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RESEARCH ARTICLE

Physiological responses of the alien macroalga *Rugulopteryx okamurae* (Phaeophyceae, Heterokontophyta) to changes in nutrients and temperature

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Abstract

The Asian brown macroalga *R. okamurae* has invaded the ultraoligotrophic areas of Mediterranean coasts since 2015, with drastic impacts on environmental conditions and socioeconomic activities in coastal areas in Europe. Therefore, it is intriguing how this species is able to grow and expand at the observed rates. In this context, the physiological responses of *R. okamurae* to changing nutrient concentrations and temperature were analyzed. Two experiments were conducted, evaluating six combinations of nitrate and phosphate concentrations and their potential interaction with temperature. Nutrient uptake efficiency (NUE) and rates (NUR), photosynthetic responses, growth rates, and biomass composition were evaluated. Photosynthesis parameters, soluble proteins, and NO_3^--NUR increased with increasing N:P ratio; however, PO_4^{3-}-NUR was very similar in all treatments. The species showed high capacity for nitrate assimilation, which was rapidly modulated by its external concentration and temperature (more than 90% of NO_3-NUE after 5 days in treatments with 5, 10, 16, 25, and 40 N:1P). Consequently, N-nutrients were removed from the water by *R. okamurae* and likely stored inside the cells. This process will allow the alga to maintain high growth rates if thalli are moved to oligotrophic areas, favoring its spreading to many marine environments. Additionally, fucoxanthin was the predominant carotenoid in this species, although its content was lower than in other brown macroalgae species (mean value of $0.51 \pm 0.05\,mg \cdot g^{-1}$ DW). However, since a huge amount of *R. okamurae* is observed recurrently on beaches, the use of this biomass might be proposed to compensate partially for its impacts.

KEYWORDS
biochemical compounds, ecophysiology, N:P ratios, photosynthesis, *Rugulopteryx okamurae*, temperature

Abbreviations: ABTS, 2.2′-azino-bis, 3-ethylbenzothiazoline-6-sulphonic sulfonic acid; AL, actinic light; ANOVAs, analysis of variances; BSA, bovine serumic albumin; chl *a*, chlorophyll *a*; chl *c*, chlorophyll *c*1 + *c*2; DPPH, 2,2-diphenyl-1-picrylhydrazyl; E_k, saturation irradiance for transporting electrons; ETR, electron transport rates; ETR_{max}, maximal electron transport rates; F_m, fluorescence after saturation pulse in light light-acclimated samples; F'_m, fluorescence after saturation pulse in dark- acclimated samples; F_o, basal fluorescence; F_t, steady- state fluorescence; F_v/F_m, photosynthetic maximum quantum yield; GR, growth rates; HPLC, high- performance liquid chromatography; LED, light emitting diodes; NUE, nutrient uptake efficiency; NUR, nutrient uptake rates; RLCs, rapid light curves; TROLOX, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Y(II), effective quantum yield; α_{ETR}, photosynthetic electron transport rates efficiency.

INTRODUCTION

Introduced species are one of the greatest threats to marine biodiversity and resource values of the world's oceans (Schaffelke et al., 2006). Regional studies have identified hundreds of non-indigenous marine species introduced by humans (Crooks, 2002; Molnar et al., 2008; Png-Gonzalez et al., 2023). Exotic species can also have invasive natures, producing extreme impacts on the marine environment. Since 2015, the Mediterranean and Atlantic coasts around the Strait of Gibraltar, including the Natural Park of El Estrecho (Spain), have been suffering a drastic invasion by the alga *Rugulopteryx okamurae* (García-Gómez et al., 2017; García-Gómez, Florido, Olaya-Ponzone, Díaz, et al., 2021). This species is a brown alga of the family Dictyotaceae (as *Dictyota* spp., De Clerck et al., 2006) and originally from east Asia (China, Japan, Korea). The first citation of its presence in Europe was in 2002 in Thau Lagoon (Mediterranean), with its introduction likely associated with oyster aquaculture (Verlaque et al., 2009). In 2016, the species was detected in the Atlantic coast close to Gibraltar strait (Altamirano et al., 2016) and in the Morocco Mediterranean coast (El Aamri et al., 2018); recently, it has been detected in French and Italian Mediterranean coasts (Bellissimo et al., 2024; Ruitton et al., 2021; Terradas-Fernández et al., 2023) and, even more recently, the Atlantic coast of the Northern of Morocco (El Madany et al., 2024), the Algarve, and Azores Islands in Portugal (Faria et al., 2021; Liulea et al., 2023). In only a few years, this species has occupied the rocky shores from 0 to 40 m depth, with maximum coverage at 10–20 m depth, in large extended coastal areas (García-Gómez et al., 2020), reducing drastically the previously reported algal diversity of ecosystems with high ecological statuses (Bermejo et al., 2015). The specimens present vegetative propagules and tetra and monosporangia, which explain its rapid propagation in the area (Altamirano et al., 2016), and its explosive growth could be enhanced by the fact that the whole photic zone (normally spreading until 60 m depth) provides temperatures that favor its growth and reproduction, as it is a subtropical species (Verlaque et al., 2009). Thallus breaking by tides and waves, linked to a high capacity for vegetative growth, is one of the most probable reasons for its rapid spread in the southern coasts of Europe. In fact, the intensive hydrodynamics in the Strait of Gibraltar, which feature the permanent flow of surface Atlantic Water entering the Mediterranean Sea and a coastal counter current displacing along the Gulf of Cádiz, might have favored its spread eastward within the Alborán Sea coast (the most western basin in the Mediterranean Sea) and westward along the coast of the Gulf of Cadiz (García-Lafuente et al., 2023; Muñoz et al., 2015).

In addition, the contribution of its bioactive compounds to the successful acclimation strategy has not been analyzed yet. The thalli of *R. okamurae* seem not to be deeply consumed by local marine herbivores (Casal-Porras et al., 2021), although it has been consumed by the urchin *Paracentrotus lividus* in laboratory. Furthermore, this last study showed that the intestinal arachidonic acid could be used as an indicator of *R. okamurae* consumption (Hachero-Cruzado et al., 2024). This can be a consequence of the presence of a potent secondary metabolism with feeding-deterrent properties due to diterpenoids (Kurata et al., 1988; Ochi et al., 1982). Paradoxically, extraction using fermentation and hydrolytic enzyme converts the biomass of *R. okamurae* into an optimal fish feed (Fonseca et al., 2023). More information about potential applications of *R. okamurae* was recently presented by Barcellos et al. (2023).

The strategy to respond to this massive invasion must be interdisciplinary within the frame of specific laws as the Spanish law on invasive alien species (RD 630/2013). The species *R. okamurae* was the first and most unique macroalga included in the list of alien invasive species of notable importance of the European Union (Tsirika, 2020). Its management is based on the principles, methodology, and guidelines for developing and managing ecological restoration projects of the Society for Ecological Restoration (SER, 2004). To this end, it is necessary to perform basic research to understand the ecophysiology of this species in the coastal area of the Strait of Gibraltar and the Mediterranean coast. The knowledge on the biology of this species and about key environmental factors that can explain its enormous success in the Mediterranean coast colonization is still extremely scarce, although the scientific interest generated by the invasion is increasing. There is an elevated number of publications on its impacts in marine ecosystems (Faria et al., 2022; García-Gómez et al., 2020; García-Gómez, Florido, Olaya-Ponzone, Díaz, et al., 2021; García-Gómez, Garrigós, & Garrigós, 2021; Sempere-Valverde et al., 2021; Verlaque et al., 2009) and also on the bioactive compounds and the possible applications of the algal biomass in agriculture, aquaculture, bioenergy, biomaterials, and cosmeceutics (Barcellos et al., 2023; Hwang et al., 2009; Navarro-Barranco et al., 2019; Patón & Garcia-Gómez, 2023; Santana et al., 2022; Vega et al., 2023; Zarraonaindia et al., 2023). However, the works dealing with its physiological and ecophysiological characterization are still scarce (Mercado et al., 2022). Thus, it is necessary to conduct basic research in order to learn its strategies of acclimation to the environment, and this research requires factorial experiments that can determine the combinations of environmental variables that are most favorable for the growth of this species and if there are interactions among different environmental factors.

In a previous report, Mercado et al. (2022) hypothesized that there is a positive relationship between the nutrient inputs into coastal areas and the development of *R. okamurae* meadows, as its nutrient uptake kinetics permit taking advantage of increases in nutrient availability (episodes that are frequent in the Strait of Gibraltar). Moreover, the possible growth-regulating role of nutrients could also be affected by other environmental factors, including the temperature. The objective of this work was to understand how this species responds to nutrient availability and the combined effect with temperature. Changes in growth, photosynthesis measured from chlorophyll *a* fluorescence, nutrient removal from the water, antioxidant capacity, and pigment and biochemical composition were determined.

MATERIALS AND METHODS

Biological material and acclimation to culture conditions

Thalli pieces of *R. okamurae* were sampled in La Caleta, Tarifa, Spain (36°00′42″ N, 5°35′53″ W). Samplings were carried out from October 16, 2019 (the first experiment) and on January 17, 2020 (the second experiment). The biological material was collected during the development of an algal bloom. Only attached thalli growing at 2 m depths were selected for the experiments and afterward transported in an icebox to the laboratory at Sciences Faculty (University of Málaga). There, the algae were washed with seawater, and epiphytes were removed. Then, algal thalli were placed into methacrylate flasks at the ratio of 2 g of algal biomass per L of seawater, with vigorous aeration. The seawater salinity was 38, and the medium was enriched with 0.05 mM $NaNO_3$ (as the N source) and 0.005 mM of Na-glycerophosphate (as the P source). The algae were illuminated with 200 μmol photons $\cdot m^{-2} \cdot s^{-1}$ emitted from white light of light emitting diodes panel (LED, 4500K), adjusted to a photoperiod of 12 h. Samples for nutrient experiments were acclimated at 22°C, while those used to evaluate the effects of combined changes in temperature and nutrients were acclimated at 15°C and 22°C. Photosynthetic maximum quantum yield (F_v/F_m) was measured every 2 days (see details about these measurements below), and when this parameter was constant, the samples were considered to be acclimated to the laboratory conditions, as the F_v/F_m decrease has been used as an indicator of stress of photosynthetic organisms (Maxwell & Johnson, 2000).

Experimental design

Two different experiments were designed. The first one was prepared to assess the effects of different N:P ratios on *R. okamurae* ecophysiology, varying the amount of $NaNO_3$ added to the seawater at 22°C. In the second one, three levels of N:P ratio were assayed at two temperatures (15°C and 22°C). Three replicates were conducted in each experiment. In both cases, initial biomass was adjusted to 2 g per L of seawater in the methacrylate flasks. Photoperiod, salinity, and irradiance were the same as described previously for acclimation.

For the first experiment, $NaNO_3$ and Na-glycerophosphate were added as N and P sources. The following six combinations of both nutrients were assayed to produce different N:P ratios: 5:1 (50 μM NO_3^-:10 μM PO_4^{2-}), 10:1 (100 μM NO_3^-:10 μM PO_4^{2-}), 16:1 (160 μM NO_3^-:10 μM PO_4^{2-}), 25:1 (250 μM NO_3^-:10 μM PO_4^{2-}), 40:1 (400 μM NO_3^-:10 μM PO_4^{2-}), and 80:1 (800 μM NO_3^-:10 μM PO_4^{2-}). The ratios below the Redfield ratio (10 N:1P) were used to mimic conditions of nitrogen limitation normally occurring in the Alborán Sea during stratification periods (5N:1P; Mercado et al., 2007) as well as the relation observed in the culture media of Von Stosch (Edwards, 1970) and Provasoli (10N:1P). The N:P ratios above 16:1 simulated conditions of nutrient inputs that occur in the coastal areas of the Strait of Gibraltar (Mercado et al., 2022). This experiment was conducted for 7 days, and the seawater enrichment was performed only at the beginning.

To conduct the second experiment, the next three combinations of $NaNO_3$ and monobasic sodium phosphate were assayed to produce the next final N:P ratios: 10:1 (56 μM NO_3^-:5.6 μM PO_4^{2-}), 25:1 (140 μM NO_3^-:5.6 μM PO_4^{2-}), and 50:1 (280 μM NO_3^-:5.6 μM PO_4^{2-}). These enrichment conditions were combined at two different temperatures: 15°C and 22°C. The nutrient enrichment was produced at the beginning of the experiment, and the enriched seawater was completely renewed in the middle of the experiment (fifth day), returning it to the initial concentrations. The second experiment was conducted for 10 days.

The following variables were evaluated in *R. okamurae* at the beginning and at the end of the first experiment: photosynthetic pigments (chlorophylls and carotenoids), antioxidant capacity, total carbohydrates, phenolic compounds, and soluble proteins. In the second experiment, only the last three biochemical variables were assessed. The algal thalli were used to measure photosynthesis (some small pieces), were weighed to calculate growth rates, and were later frozen and freeze-dried in order to perform biochemical analyses.

Nutrient analyses

Nutrient consumption in the seawater was evaluated based on the changes in their concentrations, which were determined according to the method of Grasshoff

and Johannsen (1972) in a segmented flow analyzer (SFA; Seal Analytical autoanalyzer QuAAtro). The detection limits of inorganic nutrients were 0.05 µM for nitrates and phosphates. The concentrations were assessed at days 0, 1, 5, and 7 for experiment 1 and at days 3, 5, 7, and 10 for the second experiment (in this experiment, samples were taken just before and after renewal of the culture medium on day 5). Nutrient uptake efficiency (NUE) and nutrient uptake rates (NUR) were calculated according to Massocato et al. (2023).

Physiological variables

Growth rates (GR)

The algal biomass was first weighed using an analytical balance (Sartorius, Practum-1S). Then, the excess seawater on the algal thalli was removed using a paper towel. This procedure was performed at the beginning and at the end of the first experiment. In the second experiment, the algal biomass was weighed at the middle of the experimental period, when the algal weight was again adjusted to 2 g per L, and at the end of the experimental period (day 10). Consequently, growth rates were calculated for the first (days 0–5) and second (days 5–10) phases of the experiment by following the formula of Lignell and Pedersén (1989): $GR(\%\cdot d^{-1}) = [(W_f/W_i)^{1/t} - 1] \times 100$, where W_f is the fresh weight at the final period, W_i is the fresh weight at the initial period being considered, and t is the time of the period.

Photosynthetic activity (F_v/F_m, ETR, and RLCs)

Photosynthetic activity was assessed by measuring different parameters calculated from PSII fluorescence with a MiniPAM-II (Walz Effeltrich, Germany). Maximal quantum yield, F_v/F_m, effective quantum yield, Y(II), and electron transport rates, ETR, were estimated in situ (i.e., in the culture vessels), and rapid light curves (RLCs) were conducted ex situ (using actinic light provided by the fluorometer).

The maximal quantum yield (F_v/F_m) was measured at the middle of the dark period after applying a saturating light pulse (>4000 µmol photons $\cdot m^{-2} \cdot s^{-1}$). The calculation for F_v/F_m was $F_v/F_m = (F_m - F_o)/F_m$, where F_m is the fluorescence after saturation and F_o is the basal fluorescence. These data were taken during the dark period before days 1, 2, 5, and 7 during the first experiment and before days 0, 3, 5, 7, and 10 during the second experiment.

Electron transport rates (ETRs) were measured during the light period of the day (at a minimum 2 h after lights were turned on). Small thalli pieces were placed in a Petri dish containing seawater and placed in the same area of the cultivation vessel to receive the same light amount. The fluorometer fiber was placed on, and the measuring light and actinic light (AL) of the fluorometer were switched on. The AL of 196 µmol photons $\cdot m^{-2} \cdot s^{-1}$ was chosen to perform measurements under light conditions similar to the experimental treatment. The AL remained switched on for 10 s, followed by a saturating pulse, applying the option (ACT+Y) of the fluorometer. The steady state fluorescence (F_t) of this light condition was recorded after the AL exposure period and after the saturating pulse (F'_m). The effective quantum yield was calculated following Genty et al. (1989) with the formula:

$$Y(II) = (F'_m - F_t)/F'_m$$

The absorptance of the same thalli pieces, used to estimate Y(II), was measured by covering the light sensor of a quantameter LiCor (Li-1100) with the thalli placed below a light source. Then, the irradiance values with (E_t) and without (E_o) algal thalli were recorded, allowing the calculation of Absorptance (A) according to:

$$A = 1 - (E_t/E_o).$$

Electron transport rates (ETRs) were calculated with the formula:

$$ETR = Y(II) \cdot E_{PAR} \cdot A \cdot F_{II},$$

where Y(II) is the effective quantum yield, E_{PAR} is the experimental irradiance of photosynthetic active radiation (PAR, λ = 400–700 nm), A is the absorptance, and F_{II} is the ratio of chlorophyll associated to photosystem II. A value of 0.8 for F_{II} was utilized according to Figueroa et al. (2014) for brown algae. The ETR was calculated at days 1, 2, 5, and 7 of the first experiment and at days 0, 3, 5, 7, and 10 of the second experiment.

Measurements of RLCs were performed at the beginning (day 0) and at the end of the experimental periods. The thalli were submerged in 15 mL of seawater in darkened Falcon tubes and submitted to 12 increasing AL values (25, 45, 66, 90, 125, 190, 285, 420, 625, 845, 1150, and 1500 µmol photons $\cdot m^{-2} \cdot s^{-1}$) during 20 s each provided by red light by LEDs of the MiniPAM-II (Walz GmbH, Germany). After each exposure, Y(II) was measured using the corresponding values of F_t and F'_m. Then, ETR was calculated for each light AL irradiance, as explained above. The data of ETR versus irradiance were fit to the tangential mathematical model of Platt et al. (1980) to calculate the photosynthetic parameters ETR_{max}, α_{ETR}, and E_k (maximal electron transport rates, photosynthetic electron transport rates efficiency and saturation irradiance, respectively).

Biochemical variables

Absorbance spectra and photosynthetic pigments

Twenty milligram of algal dry weight were ground with a mortar and pestle, and then, acetone 90% was added. Samples remained overnight at 4°C to complete the pigment extraction, and then, they were centrifuged at 4000 rpm for 10 min at 4°C. The absorbance spectra of the extracts were recorded from 320 to 750 nm with a UV–Vis Spectrophotometer UV-Mini 1240 (Shimadzu Europe, Duisburg). Absorbance values were used for quantification of chlorophyll *a* (chl *a*) and chlorophyll *c*1 + *c*2 (chl *c1* + *c2*) according to the formula reported by Ritchie (2006), and total carotenoids according to the formula reported by Strickland and Parsons (1972). Additionally, carotenoids were also evaluated by high performance liquid chromatography (HPLC), according to Barufi et al. (2011). Lyophilized samples were extracted in dimethylformamide during 24 h in darkness at 4°C; the extracts were filtered through 0.2 μm filters and inserted into HPLC vessels. Samples were inserted in a mobile phase composed by gradients of two solutions: A (distilled water + tetrabutyl ammonium 0.05 M and ammonium acetate 1 M + methanol) and B (acetone + methanol). The mobile-phase flow was 1 mL · min^{-1}. Carotenoids were separated using a C18 5-μm column (Symmetry® C18 of 5-μm 4.16 × 150 mm column). The pigment peaks were determined with a Waters photodiode array detector at 350–380 nm. The different carotenoids were identified by comparing the absorbance peaks with commercial standards for zeaxanthin, fucoxanthin, and violaxanthin (DHI Water and Environment, Denmark). The quantification followed standard curves of these known carotenoid concentrations versus peak absorbance area with five dilutions. Pigments were quantified only for samples obtained from the first experiment.

Soluble proteins, carbohydrates, and phenolic compounds

These procedures were performed for the two experiments. Total soluble proteins were determined according to Bradford (1976) from extracts of 20 mg of dried biomass obtained in phosphate buffer (50 mM, pH 7.0) overnight at 4°C and in darkness. The samples were homogenized using an UltraTurrax T-10 Basic (Sigma-Aldrich). In the first experiment, this same extract was used to evaluate antioxidant capacity as described below.

In brief, after centrifugation, 20 μL of extracts were added to 780 μL of phosphate buffer and 200 μL of Bio-Rad reagent. The reaction was mixed with a vortex, and remained resting for 15 min. Then, samples were read in the spectrophotometer UV-Mini 1240 (Shimadzu Europe, Duisburg) at 595 nm. Bovine serum albumin (BSA) was used to prepare a standard curve, allowing quantification of total soluble proteins equivalent to BSA per g of algal dry weight.

Total carbohydrates were determined by the method of Dubois et al. (1956). Five milligrams of freeze-dried biomass were extracted in 5 mL of H_2SO_4 1M, followed by a water bath for 1 h at 100°C. After cooling down to ambient temperature, samples were centrifuged for 10 min at 4000 rpm and 15°C. Then, 1 mL of supernatant fraction was separated into a new tube, and 1 mL of phenol 5% was added. The mixture rested for 40 min and then, 5 mL of H_2SO_4 were added, which caused the samples to heat up. When samples were again at ambient temperature, absorbance at 485 nm was recorded with a UV–Vis mini spectrophotometer against a reference blank with H_2SO_4 1M. Glucose was used to prepare a standard curve, and total carbohydrates were estimated as milligrams of glucose equivalents per gram of algal dry weight.

Phenolic compounds were quantified with the method of Folin and Ciocalteu (1927). Methanol 80% was used to prepare an extract with 20 mg of freeze-dried algal material homogenized with an UltraTurrax T-10 Basic homogenizer, and the mixture was left overnight. After centrifugation for 10 min at 4000 rpm, 100 μL of supernatant were mixed with distilled water (700 μL) and Folin reactive (50 μL). Afterward, 150 μL of Na_2CO_3, 20% was added to the mixture, and the reaction occurred in darkness for 2 h. Then, absorbance at 760 nm was recorded via a UV–Vis-MINI Shimadzu spectrophotometer. A standard curve was prepared using phloroglucinol, and phenolic compounds were determined as milligrams of phloroglucinol equivalents per gram of algal dry weight.

Antioxidant capacity—ABTS and DPPH

Antioxidant capacities of algal extracts were assessed only in samples from the first experiment, by using ABTS (2.2′-azino-bis, 3-ethylbenzothiazoline-6-sulfon ic acid), according to Re et al. (1999), and DPPH assay, according to Brand-Williams et al. (1995) with some modifications.

In the case of ABTS, the extract used was the same one prepared for the soluble proteins analyses (see above). The reaction was prepared with the addition of 50 μL of sample to 950 μL of diluted ABTS solution. The reaction stood for 8 min; the mixture was read in a spectrophotometer before and after the reaction time. Absorbances at 727 nm were read by using a UV–Vis spectrophotometer (Shimadzu UV Mini-1240). Afterward, the percentage of antioxidant capacity was estimated as explained in Schneider et al. (2020).

For the DPPH assay, 20 mg of algal (dry weight, DW) were crushed with a mortar and pestle, followed by the addition of MeOH 80%. For triggering the reaction, 200 μL of methanolic extract were mixed with 1 mL of the DPPH• (2,2-diphenyl-1-picrylhydrazyl) solution (0.06 mM of DPPH in methanol 80%). After 30 min of incubation at room temperature and in darkness, absorbance was measured at 517 nm.

Antioxidant capacity of compounds from both assays was estimated using the standard TROLOX (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) curve. Results were expressed as μM of Trolox Equivalents (TE) per gram of dry weight ($\mu M\,TE \cdot g^{-1}$ DW).

8 Statistics

The statistical significance of the differences among treatments was tested with analyses of variance (ANOVAs) for those variables that fulfilled the ANOVA assumptions. Normality and homogeneity of variances were determined with tests of Shapiro–Wilk and Cochran, respectively. In the case of the first experiment, the effects of changes in N:P ratios were determined with one-way ANOVAs. In the case of the second experiment, a two-way ANOVA was used to determine the effects of temperature (two levels, 15°C and 22°C) and nutrient ratio (N:P, three levels). Significance level was fit to $\alpha = 0.05$. In the case of photosynthetic parameters that were measured during the time period, the time was considered as a third factor, and a three-way ANOVA was conducted (to F_v/F_m and ETR in situ data). When significant differences were found in the data, means were compared with pair-wise post hoc Tukey test ($p < 0.05$). Pearson correlation analyses were conducted to data of the first experiment, considering values measured at the end of experimental period.

RESULTS

Cultivation of *R. okamurae* under different N:P ratios resulted in differences in the nutrient incorporation rates by the algal thalli. Considering the NUE parameter, N:P ratio significantly influenced the efficiency of nitrate uptake but not of phosphate (Table S1). On day 1 of the first experiment, the highest nitrate uptake efficiency was obtained at the lower N:P ratio (5N:1P and 10N:1P). In contrast, the efficiency was reduced in the treatments with N:P ratios higher than 25N:1P, while 16N:1P NUE presented intermediate data (~55%; Figure 1a). After 5 days, almost all treatments had NUEs higher than 80% with the exception of the 80N:1P treatment. This pattern was similar after 7 days, at which point the N supplied was totally removed from the water with the exception of the

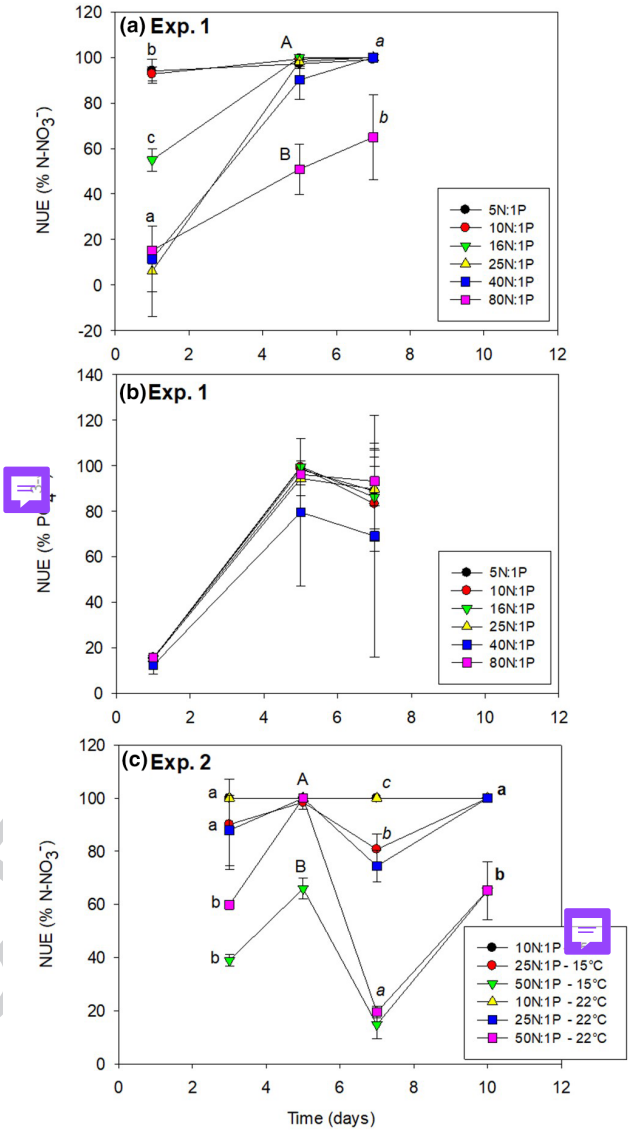

FIGURE 1 Nutrient uptake efficiency (NUE) of *R. okamurae* cultivated under two experimental conditions. The first experiment spent 1 week at six different N.P ratios (5N:1P, 10 N:1P, 16N:1P, 25N:1P, 50N:1P, and 80N:1P) at 22°C. In the second experiment, three N:P ratios (10N:1P, 25N:1P and 50N:1P) and two temperatures were applied during the period of 10 days. (a). Nitrate uptake efficiency in the first experiment. (b). Phosphate uptake efficiency in the first experiment. (c) Nitrate uptake efficiency in the second experiment. In A and C, the statistical significance ($p < 0.05$) of the differences among treatments at each time separately (Tukey post hoc comparisons) are shown with different letters (normal letters on the first day, capital letters on the fifth day, and italic letters on the seventh day) indicate significant differences after a post hoc comparison of Tukey ($p < 0.05$). Data were compared at each time, separately, and different letters (normal letters on the third day, capital letters on the fifth day, italic letters on the seventh day, and bold letters on the 10th day) indicate significant differences after a Tukey post hoc comparison ($p < 0.05$). All data are presented as means and standard deviations ($n = 3$). In (C), the values of the treatment 10N:1P at 22°C (black circle) are not visible, as all of them were 100% in the different time periods, being masked by other treatments with similar values in the figure (e.g., yellow circles of 10N:1P at 15°C). When no letter is present, the data were statistically similar.

treatment 80N:1P (Figure 1a). In the case of phosphate removal efficiency, *R. okamurae* thalli started with low efficiency after 1 day of experimentation, but phosphate was efficiently absorbed after 5 or 7 days without significant differences among treatments (Figure 1b).

In the case of the second experiment, NO_3^--NUE was influenced by the N:P ratio after 3, 7, and 10 days of experiment (Table S1). After 5 days, the interaction N:P ratio × temperature was significant, and the NUE of nitrate was lower at the 50N:1P treatment in comparison with the other N:P ratio conditions, which presented an efficiency of NO_3^- uptake between 90% and 100%, achieving the 100% after 5 days (Figure 1c), when the initial nutrient concentration was reestablished. After 7 days, N was fully removed in the 10N:1P treatment at both temperatures, and the NUE of NO_3^- was ~80% in the 25N:1P treatment. After 10 days, the total efficiency was ~60% in the 50 N:1P treatment (Figure 1c), while in all other treatments, NO_3^- was totally depleted. In the case of phosphate, it was entirely (100%) removed from the seawater at days 3 and 7.

The analysis of the NO_3^- and PO_4^{3-} removal rates indicates that the uptake dynamics of both nutrients were different. Thus, while NO_3^--NUR was influenced significantly by the N:P ratio, PO_4^{3-}-NUR was not (Table S1). In the case of NO_3^-, uptake rates increased according to the N:P ratio, and samples treated with 80N:1P achieved the highest rates (1.39 ± 0.32 µmol $NO_3^- \cdot g^{-1}$ FW $\cdot h^{-1}$; Figure 2a). The interaction between N:P ratio and temperature caused significant variability to the data of NUR in the second experiment, considering NO_3^- or PO_4^{3-} at both experimental evaluated periods (Table S1). A similar pattern of NO_3^- uptake rate was observed in the two phases of the experiment 2, with the values increasing according to the increasing N:P ratio. However, the highest NUR of NO_3^- (1.06 ± 0.1 µmol $NO_3^- \cdot g^{-1}$ FW $\cdot h^{-1}$) was obtained at 50N:1P and 22°C (Figure 2b). The average value of PO_4^{3-}-NUR after 7 days of treatment was similar at the different N:P ratios (0.022 ± 0.004 µmol $PO_4^{3-} \cdot g^{-1}$ FW $\cdot h^{-1}$; Figure 2c) indicating that the stoichiometry of N and P assimilation varied according to the N concentration. In the case of PO_4^{3-}-NUR after the second experiment, slight

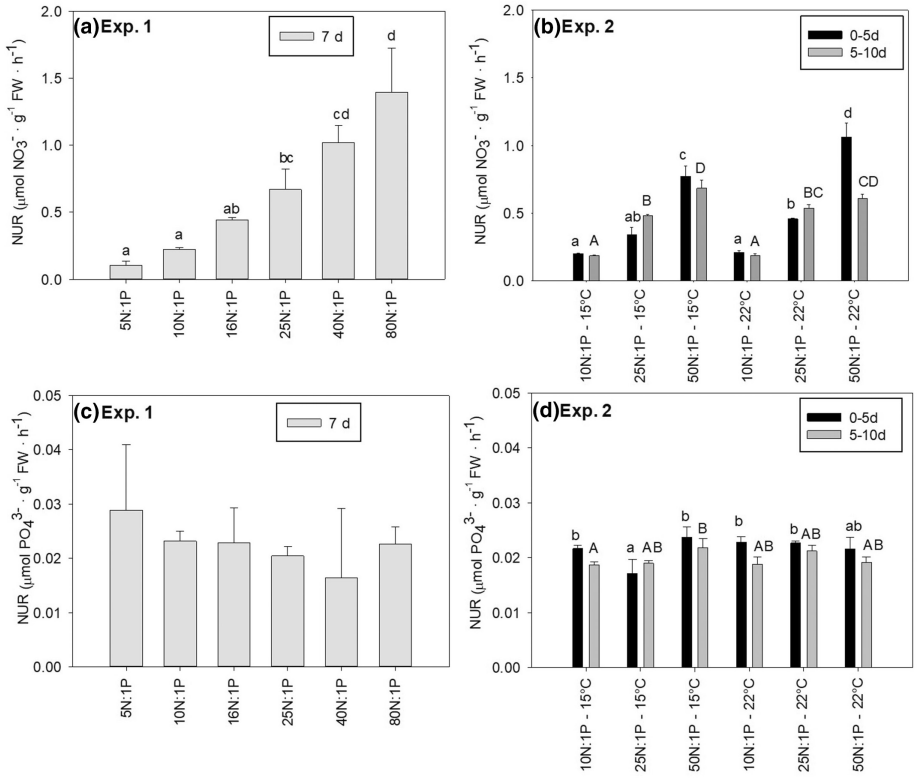

FIGURE 2 Nutrient uptake rates (NUR) of *R. okamurae* cultivated under two experimental conditions. The first experiment was conducted for 1 week at six different N:P ratios (5N:1P, 10N:1P, 16N:1P, 25N:1P, 50N:1P, and 80N:1P) at 22°C. In the second experiment, three N:P ratios (10N:1P, 25N:1P, and 50N:1P) and two temperatures were applied during a period of 10 days. (a) NUR of nitrate in the first experiment, after 7 days. Different letters indicate significant differences after a Tukey post hoc comparison ($p < 0.05$). (b) NUR of nitrate in the second experiment, between 0 and 5 days and 5–10 days. Different lowercase letters indicate significant differences to the first period (0–5 days), and different capital letters indicate differences in the second period (5–10 days), after a Tukey post hoc comparison ($p < 0.05$). (c) NUR of phosphate in the first experiment, after 7 days. (d) NUR of phosphate in the second experiment, between 0 and 5 days and 5–10 days. Different letters indicate significant differences in the first period (0–5 days), and different capital letters indicate differences in the second period (5–10 days), after a Tukey post hoc comparison ($p < 0.05$). All data are presented as means and standard deviations ($n = 3$). When no letter is present, the data were statistically similar.

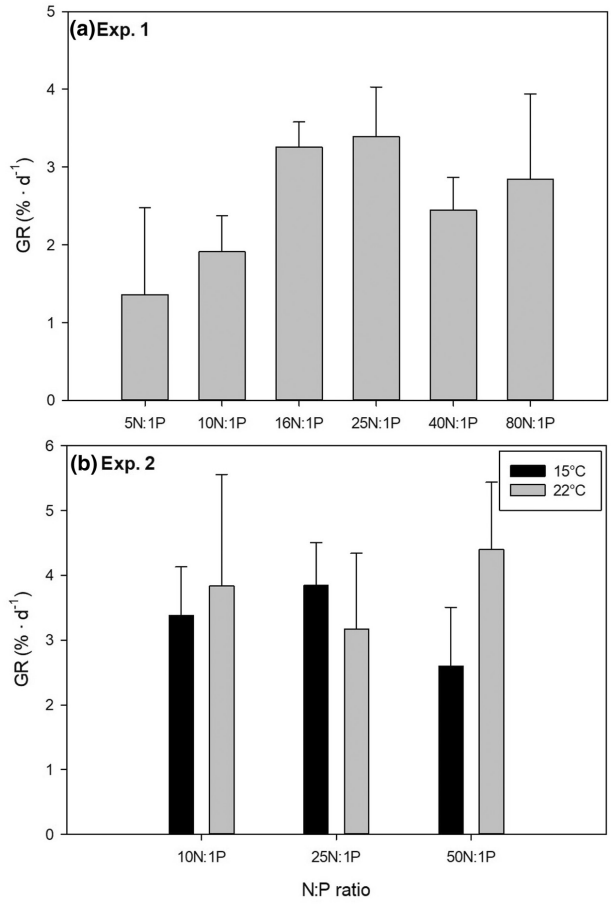

FIGURE 3 Growth rates (%·day^{-1}) of *R. okamurae* cultivated under two experimental conditions. The first experiment (a) was conducted for 1 week at six different N:P ratios (5N:1P, 10N:1P, 16N:1P, 25N:1P, 50N:1P, and 80N:1P) at 22°C. In the second experiment (b), three N:P ratios (10N:1P, 25N:1P and 50N:1P) and two temperatures were applied during a period of 10 days. Data are presented as means and standard deviations ($n=3$). When no letter is present, the data were statistically similar.

fluctuations were observed without a clear pattern, with a lower value observed at 25N:1P after the first experimental period, and higher phosphate NURs in the samples that received 50N:P at 15°C (Figure 2d).

Thalli of *R. okamurae* showed positive growth rates during the experimental time. In experiment 1, only a very slight influence of N:P ratio on the growth rates was observed ($p=0.04$, Table S2), although no significant differences were observed with Tukey's test. On average, the growth rate was 2.5%±0.97%·day^{-1} (Figure 3a). Similarly, no significant differences were observed in growth rates with temperature and N:P ratio in experiment 2, for which the average growth was 3.5%±1.09%·day^{-1} (Figure 3b).

Photosynthetic responses of *R. okamurae* were influenced by the experimental conditions. In the case of experiment 1, in situ ETR varied according to N:P ratio and time. In the case of experiment 2, this parameter was influenced significantly by the interaction between temperature and N:P ratio and between temperature and time, respectively (Table S2). Time evolution of ETR in situ is presented in Figure 4. In experiment 1, evident differences with the treatments were detected after 7 days of culturing, when the highest electron transport rates (31.52±1.84 μmol electrons·m^{-2}·s^{-1}) were obtained at 80N:1P (Figure 4a). In experiment 2, some time-related variations could be observed, although there were no clear patterns among the treatments, and the thalli presented an ETR in situ of 25.78±3.66 μmol electrons·m^{-2}·s^{-1} (Figure 4b) on average.

Photosynthesis was also assessed as potential quantum yield (F_v/F_m) during the dark periods during the experiments. In the case of samples cultivated with different N:P ratios (experiment 1), the interaction between N:P ratio and time was significant (Table S2). The general pattern of F_v/F_m responses of *R. okamurae* is shown in Figure 4. In experiment 1, this parameter varied from the minimum of 0.64 and the maximum of 0.72, observed in the treatment of 40N:1P at the days 2 and 7, respectively. The mean of potential quantum yield of *R. okamurae* considering all data was 0.67±0.02 (Figure 4c). By contrast, in the second experiment, the interactive effects of N:P ratio with time, and the interaction of N:P ratio and temperature significantly influenced F_v/F_m (Table S2). The F_v/F_m valuves decreased clearly in samples cultivated with 50N:1P at 22°C, achieving the lowest value of 0.61, while samples at 10N:1P at the same temperature remained with F_v/F_m values ~0.7 (Figure 4d).

Parameters obtained from rapid light curves performed with *R. okamurae* are presented in Table 1. In the case of experiment 1, α_{ETR} varied from 0.12±0.03 to 0.18±0.03, and E_k varied from 169.7±31.01 to 201.9±8.65 μmol photons·m^{-2}·s^{-1} (Table 1) with a mean of 187.71±12.43 μmol photons·m^{-2}·s^{-1}. However, the only variable affected significantly by changes in N:P ratio was ETR_{max}, which at the N:P ratio of 80:1 was 37.34±10.13 μmol electrons·m^{-2}·s^{-1}, that is, ~1.6 times higher compared with values obtained from the samples at the beginning of the experiment and in the treatments 5:1 and 10:1N:P (Table 1). In the second experiment, α_{ETR} and ETR_{max} were influenced by temperature (Table S2). At 15°C, the mean α_{ETR} was 0.04 units lower than at 22°C, and ETR_{max} also increased by 1.2 times from 15°C to (25.18±1.78) to 22°C (30.04±0.53 μmol electrons·m^{-2}·s^{-1}; Table 1). The E_k was neither influenced by temperature nor N:P treatments, being on average 192.48±16.9 μmol photons·m^{-2}·s^{-1}.

Photosynthetic pigments evaluated by spectrophotometer were chlorophyll *a*, chlorophyll *c* (*c*1+*c*2), and carotenoids (Table 2). Total carotenoids varied with the changes in N:P ratio, while chlorophylls remained unaffected (Table S2) in the first experiment. The mean content of chl *a* and *c* in *R. okamurae* was 2.03±0.32 mg chl *a*·g^{-1} DW and 0.30±0.04 mg chl *c* (*c*1+c2)·g^{-1}

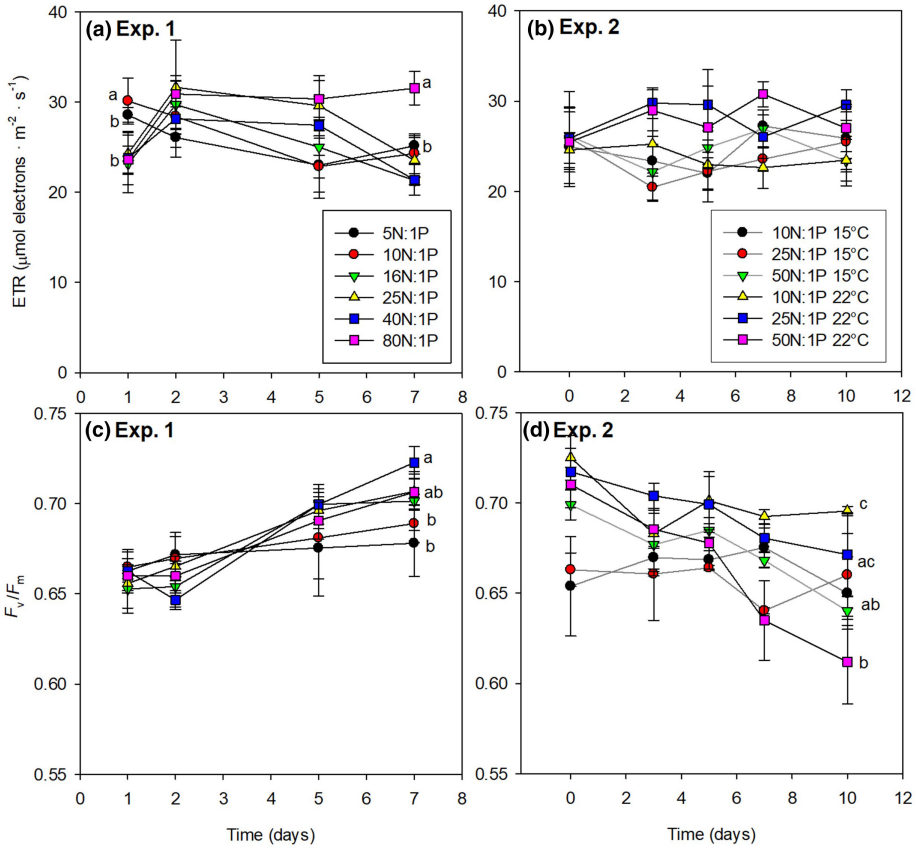

FIGURE 4 In situ electron transport rates (ETR, µmol electrons · $m^{-2} \cdot s^{-1}$) and maximum quantum yield of (F_v/F_m) of *R. okamurae* cultivated under two experimental conditions. The first experiment was conducted for 1 week receiving six different N:P ratios (5N:1P, 10N:1P, 16N:1P, 25N:1P, 50N:1P, and 80N:1P) at 22°C. In the second experiment, three N:P ratios (10N:1P, 25N:1P and 50N:1P) and two temperatures were applied during the period of 10 days. Data are presented as means and standard deviations ($n = 3$). (a) in situ ETR, first experiment. (b) in situ ETR, second experiment. (c) F_v/F_m in the first experiment. (d) F_v/F_m in the second experiment. Different letters indicate significant differences among the treatments, according to Tukey post hoc test ($p < 0.05$). When no letter is present, the data were statistically similar.

DW, respectively (Table 2). On average, the total carotenoids content was lower in the treatments with 5:1, 10:1, 16:1, and 40:1N:P (1.46 ± 0.10 mg · g^{-1} DW) compared with 80:1N:P (1.93 ± 0.17 mg · g^{-1} DW). Four different carotenoids were identified by HPLC in *R. okamurae* in the samples obtained from experiment 1: fucoxanthin, 19-hexo-fucoxanthin, violaxanthin, and zeaxanthin. The two fucoxanthins were the major carotenoids observed in *R. okamurae*. Fucoxanthin and 19-hexo-fucoxanthin of *R. okamurae* were not influenced by the experimental treatments ($F = 2.041$, $p = 0.12$; and $F = 1.64$, $p = 0.2$, respectively). The mean content of fucoxanthin was of 0.51 ± 0.05 mg · g^{-1} DW. 19-hexo-fucoxanthin was the second most abundant carotenoid, totaling 0.08 ± 0.01 mg · g^{-1} DW (Figure 5). The other two carotenoids were influenced by the treatment conditions: violaxanthin ($F = 16.96$ and $p < 0.001$) and zeaxanthin ($F = 33.49$ and $p < 0.001$). The initial amounts of violaxanthin were higher compared to the thalli after 7 days treatment. Among the N:P ratios, the treatments of lower N concentration (5 and 10N:1P) presented lower content of violaxanthin (0.04 ± 0.004 mg of violaxanthin · g^{-1} DW) in comparison with the mean content in the other treatments. In the case of zeaxanthin, content decreased with respect to the initial value in all the treatments aside from 80N:1P (0.02 ± 0.002 mg · g^{-1} DW; Figure 5).

The treatments produced changes in the *R. okamurae* thallus antioxidant capacity during experiment 1 (Table S2). The ABTS and DPPH antioxidant capacities increased by threefold to fourfold at the different N:P ratios applied. In the case of ABTS assay, for example, an antioxidant capacity of 83.4 ± 1.05 µmol $Trolox_{Eq}$ · g^{-1} DW was observed in the extracts of *R. okamurae* at the lowest N:P ratio (Figure 6a). When assessing data from DPPH analysis, a similar pattern was observed, with the lowest DPPH activity at the beginning of the treatment. After 7 days of treatment, there were significant differences, with the highest antioxidant capacity in thalli acclimated to 10N:1P and 16N:1P (164.62 ± 9.75 and 158.21 ± 17.78 µmol $Trolox_{Eq}$ · g^{-1} DW, respectively; Figure 6b).

The biochemical composition of *R. okamurae* was more stable during the two experiments. In experiment

Temperature	N:P ratio	α_{ETR}	ETR_{max}	E_k
Experiment 1				
22°C	Initial	0.13 ± 0.00	22.50 ± 4.34a	169.70 ± 31.01
	5N:1P	0.12 ± 0.03	20.64 ± 1.34a	180.81 ± 38.66
	10N:1P	0.13 ± 0.02	22.22 ± 1.08ab	176.19 ± 25.82
	16N:1P	0.13 ± 0.02	25.06 ± 4.11ab	192.34 ± 28.57
	25N:1P	0.15 ± 0.02	29.11 ± 3.83ab	191.77 ± 4.77
	40N:1P	0.15 ± 0.04	29.64 ± 9.01ab	201.90 ± 8.65
	80N:1P	0.18 ± 0.03	37.34 ± 10.13b	201.26 ± 23.04
Experiment 2				
15°C	Initial	0.09 ± 0.00	32.26 ± 7.54	345.93 ± 81.97
	10N:1P	0.12 ± 0.01	27.22 ± 5.66	224.76 ± 24.23
	25N:1P	0.13 ± 0.01	24.39 ± 2.75	189.20 ± 30.16
	50N:1P	0.13 ± 0.01	23.93 ± 3.61	182.20 ± 34.56
22°C	Initial	0.13 ± 0.02	33.58 ± 3.50	263.54 ± 20.87
	10N:1P	0.17 ± 0.05	30.02 ± 6.75	180.14 ± 14.89
	25N:1P	0.16 ± 0.01	29.53 ± 2.85	182.64 ± 17.60
	50N:1P	0.16 ± 0.02	30.58 ± 5.23	195.93 ± 25.10

TABLE 1 Photosynthetic variables (α_{ETR}, photosynthetic electron transport rates efficiency; ETR_{max}, maximum electron transport rates, and E_k, saturation irradiance) obtained from rapid light curves of *R. okamurae* cultivated under two experimental conditions. The first experiment was conducted for 1 week at six different N:P ratios (5N:1P, 10N:1P, 16N:1P, 25N:1P, 50N:1P, and 80N:1P) at 22°C.

Note: In the second experiment, three N:P ratios (10N:1P, 25N:1P, and 50N:1P) and two temperatures were applied during the period of 10 days. Data are presented as means and standard deviations ($n = 3$). Different letters indicate significant differences among the treatments, according to Tukey post hoc test ($p < 0.05$).

Temperature	N:P ratio	chl *a*	chl (*c1* + *c2*)	Total carotenoids
Experiment 1				
22°C	5N:1P	1.87 ± 0.49	0.29 ± 0.10	1.31 ± 0.06a
	10N:1P	1.77 ± 0.44	0.26 ± 0.07	1.40 ± 0.32a
	16N:1P	1.94 ± 0.25	0.28 ± 0.03	1.47 ± 0.14ab
	25N:1P	1.96 ± 0.08	0.28 ± 0.02	1.56 ± 0.06ab
	40N:1P	1.96 ± 0.28	0.28 ± 0.03	1.54 ± 0.18ab
	80N:1P	2.66 ± 0.20	0.39 ± 0.04	1.93 ± 0.17b

TABLE 2 Photosynthetic pigments (chl *a*, chlorophyll a; chl *c* (*c1* + *c2*), chlorophyll c1 + c2 and total carotenoids, in $mg \cdot g^{-1}$ DW) obtained from spectrophotometric analysis of *R. okamurae* cultivated under six different N:P ratios at 22°C after 1 week.

Note: Data are presented as means and standard deviations ($n = 3$). Different letters indicate significant differences among the treatments, according to Tukey post hoc test ($p < 0.05$).

1, only the total soluble protein was influenced by the N:P ratio, and in experiment 2, total carbohydrates were influenced by temperature (Table S2). Soluble proteins increased to the double the initial contents at the 80:1N:P ratio. In this condition, the highest amount of soluble protein was quantified in experiment 1 (4.79 ± 1.99 mg of soluble protein $\cdot g^{-1}$ DW). This value was statistically similar to the one observed at 40:1N:P ratio but higher than those obtained for the other treatments (Table 3). In the case of experiment 2, soluble protein averaged 5.43 ± 0.6 mg of soluble protein $\cdot g^{-1}$ DW and were not affected by the experimental conditions (temperature or N:P ratios). The total of carbohydrates was 137.88 ± 20.7 mg of carbohydrates $\cdot g^{-1}$ DW on average and unaffected by the N:P ratios in the experiment 1. In experiment 2, samples at 15°C showed lower amounts of carbohydrates than those cultivated at 22°C, regardless of the N:P ratio (Table 3). Phenolic compounds of *R. okamurae* were not changed by experimental conditions in both experiments (Table S2). In experiment 1, the content of phenols in *R. okamurae* was 4.34 ± 0.73 mg $\cdot g^{-1}$ DW, and in the experiment 2, the content was 6.86 ± 0.76 mg $\cdot g^{-1}$ DW (Table 3).

The biochemical and physiological responses of *R. okamurae* showed some correlations that are presented in Table S3. Antioxidant capacity, total carbohydrates, and phenolic compounds were negatively correlated with other variables. For example, antioxidant capacity was negatively correlated with F_v/F_m, fucoxanthin, violaxanthin, zeaxanthin, and soluble protein content. In the case of carbohydrates, there were negative correlations with some photosynthetic parameters, chl *a*,

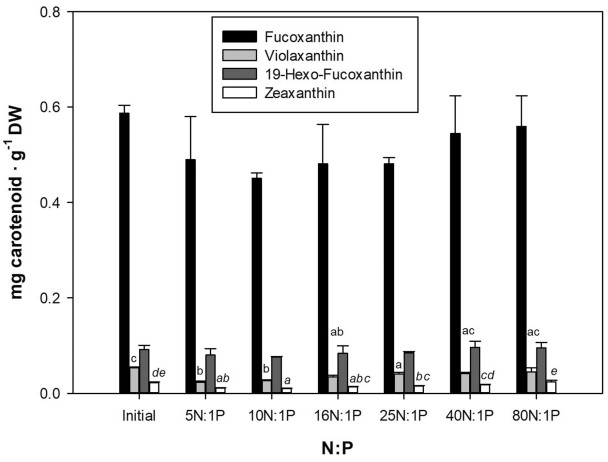

FIGURE 5 Carotenoids (fucoxanthin, 19-hexo-fucoxanthin, violaxanthin, and zeaxanthin in mg·g^{-1} DW) obtained from HPLC analysis of *R. okamurae* cultivated under six different N:P ratios at 22°C after 1 week. Data are presented as means and standard deviations ($n = 3$). Different letters indicate significant differences among the treatments, according to Tukey post hoc test ($p < 0.05$). When no letter is present, the data were statistically similar.

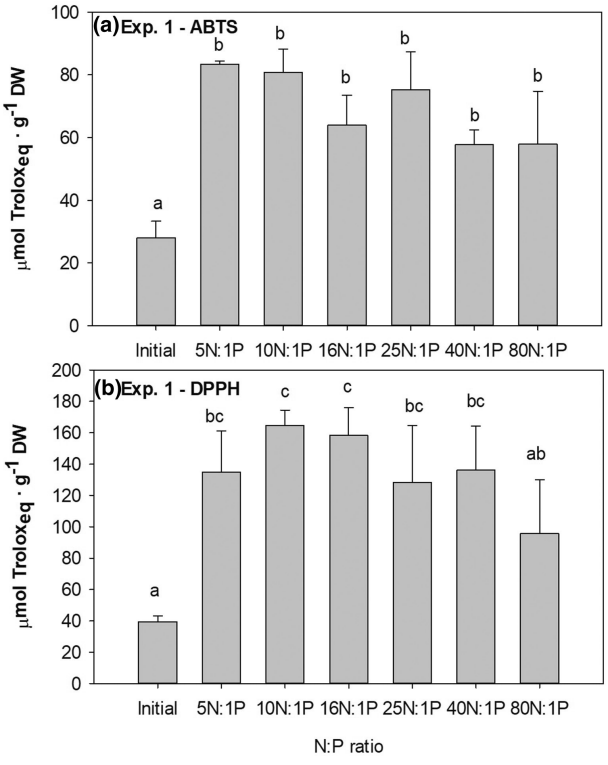

FIGURE 6 Antioxidant capacity of *R. okamurae* extracts after 1 week of cultivation under six different N:P ratios (5N:1P, 10N:1P, 16N:1P, 25N:1P, 50N:1P, and 80N:1P) at 22°C. Data are presented as means and standard deviations. Different letters indicate significant differences among the treatments, according to Tukey post hoc test ($p < 0.05$). (a) ABTS method ($n = 3$). (b) DPPH method ($n = 5$). When no letter is present, the data were statistically similar.

and total carotenoid contents. Phenolics showed negative correlations, mainly with ETR_{max} and α_{ETR}, and growth rates. The other variables presented positive relationships among them (Table S3), highlighting chl *a* and zeaxanthin positively related to photosynthetic parameters and other pigments.

DISCUSSION

R. okamurae has been recorded as an invasive macroalga in the Mediterranean coast since 2015, when the first data on its presence in the area of the Gibraltar strait (around the coast of Andalusia, Ceuta and Morocco) were reported (Altamirano et al., 2016; El Aamri et al., 2018). Later, the species started spreading along Spanish, French, and Italian Mediterranean coasts (Bellissimo et al., 2024; Ruitton et al., 2021; Terradas-Fernández et al., 2023) and, more recently, along the Atlantic coast of Northern Morocco (El Madany et al., 2024), the Algarve, and Azores Islands in Portugal (Faria et al., 2021; Liulea et al., 2023). In those areas, this species has occupied diverse ecological niches, replacing endemic and native species and surviving during the whole seasonal cycle. Moreover, it is having socioeconomical impacts on fisheries (especially artisanal fisheries) and tourism sectors due to the disturbance on beaches in Spain's southern coast (García-Gómez, Florido, Olaya-Ponzone, Sempere-Valverde, & Megina, 2021). Most of the studies published on *R. okamurae* indicate that its physiological plasticity, which permits its growth under a wide variety of environments and during the whole seasonal cycle, is probably one of the characteristics that explain its invasive success (Bermejo et al., 2015; García-Lafuente et al., 2023; Muñoz et al., 2015). Our objective and experimental design were not focused on analyzing the seasonal changes; however, our results illustrate its plasticity as thalli collected in autumn and winter presented differences in their biochemical composition, even although they were pre-acclimated to similar light and nutrient conditions. The main differences between autumn and winter thalli were related to protein and carbohydrate contents that varied by 1.4 and 3.2 times, respectively, as well as phenolics content and ETR_{max}. This different physiological status might explain some dissimilar responses to the changes in the medium N:P ratio obtained between experiments 1 and 2. However, this did not influence the N uptake rates, which were fairly high and increased with increasing N concentration in both autumn and winter thalli. The fact that photosynthetic ETR increased under higher N:P ratios in both experiments also suggests that the N assimilation is energetically supported by photosynthesis irrespective of the physiological status of the alga. Consequently, our results demonstrate that high N assimilation capacity is an intrinsic physiological feature of *R. okamurae*, as previously suggested in Mercado et al. (2022). Interestingly, this N uptake capacity is not affected by temperature or phosphate concentration.

TABLE 3 Biochemical compounds of *R. okamurae* cultivated under two experimental conditions.

Temperature	N:P ratio	Sol. Proteins	Carbohydrates	Phenolics
Experiment 1				
22°C	Initial	2.04 ± 0.51ab	110.38 ± 35.76	3.53 ± 0.23
	5N:1P	1.44 ± 0.20a	107.87 ± 22.11	4.86 ± 1.53
	10N:1P	2.30 ± 0.11ab	145.67 ± 31.67	5.54 ± 0.87
	16N:1P	2.07 ± 0.37ab	137.10 ± 25.80	4.48 ± 1.52
	25N:1P	2.90 ± 0.33abc	157.03 ± 20.53	4.01 ± 1.05
	40N:1P	3.92 ± 0.50bc	152.14 ± 16.61	4.44 ± 0.77
	80N:1P	4.79 ± 1.99c	154.94 ± 6.79	3.49 ± 1.00
Experiment 2				
15°C	10N:1P	6.52 ± 2.17	79.55 ± 9.61A	8.26 ± 2.19
	25N:1P	5.33 ± 0.15	85.18 ± 2.85A	6.64 ± 0.63
	50N:1P	5.36 ± 0.51	78.05 ± 6.88A	6.87 ± 0.64
22°C	10N:1P	5.18 ± 0.22	97.90 ± 17.03B	6.24 ± 1.35
	25N:1P	5.25 ± 0.17	99.70 ± 8.62B	6.17 ± 0.52
	50N:1P	4.92 ± 0.16	89.45 ± 3.83B	6.97 ± 1.16

Note: The first experiment was conducted for 1 week at six different N:P ratios (5N:1P, 10N:1P, 16N:1P, 25N:1P, 50N:1P, and 80N:1P) at 22°C. In the second experiment, three N:P ratios (10N:1P, 25N:1P, and 50N:1P) and two temperatures were applied during the period of 10 days. Data are presented as means and standard deviations ($n = 3$). Different letters indicate significant differences among the treatments, according to Tukey post hoc test ($p < 0.05$).

This result has important implications for the management of the alga, as it suggests that the invasive capacity would be, in part, related to the availability of dissolved inorganic N. Consequently, *R. okamurae* can be considered a nitrophilic algae, as is the case of *Ulva* species that also have high capacity of nitrate assimilation.

Higher rates of N uptake with the increasing N:P ratio did not result in increasing growth rates, suggesting that the N assimilated was allocated internally. Furthermore, the protein level was low compared to other species (Vega et al., 2023). Phenolic content was also lower than that in other brown algae, such as *Laminaria* spp., *Fucus* spp., *Sargassum* spp., or *Cystoseira* spp. (Celis-Plá et al., 2014, 2017; Connan et al., 2006; Le Lann et al., 2008). In contrast, carbohydrate concentration was relatively high under all N:P ratios tested, indicating that the photosynthetic rate favored C in contrast to N metabolism. These results indicate that N is not highly accumulated in the form of N-compounds (i.e., proteins, whereas as other biocompouds as carbohydrates are accumulated in the biomass). Alternatively, it can be speculated that the N is stocked in the form of internal nitrate, as has been described in other brown algae species such as the sugar kelp *Saccharina latissima* (Chapman et al., 1978). This nitrate would be utilized when external nutrient becomes limited (Phillips & Hurd, 2003), although this hypothesis still needs to be evaluated by conducting long-term experiments.

The main carotenoid in *R. okamurae* is fucoxanthin. This is a relevant carotenoid commonly found in brown algae and other stramenopiles (Petrushkina et al., 2017) such as haptophytes. Petrushkina et al. (2017) presented a review about the concentration of fucoxanthin, and they observed the maximum amounts in three groups of algae: diatoms (up to 21.67 $mg \cdot g^{-1}$ DW), Synurophyceae (up to 26.6 $mg \cdot g^{-1}$ DW), and Haptophytes (up to 18.23 $mg \cdot g^{-1}$ DW). The concentrations of fucoxanthin in representatives of other groups of algae (Chrysophyceae, Pelagophyceae, Phaeophyceae, Raphidophyceae) are much lower (0.02–9.01 $mg \cdot g^{-1}$ DW). Additionally, some studies have already described the variability of this carotenoid. For example, reduced light and nutrient availability down-regulated the quantity of this compound in *Undaria pinnatifida* (Endo et al., 2017). In contrast, according to our data, the fucoxanthin and 19-hexofucoxanthin are the principal carotenoid pigments found in *R. okamurae*, although they are not regulated by N availability, and their contents are not so high as in other species (from 1.27 to 2.12 $mg \cdot g^{-1}$ DW observed in six species of brown algae; *Sargassum* spp. have the highest content; Susanto et al., 2016). However, our values are higher than those reported by Zailanie and Purnomo (2011) in Indonesian species (i.e., all values reported by the authors were below of 0.3 $mg \cdot g^{-1}$ DW but in our study, it was ca. 0.5 $mg \cdot g^{-1}$ DW). We highlight that although this quantity is lower than reported in other species (Petrushkina et al., 2017), the huge amount of biomass produced by the invasive species means the extraction of this carotenoid can be highly productive; indeed, it has already proved to be very useful in diverse industrial and human biotechnology applications (see review of Myiashita et al., 2020). A relevant aspect to be taken

into account is that the fucoxanthin extraction can be done according to different procedures, and some of those procedures allow the optimal yield of this carotenoid production (Schmitz et al., 2022). Moreover, the conditions of fucoxanthin extraction have been optimized by Shannon and Abu-Ghannam (2017). These authors maximized the extraction of this carotenoid by incubating at 30°C for 36.5 min, pH 5.7, with 62.2% acetone. However, their results showed total fucoxanthin close to the amounts observed in our study, with *Alaria esculenta* having the greatest yield (0.870 $mg \cdot g^{-1}$ DW) followed by *Fucus vesiculosus* (0.699 $mg \cdot g^{-1}$ DW) and *Laminaria digitata* blade (0.650 $mg \cdot g^{-1}$ DW).

The other two carotenoids extracted from the *R. okamurae* thalli were violaxanthin and zeaxanthin. Brown algae have been reported to use xanthophyll cycles to avoid photoinhibition that is associated with the de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin in response to high light (Takaichi, 2011). Although observed in lower quantities, when *R. okamurae* received lower amounts of N:P, both carotenoids decreased. The zeaxanthin was clearly regulated by N availability, being degraded in the absence of N. However, the reduction of this carotenoid was not inversely associated with violaxanthin, indicating that the carotenoids were not being interconverted.

Photosynthetic capacity estimated as ETR presented maximal values at 80N:1P (37.34 $\mu mol\ e^- \cdot m^{-2} \cdot s^{-1}$), but the values at the other N:P ratios tested ranged from 21 to 30 $\mu mol\ e^- \cdot m^{-2} \cdot s^{-1}$. In contrast, growth rates ranged from 1.5%–2% $\cdot d^{-1}$ at low N:P ratios (5:1 and 10:1) to 2.5%–3.5% $\cdot d^{-1}$ at N:P higher than 16:1. Thus, high input of N into seawater, which might come from land activities such as excess fertilizer from intensive agriculture or sewage from urban areas, will favor the biomass increase of *R. okamurae* in the Alborán Sea. The similar productivity and growth in a wide range of N:P ratio indicated high plasticity and acclimation to different conditions that can favor bioinvasion. However, the similar physiological responses at two temperatures may indicate that the *R. okamurae* is a thermic-tolerant species, which gives it an advantage compared to other algal species that occupy the coastal areas throughout the whole year. Taking into account the huge amounts of biomass observed in the coastal areas of Mediterranean Sea, biotechnological potential is a relevant aspect to drive the biomass destination. In this sense, carotenoids and carbohydrates are two biocompouds useful for different applications, such as nutraceutics in the case of carotenoids (Meléndez-Martínez et al., 2021) or biomaterial and energy in the case of carbohydrates (De la Lama-Calvente et al., 2023; Santana et al., 2022). In addition, sometimes the extraction of a compound becomes feasible for utilization in some industrial approaches when high amount of biomass is available even though its desired contents are not high. Thus, the thoughts of utilization of *R. okamurae* biomass should be focused mainly on a biorefinery concept, with diverse and complementary destinations to the biomass.

CONCLUSIONS

The brown invasive macroalga *R. okamurae* presented high rates of N uptake and photosynthesis rates that increased at high N:P ratios. Protein levels and photosynthesis also increased with the N:P ratio. However, other parameters remained constant, revealing thermotolerant and eutrophic behavior with high potential to grow in different annual periods. This physiological performance can explain the successful growth of this alga in a scenario of eutrophication and climate change. Although contents of fucoxanthin were not so high as in other algal species, the amount of invading biomass could allow its utilization under a biorefinery concept, as it could be obtained high productivity when taking into account the biomass and the internal concentration.

Considering that our study was conducted in a laboratory providing relevant clues, an approach in the environmental areas would be very relevant to complete the understanding of *R. okamurae* ecophysiology. More investigation is necessary on the ecophysiology of this invasive exotic alga, including the combination of different physicochemical variables in the sea column and the temporal and spatial variation of biomass in the invaded coastal areas.

AUTHOR CONTRIBUTIONS

José Bonomi-Barufi: Conceptualization (equal); data curation (equal); formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); supervision (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Félix L. Figueroa:** Conceptualization (equal); data curation (equal); formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); project administration (equal); resources (equal); supervision (equal); validation (equal); visualization (equal); writing – review and editing (equal). **Julia Vega:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); resources (equal); writing – review and editing (equal). **Rubén Huesa:** Investigation (equal); methodology (equal). **Talissa B. Harb:** Investigation (equal); methodology (equal). **Antonio Avilés:** Data curation (equal); formal analysis (equal); methodology (equal). **Jesús M. Mercado:** Data curation (equal); formal analysis (equal); investigation (equal); supervision (equal); writing – review and editing (equal). **Nathalie Korbee:** Conceptualization (equal); data curation (equal); formal analysis (equal); funding acquisition (equal);

investigation (equal); methodology (equal); project administration (equal); resources (equal); supervision (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1: Summary of the results of ANOVA conducted to the analyses of nutrient uptake efficiency (NUE) 2 and nutrient uptake rates (NUR) in two experiments performed with *Rugulopteryx okamurae*. Two nutrients (nitrates and phosphates) were assayed at 1, 5, and 7 days in experiment 1, and at the 3, 5, 7, and 10 days of experiment 2. In the first experiment, one-way analyses were performed, considering N:P ratio as a factor of

influence. In the second experiment, two-way analyses were performed, with N:P ratio and temperature being the evaluated factors. Significant data are marked in bold ($p < 0.05$). df, degree of freedom; MS, Mean of squares; SS, Sum of squares.

Table S2: Summary of the results of ANOVA conducted in two experiments performed with *Rugulopteryx okamurae*. In the first experiment, one- or two-way analyses were performed, considering N:P ratio and time as factors of influence. In the second experiment, two- or three-way analyses were performed, with N:P ratio, temperature, and time being the evaluated factors. Significant data are marked in bold ($p < 0.05$). Chl *a*, chlorophyll *a* content; Chl c, chlorophyll c1 + c2 content; df, degree of freedom; E_k, saturating irradiance; ETR, electron transport rates; F_v/F_m, maximum quantum yield; MS, mean of squares; SS, sum of squares; α_{ETR}, photosynthetic electron transport rates efficiency.

Table S3: Pearson correlation between dependent variables measured in *Rugulopteryx okamurae* after the first experiment evaluating the effects of diverse N:P ratios in the physiological responses. 19-HF, 19-hexofucoxanthin; ABTS: antioxidant capacity evaluated by the ABTS method; Carb., total of carbohydrates; Chl *a*, chlorophyll *a* content; Chl *c*, chlorophyll *c1 + c2* content; E_k, saturating irradiance; ETR, electron transport rates; Fucox, fucoxanthin; F_v/F_m, maximum quantum yield; Phenol., phenolic compounds; Sol.Prot., soluble proteins; Violax., violaxanthin; Zeax., zeaxanthin; α_{ETR}, photosynthetic electron transport rates efficiency. Positive correlations are marked in green, while negatives are marked in red.

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