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THE IMPACT OF CO_2 EMISSION SCENARIOS AND NUTRIENT ENRICHMENT ON A COMMON CORAL REEF MACROALGA IS MODIFIED BY TEMPORAL EFFECTS[1]

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Future coral reefs are expected to be subject to higher pCO_2 and temperature due to anthropogenic greenhouse gas emissions. Such global stressors are often paired with local stressors thereby potentially modifying the response of organisms. Benthic macroalgae are strong competitors to corals and are assumed to do well under future conditions. The present study aimed to assess the impact of past and future CO_2 emission scenarios as well as nutrient enrichment on the growth, productivity, pigment, and tissue nutrient content of the common tropical brown alga *Chnoospora implexa*. Two experiments were conducted to assess the differential impacts of the manipulated conditions in winter and spring. *Chnoospora implexa*'s growth rate averaged over winter and spring declined with increasing pCO_2 and temperature. Furthermore, nutrient enrichment did not affect growth. Highest growth was observed under spring pre-industrial (PI) conditions, while slightly reduced growth was observed under winter A1FI ("business-as-usual") scenarios. Productivity was not a good proxy for growth, as net O_2 flux increased under A1FI conditions. Nutrient enrichment, whilst not affecting growth, led to luxury nutrient uptake that was greater in winter than in spring. The findings suggest that in contrast with previous work, *C. implexa* is not likely to show enhanced growth under future conditions in isolation or in conjunction with nutrient enrichment. Instead, the results suggest that greatest growth rates for this species appear to be a feature of the PI past, with A1FI winter conditions leading to potential decreases in the abundance of this species from present day levels.

***Key index words: Chnoospora implexa*; coral reef; macroalgae; nutrient enrichment; ocean acidification; season; temperature**

***Abbreviations*: CA, carbonic anhydrase; CCM, carbon concentrating mechanisms; PD, present day; PI, pre-industrial; RC, reaction centers; NPQ, nonphotochemical quenching; SW, seawater**

Macroalgae are an integral part of coral reef ecosystems, providing shelter and substratum for many organisms, and food for herbivorous fish and invertebrates (Diaz-Pulido et al. 2007). However, increases in macro-algal production or growth, and biomass accumulation have the potential to destabilize these ecosystems (Nyström et al. 2000) as their ability to compete for space through shading, abrasion, and the release of secondary metabolites may be enhanced (McCook et al. 2001, Smith et al. 2006). Increases in seawater (SW) pCO_2 associated with ocean acidification, and increases in eutrophication have both been identified as possible reasons for increased macroalgal productivity and growth (Done 1992, Hoegh-Guldberg et al. 2007, Hughes et al. 2007, 2010). However, algae belong to several different phyla and differ in many aspects of their anatomy and physiology, therefore, species specific approaches to algal ecology and eco-physiology are increasingly important for establishing the potential outlook for macroalgae on coral reefs (Schaffelke 1999, McCook et al. 2001, Jompa and McCook 2003, Bender et al. 2012, Cornwall et al. 2012).

Nutrients associated with eutrophication, especially nitrogen and phosphorus, are introduced to the Great Barrier Reef mainly by rivers and rain (Furnas 2003). Eutrophication, often experimentally simulated as daily/weekly pulses or as a single nutrient pulse, has been shown to increase macroalgal growth in some but not all algae (e.g., Lapointe 1987, Littler et al. 1991). In some algae, nutrients are incorpo-

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rated, without stimulating either carbon fixation or growth (Gerloff and Krombholz 1966, Schaffelke 1999, Dailer et al. 2012), but with potential implications for palatability (Chan et al. 2012). Often, initial increases in production or growth only occur under typical present-day nutrient concentrations. Kleypas et al. (1999) found that nutrient levels occur between 0–3.34 μM for NO_3 and 0–0.54 μM for PO_4^{2-} for coral reefs worldwide. Others have shown that algal growth stagnates or decreases when concentrations exceed 3.5 μM NH_4^+ and 0.35 μM PO_4^{2-} (Schaffelke and Klumpp 1998a, Dailer et al. 2012, Reef et al. 2012). Larger scale in situ experiments have shown mixed responses for biomass accumulation and productivity in response to nutrient enrichment (e.g., Larkum and Koop 1997, Miller et al. 1999, Koop et al. 2001, Smith et al. 2001), highlighting the complexity of the problem of nutrient enrichment and its ecological and physiological interactions.

Increases in atmospheric pCO_2 increase (i) global temperature, due to the greenhouse effect of CO_2 (IPCC 2007) and (ii) ocean acidification, as atmospheric CO_2 equilibrates into the oceans. CO_2 entering the oceans increases dissolved inorganic carbon, but due to the decrease in pH, CO_2 and CO_3^{2-} concentrations show the greatest percent change amongst the different carbon species with CO_2 increasing and CO_3^{2-} decreasing (Zeebe and Wolf-Gladrow 2001). Increasing ocean pCO_2 has the potential to stimulate photosynthesis by providing more substrate to Ribulose-1,5-bisphosphate carboxylase oxygenase (RUBISCO), the enzyme that fixes CO_2 into organic carbon (Beardall et al. 1998).

Brown algae, inclusive of *Chnoospora implexa* J.Agardh, most likely employ carbon concentrating mechanisms (CCM) involving either direct HCO_3^- uptake, or uptake of CO_2 following conversion from HCO_3^- by an external carbonic anhydrase (CA), to ultimately increase CO_2 concentration at the site of fixation (Surif and Raven 1989, Maberly 1990, Badger et al. 1998, Axelsson et al. 2000, Raven and Hurd 2012). The form of RUBISCO present in brown algae (type 1D) also shows a relatively high selectivity factor for CO_2 over O_2 (Raven 1997). Both CCM and type 1D RUBISCO should therefore ensure that carbon fixation is sustained at relatively high levels through RUBISCO carboxylase activity, even within an ocean deplete of CO_2. Despite this, photorespiration is still active (Larkum et al. 2004). Increasing ocean pCO_2 may eliminate some costs associated with the conversion of HCO_3^- to CO_2 (Cornwall et al. 2012), but is unlikely to further enrich CO_2 at RUBISCO because photoprotective mechanisms, such as photorespiration, whilst immediately costly in terms of carbon gain, are optimal for carbon gain over the long term in variable natural environments (Murchie and Niyogi 2011).

Within physiological limits, elevated temperatures increase the V_{max} of both carboxylase and oxygenase reactions of RUBISCO similarly. However, elevated temperatures also reduce RUBISCO's affinity for CO_2 while increasing its relative affinity for O_2 (Badger and Collatz 1977, Jordan and Ogren 1984, Badger et al. 2000). As a consequence, the elevation of temperature has the potential to negate or counterbalance potential changes in the rate of carbon fixation by algae residing in CO_2 enriched oceans. Outside physiologically acceptable temperature and pH ranges, cellular metabolism is negatively impacted. Often, these physiologically acceptable ranges tend to be associated with local adaptation to the long-term dynamics of a specific habitat and coral reef algae may be living relatively close to their upper thresholds (Humphrey 1975, Mathieson and Dawes 1986). Organisms of the future will have to deal with both warmer and more acidified oceans that may take them outside physiologically acceptable ranges for all or part of the year. Future scenarios based on "reduced" CO_2 emission or "business-as-usual" CO_2 emission profiles over the next decades tend to define warming as offsets from past or present temperature (IPCC 2007); likewise, it is possible to do the same for future ocean pCO_2. By jointly applying these offsets to diurnally and seasonally variable local present conditions, it becomes possible to make relatively sound prediction regarding the fate of these organisms under the different scenarios. Such predictions are needed to inform risk assessments concerning current CO_2 emission levels (Harvey et al. 2013).

The present study aimed to assess the response of *C. implexa*, a brown alga common to the GBR (Rogers 1997, Schaffelke 1999) to combined ocean warming and acidification levels. *C. implexa* is a mat-forming, corticated and relatively unpalatable alga (Jones 1968) whose main impact on corals is likely to be due to smothering of adult corals and/or inhibition of coral recruits (Birrell et al. 2008). Few herbivores appear to eat it (Jones 1968) making growth rates the most significant feature with respect to its effect on coral reef ecosystems. *C. implexa* is therefore a good representative for an algae associated with deleterious effects on reefs, irrespective of fishing impacts on herbivores. For the present study, this species was subjected to pre-industrial (PI) conditions and two future IPCC scenarios: a "reduced" CO_2 emission scenario (B1); and a "business-as-usual" CO_2 emission scenario such as A1FI (IPCC 2007). The study was conducted under ambient levels of inorganic nitrogen and phosphorus, and under elevated levels periodically associated with flood plumes. The experiment was conducted in two different seasons to account for possible temporal differences, as a first step toward understanding the potential annual response of this common macroalga to predicted changes in its local environment.

MATERIALS AND METHODS

Sample collection and experimental set-up. The aim of the present study was to assess the effect of elevated nutrients

and combined warming and acidification of SW associated with a range of CO_2 emission scenarios, on *C. implexa* over two distinct periods of time that happened to fall within spring and winter. *C. implexa* is presently abundant on the reef flat of Heron Island Research Station (HIRS, 23°26′ S 151°52′ E) in all seasons with the exception of autumn (Rogers 1997, D. Bender personal observation). The first experiment was conducted in the austral winter of 2011 (August–September, referred to as the August experiment), the second experiment was conducted in the austral spring of 2011 (November, referred to as the November experiment). An orthogonal design was used for the experiments, which allowed for the interaction between CO_2 emission scenario treatments at four levels and nutrient concentration treatments at two levels, with three replicate tanks per treatment combination and a total of 24 tanks. Temperature and pCO_2 anomalies associated with each scenario were applied as offsets to seasonally varying baseline data collected from Heron Island.

The algal thalli where collected on the reef flat and subsequently cleaned of epiphytes using forceps and soft brushes. Each thallus was attached to the bottom of the tank (glass aquarium, 35 L) using cable ties, avoiding exposure to air and shading. The tanks and their lids were covered in blue filter (LEE Filters, #725 "old steel blue") to provide a light environment similar to the shallow sandy region from where the algae were collected.

The algae were introduced into one of four scenarios by steadily increasing the ratio of scenario SW to Heron Island intake SW over 3 d. Temperature and pCO_2 concentrations in a present day (PD) or control treatment were determined from three hourly measurements observed at Harry's Bommie (23°27′ S, 151°55′ E (http://www.pmel.noaa.gov/co2/story/Heron+Island) over the same temporal period but in the previous year (2010). All other scenarios were then achieved by applying fixed offsets to PD levels, where the offsets reflect the projected anomalies for the distinct scenarios. In this way, natural diurnal and seasonal fluctuations are accommodated across treatments. The four CO_2/temperature scenarios obtained were: (i) a B1 (or RCP4.5), "reduced" CO_2 emission scenario (set-point: +217 μatm pCO_2, +1.8°C); (ii) a A1FI (or RCP8.5), "business-as-usual" CO_2 emission scenario (set-point: +681 μatm pCO_2, +4.0°C; IPCC 2007, Rogelj et al. 2012); (iii) a PI scenario (set-point: −100 μatm pCO_2, −1°C); and (iv) an August PD scenario averaging 379 μatm pCO_2, and 22.5°C, and November PD scenario averaging 434 μatm pCO_2 and 25.2°C (Table 1).

To achieve these settings, SW from the HIRS intake system was used to continuously fill the four scenario sumps (each 8,000 L), with the conditions in each sump subsequently manipulated by a computer controlled feed-back system (SCIWARE Software Solutions, Springwood, NSW, Australia). Correct temperatures were obtained by the use of industrial scale heater chillers (Rheem HWPO17-1BB; Accent Air, Liverpool, NSW, Australia), responding to temperatures measured in the experiment aquaria. pCO_2 was monitored by a pCO_2 sensor (CO_2-PRO; Pro-Oceanus Systems, Bridgewater, Nova Scotia, Canada) and adjusted using the required mix of 30% CO_2 enriched (Gas mixer, Mg100-2ME; Witten, Nordrhein-Westfalen, Germany) and CO_2 deplete air (Spherasorb Soda Lime; Mayo Healthcare, Moorebank, NSW, Australia). The scenario water was pumped from the sumps into the experimental aquaria at a flow rate of 0.8 $L \cdot min^{-1}$. Mean pCO_2 and temperatures attained for all scenarios in the distinct experimental months are provided in Table 1.

To achieve the elevated nutrient treatment a solution made from HIRS reef flat SW, NH_4Cl, and NaH_2PO_4 (Sigma-Aldrich, St. Louis, MO, USA) was prepared and kept in 25 L nutrient-carboys, from where it was pumped into three aquaria per CO_2 emission scenario. The three ambient nutrient treatment tanks per CO_2 emission scenario received HIRS reef flat SW likewise pumped from 25 L nutrient-carboys. The solutions in the elevated nutrient-carboys and control nutrient-carboys were replenished twice a day. The ammonium and phosphate concentrations aimed for in the aquaria were selected to be in the range of data reported from river plumes reaching Heron Island during heavy rain and flooding events and were set to be ~2.5 μM ammonium and 1.25 μM phosphate respectively (Devlin et al. 2001). Ammonium was chosen as it is both abundant in river plumes (Devlin et al. 2001) and also bio-available for algal metabolism. Other forms of nitrogen, such as nitrate, require conversion to ammonium prior to assimilation into amino acids, demanding additional energy (Lobban and Harrison 1994), additionally it seems that ammonium is the preferred nitrogen species (Phillips and Hurd 2004). Ambient nitrogen concentrations were consistent between winter and spring experiment and five times greater under nutrient enrichment (Table 1). Ambient phosphorus concentrations were, however, 50% less in spring than in winter, leading to differential enrichment in winter and spring of four times and 12 times, respectively, (Table 1) when concentrations were elevated to

TABLE 1. Mean CO_2 concentration, total alkalinity (A_T), temperature (T), ammonium and phosphate concentration as well as the average light intensity (mean ± SD/SE) of the experiments.

	August (winter)		November (spring)	
	CO_2 (μatm)	SD	CO_2 (μatm)	SD
PI	294	61	345	47
PD	379	49	434	69
B1	562	32	617	50
A1FI	990	175	1,014	45
	A_T ($\mu mol \cdot L^{-1}$)	SD	A_T ($\mu mol \cdot L^{-1}$)	SD
PI	2,273	8	2,278	8
PD	2,271	12	2,285	4
B1	2,273	11	2,291	6
A1FI	2,266	25	2,286	8
	T (°C)	SD	T (°C)	SD
PI	21.3	0.7	24.4	0.9
PD	22.5	1.1	25.2	1.0
B1	24.5	0.7	27.4	0.8
A1FI	26.2	0.9	28.9	1.2
	NH_4^+ ($\mu mol \cdot L^{-1}$)	SE	NH_4^+ ($\mu mol \cdot L^{-1}$)	SE
Ambient	0.49	0.03	0.52	0.02
Elevated	2.49	0.12	2.54	0.18
	PO_4^{3-} ($\mu mol \cdot L^{-1}$)	SE	PO_4^{3-} ($\mu mol \cdot L^{-1}$)	SE
Ambient	0.26	0.01	0.13	0.01
Elevated	1.00	0.06	1.60	0.07
	Light intensity (μmol photons $\cdot m^{-2} \cdot s^{-1}$)	SE	Light intensity (μmol photons $\cdot m^{-2} \cdot s^{-1}$)	SE
	320.3	5	402	7

Temperature and CO_2 concentration were measured in the sumps where the water was prepared, while the other parameters were sampled within the experimental tanks.

All parameters but A_T were measured continuously or daily.

PI, pre-industrial scenario; PD, present-day scenario.

those typically observed in flood events at site (1–1.6 μM phosphorus; Devlin et al. 2001). Nutrient concentrations in all aquaria were measured daily using a photometric approach (see Parsons et al. (1984); ammonium assay: pp. 14–17, phosphate assay: pp. 22–25). The aquarium temperature was monitored using loggers (Onset HOBO) and pH (NIST scale) was recorded in one randomly selected tank per treatment in 24 h increments (Mettler Toledo, Port Melbourne, Victoria, Australia, InPro4501VP X connected to a Aquatronica Aquarium Controller ACQ110). Samples for daytime tank alkalinity measurements were taken on September 3, 2012 and November 13, 2012. Total alkalinity was established using Gran titration method (Kline et al. 2012), see Table 1. The light intensity was recorded in air adjacent to the experimental tanks and calculated as the mean over 24 h (Table 1).

Biomass and productivity measurements. The experiments lasted 32 and 29 d in winter and spring respectively. At the beginning and at the end of each experiment, the algal thalli were weighed after drying by spinning each thallus in a salad spinner for 7 s. The growth rate was then calculated as biomass percent change per week. Oxygen flux measurements were conducted following the methodology of Crawley et al. (2010) to establish maximum daytime net productivity (P_{nmax}) and dark respiration (R_{dark}). The maximum quantum efficiency of photosystem II by fluorescence (dark-adapted F_v/F_m) of the algae was averaged over each whole algal thallus (Walz Imaging PAM, IMAG-MAX/L and MAX/K, Effeltrich, Germany). Oxygen flux and dark-adapted F_v/F_m measurements were conducted on the same five samples per tank, with measurements taken under appropriate scenario and nutrient treatments.

Pigments and tissue nutrient content. The samples were snap frozen in darkness after the oxygen flux and fluorescence measurements. Samples were then stored at −70°C prior to extracting thalli pigments once in 100% DMSO, followed by 100% acetone extractions until no visible pigmentation remained in the thalli. Pigment extractions were performed on thalli tips, with approximately the same biomass extracted per thalli ($n = 3$ per tank and $n = 9$ per nutrient and emission scenario treatment combination). The extracted samples were filtered and the pigment content established using HPLC following Dove et al. (2006) and Zapata et al. (2000) using an 0.25 M aqueous ammonium acetate solution at pH 5 as solution A. The thalli nutrient concentrations, expressed as percent algal tissue weight (Wt%), were obtained either by combustion and digestion (carbon and nitrogen) or by Inductively Coupled Plasma-Optical Emission Spectroscopy (phosphorus). All analyses were conducted by the Analytical Services Unit of the School of Agriculture and Food Science at the University of Queensland.

Statistical analyses. For all response variables, tank was used as the unit of replication, with tank values obtained from the average response of thalli held within the tank. The statistical analyses were conducted using Statistica 10 (StatSoft, Tulsa, OK, USA). Three-way ANOVAs were run on data obtained for oxygen flux, PAM fluorometry, biomass, and tissue nutrient concentration. The three factors were Time (T, 2 levels: August and November), Nutrients (N, 2 levels: ambient and elevated), and Scenario (S, 4 levels: PI, PD, B1, A1FI). Tukey's post-hoc tests were performed for significant single factor or two factor interactions. Significant three-way interactions were explored by running separate two-way ANOVAs for each experiment separately. The data were log or square root-transformed to meet the assumptions of homogeneity and normality.

RESULTS

Biomass and productivity. All response variables were affected by at least two of the factors tested.

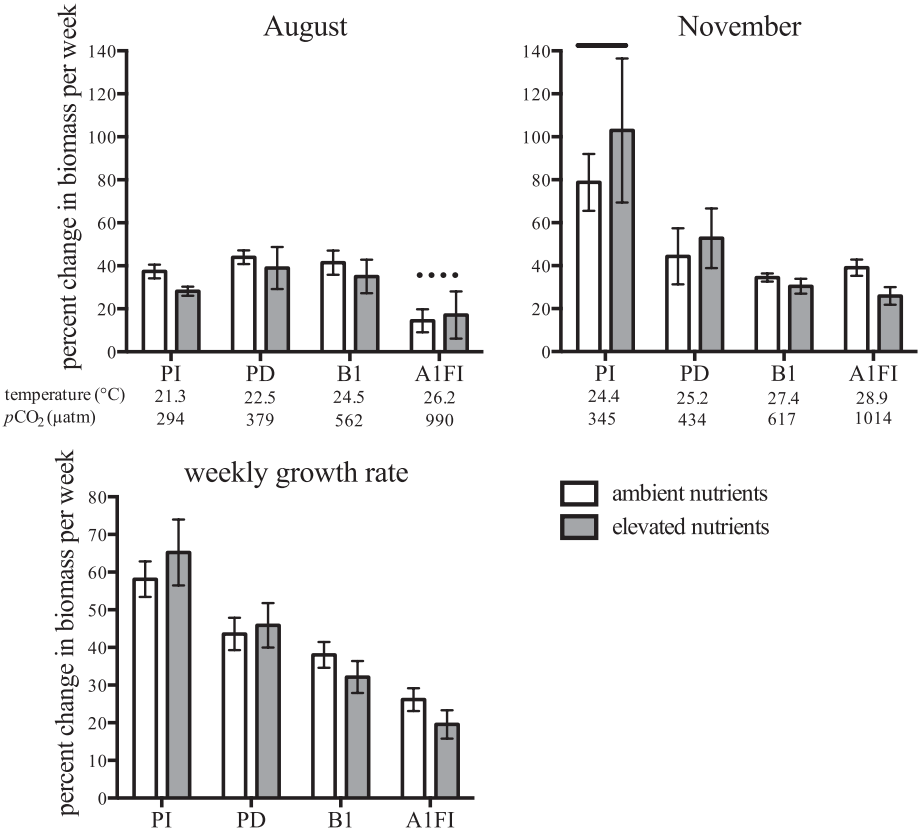

FIG. 1. Percent change in biomass per week (growth rate) of *Chnoospora implexa* during the August experiment (austral winter), the November experiment (austral spring) and the weekly growth rates averaged over both experiments (mean ± SE) under different temperature/acidity treatments as well as ambient and elevated nutrient conditions. Mean temperature and pCO_2 for each treatment are noted below graphs. Solid line over data = significantly different to all others, dotted line over data = significantly different to PD and PI scenario of the November experiment. PI, preindustrial scenario; PD, present-day scenario.

The rate of algal growth, P_{nmax}, dark-adapted F_v/F_m, and R_{dark} tended to be at their lowest values in August, and were mostly governed by the interaction of Time (August vs. November) and Scenario. Growth was enhanced by the PI treatment in November, and slightly reduced in August in response to the A1FI treatment (three-way factorial ANOVAs, $F_{(3,32)} = 6$, $P < 0.002$; post hoc: November-PI > Other, August-A1FI < November-PD, Fig. 1). The average values for temperature and pCO_2 were 26.2° ± 0.9°C (mean ± SD) and 990 ± 175, respectively, under August-A1FI treatments, and 24.4° ± 0.9°C and 345 ± 47, respectively, under November-PI treatments (Table 1). Weekly growth rates, calculated as an average between August and November experiments, decreased with increasing temperature and acidification offsets, and was lowest under A1FI treatment (+4.0°C, +681 μatm) and highest under the PI treatment (−1°C, −100 μatm).

The mean dark-adapted F_v/F_m showed a significant temporal effect and an interaction between Time × Scenario (Fig. 2; Table 2). This interaction (three-way factorial ANOVA, $F_{(3,32)} = 4$, $P = 0.02$) led to significantly elevated dark-adapted F_v/F_m in November PI grown algae compared to either August-PI or August-A1FI grown algae (Fig. 2).

P_{nmax} ($\mu mol \cdot h^{-1} \cdot g_{fw}^{-1}$) was governed by Scenario (Fig. 2, three-way factorial ANOVA, $F_{(3,32)} = 5.3$, $P = 0.004$; Table 2). P_{nmax} was greatest under A1FI, having significantly higher values than those obtained under PD and PI scenarios. The response of algal R_{dark} ($\mu mol \cdot h^{-1} \cdot g_{fw}^{-1}$) was dominated by a three-way interaction (Fig. 2, three-way factorial ANOVA, $F_{(3,32)} = 4.2$, $P = 0.01$; Table 2). As a main effect, algae grown under the November-A1FI scenario had significantly higher dark respiration rates than algae grown under November-PI and November-PD scenarios (two-way factorial ANOVA, $F_{(3,16)} = 5.2$, $P = 0.01$; Table 2). However, a weak interaction between Nutrients and Scenario led to increased respiration under A1FI ambient nutrients compared with the nutrient enriched PI and ambient nutrient PD thalli (two-way

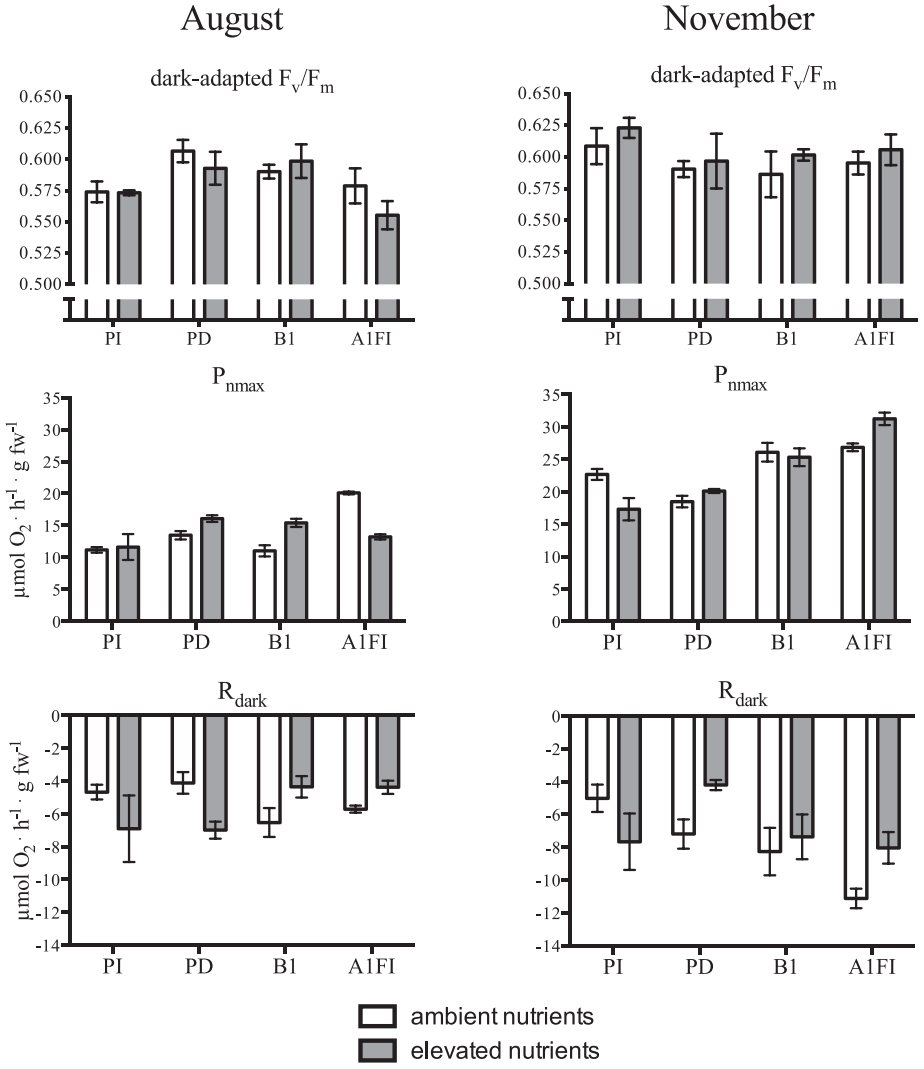

FIG. 2. Dark-adapted F_v/F_m, maximum net productivity (P_{nmax}, $\mu mol \cdot h^{-1} \cdot g_{fw}^{-1}$) and dark respiration ($R_{dark}$, $\mu mol \cdot h^{-1} \cdot g_{fw}^{-1}$) for *Chnoospora implexa* after the August and November experiments (austral winter and spring, respectively; mean ± SE). During these experiments the thalli were exposed to different temperature/acidity treatments as well as ambient and elevated nutrient conditions. The productivity measurements were conducted in the respective treatment conditions. PI, pre-industrial scenario; PD, present-day scenario.

TABLE 2. Results of the statistical analysis (two- and three-way ANOVAs) for growth, F_v/F_m, P_{nmax}, and R_{dark}.

Response variable	Source of variation	df	F	P	Conclusions/post hoc test
Growth (percent change in biomass per week)	Time (T)	1	13.0	0.0010	Nov > Aug
	Nutrients (N)	1	0.2	0.69	
	Scenario (S)	3	9.4	0.0001	PI > all, A1FI < PD = PI
	$T \times N$	1	0.4	0.53	
	$T \times S$	3	6.1	0.0022	PI_{Nov} > all, $A1FI_{Aug}$ < PD_{Nov}
	$N \times S$	3	0.2	0.88	
	$T \times N \times S$	3	0.8	0.48	
Dark-adapted F_v/F_m	Time (T)	1	8.7	0.0059	
	Nutrients (N)	1	0.2	0.67	
	Scenario (S)	3	0.9	0.44	
	$T \times N$	1	2.8	0.11	
	$T \times S$	3	4.0	0.0163	$PI_{Nov} > PI_{Aug} = A1FI_{Aug}$
	$N \times S$	3	0.5	0.71	
	$T \times N \times S$	3	0.3	0.86	
P_{nmax} ($\mu mol \cdot h^{-1} \cdot g_{fw}^{-1}$)	Time (T)	1	60.6	<0.0001	Nov > Aug
	Nutrients (N)	1	0.004	0.95	
	Scenario (S)	3	5.3	0.0042	A1FI > PD = PI
	$T \times N$	1	0.4	0.53	
	$T \times S$	3	1.8	0.16	
	$N \times S$	3	1.0	0.41	
	$T \times N \times S$	3	2.6	0.07	
R_{dark} ($\mu mol \cdot h^{-1} \cdot g_{fw}^{-1}$)	Time (T)	1	14.5	0.0006	Nov > Aug
	Nutrients (N)	1	0.4	0.52	
	Scenario (S)	3	2.1	0.12	
	$T \times N$	1	1.6	0.21	
	$T \times S$	3	3.5	0.0272	
	$N \times S$	3	4.3	0.0115	
	$T \times N \times S$	3	4.2	0.0125	See two-way ANOVAs
Aug	Nutrients (N)	1	0.2	0.66	
	Scenario (S)	3	0.1	0.94	
	N × S	3	5.1	0.0114	
Nov	Nutrients (N)	1	1.8	0.20	
	Scenario (S)	3	5.2	0.0108	A1FI > PD = PI
	N × S	3	3.5	0.0393	$PD_A = PI_E < A1FI_A$

PI, pre-industrial scenario; PD, present-day scenario; E, elevated nutrients; A, ambient nutrients; Aug, August experiment; Nov, November experiment.

Significant three-way interactions between the Time, Scenario, and Nutrients were explored with two-factorial ANOVAs for each experiment separately.

factorial ANOVA, $F_{(3,16)} = 3.5$, $P = 0.04$; Table 2). In August, an interaction between Scenario and Nutrients led to opposing effects. This interaction had a tendency to lead to reduced R_{dark} under ambient nutrients combined with PD or PI scenarios, but nutrient enrichment increased R_{dark} under these same scenarios. Under B1 and A1FI scenarios, nutrient enrichment led to lower respiration rates, while R_{dark} was increased under ambient nutrients.

Pigment and nutrient content. Pigments showed complex responses to the different treatments (Fig. 3; Table 3). Pigment responses were governed by interactions amongst the factors. Chlorophyll *a* (Chl *a*, $\mu g_{pigment} \cdot g_{fw}^{-1}$) was affected by both Scenario × Time and Nutrients × Time interactions. Algae grown in August under nutrient enrichment had significantly lower values of Chl *a* per unit biomass than those detected amongst all other treatment combinations (three-way factorial ANOVA, $F_{(3,32)} = 23.9$, $P < 0.0001$; Table 2). Also, the interaction of Time and Scenario was explained by a decrease in Chl *a* concentrations for algae grown under the August-B1 treatment compared with all algae grown in November. However, in November, algae kept under the B1 scenario contained more chlorophyll *a* than algae grown under August-A1FI conditions (three-way factorial ANOVA, $F_{(3,32)} = 3.6$, $P < 0.02$, post hoc: Aug_E < all; $B1_{Aug}$ < Nov; $A1FI_{Aug} < B1_{Nov}$).

The combined concentration of the xanthophylls antheraxanthin and violaxanthin normalized to Chl *a* ($\mu g_{pigment} \cdot mg_{Chl\,a}^{-1}$) was significantly affected by the interaction between Nutrients and Time (three-way factorial ANOVA, $F_{(3,128)} = 7.5$, $P < 0.01$). This was due to an increase in the relative concentration of these xanthophylls in August under elevated nutrients compared with all other treatment conditions. Zeaxanthin was not detected in these dark-adapted samples.

β-carotene ($\mu g_{pigment} \cdot g_{fw}^{-1}$; Fig. 3) was generally at its lowest value in August (three-way factorial ANOVA, $F_{(1,32)} = 59.6$, $P < 0.0001$). An interaction between Nutrients × Scenario was observed (three-way factorial ANOVA, $F_{(3,32)} = 3.2$, $P = 0.04$), with post hoc analysis suggesting that β-carotene concentrations were higher for algae grown under A1FI, as opposed to PD scenarios, when in the presence of ambient nutrient concentrations.

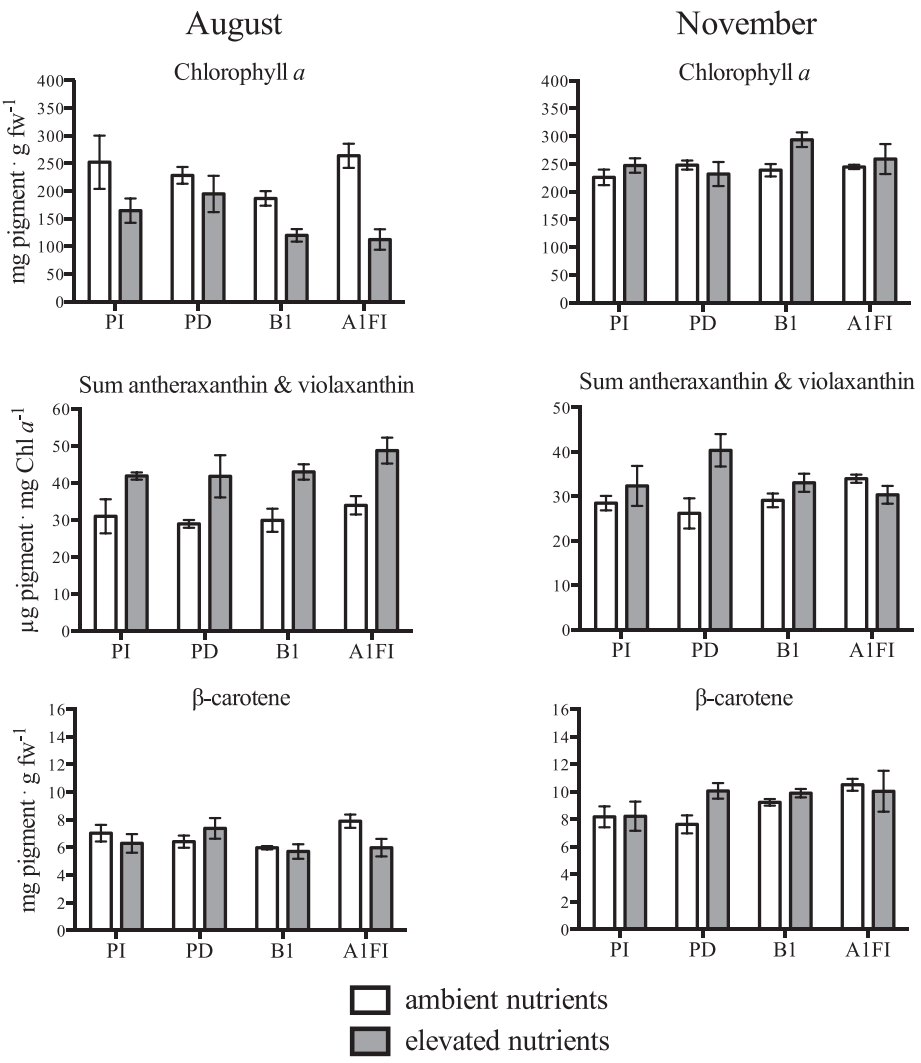

FIG. 3. Pigment concentrations extracted from the tissue of *Chnoospora implexa* the August and November experiments (austral winter and spring, respectively; mean ± SE). The thalli were exposed to different temperature/acidity treatments as well as ambient and elevated nutrient conditions. All data were normalized to biomass ($\mu g_{pigment} \cdot g_{fw}^{-1}$), except for the sum of violaxanthin and antheraxanthin, which was normalized to chlorophyll *a* ($\mu g_{pigment} \cdot mg_{Chl\,a}^{-1}$). PI, pre-industrial scenario; PD, present-day scenario.

Nutrient concentrations within the algal tissue differed significantly between treatments. Carbon tissue concentrations involved a significant three-way interaction amongst the factors (carbon: three-way factorial ANOVA, $F_{(3,32)} = 3.5$, $P = 0.03$, Fig. 4; Table 4). In both August and November, nutrient addition had a detrimental effect on carbon tissue content irrespective of Scenario (three-way factorial ANOVA, $F_{(3,32)} = 86$, $P < 0.0001$). In November, adding nutrients tended to have a detrimental effect that was more pronounced for PI and PD scenarios than for either B1 or A1FI scenario (two-way factorial ANOVA, $F_{(3,16)} = 5.4$, $P < 0.0001$).

Nitrogen and phosphorus tissue concentrations were elevated in algae grown in enriched nutrient environments (three-way factorial ANOVA, nitrogen: $F_{(1,32)} = 402$, $P < 0.0001$, phosphorus: $F_{(1,32)} = 223$, $P < 0.0001$; Fig. 4). Nitrogen, like carbon, concentrations, showed a complex three-way interaction (three-way factorial ANOVA, $F_{(3,32)} = 5.2$, $P = 0.005$). In November, a significant Nutrient × Scenario interaction (two-way factorial ANOVA, $F_{(3,16)} = 6.9$, $P = 0.004$) followed by post hoc analysis confirmed that higher nitrogen was stored in samples from nutrient enriched treatments. The results also suggested a significantly higher nitrogen content for algae grown under A1FI, as opposed to B1, when nutrients were enriched. By contrast, in August, nitrogen tissue content for A1FI-grown algae was significantly lower than under all other treatments (two-way factorial ANOVA, $F_{(3,16)} = 5.8$, $P = 0.007$).

For tissue phosphorus content, a significant Scenario × Time interaction was found (three-way factorial ANOVA, $F_{(3,32)} = 3.5$, $P = 0.03$). Algae grown in August, with the exception of the A1FI scenario, had significantly higher phosphorus content than algae grown in November. In November, PD and A1FI scenario-grown algae had lower phosphorus content, than found under PI or B1 scenarios. A significant interaction for Time × Nutrients was also detected for phosphorus tissue content. The interaction was driven by the fact that nutrient enrichment in August led to higher tissue concentrations of phosphorus than those observed under ambient

TABLE 3. Results of the statistical analysis (two- and three-way ANOVAs) for the pigments within the algal tissue.

Response variable	Source of variation	df	F	P	Conclusions/post hoc test
Chlorophyll *a* ($\mu g_{pigment} \cdot g_{fw}^{-1}$)	Time (T)	1	30.3	<0.0001	Nov > Aug
	Nutrients (N)	1	9.8	0.004	E < A
	Scenario (S)	3	0.4	0.74	
	$T \times N$	1	23.9	<0.0001	Aug_E < all
	$T \times S$	3	3.6	0.024	$B1_{Aug}$ < Nov, $A1FI_{Aug}$< $B1_{Nov}$
	$N \times S$	3	1.5	0.23	
	$T \times N \times S$	3	2.2	0.11	
Violaxanthin & antheraxanthin ($\mu g_{pigment} \cdot mg_{Chla}^{-1}$)	Time (T)	1	13.9	<0.0001	Aug > Nov
	Nutrients (N)	1	33.3	<0.0001	E > A
	Scenario (S)	3	1.0	0.41	Ns
	$T \times N$	1	7.5	0.010	Aug_E > all
	$T \times S$	3	0.9	0.44	Ns
	$N \times S$	3	1.3	0.30	Ns
	$T \times N \times S$	3	1.3	0.30	Ns
β-carotene ($\mu g_{pigment} \cdot g_{fw}^{-1}$)	Time (T)	1	59.6	<0.0001	Nov > Aug
	Nutrients (N)	1	0.1	0.80	Ns
	Scenario (S)	3	2.2	0.11	Ns
	$T \times N$	1	2.9	0.10	Ns
	$T \times S$	3	2.4	0.09	Ns
	$N \times S$	3	3.2	0.038	$A1FI_A$ > PD_A
	$T \times N \times S$	3	0.1	0.98	Ns

PI, pre-industrial scenario; PD, present-day scenario; E, elevated nutrients; A, ambient nutrients; Aug, August experiment (winter); Nov, November experiment (spring).

Significant three-way interactions between the Time, Scenario, and Nutrients were explored with two-factorial ANOVAs for each experiment separately.

nutrient doses in August or either nutrient levels in November (three-way factorial ANOVA, $F_{(3,32)} = 19$, $P < 0.0001$). Tissue phosphorus occurred at its lowest value in November under ambient nutrient doses.

DISCUSSION

In the present study, the response of the brown alga *C. implexa* to predicted changes in ocean temperature and acidification was explored. The future growth rate of *C. implexa* was found to be either unchanged, or significantly reduced from present, depending on whether the experiment was performed in the spring month of November or in the winter month of August. Significantly, the results further suggested that optimal growth conditions for this mat-forming alga occurred in the PI past, countering suggestions that algae will "bloom" in the future (e.g., Hoegh-Guldberg et al. 2007, Hughes et al. 2010). Therefore, it seems that not all macroalgal species have similar responses to ocean acidification and warming.

Other studies have investigated the effects of acidification on brown algal growth and have come to opposing conclusions. For example, Diaz-Pulido et al. (2011) found that A1FI-like acidification levels led to decreased growth in *Lobophora papenfussii*, while Israel and Hophy (2002) found no effect on *Sargassum vulgare*. It is not clear whether the different responses are species specific or associated with different, but undefined background temperatures, nutrient, and light conditions. Our data, however, suggest that limited or no differential responses between A1FI and present-day are derived because growth has already been significantly impacted since PI times. In the present study, *C. implexa*, experienced slight reductions in growth in winter under the dual impact of future A1FI warming and acidification. The data suggest, that prior to industrialization, *C. implexa* potentially exhibited much greater seasonal dynamics than it does today, potentially flourishing in November and hence at a time when its impact on coral recruitment may be at its greatest (Babcock et al. 1986). Clearly, further experiments need to be conducted at more time points and nested within seasons to gather a more accurate picture. The assumption that these algae will be relatively resilient to future conditions, however, appears based on an already shifted baseline.

In contrast with other studies on brown algae, inclusive of *C. implexa* (e.g., Larned 1998, Schaffelke and Klumpp 1998a,b, Schaffelke 1999), nutrient enrichment appeared to play no role in the elevated growth rates. This disparity may be due to the different experimental designs used: in previous studies nutrients were added as pulses and the experimental period was considerably shorter, whereas in the present study, press nutrient treatments were applied over 1 month. The mean growth rates of *C. implexa* under enriched November-PI scenarios over nonenriched treatments are slightly but not significantly elevated. This difference in growth rate between enriched- and ambient-PI scenarios is surpassed by the stimulation of growth observed in November-PI scenarios compared with all other scenarios. Potentially, it is the interaction between light, temperature and SW pCO_2 that is driving the

TABLE 4. Results of the statistical analysis (two- and three-way ANOVAs) for carbon, nitrogen, and phosphorus (Wt%) within the algal tissue.

Response variable	Source of variation	df	*F*	*P*	Conclusions/post hoc test
%Carbon (Wt%)	Time (*T*)	1	28	<0.0001	Aug < Nov
	Nutrients (*N*)	1	86	<0.0001	A > E
	Scenario (*S*)	3	13	<0.0001	A1FI = B1 > PD, A1FI > PI
	$T \times N$	1	0	0.60	
	$T \times S$	3	3	0.0385	
	$N \times S$	3	2	0.18	
	$T \times N \times S$	3	4	0.0255	See two-way ANOVAs below
Aug	Nutrients (N)	1	37	<0.0001	A > E
	Scenario (S)	3	1	0.31	
	N × S	3	2	0.24	
Nov	Nutrients (N)	1	54	<0.0001	A > E
	Scenario (S)	3	21	<0.0001	PD = PI < B1 = A1FI
	N × S	3	5	0.0094	PD_E < all, PI_E < all but $PI_A = PD_A = B1_E$
%Nitrogen (Wt%)	Time (*T*)	1	34	<0.0001	Aug > Nov
	Nutrients (*N*)	1	402	<0.0001	E > A
	Scenario (*S*)	3	3	0.0291	PI > A1FI
	$T \times N$	1	0.01	0.0017	
	$T \times S$	3	6	0.0018	
	$N \times S$	3	2	0.0867	
	$T \times N \times S$	3	5	0.0048	See two-way ANOVAs below
Aug	Nutrients (N)	1	129	<0.0001	E > A
	Scenario (S)	3	6	0.0069	A1FI < all
	N × S	3	1	0.34	
Nov	Nutrients (N)	1	299	<0.0001	E > A
	Scenario (S)	3	4	0.0332	PI > PD
	N × S	3	7	0.0035	E > A, $A1FI_E > B1_E$
%Phosphorus (Wt%)	Time (*T*)	1	182	<0.0001	Aug > Nov
	Nutrients (*N*)	1	223	<0.0001	E > A
	Scenario (*S*)	3	10	0.0001	A1FI < all
	$T \times N$	1	19	0.0001	$Aug_E > Aug_A = Nov_E > Nov_A$
	$T \times S$	3	3	0.0272	Aug > Nov but $A1FI_{Aug}$, within Nov: PD = A1FI < all
	$N \times S$	3	1	0.45	
	$T \times N \times S$	3	1	0.47	

PI, pre-industrial scenario; PD, present-day scenario; E, elevated nutrients; A, ambient nutrients; Aug, August experiment (winter); Nov, November experiment (spring).

Significant three-way interactions between the Time, Scenario, and Nutrients were explored with two-factorial ANOVAs for each experiment separately.

response, with light levels 20% greater in November than those observed in August, but the photosynthetic apparatus seemingly only able to take advantage of the greater light availability when temperature and pCO_2 are both relatively low. At present, *C. implexa* cover at the study site is highest in the month of December (Rogers 1997), but the present data also suggest that the late spring period is not necessarily also the period of greater growth under present-day conditions.

The importance of the timing of the experiment as well as the applied scenario conditions is reflected in all productivity measurements (dark-adapted F_v/F_m, P_{nmax} and R_{dark}). The dark-adapted F_v/F_m showed a similar trend as the growth data, due to its tendency to be elevated in the November-PI scenario and relatively low in the August-A1FI scenario. The opposing patterns observed for dark-adapted F_v/F_m and O_2 flux (P_{nmax} and P_{gross}, P_{gross} not shown) are unexpected. In the short term, dark-adapted F_v/F_m is typically reduced following closure of reaction centers (RC) and under conditions that lead to an imbalance between light harvested and photochemical quenching capability (Genty et al. 1989). Frequently, the response to such conditions is to increase nonphotochemical quenching (NPQ); rerouting captured light energy to heat prior to its activation of the RC and hence O_2 evolution (Müller et al. 2001). Both the closure of RCs and the activation of NPQ should reduce O_2 evolution, that is, F_v/F_m and O_2 evolution should work in concert. Decoupling of dark-adapted F_v/F_m and O_2 flux responses has previously been observed for *Palmaria palmata*, in this case constant O_2 flux gave way to decreased O_2 flux only when F_v was reduced by 40% (Hanelt and Nultsch 1995). In the present case, however, the relationship between F_v/F_m and O_2 flux are opposite for different PI- and A1FI-scenarios in different seasons and established over a 4 week period, suggesting the establishment of significant differences in photosystem dynamics between treatments. Furthermore, the disparity between fluorescent and O_2 flux measurement is not solved by reference to respiration rates (R_{dark} or Light-enhanced dark respiration, results not shown for the latter) as they follow a similar trend as P_{nmax} and P_{gross}.

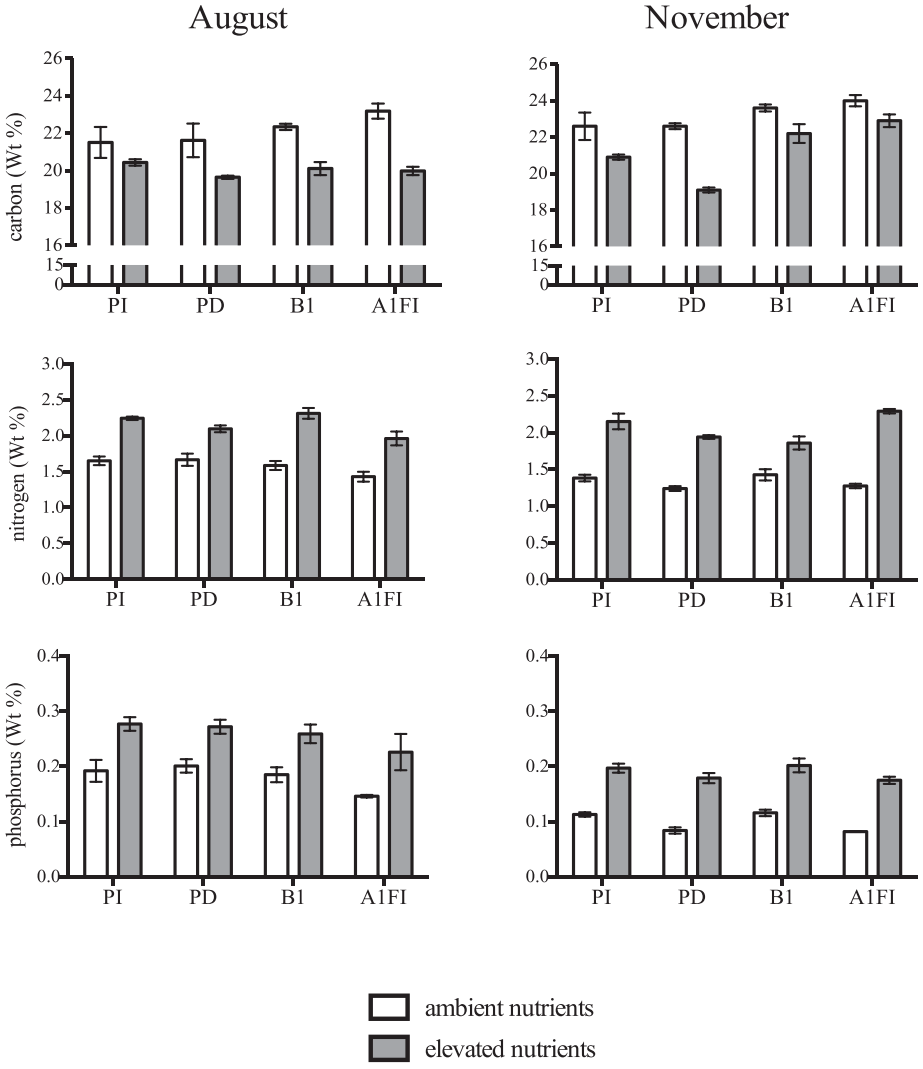

FIG. 4. Mean carbon, nitrogen, and phosphorus concentration (Wt%, normalized to dry weight) of the algal tissue after the August and November experiments (austral winter and spring, respectively; mean ± SE). The thalli were exposed to different temperature/acidity treatments as well as ambient and elevated nutrient conditions. PI, pre-industrial scenario; PD, present-day scenario.

C. implexa grew profusely under November-PI conditions, but, P_{nmax} was greatest under November-A1FI conditions. An uncoupling between biomass accumulation and growth rate has been observed in other studies (e.g., Israel et al. 1999 and Xu and Gao 2012) and in at least one species this has been attributed to changes in carbon allocation (Gordillo et al. 2001). Likewise, changes in resource allocation may have occurred in *C. implexa*, where R_{dark} tended to be greater under November-A1FI, suggesting that much of the carbon accumulated by day is respired by night, as opposed to converted into biomass for growth. Furthermore, there is a tendency for the amount of carbon per dry weight of tissue to be less in the PI and present-day treatments than in the B1 or A1FI treatments, suggesting a bias against the formation of carbon storage compounds such as laminarin and fatty acids (Michel et al. 2010, Gardner et al. 2013). This bias is especially noticeable in the contrast between nutrient-enriched versus ambient treatments. In this case, algal tissue from enriched treatments, irrespective of experimental time point or scenario, are relatively deplete in carbon and enriched in both nitrogen and phosphorus, clearly demonstrating that the enrichment was assimilated by the algae, even if it did not lead to differential growth. The reduction in tissue carbon content observed under nutrient addition may have been caused by its release as dissolved organic carbon; this has been suggested for various other tropical algal species under seasonal nutrient enrichment (Wild et al. 2008).

The nitrogen assimilated into the tissue of *C. implexa* can be stored as inorganic nitrogen, used in nitrogen rich pigments such as Chl *a*, or amino acids and proteins (Chapman and Craigie 1977, Wheeler and North 1980, Bird et al. 1982). The present results suggest that the additional nitrogen is not used for Chl *a* synthesis in *C. implexa* because (i) no increase was observed with nutrient addition and (ii) winter Chl *a* concentration decreased under nutrient addition. This leaves proteins, amino acids, and inorganic nitrogen storage as possible nutrient sinks. The relative xanthophyll pool, that does not

include nitrogen as a component, increased with nutrient enrichment, but this response was principally driven by the reduction in Chl *a* levels, rather than an increase in xanthophyll synthesis. Interestingly, neither reduction in Chl *a* nor the increase in the relative xanthophyll pool appeared to have consistent effects on either dark-adapted F_v/F_m or P_{nmax}. This possibly suggests that NPQ in the light-harvesting antennae may not be the dominant photoprotective mechanism employed by this alga (Niyogi 1999). The increase in β-carotene concentration may be related to several factors such as light intensity and quality and has also been correlated with the biosynthesis of chlorophylls (Bohne and Linden 2002).

The nutritional status of the algal tissue not only alters concentrations of inorganic and organic compounds within the organism, but also changes its nutritional value. In *C. implexa*, the addition of ammonium and phosphate mainly led to more tissue nitrogen and phosphorus in both experiments, indicating luxury nutrient uptake as opposed to investment into new tissue as observed by Schaffelke (1999). A trade-off between new tissue synthesis (growth) versus nutrient enrichment of current tissue was observed between November and August experiments with growth promoted in November and tissue enrichment promoted in August. Higher nutrient content in algae has also been correlated with higher palatability for herbivores and even species with relatively low palatability have recently been shown to follow this trend (Diaz-Pulido 2003, Chan et al. 2012). Therefore, it is possible that this species may be increasingly grazed upon following nutrient enrichment and this may be more pronounced in winter than in spring. Further studies involving behavioral feeding experiments with herbivores are required to support this hypothesis.

Growth is the key response variable examined in this study on the effect of CO_2 emission scenarios and nutrient enrichment on *C. implexa*. Growth was greatest under past spring conditions, a finding that is in contrast with current predictions (Hoegh-Guldberg et al. 2007, Hughes et al. 2010). This suggests that *C. implexa* is not likely to pose an increasing threat in the future. Furthermore, nutrient enrichment led to comparatively small changes in the measured parameters and did not cause significant biomass increases. Under A1FI conditions, winter growth rates were further reduced from PD and B1 scenarios, suggesting further reductions to the threat posed to reefs by this alga. Clearly, other coral competitors may fill the void, either other algae such as cyanobacteria (Paerl and Huisman 2009, Diaz-Pulido et al. 2011), or potentially other organisms such as soft corals, or sponges, inclusive of bioeroding sponges, that may have even greater negative impacts on aspects of reefs such as their carbonate balance (Nyström et al. 2008, Wisshak et al. 2012, Gabay et al. 2013, Fang et al. 2013). The assumed persistence of macroalgae as a group and their inferred superiority to cope with future conditions is not universal, and needs to be reassessed relative to other competitor groups to more reliably predict the fate of future reefs.

The present results provide insights into the way *C. implexa* may be affected by future changes, whilst highlighting the importance of temporal effects. In light of this study, it is important to expand the scope of future studies to include all seasons, as impacts and interactions are likely to vary throughout the year. Interactions amongst environmental factors appear to dominate the response of this alga, highlighting the necessity to investigate the impact of environmental factors in conjunction, rather than in an isolated fashion, especially if our aim is to gain insight into the future fate of coral reefs (Harvey et al. 2013).

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