

Ulva rigida in the future ocean: potential for carbon capture, bioremediation and biomethane production

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Abstract

Ulva species have been considered as ideal candidates for carbon capture, bioremediation and biofuel production. However, little is known regarding the effects of simultaneous ocean warming, acidification and eutrophication on these capacities. In this study, *Ulva rigida* was cultivated under two levels of: temperature (14 °C (LT) and 18 °C (HT)); pH (8.10 and 7.70) by controlling $p\text{CO}_2$ (LC, HC respectively); and nutrients (low (LN) – 50 μM N and 2.5 μM P and high (HN) – 1000 μM N and 50 μM P) for 6 weeks. During the first week of cultivation, HT, HC and HN increased biomass by 38.1%, 17.1% and 20.8%, respectively, while the higher temperature led to negative growth in weeks 2, 4 and 6 due to reproductive events. By the end of the cultivation, biomass under HTHCHN was 130.4% higher than the control (LTLCLN), contributing to a higher carbon capture capacity. Although the thalli at HT released nutrients to seawater in weeks 2, 4 and 6, the HTHCHN treatment increased the overall nitrate uptake rate over the cultivation period by 489.0%. The HTHCHN treatment also had an increased biochemical methane potential and methane yield (47.3% and 254.6%, respectively). Our findings demonstrate that the capacities for carbon and nutrient capture, and biomethane production of *U. rigida* in the future ocean may be enhanced, providing important insight into the interactions between global change and seaweeds.

Keywords: biofuel, carbon capture, chemical composition, eutrophication, green tide, growth, ocean acidification, ocean warming, reproduction, *Ulva*

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Introduction

The burning of fossil fuels combined with changes in land use have been the main drivers behind increased atmospheric and oceanic CO_2 levels, which having risen by more than 40% since the industrial revolution (270–400 ppm) are currently increasing by approximately 2 ppm per year (Moreira & Pires, 2016). Our CO_2 -enriched world contributes to global warming and ocean acidification, both of which directly and indirectly impact a wide range of organisms within the biosphere (Doney *et al.*, 2009; Joos, 2015). The global ocean will continue to warm during the 21st century (IPCC, 2013) with the global mean sea surface temperatures for the months of February and August projected to increase by 1.9 °C by the end of the 21st century. The maximum summer warming of around 4 °C is predicted for high northern latitudes (Bartsch *et al.*, 2012). In addition, sea surface pH is predicted to decrease by 0.4 units based

on Representative Concentration Pathway (RCP) 8.5 (Gattuso *et al.*, 2015). To meet the Paris Agreement goal of maintaining the global temperature rise to below 2 °C above preindustrial levels (Schreurs, 2016) will necessitate a reduction in CO_2 emissions and a sweeping programme of CO_2 capture and storage (Archer *et al.*, 2009). In addition to chemical absorption, biological approaches to CO_2 capture remain research and policy priorities.

Added to the immense challenges posed by rising CO_2 levels, eutrophication presents another macroscale problem that has yet to be effectively mitigated, particularly in rapidly developing nations with increasing coastal populations (Fleming-Lehtinen *et al.*, 2015; Liu & Wang, 2016). Excessive nutrient enrichment drives cycles of algal blooms and crashes (so-called red and green tides) that risk the proliferation of potentially toxic species, and also drive destructive hypoxia events over vast spatial scales (Smith *et al.*, 1999; Smetacek & Zingone, 2013; Farmaki *et al.*, 2014).

Despite algae proliferation (both micro- and macroalgae) being a symptom of the eutrophication dilemma, they are also gaining attraction as CO_2 and nutrient

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biocapture candidates. Due to their large biomass and relatively long turnover time, marine macrophytes (e.g. seagrasses and macroalgae) are more effective carbon sinks than microalgae (Smith, 1981). Species of *Ulva* (Chlorophyta) exhibit high growth rates with strong CO₂ capture capacities and a high affinity for nutrients. For instance, *U. fasciata* has the highest CO₂ capture rate among marine or freshwater macrophytes (Alwis & Jayaweera, 2011).

Ocean warming, acidification and eutrophication usually promote *Ulva*'s growth and hence increase the overall carbon capture capacity. For instance, the growth rate of *U. fasciata* increased as temperature rose from 15 to 25 °C (Mohsen *et al.*, 1973). The growth of *U. prolifera* was also enhanced when cultured under ocean acidification conditions compared with controls (Xu & Gao, 2012). In addition, the growth rate of *U. rigida* was positively related to dissolved inorganic nitrogen (DIN) levels when DIN varied from 3 to 75 µmol L⁻¹ (Viaroli *et al.*, 1996). Ocean warming, acidification and eutrophication can also enhance the nutrient uptake rate of *Ulva* species (Gordillo *et al.*, 2001; Fan *et al.*, 2014).

Increasingly, the carbon capture community has divided into two camps; advocates of carbon capture and storage (i.e. long-term burial of the captured carbon) and advocates of carbon capture and reuse (i.e. recycling the captured carbon into a valorised form, e.g. biofuel; Rao & Rubin, 2002; Brune *et al.*, 2009). An exposition of the relative merits of both approaches is beyond the scope of this study. However, we have investigated the response of a major green tide-forming alga, *U. rigida* to simulated climate change and eutrophication conditions, with a focus on growth rate, reproductive response and carbon and nutrient capture; feeding into the potential to further exploit *Ulva* species as a biofuel source.

Macroalgae are a promising biofuel feedstock owing to their high carbohydrate content that can readily be converted by microbes to biomethane or bioethanol (Kraan, 2010; Hinks *et al.*, 2013). *Ulva* species are an attractive feedstock due to their wide distribution, rapid growth rate and high carbohydrate content (Bruhn *et al.*, 2011). Married to their strong propensity for nutrient capture (particularly nitrogen and phosphorous), *Ulva* species are increasingly at the forefront of applied phyecology (Bolton *et al.*, 2009; Cruz-Suárez *et al.*, 2010; Lawton *et al.*, 2013; Korzen *et al.*, 2016); however, uncertainties remain over *Ulva*'s responses to an ever more carbon and nitrogen impacted future ocean.

Until now, most studies regarding the effects of ocean warming, acidification and eutrophication on *Ulva* species have been either single- or two-factor trials. The combined effects of global change factors need to be examined simultaneously as they are co-occurring

(Koch *et al.*, 2013). To the best of our knowledge, no study has yet investigated the interactive effects of ocean warming, acidification and eutrophication on *Ulva* species from the perspective of their potential for carbon and nutrient capture for biofuel production. In this study, *U. rigida* was cultured at current and simulated future ocean conditions to test the hypothesis that the combined influence of ocean warming, acidification and eutrophication would enhance *Ulva*'s biocapture propensity and utility as a biofuel feedstock, resulting in a negative feedback (Fig. 1).

Materials and methods

Sample collection and culture conditions

Vegetative *U. rigida* of 25–30 mm in length were collected in July 2015 from the low intertidal of Cullercoats Bay, UK (55.03° N, 1.43° W) after a spring tide. The thalli were placed in a zip-lock plastic bag and transported to the laboratory within one hour where they were gently rinsed in one micron filtered natural seawater to remove any contaminating sediment, epiphytes and small grazers. The 720 healthy individual *Ulva* plants were randomly assigned to 24 Perspex® tanks of 13.5 L in volume, each containing 10 L of natural seawater. The interactive effects of ocean warming, acidification and eutrophication were investigated using a fully crossed factorial design, wherein the thalli were incubated under combinations of two pH levels (8.10, 7.70; coded as low pCO₂, LC and high pCO₂, HC, respectively), two temperatures (14, 18 °C; coded as LT and HT, respectively) and two nutrient conditions (50 µM N as nitrate and 2.5 µM P as phosphate, 1000 µM N and 50 µM P; coded as LN and HN, respectively). Three replicate tanks (30 plants per tank) were set up for each treatment. The summer

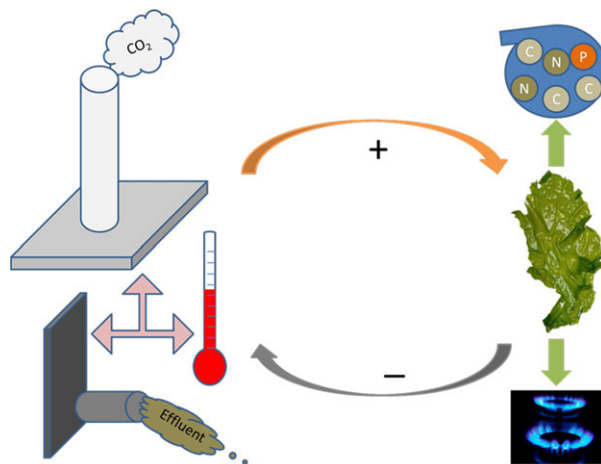


Fig. 1 Interactions between global change variables (ocean warming, acidification and eutrophication) and *Ulva rigida* in terms of carbon and nutrient capture, and biomethane production. The plus sign represents strengthening and the minus sign represents weakening.

average surface seawater temperature in the coastal waters of the central North Sea (14 °C; Mathis *et al.*, 2015) and the ambient pH of natural seawater (8.10) were set as LTLC. The reduced pH and elevated temperature represent the predicted levels by the year 2100 (Baede *et al.*, 2001). The low (50 µM N and 2.5 µM P) and high (1000 µM N and 50 µM P) nutrient levels were chosen as they could represent nutrient limited and replete conditions based on a preliminary experiment. Nutrient concentrations were maintained daily by adding the consumed amount after direct measurement. The culture temperatures were controlled using research grade laboratory incubators while pH was maintained using a custom-built, Aqua-medic™ computer-controlled pH system (Loveland, Colorado) to add CO₂ into an air stream via solenoid valves. The thalli received a light intensity of 80 µmol photons m⁻² s⁻¹, with a photoperiod of 16L: 8D. The culture lasted for 6 weeks and seawater media were fully renewed every 3–4 days.

Carbonate chemistry

Total alkalinity (TA) was measured by titrations prior to seawater changes. Carbonate system parameters, which were not directly measured, were calculated using CO2SYS (Pierrot *et al.*, 2006), using the equilibrium constants of K1 and K2 for carbonic acid dissociation from Roy *et al.* (1993) and the KSO₄⁻ dissociation constant from Dickson (1990).

Biomass and growth

Biomass of *U. rigida* was determined each week by weighing fresh thalli. *Ulva* thalli were blotted gently with tissue paper to remove surface water before weighing. Specific growth rate (SGR) was calculated by the formula:

$$\text{SGR (\%)} = \left(\ln \frac{W_t}{W_0} \right) \times \frac{100}{t},$$

where W_t is the weight after t days, W_0 is the initial weight and t is the number of culture days. The mean SGR over 6 weeks of cultivation was based on the initial weight and the final weight at the end of the cultivation period.

Carbon capture

Carbon capture rate (CCR) over 42 days of culture was determined by the following equation:

$$\text{CCR} = \frac{W_t \times C_t - W_0 \times C_0}{W_0 \times t},$$

where W_t and C_t are, respectively, the algal weight and carbon content after t days of culture, W_0 and C_0 are, respectively, the initial weight and carbon content and t is culture time (42 days). Total carbon content was measured by a CHN elemental analyzer (PerkinElmer 2400, Shelton, CT, USA).

Reproduction

Fertile thalli were recognized by their colour. The formation of reproductive cells in *U. rigida* is accompanied by a change in

thallus colour from green (vegetative state) to yellowish (reproductive state) and then to white (after the release of swimmers; Gao *et al.*, 2017a). This was verified by microscope observation. The reproduction rate at each week was expressed as the ratio of fertile thalli to all thalli in a tank.

Nitrate and phosphate uptake determination

The nitrate or phosphate uptake rate (NUR) was estimated from the change of NO₃⁻ or PO₄³⁻ concentrations in the culture medium over a given time interval (24 h) using the following equation:

$$\text{NUR} = \frac{(N_0 - N_t) \times V}{W},$$

where N_0 is the initial concentration of NO₃⁻ or PO₄³⁻, N_t is the concentration after 24 h, V is the volume of the culture medium and W is the fresh weight of the thalli in culture. Nitrate concentration was measured by a rapid spectrophotometer method (Collos *et al.*, 1999), and phosphate was determined by the phosphomolybdenum blue colorimetry method (Murphy & Riley, 1962).

The overall nitrate rate (ONR) over 42 days of culture was determined by the following equation:

$$\text{ONR} = \frac{(W_t \times N_t - W_0 \times N_0)}{W_0 \times t},$$

where W_t and C_t are, respectively, the algal weight and nitrogen content after t days of culture, W_0 and C_0 are, respectively, the initial weight and nitrogen content, and t is culture time (42 days). Total nitrogen content was measured by a CHN elemental analyzer (PerkinElmer 2400, USA).

Biochemical composition

At the end of 42 days of culture, dry weight was obtained by oven drying fresh thalli at 50 °C until a consistent weight was attained (for 24 h). Ash content was measured by burning dried seaweed samples at 550 °C for 24 h, and volatile solids (VS) were calculated as the ash-free dry weight. Total protein content was estimated by the Kjeldahl method using nitrogen contents multiplied by 5.45 based on the mean value of three species of *Ulva* (Shuuluka *et al.*, 2013). Lipid was extracted according to a modified Folch method (Gao *et al.*, 2017b), and carbohydrate was estimated by an approximation, subtracting the content of protein, lipid and ash from the total content.

Biochemical methane potential and biomethane yield

At the end of 42 days of culture, the biochemical methane potential (BMP) of thalli was determined according to the modified method of Jard *et al.* (2013). Dry thalli (~2 g DW) from each treatment were placed in a 500 mL Duran bottle. Each bottle was inoculated with 40 g of effluent from a 5.0 L laboratory-scale reactor treating cattle manure (5.5% VS). Bottles were filled to 400 mL with distilled water. Blanks (without thalli) were carried out simultaneously to account for the biogas produced from the inoculum alone. The bottles were rapidly

sealed with butyl-rubber stoppers and held using clamped aluminium collars. Pure nitrogen gas (99.999%) was flushed into the headspace to create an anaerobic condition. Afterwards, the bottles were incubated at 35 °C and shaken throughout the 45-day incubation period. The butyl-rubber stopper in each Duran bottle was perforated with a needle attached to a pressure transducer (Type 453A, Bailey and Mackey Ltd, Birmingham, UK) which recorded the gas pressure within the bottle. Methane production was measured with a gas chromatograph equipped with a flame ionization detector (6890 N, Agilent Technologies, Santa Clara, CA, USA). The background methane production from the blank was subtracted from sample methane production obtained in the substrate assays. The biochemical methane potential was calculated by dividing the corrected methane volume (standard pressure and temperature) with the weight of sample VS added to each bottle. The methane yield (MY) was estimated by the following equation:

$$MY = \frac{W_t \times VS \times BMP}{W_0 \times t},$$

where W_t is the weight after t days of culture, VS is the volatile solid per cent, BMP is biochemical methane potential, W_0 is the initial weight and t is the culture time (42 days).

Statistical analysis

The results were expressed as means of replicates $\pm$ standard deviation. Data were analysed using SPSS v.21 (IBM, Armonk, NY, USA). The data under every treatment conformed to a normal distribution (Shapiro–Wilk, $P > 0.05$) and the variances could be considered equal (Levene's test, $P > 0.05$). Repeated-measures ANOVAS (RM-ANOVAS) were conducted to assess the effects of temperature, $p\text{CO}_2$ and nutrients on biomass, specific growth rate, reproduction rate and nitrate and phosphate uptake rate of *U. rigida* over cultivation time. Three-way ANOVAS were conducted to assess the effects of temperature, $p\text{CO}_2$ and nutrients on mean growth rate, carbon content, carbon capture rate, overall nitrate uptake rate, BMP and methane yield. Three-way multivariate ANOVAS (MANOVAS) were conducted to assess the effects of temperature, $p\text{CO}_2$ and nutrients on seawater carbonate parameters and biochemical composition (protein, lipid, carbohydrate and ash). Tukey's honest significant difference test was conducted for *post hoc* investigation. A confidence interval of 95% was set for all tests.

Results

Seawater carbonate chemistry

The decrease of 0.4 pH units led to $p\text{CO}_2$ increases of 187.4%, DIC of 8.8%, HCO_3^- of 11.9% and CO_2 of 188.0%, with a decrease in CO_3^{2-} of 56.5% ($P < 0.001$; Table 1). There was no significant difference in TA between the pH levels. The higher temperature enhanced $p\text{CO}_2$ by 14.9%, DIC by 9.1%, HCO_3^- by 8.7%, CO_3^{2-} by 19.0% and TA by 9.4% ($P < 0.05$). Nutrient enrichment did not affect seawater carbonate chemistry.

Table 1 Seawater carbonate parameters in the different treatments. Data are the means $\pm$ SD ($n = 3$)

Treatment	pH	Temperature (°C)	$p\text{CO}_2$ (μatm)	DIC ($\mu\text{mol kg}^{-1}$)	HCO_3^- ($\mu\text{mol kg}^{-1}$)	CO_3^{2-} ($\mu\text{mol kg}^{-1}$)	CO_2 ($\mu\text{mol kg}^{-1}$)	TA ($\mu\text{mol kg}^{-1}$)
LTLCLN	8.10 $\pm$ 0.05	14.0 $\pm$ 0.5	408.3 $\pm$ 46.2	2018.4 $\pm$ 81.0	1865.0 $\pm$ 72.8	137.5 $\pm$ 16.8	15.9 $\pm$ 1.8	2210.1 $\pm$ 94.6
LTHCLN	7.70 $\pm$ 0.05	14.0 $\pm$ 0.5	1317.3 $\pm$ 181.6	2236.9 $\pm$ 65.5	2130.0 $\pm$ 62.9	55.6 $\pm$ 5.1	51.3 $\pm$ 7.1	2270.4 $\pm$ 54.3
LTLCHN	8.10 $\pm$ 0.05	14.0 $\pm$ 0.5	460.5 $\pm$ 70.0	2008.7 $\pm$ 88.6	1868.4 $\pm$ 90.8	122.4 $\pm$ 9.9	17.9 $\pm$ 2.7	2227.8 $\pm$ 79.0
LTHCHN	7.70 $\pm$ 0.05	14.0 $\pm$ 0.5	1286.2 $\pm$ 155.6	2187.6 $\pm$ 50.3	2083.1 $\pm$ 47.5	54.5 $\pm$ 6.4	50.1 $\pm$ 6.1	2269.0 $\pm$ 51.9
HTLCLN	8.10 $\pm$ 0.05	18.0 $\pm$ 0.5	536.1 $\pm$ 101.7	2246.9 $\pm$ 154.1	2074.1 $\pm$ 156.6	154.2 $\pm$ 6.1	18.5 $\pm$ 3.5	2452.2 $\pm$ 137.9
HTHCLN	7.70 $\pm$ 0.05	18.0 $\pm$ 0.5	1481.2 $\pm$ 144.6	2417.1 $\pm$ 44.1	2297.4 $\pm$ 40.2	68.5 $\pm$ 9.0	51.2 $\pm$ 5.0	2466.3 $\pm$ 61.0
HTLCHN	8.10 $\pm$ 0.05	18.0 $\pm$ 0.5	521.3 $\pm$ 83.6	2189.8 $\pm$ 101.0	2021.1 $\pm$ 105.5	150.6 $\pm$ 10.6	18.0 $\pm$ 2.9	2443.6 $\pm$ 86.1
HTHCHN	7.70 $\pm$ 0.05	18.0 $\pm$ 0.5	1450.5 $\pm$ 159.7	2365.9 $\pm$ 76.2	2248.8 $\pm$ 71.4	67.0 $\pm$ 8.9	50.1 $\pm$ 5.5	2462.9 $\pm$ 85.2

LTLCLN, lower temperature, lower $p\text{CO}_2$ and lower nitrate; LTHCLN, lower temperature, higher $p\text{CO}_2$ and lower nitrate; LTLCHN, lower temperature, lower $p\text{CO}_2$ and higher nitrate; LTHCHN, lower temperature, higher $p\text{CO}_2$ and higher nitrate; HTLCLN, higher temperature, lower $p\text{CO}_2$ and lower nitrate; HTHCLN, higher temperature, higher $p\text{CO}_2$ and lower nitrate; HTLCHN, higher temperature, lower $p\text{CO}_2$ and higher nitrate; HTHCHN, higher temperature, higher $p\text{CO}_2$ and higher nitrate; DIC, dissolved inorganic carbon; TA, total alkalinity.

Biomass and growth

The biomass and specific growth rate varied with cultivation time and the variation patterns under different treatments were heterogeneous ($P < 0.001$; Fig. 2). For example, the biomass in the low temperature treatment had two peaks; $26.7 \pm 4.3 \text{ g tank}^{-1}$ by week 2 and $41.0 \pm 10.2 \text{ g tank}^{-1}$ by week 5. The high temperature treatment, while also having two peaks, was slightly out of phase from the low temperature treatment, with peaks of $37.8 \pm 8.5 \text{ g tank}^{-1}$ by week 3 and $47.5 \pm 12.3 \text{ g tank}^{-1}$ by week 5 (Fig. 2a). The fluctuation in biomass with cultivation time was a function of growth rate. The specific growth rate at the lower temperature was negative in weeks 3 and 6, whereas negative growth occurred in weeks 2, 4 and 6 in the high temperature treatment (Fig. 2b).

In terms of the effect of temperature, $p\text{CO}_2$ and nutrients on biomass in each week, high temperature, high $p\text{CO}_2$ and high nutrients increased biomass by 38.1%, 17.1% and 20.8%, respectively, by week 1 ($P < 0.001$). By week 2, the higher temperature had reduced the biomass by 42.3% ($P < 0.001$). High $p\text{CO}_2$ and nutrients, respectively, increased biomass by 18.4% and 26.3% at

the lower temperature ($P < 0.01$) but not at the higher temperature. By week 3, high temperature, high $p\text{CO}_2$ and high nutrient treatments had increased biomass by 79.1%, 15.0% and 38.9%, respectively ($P < 0.001$). By week 4, temperature did not affect biomass while high $p\text{CO}_2$ and nutrients increased it by 16.4% and 36.3%, respectively ($P < 0.001$). By week 5, high temperature, high $p\text{CO}_2$ and high nutrients increased biomass by 15.9%, 24.1% and 55.0%, respectively ($P < 0.001$). The stimulating effects of high temperature (27.1%), high $p\text{CO}_2$ (22.1%) and high nutrient (45.3%) on biomass continued into week 6 ($P < 0.001$).

The effects of these three factors on specific growth rate were similar to the biomass yield (Fig. 2b). There were two trends that are worthy of note. First, growth rate generally decreased with cultivation time. Secondly, the negative growth effect of high temperature decreased with cultivation time and the negative specific growth rate at the lower temperature did not change with cultivation time. To evaluate the growth effects of temperature, $p\text{CO}_2$ and nutrients over the whole cultivation period, the mean growth rate over 6 weeks of cultivation was calculated (Fig. 2c). All three factors positively affected the mean growth rate. High

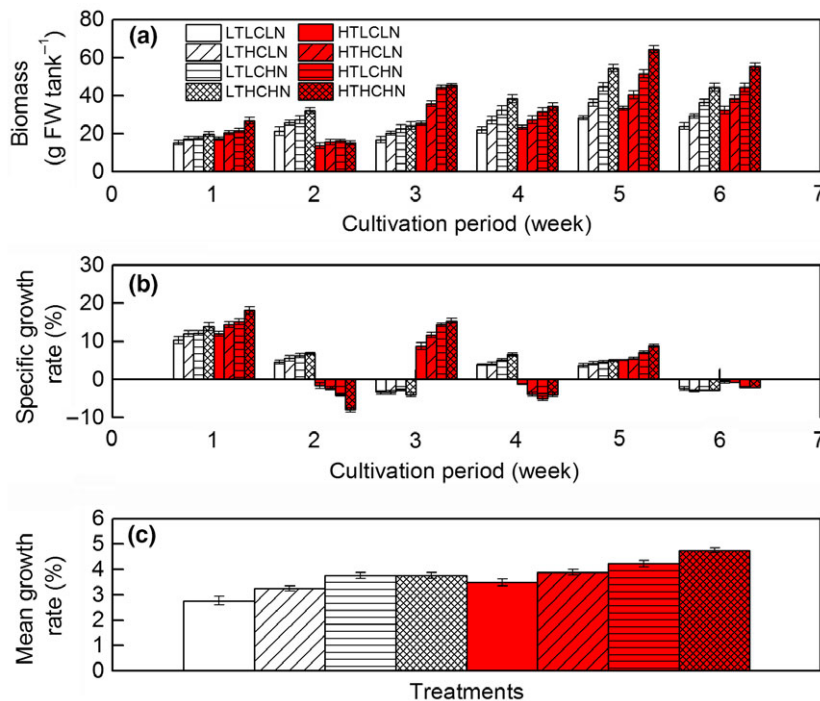


Fig. 2 Biomass (a), specific growth rate (b) and mean growth rate (c) of *Ulva rigida* cultured under the experimental conditions for 6 weeks. The mean growth rate is based on the initial biomass and final biomass at the end of the cultivation. The error bars indicate the standard deviations ($n = 3$). LT = lower temperature (14 °C); HT = higher temperature (18 °C); LC = lower $p\text{CO}_2$ (pH 8.10); and HC = higher $p\text{CO}_2$ (pH 7.70); LN = lower nutrients ($50 \mu\text{mol L}^{-1}$ N and $2.5 \mu\text{mol L}^{-1}$ P); HN = higher nutrients ($1000 \mu\text{mol L}^{-1}$ N and $50 \mu\text{mol L}^{-1}$ P).

temperature, $p\text{CO}_2$ and nutrients increased it by 17.1%, 11.1% and 23.5%, respectively ($P < 0.001$).

Reproduction

The reproduction rate of thalli during cultivation was observed to investigate the reasons behind the periodic decrease in biomass and growth (Fig. 3). The thalli grown at the higher temperature had reproductive events in weeks 2, 4 and 6, whereas those grown at the lower temperature were reproductive in weeks 3 and 6, indicating that the higher temperature shortened the reproductive period from three to two weeks. Another temperature trend was that the reproduction rate at the lower temperature did not change with cultivation time ($43.9 \pm 5.8\%$ in week 3 and $42.5 \pm 6.8\%$ in week 6), while it decreased from $63.9 \pm 18.5\%$ (week 2) to $42.2 \pm 9.0\%$ (week 6; $P = 0.003$) at the higher temperature; although the differences between weeks 2 and 4 ($P = 0.086$) or weeks 4 and 6 ($P = 0.328$) were not significant. $p\text{CO}_2$ and nutrients also affected reproduction. High $p\text{CO}_2$ increased reproduction by 25.4%, 17.8% and 15.0% in weeks 2, 4 and 6, respectively. High nutrients increased reproduction by 64.4%, 16.5%, 65.3% and 29.5% in weeks 2, 3, 4 and 6, respectively.

Carbon capture

The thalli carbon content is presented in Fig. 4a. By the end of the cultivation period, temperature ($P < 0.001$)

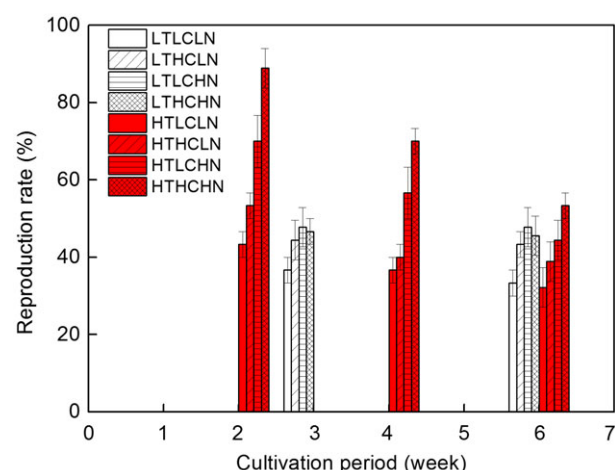


Fig. 3 Reproduction rate of *Ulva rigida* cultured under the experimental conditions for 6 weeks. The error bars indicate the standard deviations ($n = 3$). LT = lower temperature (14°C); HT = higher temperature (18°C); LC = lower $p\text{CO}_2$ (pH 8.10); and HC = higher $p\text{CO}_2$ (pH 7.70); LN = lower nutrients ($50\ \mu\text{mol L}^{-1}\text{N}$ and $2.5\ \mu\text{mol L}^{-1}\text{P}$); HN = higher nutrients ($1000\ \mu\text{mol L}^{-1}\text{N}$ and $50\ \mu\text{mol L}^{-1}\text{P}$).

and $p\text{CO}_2$ ($P = 0.029$) had main effects, and $p\text{CO}_2$ had interactive effects with temperature ($P = 0.001$) or nutrients ($P = 0.005$). Additionally, these three factors interacted ($P = 0.004$) to affect the thallus carbon content. The higher temperature increased the carbon content by 10.9–23.0% ($P < 0.01$). High $p\text{CO}_2$ increased the carbon content by 4.1–4.3% at the higher temperature ($P < 0.05$), but did not affect it in the LTHN treatment and decreased it by 5.98% in the LTLN treatment ($P = 0.027$). In terms of the carbon capture rate (Fig. 4b), all three factors had positive effects ($P < 0.001$). High temperature, $p\text{CO}_2$ and nutrients increased carbon capture by 61.8%, 31.3% and 60.6%, respectively. In addition, $p\text{CO}_2$ interacted with temperature ($P = 0.007$) or nutrients ($P = 0.002$). For instance, high $p\text{CO}_2$ increased the carbon capture rate by 29.2% at the lower temperature and by 32.7% at the higher temperature, and by 26.7% in the low nutrient treatment and by 34.3% in the high nutrient treatment. Due to the main and interactive effects of temperature, $p\text{CO}_2$ and nutrients, the carbon capture rate at HTHCHN was 245.1% higher than that at LTLCLN.

Uptake of nitrate and phosphate

Nitrate and phosphate uptake rates were measured to investigate bioremediation capacity (Fig. 5). A RM-ANOVA showed that nitrate uptake varied with cultivation time, and variation patterns were different under each treatment ($P < 0.001$). For instance, the nitrate uptake rate at the lower temperature decreased from $211.4 \pm 65.9\ \mu\text{M g DW}^{-1}\text{day}^{-1}$ (week 1) to $134.3 \pm 33.2\ \mu\text{M g DW}^{-1}\text{day}^{-1}$ (week 2) further to $-47.7 \pm 4.2\ \mu\text{M g DW}^{-1}\text{day}^{-1}$ (week 3), then increased to $119.0 \pm 25.7\ \mu\text{M g DW}^{-1}\text{day}^{-1}$ (week 4), did not change in week 5 ($101.3 \pm 29.7\ \mu\text{M g DW}^{-1}\text{day}^{-1}$) and finally decreased to $-37.4 \pm 6.1\ \mu\text{M g DW}^{-1}\text{day}^{-1}$ (week 6; Fig. 5a). In contrast, the nitrate uptake rate at the higher temperature decreased from $267.5 \pm 143.0\ \mu\text{M g DW}^{-1}\text{day}^{-1}$ (week 1) to $-50.6 \pm 33.9\ \mu\text{M g DW}^{-1}\text{day}^{-1}$ (week 2), increased to $279.4 \pm 44.5\ \mu\text{M g DW}^{-1}\text{day}^{-1}$ (week 3), decreased to $-52.9 \pm 8.8\ \mu\text{M g DW}^{-1}\text{day}^{-1}$ (week 4), increased to $150.9 \pm 83.6\ \mu\text{M g DW}^{-1}\text{day}^{-1}$ (week 5), and finally decreased to $-27.3 \pm 17.1\ \mu\text{M g DW}^{-1}\text{day}^{-1}$ (week 6). The effects of temperature, $p\text{CO}_2$ and nitrate on the nitrate uptake in each week were analysed by a MANOVA. By week 1, high temperature and high $p\text{CO}_2$ increased nitrate uptake in thalli grown in the high nutrient treatment by 51.1% ($P < 0.001$) and 37.6% ($P = 0.002$), respectively, with high nutrient having a larger promoting effect of 118.0% ($P < 0.001$). By week 2, thalli grown at the higher temperature had negative nitrate uptake rates, suggesting nitrate release from thalli to the

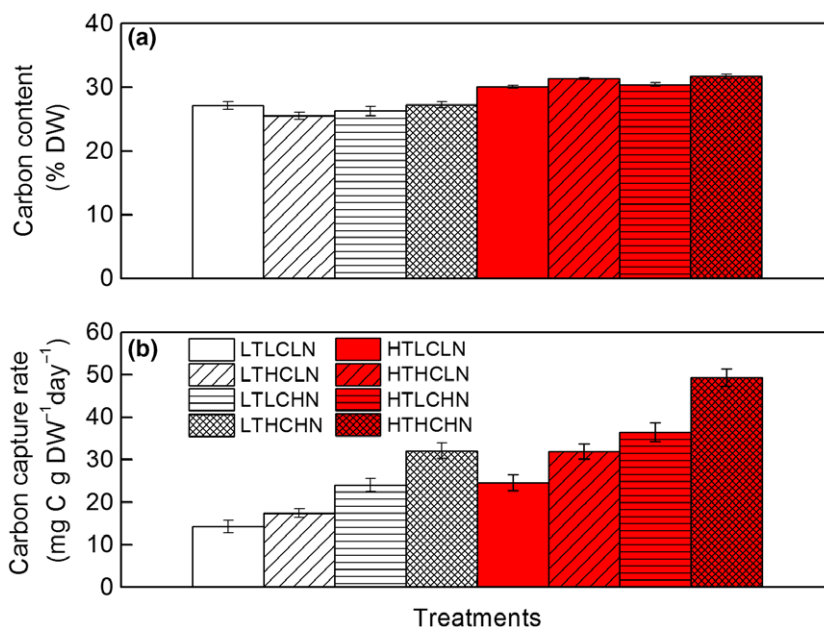


Fig. 4 Carbon content (a) and carbon capture rate (b) of *Ulva rigida* grown at the experimental conditions by the end of 6 weeks of cultivation. The error bars indicate the standard deviations ($n = 3$). LT = lower temperature (14 °C); HT = higher temperature (18 °C); LC = lower $p\text{CO}_2$ (pH 8.10); and HC = higher $p\text{CO}_2$ (pH 7.70); LN = lower nutrients (50 $\mu\text{mol L}^{-1}$ N and 2.5 $\mu\text{mol L}^{-1}$ P); HN = higher nutrients (1000 $\mu\text{mol L}^{-1}$ N and 50 $\mu\text{mol L}^{-1}$ P).

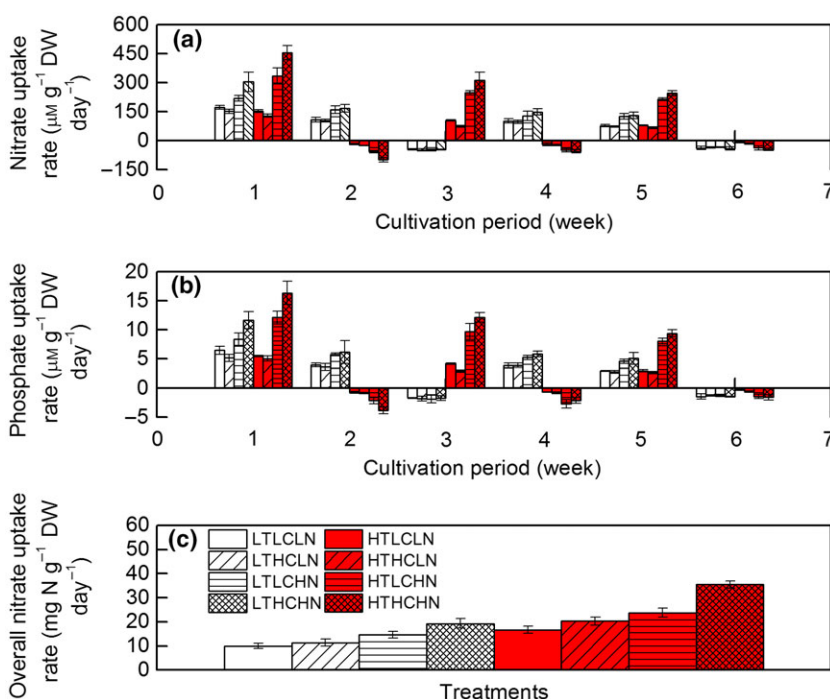


Fig. 5 Changes of nitrate (a) and phosphate (b) uptake rate of *Ulva rigida* with cultivation time. The overall nitrate uptake rate (c) is based on the initial nitrogen content and final nitrogen content at the end of the cultivation. The error bars indicate the standard deviations ($n = 3$). LT = lower temperature (14 °C); HT = higher temperature (18 °C); LC = lower $p\text{CO}_2$ (pH 8.10); and HC = higher $p\text{CO}_2$ (pH 7.70); LN = lower nutrients (50 $\mu\text{mol L}^{-1}$ N and 2.5 $\mu\text{mol L}^{-1}$ P); HN = higher nutrients (1000 $\mu\text{mol L}^{-1}$ N and 50 $\mu\text{mol L}^{-1}$ P).

seawater. High nutrients increased nitrate uptake by 54.7% at low temperature ($P < 0.001$) but it led to a quicker nitrate release of 253.4% at the higher temperature ($P < 0.001$). By week 3, thalli grown at the lower temperature released nitrate to the seawater. High nutrients increased nitrate uptake by 214.3% at the higher temperature ($P < 0.001$) but did not affect it at the lower temperature. By week 4, the high temperature led to a negative nitrate uptake. High nutrients increased nitrate uptake by 38.7% at the lower temperature ($P = 0.003$) but lead to a quicker nitrate release of 147.2% at the higher temperature ($P < 0.001$). By week 5, the higher temperature increased nitrate uptake in thalli grown with high nutrients by 79.6% ($P < 0.001$). High nutrient levels increased nitrate uptake rate by 143.6% ($P < 0.001$). By week 6, the higher temperature reduced nitrate release by 27.0% ($P = 0.002$). The high nutrients treatment increased nitrate releases of thalli grown at the higher temperature by 236.8% ($P < 0.001$).

The pattern of phosphate uptake (Fig. 5b) was the same as the nitrate uptake. When the effects of these three factors were normalized to 6 weeks (Fig. 5c), all of them showed positive effects on nitrate uptake. High temperature, $p\text{CO}_2$ and nutrients increased the overall nitrate uptake rate by 104.2%, 17.6% and 108.3%, respectively ($P < 0.001$), making the overall nitrate uptake rate at HTHCHN ($7.1 \pm 0.4 \mu\text{M g DW}^{-1} \text{ day}^{-1}$) almost six times greater than that at LTLCLN.

Chemical composition

By the end of 6 weeks of culture, the biochemical composition of *U. rigida* cultivated under different conditions was also investigated (Fig. 6). The high temperature and high nutrients treatments increased the protein content by 38.9% ($P < 0.001$) and 25.2% ($P < 0.001$), respectively. $p\text{CO}_2$ did not affect protein content (Fig. 6a). All three factors had a positive effect on the lipid content. Higher temperature, $p\text{CO}_2$ and nutrients increased lipid content by 26.8% ($P < 0.001$), 16.3% ($P < 0.001$) and 24.4% ($P < 0.001$), respectively (Fig. 6b). High temperature and nutrients decreased the carbohydrates content by 5.9% ($P < 0.001$) and 14.1% ($P < 0.001$), respectively, with no effect of $p\text{CO}_2$ (Fig. 6c). Temperature interacted with nutrients ($P = 0.029$) or $p\text{CO}_2$ ($P = 0.004$) to affect ash content (Fig. 6d). The higher temperature decreased the ash content by 6.92–18.49%, with the decrease being more pronounced at HCHN (14.9–18.5%) than at LCLN (6.92–12.20%).

Biochemical methane potential and methane production rate

The biochemical methane potential (Fig. 7a) and methane yield (Fig. 7b) of *U. rigida* grown at different conditions were investigated by the end of 6 weeks of

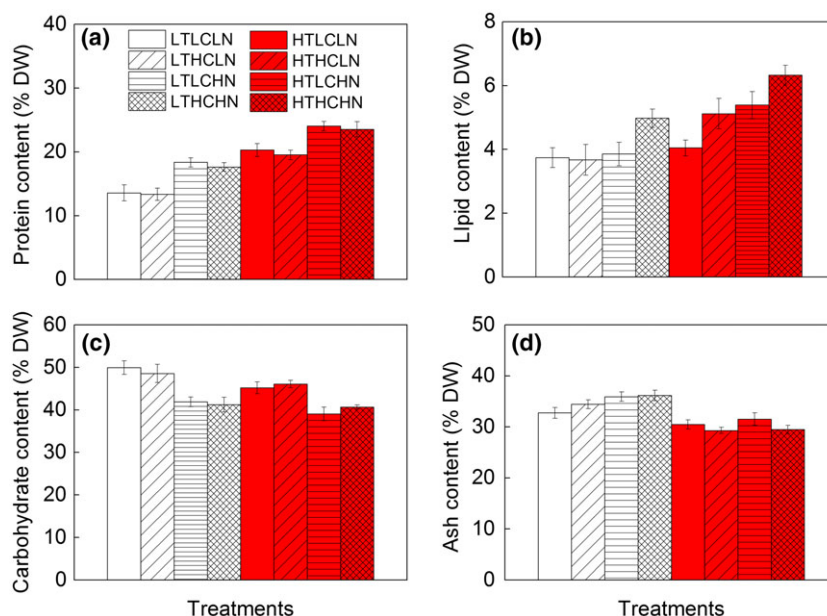


Fig. 6 Content of protein (a), lipid (b), carbohydrate (c) and ash (d) in *Ulva rigida* grown at the experimental conditions by the end of 6 weeks of cultivation. The error bars indicate the standard deviations ($n = 3$). LT = lower temperature (14 °C); HT = higher temperature (18 °C); LC = lower $p\text{CO}_2$ (pH 8.10); and HC = higher $p\text{CO}_2$ (pH 7.70); LN = lower nutrients ($50 \mu\text{mol L}^{-1} \text{ N}$ and $2.5 \mu\text{mol L}^{-1} \text{ P}$); HN = higher nutrients ($1000 \mu\text{mol L}^{-1} \text{ N}$ and $50 \mu\text{mol L}^{-1} \text{ P}$).

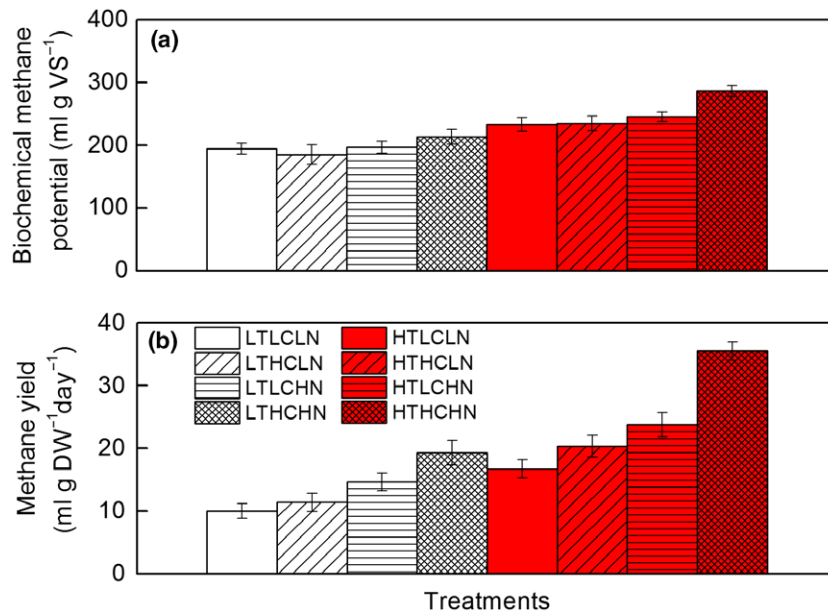


Fig. 7 Biochemical methane potential (a) and methane yield (b) of *Ulva rigida* grown at the experimental conditions by the end of 6 weeks of cultivation. The error bars indicate the standard deviations ($n = 3$). LT = lower temperature (14 °C); HT = higher temperature (18 °C); LC = lower $p\text{CO}_2$ (pH 8.10); and HC = higher $p\text{CO}_2$ (pH 7.70); LN = lower nutrients (50 $\mu\text{mol L}^{-1}$ N and 2.5 $\mu\text{mol L}^{-1}$ P); HN = higher nutrients (1000 $\mu\text{mol L}^{-1}$ N and 50 $\mu\text{mol L}^{-1}$ P).

culture. A three-way ANOVA showed that temperature ($P < 0.001$), $p\text{CO}_2$ ($P = 0.014$) and nutrients ($P < 0.001$) had main effects on the biochemical methane potential, and $p\text{CO}_2$ had an interactive effect with nutrients ($P = 0.002$). The higher temperature increased the biochemical methane potential by 19.9–34.3% ($P < 0.001$). The higher nutrient treatment did not affect the biochemical methane potential at LC, but increased it by 15.3–22.2% at HC ($P < 0.001$).

In terms of methane yield (Fig. 7b), all three factors had main effects ($P < 0.001$) and any two of them had an interactive effect ($P < 0.01$). For instance, high $p\text{CO}_2$ did not affect methane yield at LN but increased it by 42.7% at HN. HN increased methane yield at LC by 44.4%, while it was 73.4% at HC. The stimulating effects of these three factors made the methane yield ($35.5 \pm 1.5 \text{ mL g DW}^{-1} \text{ day}^{-1}$) at HTHCHN 254.5% higher than that at LTLCLN.

Discussion

Growth and reproduction

In the present study, the higher temperature enhanced the specific growth rate in *U. rigida* in weeks 1, 3 and 5. It has been widely reported that high temperatures could promote the growth of *Ulva*. For instance, a 5 °C increase (from 20 to 25 °C) more than doubled the growth rate of *U. fasciata* when salinity was 25 (Mantri

et al., 2011). The results in the present study indicate that the lower temperature at high latitudes limits the growth of *U. rigida* even in summer, and therefore, *Ulva* species may benefit from future ocean warming. In terms of the effect of CO_2 , although it has been reported that *Ulva* species have efficient carbon concentrating mechanisms (CCMs) and their photosynthesis could be saturated at the current CO_2 level (Axelsson *et al.*, 1999; Gao *et al.*, 2016), growth could still be promoted by elevated CO_2 (Gao *et al.*, 2016, 2017b,d). Our results were consistent with these studies. Such an increase in growth may be due to enhanced nitrogen assimilation at the elevated CO_2 concentration (Gordillo *et al.*, 2003; Xu *et al.*, 2017). In addition, nitrogen and phosphorus, two key nutrients supporting algal growth, are generally thought to be limiting in marine systems (Müller & Mitrovic, 2015). Accordingly, their enrichment can promote algal growth (Gao *et al.*, 2017b; Xu *et al.*, 2017). In the present study, adding nitrogen and phosphorus increased the growth of *U. rigida*. A similar result was also documented in nitrogen and phosphorus enrichment experiments for *Ulva* spp. conducted in the field (Teichberg *et al.*, 2010).

On the other side, the effect of temperature on the specific growth rate was reversed, the positive effect turning to negative in weeks 2, 4 and 6. Decreased growth at the higher temperature could be attributed to induced reproductive events at the higher temperature. Reproduction can stop vegetative growth, and the

release of spores leads to a loss of thallus mass (Gao *et al.*, 2017b). Moderate temperatures can accelerate reproductive processes by increasing the metabolic activity to produce essential materials, such as nucleotides and proteins (Iken, 2012). For instance, the reproductive period of *U. fenestrata* in the laboratory decreased from 30 to 5 days when temperature increased from 10 to 20 °C (Kalita & Titlyanov, 2011). The effect of temperature on rhythms of *Ulva* reproduction was also found in the field. *U. pseudocurvata* in the North Sea was reported to have biweekly peaks of gametophytic reproduction during the colder seasons and approximately weekly peaks during summer (Lüning *et al.*, 2008). The high temperature also reduced the reproductive period of *U. rigida* from three to two weeks compared to the low temperature in the present study. Taken together, these findings indicate that higher temperature could commonly shorten the reproductive period in *Ulva* species. It is noteworthy that the reproduction rate at high temperature declined with cultivation time, indicating acclimation of reproduction to ocean warming. This also led to the decreasing negative effect of high temperature on the specific growth rate with cultivation time. Most studies on algal acclimation to temperature rise are confined to photosynthesis, growth and respiration (Eggert *et al.*, 2006; Zou & Gao, 2013; Graiff *et al.*, 2015; Al-Janabi *et al.*, 2016; Gao *et al.*, 2017c). Our study suggests algal reproduction could also acclimatize to global warming.

In addition to temperature, high $p\text{CO}_2$ and nitrate also induced more reproduction in *U. rigida*. This finding was consistent with our previous study based on a short-term cultivation (Gao *et al.*, 2017b). The promoting effects of higher CO_2 and nutrients on *U. rigida* may be due to increased carbon and nitrogen assimilation and thus the necessary materials for reproduction. Compared to $p\text{CO}_2$, temperature and nutrients seem to play a more important independent role in controlling both growth and reproduction of *U. rigida*. This finding is consistent with field studies (Keesing *et al.*, 2011; Liu *et al.*, 2013; Smetacek & Zingone, 2013). Eutrophication is deemed as the primary reason for green tides (Smetacek & Zingone, 2013). In addition, the coverage of green tides formed by *Ulva* species extends with the rise of seawater temperature but begins to shrink and then disappears when seawater temperature increases further and nutrients are exhausted (Keesing *et al.*, 2011; Liu *et al.*, 2013).

Carbon capture capacity

The carbon capture rate depends on the growth rate and carbon content. In the present study, high temperature, $p\text{CO}_2$ and nitrate induced more reproductive

events, leading to reduced growth rate in the shorter term. This phenomenon was also found in our previous study (Gao *et al.*, 2017b). But in a longer-term cultivation, the quicker growth at the high temperature, $p\text{CO}_2$ and nutrients could offset the negative effect of reproduction on growth. Accordingly, high temperature, $p\text{CO}_2$ and nutrients increased the mean growth rate over 7 weeks of cultivation. In addition, high temperature and $p\text{CO}_2$ also increased the carbon content in *U. rigida*. These resulted in the highest carbon capture rate at HTHCHN, which is more than three times higher than at LTLCLN. Chung *et al.* (2011) showed that *Ulva* species had the highest carbon capture capacity compared to seaweeds belonging to the Chlorophyta, Phaeophyta and Rhodophyta. Our findings suggest that the future ocean environment may strengthen the carbon capture capacity of *Ulva* species.

Bioremediation capacity

The nitrate and phosphate uptake of algae commonly increases with temperature (Pedersen *et al.*, 2004; Smith *et al.*, 2009; Fan *et al.*, 2014). In the present study, the higher temperature also increased nitrate and phosphate uptake in *U. rigida* when thalli were vegetative. However, the higher temperature led to a negative nutrient uptake when reproductive events occurred, indicating that thalli were releasing rather than absorbing nutrients from the seawater. The reasons for this may be twofold. When thalli release spores, the nutrients in the cell could also be discharged to the seawater. Meanwhile, the decomposition of debris after spore release also contributes to the increase in nutrients in the seawater. This is supported by studies in which the seawater was enriched with nitrate and phosphate when macrophytes were decomposing (Hanisak, 1993; Gao *et al.*, 2013). Furthermore, the higher temperature could increase the decomposition rate and thus the nutrient release rate (Hanisak, 1993; Da *et al.*, 2014), which may explain the negative nutrient uptake rate at the higher temperature in the present study. This suggests that *Ulva* species can actually be a source of nutrients when they are reproducing, which has implications for the biofilter efficiency of *Ulva* in the future ocean. Maintaining a long-term vegetative state seems to be critical for promoting the biofiltering efficiency of *Ulva* species.

The higher $p\text{CO}_2$ in the present study also increased the nitrate and phosphate uptake during the first week. Xu *et al.* (2017) demonstrated that a higher $p\text{CO}_2$ level (1017 μatm) increased the nitrate reductase activity and thus the nitrate uptake in *Sargassum muticum* during a 13-day cultivation. Therefore, the increased nutrient uptake at elevated $p\text{CO}_2$ in the present study may be

due to the activation of nitrate reductase at the higher $p\text{CO}_2$ levels (Gordillo *et al.*, 2001; Xu *et al.*, 2017).

Biomethane production

In the present study, *U. rigida* cultivated at the conditions of high temperature and high nutrients had a higher biochemical methane potential. When measuring the biochemical composition, it was found that the culture conditions of higher temperature and nutrients resulted in increased lipid and protein content and decreased carbohydrate content. The theoretical methane production for lipid, protein and carbohydrate is 1014, 496 and 415 mL CH_4 g VS^{-1} , respectively (Møller *et al.*, 2004). Therefore, the greater proportion of lipid and protein in thalli grown under the conditions of high temperature and high nutrient will produce a higher biochemical methane potential. The biochemical methane potential (286.3 ± 8.9 mL CH_4 g VS^{-1}) of thalli cultured under the conditions of high temperature, high $p\text{CO}_2$ and high nutrients was 47.3% higher than the control and was also above the range of 120–271 mL CH_4 g VS^{-1} reported in *Ulva* species using fresh or ground thalli (Briand & Morand, 1997; Peu *et al.*, 2011; Allen *et al.*, 2013; Jard *et al.*, 2013). The findings above indicate that the future ocean could increase the biochemical methane potential of *U. rigida* by altering its biochemical composition. The present study is the first to document the impacts of global change factors on biochemical methane potential in seaweeds.

In addition to biochemical methane potential, methane yield was also determined by growth rate. High temperature, $p\text{CO}_2$ and nutrients increased mean growth rate over 6 weeks of cultivation. Combined with the positive effect of these three factors on biochemical methane potential, methane yield at HTLCHN was 2.5 times higher than at LTLCLN. Ignoring the high levels of sulphur, *Ulva* species are considered as ideal seaweeds for biomethane production due to their abundant availability, quick growth and high biochemical methane potential (Bruhn *et al.*, 2011; Amosu, 2016; Karray *et al.*, 2017). Our finding indicates that the future ocean may further improve *Ulva*'s advantages as a biofuel feedstock.

Interactions between climate change and seaweeds

It has been suggested that CO_2 -induced global warming would lead to increased stratification of the water column, resulting in decreasing nutrient transport from the deep ocean to the upper ocean and increasing light exposure (Falkowski *et al.*, 2000; Gao *et al.*, 2012). This can reduce the carbon fixation of phytoplankton and thereby the rate of oceanic uptake of anthropogenic CO_2

(Gao *et al.*, 2012, 2017c). On the other hand, most seaweeds inhabit the tide zones and are not affected by stratification of the water column. Our results demonstrate that ocean warming, acidification and eutrophication significantly enhanced the capacities for carbon and nutrient capture and biomethane production in *U. rigida*. The increased capacity for carbon and nutrient capture seems to be a negative feedback in response to the environmental changes caused by human activity and thus could alleviate some aspects of global climate change. Although seaweeds are restricted to a narrow zone of the oceans, they contribute to about 10% of the total world marine productivity (Israel *et al.*, 2010). Further investigations into other seaweeds are needed to understand whether this negative feedback applies to the whole seaweed community and to have a more comprehensive view of the interactions of climate change and marine primary producers.

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