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Eutrophication and warming-driven green tides (*Ulva rigida*) are predicted to increase under future climate change scenarios



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ABSTRACT

The incidence and severity of extraordinary macroalgae blooms (green tides) are increasing. Here, climate change (ocean warming and acidification) impacts on life history and biochemical responses of a causative green tide species, *Ulva rigida*, were investigated under combinations of pH (7.95, 7.55, corresponding to lower and higher pCO_2), temperature (14, 18 °C) and nitrate availability (6 and 150 µmol L⁻¹). The higher temperature accelerated the onset and magnitude of gamete settlement. Any two factor combination promoted germination and accelerated growth in young plants. The higher temperature increased reproduction, which increased further in combination with elevated pCO_2 or nitrate. Reproductive success was highest (64.4 ± 5.1%) when the upper limits of all three variables were combined. Biochemically, more protein and lipid but less carbohydrate were synthesized under higher temperature and nitrate conditions. These results suggest that climate change may cause more severe green tides, particularly when eutrophication cannot be effectively controlled.

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1. Introduction

Due largely to fossil fuels burning and land use change, the atmospheric concentration of carbon dioxide (CO_2) has increased by over 40% since 1750 and currently exceeds 400 ppm, a rate of increase unprecedented within the last 800,000 years (Gattuso et al., 2015). These emissions are driving global climate change, particularly by increasing mean global temperatures and reducing the pH of seawater. More than 90% of the thermal energy accumulated between 1971 and 2010 was absorbed by the oceans, with surface waters (upper 75 m) warming at the greatest rate (increasing between 0.09 and 0.13 °C per decade over the period 1971 to 2010; IPCC, 2013). The global mean sea surface temperatures for the months of February and August are projected to increase by 1.9 °C by the end of the 21st century. The maximum warming of around 4 °C is predicted for high latitudes of the northern hemisphere in summer (Bartsch et al., 2012).

Aside from thermal storage, the oceans are also major sinks for CO₂. When CO₂ dissolves in seawater it forms carbonic acid which decreases pH - a process termed ocean acidification. The mean surface ocean pH has already decreased by 0.1 units since the beginning of the industrial era, corresponding to a 26% increase in hydrogen ion concentration (IPCC, 2013). It is predicted that by 2100 the average surface ocean pH may decrease by 0.5 units below pre-industrial levels if CO_2 emissions continue at current trajectories (Raven et al., 2005). Seawater at high latitudes is expected to experience more serious acidification since more CO_2 can dissolve in cold waters compared to tropical regions (McNeil and Matear, 2008; Roleda et al., 2012). Coastal waters are also more susceptible to acidification than the pelagic ocean due to eutrophication processes as bacterial respiration of algae biomass further depresses seawater pH (Cai et al., 2011).

Anthropogenic eutrophication driven by increased urbanization and use of the coastal zone, as well as rising fertilizer use, has led to accelerated nutrient inputs to coastal waters (Carpenter et al., 1998; Smith et al., 1999). Eutrophication poses a growing threat for many coastal ecosystems (Bricker et al., 2008). One consequence of eutrophication is the promotion of green tide events – extraordinary blooms of macroalgae biomass. Green tides are of growing global concern due to their substantial ecological and economic impacts (Smetacek and Zingone, 2013); for example, the cost of maintaining an algae-free sea area near Qingdao for the 2008 Beijing Olympics sailing competition exceeded US\$100 million (Wang et al., 2009). Curiously, *Ulva* is the dominant genus contributing the majority of green tide events (Fletcher, 1996).

The environmental changes caused by human activities would pose an effect on the physiological and biochemical traits of *Ulva*, an ecologically and economically important genus. However, little has been studied on the physiological traits and chemical composition of *Ulva* in the

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context of the effects of ocean acidification, warming and eutrophication but some indications can be obtained from the effects of higher CO₂, temperature and nitrate levels.

Higher temperatures can usually stimulate the physiological performances of Ulva. For instance, the number of settled zoospores in U. intestinalis increased with temperature with the maximum at 23 °C (Christie and Shaw, 1968). Likewise, the bound zoospores of U. compressa increased from ~150 cells mm^{-2} to ~450 cells mm^{-2} when the temperature rose from 5 °C to 25 °C (Callow et al., 1997). The germination rate of *U. fasciata* was also enhanced by higher temperature, with the highest germination rate (78.53 \pm 10.05%) at 25 °C (Mantri et al., 2011). In terms of growth and reproduction, the growth rate of U. *fenestrata*, collected from 6 °C seawater in Japan, was $3.349 \pm 0.398\%$ at 5 °C and 40 μ mol photons m $^{-2}$ s $^{-1}$ while it was 6.559 \pm 0.312% at 10 °C and 40 μ mol photons m⁻² s⁻¹ (Kalita and Tytlianov, 2003). The reproduction rate of U. fenestrata increased from 6.1 \pm 3.6% to 71.3 \pm 31.8% when the temperature was increased from 10 to 15 °C (Kalita and Tytlianov, 2003). Regarding biochemical composition, the content of sugars and amino acids in U. fasciata increased with the rise of temperature (from 15 to 25 °C), reaching their maximum around 25 °C (Mohsen et al., 1973). The high temperature of 25 °C decreased the total lipid of U. pertusa from 2.7-3.6% dry weight to 2.6-2.7% dry weight compared to the low temperature of 15 °C (Floreto et al., 1993).

As for most organisms studied from an ocean acidification context, the experimental outcomes vary and appear to be species-dependent. For instance, growth of Porphyra yezoensis juveniles increased with CO₂ (350 to 1600 ppm) (Gao et al., 1991) as did growth of U. prolifera (1000 ppm) following an acclimation period (Xu and Gao, 2012). On the other hand, negative effects were observed on photosynthesis in Ulva spp. (Bjork et al., 1993), as well as growth in Gracilaria tenuistipitata (García-Sánchez et al., 1994), P. leucostica (Mercado et al., 1999), P. linearis (Israel et al., 1999) and Fucus vesiculosus (Gutow et al., 2014). In addition, recent studies have demonstrated that U. rigida (Rautenberger et al., 2015) and the giant kelp Macrocystis pyrifera (Fernández et al., 2015) are insensitive to ocean acidification (~1220 μ atm pCO₂). Effects of high CO₂ levels on the settlement, germination and reproduction of Ulva have not yet been studied. In terms of biochemical composition, high CO₂ concentration (10,000 ppm) did not significantly affect total internal carbon, nitrogen or soluble carbohydrate in U. rigida but reduced soluble protein compared with the normal CO₂ level (350 ppm, Gordillo et al., 2001a, 2001b).

Nitrate is one of most important factors affecting Ulva growth. Research by Steffensen (1976) demonstrated that the addition of nitrate stimulated growth of U. lactuca with optimum levels being 43 μ mol L⁻¹. The specific growth rate of U. rigida is also positively related to dissolved inorganic nitrogen (DIN) in the water column when DIN varies from 3 to 75 μ mol L⁻¹ (Viaroli et al., 1996). The only literature reporting nutrient effects on Ulva reproduction is from Mohsen et al. (1974). Their research demonstrated that nitrogen enrichment induced rapid sporogenesis and sporulation, whereas depleted nitrogen led to zygospore formation. Higher nitrate levels commonly stimulate the synthesis of amino acids and then protein content of Ulva (Naldi and Wheeler, 1999; Msuya and Neori, 2008; Angell et al., 2014). For instance, the total amino acid content of U. ohnoi increased linearly with internal nitrogen content (r = 0.987) with a range from 2.98 g 100 g⁻¹ dry weight to 18.72 g 100 g⁻¹ dry weight (Angell et al., 2014). Nitrogen concentration in the culture medium can regulate the degree of cellular lipid accumulation (Brennan and Owende, 2010). Nitrogen limitation enhanced total lipid of U. rigida from 64 mg g^{-1} dry weight to 72 mg g^{-1} dry weight at ambient CO₂ concentration (350 ppm) (Gordillo et al., 2001a). No reports on high nitrate levels affecting settlement and germination of Ulva have been found.

The findings of previous studies are helpful in understanding how ocean acidification, warming, or eutrophication alone affects the physiological or biochemical traits of seaweeds. However, to the best of our knowledge, none of the previous studies have examined the outcomes of the interactive effects of multiple climate change variables on life history and biochemical traits of *Ulva*. Neither ocean warming nor acidification are proceeding in isolation, rather there are also concurrent changes in nutrient levels. Given the ecological and socio-economic impacts of *Ulva* green tides we examined the interactive effects of ocean warming, acidification, and eutrophication on a selection of life history (gamete settlement, germination, growth, and reproduction) and key biochemical traits of *U. rigida*, a major green tide species (Fletcher, 1996). This research was undertaken with a view to predicting the future responses of green tides to ongoing global climate change.

2. Materials and methods

2.1. Sample identification, preparation and culture conditions

Ulva plants of 50–60 mm in length were collected from the low intertidal zone of Cullercoats Bay, Tyne and Wear, UK (55.03° N, 1.43° W) after a spring tide in May 2014. The fronds were placed in zip-lock plastic bags and transported to the laboratory within 1 h where they were gently rinsed in filtered (1 µm) natural seawater to remove any sediment, epiphytes and small grazers. The *Ulva* species used in this study was identified by DNA barcoding at the Institute of Oceanology, Chinese Academy of Sciences. It was found that the sequence, excluding the primers at both ends, fully matched (100%) to *U. rigida* SSB00102 isolated from Skara Brae, Orkney, Scotland (Gao, 2016).

To determine whether life stage affects responses to the experimental factors assayed here, both adults and gametes of U. rigida were used. Seven hundred and twenty adult vegetative U. rigida fronds of 50-60 mm in length were haphazardly assigned to 24 identical Perspex tanks, each containing 10 L of natural seawater. Natural seawater was collected from the Blue Reef Aquarium®, Tynemouth, Tyne and Wear, UK (55.03°N, 1.43°W), very close to the U. rigida collection site. Gametes were obtained from fertile plants collected during a spring tide in June 2014 and treated as above. Gametes were released into suspension after exposing the fronds to light (fluorescent tubes, 80 µmol photons $m^{-2} s^{-1}$) for 1–2 h. The gamete suspension was transferred to a 500 mL beaker and the cells were concentrated by phototaxis to a point source light. They were then collected by pipette and transferred to filtered seawater. This step, which selected for healthy gametes and excluded most non-phototactic organisms, was repeated three times. Gametes were checked microscopically for the presence of two flagella and were positively identified as gametes given their positive phototaxis. Afterwards, collected gametes were used for settlement, germination and growth experiments.

The interactive effects of ocean acidification and warming under nitrate-limited and replete conditions were investigated using a fully crossed factorial design. The mature plants and gametes were cultured separately under the same treatment conditions in combinations of two pH (7.95, 7.55; coded as low carbon, LC and high carbon, HC respectively), temperature (14, 18 °C; coded as low temperature, LT and high temperature, HT respectively) and nitrate (6, 150 μ mol L⁻¹; coded as low nitrate, LN and high nitrate, HN respectively) levels. The phosphate concentration was arbitrarily set at 50 μ mol L⁻¹ to obviate phosphorus limitation. Three replicate tanks were run for each treatment. Temperature was controlled using laboratory incubators with a photoperiod of 16 h light:8 h dark. Light intensity was 80 μ mol photons m⁻² s⁻¹. The ambient pH (7.95), nitrate concentration (6 μ mol L⁻¹) and summer average surface seawater temperature (14 °C) of the North Sea (Mathis et al., 2015) were set as the control conditions. The reduced pH and elevated temperatures used represent the predicted levels by the year 2100 (Baede et al., 2001). The available nitrate concentration was maintained daily by addition of NaNO₃ following measurement by a rapid spectrophotometer method (Collos et al., 1999). Seawater was renewed every three days.

2.2. Carbonate chemistry

Culture pH was maintained using a computer-controlled pH system (Aqua-medicTM; Loveland, Colorado) that dosed CO₂ into an air stream using solenoid valves. Temperature and salinity were recorded daily. Total alkalinity was determined by titrations prior to water changes. Carbonate system parameters, which were not directly measured, were calculated using CO2SYS (Pierrot et al., 2006) using constants from Mehrbach et al. (1973), Dickson and Millero (1987) and the KSO dissociation constant from Dickson (1990).

2.3. Gamete settlement

Gamete density was determined by haemocytometer counts following immobilisation with 4% formaldehyde. Gametes were added to 85 mm diameter Petri dishes containing 20 mL of treatment-adjusted filtered seawater to a final concentration of 1.2×10^6 mL⁻¹. The dishes were incubated in the dark to promote settlement. Settled gametes were counted at 4, 8, 16, 24, 32, and 40 h after washing with seawater to remove unattached spores. The variation in pH during the 40 h dark incubation was <0.05 units, along with a roughly 1% nitrate fluctuation. The number of settled spores was counted at ×400 magnification using an eyepiece counting graticule. The mean of nine counts ± SD was calculated.

2.4. Germination and growth of young U. rigida

After settlement, the Petri dishes were placed into 5-L tanks containing pH-, temperature- and nitrate-adjusted seawater. The regeneration rate was determined by counting the number of cells that had divided versus those that had not after two, four, six, and eight incubation days from three randomly selected microscopic fields of view per dish. Once the germlings had attained a length of 5 mm they were detached from the dishes and dispersed directly into the treatment tanks where they were cultured for 60 days.

2.5. Growth quantification

The weight of a single gamete, which served as the initial weight, was determined from the dry weight of a known number (2×10^8) of gametes. Changes in biomass (fresh weight) of adult *Ulva* were recorded every four days for a period of 12 days. The fresh weight was determined after removing excess water by gently blotting the thalli with tissue paper. Specific growth rates (SGR) of young and adult *Ulva* were calculated by the formula: SGR (%) = $[\ln(W_2 / W_1)] / t \times 100$, where W_2 is the final weight, W_1 is the initial weight and t is the number of culture days. The dry weight of individual plants was measured after 60 incubation days.

2.6. Determination of reproduction

Reproductive thalli were recognized by their colour, as the formation of reproductive cells in *Ulva* is accompanied by a colour change from green (vegetative state) to yellowish (reproductive state) and then to white (after gamete release). This was verified in this study by microscope observation. The reproduction rate was expressed as the ratio of reproductive thalli to all thalli in a tank.

2.7. Bulk biochemical content

Total protein was determined by the Kjeldahl method. The protein content was calculated using a nitrogen conversion factor of 5.45 based on the mean value of three species of *Ulva* (Shuuluka et al., 2013). Total nitrogen was measured by an Elementar Vario Macro Cube (Elementar, Hanau Germany).

Lipid was extracted according to the Folch gravimetric method (Folch et al., 1957) with some modifications. Briefly, 3 g of homogenized sample were extracted with 60 mL chloroform:methanol (2:1) solution. After vortexing for 20 min at room temperature, 12 mL NaCl (0.88%) was added to the aqueous phase. The samples were then centrifuged for 5 min at 1000g and the upper phase was removed. Sixty milliliters of methanol:water (1:1) was used to rinse the tubes. After repeating the centrifugation step, the upper phase was again removed and the lower phase was dried under a steady stream of nitrogen. After the chloroform had completely evaporated, the crude lipid was weighed. Results were expressed as percentage of dry weight.

Ash was determined by incinerating samples in a muffle furnace at 550 °C for 24 h. The total ash content was expressed as percent of dry weight.

Carbohydrate was estimated as the remaining unaccounted for bulk biochemicals.

2.8. Statistical analysis

Data were analyzed using SPSS v.21. Shapiro-Wilk and Levene's tests were used to check normality and homogeneity of variance, respectively. All data sets conformed to a normal distribution (Shapiro-Wilk, P > 0.05) and the variances of all samples could be considered equal (Levene's test, F < 2.378, P > 0.05) except the settlement rates at four (Levene's test, F = 2.776, P = 0.022), eight (Levene's test, F = 2.964, P = 0.010), 16 h (Levene's test, F = 2.653, P = 0.028) and the reproduction rates on days eight (Levene's test, F = 3.644, P = 0.015) and 12 (Levene's test, F = 4.755, P = 0.009). A three-way MANOVA was conducted to compare the seawater carbonate parameters at different conditions. Three-way ANOVAs were used to assess the effects of pCO_2 , temperature and nitrate on settlement, germination, growth, reproduction, and biochemical properties (protein, lipid, carbohydrate, and ash) of U. rigida considering that ANOVA is reasonably robust to violations of normal distribution and homogeneity of variance particularly when the group sizes are equal (Pallant, 2010). A confidence interval of 95% was set for all tests where data fulfilled the assumptions of normality and homogeneity and 99% for all tests where data did not fulfil the assumptions of homogeneity.

3. Results

3.1. Changes in seawater carbonate chemistry

The changes in the seawater carbonate system under the different treatments are summarized in Table 1. There were no interactive effects of nitrate, temperature, and pCO_2 on seawater carbonate parameters while temperature (MANOVA, F (6, 11) = 30.922, P < 0.001) and pCO_2 (MANOVA, F (6, 11) = 100.999, P < 0.001) had the main effects. The higher temperature enhanced dissolved inorganic carbon (DIC) concentrations by 6.5%, HCO_3^- by 6.8%, CO_3^{2-} by 28.8%, and total alkalinity by 7.8%. The decrease of 0.4 pH units lead to increases of 180.9% in pCO_2 , 7.8% in DIC, 9.7% in HCO_3^- , and 181.3% in CO_2 . Meanwhile, the lower pH decreased the concentration of CO_3^{2-} by 58.1%.

3.2. Settlement and germination

The effects of nitrate, temperature, and pCO_2 on settlement rates are presented in Fig. 1. There were no interactive effects between the factors within the first 4 h. The higher temperature significantly accelerated the onset of settlement (ANOVA, F (1, 64) = 271.572, P < 0.001) with gametes beginning to settle ($0.45 \pm 0.17\%$) within 4 h (Fig. 1). Nitrate, temperature, and pCO_2 had an interactive effect on settlement at eight, 16, and 24 h (ANOVA, F (1, 64) > 12.286, P < 0.01), suggesting that pCO_2 effects may vary at differing temperatures and nitrate levels. For instance, gametes had not settled by hour eight in the LNLTLC, LNLTHC, HNLTLC, or HNLTHC treatments, whereas settlement rates were 1.49 \pm 0.27%

Table 1

Seawater carbonate parameters of the different treatment combinations. Measurements and estimation of the parameters are described in Section 2. Data are the means \pm SD (n = 6). LTLC, lower temperature and lower *p*CO₂; LTHC, lower temperature and higher *p*CO₂; HTLC, higher temperature and lower *p*CO₂; HTHC, higher temperature and higher *p*CO₂. DIC = dissolved inorganic carbon, TA = total alkalinity.

Treatment	рН	Temperature (°C)	pCO ₂ (µatm)	DIC (µmol kg ⁻¹)	HCO ₃ (μmol kg ⁻¹)	СО ₃ ²⁻ (µmol kg ⁻¹)	CO_2 (µmol kg ⁻¹)	TA (μmol kg ⁻¹)
LNLTLC	7.95 ± 0.05	14.0 ± 0.5	669.4 ± 103.9	2073.2 ± 187.4	1902.1 ± 103.7	95.2 ± 10.2	26.1 ± 4.5	2190.5 ± 95.3
LNLTHC	7.55 ± 0.05	14.0 ± 0.5	2001.8 ± 117.0	2226.0 ± 53.6	2109.2 ± 51.6	38.9 ± 3.0	77.9 ± 5.1	2254.0 ± 55.0
LNHTLC	7.95 ± 0.05	18.0 ± 1.0	764.7 ± 57.3	2231.1 ± 121.6	2083.8 ± 107.1	120.9 ± 15.8	26.4 ± 1.2	2428.6 ± 142.9
LNHTHC	7.55 ± 0.05	18.0 ± 1.0	2073.3 ± 269.6	2361.7 ± 56.9	2238.6 ± 53.6	51.6 ± 4.0	71.5 ± 7.4	2412.7 ± 55.0
HNLTLC	7.95 ± 0.05	14.0 ± 0.5	671.3 ± 75.9	2034.5 ± 72.0	1912.4 ± 77.0	95.9 ± 11.5	26.1 ± 3.0	2202.2 ± 80.3
HNLTHC	7.55 ± 0.05	14.0 ± 0.5	1851.7 ± 224.8	2217.8 ± 59.5	2103.6 ± 55.3	41.8 ± 5.2	72.5 ± 9.5	2255.6 ± 56.4
HNHTLC	7.95 ± 0.05	18.0 ± 1.0	741.4 ± 76.4	2158.4 ± 73.5	2015.7 ± 62.1	126.1 ± 11.5	25.6 ± 2.7	2352.9 ± 92.8
HNHTHC	7.55 ± 0.05	18.0 ± 1.0	2068.7 ± 286.6	2355.2 ± 59.7	2232.2 ± 54.3	51.5 ± 4.8	71.5 ± 9.9	2406.2 ± 43.8

and 2.45 \pm 0.26% in the LNHTLC and LNHTHC treatments but 1.71 \pm 0.39% and 1.44 \pm 0.34% in the HNHTLC and HNHTHC treatments respectively (Fig. 1). Temperature and nitrate levels were the main affecters of settlement at eight, 16, 24, and 32 h (ANOVA, F (1, 64) > 13.542, P < 0.001). Settlement under the higher temperature was higher than the lower temperature at eight (1.77 \pm 0.51% versus 0.00 \pm 0.00%), 16 (2.58 \pm 0.60% versus 0.65 \pm 0.68%), and 24 (3.62 \pm 0.74% versus 1.82 \pm 0.46%) hours (Fig. 1). In contrast, settlement under higher nitrate levels was less than the lower nitrate at eight (0.79 \pm 0.84% versus 0.98 \pm 1.07%), 16 (1.09 \pm 1.15% versus 2.14 \pm 0.92%), and 24 (2.31 \pm 0.93% versus 3.13 \pm 1.11%) hours (Fig. 1). The interactive effects disappeared by hours 32 and 40 whereby temperature was the main driver of settlement (ANOVA, F (1, 64) > 43.877, P < 0.001).

Each factor enhanced germination rates in isolation (ANOVA, F (1, 64) > 8.966, P < 0.01), and any of two factors in combination contributed to a positive interactive effect (ANOVA, F (1, 64) > 4.399, P < 0.05), with a further significant increase in germination rate when all three factors acted together (ANOVA, F (1, 64) = 4.898, P < 0.05) (Fig. 2). The higher temperature, nitrate and pCO_2 treatments increased germination rates by 175.22%, 60.09%, and 15.13% respectively; however, in combination these factors increased germination by 440.35% on day two. By day four, only temperature had interactive effects with pCO_2 (ANOVA, F (1, 64) = 14.706, P < 0.001) or nitrate (ANOVA, F (1, 64) = 8.463, P = 0.005), although each single factor still increased the germination rate (ANOVA, F (1, 64) > 15.738, P < 0.001). By day six, the germination-promoting effect of the elevated pCO_2 was lost, both in isolation and in combination with the other factors. There were no significant differences in germination rates across all treatments by day eight.

3.3. Growth of young U. rigida

There were no interactive effects on the specific growth rate (daily mean value over the 60-day culture period) of young plants when all



3.4. Growth and reproduction of adult U. rigida

During the first four days, temperature and pCO_2 had interacted synergistically to increase the specific growth rate of mature plants (ANOVA, F (1, 64) = 5.565, P < 0.05). The warmer temperature contributed most to growth (increased by 80.78%; ANOVA, F (1, 64) = 257.017, P < 0.001, Fig. 4a) followed by higher nitrate (45.35%, ANOVA, F (1, 64) = 105.704, P < 0.001), then pCO_2 (19.86%; ANOVA, F (1, 64) = 25.111, P < 0.001). There were no interactive effects detected by day eight. Nitrate replaced temperature as the most effective factor, with an increase in the specific growth rate of 58.13% (ANOVA, F (1, 64) = 169.594, P < 0.001, Fig. 4b) compared with 29.74% for temperature (ANOVA, F (1, 64) = 55.749, P < 0.001, Fig. 4b). pCO_2 continued to have the smallest effect (increase of 17.53%, ANOVA, F (1, 64) =



Fig. 1. Time course of gamete settlement (mean \pm standard deviation) under different treatment combinations. LN = lower nitrate (6 µmol L⁻¹); HN = higher nitrate (150 µmol L⁻¹); LT = lower temperature (14 °C); HT = higher temperature (18 °C); LC = lower pCO₂ (pH 7.95); and HC = higher pCO₂ (pH 7.55).



Fig. 2. Percentage germination success (mean \pm standard deviation) of settled gametes with time under different treatment combinations. LN = lower nitrate (6 µmol L⁻¹); HN = higher nitrate (150 µmol L⁻¹); LT = lower temperature (14 °C); HT = higher temperature (18 °C); LC = lower pCO₂ (pH 7.95); and HC = higher pCO₂ (pH 7.55).



Fig. 3. Interactive effects of ocean acidification and warming on individual weight (a) and growth rate (b) of young *Ulva* plants (mean \pm standard deviation) under lower and higher nitrate conditions over 60-day culture. LN = lower nitrate (6 µmol L⁻¹); HN = higher nitrate (150 µmol L⁻¹); LT = lower temperature (14 °C); HT = higher temperature (18 °C); LC = lower *p*CO₂ (pH 7.95); and HC = higher *p*CO₂ (pH 7.55).

21.740, P < 0.01, Fig. 4b). By day 12, any two-factor combinations had an interactive effect (ANOVA, F (1, 64) = 13.680, P < 0.01). However, the higher temperature decreased the specific growth rate by 8.83% at LC and by 28.95% at HC (Fig. 4c).

No reproduction of *U. rigida* occurred during the first four days of culture regardless of conditions (Fig. 4d). Temperature had an interactive effect with nitrate (ANOVA, F (1, 64) = 31.500, P < 0.001) or pCO_2 (ANOVA, F (1, 64) = 25.786, P < 0.001). The higher temperature (HT) alone resulted in a 6.67% increase in reproduction by day eight (ANOVA, F (1, 64) = 283.500, P < 0.001, Fig. 4e), which was higher still in combination with elevated pCO_2 (16.67%) or the higher nitrate treatment (17.77%). This trend continued to day 12 with all factors having interactive effects (ANOVA, F (1, 64) = 29.762, P < 0.001). The highest reproductive rate (64.44 ± 5.09%) occurred in the higher nitrate, temperature, and pCO_2 treatment (HNHTHC, Fig. 4f).

3.5. Biochemical responses

The percentage protein content had a large variation when grown under simulated climate change conditions (Fig. 5a). The lowest content of 11.17 \pm 1.64% was found in the LNLTLC treatment, while the highest value (24.14 \pm 0.76%) occurred when *U. rigida* was grown at HNHTLC. No interactive effects were found. Temperature was the main factor (ANOVA, F (1, 16) = 291.977, *P* < 0.001), with the warmer treatment enhancing protein content by 49.13% (Fig. 5a). The higher nitrate treatment increased protein content by 31.06% (ANOVA, F (1, 16) = 135.916, *P* < 0.001) and elevated *p*CO₂ by 6.90% (ANOVA, F (1, 16) = 9.567, *P* < 0.01) (Fig. 5a).

Total lipid content ranged from $3.84 \pm 0.35\%$ to $6.31 \pm 0.32\%$ (Fig. 5b), with temperature, nitrate, and *p*CO₂ all interacting (ANOVA, F (1, 16) = 5.222, *P* < 0.05). Treatment HNHTHC enhanced lipid content by 75.14% compared with LNLTLC (Fig. 5b). The higher temperature enhanced lipid content under high nitrate (30.29%) or high *p*CO₂ (30.32%, ANOVA, F (1, 16) = 51.114, *P* < 0.001). The higher nitrate treatment increased lipid content under the warmer conditions (26.90%) and high *p*CO₂ (28.54%, ANOVA, F (1, 16) = 41.497, *P* < 0.001). The higher *p*CO₂ treatment increased lipid content under the high temperature (22.55%) or high nitrate conditions (23.81%, ANOVA, F (1, 16) = 29.278, *P* < 0.001).

The carbohydrate content ranged from $39.02 \pm 1.56\%$ to $51.15 \pm 0.24\%$ (Fig. 5c). The higher temperature treatment reduced carbohydrate by 10.75% (ANOVA, F (1, 16) = 61.829, P < 0.001) and the higher nitrate decreased it by 17.77% (ANOVA, F (1, 16) = 250.333, P < 0.001). pCO_2 did not affect carbohydrate content (ANOVA, F (1, 16) = 2.770, P = 0.116). The higher nitrate (ANOVA, F (1, 16) = 10.834, P < 0.05) or pCO_2 (ANOVA, F (1, 16) = 5.400, P < 0.05) alleviated the negative effects of the higher temperature on carbohydrate content. For instance, the higher temperature reduced carbohydrate by 7.60% in the low pCO_2 and 3.66% in the high pCO_2 treatments under the higher nitrate conditions; while they were 12.56% (LC) and 7.29% (HC) under the lower nitrate conditions.

Ash content ranged from 29.15 \pm 0.46% to 36.19 \pm 1.12% (Fig. 5d). The higher temperature treatments reduced the ash content by 12.4–



Fig. 4. Effects of ocean acidification and warming on growth (a, b, c) and reproduction (d, e, f) of adult *Ulva* plants (mean \pm standard deviation) under low and high nitrate conditions over 12-day culture. LN = lower nitrate (6 µmol L⁻¹); HN = higher nitrate (150 µmol L⁻¹); LT = lower temperature (14 °C); HT = higher temperature (18 °C); LC = lower *p*CO₂ (pH 7.95); and HC = higher *p*CO₂ (pH 7.55).



Fig. 5. Bulk biochemical content (mean \pm standard deviation) of *Ulva rigida* grown under different treatment combinations for 12 days. (a) protein; (b) lipid; (c) carbohydrate; (d) ash. LN = lower nitrate (6 µmol L⁻¹); HN = higher nitrate (150 µmol L⁻¹); LT = lower temperature (14 °C); HT = higher temperature (18 °C); LC = lower *p*CO₂ (pH 7.95); and HC = higher *p*CO₂ (pH 7.55).

18.6% (ANOVA, F (1, 16) = 106.570, P < 0.001). In contrast, the higher nitrate treatment increased ash by 9.9% (ANOVA, F (1, 16) = 11.883, P < 0.01). Temperature had interactive effects with and nitrate (ANOVA, F (1, 16) = 5.655, P < 0.05) or pCO_2 (ANOVA, F (1, 16) = 11.144, P < 0.01).

4. Discussion

4.1. Settlement and germination

Ulva release swarmers (either biflagellate gametes or quadriflagellate zoospores) from fertile thalli that subsequently disperse and adhere to a surface to complete their life history (Callow and Callow, 2011). In the present study, the higher temperature (18 °C) significantly enhanced U. rigida gamete settlement. Zoospore settlement of U. propagule was similarly increased by temperature (from 5 to 25 °C) (Christie and Shaw, 1968; Callow et al., 1997). Germination success also increased with temperature, although there was no significant difference after eight days. The positive effect of temperature on germination was magnified further under the higher pCO₂ during the first four days, suggesting that ocean warming and acidification will synergistically support green tide development. The higher nitrate reduced gamete settlement, particularly within the first 24 h. This may be due to toxicity at the higher nitrogen levels as it has been reported that Ulva spores are more sensitive to changes in the external nutrient environment than adult plants (Sousa et al., 2007). In contrast, the higher nitrate increased germination success. As Ulva swarmers are naked (i.e. lacking cell walls), they are vulnerable to environmental change (Callow and Callow, 2006). After settlement, a new cell wall is produced, improving the cell's ability to deal with stress.

4.2. Different responses of young and adult U. rigida

The effects of pCO_2 , temperature, and nitrate on the growth of young and adult *U. rigida* were studied for the first time. Young *U. rigida* grew much faster than adult plants irrespective of culture conditions. The growth rate of young *Ulva* in this study was higher than the 22% reported by Xu and Gao (2012) for *U. prolifera* over a 50-day period. The four hour illumination period used in the present study may account for this. An increase of 43% was reported for *U. intestinalis* over 14 days (Kim and Lee, 1996). The low adult growth was consistent with previous studies (Ale et al., 2011; Mantri et al., 2011). The growth differences may mainly be due to reproduction that leads to a loss of thallus mass through the production and release of swarmers. In this study, the young plants did not reproduce, while adults became reproductive on day eight. Reproduction is suppressed in young *Ulva* by the excretion of an extracellular sporulation inhibitor; the excretion of which decreases as the thallus matures thereby allowing vegetative cells to transform into reproductive cells (Stratmann et al., 1996).

Temperature is vital for *U. rigida* growth (Liu et al., 2013). The higher temperature enhanced growth of young and adult plants during the first eight culture days. Growth acceleration at moderately elevated temperature is common among seaweeds due to increased metabolic activity (Kim and Lee, 1996; Mantri et al., 2011). Yet the higher temperature decreased growth in adults by day 12. This is likely due to biomass loss to reproduction, particularly given that thalli cultured at the lower temperature showed no signs of reproduction. For example, 15 °C induced most sporogenesis and gametogenesis in *U. fenestrata* while 10 °C was optimal for vegetative growth (Kalita and Tytlianov, 2003). Likewise, the reproductive rhythm of *U. fenestrata* decreased from 30 to five days when temperature increased from 10 to 20 °C (Kalita and Titlyanov, 2011).

The higher pCO_2 only stimulated growth of young *U. rigida* when nitrate was replete, while adult growth was enhanced by the higher pCO_2 at both nitrate conditions. The higher nitrate supports growth through higher levels of amino acid and protein synthesis but also by triggering widespread changes in carbon metabolism gene expression (Gordillo et al., 2003). This may explain why the higher pCO_2 did not increase growth of young plants under the nitrate-limited condition but did so in adults by virtue of their internal nitrate reserves (Viaroli et al., 1996).

Nitrate played an important role in the growth of young *U. rigida*. The mass of individuals grown under the lower nitrate were only 2.8–7.7% of those cultured under the higher nitrate (Fig. 3b). The effect of nitrate on adults changed with time. Nitrate was less important than temperature at promoting growth during the first four culture days but it subsequently replaced temperature as the most important factor. These differential responses to nitrate. Gametes may not have sufficient nitrate reserves to maintain fast propagation and energy reserves might be consumed during swimming and settling; therefore low nitrate may inhibit germling growth. In contrast, adults have developed low nitrate coping strategies including exploiting internal nitrate reserves during short-term nitrate limitation (Viaroli et al., 1996); however, the effects of nitrate limitation emerge when the internal stores are depleted.

Any two factors interacted to affect growth of both young and adult plants (day 12) but in different directions. All interactive effects in young *U. rigida* were positive. Both nitrate and CO_2 are essential for plant growth, as they are the substrates of nitrogen and carbon assimilation, respectively. Based on metabolic theory, rising temperature can increase metabolic rates of seaweed within a certain range, nitrogen and carbon assimilation included (Iken, 2012). Therefore, it is unsurprising that temperature interacted with nitrate or pCO_2 . On the other hand, the higher temperature and pCO_2 decreased adult growth synergistically by day 12 – mainly due to reproduction. This is the first report as far as we are aware, of the higher pCO_2 contributing to the induction of reproduction of seaweed.

4.3. Biochemical content

Metabolic rates generally rise exponentially with temperature within a certain range (Iken, 2012), leading to higher rates for most physiological processes, including nitrate assimilation. In the present study, the higher temperature increased protein content, which is consistent with Mohsen et al. (1973) who found the optimal temperature for amino acid synthesis in *U. fasciata* was 20–25 °C. A higher *p*CO₂ commonly leads to a lower soluble protein content in higher plants (Spencer and Bowes, 1986; Van Oosten et al., 1992; Sicher et al., 1994) due to an increase of soluble carbohydrates (Van Oosten et al., 1992). Whereas the higher pCO_2 treatment did decrease the protein content of *U. rigida*, there was no enhanced carbohydrate production at the higher pCO_2 condition, which indicates that the mechanism of interaction between carbon and nitrogen assimilation might be different between seaweeds and higher plants.

Temperature affects lipid content of photosynthetic organisms (Guschina and Harwood, 2009). In the present study, the higher temperature enhanced total lipid content compared to the lower temperature except under LNLC. However, the total lipid content of U. pertusa grown at 25 °C decreased compared with that at 15 °C (Floreto et al., 1993). Apart from species differences, the opposite effects of temperature may be due to the temperature ranges used (4 °C versus 10 °C range). Mohsen et al. (1973) reported that the largest lipid yield in U. fasciata was at 20 °C, followed by 15 °C, and 25 °C. This is broadly consistent with both Floreto et al. (1993) and our data, indicating the existence of an optimal temperature for lipid biosynthesis. Fatty acid biosynthesis to a large extent depends on CO₂ assimilation (Gordillo et al., 2003). In the present study, the higher pCO_2 increased total lipid content except under the LNLT condition. A similar trend was also found for microalgae (Pratt and Johnson, 1964; Gordillo et al., 1998). Nitrogen availability can regulate cellular lipid accumulation, with nitrogen deficiency regarded as an effective approach to increase lipid content in microalgae (Roessler, 1990; Thompson, 1996; Rodolfi et al., 2009; Brennan and Owende, 2010). In regard to Ulva, nitrogen limitation enhanced the total lipid content of U. rigida (Gordillo et al., 2001a). On the other hand, nitrogen limitation did not enhance the total lipid content in U. lactuca compared with nitrogen enriched conditions (Kumari et al., 2014). Furthermore, the higher nitrate increased the total lipid content in U. rigida compared to the lower nitrate except under the LTLC condition in the present research. A possible reason for this positive effect of the higher nitrate might be due to the stage at which the U. rigida was harvested. U. rigida was harvested at the end of 12 culture days, when the higher nitrate induced more reproduction. More lipids might be required when vegetative cells transit into reproductive cells. Apart from the massive synthesis of lipid during mitosis and meiosis, swarmers may contain more lipid than vegetative cells since they are in great need of energy to support swimming and settlement. The total lipid content was 56.7% normalised to carbon in U. intestinalis spores and 84.0% in Zonaria farlowii spores (Reed et al., 1999). In addition, lipid content decreased from 176.0 to 123.5 μ g 10⁻⁷ per spore during 10 days development from spores to gametophytes of Saccharina latissima (Steinhoff et al., 2011).

Carbohydrate synthesis is commonly favoured by increasing temperature (Rosenberg and Ramus, 1982; Rotem et al., 1986; Marinho-Soriano et al., 2006). However, the higher temperature decreased carbohydrate content in the present study. As per lipids, this may be a consequence of the switch to reproduction as the photosynthetic capacity of reproductive cells is usually lower than vegetative cells (Kain and Erin, 1964). Nitrogen limitation can increase seaweed carbohydrate content (Rosenberg and Ramus, 1982; Rotem et al., 1986; Marinho-Soriano et al., 2006). This is related to the decline of protein synthesis (Mouradi-Givernaud et al., 1993; Marinho-Soriano et al., 2006) and is consistent with the present study. The effect of elevated pCO₂ on carbohydrate is species-dependant. High CO₂ (1% in air) enhanced carbohydrate content of *Porphyra leucosticte* from 5.3 mg g⁻¹ fresh weight to 15.1 mg g⁻¹ fresh weight compared with ambient CO₂ (Mercado et al., 1999). On the other hand, the same CO₂ concentration did not increase the soluble carbohydrate content of U. rigida (Gordillo et al., 2001b) - similar to the present study. The various effects of CO₂ on carbohydrate may be attributed to different strengths of the carbon-concentrating mechanisms (CCMs). Algae with robust CCMs are less sensitive to CO₂ change as photosynthesis is already saturated at ambient CO₂.

4.4. Life cycle and green tide

The higher temperature, pCO_2 and nitrate conditions induced markedly greater reproduction in *U. rigida*, likely through an overall acceleration in metabolic rate. The higher pCO₂ and nitrate may also promote CO₂ fixation and nitrate assimilation since seawater is CO₂ and nitrate limited for seaweed. Both CO₂ fixation and nitrate assimilation may supply essential materials to convert vegetative cells into reproductive cells. However, it is important to note that the enhanced formation and discharge of swarmers under increased temperature, pCO₂, and nitrate might be an *Ulva* survival strategy in response to environmental stress. To meet the demand of producing numerous swarmers, more protein and lipid were synthesized. Apart from enhanced reproduction, the higher temperature shortened settlement time. The high temperature, pCO_2 and nitrate increased germination rate, and these three factors promoted growth of young Ulva and early-stage growth of adult Ulva. Therefore, this indicates that Ulva may adapt to climate change by shortening its life cycle. Shorter generation times mean more opportunities to adapt phenotypically and genetically to climate change. This adaptive strategy may improve Ulva's competitiveness against other seaweeds as a gradual reduction in the competency of either reproduction, recruitment, or recruit survival with increasing ocean temperature was found in kelp (Wernberg et al., 2010).

The predicted effects of ocean warming, acidification, and eutrophication on settlement, germination, growth and reproduction of *U. rigida* may lead to increased green tides as high concentrations of released swarmers are a prerequisite for blooms (Zhang et al., 2011). Moreover, these swarmers may be able to propagate faster in a future ocean. Nevertheless, the present study offers a clue on how to deal with green tides, namely to carefully control nitrate levels since *Ulva* gametes are very sensitive to low nitrate. If nitrate can be limited, it would effectively inhibit germling growth.

Author contributions

The study was conceived by GSC, ASC and CR. Experiments were designed and conducted by GG, data was analyzed by GG. The manuscript was written by GG and GSC and edited by ASC and CR.

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