

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

Débora Tomazi Pereira

debora.tomazi@uma.es

University of Málaga, University Institute of Blue Biotechnology and Development (IBYDA)

Nathalie Korbee

University of Málaga, University Institute of Blue Biotechnology and Development (IBYDA)

Julia Vega

University of Málaga, University Institute of Blue Biotechnology and Development (IBYDA)

Félix L. Figueroa

University of Málaga, University Institute of Blue Biotechnology and Development (IBYDA)

Research Article

Keywords: nitrate assimilation, bioremediation, integrated multi-trophic aquaculture, photosynthetic antennae, pigments

Posted Date: March 25th, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-4139150/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Additional Declarations: No competing interests reported.

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. *P. 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 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 ($E_{opt_{ETR}}$) and saturated irradiance for photosynthesis ($E_{k_{ETR}}$) and between saturated irradiance for non-photochemical quenching ($E_{k_{NPQ}}$) and $E_{k_{ETR}}$, 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.

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 (Errat 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 2023).

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

Porphyra sensu lato, a red macroalgae, 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 et al. 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 algae. Fortune Business Insights reports that the global market for commercial seaweed stood at \$14.11 billion in 2020 and is projected to grow to \$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 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 *P. 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 Muíños' 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, $\lambda = 400-700$ nm) of $120 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (White LED light, 5000K, 54W, NU-8416, Nuovo), temperature of $15 \pm 2^\circ\text{C}$, 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} (Supp. Table 1).

The optimal or maximal quantum yield (F/F_0), effective quantum yield ($Y(II)$) (Supp. Figure 1), electron transport rate (ETR) and non-photochemical quenching (NPQ) by rapid light curves (RLC) were determined (Supp. Figure 2). Growth rate, content of phycobiliproteins (PC: phycocyanin and PE: phycoerythrin), phenolic compounds, and antioxidant activity (Supp. 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) (Supp. 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 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 of 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 $24 \mu\text{M}$ 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 replicates, and after this, the samples were frozen at -80°C 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 HCl 1.3N (1% w/v) and N-(1-naphthyl)-ethylenediamine dihydrochloride (NED) in MilliQ water 0.005%, 1:1) and 100 μL VCl_3 -reagent (Vanadium (III) chloride in HCl 6N (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 down to room temperature in a water-bath and the absorbance was measured at 540 nm (García Robledo et al. 2014). The quantification of nitrate total 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 and Anton (2013): $\text{RGR \% day}^{-1} = [(W_f/W_0)^{1/t} - 1] \times 100$, where RGR is relative growth rate ($\% \text{ day}^{-1}$), W_f is final fresh weight in grams, W_0 is initial fresh weight in grams, and time in days.

Light microscopy

On the seventh day of the experiment, fresh material of all replicates was extended between slide and coverslip, and observed under a light microscope (Leica DME, Wetzlar, 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.

Pigment lipophilic quantification

The extractions, after 7 days of experiment, of lipophilic pigments (chlorophyll *a* and carotenoids) were performed from 100 mg of fresh algae and 1.5 mL of extraction solvent (methanol 100%). After combining the algae with the extraction solvent, they were macerated by 30 seconds using an UltraTurrax® (T25, IKA, Germany) (18000 rpm) and then subjected to extraction at 4°C for 3 hours. Following extraction, the samples were centrifuged at $2721.6 \times g$, 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 $0.2 \mu\text{m}$ membrane before chromatographic analysis using a HPLC system (1260 Agilent InfinityLab Series, Santa Clara, CA, 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 ($5 \mu\text{m}$; 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 standard 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% of sodium carbonate (SC)) added, performing alkaline hydrolysis. After combining the algae with the extraction solvent, they were macerated by 30 seconds using an UltraTurrax® (18000 rpm) and then subjected to extraction at 80°C for 1.5 hour. Following extraction, the samples were centrifuged at $2721.6 \times g$, the supernatant was collected, and the pH was adjusted using lactic acid to achieve a pH of 7.0 (pHmeter Horiba LaquaTwin.). Thus, the algal extracts were prepared 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 by using a UV–visible spectrophotometer (Shimadzu UV-2600, $\lambda = 498, 615, \text{ and } 651 \text{ nm}$), following the formulas described by Kursar et al. (1983). The analyses were conducted in triplicates, and the results were expressed in mg of pigments 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 Ciocalteu (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 hours at 4 °C. Subsequently, readings were taken at 760 nm using a UV–visible spectrophotometer. The quantification of total phenolic compounds was determined using a standard curve of phloroglucinol ($1 \text{ to } 20 \mu\text{g mL}^{-1}$ - $R^2 = 0.99$; $y = 0.0586x$; where y represents the absorbance and x represents the concentration of phenols). The analyses were performed in triplicate, and the results were expressed in mg of phloroglucinol per g of DW.

Antioxidant activity

Antioxidant capacity was determined by the ABTS radical scavenging assay. Firstly, the radical cation $\text{ABTS}^{+\bullet}$ 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 ($\text{K}_2\text{S}_2\text{O}_8$) 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 $\text{ABTS}^{+\bullet}$ was diluted with phosphate buffer until absorbance at 727 nm were 0.75 ± 0.05 . The assay was done by adding 950 μL of the diluted $\text{ABTS}^{+\bullet}$ solution and 50 μL of algal extract, according to Re et al. (1999). The samples were shaken, and absorbance was recorded by a UV–visible spectrophotometer at 727 nm at the beginning of the reaction (DO_i) and after 8 minutes of incubation (DO_f). The percentage of antioxidant activity (AA%) was calculated according to the formula:

$$\text{AA\%} = [(\text{absDO}_i - \text{absDO}_f) / \text{absDO}_i] \times 100$$

The antioxidant compounds concentration was calculated using a standard curve of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma-Aldrich) ($20 \text{ to } 100 \mu\text{g mL}^{-1}$ - $R^2 = 0.99$; $y = 15.8928x$; 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 internal carbon, nitrogen, and sulfur

Total internal carbon (C), nitrogen (N), and sulfur (S) contents of the dry algal biomass were evaluated using a LECO-932 CNHS elemental analyzer (St. Joseph, MI, USA) 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 chlorophyll *a* fluorescence was measured *in situ*, directly under the cultivation conditions in the culture chambers. To achieve this, saturating pulses ($> 3000 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) 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_0), 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 (Diving-PAM II (Walz®, Germany)). The optimal quantum yield (F_t / F_0) and the effective quantum yield ($Y(\text{II})$) were assessed at on the seventh day. Calculations for F_t / F_0 and $Y(\text{II})$ were conducted using their respective standard formulas:

$$F_v / F_m = (F_m - F_0) / F_m$$

$$Y(\text{II}) = (F_m' - F_t) / F_m'$$

The electron transport rate (ETR) was determined through rapid light curves (RLCs) by exposing samples to thirteen ascending irradiances, ranging from 0 to 1500 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, of actinic red light from Diving-PAM II. After 30 seconds of incubation at each irradiance level, a saturating pulse was applied to the samples. At each irradiance level of the RLCs, different parameters were calculated including the $Y(\text{II})$, ETR, and two different types of yield losses: the yield for non-photochemical quenching ($Y(\text{NPQ})$) calculated as $(F_t / F_0) - (F_t / F_m)$ and the yield for non-regulated energy dissipation ($Y(\text{NO})$) calculated as F_0 / F_m . Additionally, nonphotochemical quenching (NPQ) was calculated using the formula $Y(\text{NPQ}) / Y(\text{NO})$. The energy dissipation rate (EDR) was also evaluated at 124 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (intensity closest to that used in cultivation), at 631 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (median intensity of the RLC), and at 1509 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (maximum intensity of the RLC). The ETR (Schreiber et al. 1995) and EDR (Vega et al. 2023) was calculated as follows:

$$\text{ETR} (\mu\text{mol e}^- \text{ m}^{-2} \text{ s}^{-1}) = Y(\text{II}) \times E_{\text{PAR}} \times A \times F_{\text{II}}$$

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

where $Y(\text{II})$ is the effective quantum yield, $Y(\text{NO})$ is the yield loss related to passive thermal dissipation, and $Y(\text{NPQ})$ is the yield loss related to photoregulated process; E_{PAR} is the incident PAR irradiance expressed in $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, A is the absorptance that was calculated as $A = 1 - (E_t / E_0)$ (E_t is the irradiance that pass 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 ($E_{\text{K}_{\text{ETR}}}$) and NPQ ($E_{\text{K}_{\text{NPQ}}}$) and optimal irradiance ($E_{\text{opt}_{\text{ETR}}}$, respectively). The $E_{\text{K}_{\text{ETR}}}$ was subtracted to the E_{opt} values to detect the range of supported light intensities, without leading to photoinhibition, and $E_{\text{K}_{\text{NPQ}}}$ was subtracted to the $E_{\text{K}_{\text{ETR}}}$ 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 during 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/F_0) did not show significant differences among the three salt types across all days analyzed (days 4, 7, 11, and 14) (Supp. Figure 1a). Considering the effective quantum yield ($\Phi(II)$), 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 (Supp. Figure 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. Figure 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 (Supp. Figure 2c,d).

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 absorptions. In terms of the daily uptake rate, for seven days of exposure, the daily absorption rate was highest 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 ($r = 0.8440$, $p = 0.001$).

Table 1

Nitrate uptake efficiency (NUE, %) and in nitrate uptake rate (NUR, mmol of $\text{NO}_3^- \text{ g}^{-1} \text{ DW day}^{-1}$) in *P. linearis* after exposure to different nitrate concentrations for seven days ($n = 3$; mean $\pm$ 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 $\text{NO}_3^- \text{ g}^{-1} \text{ DW day}^{-1}$ - 7 days)
0 mM	-	-
0.15 mM	95.36 $\pm$ 4.40 ^a	0.12 $\pm$ 0.01 ^d
0.5 mM	99.51 $\pm$ 0.16 ^a	0.43 $\pm$ 0.15 ^c
1.5 mM	81.68 $\pm$ 7.07 ^b	1.02 $\pm$ 0.13 ^{ab}
3 mM	53.99 $\pm$ 3.74 ^c	1.26 $\pm$ 0.05 ^a
5 mM	27.66 $\pm$ 1.67 ^d	1.21 $\pm$ 0.06 ^a
6.5 mM	16.09 $\pm$ 2.13 ^e	0.92 $\pm$ 0.13 ^b

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 ($r = 0.6545$, $p = 0.001$); however, no statistical differences were found among them (Table 2).

Table 2

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

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

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 (Fig. 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 ($r = 0.5685$, $p = 0.007$).

Table 3
Protoplast length (μm) in *P. linearis* exposed to different nitrate concentrations ((n = 50; mean $\pm$ 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 $\pm$ 2.59 ^{ab}
0.15 mM	16.42 $\pm$ 3.97 ^{ab}
0.5 mM	15.17 $\pm$ 3.09 ^b
1.5 mM	19.40 $\pm$ 3.86 ^a
3 mM	19.24 $\pm$ 3.35 ^a
5 mM	19.72 $\pm$ 3.79 ^a
6.5 mM	19.23 $\pm$ 4.36 ^a

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 correlation 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).

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 ($r = 0.5280$, $p = 0.014$), 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 e 6.5 mM nitrate concentrations displayed the highest ETRs, whereas the 0 mM nitrate sample consistently showed the lowest ETR (Suppl. Figure 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. Figure 3b).

After seven days, the lowest F/F_0 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/F_0 ($r = 0.4731$, $p = 0.03$). For the photosynthetic electron transport rate efficiency (α_{ETR}) and ETR saturated irradiance ($E_{k_{\text{ETR}}}$), no statistical differences were observed across all treatments. However, 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. $E_{k_{\text{ETR}}}$ and ETR_{max} exhibited positive Pearson correlation with the nitrate concentration ($r = 0.4391$, $p = 0.046$ and $r = 0.5397$, $p = 0.012$, respectively), whereas α_{ETR} did not demonstrate any correlation.

For the optimal irradiance for ETR ($E_{\text{opt}_{\text{ETR}}}$), 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 ($E_{k_{\text{NPQ}}}$), 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 $E_{\text{opt}_{\text{ETR}}}$ and $E_{k_{\text{ETR}}}$, the greatest range of intensity 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 $E_{K_{NPQ}}$ and $E_{K_{ETR}}$, 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). $E_{K_{NPQ}}$ and the difference between $E_{K_{NPQ}}$ and $E_{K_{ETR}}$ displayed a positive Pearson correlation with the nitrate concentration ($r = 0.5771$, $p = 0.006$ and $r = 0.5078$, $p = 0.019$, respectively), while $E_{opt_{ETR}}$ and the difference between $E_{opt_{ETR}}$ and $E_{K_{ETR}}$ did not show any correlation with the nitrate concentration.

Table 4

Photosynthetic parameters (optimal quantum yield (F_v/F_m), photosynthetic electron transport rate efficiency (α_{ETR}), maximal ETR (ETR_{max} - $\mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$), ETR saturated irradiance ($E_{K_{ETR}}$ - $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), ETR optimal irradiance ($E_{opt_{ETR}}$ - $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), NPQ saturated irradiance ($E_{K_{NPQ}}$ - $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), and difference between $E_{opt_{ETR}}$ and $E_{K_{ETR}}$, and between $E_{K_{NPQ}}$ and $E_{K_{ETR}}$ ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$) 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 $\pm$ 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	F_v/F_m	α_{ETR}	ETR_{max}	$E_{K_{ETR}}$	$E_{opt_{ETR}}$	$E_{opt_{ETR}} - E_{K_{ETR}}$	$E_{K_{NPQ}}$	$E_{K_{NPQ}} - E_{K_{ETR}}$
0 mM	0.41 $\pm$ 0.04 ^b	0.045 $\pm$ 0.02 ^a	2.42 $\pm$ 0.33 ^b	56.07 $\pm$ 12.13 ^a	313.37 $\pm$ 82.99 ^b	269.00 $\pm$ 77.32 ^b	77.65 $\pm$ 24.29 ^b	21.58 $\pm$ 17.45 ^b
0.15 mM	0.55 $\pm$ 0.03 ^a	0.052 $\pm$ 0.02 ^a	3.71 $\pm$ 0.98 ^{ab}	58.03 $\pm$ 26.85 ^a	361.95 $\pm$ 70.36 ^{ab}	280.98 $\pm$ 34.96 ^b	175.93 $\pm$ 79.62 ^{ab}	94.96 $\pm$ 89.43 ^{ab}
0.5 mM	0.55 $\pm$ 0.03 ^a	0.054 $\pm$ 0.02 ^a	4.01 $\pm$ 0.49 ^{ab}	76.71 $\pm$ 13.03 ^a	389.20 $\pm$ 63.46 ^{ab}	345.82 $\pm$ 130.08 ^{ab}	173.24 $\pm$ 83.32 ^{ab}	96.53 $\pm$ 71.77 ^{ab}
1.5 mM	0.59 $\pm$ 0.03 ^a	0.086 $\pm$ 0.03 ^a	4.14 $\pm$ 0.57 ^{ab}	53.30 $\pm$ 18.82 ^a	494.78 $\pm$ 70.24 ^a	448.86 $\pm$ 72.76 ^a	250.94 $\pm$ 54.63 ^a	197.64 $\pm$ 42.71 ^a
3 mM	0.59 $\pm$ 0.05 ^a	0.049 $\pm$ 0.01 ^a	4.21 $\pm$ 1.10 ^{ab}	84.86 $\pm$ 9.67 ^a	355.59 $\pm$ 20.70 ^{ab}	270.73 $\pm$ 20.99 ^b	232.41 $\pm$ 21.46 ^a	153.01 $\pm$ 27.12 ^{ab}
5 mM	0.58 $\pm$ 0.01 ^a	0.058 $\pm$ 0.01 ^a	4.39 $\pm$ 1.02 ^{ab}	77.72 $\pm$ 20.07 ^a	423.10 $\pm$ 64.47 ^{ab}	312.04 $\pm$ 106.60 ^{ab}	231.66 $\pm$ 30.07 ^a	153.94 $\pm$ 40.51 ^{ab}
6.5 mM	0.60 $\pm$ 0.02 ^a	0.061 $\pm$ 0.01 ^a	4.85 $\pm$ 1.12 ^a	82.52 $\pm$ 29.72 ^a	377.21 $\pm$ 51.16 ^{ab}	261.36 $\pm$ 80.39 ^{ab}	256.19 $\pm$ 22.49 ^a	173.67 $\pm$ 52.21 ^a

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 ($r = 0.7311$, $p = 0.0001$).

At 124 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 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 ($r = 0.6201$, $p = 0.003$), whereas a negative Pearson correlation was noted in EDR in this same irradiance and the nitrate quantity ($r = -0.4402$, $p = 0.046$).

At 631 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, no statistical difference in ETR was noted among all samples, but a positive Pearson correlation was detected with the nitrate concentration ($r = 0.4393$, $p = 0.046$). 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 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 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).

Table 5

Electron transport rate *in situ* (ETR_{in situ} - $\mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$), ETR and energy dissipation rate (EDR - $\mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$), and ETR:EDR ratio obtained after the adjustment of the rapid light curves (RLCs) at 124, 631, and 1509 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in *P. linearis* after exposure to different nitrate concentrations for seven days (n = 3; mean $\pm$ 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 $\pm$ 0.32 ^b	1.96 $\pm$ 0.10 ^b	9.95 $\pm$ 0.67 ^a	0.20	2.23 $\pm$ 0.49 ^a	59.77 $\pm$ 4.90 ^{ab}	0.04	1.86 $\pm$ 0.83 ^a	172.10 $\pm$ 28.56 ^a	0.01
0.15 mM	4.43 $\pm$ 0.30 ^b	2.85 $\pm$ 0.58 ^{ab}	9.18 $\pm$ 0.87 ^a	0.31	3.50 $\pm$ 0.99 ^a	61.33 $\pm$ 2.36 ^{ab}	0.06	2.49 $\pm$ 0.71 ^a	155.87 $\pm$ 24.46 ^a	0.02
0.5 mM	5.15 $\pm$ 1.23 ^{ab}	3.10 $\pm$ 0.39 ^{ab}	9.03 $\pm$ 0.46 ^a	0.34	3.82 $\pm$ 0.63 ^a	60.93 $\pm$ 1.90 ^{ab}	0.06	2.93 $\pm$ 0.97 ^a	151.36 $\pm$ 13.08 ^a	0.02
1.5 mM	5.79 $\pm$ 1.64 ^{ab}	3.29 $\pm$ 0.30 ^a	8.27 $\pm$ 0.56 ^a	0.40	4.11 $\pm$ 0.58 ^a	66.90 $\pm$ 6.17 ^a	0.06	3.68 $\pm$ 0.49 ^a	141.56 $\pm$ 9.98 ^a	0.03
3 mM	6.67 $\pm$ 0.14 ^{ab}	3.25 $\pm$ 0.75 ^{ab}	8.67 $\pm$ 0.57 ^a	0.37	3.90 $\pm$ 1.05 ^a	59.97 $\pm$ 1.92 ^{ab}	0.07	2.66 $\pm$ 0.74 ^a	138.31 $\pm$ 12.61 ^a	0.02
5 mM	6.48 $\pm$ 0.23 ^{ab}	3.40 $\pm$ 0.48 ^a	8.97 $\pm$ 0.88 ^a	0.38	4.11 $\pm$ 1.22 ^a	55.60 $\pm$ 2.76 ^b	0.07	3.05 $\pm$ 1.29 ^a	158.84 $\pm$ 17.05 ^a	0.02
6.5 mM	6.79 $\pm$ 0.32 ^a	3.75 $\pm$ 0.37 ^a	8.23 $\pm$ 0.102 ^a	0.46	4.43 $\pm$ 1.43 ^a	57.84 $\pm$ 2.41 ^{ab}	0.08	3.03 $\pm$ 1.18 ^a	142.65 $\pm$ 20.66 ^a	0.02

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, 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 ($1.26 \text{ mmol NO}_3^- \text{g}^{-1} \text{DW day}^{-1}$), 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) across 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% per day (Carmona et al. 2000, 2006), whereas the growth rate in the current study was around 2% per day. The light intensity used in the experiment ($120 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) is the same as that employed in controlled laboratory 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 Duke 1984). 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; Tala and Chow 2014; Pereira et al. 2018, 2020). 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 of 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 (Fv/Fm) (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-Pasin 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 higher 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. (2023) also observed a decrease in cell size and increase in S/V in *Pyropia leucosticta* grown under 50 and 250 μ M compared to 500 μ M 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. 2018). Increase of CO₂, 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. 2018). CO₂ 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 *Gracilariopsis longissima* (Bermejo et al. 2020) or *Gracilaria cornea* (Figueroa et al. 2022).

In this article, the presence of nitrate led to an increase in $ETR_{in situ}$, reaching 6.79 μ mol e⁻ m⁻² s⁻¹ at the highest nitrate concentration tested. The same pattern is observed at the light intensity of 124 μ mol photons m⁻² s⁻¹, achieving a maximum of 3.75 μ mol 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 E_{optETR} 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 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 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 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 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 *Porphyra linearis* 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 enhance the photosynthetic response of *P. linearis*, supporting their potential application in IMTA cultures and bioremediation processes.

Declarations

Funding: The authors acknowledge the European Union for the financial support to Débora Tomazi Pereira ('NUVAPY-BLUE' HORIZON-MSCA-2022-101106349) and by the Project 'AlgaHub: Algae for more sustainable and healthy functional foods' (TED2021-131555B-C22) by the Ministry of Science and Innovation of Spanish Government. The first author is funded by the European Union under the Marie Skłodowska-Curie grant agreement N° 101106349. Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of the European Union or the European Commission (granting authority). Neither the European Union nor the granting authority can be held responsible for them'.

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.

Availability of data and material: All data generated or analyzed during this study are included in this published article.

Code availability: 'Not applicable'.

Authors' contributions: **Débora Tomazi Pereira:** Conceptualization, Methodology, Investigation, Writing, Review, Editing - original draft, Project administration. **Nathalie Korbee:** Conceptualization, Methodology, Investigation, Review, Editing - original draft. **Júlia Vega:** Methodology, Investigation. **Félix López Figueroa:** Conceptualization, Methodology, Investigation, Writing, Review, Editing - original draft, Supervision, Project administration.

Acknowledgments: We tank to Porto Muiños company the harvesting of *Porphyra linearis* in Galicia (Northwestern Spain), as well as the Laboratory of Fotobiología y Biotecnología de Organismos Acuáticos (FYBOA-UMA) and the University Institute of Blue Biotechnology and Development at Grice Hutchinson Research Center (IBYDA-UMA) for providing the facilities to conduct the experiments and analyses.

References

1. Aple K, Hirt H (2004) Reactive oxygen species, metabolism, oxidative stress, and signal transduction. *Ann Rev Plant Biol* 55:373–399.
2. Barceló-Villalobos M, Figueroa FL, Korbee N, Álvarez-Gómez F, Abreu MH (2017) Production of mycosporine-like amino acids from *Gracilaria vermiculophylla* (Rhodophyta) cultured through one year in an Integrated Multi-trophic Aquaculture (IMTA) system. *Mar Biotechnol* 19:246–254.
3. Barufi, JB, Korbee N, Oliveira MC, Figueroa FL (2011) Effects of N supply on the accumulation of photosynthetic pigments and photoprotectors in *Gracilaria tenuistipitata* (Rhodophyta) cultured under UV radiation. *J App Phycol* 23:457–466.
4. Bermejo R, Macías M, Sánchez-García F, Love R, Varela-Álvarez E, Hernández I (2020) Influence of irradiance, dissolved nutrients and salinity on the colour and nutritional characteristics of *Gracilariopsis longissima* (Rhodophyta) *Algal Res* 42:102121.
5. Blouin, N, Xiugeng F, Peng J, Yarish C, Brawley SH (2007) Seeding nets with neutral spores of the red alga *Porphyra umbilicalis* (L.) Kützinger for use in integrated multi-trophic aquaculture (IMTA). *Aquaculture* 270(1–4):77–91.
6. Blouin NA, Brodie JA, Grossman AC, Xu P, Brawley SH (2011) *Porphyra*: a marine crop shaped by stress. *Trends in Plant Scie* 16(1):29–37.
7. Cabello-Pasini A, Figueroa FL (2005) Effect of nitrate concentration on the relationship between photosynthetic oxygen evolution and electron transport rate in *Ulva rigida* (Chlorophyta). *J Phycol* 41(6):1169–1177.
8. Carmona R, Chanes L, Kraemer G, Chopin T, Neefus C, Zertuche JA, Yarish C (2000) Nitrogen uptake by *Porphyra purpurea*: its role as a nutrient scrubber. In: 5th Biennial Long Island Sound Research Conference Proceedings 87–91.
9. Carmona R, Kraemer GP, Yarish C (2006) Exploring northeast american and asian species of *Porphyra* for use in an integrated finfish–algal aquaculture system. *Aquaculture* 252(1):54–65.
10. Cunningham SD, Berti WR (1993) Remediation of contaminated soils with green plants: an overview. *In Vitro Cell Dev Biol Plant* 29:207–212.

11. Eilers PHC, Peeters JCH (1988) A model for the relationship between light intensity and the rate of photosynthesis in phytoplankton. *Ecol Modell* 42:199–215.
12. Erratt KJ, Creed IF, Trick CG (2018) Comparative effects of ammonium, nitrate and urea on growth and photosynthetic efficiency of three bloom-forming cyanobacteria. *Freshwater Biol* 63(7):626–638.
13. Fatini MA, Basri EM, Maznah WW (2021) Effect of different nitrogen sources on cell growth and biochemical compositions of *Chlorococcum* sp. cultivated under laboratory conditions. In: IOP Conference Series: Earth and Environmental Science. IOP Publishing, 012010.
14. Figueroa FL, Aguilera J, Niell FX (1995). Red and blue light regulation of growth and photosynthetic metabolism in *Porphyra umbilicalis* (L.) Kützinger (Bangiales, Rhodophyta). *Europ J Phycol* 30:11–18.
15. Figueroa FL, Escassi L, Pérez-Rodríguez E, Korbee N, Giles AD, Johnsen G (2003) Effects of short-term irradiation on photoinhibition and accumulation of mycosporinlike amino acids in sun and shade species of the red algal genus *Porphyra*. *J Photochem Photobiol B* 69:21–30.
16. Figueroa FL, Israel A, Neori A, Martínez B, Malta EJ, Put A, Inken S, Marquardt R, Abdala-Díaz R, Korbee N (2010) Effect of nutrient supply on photosynthesis and pigmentation to short-term stress (UV radiation) in *Gracilaria conferta* (Rhodophyta). *Mar Pollut Bull* 60:1768–1778.
17. Figueroa FL, Korbee N, Abdala-Díaz R, Jerez CG, López de la Torre M, Güenaga L, Larrubia MA, Gómez-Pinchetti JL (2012) Biofiltration of fishpond effluents and accumulation of N-compounds (phycobiliproteins and mycosporine-like amino acids) versus compounds (polysaccharides) in *Hydropuntia cornea* (Rhodophyta). *Mar Pollut Bull* 64:310–318.
18. Figueroa FL, Domínguez-González B, Korbee N (2014) Vulnerability and acclimation to increased UVB radiation in three intertidal macroalgae of different morpho-functional groups. *Mar Environ Res* 97:30–38.
19. Figueroa FL, Álvarez-Gómez F, Bonomi-Barufi J, Vega J, Massocato TF, Gómez-Pinchetti JL, Korbee N (2022) Interactive effects of solar radiation and inorganic nutrients on biofiltration, biomass production, photosynthetic activity and the accumulation of bioactive compounds in *Gracilaria cornea* (Rhodophyta). *Algal Res* 68: 102890.
20. Folin O, Ciocalteu V (1927) On tyrosine and tryptophane determinations in proteins. *J Biol Chem* 73(2):627–650.
21. Gao G, Gao Q, Nao M, Xu J, Li X (2019) Nitrogen availability modulates the effects of ocean acidification on biomass yield and food quality of a marine crop *Pyropia yezoensis*. *Food Chemistry* 271:623–629.
22. García-Robledo E, Corzo A, Papaspyrou S (2014) A fast and direct spectrophotometric method for the sequential determination of nitrate and nitrite at low concentrations in small volumes. *Mar Chem* 162:30–36.
23. Gheysen L, Demets R, Devaere J, Bernaerts T, Goos P, Van Loey A, Foubert I (2019) Impact of microalgal species on the oxidative stability of n-3 LC-PUFA enriched tomato puree. *Algal Res* 40:101502.
24. Guedes ÉA, Silva TGD, Aguiar JS, Barros LDD, Pinotti LM, Sant'Ana AE (2013) Cytotoxic activity of marine algae against cancerous cells. *Rev Bras Farmacogn* 23:668–673.
25. He P, Xu S, Zhang H, Wen S, Dai Y, Lin S, Yarish C (2008) Bioremediation efficiency in the removal of dissolved inorganic nutrients by the red seaweed, *Porphyra yezoensis*, cultivated in the open sea. *Water Res* 42(4–5):1281–1289.
26. Holdt SL, Edwards MD (2014) Cost-effective IMTA: a comparison of the production efficiencies of mussels and seaweed. *J Appl Phycol* 26:933–945.
27. Hooda PS, Edwards AC, Anderson HA, Miller A (2000) A review of water quality concerns in livestock farming areas. *Sci Total Environ* 250(1–3):143–167.
28. Hu X, Gu T, Khan I, Zada A, Jia T (2021) Research progress in the interconversion, turnover and degradation of chlorophyll. *Cells* 10(11):3134.
29. Jerez CG, Malapascua JR, Sergejevoá M, Masojídek J, Figueroa FL (2016) *Chlorella fusca* (Chlorophyta) grown in thin-layer cascades: Estimation of biomass productivity by in-vivo chlorophyll *a* fluorescence monitoring. *Algal Res* 17:21–30.
30. Kim JK, Kraemer GP, Neefus CD, Chung IK, Yarish C (2007) Effects of temperature and ammonium on growth, pigment production and nitrogen uptake by four species of *Porphyra* (Bangiales, Rhodophyta) native to the New England coast. *J Appl Phycol* 19:431–440.
31. Kim JH, Choi SJ, Lee S (2019) Effects of temperature and light on photosynthesis and growth of red alga *Pyropia dentata* (Bangiales, Rhodophyta) in a conchocelis phase. *Aquaculture* 505:167–172.
32. Korbee N, Figueroa FL, Aguilera J (2005) Effect of light quality on the accumulation of photosynthetic pigments, proteins and mycosporine-like amino acids in the red alga *Porphyra leucosticta* (Bangiales, Rhodophyta). *J Photochem Photobiol B* 80(2):71–78.
33. Kottuparambil S, Shin W, Brown MT, Han T (2012) UV-B affects photosynthesis, ROS production and motility of the freshwater flagellate, *Euglena agilis* Carter. *Aquat Toxicol* 122:206–213.
34. Kraemer GP, Carmona R, Chopin T, Neefus C, Tang X, Yarish C (2004) Evaluation of the bioremediatory potential of several species of the red alga *Porphyra* using short-term measurements of nitrogen uptake as a rapid bioassay. *J Appl Phycol* 16:489–497.
35. Kursar TA, Van der Meer, J, Alberte RS (1983) Light-harvesting system of the red alga *Gracilaria tikvahiae*. I. Biochemical analyses of pigment mutations. *Plant Physiol* 73:353–360.
36. Lanfer-Marquez UM, Barros RM, Sinnecker P (2005) Antioxidant activity of chlorophylls and their derivatives. *Int Food Res* 38(8–9):885–891.
37. Lapointe BE, Ryther JH (1979) The effects of nitrogen and seawater flow rate on the growth and biochemical composition of *Gracilaria foliifera* v. *angustissima* in mass outdoor culture. *Bot Mar* 22:529–537.
38. Li L, Saga N, Mikami K (2009) Ca²⁺ influx and phosphoinositide signalling are essential for the establishment and maintenance of cell polarity in monospores from the red alga *Porphyra yezoensis*. *J Exp Bot* 60(12):3477–3489.
39. Liu C, Zou D (2015) Effects of elevated CO₂ on the photosynthesis and nitrate reductase activity of *Pyropia haitanensis* (Bangiales, Rhodophyta) grown at different nutrient levels. *Chin J Oceanol Limn* 33(2):419–429.

40. Maxwell K, Johnson GN (2000). Chlorophyll fluorescence—a practical guide. *J Exp Bot* 51:659–668.
41. Mercurio DG, Wagemaker TAL, Alves VM, Benevenuto CG, Gaspar LR, Campos PM (2015) In vivo photoprotective effects of cosmetic formulations containing UV filters, vitamins, Ginkgo biloba and red algae extracts. *J Photochem Photobiol B* 153:121–126.
42. Morais T, Cotas J, Pacheco D, Pereira L (2021) Seaweeds compounds: an ecosustainable source of cosmetic ingredients? *Cosmetics* 8(1):8.
43. Parmar A, Singh NK, Dhoke R, Madamwar D (2013) Influence of light on phycobiliprotein production in three marine cyanobacterial cultures. *Acta Physiol Plant* 35:1817–1826.
44. Pereira R, Kraemer G, Yarish C, Sousa-Pinto I (2008) Nitrogen uptake by gametophytes of *Porphyra dioica* (Bangiales, Rhodophyta) under controlled-culture conditions. *Eur J Phycol* 43(1):107–118.
45. Pereira DT, Filipin EP, Ramlov F, Maraschin M, Bouzon ZL, Simioni C (2018) *Pyropia acanthophora* var. *brasiliensis* EC Oliveira and Coll (Rhodophyta: Bangiales) cultivated in seawater under laboratory conditions favors the production of economically important secondary metabolites. *Braz J Biol Sci* 5(9):85–93.
46. Pereira DT, Ouriques LC, Bouzon ZL, Simioni C (2020) Effects of high nitrate concentrations on the germination of carpospores of the red seaweed *Pyropia acanthophora* var. *brasiliensis* (Rhodophyta, Bangiales). *Hydrobiologia* 847:217–228.
47. Pérez-Gálvez A, Viera I, Roca M (2020) Carotenoids and chlorophylls as antioxidants. *Antioxidants* 9(6):505.
48. Pliego-Cortés H, Bedoux G, Boulho R, Taupin L, Freile-Pelegrín Y, Bourgougnon N, Robledo D (2019) Stress tolerance and photoadaptation to solar radiation in *Rhodomenia pseudopalmeta* (Rhodophyta) through mycosporine-like amino acids, phenolic compounds, and pigments in an Integrated Multi-Trophic Aquaculture system. *Algal Res* 41:101542.
49. Quinlan RF, Shumskaya M, Bradbury LM, Beltrán J, Ma C, Kennelly EJ, Wurtzel ET (2012) Synergistic interactions between carotene ring hydroxylases drive lutein formation in plant carotenoid biosynthesis. *Plant Physiol* 160(1):204–214.
50. Rani V, Maróti G (2022) Light-dependent nitrate removal capacity of green microalgae. *Int J Mol Sci* 24(1):77.
51. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med* 26:1231–1237.
52. Schreiber U, Endo T, Mi H, Asada K (1995) Quenching analysis of chlorophyll fluorescence by the saturation pulse method: particular aspects relating to the study of eukaryotic algae and cyanobacteria. *Plant Cell Physiol* 36(5):873–882.
53. Simkin AJ, Kapoor L, Doss CGP, Hofmann TA, Lawson T, Ramamoorthy S (2022) The role of photosynthesis related pigments in light harvesting, photoprotection and enhancement of photosynthetic yield in planta. *Photosynth Res* 152(1):23–42.
54. Tala F, Chow F (2014) Ecophysiological characteristics of *Porphyra* spp. (Bangioiphyceae, Rhodophyta): seasonal and latitudinal variations in northern-central Chile. *J Appl Phycol* 26:2159–2171.
55. Talarico L, Maranzana G (2000) Light and adaptive responses in red macroalgae: an overview. *J Photochem Photobiol B* 56(1):1–11.
56. Vega JS (2023) Blue cosmeceutical applications of uv screen and antioxidant compounds from photosynthetic organisms. PhD Thesis, University of Malaga, Spain 235, 269 pp.
57. Wu Q, Fu XP, Sun LC, Zhang Q, Liu GM, Cao MJ, Cai QF (2015) Effects of physicochemical factors and in vitro gastrointestinal digestion on antioxidant activity of R-phycoerythrin from red algae *Bangia fusco-purpurea*. *Int J Food Sci Tech* 50(6):1445–1451.
58. Yong YS, Yong WTL, Anton A (2013) Analysis of formulae for determination of seaweed growth rate. *J Appl Phycol* 25:1831–1834.
59. Zhou C, Yu X, Zhang Y, He R, Ma H (2012) Ultrasonic degradation, purification and analysis of structure and antioxidant activity of polysaccharide from *Porphyra yezoensis* Ueda. *Carbohydr Polym* 87(3):2046–2051.

Figures

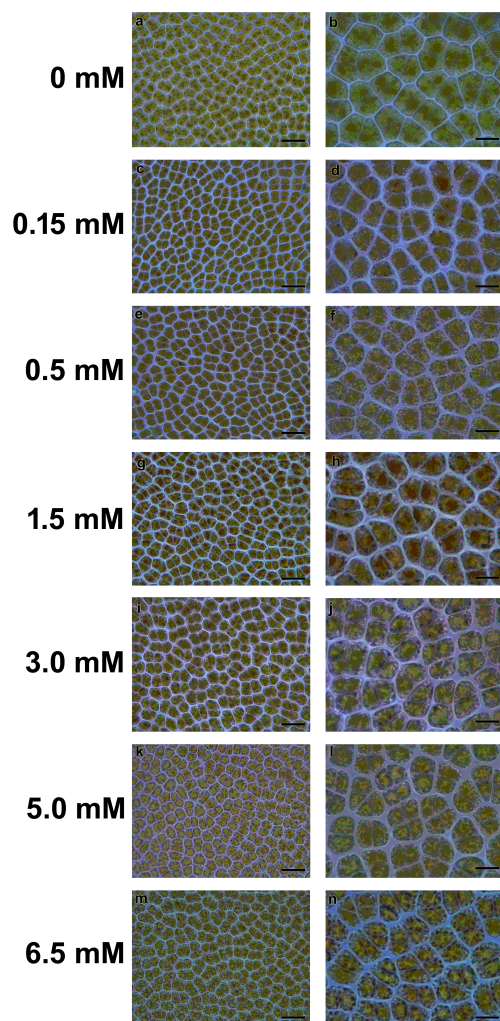


Figure 1
Thalli of *P. linearis* exposed to different nitrate concentrations observed under light microscopy (scale bars in the first column = 50 μm, and in the second column = 20 μm)

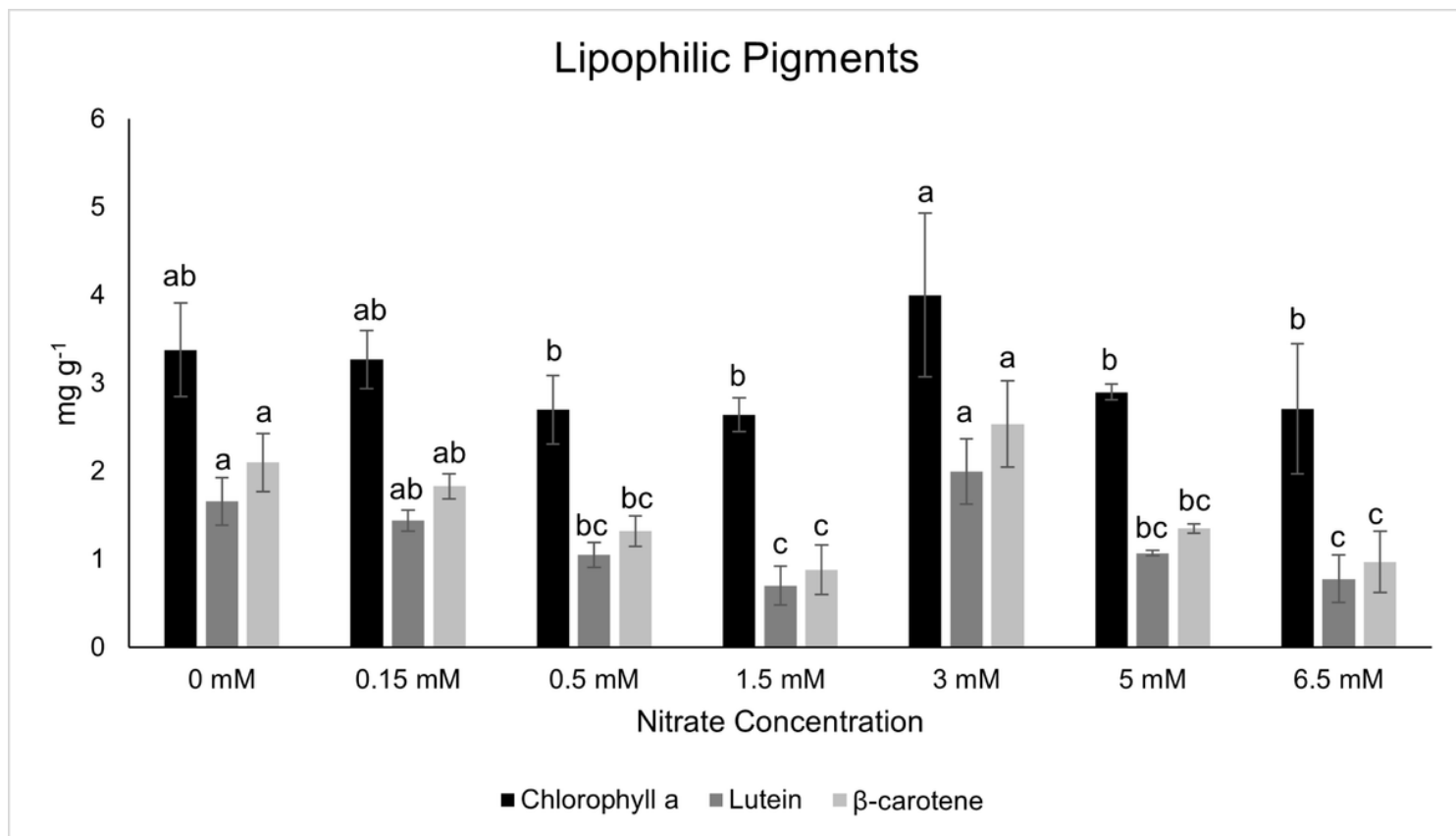


Figure 2

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 ≤ 0.05)

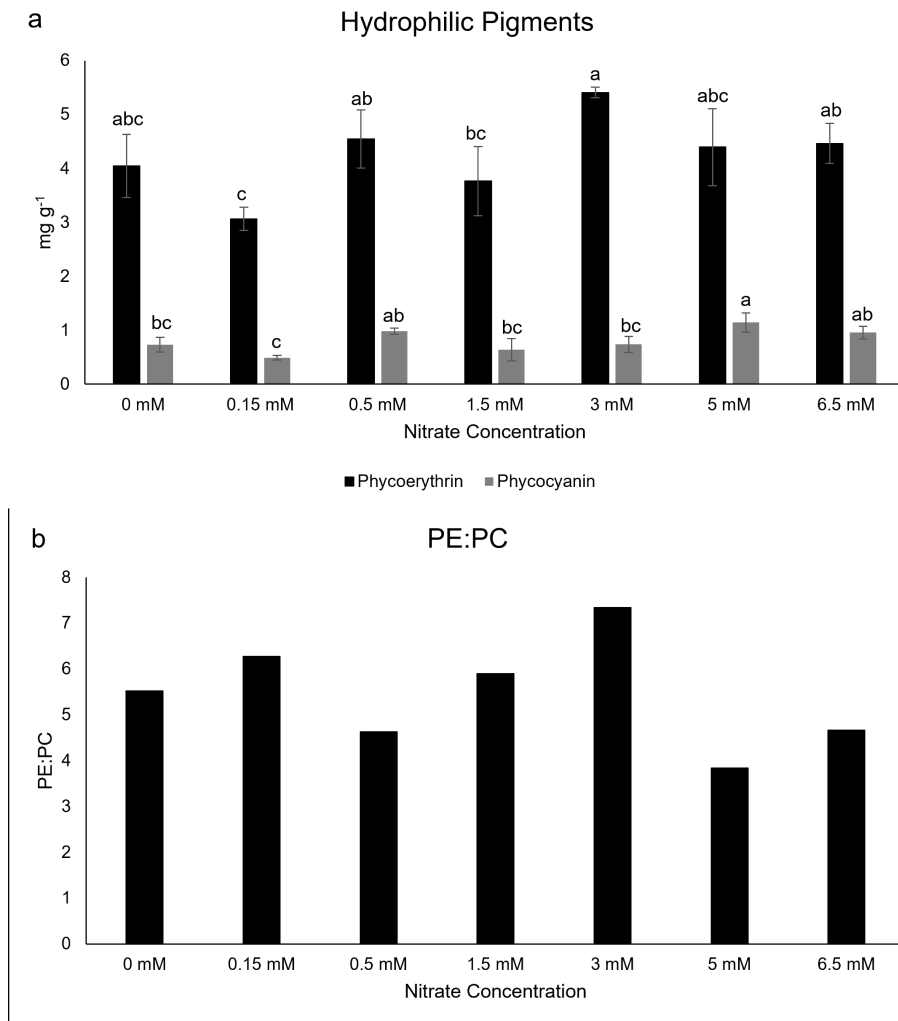


Figure 3

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 ≤ 0.05)

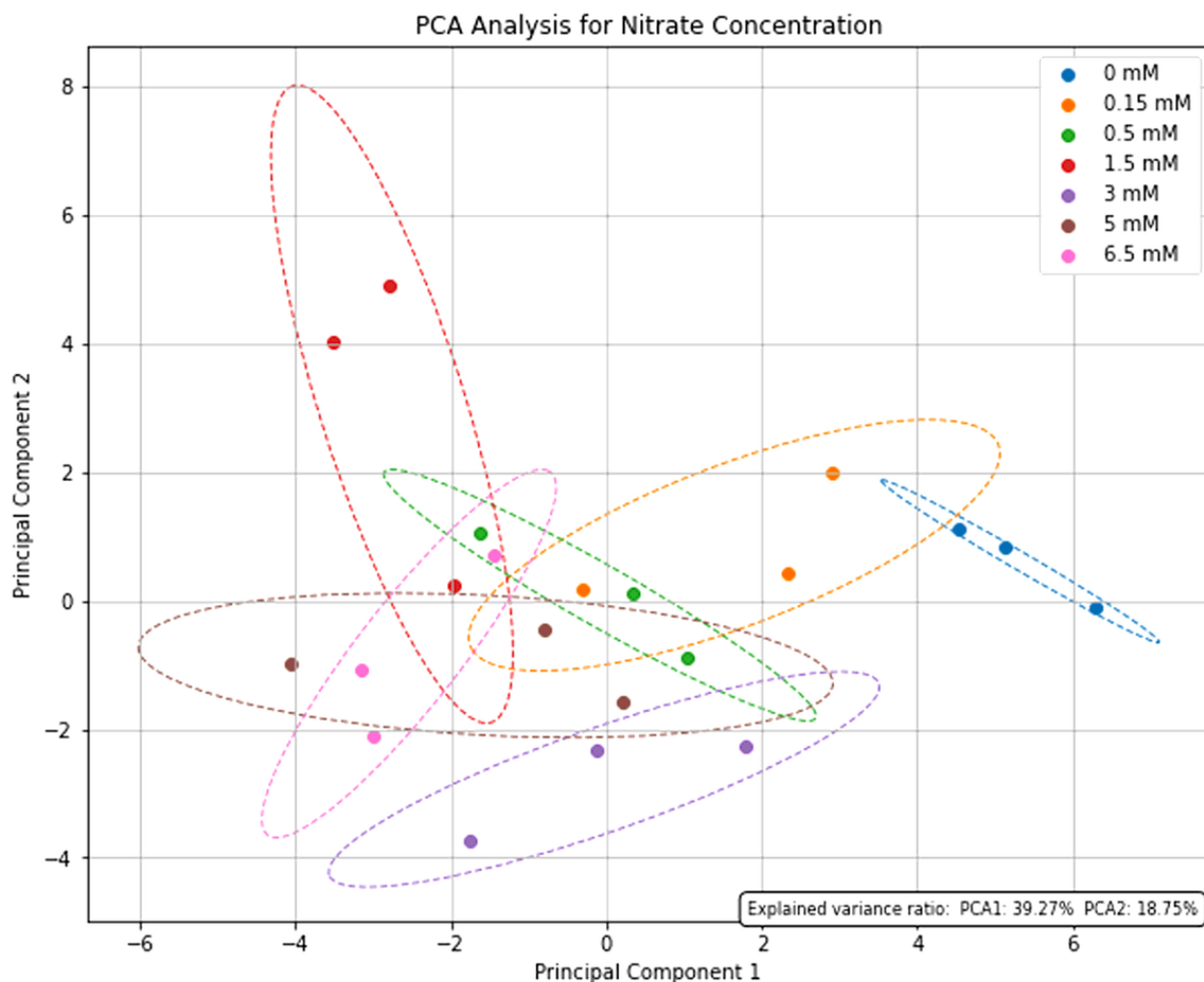


Figure 4

Principal Component Analysis (PCA) of relative growth rate and photosynthetic parameters (chlorophyll *a*, lutein, β -carotene, phycobiliproteins (PC and PE), F/F_0 , $ETR_{in situ}$, α_{ETR} , E_{optETR} , E_{kETR} , $E_{kETRmax}$, ETR , E_{kNPQ} , ETR and EDR in 124, 631, and 1509 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, $E_{optETR} - E_{kETR}$ and $E_{kNPQ} - E_{kETR}$) of *P. linearis*, considering nitrate concentration after seven days.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SUPPLEMENTARYMATERIALS.docx](#)
- [SuppFig1.tif](#)
- [SuppFig2.tif](#)
- [SuppFig3.tif](#)