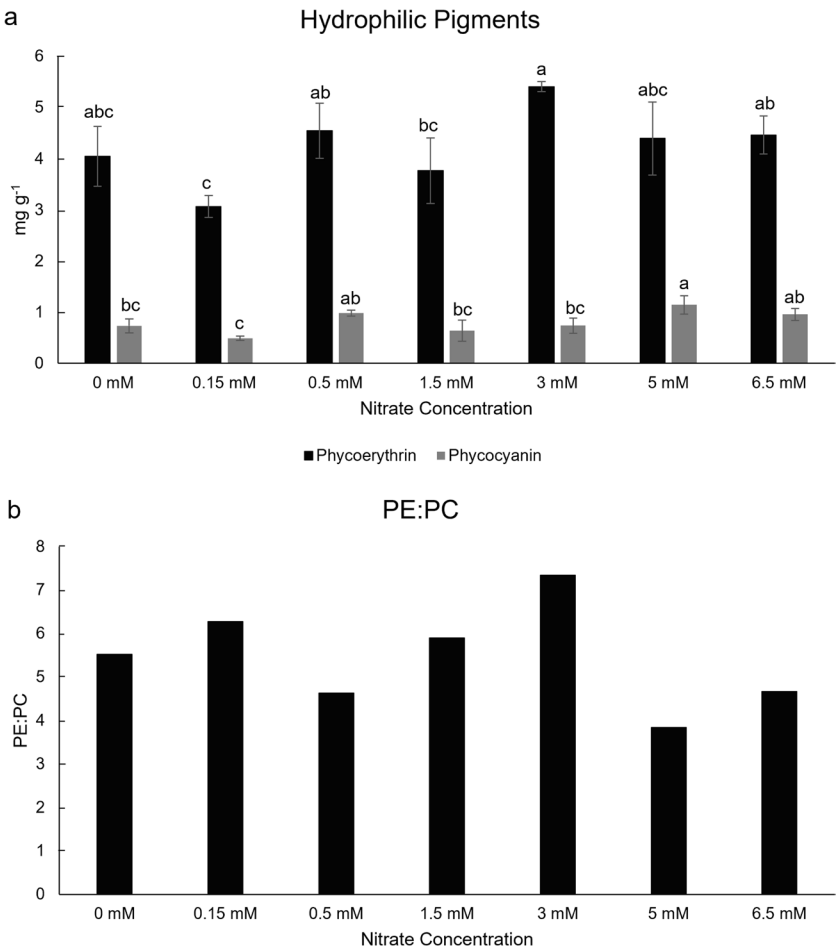

Fig. 3 Concentrations of phycobiliproteins (PE: phycoerythrin and PC: phycocyanin (**a**)) (mg g^{-1} dry weight) and PE:PC ratio (**b**) in *P. linearis* after exposure to different nitrate concentrations for seven days (n = 3; mean ± SD). Different letters indicate significant differences according to the one-way analysis of variance and Tukey's test ($p \leq 0.05$)

was noted in the sample exposed to 1.5 mM, whereas the smallest ranges were seen in the samples with 0, 0.15, and 3 mM. Finally, upon calculating the difference between Ek_{NPQ} and Ek_{ETR}, a greater range of intensity was observed in the 1.5 and 6.5 mM sample, and a smaller range was noted in the 0 mM exposed sample (Table 4). Ek_{NPQ} and the difference between Ek_{NPQ} and Ek_{ETR} displayed a positive Pearson correlation with the nitrate concentration ($r = 0.577$, $p = 0.006$ and $r = 0.508$, $p = 0.019$, respectively), while $Eopt_{ETR}$ and the difference between $Eopt_{ETR}$ and Ek_{ETR} did not show any correlation with the nitrate concentration.

The $ETR_{in\ situ}$ was statistically higher in algae exposed to 6.5 mM of nitrate, while samples at 0 and 0.15 mM exhibited the lowest $ETR_{in\ situ}$ values. All other samples showed $ETR_{in\ situ}$ results that were statistically similar to the highest and lowest observed rates (Table 5). A strong and a positive Pearson correlation was observed in this parameter and the nitrate concentration ($r = 0.731$, $p = 0.0001$).

At 124 µmol photons m^{-2} s^{-1} the highest ETR was noted in 1.5, 5 and 6.5 mM samples, whereas the lowest value was detected in the sample exposed to 0 mM of nitrate. For this same irradiance, no statistical differences were observed for EDR across all samples. The ETR:EDR ratio was highest in samples with 6.5 mM nitrate, and it was observed to be the lowest in samples with 0 mM nitrate (Table 5). A positive Pearson correlation was exhibited in ETR_{124} and the nitrate concentration ($r = 0.620$, $p = 0.003$), whereas a negative Pearson correlation was noted in EDR in this same irradiance and the nitrate quantity ($r = -0.440$, $p = 0.046$).

At 631 µmol photons m^{-2} s^{-1} no statistical difference in ETR was noted among all samples, but a positive Pearson correlation was detected with the nitrate concentration ($r = 0.439$, $p = 0.046$). Considering the EDR at this irradiance, the lowest response was in the 5 mM nitrate sample, while the highest was in the sample exposed to 1.5 mM. All other samples showed a similar statistical profile, falling between the highest and lowest responses. No correlation was observed in this parameter and the nitrate quantity. The ETR:EDR ratio was higher in samples with 6.5 mM nitrate, while it was lower in the 0 mM nitrate samples (Table 5).

The maximal irradiance, 1509 µmol photons m^{-2} s^{-1}, neither ETR nor EDR exhibited statistical differences among any of the samples nor did they exhibit any correlation with the nitrate concentration. The ETR:EDR ratio was highest in samples with 1.5 mM nitrate and lowest in those with 0 mM nitrate. Samples with other nitrate concentrations exhibited identical ETR:EDR ratios (Table 5).

Principal Component Analysis (PCA) of photosynthetic parameters from *P. linearis* extracts with different nitrate concentrations The PC1 and PC2 explained 58.02% of the variability in the samples across different nitrate concentrations after seven days. Regarding nitrate concentration,

Table 4 Photosynthetic parameters (optimal quantum yield (F_v/F_m), photosynthetic electron transport rate efficiency (αETR), maximal ETR (ETR_{max} - µmol e^- m^{-2} s^{-1}), ETR saturated irradiance (Ek_{ETR} - µmol photons m^{-2} s^{-1}), ETR optimal irradiance ($Eopt_{ETR}$ - µmol photons m^{-2} s^{-1}), NPQ saturated irradiance (Ek_{NPQ} - µmol photons m^{-2} s^{-1}), and difference between $Eopt_{ETR}$ and Ek_{ETR}, and between Ek_{NPQ} and Ek_{ETR} (µmol photons m^{-2} s^{-1}) obtained after the adjustment of the rapid light curves (RLCs) in *P. linearis* after exposure to different nitrate concentrations for seven days. Values are calculated as mean ± standard deviation (SD) (n = 3). Different letters indicate significant differences according to the one-way analysis of variance (nitrate concentrations), followed by Tukey's post-hoc test ($p \leq 0.05$)

Nitrate concentration	Fv/Fm	α_{ETR}	ETR_{max}	Ek_{ETR}	$Eopt_{ETR}$	$Eopt_{ETR}$ - Ek_{ETR}	Ek_{NPQ}	Ek_{NPQ} - Ek_{ETR}
0 mM	0.41 ± 0.04^{b}	0.045 ± 0.02^{a}	2.42 ± 0.33^{b}	56.07 ± 12.13^{a}	313.37 ± 82.99^{b}	269.00 ± 77.32^{b}	77.65 ± 24.29^{b}	21.58 ± 17.45^{b}
0.15 mM	0.55 ± 0.03^{a}	0.052 ± 0.02^{a}	3.71 ± 0.98^{ab}	58.03 ± 26.85^{a}	361.95 ± 70.36^{ab}	280.98 ± 34.96^{b}	175.93 ± 79.62^{ab}	94.96 ± 89.43^{ab}
0.5 mM	0.55 ± 0.03^{a}	0.054 ± 0.02^{a}	4.01 ± 0.49^{ab}	76.71 ± 13.03^{a}	389.20 ± 63.46^{ab}	345.82 ± 130.08^{ab}	173.24 ± 83.32^{ab}	96.53 ± 71.77^{ab}
1.5 mM	0.59 ± 0.03^{a}	0.086 ± 0.03^{a}	4.14 ± 0.57^{ab}	53.30 ± 18.82^{a}	494.78 ± 70.24^{a}	448.86 ± 72.76^{a}	250.94 ± 54.63^{a}	197.64 ± 42.71^{a}
3 mM	0.59 ± 0.05^{a}	0.049 ± 0.01^{a}	4.21 ± 1.10^{ab}	84.86 ± 9.67^{a}	355.59 ± 20.70^{ab}	270.73 ± 20.99^{b}	232.41 ± 21.46^{a}	153.01 ± 27.12^{ab}
5 mM	0.58 ± 0.01^{a}	0.058 ± 0.01^{a}	4.39 ± 1.02^{ab}	77.72 ± 20.07^{a}	423.10 ± 64.47^{ab}	312.04 ± 106.60^{ab}	231.66 ± 30.07^{a}	153.94 ± 40.51^{ab}
6.5 mM	0.60 ± 0.02^{a}	0.061 ± 0.01^{a}	4.85 ± 1.12^{a}	82.52 ± 29.72^{a}	377.21 ± 51.16^{ab}	261.36 ± 80.39^{ab}	256.19 ± 22.49^{a}	173.67 ± 52.21^{a}

Table 5 Electron transport rate *in situ* ($ETR_{in\ situ}$ - µmol e^- m^{-2} s^{-1}), ETR and energy dissipation rate (EDR - µmol e^- m^{-2} s^{-1}), and ETR:EDR ratio obtained after the adjustment of the rapid light curves (RLCs) at 124, 631, and 1509 µmol photons m^{-2} s^{-1} in *P. linearis* after exposure to different nitrate concentrations for seven days (n = 3; mean ± SD). Different letters indicate significant differences according to the one-way analysis of variance (nitrate concentrations), followed by Tukey's post-hoc test ($p \leq 0.05$)

Nitrate concentration	$ETR_{in\ situ}$	ETR_{124}	EDR_{124}	ETR_{124}:EDR_{124}	ETR_{631}	EDR_{631}	ETR_{631}:EDR_{631}	ETR_{1509}	EDR_{1509}	ETR_{1509}:EDR_{1509}
0 mM	4.44 ± 0.32^{b}	1.96 ± 0.10^{b}	9.95 ± 0.67^{a}	0.20	2.23 ± 0.49^{a}	59.77 ± 4.90^{ab}	0.04	1.86 ± 0.83^{a}	172.10 ± 28.56^{a}	0.01
0.15 mM	4.43 ± 0,30^{b}	2.85 ± 0.58^{ab}	9.18 ± 0.87^{a}	0.31	3.50 ± 0.99^{a}	61.33 ± 2.36^{ab}	0.06	2.49 ± 0.71^{a}	155.87 ± 24.46^{a}	0.02
0.5 mM	5.15 ± 1.23^{ab}	3.10 ± 0.39^{ab}	9.03 ± 0.46^{a}	0.34	3.82 ± 0.63^{a}	60.93 ± 1.90^{ab}	0.06	2.93 ± 0.97^{a}	151.36 ± 13.08^{a}	0.02
1.5 mM	5.79 ± 1.64^{ab}	3.29 ± 0.30^{a}	8.27 ± 0.56^{a}	0.40	4.11 ± 0.58^{a}	66.90 ± 6.17^{a}	0.06	3.68 ± 0.49^{a}	141.56 ± 9.98^{a}	0.03
3 mM	6.67 ± 0.14^{ab}	3.25 ± 0.75^{ab}	8.67 ± 0.57^{a}	0.37	3.90 ± 1.05^{a}	59.97 ± 1.92^{ab}	0.07	2.66 ± 0.74^{a}	138.31 ± 12.61^{a}	0.02
5 mM	6.48 ± 0.23^{ab}	3.40 ± 0.48^{a}	8.97 ± 0.88^{a}	0.38	4.11 ± 1.22^{a}	55.60 ± 2.76^{b}	0.07	3.05 ± 1.29^{a}	158.84 ± 17.05^{a}	0.02
6.5 mM	6.79 ± 0.32^{a}	3.75 ± 0.37^{a}	8.23 ± 0.102^{a}	0.46	4.43 ± 1.43^{a}	57.84 ± 2.41^{ab}	0.08	3.03 ± 1.18^{a}	142.65 ± 20.66^{a}	0.02

there is not a clear distinction in group formation, with an overlap observed among all groups; however, the samples exposed to 0, 1.5, and 3 mM tend to be more separated than the others (Fig. 4).

Discussion

Based on the results of this study, the use of commercial salt (InstantOcean) or sea salt, with or without supplementation for the production of artificial seawater for the cultivation of *P. linearis*, did not cause significant differences in all evaluated photosynthetic and biochemical parameters. This reveals a low-cost alternative source (saving approximately €15.45 per liter of produced seawater) that is commercially very appealing for large-scale production of this algae. Therefore, the use of sea salt and different nitrate concentrations in the cultivation water of *P. linearis* were evaluated and showed significant differences in the photosynthetic response.

At the end of seven days of exposure, algae exposed to 0.15 and 0.5 mM nitrate absorbed nearly all the nitrate present in the water (95.36% and 99.51%, respectively). However, as the nitrate concentration increased, the efficiency of nitrate absorption from the water decreased. Nonetheless, when calculating the total amount of nitrate absorbed, samples exposed to 3 mM absorbed the greatest quantity of nitrate by the end of the experiment, approximately 1.62 mmol. It was in this same treatment that the highest daily nitrate uptake rate was observed (1.26 mmol NO_3^- g^{-1} DW day^{-1}), as well as in samples with medium and high nitrate concentrations, whereas a low rate was noted in samples with minimal nitrate availability. Carmona et al. (2006) tested nitrate absorption (0.025 to 0.3 mM) in six different *Porphyra* species, finding the best nitrate uptake rates at higher nitrate concentrations, similar to the results observed in the current study. However, it may be considered that at the highest nitrate concentration (6.5 mM), an even greater increase in nitrate absorption was not observed because these elevated concentrations could be causing saturation and toxicity. It is known that *Porphyra* has the characteristics of an opportunistic species, with rapid growth and the ability to survive with high nutrient concentrations (Pereira et al. 2008). The presence of nitrate, regardless of the concentration, enhances the alga's growth, and even without a statistical difference between the nitrate treatments, there is a tendency for higher nitrate availability to correlate with increased growth rates. Growth rates in different *Porphyra* species exposed to various nitrate concentrations range from 5 to 10% day^{-1} (Carmona et al. 2000, 2006), whereas the growth rate in the current study was around 2% day^{-1}. The light intensity used in the experiment (120 µmol photons m^{-2} s^{-1}) is the same as that employed in controlled laboratory

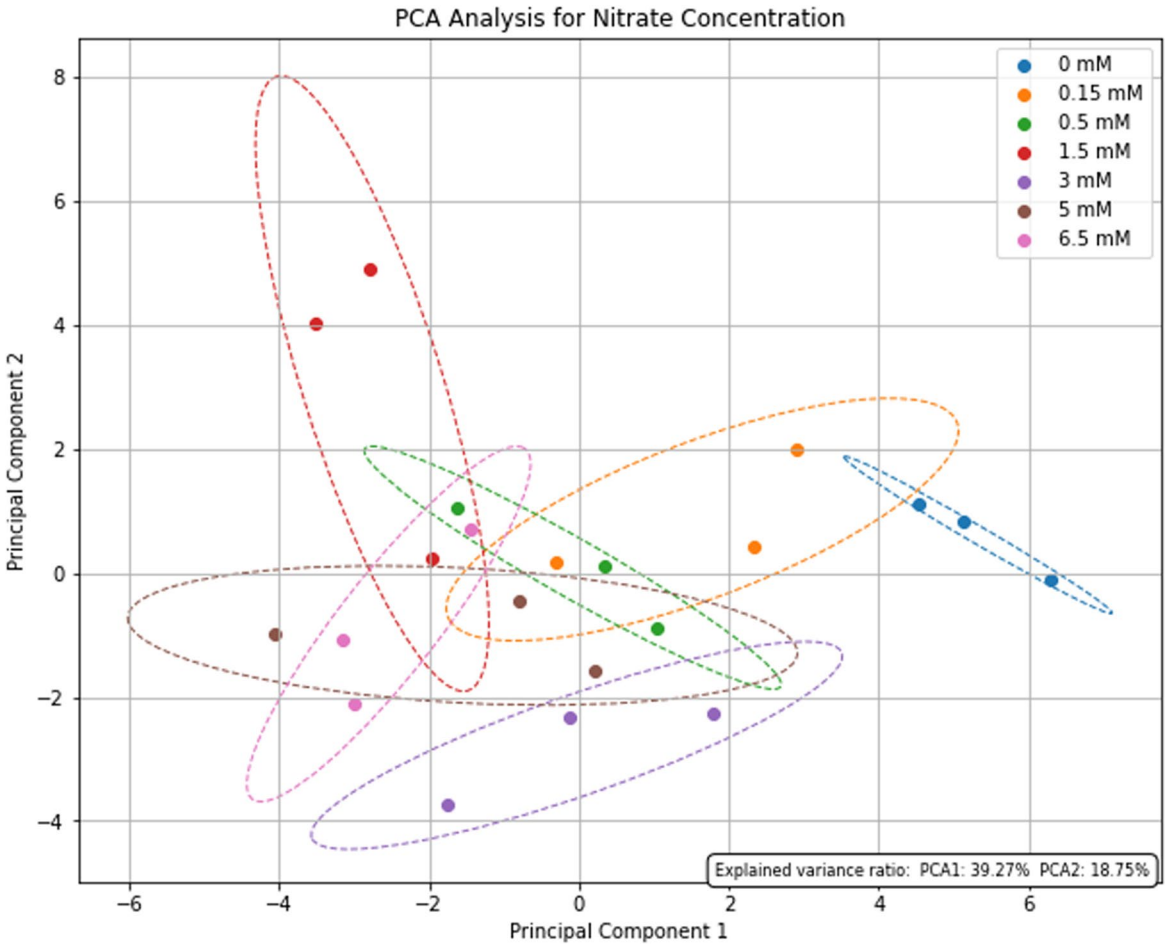

Fig. 4 Principal Component Analysis (PCA) of relative growth rate and photosynthetic parameters (chlorophyll *a*, lutein, β-carotene, phycobiliproteins (PC and PE), F_v/F_m, $ETR_{in\ situ}$, α_{ETR}, $Eopt_{ETR}$, Ek_{ETR}, $_{max}ETR$, Ek_{NPQ}, ETR and EDR in 124, 631, and 1509 µmol photons m^{-2} s^{-1}, $Eopt_{ETR}$ - Ek_{ETR}, and Ek_{NPQ} - Ek_{ETR}) of *P. linearis*, considering nitrate concentration after seven days.

experiments for *Porphyra* (Blouin et al. 2007; Kim et al. 2007). However, a higher intensity and/or quality of cultivation light to achieve a higher photosynthetic ETR could have favored absorption and consequently growth in *P. linearis*, especially since growth rates and photosynthesis are significantly influenced by the light source's wavelength. In particular blue and blue+red lights have shown higher growth rates than white light and red lights (Kim et al. 2019).

Phycobiliproteins are nitrogenous molecules that act as photosynthesizing pigments and are known to be a nitrogen storage mechanism when nitrogen is abundant, and a nitrogen source when it is limited (Lapointe and Ryther 1979). However, in the present study, the phycobiliprotein contents of *P. linearis* exhibited resistance during a no-nutrient period, which did not cause a significant decline in the pigment content, indicating that even in the absence of nitrate, the algae had a high concentration of these pigments compared to other *Porphyra sensu lato* species (Carmona et al. 2006; Pereira et al. 2008, 2018, 2020; Tala and Chow 2014). It is known that a relatively high phycobiliprotein content does not necessarily indicate 'well-assembled and functional antennae', it could also signify nitrogen storage or the dissipation of excess energy during photoinhibition (Talarico and Maranzana 2000), as observed in this current study. For most samples, by the seventh day of exposure, the amount of phycocyanin and phycoerythrin were not significantly increased when compared to the sample exposed to 0 mM of nitrate, but exhibited greater functionality, as manifested by the enhanced photosynthetic parameters that were analyzed. On the other hand, samples exposed to 3, 5, and 6.5 mM nitrate, exhibited an increase, albeit with minor statistical differences, in the quantity of these pigments. A longer experiment duration or increased light intensity might favor not only the growth rate, but also the production of phycobiliproteins.

Chlorophyll *a* is a primary metabolite and the principal photosynthetic pigment in algae, located in the most protected site of the photosynthetic antennae (Parmar et al. 2013;

Hu et al. 2021). Due to its importance and location, it is one of the last pigments to degrade under stress conditions, such as nutrient deficiency. It was observed that a 3 mM nitrate condition favored the highest production of this pigment, while the lower concentrations (0 and 0.15 mM) exhibited the next highest concentrations, corroborating with results observed in *Ulva rigida* (Cabello-Passini and Figueroa 2005) and *Gracilaria tenuistipitata* (Barufi et al. 2011). Chlorophyll *a* is a large molecule containing only four nitrogen atoms in its structure, so it is not considered highly dependent on external nitrogen for its synthesis. However, optimal culture conditions and nitrogen availability can enhance its production, as seen at 3 mM nitrate. Moreover, chlorophyll *a* can act as an antioxidant agent (Lanfer-Marquez et al. 2005; Pérez-Gálvez et al. 2020) and its high concentrations in samples under stress conditions (absence and/or low availability of nitrate) may function as a defense molecule.

Carotenoids are secondary metabolites that, besides capturing light for the photosynthetic antennae, can also act as antioxidant agents (Aple and Hirt 2004; Kottuparambil et al. 2012). Among the two detected carotenoids, lutein and β-carotene, the highest quantities were observed in algae exposed to 0 and 3 mM nitrate, while concentrations of 1.5 and 6.5 mM showed the lowest amounts of these carotenoids. Carotenoids consist of long carbon chains, and their production may be associated with stress conditions aimed at preventing cellular oxidation, as might be the case in samples with 0 and 0.15 mM nitrate. Alternatively, they can be produced under optimal metabolic conditions to aid in capturing photosynthetic light (Quinlan et al. 2012), as observed in the algae exposed to 3 mM. Data indicating the physiological state of photosynthetic organisms (F_v/F_m) (Maxwell and Johnson 2000) and productivity (ETR_{max} and $ETR_{in\ situ}$) (Jerez et al. 2016; Figueroa et al. 2022) support these results. Samples exposed to higher nitrate concentrations exhibited enhanced photosynthetic parameters, indicating that nitrate presence favored the development of a photosynthetic apparatus available for electron transport, without causing photoinhibition. On the other hand, different nitrate concentrations did not cause statistical differences in αETR or Ek_{ETR}, demonstrating that nitrate did not interfere with photosynthetic efficiency or the intensity of photosynthetic saturation radiation.

The absence of nitrate resulted in cell depigmentation, turning them greenish, with degraded chloroplast branches, while other concentrations maintained the brown color and intact chloroplast, a similar result observed in *Pyropia acanthophora* var. *brasiliensis* under nutrient deprivation (Pereira et al. 2020). In addition to photoprotection mechanisms, nitrogen limitation affects various processes, including photosynthetic capacity (Cabello-Passini and Figueroa 2005) and protein content (Korbee et al. 2005; Pereira et al. 2020), and it leads to reduced cell size (Fatini et al. 2021), as observed in the current study, where higher nitrate availability correlated with larger cell size. Due to the increased surface area of the cells, the algae are capable of assimilating more nutrients, which explains the high daily nitrate uptake rate in these algae. On the effects of nitrate supply on the cell size, the decrease of protoplast length under the lowest nitrate level (0-0.5 mM) can be a morphogenesis acclimation since the smaller cells are related to increase in surface/volume ratio (S/V) in order to increase the uptake of nitrate (Figueroa et al. 1995) i.e., in *Porphyra umbilicalis* grown under blue-light the cell size was greater than that under red-light, however S/V was the reverse and consequently growth rate and biomass productivity was much higher in red-light than blue-light. Vega et al. (2024) also observed a decrease in cell size and increase in S/V in *Pyropia leucosticta* grown under 50 and 250 µM compared to 500 µM nitrate as acclimation response to the decrease of nitrate level. The color is taken into account in the selection of *Porphyra* strains for culinary uses (Gao et al. 2019). Increase of CO_2, i.e ocean acidification may reduce the biomass of *Porphyra yezoensis* but increase the color and flavor, which was regulated by nitrogen availability (Gao et al. 2019). CO_2 and nitrate availability and light quality radiation have effects on the color variation and biochemical composition in seaweeds as in the case of *Porphyra* sp. (Figueroa et al. 1995) and in other red algae as *Gracilariopis longissima* (Bermejo et al. 2020) or *Gracilaria cornea* (Figueroa et al. 2022).

In this study, the presence of nitrate led to an increase in $ETR_{in\ situ}$, reaching 6.79 µmol e^- m^{-2} s^{-1} at the highest nitrate concentration tested. The same pattern is observed at the light intensity of 124 µmol photons m^{-2-1}, achieving a maximum of 3.75 µmol e^- m^{-2} s^{-1}. This difference was found because ETR_{max} is calculated using a rapid light curve (RLC) through PAM equipment with a red-light pulse, whereas $ETR_{in\ situ}$ was calculated in algae grown under white light. Chlorophyll and accessory pigments can absorb light from different wavelengths, thus achieving higher photosynthetic capacity when analyzed under white light/different wavelengths (Simkin et al. 2022).

The absence of nitrate resulted in a narrow range between $Eopt_{ETR}$ and Ek_{ETR}, not supporting a wide variety of light intensities and leading to photoinhibition at low light intensities. On the other hand, algae exposed to 1.5 mM nitrate exhibited the widest possible cultivation range, becoming more adaptable concerning the supported light intensities. Additionally, the analysis of the intensity range between Ek_{NPQ} and Ek_{ETR} demonstrates the conditions under which the algae perform photosynthesis and dissipation has not yet begun; in other words, it would be the optimal range for conducting photosynthesis. Furthermore, this study showed the widest range between these two parameters for algae exposed to 1.5 mM nitrate, while the absence of nitrate resulted in the

narrowest range. Interestingly, the level of nitrate in IMTA systems by using *Sparus aurata* effluents from growing different red algae ranged from 0.2 to 2 mM (Figueroa et al. 2010, 2012; Barceló-Villalobos et al. 2017). The dissipation of light energy is essential for photosynthesis as it protects the photosynthetic apparatus from damage due to excess light. The absence of nitrate caused this dissipation to occur at lower light intensities, whereas algae exposed to 1.5, 3, 5, and 6.5 mM of nitrate began to dissipate energy at higher intensities, indicating that the photosynthetic apparatus was functional and extensive. Carotenoids and phycobiliproteins, as mentioned above, can act as light-harvesting agents for the photosynthetic apparatus, as antioxidant agents, and also as energy dissipators (Talarico and Maranzana 2000; Li et al. 2009; Simkin et al. 2022). This could also explain the elevated levels of biliproteins, lutein and β-carotene found in the samples without nitrate.

When analyzing the relationship between the ETR and the EDR at 124 and 631 µmol photons m^{-2} s^{-1}, a higher result is observed with an increase in nitrate concentration. This suggests that with more nitrate available, the algae dissipate less energy, once again, indicating better photosynthetic apparatus health due to the quantity and functionality of the pigments. However, at 1509 µmol photons m^{-2} s^{-1} this stoichiometry shows no differences in relation to the nitrate quantity, indicating that at such high light intensity, the presence of nitrate does not confer photosynthetic protection.

Thus, analyzing all the photosynthetic parameters evaluated in this study and considering the different nitrate concentrations after seven days of exposure, PCA results indicate a distinct group formation is observed for samples exposed to 0 mM of nitrate, highlighting that the absence of nitrate is the determining factor for the distinction between treatments.

In conclusion *P. linea*ris showed a wide range of nitrate use without variation in pigment composition in contrast to photosynthetic capacity. Upon completing all analyses in this study, it is concluded that these specific nitrate concentrations in cultivation significantly enhance the photosynthetic response of *P. linearis*. The 1.5 and 3 mM of nitrate in cultivation significantly enhanced the photosynthetic response of *P. linearis*, supporting their potential application in IMTA cultures and bioremediation processes.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

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

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