Gao G, Clare AS, Rose C, Caldwell GS.

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

***Marine Pollution Bulletin* (2016)**

DOI: http://dx.doi.org/10.1016/j.marpolbul.2016.10.003

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

Running head: EFFECTS OF CLIMATE VARIABLES ON GREEN TIDES

Authors: Guang Gao[1], Anthony S. Clare[1], Craig Rose[2], and Gary S. Caldwell[1]*

[1]School of Marine Science and Technology, Ridley Building, Newcastle University, Newcastle upon Tyne, NE1 7RU, England, UK

[2]Seaweed & Co. Ltd, Office 2.3 North Tyneside Business Centre, 54a Saville Street, North Tyneside, NE30 1NT, England, UK.

*Correspondence: Gary Caldwell, tel. +44 (0)191 208 6660, e-mail: gary.caldwell@ncl.ac.uk

Type of paper: Research article

Present address for Guang Gao: Marine Resources Development Institute of Jiangsu, Lianyungang, 222001, China

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 ^oC) 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 synthesised 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.

Keywords: eutrophication, germination, ocean acidification, ocean warming, reproduction, settlement

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 to 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 CO_2. When CO_2 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 & 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 & 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 context of the effects of ocean acidification, warming and eutrophication but some indications can be obtained from the effects of higher CO_2, 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 ^{o}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 ^{o}C to 25 ^{o}C (Callow *et al*., 1997). The germination rate of *U. fasciata* was also enhanced by higher temperature, with the highest germination rate (78.53 ± 10.05%) at 25 °C (Mantri *et al*., 2011). In terms of growth and reproduction, the growth rate of *U. fenestrata*, collected from 6 ^{o}C seawater in Japan, was 3.349 ± 0.398% at 5 ^{o}C and 40 μmol photons m^{-2} s^{-1} while it was 6.559 ± 0.312 at 10 ^{o}C and 40 μmol photons m^{-2} s^{-1} (Kalita & Tytlianov, 2003). The reproduction rate of *U. fenestrata* increased from 6.1 ± 3.6% to 71.3 ± 31.8% when the temperature was increased from 10 to 15 ^{o}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 15–25 °C), reaching their maximum around 25 °C (Mohsen *et al*., 1973). The high temperature of 25 ^{o}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 ^{o}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 CO_2 (350 to 1600 ppm) (Gao *et al.,* 1991) as did growth of *U. prolifera* (1000 ppm) following an acclimation period (Xu & Gao, 2012). On the other hand, negative effects 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 µatm pCO_2). Effects of high CO_2 levels on the settlement, germination and reproduction of *Ulva* have not yet been studied. In terms of biochemical composition, high CO_2 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 CO_2 level (350 ppm, Gordillo *et al*., 2001a, b).

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 µmol 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 3–75 µmol 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 & Wheeler, 1999; Msuya & 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} DW 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 & 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 CO_2 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.

Materials and methods

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 (55.03ºN, 1.43ºW) after a spring tide in May 2014. The fronds were placed in zip-lock plastic bags and transported to the laboratory within one hour 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* SSBO0102 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 (55.03ºN, 1.43ºW), 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 hours. 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 ºC; coded as low temperature, LT and high temperature, HT respectively) and nitrate (6, 150 μmol L^{-1}; coded as low nitrate, LN and high nitrate, HN respectively) levels. The phosphate concentration was arbitrarily set at 50 μmol 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 μmol photons m^{-2} s^{-1}. The ambient pH (7.95), nitrate concentration (6 μmol L^{-1}) and summer average surface seawater temperature (14 ºC) 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 $NaNO_3$ following measurement by a rapid spectrophotometer method (Collos *et al.* 1999). Seawater was renewed every three days.

Carbonate chemistry

Culture pH was maintained using a computer-controlled pH system (Aqua-medic™; Loveland, Colorado) that dosed CO_2 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 Milero (1987) and the KSO dissociation constant from Dickson (1990).

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 x 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 hours after washing with seawater to remove unattached spores. The variation in pH during the 40-hour dark incubation was less than 0.05 units, along with a roughly 1% nitrate fluctuation. The number of settled spores was counted at x400 magnification using an eyepiece counting graticule. The mean of nine counts ± SD was calculated.

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.

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.

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.

Bulk biochemcial 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 minutes at room temperature, 12 ml NaCl (0.88%) was added to the aqueous phase. The samples were then centrifuged for 5 minutes at 1,000 *g* 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 ^{o}C for 24 hours. The total ash content was expressed as percent of dry weight.

Carbohydrate was estimated by rounding up.

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 hours (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.

Results

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 whilst 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%.

Settlement and germination

The effects of nitrate, temperature, and pCO_2 on settlement rates are presented in Figure 1. There were no interactive effects between the factors within first four hours. 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 ± 0.17%) within four hours (Fig.

1). Nitrate, temperature, and pCO_2 had an interactive effect on settlement at eight, 16, and 24 hours (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 ± 0.27% and 2.45 ± 0.26% in the LNHTLC and LNHTHC treatments but 1.71 ± 0.39% and 1.44 ± 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 hours (ANOVA, F (1, 64) > 13.542, $P < 0.001$). Settlement under the higher temperature was higher than the lower temperature at eight (1.77 ± 0.51% versus 0.00 ± 0.00%), 16 (2.58 ± 0.60% versus 0.65 ± 0.68%), and 24 (3.62 ± 0.74% versus 1.82 ± 0.46%) hours (Fig. 1). In contrast, settlement under higher nitrate levels was less than the lower nitrate at eight (0.79 ± 0.84% versus 0.98 ± 1.07%), 16 (1.09 ± 1.15% versus 2.14 ± 0.92%), and 24 (2.31 ± 0.93% versus 3.13 ± 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.

Growth of young U. rigida

There were no interactive effect on the specific growth rate (daily mean value over the 60-day culture period) of young plants when all three factors were tested together; however, any two factors in combination did interact synergistically (ANOVA, $F(1, 64) > 4.395$, $P < 0.05$) to stimulate growth (Fig. 3a). For example, the specific growth rate of the LNLTLC treatment was 34.07 ± 0.67% from which the higher pCO_2 (LNLTHC) and higher temperature (LNHTLC) treatments increased growth by 0.27% and 2.14% respectively, while the higher pCO_2 and higher temperature combination (LNHTHC) increased growth rate by 2.81% (Fig. 3a). Based on the F values, nitrate had the strongest effect on growth (increasing by 14.75% compared to the lower nitrate; ANOVA, $F(1, 64) = 1989.527$, $P < 0.001$), followed temperature (5.05% increase; ANOVA, $F(1, 64) = 254.515$, $P < 0.001$), while the higher pCO_2 only promoted growth by 1.1-1.6% under the higher nitrate condition (ANOVA, $F(1, 64) = 60.766$, $P < 0.001$). The positive effects of nitrate, temperature or pCO_2 on growth translated into large differences in mass yield (Fig. 3b). For example, the mass of individual germlings was only 0.018 ± 0.007 mg under LNLTLC conditions but 2.111 ± 0.366 mg under HTHNHC, a greater than 100-fold increase over the 60-day culture period.

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) = 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 have 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).

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 ± 1.64% was found in the LNLTLC treatment while the highest value (24.14 ± 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 pCO_2 by 6.90% (ANOVA, F (1, 16) = 9.567, $P < 0.01$) (Fig. 5a).

Total lipid content ranged from 3.84 ± 0.35% to 6.31 ± 0.32% (Fig. 5b) with temperature, nitrate, and pCO_2 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 pCO_2 (30.32%, ANOVA, F (1, 16) = 51.114, $P < 0.001$). The higher nitrate treatment increased lipid content under the warmer conditions (26.90%) or high pCO_2 (28.54%, ANOVA, F (1, 16) = 41.497, $P < 0.001$). The higher pCO_2 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 ± 1.56% to 51.15 ± 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 ± 0.46% to 36.19 ± 1.12% (Fig. 5d). The higher temperature treatment reduced the ash content by 12.4-18.6% (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 either nitrate (ANOVA, F (1, 16) = 5.655, $P < 0.05$) or pCO_2 (ANOVA, F (1, 16) = 11.144, $P < 0.01$).

Discussion

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 & Callow, 2011). In the present study, the higher temperature (18 ^{o}C) significantly enhanced *U. rigida* gamete settlement. Zoospore settlement of *U. propagule* was similarly increased by temperature (from 5 to 25 ^{o}C) (Christie & 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 pCO_2 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 hours. 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 & Callow, 2006). After settlement, a new cell wall is produced, improving the cell's ability to deal with stress.

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 & 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 & 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 & Tytlianov, 2003). Likewise, the reproductive rhythm of *U. fenestrata* decreased from 30 to five days when temperature increased from 10 to 20 °C (Kalita & 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*. Masses 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 in young and adult plants may be due to different tolerances to low 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 of the higher pCO_2 contributing to the induction of reproduction of seaweed.

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 ^{o}C. A higher pCO_2 commonly leads to a lower soluble protein content in higher plants (Spencer & 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 & 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 ^{o}C decreased compared with that at15 ^{o}C (Floreto *et al.,* 1993). Apart from species differences, the opposite effects of temperature may be due to the temperature ranges used (4 ^{o}C versus 10 ^{o}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 CO_2 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 & 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 & 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* were 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 µg 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 & 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 & Erin, 1964). Nitrogen limitation can increase seaweed carbohydrate content (Rosenberg & 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 pCO_2 on carbohydrate is species-dependant. High CO_2 (1% in air) enhanced carbohydrate content of *Porphyra leucosticte* from 5.3 mg/g fresh weight to 15.1 mg/g fresh weight compared with ambient CO_2 (Mercado *et al.*, 1999). On the other hand, the same CO_2 concentration did not increase the soluble carbohydrate content of *U. rigida* (Gordillo *et al.*, 2001b) – similar to the present study. The various effects of CO_2 on carbohydrate may be attributed to different strengths of the carbon-concentrating mechanisms

(CCMs). Algae with robust CCMs are less sensitive to CO_2 change as photosynthesis is already saturated at ambient CO_2.

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 pCO_2 and nitrate may also promote CO_2 fixation and nitrate assimilation since seawater is CO_2 and nitrate limited for seaweed. Both CO_2 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, pCO_2, 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.

Acknowledgements

This research was funded by a School of Marine Science and Technology doctoral scholarship awarded to GG. We thank David Whitaker and Eleni Charzidimitriou for their technical assistance over the duration of this project. CR is Director of Seaweed & Co. Ltd which advises on the supply and quality control of seaweeds into the UK marketplace.

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

References

Ale MT, Mikkelsen JD, Meyer AS. 2011. Differential growth response of *Ulva lactuca* to ammonium and nitrate assimilation. *Journal of Applied Phycology* **23**: 345–351.

Angell AR, Mata L, Nys R, Paul NA. 2014. Variation in amino acid content and its relationship to nitrogen content and growth rate in *Ulva ohnoi* (Chlorophyta). *Journal of Phycology* **50**: 216–226.

Baede APM, Ahlonsou E, Ding Y, Schimel D, Bolin B, Pollonais S. 2001. The climate system: an overview. In: Houghton JT, ed. *Climate change 2001: the scientific basis.* Cambridge, UK: Cambridge University Press, 87–98.

Bartsch I, Wiencke C, Laepple T 2012. Global seaweed biogeography under a changing climate: the prospected effects of temperature. In: Christian Wiencke KB, ed. *Seaweed biology*: Berlin, Germany: Springer, 383–406.

Bjork M, Haglund K, Ramazanov Z, Pedersen M. 1993. Inducible Mechanisms for HCO_3^- Utilization and Repression of Photorespiration in Protoplasts and Thalli of 3 Species of *Ulva* (Chlorophyta). *Journal of Phycology* **29**: 166–173.

Brennan L, Owende P. 2010. Biofuels from microalgae—a review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews* **14**: 557–577.

Bricker SB, Longstaff B, Dennison W, Jones A, Boicourt K, Wicks C, Woerner J. 2008. Effects of nutrient enrichment in the nation's estuaries: a decade of change. *Harmful Algae* **8**: 21–32.

Cai W-J, Hu X, Huang W-J, Murrell MC, Lehrter JC, Lohrenz SE, Chou W-C, Zhai W, Hollibaugh JT, Wang Y. 2011. Acidification of subsurface coastal waters enhanced by eutrophication. *Nature Geoscience* **4**: 766–770.

Callow JA, Callow ME 2006. The *Ulva* spore adhesive system. In: Smith AMaC, J A ed. *Biological adhesives*. Berlin, Germany: Springer, 63–78.

Callow JA, Callow ME. 2011. Trends in the development of environmentally friendly fouling-resistant marine coatings. *Nature Communications* **2**: 244.

Callow ME, Callow JA, Pickett−Heaps JD, Wetherbee R. 1997. Primary adhesion of *Enteromorpha* (Chlorophyta, Ulvales) propagules: quantitative settlement studies and video microscopy. *Journal of Phycology* **33**: 938–947.

Carpenter SR, Caraco NF, Correll DL, Howarth RW, Sharpley AN, Smith VH. 1998. Nonpoint pollution of surface waters with phosphorus and nitrogen. *Ecological Applications* **8**: 559–568.

Christie AO, Shaw M. 1968. Settlement experiments with zoospores of *Enteromorpha intestinalis* (L.) Link. *British Phycological Bulletin* **3**: 529–534.

Collos Y, Mornet F, Sciandra A, Waser N, Larson A, Harrison, PJ. 1999. An optical method for the rapid measurement of micromolar concentrations of nitrate in marine phytoplankton cultures. *Journal of Applied Phycology* **11:** 179–184.

Dickson AG. 1990. Thermodynamics of the dissociation of boric acid in potassium chloride solutions from 273.15 K to 318.15 K. *Journal of Chemical and Engineering Data* **35**: 253–257.

Dickson AG, Millero FJ. 1987. A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. *Deep Sea Research Part A, Oceanographic Research Papers* **34**: 1733–1743.

Fernández PA, Roleda MY, Hurd CL. 2015. Effects of ocean acidification on the photosynthetic performance, carbonic anhydrase activity and growth of the giant kelp Macrocystis pyrifera. *Photosynthesis Research* **124:** 293–304.

Fletcher RL 1996. The occurrence of "green tides"—a review. In: Schramm W, Nienhuis NPH, eds. *Marine benthic vegetation*. Berlin, Germany: Springer, 7–43.

Floreto EAT, Hirata H, Ando S, Yamasaki S. 1993. Effects of temperature, light intensity, salinity and source of nitrogen on the growth, total lipid and fatty acid composition of *Ulva pertusa* Kjellman (Chlorophyta). *Botanica Marina* **36**: 149–158.

Folch J, Lees M, Sloane-Stanley GH. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *The Journal of Biological Chemistry* **226**: 497–509.

Gao G. 2016. Developing Systems for the Commercial Culture of *Ulva* Species in the UK. Ph. D. Thesis, Newcastle University, UK.

Gao K, Aruga Y, Asada K, Ishihara T, Akano T, Kiyohara M. 1991. Enhanced growth of the red alga *Porphyra yezoensis* Ueda in high CO_2 concentrations. *Journal of Applied Phycology* **3**: 355–362.

García-Sánchez MJ, Fernández JA, Niell X. 1994. Effect of inorganic carbon supply on the photosynthetic physiology of *Gracilaria tenuistipitata*. *Planta* **194**: 55–61.

Gattuso JP, Magnan A, Billé R, Cheung WWL, Howes EL, Joos F, Allemand D, Bopp L, Cooley SR, Eakin CM. 2015. Contrasting futures for ocean and society from different anthropogenic CO_2 emissions scenarios. *Science* **349**: aac4722.

Gordillo FJL, Figueroa FL, Niell FX. 2003. Photon-and carbon-use efficiency in *Ulva rigida* at different CO_2 and N levels. *Planta* **218**: 315–322.

Gordillo FJL, Goutx M, Figueroa FL, Niell FX. 1998. Effects of light intensity, CO_2 and nitrogen supply on lipid class composition of Dunaliella viridis. *Journal of Applied Phycology* **10**: 135–144.

Gordillo FJL, Jiménez C, Goutx M, Niell FX. 2001a. Effects of CO_2 and nitrogen supply on the biochemical composition of *Ulva rigida* with especial emphasis on lipid class analysis. *Journal of Plant Physiology* **158**: 367–373.

Gordillo FJL, Niell FX, Figueroa FL. 2001b. Non-photosynthetic enhancement of growth by high CO_2 level in the nitrophilic seaweed *Ulva rigida* C. Agardh (Chlorophyta). *Planta* **213**: 64–70.

Guschina IA, Harwood JL 2009. Algal lipids and effect of the environment on their biochemistry. In: Kainz M, Brett M, Arts M, eds. *Lipids in aquatic ecosystems*. Dordrecht, Netherland: Springer, 1–24.

Gutow L, Rahman MM, Bartl K, Saborowski R, Bartsch I, Wiencke C. 2014. Ocean acidification affects growth but not nutritional quality of the seaweed Fucus vesiculosus (Phaeophyceae, Fucales). *Journal of Experimental Marine Biology and Ecology* **453**: 84–90.

Iken K 2012. Grazers on benthic seaweeds. In: Wiencke C, Bischof K, eds. *Seaweed Biology*. Berlin, Germany: Springer, 157–175.

IPCC 2013. Stocker TF, Qin D, Plattner G-K, Tignor M, Allen SK, Boschung J, Nauels A, Xia Y, Bex V, Midgley PM, eds. *Climate change 2013: the physical science basis.Working Group I Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. New York: Cambridge Univ Press. 6–10.

Israel A, Katz S, Dubinsky Z, Merrill JE, Friedlander M. 1999. Photosynthetic inorganic carbon utilization and growth of *Porphyra linearis* (Rhodophyta). *Journal of Applied Phycology* **11**: 447–453.

Kain JM, Erin P. 1964. Aspects of the biology of *Laminaria hyperborea*. *Marine Biological Association of the UK* **44**: 415–433.

Kalita TL, Titlyanov EA. 2011. The effect of temperature on infradian rhythms of reproduction in *Ulva fenestrata* postels et ruprecht, 1840 (Chlorophyta: Ulvales). *Russian Journal of Marine Biology* **37**: 52–61.

Kalita TL, Tytlianov EA. 2003. Effect of temperature and illumination on growth and reproduction of the green alga *Ulva fenestrata*. *Russian Journal of Marine Biology* **29**: 316–322.

Kim KY, Lee IK. 1996. The germling growth of *Enteromorpha intestinalis* (Chlorophyta) in laboratory culture under different combinations of irradiance and salinity and temperature and salinity. *Phycologia* **35**: 327–331.

Kumari P, Kumar M, Reddy CRK, Jha B. 2014. Nitrate and phosphate regimes induced lipidomic and biochemical changes in the intertidal macroalga *Ulva lactuca* (Ulvophyceae, Chlorophyta). *Plant and Cell Physiology* **55**: 52–63.

Liu D, Keesing JK, He P, Wang Z, Shi Y, Wang Y. 2013. The world's largest macroalgal bloom in the Yellow Sea, China: formation and implications. *Estuarine, Coastal and Shelf Science* **129**: 2–10.

Mantri VA, Singh RP, Bijo AJ, Kumari P, Reddy CRK, Jha B. 2011. Differential response of varying salinity and temperature on zoospore induction, regeneration and daily growth rate in *Ulva fasciata* (Chlorophyta, Ulvales). *Journal of Applied Phycology* **23**: 243–250.

Marinho-Soriano E, Fonseca PC, Carneiro MAA, Moreira WSC. 2006. Seasonal variation in the chemical composition of two tropical seaweeds. *Bioresource Technology* **97**: 2402–2406.

Mathis M, Elizalde A, Mikolajewicz U, Pohlmann T. 2015. Variability patterns of the general circulation and sea water temperature in the North Sea. *Progress in Oceanography* 135**:** 91–112.

McNeil BI, Matear RJ. 2008. Southern Ocean acidification: A tipping point at 450-ppm atmospheric CO_2. *Proceedings of the National Academy of Sciences* **105**: 18860–18864.

Mehrbach C, Culberson CH, Hawley JE, Pytkowicz RM. 1973. Measurements of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. *Limnology and Oceanography* **18**: 897–907.

Mercado JM, Javier F, Gordillo L, Niell FX, Figueroa FL. 1999. Effects of different levels of CO_2 on photosynthesis and cell components of the red alga *Porphyra leucosticta*. *Journal of Applied Phycology* **11**: 455–461.

Mohsen AF, Khaleata AF, Hashem MA, Metwalli A. 1974. Effect of different nitrogen sources on growth, reproduction, amino acid, fat and sugar contents in *Ulva fasciata* Delile (Part III). *Botanica Marina* **17:** 218–222.

Mohsen AF, Nasr AH, Metwalli AM. 1973. Effect of temperature variations on growth, reproduction, amino acid synthesis, fat and sugar content in *Ulva fasciata* delile plants. *Hydrobiologia* **42**: 451–460.

Mouradi-Givernaud A, Givernaud T, Morvan H, Cosson J. 1993. Annual variations of the biochemical composition of *Gelidium latifolium* (Greville) Thuret et Bornet. *Hydrobiologia* **260**: 607–612.

Msuya FE, Neori A. 2008. Effect of water aeration and nutrient load level on biomass yield, N uptake and protein content of the seaweed *Ulva lactuca* cultured in seawater tanks. *Journal of Applied Phycology* **20**: 1021–1031.

Naldi M, Wheeler PA. 1999. Changes in nitrogen pools in *Ulva fenestrata* (Chlorophyta) and *Gracilaria pacifica* (Rhodophyta) under nitrate and ammonium enrichment. *Journal of Phycology* **35**: 70–77.

Pallant J. 2010. SPSS survival manual: a step by step guide to data analysis using IBM SPSS. Buckingham, UK: Open University Press.

Pierrot D, Lewis E, Wallace DWR. 2006. *MS excel program developed for CO_2 system calculations. ORNL/CDIAC-105a.* Oak Ridge: Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, US Department of Energy, Tennessee.

Pratt R, Johnson E. 1964. Lipid content of *Chlorella* "aerated" with a CO_2—nitrogen versus a CO_2—air mixture. *Journal of Pharmaceutical Sciences* **53**: 1135–1136.

Rautenberger R, Fernández PA, Strittmatter M, Heesch S, Cornwall CE, Hurd CL, Roleda MY. 2015. Saturating light and not increased carbon dioxide under ocean acidification drives photosynthesis and growth in *Ulva rigida* (Chlorophyta). *Ecology and Evolution* **5**: 874–888.

Raven J, Caldeira K, Elderfield H, Hoegh-Guldberg O, Liss P, Riebesell U, Shepherd J, Turley C, Watson A. 2005. *Ocean acidification due to increasing atmospheric carbon dioxide*. London: The Royal Society.

Reed DC, Brzezinski MA, Coury DA, Graham WM, Petty RL. 1999. Neutral lipids in macroalgal spores and their role in swimming. *Marine Biology* **133**: 737–744.

Rodolfi L, Chini Zittelli G, Bassi N, Padovani G, Biondi N, Bonini G, Tredici MR. 2009. Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low−cost photobioreactor. *Biotechnology and Bioengineering* **102**: 100–112.

Roessler PG. 1990. Environmental control of glycerolipid metabolism in microalgae: commercial implications and future research directions. *Journal of Phycology* **26**: 393–399.

Roleda MY, Morris JN, McGraw CM, Hurd CL. 2012. Ocean acidification and seaweed reproduction: increased CO_2 ameliorates the negative effect of lowered pH on

meiospore germination in the giant kelp *Macrocystis pyrifera* (Laminariales, Phaeophyceae). *Global Change Biology* **18**: 854–864.

Rosenberg C, Ramus J. 1982. Ecological growth strategies in the seaweeds *Gracilaria foliifera* (Rhodophyceae) and *Ulva* sp.(Chlorophyceae): soluble nitrogen and reserve carbohydrates. *Marine Biology* **66**: 251–259.

Rotem A, Roth−Bejerano N, Arad SM. 1986. Effect of controlled environmental conditions on starch and agar contents of *Gracilaria* sp. (Rhodophyceae). *Journal of Phycology* **22**: 117–121.

Shuuluka D, Bolton JJ, Anderson RJ. 2013. Protein content, amino acid composition and nitrogen-to-protein conversion factors of *Ulva rigida* and *Ulva capensis* from natural populations and *Ulva lactuca* from an aquaculture system, in South Africa. *Journal of Applied Phycology* **25**: 677–685.

Sicher RC, Kremer DF, Rodermel SR. 1994. Photosynthetic acclimation to elevated CO_2 occurs in transformed tobacco with decreased ribulose-1, 5-bisphosphate carboxylase/oxygenase content. *Plant Physiology* **104**: 409–415.

Smetacek V, Zingone A. 2013. Green and golden seaweed tides on the rise. *Nature* **504**: 84–88.

Smith VH, Tilman GD, Nekola JC. 1999. Eutrophication: impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. *Environmental Pollution* **100**: 179–196.

Sousa AI, Martins I, Lillebø AI, Flindt MR, Pardal MA. 2007. Influence of salinity, nutrients and light on the germination and growth of *Enteromorpha* sp. spores. *Journal of Experimental Marine Biology and Ecology* **341**: 142–150.

Spencer W, Bowes G. 1986. Photosynthesis and growth of water hyacinth under CO_2 enrichment. *Plant Physiology* **82**: 528–533.

Steffensen DA. 1976. The effect of nutrient enrichment and temperature on the growth in culture of *Ulva lactuca* L. Aquatic Botany 2: 337–351.

Steinhoff FS, Graeve M, Wiencke C, Wulff A, Bischof K. 2011. Lipid content and fatty acid consumption in zoospores/developing gametophytes of *Saccharina latissima* (Laminariales, Phaeophyceae) as potential precursors for secondary metabolites as phlorotannins. *Polar Biology* **34**: 1011–1018.

Stratmann J, Paputsoglu G, Oertel W. 1996. Differentiation of *Ulva mutabilis* (Chlorophyta) gametangia and gamete release are controlled by extracellular inhibitors. *Journal of Phycology* **32**: 1009–1021.

Thompson GA. 1996. Lipids and membrane function in green algae. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism* **1302**: 17–45.

Van Oosten JJ, Afif D, Dizengremel P. 1992. Long-term effects of a CO_2 enriched atmosphere on enzymes of the primary carbon metabolism of spruce trees. *Plant Physiology and Biochemistry* **30**: 541–547.

Viaroli P, Naldi M, Bondavalli C, Bencivelli S. 1996. Growth of the seaweed *Ulva rigida* C. Agardh in relation to biomass densities, internal nutrient pools and external nutrient supply in the Sacca di Goro lagoon (Northern Italy). *Hydrobiologia* **329**: 93–103.

Wang XH, Li L, Bao X, Zhao LD. 2009. Economic cost of an algae bloom cleanup in China's 2008 Olympic sailing venue. *Eos, Transactions American Geophysical Union* **90**: 238.

Wernberg T, Thomsen MS, Tuya F, Kendrick GA, Staehr PA, Toohey BD. 2010. Decreasing resilience of kelp beds along a latitudinal temperature gradient: potential implications for a warmer future. *Ecology Letters* **13**: 685–694.

Xu J, Gao K. 2012. Future CO_2-induced ocean acidification mediates the physiological performance of a green tide alga. *Plant Physiology* **160**: 1762–1769.

Zhang X, Xu D, Mao Y, Li Y, Xue S, Zou J, Lian W, Liang C, Zhuang Z, Wang Q. 2011. Settlement of vegetative fragments of *Ulva prolifera* confirmed as an important seed source for succession of a large-scale green tide bloom. *Limnology and Oceanography* **56**: 233–242.

Table 1 Seawater carbonate parameters of the different treatment combinations. Measurements and estimation of the parameters are described in Materials and Methods. Data are the means ± SD (n=6). LTLC, lower temperature and lower pCO_2; LTHC, lower temperature and higher pCO_2; HTLC, higher temperature and lower pCO_2; HTHC, higher temperature and higher pCO_2. DIC = dissolved inorganic carbon, TA = total alkalinity. Different superscript letters indicate significant differences ($P < 0.05$, by independent samples t-test).

Treatment	pH	Temperature (°C)	pCO_2 (μatm)	DIC (μmol kg^{-1})	HCO_3^- (μmol kg^{-1})	CO_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

Figure Legends

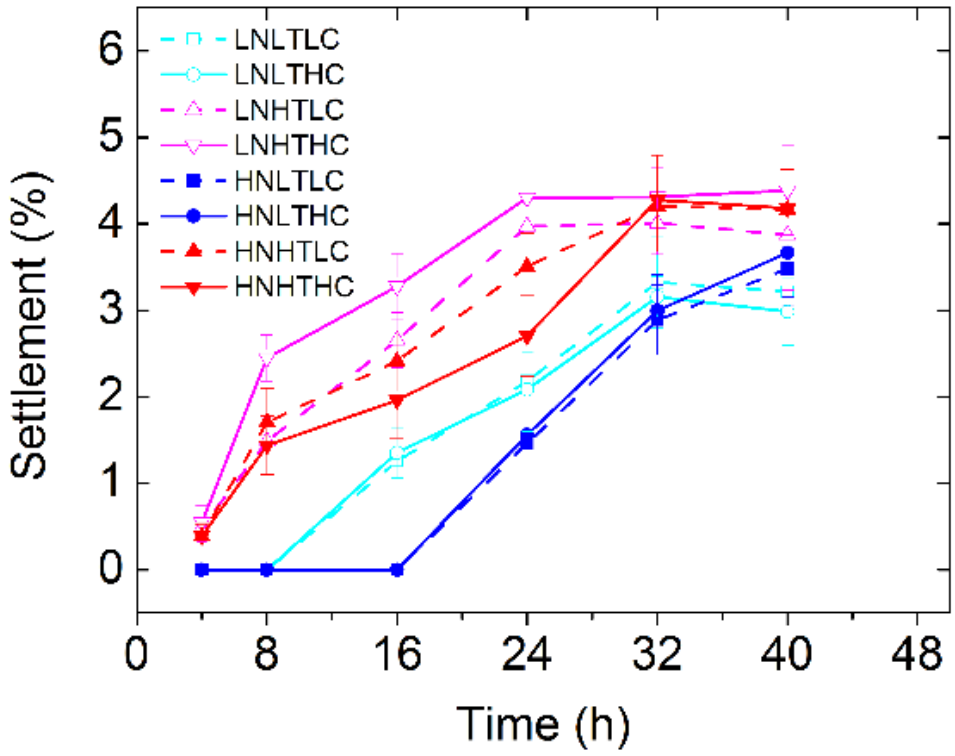
Fig. 1 Time course of gamete settlement (mean ± standard deviation) under different treatment combinations. LN = lower nitrate (6 μmol L^{-1}); HN = higher nitrate (150 μmol L^{-1}); LT = lower temperature (14 °C); HT = higher temperature (18 °C); LC = lower pCO_2 (pH 7.95); and HC = higher pCO_2 (pH 7.55).

Fig. 2 Percentage germination success (mean ± 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 pCO_2 (pH 7.95); and HC = higher pCO_2 (pH 7.55).

Fig. 3 Interactive effects of ocean acidification and warming on growth rate (a) and individual weight (b) of young *Ulva* plants (mean ± 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 pCO_2 (pH 7.95); and HC = higher pCO_2 (pH 7.55).

Fig. 4 Effects of ocean acidification and warming on growth (a, b, c) and reproduction (d, e, f) of adult *Ulva* plants (mean ± 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 pCO_2 (pH 7.95); and HC = higher pCO_2 (pH 7.55).

Fig. 5 Bulk biochemical content (mean ± 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 pCO_2 (pH 7.95); and HC = higher pCO_2 (pH 7.55).


LNLTLC
LNLTHC
LNHTLC
LNHTHC
HNLTLC
HNLTHC
HNHTLC
HNHTHC
Settlement (%)
Time (h)
0
1
2
3
4
5
6
0
8
16
24
32
40
48

25

26 Fig. 1

27

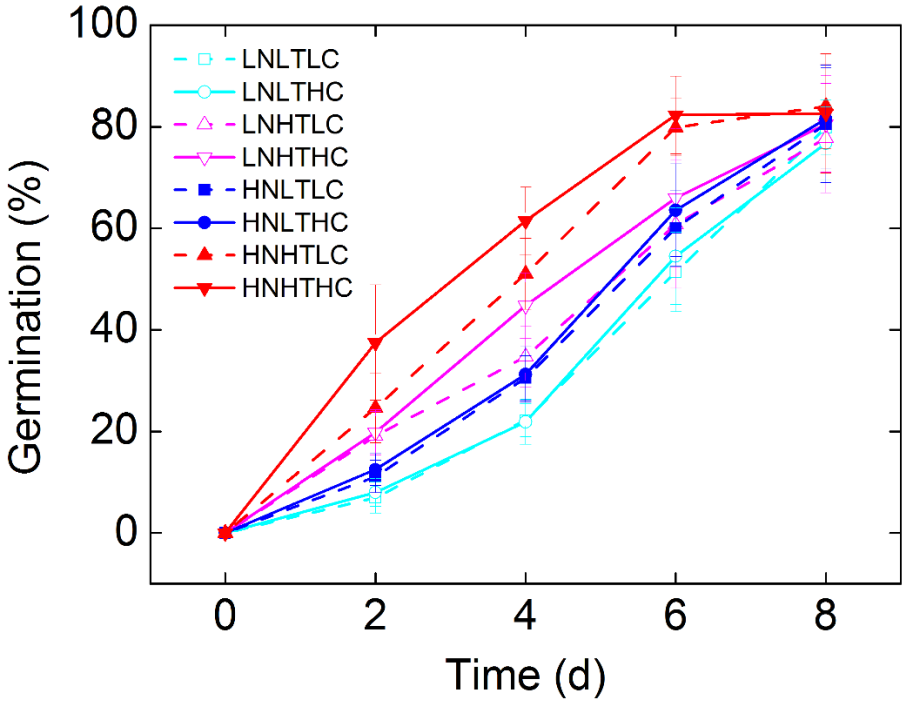
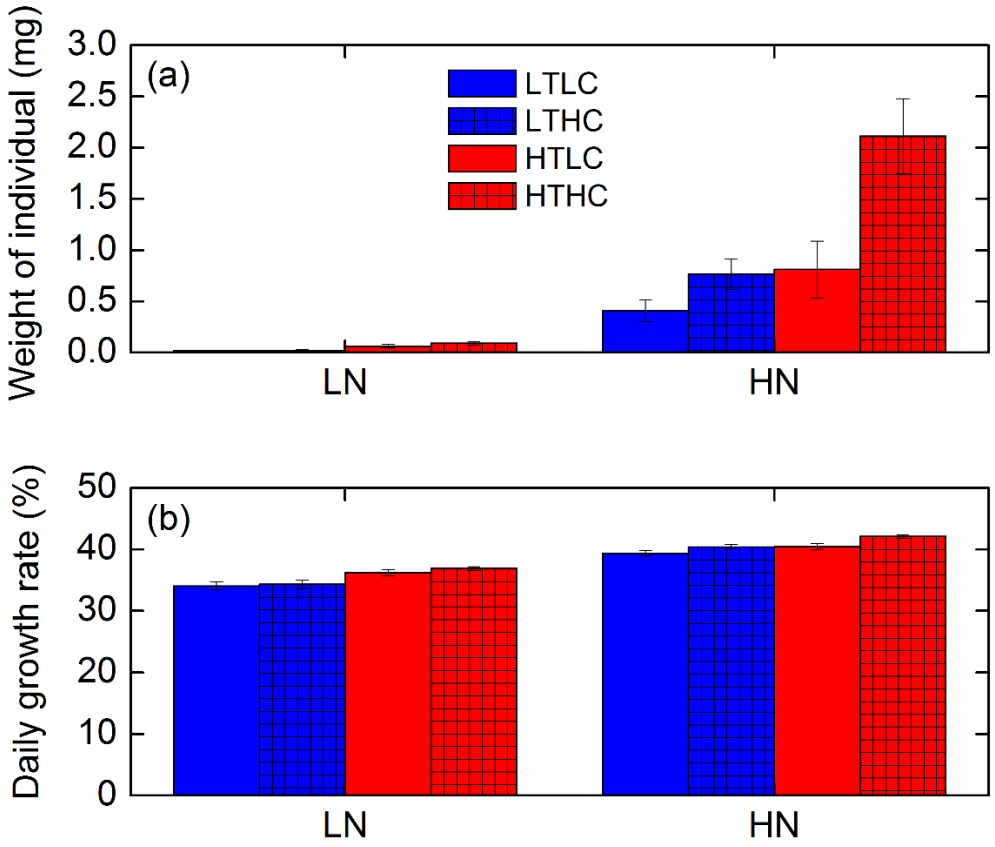

100
80
60
40
20
0
Germination (%)
0
2
4
6
8
Time (d)
LNLTLC
LNLTHC
LNHTLC
LNHTHC
HNLTLC
HNLTHC
HNHTLC
HNHTHC

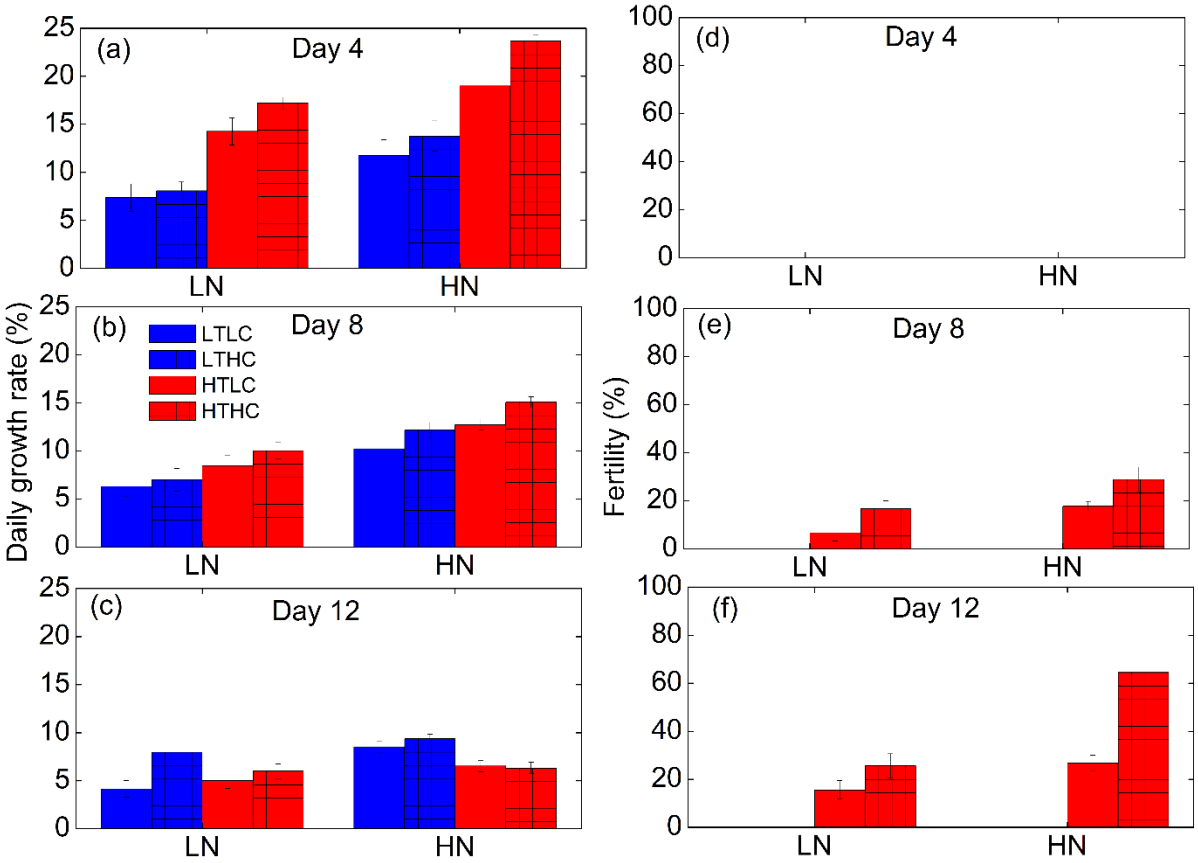
Fig. 2


(a)
LTLC
LTHC
HTLC
HTHC
Weight of individual (mg)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
LN
HN
(b)
Daily growth rate (%)
50
40
30
20
10
0
LN
HN

32

33 Fig. 3

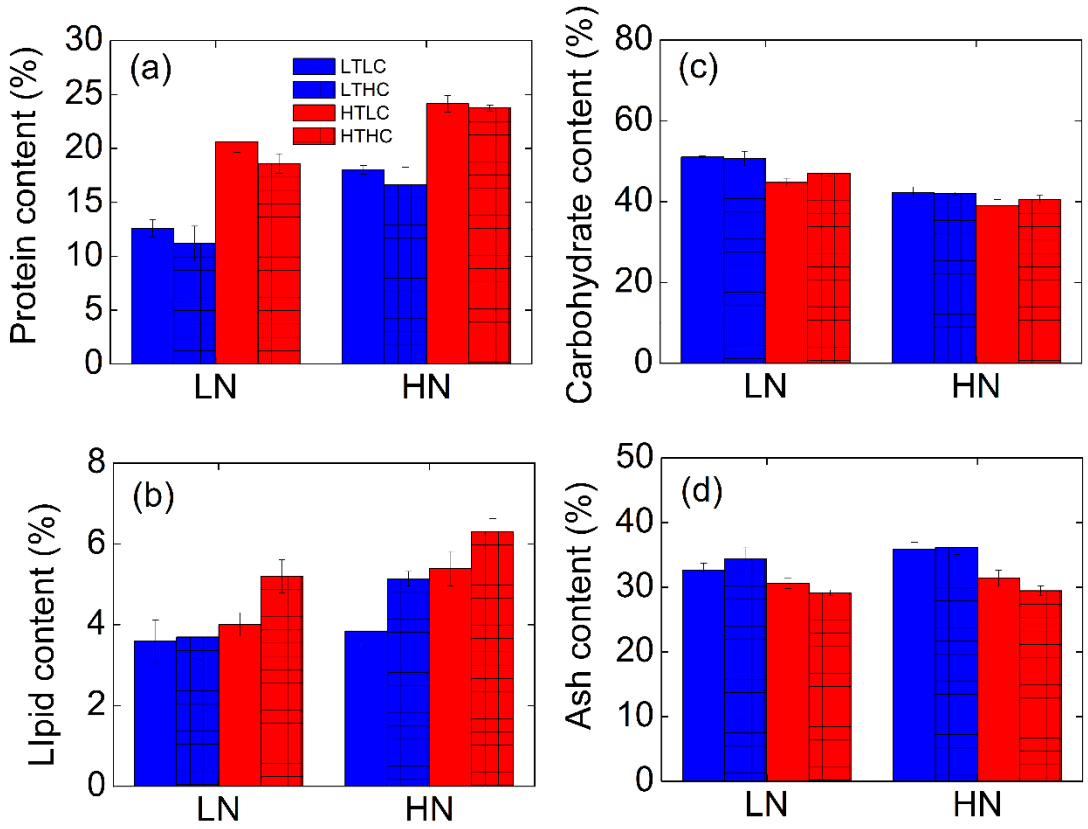
34


(a)
Day 4
(b)
Day 8
(c)
Day 12
(d)
Day 4
(e)
Day 8
(f)
Day 12
LTLC
LTHC
HTLC
HTHC
Daily growth rate (%)
Fertility (%)
LN
HN

35

36 Fig. 4

37


(a)
LTLC
LTHC
HTLC
HTHC
Protein content (%)
30
25
20
15
10
5
0
LN
HN
(c)
Carbohydrate content (%)
80
60
40
20
0
LN
HN
(b)
LIpid content (%)
8
6
4
2
0
LN
HN
(d)
Ash content (%)
50
40
30
20
10
0
LN
HN

38

39 Fig. 5