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Interactive effects of marine heatwaves and eutrophication on the ecophysiology of a widespread and ecologically important macroalga

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Abstract

Extreme temperature events are becoming more recurrent and are more frequent with major impacts on coastal ecosystems, which are additionally impacted by increasing urbanization, resulting in high nutrient load. So far little is known about cumulative and/or interactive effects of global and local stressors on species' performance. Here, we evaluated the single and combined effects of simulated heatwaves and eutrophication on the ecophysiology of *Laurencia catarinensis* in a bi-factorial mesocosm experiment. The algae were exposed for 30 d to three different temperatures (20°C, 24°C, 28°C) and nutrient concentrations (low, intermediate, high) and their nutrient uptake rates, photosynthetic performance, growth rates, pigment and phenolic compound concentrations, as well as antioxidant capacity was determined and compared between treatments. Under low nutrient concentration, physiological performance and growth decreased with increasing temperatures. In contrast, they increased with higher nutrient availability and moderate temperature increase close to the summer average temperature, but largely declined upon exposure to higher temperature (28°C). This decline seemed to be related to oxidative stress, as indicated by an increase of compounds with antioxidant properties (lutein, zeaxanthin, phenols). Our data show that all measured parameters were affected by temperature and nutrient availability, with an interactive effect between these two factors, indicating that increasing temperature will influence macroalgal performance, and even more dramatically in coastal systems that are highly impacted by urbanization. However, the direction of the response will be determined by nutrient availability and will also depend on the magnitude of the temperature increase, that is, whether it surpasses the thermal threshold of the species.

Marine macroalgae are dominant and essential components of coastal ecosystems around the world (Gattuso et al. 1998; Harley et al. 2012) and are currently threatened by a multitude of stressors, such as those related to global climate change (Harley et al. 2006, 2012), but also locally by increasing nutrient and sediment loading, the overfishing and spread of invasive species (Worm and Lotze 2006; Schmidt and Scheibling 2007). Current changes in seawater temperature, due to climatic change, predict for the end of this century a continuous warming of near-surface air temperature, on the order of 2–7°C with regional, seasonal and diurnal

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Additional Supporting Information may be found in the online version of this article.

variations (Christensen et al. 2007), causing continuing temperature rise in surface oceans. Already, the effects of ocean warming have been detected in marine ecosystems along the Australian west coast and in the Mediterranean, which experienced unprecedented heatwaves, causing mass mortalities (Garrabou et al. 1998; Smale and Wernberg 2013; Wernberg et al. 2013; Short et al. 2015), as well as ecosystem-scale reconfigurations toward warm-water species (Wernberg et al. 2016). Recent analyses show that the frequency of this anomalously high seawater temperatures has increased by 38% along the world's coastlines, including the Brazilian Atlantic coast (Lima and Wethey 2012), and a further increase over the 21st century is predicted (Easterling et al. 2000; Meehl et al. 2007). So far, the influence of these extreme events, rather than gradual warming trends, on marine ecosystems is poorly understood (Hobday et al. 2016). However, it is clear that the changes in temperature will have a great impact on costal ecosystems dominated by seaweeds, as it is a major factor controlling macroalgal metabolic and growth rates (Davison 1991; Davison and Pearson 1996). Temperature is thus considered as one of the main environmental drivers of macroecological and evolutionary processes and as a key factor for the interpretation of seaweed community shifts observed throughout world oceans (Wernberg et al. 2011*a*,*b*).

In combination with an increase in temperature, related to global climate change, marine coastal ecosystems are also affected by rapidly changing stressors that originate and act at regional and local scales, such as eutrophication (see Halpern et al. 2008). Besides additive effects between local and global stressors (where the response can be predicted based on the effects of individual stressors), recently, more attention has been drawn to the effects of possible interactions between stressors (Crain et al. 2008; Gunderson et al. 2016). The nature of these interactions can range from synergistic to antagonistic (where the response is greater or lesser than would be predicted from adding the independent effects of stressors, respectively). Here, especially synergisms between stressors are of particular concern, as the future impacts on ecosystems predicted on the basis of individual stressor effects will be underestimated if there are synergistic interactions between stressors. Interactions between global and local stressors are predicted to have a great impact on marine ecosystems, resulting in community shifts toward dominance of opportunistic species (e.g., Harley et al. 2006) or influencing transitions to novel habitats (Hobbs et al. 2009). This consequently will lead to changes in the structure, function, and services that these ecosystems provide (Harley et al. 2006, 2012; Wernberg et al. 2010, 2011*a*,*b*).

For marine seaweed communities, temperature is recognized as a major factor influencing their biology (Davison 1991; Eggert 2012). Latitudinal and vertical changes in this factor represent key ecological and evolutionary drivers in the marine environment, which can result in molecular divergences in seaweeds with broad ranges of distribution (Sissini et al. 2014). These divergences manifest mainly by different physiological tolerance limits imposed by temperature in their populational edges (Bolton et al. 2004; Harley et al. 2012).

The algal response to changes in temperature usually displays a maximum, which indicates the thermal optimum, with a decline in algal performance below and above this optimum (e.g., Hodgson 1981; Kuebler et al. 1991; Pakker and Breeman 1996; Nejrup et al. 2013; Flukes et al. 2015; Wilson et al. 2015). The algal physiological response to high temperatures is related to cellular processes, as thermal stress is known to affect membrane-associated processes, to cause decrease in enzyme activity or even inactivation, to trigger the production of reactive oxygen species (e.g., Larkindale et al. 2005) and to affect the efficiency of antioxidant systems (Bischof and Rautenberger 2012), all of them causing reduction in photosynthesis, and consequently growth. Thus, secondary metabolites (e.g., phenolic compounds, carotenoids), which are associated with protective strategies against environmental stressors, such as excess irradiance, temperature, etc. (Logan et al. 2006; Hargrave et al. 2016), have been reported to be up-regulated under thermal stress, resulting in large changes in gene expression and resource allocation, including antioxidant proteins and detoxifying enzymes (Collén et al. 2007).

Despite temperature, increased nutrient concentrations can severely impact coastal ecosystems, causing increases in growth and abundance of opportunistic species of macroalgae (e.g., Morand and Briand 1996; Morand and Merceron 2005; Dailer et al. 2012). On the other hand, the excess of nutrients can also be toxic to some species, which can cause decreases in species richness with increasing anthropogenic pressure (Portugal et al. 2016). However, it has been shown that the macroalgal responses to nutrient addition are species-specific, ranging from negative responses to increases in photosynthesis and growth (Schaffelke 1999). In addition, excess nutrient concentrations can compromise seaweed ecophysiology, reducing the wealth and diversity of species in coastal ecosystems (e.g., Halpern et al. 2008; Martins et al. 2012; Scherner et al. 2013) and/or inducing community shifts due to differential impacts on benthic macroalgae (Portugal et al. 2016). Changes in nutrient availability not only alter the physiological performance of macroalgae, thus affecting growth rates, but can also affect the production of primary and secondary metabolites. For example, the carbohydrate content in macroalgae has been shown to be inversely related to nutrient concentrations (Macler 1986; Rotem et al. 1986; Marinho-Soriano et al. 2006) due to reallocation of carbohydrates from storage reserves toward the production of energy and carbon skeletons necessary to reduce nitrate and form amino acids, which can also cause starch degradation (Turpin 1991; Huppe and Turpin 1994). On the other hand, in red algae, the main antenna

pigments, chlorophyll *a* (Chl *a*) and phycobiliproteins, which are considered nitrogen reserves, increase their concentration when subjected to high nitrogen availability (Bird et al. 1982; Lapointe and Duke 1984; García-Sánchez et al. 1993; Andria et al. 1999; Kim et al. 2007). Under these conditions, an increase in algal photosynthetic rates, and ultimately growth has been reported (e.g., Lapointe and Duke 1984; Littler and Littler 1992; Kim et al. 2007). Also, an increase in secondary metabolites, e.g., phenolic compounds, has been reported in response to increased nutrient availability (Cabello-Pasini et al. 2011).

Little is known about the effects of increasing temperature when paired with increased nutrient concentrations in macroalgae. However, as carbon and nitrogen metabolisms are closely coordinated (Turpin 1991; Huppe and Turpin 1994), the nutritional status of the algae can markedly influence photosynthetic and respiratory processes in response to temperature changes (Raven and Geider 1988; Turpin 1991; Huppe and Turpin 1994). In addition, increasing temperature can increase nutrient uptake rates (e.g., Pedersen et al. 2004; Du et al. 2013), but it has been suggested that above and below the optimal range of temperature of the algae, over which uptake occurs, rates are likely to decrease (Hanisak and Harlin 1978; Topinka 1978; Wheeler and Srivastava 1984; Reay et al. 1999). The few studies investigating possible interactive effects of temperature and nutrient availability showed contrary results that might be related to species-specific differences. In brown seaweed species, no interactive effect between temperature and nutrient availability was found either in physiological performance, growth, reproduction, development nor survival (Steen and Rueness 2004; Mabin et al. 2013; Flukes et al. 2015; Kay et al. 2016). In contrast, in the green macroalgae *Enteromorpha intestinalis* and *Ulva rigida*, a positive synergistic effect of temperature and nutrient increase on recruitment was recorded (Lotze and Worm 2002; Gao et al. in press), as well as an effect of these factors on biochemical compounds, such as proteins, lipids and carbohydrates (Gao et al. in press). In the red alga *Hypnea musciformis*, a positive synergistic effect has been reported for the physiological and growth performance under moderate temperature and high nutrient availability, while under high temperature stress (+10°C over mean summer temperature), a large negative effect was observed (De Faveri et al. 2015).

In Brazil, frequent episodes of high temperature peaks at different locations are recorded, including the subtropical Santa Catarina coast (Fig. 1). As already reported in other regions around the world, these heatwaves have great impacts on marine benthic communities, which along the Brazilian Southwestern coast are predominantly composed of seaweeds. In these coastal environments, high nutrient load due to increasing urbanization (Pagliosa et al. 2006) has already lead to species loss and community shifts (Scherner et al. 2012, 2013; Portugal et al. 2016), and it can be expected that macroalgae in these impacted coastal areas will be even more dramatically affected under extreme events, such as heatwaves (Hobday et al. 2016).

The rhodophyte species studied here, *Laurencia catarinensis*, is an ecologically and economically important seaweed with a broad distribution in the Atlantic Ocean. Its habitat ranges from the Northeastern to the Southeastern Atlantic coast, and from the lower mesolittoral up to 3 m deep (Cassano 2009), an environment which currently is becoming subjected to more extreme and frequent temperature anomalies (Lima and Wethey 2012), in addition to anthropogenic impacts due to increasing urbanization. Thus, the goal of this study was to evaluate the single and combined effects of conditions, simulating heatwaves and increasing nutrient load in *L. catarinensis*, by measuring the changes in algal physiological performance, growth, pigment content, carbohydrates, and secondary metabolites. This evaluation of the impacts of these global and local stressors and their possible interactions will aid in predicting possible shifts in distribution and dominance of this species along the Southwestern Brazilian coast, and hence, potential impacts at the community and ecosystem level.

Material and methods

Collection site and algal sampling

L. catarinensis samples were collected in March 2014 from the intertidal zone of the Southwestern Atlantic coast of Brazil (Xavier Archipelago, 27°36′33″S 48°23′09 ′W, 27°36′38″S 48°23′15″W), localized at 4.5 km east from Santa Catarina Island. This region, representing the southern distributional limit of the species, is regarded as warm temperate biogeographical province (Horta et al. 2001; Spalding et al. 2007), exhibiting a well-defined seasonal pattern with an annual mean sea-surface temperature (2004–2014) of 21.9 ± 3°C, a maximum monthly mean value of 25.6 ± 0.5°C in summer and a mean maximum value of 27.0 ± 0.9°C in summer (Fig. 1a; http://podaac.jpl.nasa.gov/dataset/NCDC-L4LRblend-GLOB-AVHRR_OI/).

The region periodically experiences prolonged thermal events with temperatures substantially hotter than normal (heatwaves). Recently, a standardized definition for marine heatwaves has been constructed, based on consecutive calendar days exceeding the 90th percentile of temperature for at least five consecutive days (Hobday et al. 2016). From this definition, a set of metrics can be computed that measure marine heatwave intensity, duration, cumulative intensity and rate of onset/decline, using the free software package in R Program, RmarineHeatWaves using (Smit et al. 2016). Here, we used daily mean Sea Surface Temperature of time series data from 1985 to 2014 (http://podaac.jpl.nasa.gov/dataset/NCDC-L4LRblend-GLOB-AVHRR_OI/) for Xavier Archipelago to estimate duration, intensity and frequency of heatwave events for the studied region. According to the Hobday et al. (2016) definition of a marine heatwave, we

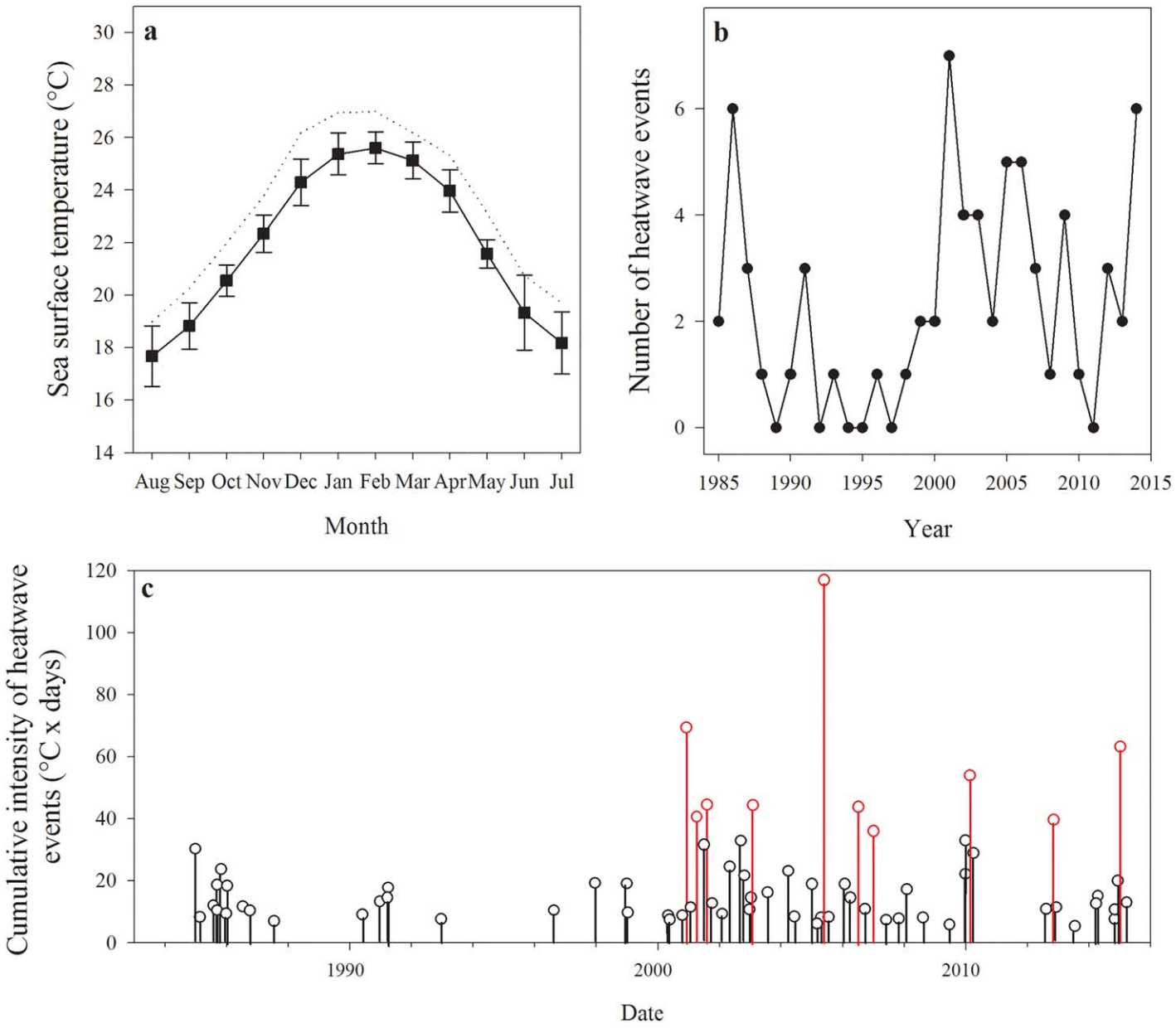

Fig. 1. (**a**) Sea surface temperature at Xavier Archipelago (2004–2014; http://podaac.jpl.nasa.gov/dataset/NCDC-L4LRblend-GLOB-AVHRR_OI/): Monthly mean (solid line) and maximum temperature (dotted line), and (**b**) the frequency and (**c**) cumulative intensity of heatwave events between 1985 and 2014 (most intense events are indicated in red), derived from the analysis of marine heatwaves according to Hobday et al. (2016). [Color figure can be viewed at wileyonlinelibrary.com]

estimated 70 events between 1985 and 2014, with an increasing frequency over the last 15 yr (Fig. 1b). This increase in frequency was accompanied by an increase in intensity, calculated as the product of duration and mean intensity of the event (Fig. 1c; Table 1). The most intense event was detected during winter 2005, followed by summer 2001 and spring 2014, with maximum temperature (peak) intensities between 2.6°C and 3.7°C (Table 1).

In addition to marine heatwave events, the region is under the influence of frequent upwelling events, resulting in nutrient enrichment, but nitrogen and phosphate values can reach even higher values (1–49 μM and 0.4–12 μM, respectively) due to urban run-off (Pagliosa et al. 2006). In addition, the Brazilian southwestern coast is impacted by the La Plata river discharge (Piola et al. 2005), which has a very high nitrogen load (1.0 Tg N) due to agriculture activities (Watanabe et al. 2012).

After collection, the algae were transported in coolers with seawater to the laboratory, where they were cleaned to remove epiphytes and epifauna. Afterwards, the samples

Table 1. Most intense heatwave events [according to their cumulative intensity: Mean intensity (°C) × duration (d)] estimated between 1985 and 2014 at Xavier Island, Southwestern Atlantic coast of Brazil. The events were estimated according to the definition of Hobday et al. (2016), using RmarineHeatWaves (Smit et al. 2016). Mean and maximum intensity refer to °C above threshold (calendar day 90th percentile).

Date						
Start	Stop	Peak	Duration (d)	Mean intensity (°C)	Max. intensity (°C)	Cumulative intensity
30.05.2005	09.07.2005	18.06.2005	41	2.84	3.71	116.4
06.02.2001	08.03.2001	14.02.2001	31	2.21	2.86	68.6
08.10.2014	13.11.2014	04.11.2014	37	1.73	2.66	63.9
19.12.2009	13.01.2010	30.12.2009	26	2.08	3.54	54.0
14.07.2006	03.08.2006	28.07.2006	21	2.13	2.84	44.7
05.10.2001	29.10.2001	11.10.2001	25	1.78	2.58	44.5
28.02.2003	22.03.2003	13.03.2003	23	1.92	2.68	44.1
02.06.2001	21.06.2001	17.06.2001	20	2.03	2.74	40.7
08.09.2012	26.09.2012	20.09.2012	19	2.12	3.22	40.3
15.12.2006	02.01.2007	22.12.2006	19	1.95	2.41	37.1

were transferred to a mesocosm system, where they were maintained for 24 h under similar light, temperature, and salinity conditions as measured in the field (max. light intensity determined in the field: 690 μmol quanta m^{-2} s^{-1}; T = 20°C; salinity: 32 psu).

Experimental design

The bi-factorial experiment was performed in a mesocosm system consisting of opaque reservoir tanks ($n = 9$, V = 300 L), equipped with a pump (Sarlo Better, Brazil) for internal water circulation, in which the temperature was controlled, using chillers (Radical 1 HP, Brazil). Before entering the mesocosm system, the seawater passed through sand-filters (25 μm, 20 μm, and 5 μm) and were exposed to ultraviolet light.

Each reservoir tank supplied four transparent small plastic tanks (V = 2.5 L), which contained each 15 g of *L. catarinensis* clumps fixed on glass slides. During the experiment, the algae were maintained for 30 d under three different temperature regimes (20°C, 24°C, and 28°C) and nutrient conditions (low, intermediate, and high). At the beginning of the experiment, the temperature was gradually increased (2°C every 2 h) until reaching the respective treatment temperature.

The lowest nutrient condition (LNC) represented the field condition without additional nutrients (0.96 μM - NH_4, 0.46 μM PO_4, 0.38 μM NO_3). To simulate increased nutrient availability, $(NH_4)_2SO_4$, Na_2HPO_4, and $NaNO_3$ were added to reach intermediate (INC; 40 μM NH_4, 2 μM PO_4, 10 μM NO_3) and high nutrient levels (HNC; 80 μM NH_4, 5 μM PO_4, 40 μM NO_3). At the beginning of the experiment, nutrient concentrations were monitored every 2 d by sampling 0.5 L seawater from three tanks of each treatment ($n = 3$ per treatment). The data showed that the concentrations were stable for at least 8 d and only showed decline at the tenth day (see Supporting Information Table S1). Thus, new nutrients were added every 10 d to maintain the desired concentration. Before each new addition of nutrients, samples were taken from the treatment tanks ($n = 3$ per treatment) to determine the amount of nutrients that had to be added in order to maintain the desired concentration (see Supporting Information Table S2). Additionally, dissolved O_2 (Oxi 315i Oximeter, WTW GmbH, Germany), pH (AT-315 pH meter, Alfakit, Brazil), salinity (SZJ-S10 Refractometer) and irradiance (LI-1400, LI-COR, U.S.A.) were monitored daily.

At the end of the experiment, the photosynthetic performance and growth rates of the algae under different treatments were determined. Also, algal samples were frozen at −80°C for posterior pigment, phenol, sugar, and starch analysis.

Nutrient analysis and determination of nutrient uptake rates

Seawater samples from all experimental units were passed through GF/F Whatman 0.45 μm filters before analysis. The dissolved inorganic nutrients (NO_3^{-2}, NH_4^+, PO_4^{-3}) were determined colorimetrically, according to Tréguer and Le Corre (1976) and Grasshoff et al. (1983), using a spectrophotometer (UV-1100, Pró-Análise ISE Química e Diagnóstica Ltda, Brazil).

In order to determine nutrient uptake rates, water samples from the experimental treatment tanks were collected every 10 d before the addition of new nutrients, as described above. These samples were used to determine differences in nutrient uptake rates (μmol g^{-1} FW d^{-1}) along the experiment and between treatments.

Photosynthesis

The photosynthetic performance of samples ($n = 4$ for each treatment) was estimated at the end of the experiment by measuring maximum electron transport rates (ETR) through in vivo Chl *a* fluorescence of PSII, using a portable fluorometer (Junior-PAM, Walz, Germany). ETR_{max} was calculated according to Genty et al. (1989), using the equation: $ETR = \Delta F/F_{m'} \times A \times 0.5 \times PAR$, where $\Delta F/F_{m'}$ represents the effective quantum yield, A represents the tissue absorptance of the sample and PAR represents the photosynthetically active radiation in μmol quanta m^{-2} s^{-1}.

The absorptance of the algal samples was determined according to Beer and Björk (2000), using a cosine light sensor (LI-192 Underwater Quantum Sensor, LI-COR, U.S.A.) connected to a datalogger (LI-1400, LI-COR, U.S.A.) and a high-pressure sodium lamp (Osram, 200W) as light source, located in a fixed position above the surface of the sensor. Absorptance was calculated using the equation: $A = 1 - (E_t/E_o)$ (relative units), where E_o is the incident irradiance and E_t is the transmitted irradiance when the alga was located on the light sensor. In each case, the incident irradiance (E_o) was measured first. The algal thallus was placed over the sensor and the amount of light transmitted through the thallus (E_t) was determined.

Relative growth rate (RGR)

The effects of different temperature and nutrient concentrations on the growth of *L. catarinensis* were evaluated by determining the difference between wet weight at the beginning and at the end of the experiment ($n = 4$ per treatment), using the following equation (Lignell and Pedersén 1989):

$$RGR\ \left[\%\ d^{-1}\right] = \left[(W_t/W_i)^{1/t} - 1\right] * 100 \qquad (1)$$

where W_i = initial wet weight, W_t = wet weight after 30 d, and t = internal time in days.

Photosynthetic pigments

For pigment extractions, samples (0.3 g fresh weight, $n = 3$ for each treatment) were ground to powder with liquid nitrogen and extracted with 50 mM phosphate buffer (pH 5.5). The homogenates were centrifuged at 4000 rpm for 20 min at 4°C and the supernatant was used to determine phycobilipigment concentrations. Chl *a* was extracted after resuspending the remaining pellet in 90% acetone and posterior centrifugation (EPPENDORF 5810R, Eppendorf AG, Germany) at 4000 rpm for 15 min at 4°C. Pigments were quantified spectrophotometrically (Bel Spectro LGS53, BEL Analytical Equipment Ltd., Brazil), and phycobilipigment and Chl *a* concentrations were calculated according to Kursar et al. (1983) and Ritchie (2008), respectively.

Carotenoids were extracted from samples (0.5 g dry mass, $n = 3$) using methanol. Extracts were centrifuged (10 min, 4000 rpm), and an aliquot (10 μL, $n = 3$) was injected onto the liquid chromatograph (Shimadzu LC-10A) equipped with a C_{18} reverse-phase column (Vydac 218TP54; 250 mm x 4.6 mm ∅, 5 μm, 30°C), protected by a 5 μm C_{18} reverse-phase guard column (Vydac 218GK54) and a UV-visible detector (450 nm). Elution was performed with MeOH: CH_3CN (90: 10, v/v) at a flow rate of 1 mL min^{-1}. Carotenoid identification (lutein) was performed using retention times and co-chromatography of standard compounds (Sigma-Aldrich, St. Louis, Missouri, U.S.A.), as well as through comparison with other reports of carotenoid analysis by RP-HPLC-UV-vis under similar conditions (Scott and Eldridge 2005; Hulshof et al. 2007).

Total sugar and starch

The extraction of total soluble sugars was performed according to Shannon (1968). Samples (0.05 g dry weight, $n = 3$ for each treatment) were extracted with 2 mL of methanol : chloroform : water (MCW; 12 : 5 : 3) and centrifuged (EPPENDORF 5810R, Eppendorf AG, Germany) at 3000 rpm for 5 min at 25°C. The supernatant was recovered and the pellet was re-extracted using 2 mL of MCW. One-part chloroform and 1.5-part water were added to the supernatant, followed by centrifuging at 3000 rpm for 5 min, from which two phases were obtained. The upper aqueous phase was collected and anthrone 0.2% was added, in accordance with Umbreit and Burris (1957).

The starch extraction was performed according to McCready et al. (1950). The pellets used in the total soluble sugar extraction were ground with perchloric acid ($HClO_4$) 30% (v/v) and centrifuged at 3000 rpm for 5 min at 25°C. The supernatant was collected, and the pellet was extracted again as specified above. The extract was again centrifuged and the supernatants of both extractions were pooled and analyzed according to Umbreit and Burris (1957), using the reagent anthrone 0.2% (w/v). Afterwards the samples were measured spectrophotometrically (Bel Spectro LGS53, BEL Analytical Equipment Ltd., Brazil) and sugar and starch concentrations were calculated using D-glucose as standard.

Total phenolic compounds

Polyphenolics were extracted from samples (0.5 g dry mass, $n = 3$ for each treatment) using 80% methanol. Extracts were centrifuged (10 min, 4000 rpm), and injected onto the liquid chromatograph (Shimadzu LC-10A), equipped with a C_{18} reverse-phase column (Shim-pack C18; 250 mm × 4.6 mm Ø column, 5 μm, 30°C), protected by a 5 μm C_{18} reverse-phase guard column and a UV-vis detector (280 nm). Elution was performed with water : acetic acid : n-butanol (350 : 1 : 10, v/v/v), at a flow rate of 0.8 mL min^{-1}. Polyphenolic identification (epicatechin and gallocatechin) was performed using retention times and chromatography of standard compounds (Sigma-Aldrich, St. Louis, Missouri, U.S.A.).

Radical scavenging activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was determined ($n = 3$ per treatment) according to Tait et al. (1996). For quantitative evaluation, 100 μL of each extract (0.1 mg mL^{-1}) were mixed with 2.9 mL of DPPH solution (200 μg mL^{-1}) (Sigma-Aldrich, U.S.A.), which was prepared daily. After 1 h in the dark at 25°C, the absorbance was measured spectrophotometrically at 517 nm (SP-220, Biospectro, Brazil). The DPPH radical scavenging activity was determined using the following equation:

$$\mathrm{DPPH\%} = \left(\mathrm{Absorbance_{control}} - \mathrm{Absorbance_{sample}}\right) / \mathrm{Absorbance} \times 100\% \quad (2)$$

A positive control of quercetin was also measured in the same concentrations.

Estimation of algal biomass

The biomass of *L. catarinensis* was determined at the sampling site in summer and winter between 2012 and 2014. The algae were collected during low tides at three locations at Xavier Archipelago, using 10 × 10 cm quadrats ($n = 5$ per site), and dried at 60°C for determination of biomass per area.

Molecular evaluation of tropical and warm temperate populations

To determine possible molecular divergences between populations along the Brazilian coast, which might result in differences in their physiological thermal limits, *L. catarinensis* specimens were collected in Fernando de Noronha and Rio Grande do Norte, and from Santa Catarina, São Paulo and Rio de Janeiro, representing tropical and warm temperate populations (Horta et al. 2001), respectively (Supporting Information Tables S4, S5). After sampling, the specimens were dried and transported to the laboratory for molecular analyses. DNA extraction, PCR and sequencing protocols were performed as described in Machín-Sánchez et al. (2012). The neighbor-joining (NJ) and maximum likelihood (ML) analyses were performed in PAUP* 4.0b10 (Sinauer Associates, Publishers, Sunderland, Massachusetts, U.S.A.) (Swofford 2003) with 2000 and 100 bootstrap replicates, respectively. For the Bayesian Inference (BI) analysis the program MrBayes v.3.0 beta 4 (San Diego, California, U.S.A.) was used (Huelsenbeck and Ronquist 2001). For both, ML and BI analyses, the model used was the general-time-reversible model of nucleotide substitution with invariant sites and gamma distributed rates for the variable sites (GTR + I+G). This model was selected based on maximum-likelihood ratio tests implemented in Modeltest version 3.06 (Posada and Crandall 1998) using the Akaike information criterion (AIC). For Bayesian analysis, four chains of the Markov chain Monte Carlo (one hot and three cold) were used, sampling one tree every 100 generations for 4 000 000 generations starting with a random tree. Log-likelihood values stabilized at around 30 000 generations, which were discarded as "burn in." A 50% consensus tree (majority rule as implemented by PAUP) was computed after the 'burn in' point. The range of *rbc*L divergence values within and among species was calculated using uncorrected "p" distances using PAUP. This analytical approach permits the characterization of molecular particularities of southern populations fostering discussion regarding segregation of ecotypes shaped by environmental factors related with warm temperate provinces (e.g., Hammann et al. 2016; Marín-Guirao et al. 2016; Pfaff et al. 2016).

Statistical analysis

Statistical analyses were performed using the software STATISTICA 6.0 (Statsoft). Interactive and isolated effects between temperature and nutrient concentrations were evaluated for ETR_{max}, relative growth rate, pigment concentrations, phenols, DPPH activity, starch and total sugars, using two-way ANOVA. For nutrient uptake rate, three-way ANOVA was used to determine isolated and interactive effects between temperature, nutrients, and time. For algal biomass, one-way ANOVA was used to determine differences between sampling dates. Newman Keuls Significant Difference post hoc tests were used to identify the statistically different groups. Homogeneity of the variance was tested a priori using Cochran's test.

Results

Nutrient uptake

The nutrient uptake rates in *L. catarinensis* showed a significant increase (ANOVA, $p < 0.00001$; Supporting Information Table S3) with time of the experiment and nutrient concentration (Fig. 2a). There was little effect of the temperature increase from 20°C to 24°C on the uptake rates, but a large decline at 28°C under intermediate (INC) and high nutrient concentrations (HNC) for nitrate and ammonium uptake rates (Fig. 2a,b). In contrast, the phosphate uptake rates did not show a time or temperature effect under INC, while under HNC, the uptake rates followed a similar pattern as found for nitrate and ammonium (Fig. 2c).

Photosynthetic performance and relative growth rate (RGR)

Photosynthesis in *L. catarinensis*, measured as maximum electron transport rate (ETR_{max}), showed significant differences in algae exposed to different temperature and nutrient conditions, as well as a significant interactive effect between these two abiotic parameters ($p \leq 0.05$, Table 2). In general, ETR_{max} exhibited highest values at 24°C, which were positively related to nutrient availability, a pattern also found under 20°C (Fig. 3a). On the other hand, at 28°C ETR_{max} decreased dramatically in the algae under HNC (Fig. 3a).

The responses in growth rate of *L. catarinensis* were in concordance with the photosynthetic responses, with a

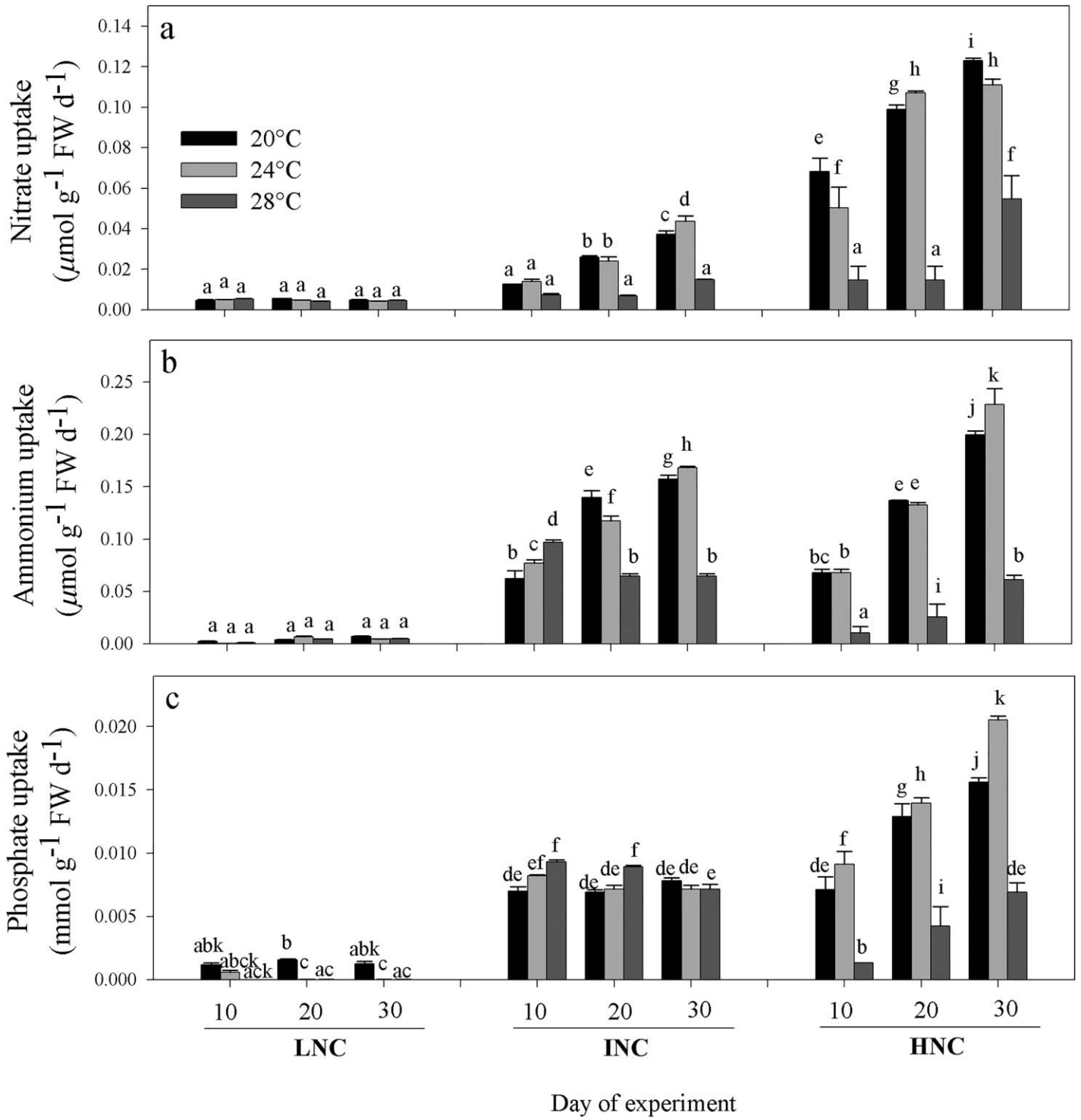

Fig. 2. Changes in nutrient uptake rates (**a**, nitrate; **b**, ammonium; **c**, phosphate) of *L. catarinensis* under different temperature (20°C, 24°C, and 28°C) and nutrient concentrations (low, LNC; intermediate, INC and high nutrient concentration, HNC), determined for different time points during the experiment (day 10, 20, and 30). Uptake rates were derived from nutrient concentrations measured every 10 d before addition of new nutrients in order to maintain the desired nutrient level (see Supporting Information Table S2). Data are shown as mean ± SD ($n = 3$) and different letters indicate significant differences (Newman–Keulen test, two-way ANOVA).

Table 2. Results of two-way ANOVA for different parameters determined in *L. catarinensis* samples after exposure for 30 d to different temperature and nutrient conditions. Significant effects are indicated in bold.

Variation source df = 2	Nutrients			Temperature			Nutrients × temperature		
Variables	MS	*F*	*p*	MS	*F*	*p*	MS	*F*	*p*
RGR	15.35	1746.79	**<0.001**	9.31	1059.80	**<0.001**	3.10	353.35	**<0.001**
ETR_{max}	542.5	23.1	**<0.001**	208.2	8.9	**0.002**	71.3	3.0	**0.046**
Chl *a*	0.19	70.36	**<0.001**	0.07	25.31	**<0.001**	0.019	7.23	**0.0013**
Phycocyanin (PC)	0.21	1.67	0.22	2.47	19.213	**<0.001**	0.66	5.16	0.009
Phycoerythrin (PE)	3.41	6.65	**0.007**	10.85	21.15	**<0.001**	4.62	9.00	**<0.001**
ΣPE+PC	5.28	4.80	**0.022**	24.02	21.83	**<0.001**	9.38	8.52	**<0.001**
Total sugars	6.61	1139.86	**<0.001**	43.73	7533.82	**<0.001**	12.32	2123.2	**<0.001**
Starch	0.50	5229.50	**<0.001**	0.28	2930.45	**<0.001**	0.56	5792.9	**<0.001**
Total phenolic	67.19	24.4005	**<0.001**	53.82	19.54	**<0.001**	60.187	21.85	**<0.001**
DPPH	177.60	202.61	**<0.001**	90.20	102.9	**<0.001**	56.54	64.50	**<0.001**
Lutein	12679.32	128.62	**<0.001**	2097.29	21.27	**<0.001**	3239.50	32.9	**<0.001**
Zeaxanthin	302.55	3.63	**<0.001**	2214.48	26.57	**<0.001**	1139.2	13.67	**<0.001**

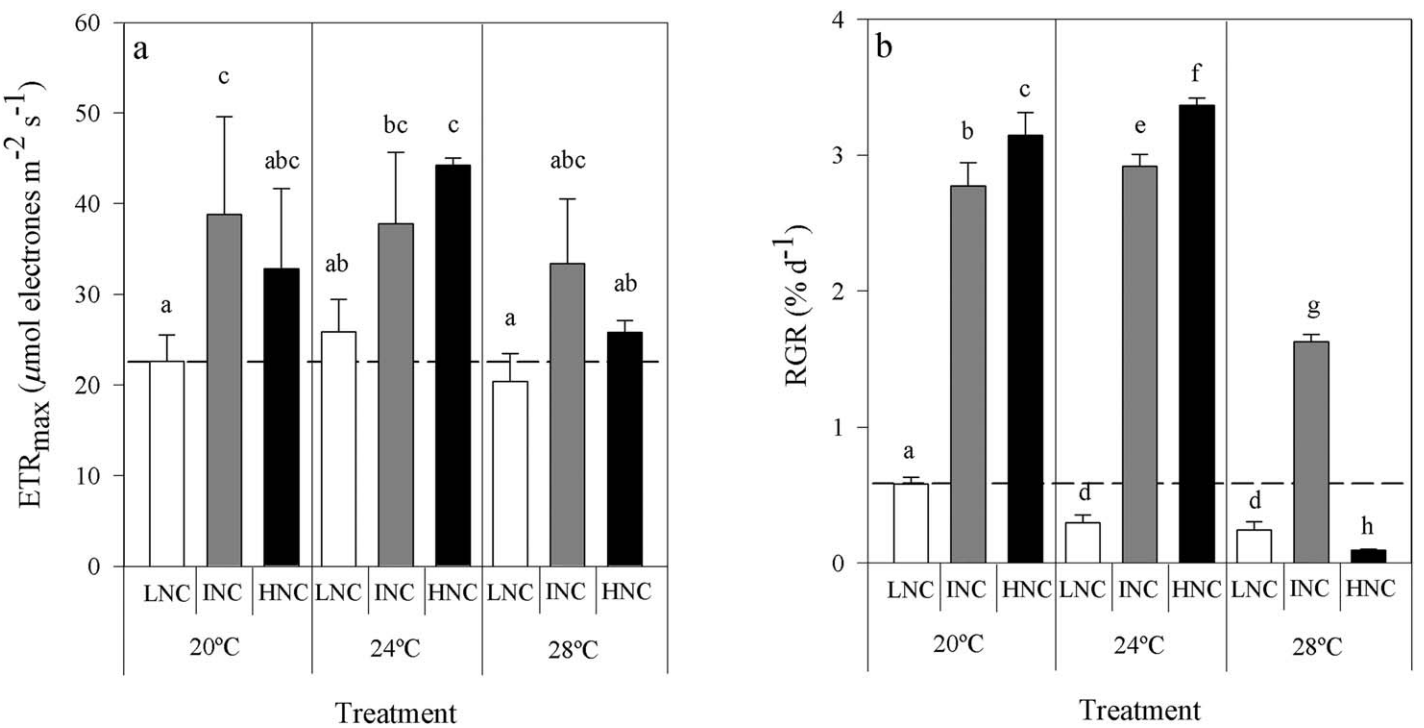

Fig. 3. Comparison of (**a**) photosynthetic rate, measured as maximum electron-transport rate (ETR_{max}) and (**b**) growth response (RGR, % d^{-1}) of *L. catarinensis* after exposure to different temperature (20°C, 24°C, and 28°C) and nutrient conditions (low, LNC; intermediate, INC and high nutrient concentration, HNC) for 30 d. Data are shown as mean ± SD ($n = 3$ for ETR_{max}, $n = 4$ for RGR). Different letters indicate significant differences (Newman–Keulen test, two-way ANOVA) and broken line indicates control value (LNC at 20°C).

significant increase under higher nutrient availability (INC, HNC), with the highest values at 20°C (~ 5-fold compared to LNC) and 24°C (9- to 11-fold compared to LNC), while at these temperatures the lowest growth was recorded under LNC (Fig. 3b). In contrast, at 28°C there was a large decline in RGR under elevated nutrient availability (INC and HNC), which was more dramatic under HNC (Fig. 3b).

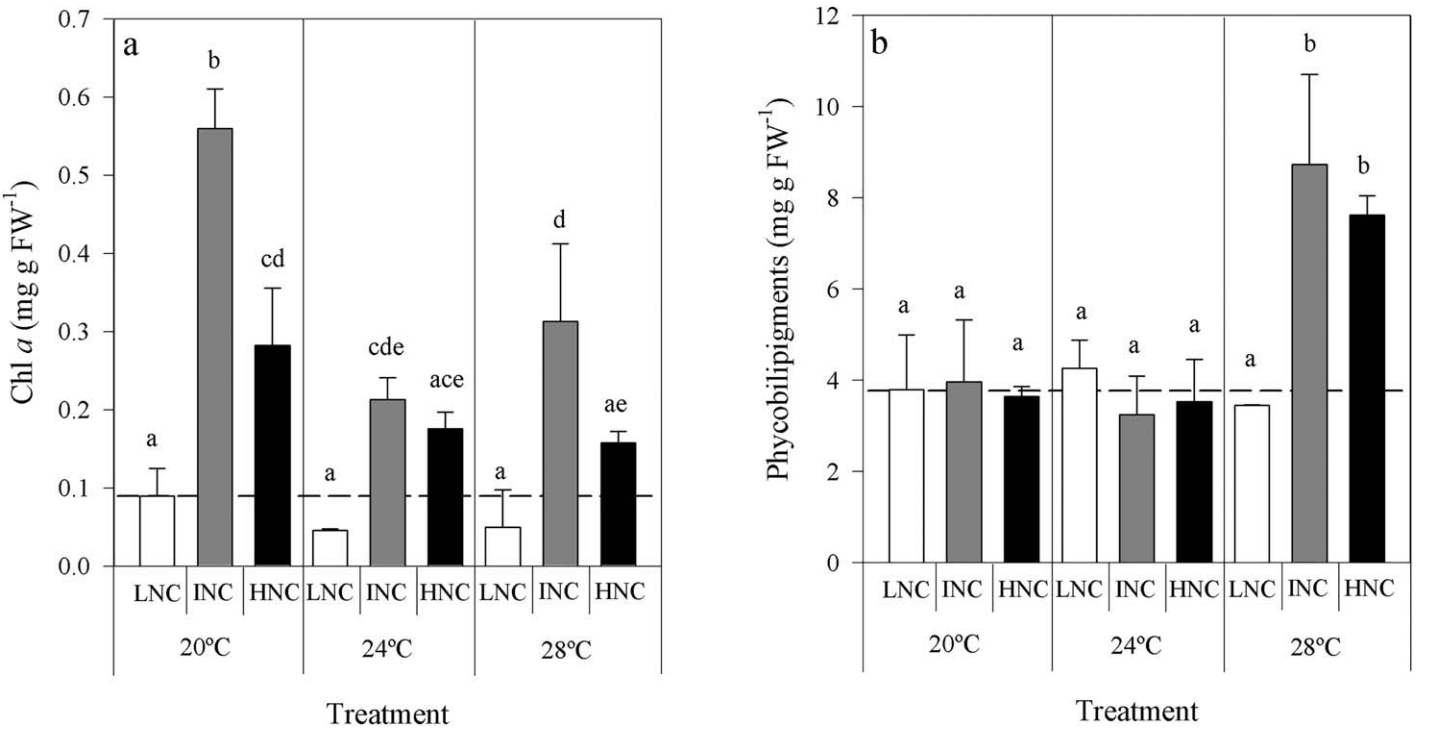

Fig. 4. (**a**) Chl *a* and (**b**) phycobilipigment content in *L. catarinensis* after exposure to different temperature (20°C, 24°C, and 28°C) and nutrient conditions (low, LNC; intermediate, INC and high nutrient concentration, HNC) for 30 d. Data are shown as mean ± SD ($n = 3$). Different letters indicate significant differences (Newman–Keulen test, two-way ANOVA) and broken line indicates control value (LNC at 20°C).

Photosynthetic pigments

The different temperature and nutrient treatments, as well as the interaction between these two parameters, produced a significant response in the concentrations of Chl *a* and phycobilipigments (Fig. 4). Under all temperature conditions, the Chl *a* concentration was highest in the INC treatment and lowest under LNC (Fig. 4a). In contrast, phycobilipigments did not respond to increased nutrient availability, except under the highest temperature, where their concentration was increased more than twice under INC and HNC (Fig. 4b).

Total sugars and starch

In general, the total sugar content in *L. catarinensis* decreased with increased nutrient availability and temperature, except under 24°C, where the HNC-treatment showed the highest sugar content (Fig. 5a). Both temperature and nutrient availability had a significant effect on the sugar content, when isolated and in combination (Table 2).

The response of the starch content to the different temperature and nutrient treatments was significant (Table 2), but more complex compared to the response in sugar content (Fig. 5). While under 20°C and 24°C the starch content was highest under low nutrient availability, at 28°C it was lowest, with the highest content found under INC (Fig. 5b).

Compounds with antioxidant activity

Different compounds for which an antioxidant function has been described, such as certain carotenoids and phenolic compounds, have been quantified here, as well as the radical scavenging activity of the algae.

The carotenoids lutein and zeaxanthin showed a significant response to the different temperature and nutrient conditions, generally with increased concentration at higher temperature and nutrient availability (Fig. 6a,b). The lutein and zeaxanthin content was lowest under HNC only at 20°C, while at higher temperature the concentration was higher under HNC or similar between INC and HNC for lutein and zeaxanthin, respectively (Fig. 6a,b).

A very similar pattern as in lutein and zeaxanthin was also found for the phenolic compounds and the DPPH scavenging activity, with significant increases at higher temperatures and nutrient availabilities, except at 20°C (Fig. 6c,d).

Algal biomass in the field

The biomass of *L. catarinensis* at the sampling site varied between 414 and 455 g DW m^{-2} and did not show significant seasonal variation between summer and winter, with the exception of summer 2014 (Table 3). This last sampling was performed shortly after a major heatwave event (see Table 1), which might explain the up to 50% lower biomass compared to the former samplings.

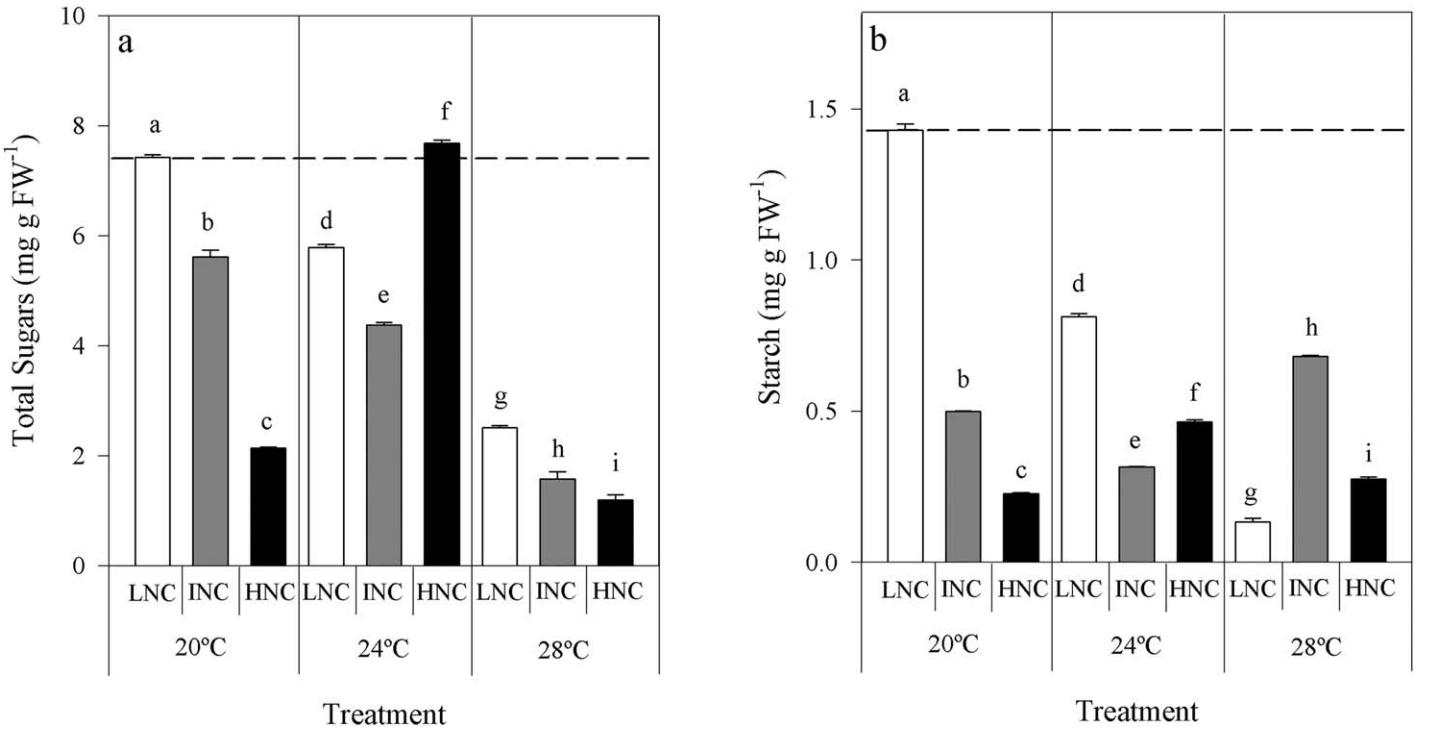

Fig. 5. Comparison of (**a**) total sugar and (**b**) starch concentrations of *L. catarinensis* samples after 30 d under different temperature (20°C, 24°C, and 28°C) and nutrient conditions (low, LNC; intermediate, INC and high nutrient concentration, HNC). Data are shown as mean ± SD ($n = 3$). Different letters indicate significant differences (Newman–Keulen test, two-way ANOVA) and broken line indicates control value (LNC at 20°C).

Molecular evaluation of tropical and warm temperate populations

We sequenced the *rbc*L gene for two specimens of *L. catarinensis*; the other sequences of this species were provided by Cassano (2009). By adding sequences from GenBank, an alignment of 28 sequences was assembled, including *Chondria collinsiana* as outgroup (Supporting Information Tables S4, S5). A total of 274 nucleotides was removed from the alignment at the beginning and end, as many sequences from the GenBank were incomplete, producing a data set of 1193 base pairs. Based on the 1193 bp alignment, the tropical (Fernando do Noronha, Rio Grande do Norte) and warm temperate samples (Santa Catarina, São Paulo, Rio de Janeiro) exhibited 0.5% genetic divergence (Fig. 7).

Discussion

Temperature is a major factor influencing marine seaweed communities (Davison 1991; Eggert 2012), and thus has been included in most of the efforts regarding multiple stressors through marine experimental evaluations (Gunderson et al. 2016). Our results show that this global stressor will influence even more dramatically macroalgal performance in coastal systems that are highly impacted by urbanization and agriculture. In the species studied here, *L. catarinensis*, the direction of the algal response will be determined by nutrient availability, but more importantly, will depend on the magnitude of the temperature increase, i.e., if it surpasses the thermal threshold of the species.

The present study shows that in *L. catarinensis*, temperature and nutrient availability are factors regulating the physiological performance, and consequently growth, with a significant interaction between these two abiotic factors, which is similar to findings in *Laurencia papillosa* (Tsai et al. 2005). The physiological performance of *L. catarinensis* was highest at 24°C, which is close to the summer average of ~25°C, and declined largely upon exposure to +3°C above this average value, which represented ~ 1°C above summer maximum (see Fig. 1a). The sensitivity of this species to temperatures greater than those it normally experiences in its natural environment agrees with studies in other macroalgal species (Zou and Gao 2014; De Faveri et al. 2015; Flukes et al. 2015). It is also supported by field observation, showing that *L. catarinensis* was heavily affected by an intense heatwave event that occurred in late spring 2014, reflected in an almost 50% lower biomass in the following summer (see Table 3).

However, in view of *L. catarinensis*' amphi-Atlantic distribution, inhabiting tropical, subtropical, and warm temperate regions (Machín-Sánchez et al. 2012), which suggests a tolerance to high temperatures, its high sensitivity to 28°C in our experimental study was somewhat unexpected. The

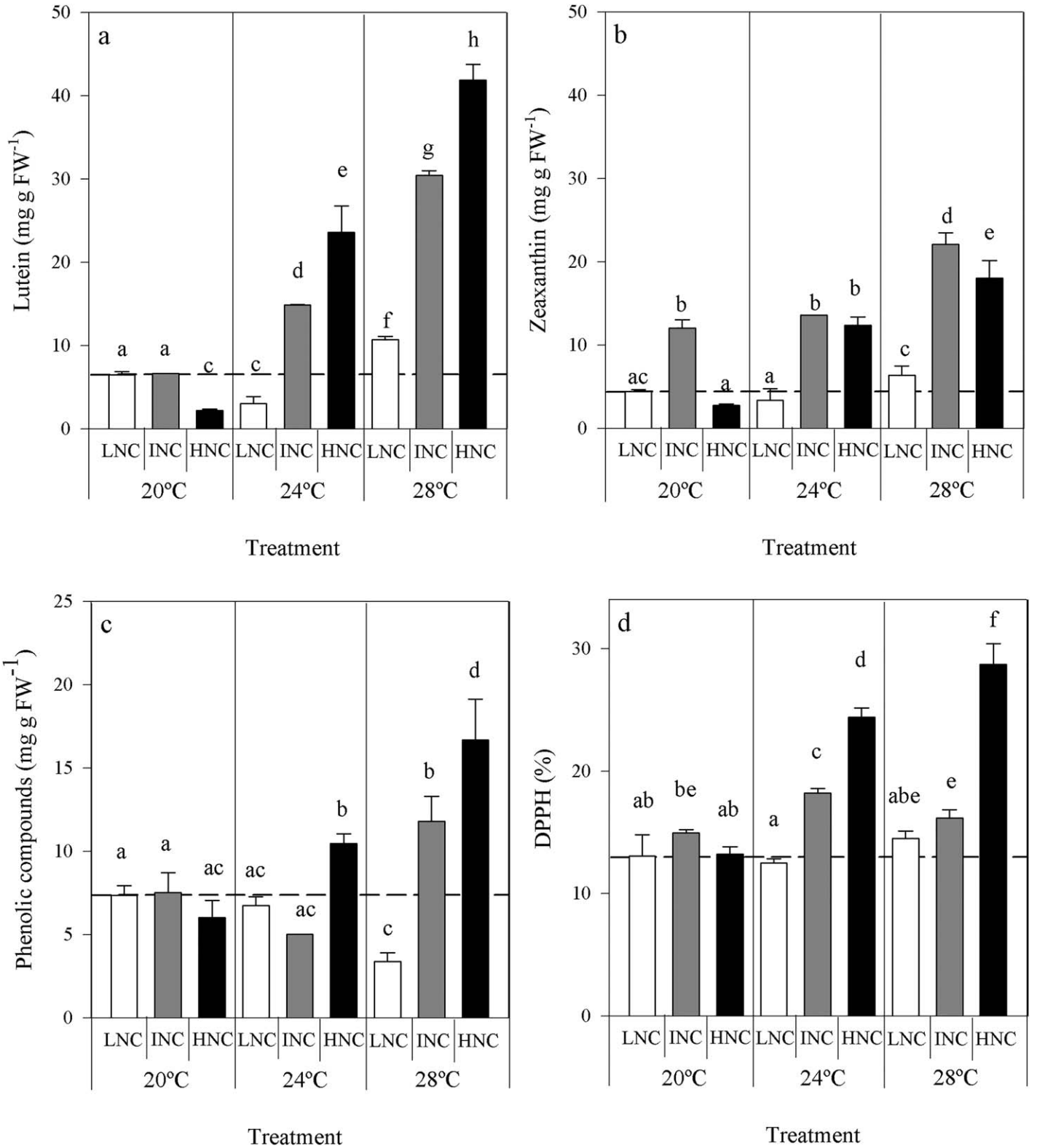

Fig. 6. Comparison of compounds with antioxidant activity and antioxidant activity of *L. catarinensis* samples after 30 d under different temperature (20°C, 24°C, and 28°C) and nutrient conditions (low, LNC; intermediate, INC and high nutrient concentration, HNC). (**a**) Lutein, (**b**) zeaxanthin, (**c**) total phenolic compounds, and (**d**) DPPH radical scavenging activity. Data are shown as mean ± SD ($n = 3$). Different letters indicate significant differences (Newman–Keulen test, two-way ANOVA) and broken line indicates control value (LNC at 20°C).

Table 3. Seasonal variation in biomass of *L. catarinensis*. Data are shown as mean ± SE and different letters indicate significant differences (Newman–Keulen test, ANOVA).

Year	Season	Biomass (g DW m^{-2})
2012	Winter	455 ± 57^{a}
2013	Summer	414 ± 52^{a}
2013	Winter	443 ± 56^{a}
2014	Summer*	240 ± 34^{b}

* After heatwave event.

sensitivity to high temperature in this warm-temperate population might be due to adaptation to the local temperature conditions that can strongly influence the species' temperature tolerance, resulting in an ecotype with different optimal temperatures for photosynthesis and growth, compared to tropical populations. Previous studies support the suggestion of differences in thermal acclimation between ecotypes from environments with different temperature regimes (Gerard and Du Bois 1988; Pakker et al. 1996). In addition, our molecular analysis comparing the *L. catarinensis* population at our studied location with more northern populations in the Brazilian tropical region (Rio Grande do Norte and Fernando de Noronha) showed a molecular divergence of 0.5% between these populations, which indicates different ecotypes. Similar phylogeographical patterns were observed in other groups of red algae (Sissini et al. 2014), reinforcing that different temperature regimes, among other oceanographic environmental factors, can impose physiological barriers and lead to genetic variability as indicated by the minor but significant differences between tropical and subtropical *L. catarinensis* populations.

Below the species' thermal threshold, photosynthesis, nutrient uptake rates, and growth increased under higher nutrient availability and temperature, which is similar to previous studies (Hanisak and Harlin 1978; Lapointe and Duke 1984; Nishihara et al. 2005; Teichberg et al. 2008, 2010). This response might be related to an increased activity of enzymes related to the metabolism of nitrogen, such as nitrate reductase and enzymes related to carbon metabolism (RUBISCO) (Rosenberg and Ramus 1982*b*; Wheeler and Weidner 1983).

However, as also found in this and other studies, compared to nutrient concentration, temperature has a minor effect on algal growth and nutrient uptake rates (see Figs. 2, 3; Duke et al. 1989; Pedersen et al. 2004), except when the temperature increase surpassed the species thermal threshold, which can result in a large decline in these parameters (see Figs. 2, 3; Hanisak and Harlin 1978). In contrast, other studies showed that only temperature, but not nutrient availability, was the critical factor regulating growth in different seaweed species (e.g., Lotze and Worm 2002; Flukes et al. 2015). These differences between studies might be related to species-specific differences in nutrient requirements and storage capacities (Pedersen and Borum 1996; Teichberg et al. 2008). The studied species, *L. catarinensis*, seemed to be nutrient-limited under ambient conditions, as shown by the observed positive effect of nutrient addition on nutrient uptake and algal metabolic and growth rates (see Figs. 2, 3). Also, the pigment response of *L. catarinensis* indicates low nutrient storage capacity. Generally, it is assumed that pigment concentrations in algal tissue are strongly associated with the nitrogen concentration available in the water column, showing an increase in pigments in response to nutrient enrichment (DeBoer and Ryther 1977; Lapointe and Ryther 1979; Morgan et al. 1980). In red algae, the phycobilipigments are considered important for nitrogen storage (Lapointe 1981; Bird et al. 1982; Rosenberg and Ramus 1982*a*; Vergara and Niell 1993) and their increase has been related to increases in photosynthetic capacity and, thus, the fixed carbon available for growth (Chapman et al. 1978; Lapointe and Ryther 1979; Lapointe 1981; Friedlander et al. 1991). In this study, however, even though an increase in Chl *a* content under higher nutrient availability was found, phycobilipigments did not respond to changes in nutrient concentration, except under the highest temperature, when photosynthetic and growth rate dramatically declined, the phycobilipigment content increased (see Fig. 4b). This might be related to the impairment/restriction of electron flow, indicated by the decrease in ETR_{max}, resulting in an accumulation of nitrogen in form of phycobilipigments.

Both temperature and nutrient concentrations also influenced the starch and carbohydrate content in *L. catarinensis*. In general, the levels of reserve carbohydrates and starch reflect a balance between accumulation from photosynthesis and consumption for energy production, biosynthesis, and growth. Hence, an inverse relationship between starch content and growth can be found, as reported in *Gracilaria foliifera* and *Ulva* spp. (Rosenberg and Ramus 1982*b*), as carbon reserves are mobilized during nutrient assimilation (e.g., Vergara et al. 1995) to supply amino acid biosynthesis (see Huppe and Turpin 1994). This agrees with the observed accumulation of carbohydrates under the low ambient nutrient level in this study (see Fig. 5). However, environmental factors, such as temperature and nutrient availability, might uncouple this correlation, as it seemed to be the case in *L. catarinensis*. It could be related to the temperature effect on respiratory activity and the link between carbon and nitrogen assimilation (see Huppe and Turpin 1994). The internal carbon reserves declined with increasing temperature and nutrient availability, indicative for decreased net C fixation under thermal stress, probably resulting from increased respiration under higher temperatures and/or re-allocation of reducing power and energy derived from photosynthesis toward nutrient assimilation (see Huppe and Turpin 1994). Additionally, energy might also be re-allocated to enhance the algas' photoprotective capacity (e.g., Collén et al. 2007).

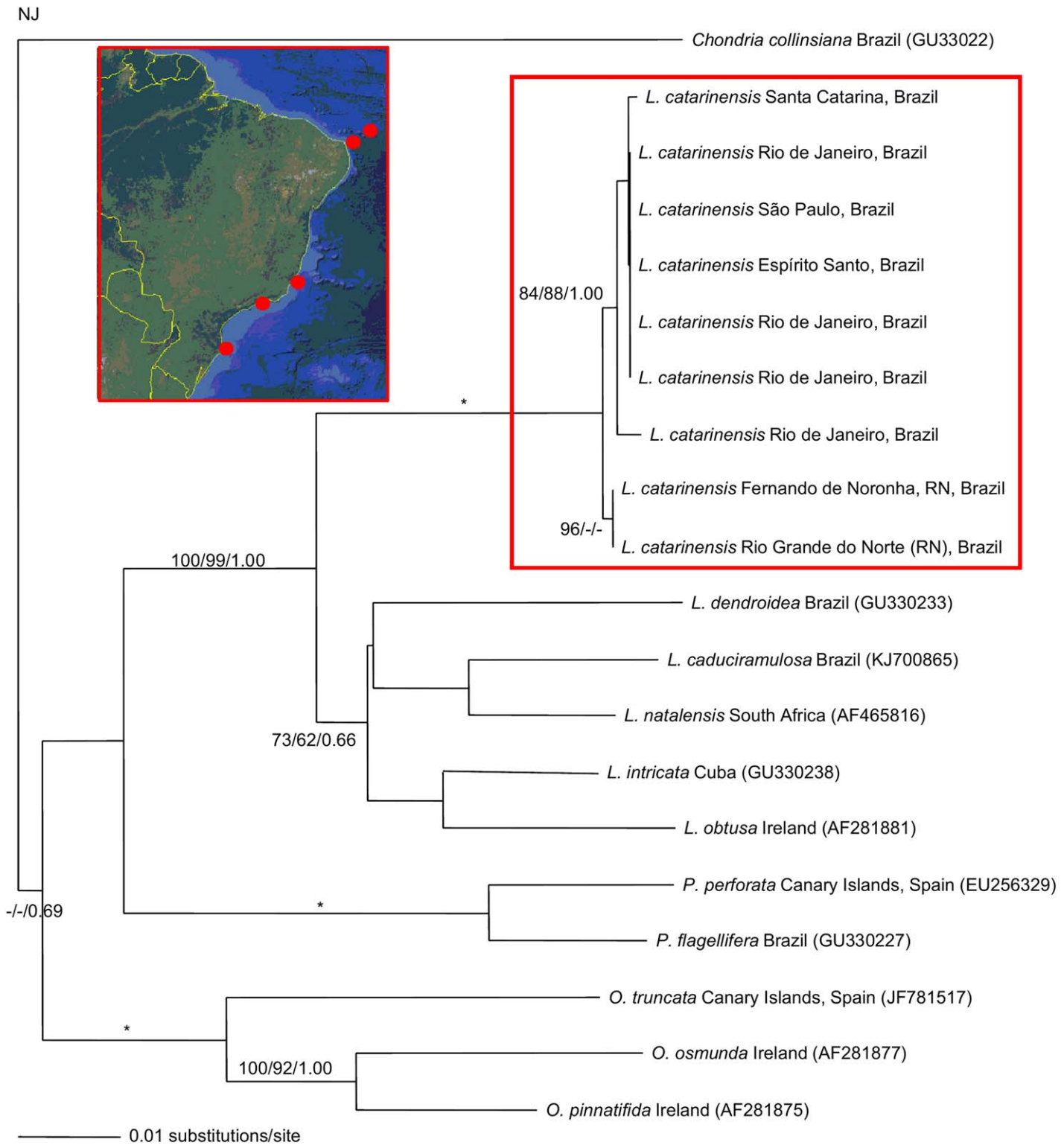

Fig. 7. Sequence the *rbc*L gene for 11 specimens of *L. catarinensis*. By adding sequences from GenBank, an alignment of 28 sequences was assembled, including *C. collinsiana* as outgroup. Based on the 1193 bp alignment, the tropical (Fernando do Noronha, Rio Grande do Norte) and warm temperate samples (Santa Catarina, São Paulo, Rio de Janeiro) exhibited 0.5% genetic divergence. [Color figure can be viewed at wileyonlinelibrary.com]

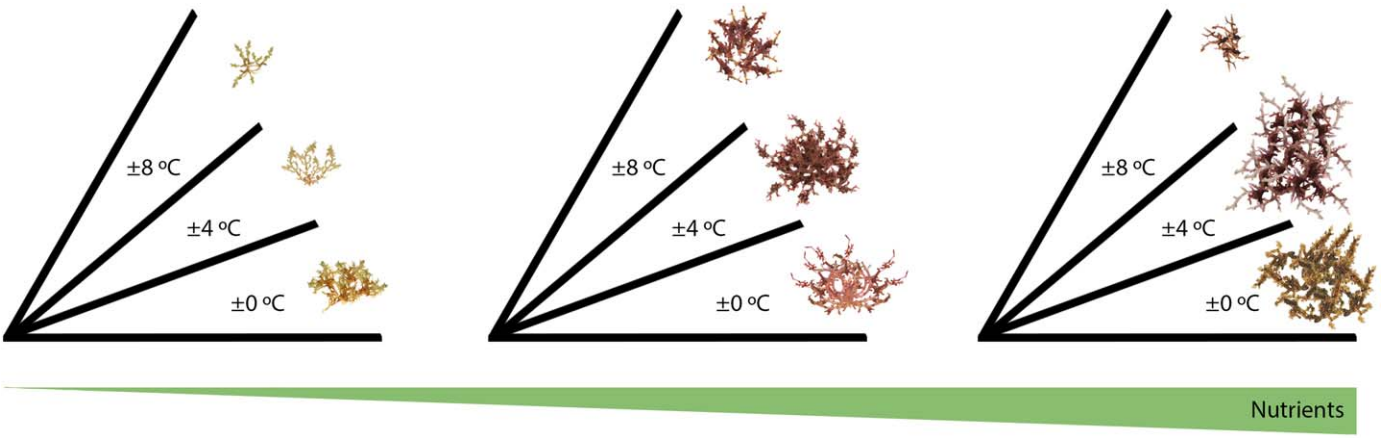

Fig. 8. Summary of the effects of increasing temperature and nutrient availability on *L. catarinensis* biomass. Photos were taken from algae after 30 d under different temperature (20°C, 24°C, and 28°C) and nutrient conditions (low, LNC; intermediate, INC and high nutrient concentration, HNC). [Color figure can be viewed at wileyonlinelibrary.com]

Environmental stresses, such as temperature increases, not only affect the activity of enzymes involved in photosynthesis, but can also produce damage to the photosynthetic apparatus through the increase in reactive oxygen species. Thus, reported seaweed responses to thermal stress include alterations in the content of photosynthetic/photoprotective pigments (Staehr and Wernberg 2009), phenolic compounds, and large changes in the expression of genes encoding antioxidant proteins and detoxifying enzymes (Collén et al. 2007) and the efficiency of the antioxidant systems (Bischof and Rautenberger 2012). In *L. catarinensis*, carotenoids, such as zeaxanthin and lutein, and phenolic compounds have been found, each of them with reported antioxidant properties (Havaux and Niyogi 1999; Cornish and Garbary 2010). These compounds, as well as the radical scavenging activity performed by DPPH, showed an increase with temperature, specifically in the algae exposed to higher nutrient availability (see Fig. 6), which indicates an increase in oxidative stress, and hence increase in antioxidants under these conditions.

In summary, this study demonstrates synergistic effects of eutrophication and climate warming on macroalgal performance in the field, as the nutrient availability can influence their response to increasing temperatures (Fig. 8). The growth of nutrient-limited algae can be negatively impacted already at moderate temperature increases, a response that agrees with the interactive effect of super-optimal temperature and nutrient limitation in seaweeds during warm summer periods of strong stratification or El Niño events (see Gerard 1997 and references therein). It suggests that large-scale declines in algal production formerly attributed to N limitation may actually be due to the interactive effects of N limitation and heat stress. On the other hand, reported community shifts that have been attributed to nutrient pollution (Scherner et al. 2012; Portugal et al. 2016) might also be related to some level with its interaction with recent warming or heatwaves. Thus, to improve the global perception about the future of benthic communities, species-specific differences in their capacity for luxury N assimilation and storage should be evaluated in relation to varying temperatures. Also, for the development of coastal management strategies and conservation policies, interactions between global and local stressors in mitigation and adaptation programs against climate change should be considered, using seaweeds like *Laurencia catarinensis*, that respond quickly to environmental changes, as a warning system for global and local ecosystem impacts (Harley et al. 2006; Lima et al. 2007).

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Conflict of Interest

None declared.

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