Changes in morphological plasticity of Ulva prolifera under different environmental conditions: A laboratory experiment

Article *in* Harmful Algae · November 2016

DOI: 10.1016/j.hal.2016.09.004

4 authors, including:

Guang Gao
Newcastle University
13 PUBLICATIONS **141** CITATIONS
SEE PROFILE

Juntian Xu
HuaiHai Institute of Technology
29 PUBLICATIONS **241** CITATIONS
SEE PROFILE

Harmful Algae 59 (2016) 51–58

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

Changes in morphological plasticity of *Ulva prolifera* under different environmental conditions: A laboratory experiment

Guang Gao, Zhihai Zhong, Xianghong Zhou, Juntian Xu *

Marine Resources Development Institute of Jiangsu, Huaihai Institute of Technology, Lianyungang 222005, China

ARTICLE INFO

Article history:
Received 2 June 2016
Received in revised form 11 September 2016
Accepted 15 September 2016

Keywords:
Growth
Morphology
Salinity
Temperature
Nitrate reductase
Ulva prolifera

ABSTRACT

The large-scale green tides, consisting mainly of *Ulva prolifera*, have invaded the coastal zones of western Yellow Sea each year since 2008, resulting in tremendous impacts on the local environment and economy. A large number of studies have been conducted to investigate the physiological traits of *U. prolifera* to explain its dominance in the green tides. However, little has been reported regarding the response of *U. prolifera* to changing environmental factors via morphological variation. In our experiments, we found remarkable morphological acclimation of *U. prolifera* to various temperature (20 and 25 °C) and salinity (10, 20, and 30) conditions. *U. prolifera* had more, but shorter branches when they were cultured at lower temperature and salinity conditions. To investigate the significance of these morphological variations in its acclimation to changes of environmental factors, physiological and biochemical traits of *U. prolifera* grown under different conditions were measured. Higher temperature increased the relative growth rate while salinity did not affect it. On the other hand, higher temperature did not enhance the net photosynthetic rate whilst lower salinity did. The increased net photosynthetic rate at lower salinity conditions could be attributed to more photosynthetic pigments—chlorophyll *a*, chlorophyll *b*, and carotenoids—in thalli due to there being more branches at lower salinity conditions. Increased numbers of branches and thus an increased intensity of thalli may be helpful to protect thalli from increased osmotic pressure caused by lower salinity, but it led to more shading. In order to capture enough light when being shaded, thalli of *U. prolifera* synthesized more photosynthetic pigments at lower salinity levels. In addition, higher temperature increased nitrate reductase activity and soluble protein content but variations in salinity did not impose any effect on them. Our results demonstrate conclusively that *U. prolifera* can acclimatize in the laboratory to the changes of environmental factors (salinity and temperature) by morphology-driven physiological and biochemical variation. We suggest that the morphological plasticity of *U. prolifera* may be an important factor for it to outcompete other algal species in a changing ocean.

1. Introduction

Green seaweeds started to grow excessively and became a nuisance along the shores of industrialized countries in the 1970s (Fletcher, 1996). This is commonly termed "green tide" because of the color imparted by seaweed suspended in the water. The large-scale green tides, have consecutively invaded the coastal zones of western Yellow Sea since 2008 (Hu and He, 2008; Hu et al., 2010). Due to their frequent occurrences, the consequences of green tides have drawn considerable attention (Liu et al., 2013; Smetacek and Zingone, 2013). Firstly, green tides change the chemistry of seawater (Van Alstyne et al., 2015). The quick absorption of nutrients from seawater leads to states of nutrient limitation for other photosynthetic organisms. The active photosynthesis occurring during the day combined with respiration at night results in large pH fluctuations which can affect other marine organisms (Van Alstyne et al., 2015). Secondly, seaweeds that form green tides commonly produce allelopathic compounds, of which the best documented are dimethylsulfoniopropionate (DMSP), dopamine, reactive oxygen species (ROS) and their breakdown products. DMSP and dopamine function as a defense against herbivores while ROS can be allelopathic (Sieburth, 1964), or toxic to other organisms (Van Alstyne et al., 2015). Therefore, green tides can impose negative effects on other marine organisms including inhibiting the germination of seaweed zygotes (Nelson et al., 2003) and decreasing the growth rates of other seaweed species (Xu et al.,

* Corresponding author. Fax: +86 0518 8558 5003.
E-mail address: jtxu@hhit.edu.cn (J. Xu).

http://dx.doi.org/10.1016/j.hal.2016.09.004
1568-9883/

2013). Further impacts are felt in both benthic (Nelson et al., 2003) and planktonic ecosystems (Jin and Dong, 2003; Wang et al., 2009; Tang and Gobler, 2011), for example, inhibiting the growth of marine bacteria (Sieburth, 1964) and viruses (Lu et al., 2008), reducing the settlement rates of barnacles (Magre, 1974), and increasing mortality rates of crab (Johnson and Welsh, 1985) and oyster larvae (Nelson and Gregg, 2013). Consequently, green tides can have significant impacts on aquaculture and may lead to substantial aquaculture losses, mainly due to toxin production by green tide seaweeds (Ye et al., 2011). For instance, the economic losses during the green tide (2008) in China were approximately 300–400 million RMB for one sea cucumber (*Apostichopus japonicus*) farm in Haiyang, approximately 300 million RMB for one Rushan *Ruditapes philippinarumin* (Japanese carpet shell) farm and around 160 million RMB for a Rushan *Placopecta magellanicus* (American sea scallop) aquafarm (Ye et al., 2011).

Apart from the effects on marine organisms, green tides have detrimental effects on terrestrial animals. The green tides can turn into a sinking morass if they are not removed quickly, and produce toxic hydrogen sulphide (H_2S) from their anoxic interior, which led to the death of a horse on a Brittany beach in 2009 and the death of around 30 wild boars in 2011 (Smetacek and Zingone, 2013). These two incidents were widely reported by the media with some headlines producing the impression that the algae were toxic to human beings. Consequently, the local economy incurred severe losses from the tourist industry, in addition to the costs of removing and disposing of the 100,000 tonnes of beached algae (Charlier et al., 2008). Furthermore, the cost of coping with the green tide occurring in Qingdao before the 2008 Beijing Olympic Games sailing competition was estimated at up to 2 billion RMB, accounting for 5.84% of the total budget (an itemized summary of estimated or intended expenditures for a given period) of Qingdao Municipality in 2008 (Ye et al., 2011).

Ulva is the main genus in the majority of green tides reported (Fletcher, 1996) and *U. prolifera* in particular is the causative species of the world's largest recorded green tide which occurred in Qingdao of China in 2008 (Liu et al., 2009). The fact that it can outcompete other species is usually attributed to its strong nutrient uptake capability. *Ulva* has been considered as an opportunist, characterized by high rates of nutrient uptake and growth (Scanlan et al., 2007). Nutrient uptake and thus growth rate of *Ulva* are commonly quicker than other macroalgae (Rosenberg and Ramus, 1981; Gao et al., 2014; Chen et al., 2015). Meanwhile, the maximal rate of nitrate uptake by *U. prolifera* was observed to be higher than other *Ulva* species (Luo et al., 2012). In addition, *Ulva* has diverse and efficient reproductive strategies that supply a very large number of seeds. *Ulva* can reproduce both sexually and asexually (Lin et al., 2008). Propagation of asexual zoospores and vegetative fragments are particularly effective reproductive routes that give rise to the rapid proliferation of the seaweed under field conditions (Ye et al., 2008; Gao et al., 2010; Zhang et al., 2011). *Ulva* also has a robust capacity to acclimatize itself to a fluctuating environment. For instance, seaweeds generally perform C3-type carbon metabolism while *U. profilera* can operate both C3 and C4 photosynthesis (Xu et al., 2012)—the existence of C4 photosynthesis in algae is rare. This unusual advantage enhances its capacity for carbon fixation and growth under a changing CO_2 environment.

Previous studies have demonstrated the close relationship between morphology variation of *Ulva* and bacteria, in which *Ulva* loses its typical morphology when grown under axenic conditions (Matsuo et al., 2005; Wichard, 2015). In addition, the same species of *Ulva* may have various morphologies at different locations. Leskinen et al. (2004) observed that all true marine samples except one of *U. compressa* were branched, while *U. intestinalis* were never branched in a marine area, but unbranched *U. compressa* and branched *U. intestinalis* were found even in areas of moderately reduced salinity. In addition, Zhang et al. (2013) found four morphological forms of *U. prolifera*, including filamentous, tubular, cystic and folded blades, in the Yellow Sea of China during green tides. Filamentous thalli were the dominant morphology of *U. prolifera* in the South Yellow Sea, while they were the tubular and cystic thalli in the North Yellow Sea. In spite of these studies, little is known about the physiological and ecological significance of the morphological plasticity of *Ulva*. In our study, we found contrasting changes in *Ulva* morphology when they were cultured at various conditions of temperature and salinity. Then we hypothesized that those changes must serve for addressing the environmental variation. To test that, physiological and biochemical traits (growth, net photosynthetic rate, pigments, nitrate reductase, soluble protein, etc.) of *U. prolifera* in various morphological forms were analyzed. Our findings can explain the ecological success of *U. prolifera* from a fresh angle.

2. Materials and methods

2.1. Collection and culture of U. prolifera

Thalli of *U. prolifera* of 5–8 cm in length were collected from the coastal water of Lianyungang (119.3° E, 34.5° N), Jiangsu province, China, where the alga cause green tides (Keesing et al., 2011; Liu et al., 2013; Yuanzi et al., 2014). The temperature was 22 °C and salinity was 28 at the sampling site. The thalli were transported to the lab in a cool box (4–6 °C) within 3 h and then washed gently with 0.2 μm filtered seawater to remove any sediment, epiphytes or small grazers. *U. prolifera* thalli were placed in 500 ml conical flasks (one thallus per flask) and cultured in GXZ-300C intelligent illumination incubators (Jiangnan, Ningbo, China) at fully crossed temperature (20 °C and 25 °C) and salinity (10, 20, and 30) conditions with continuous aeration for four weeks. Therefore, *U. prolifera* were grown under six treatment conditions: low temperature and low salinity (LTLS, 20 °C and 10), low temperature and medium salinity (LTMS, 20 °C and 20), low temperature and high salinity (LTHS, 20 °C and 30), high temperature and low salinity (HTLS, 25 °C and 10), high temperature and medium salinity (HTMS, 25 °C and 20), and high temperature and high salinity (HTHS, 25 °C and 30). The diameter of the 500 ml conical flask used here is 10 cm. It would not limit the growth of *U. prolifera* as the filament of the plant is quite soft and plastic and it usually grows curly rather than straight (Fig. 1). Media were made from natural seawater with the addition of 60 μM $NaNO_3$ and 8 μM KH_2PO_4. The 2 unit gap in salinity between seawater in situ (28) and seawater in laboratory (30) should be due to the evaporation of water during seawater transport and storage. Salinity was adjusted by diluting natural seawater with distilled water. Media were renewed every two days. Natural seawater was collected from the same location as the thalli of *U. prolifera*. Light intensity was set as 240 μmol photons m^{-2} s^{-1}, with a 12L:12D of light and darkness cycle. The light dose used here is comparative to the ambient sunlight dose at Sheyang, Jiangsu province in March (Keesing et al., 2016), as well as that at 0–1 m depth of coastal water at Xiangshui, Jiangsu province in April (Liu et al., 2012). Both sites are where green tides occur and are very close to our sampling site. The experiment was done in triplicate.

2.2. Morphology observation and analysis

After they had acclimatized to various temperature and salinity conditions for four weeks, the morphology of *U. prolifera* was photographed by a Canon camera (EOS 70D, China). Then the number of branches per centimeter primary axis and length of the branches in thalli of *U. prolifera* were measured with photo processing software IMAGE J 1.41. The procedure of using IMAGE J

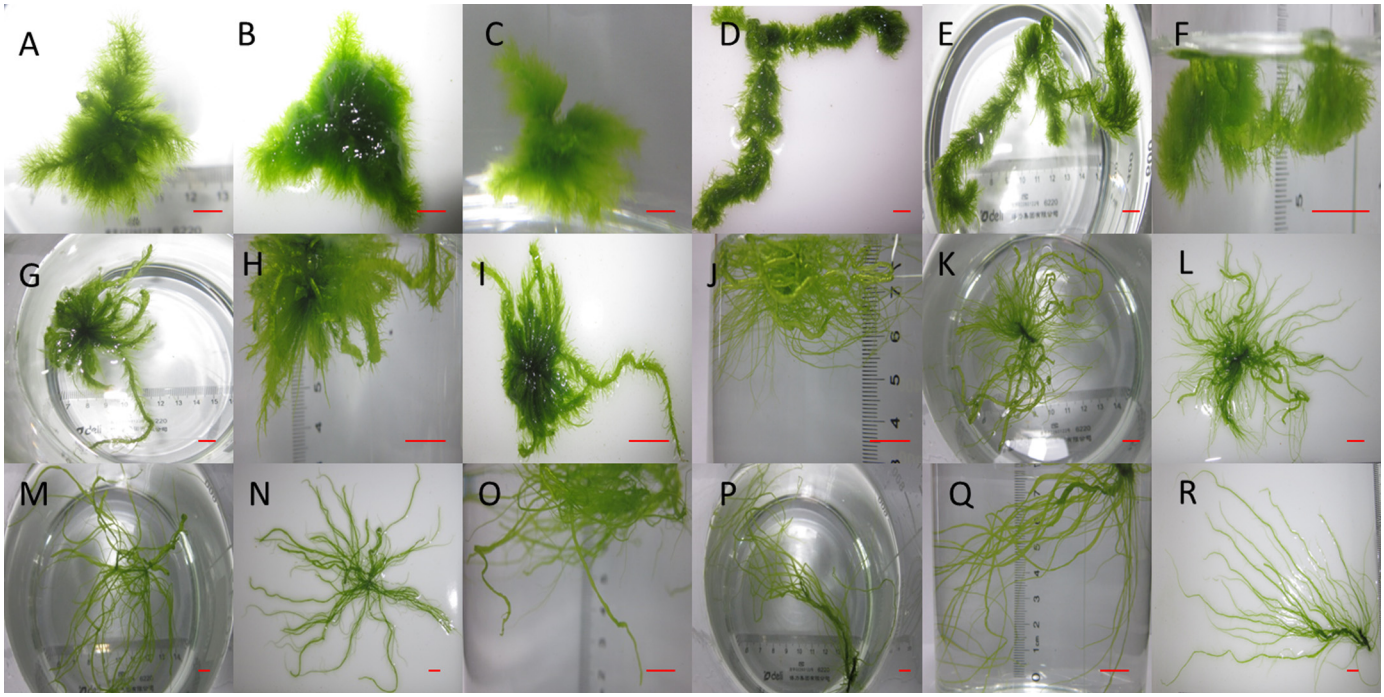

Fig. 1. Morphological variation of *U. prolifera* grown under different temperature and salinity conditions. (A–C) LTLS (20 °C and 10); (D–F) LTMS (20 °C and 20); (G–I) LTHS (20 °C and 30); (J–L) HTLS (25 °C and 10); (M–O) HTMS (25 °C and 20); (P–R) HTHS (25 °C and 30). The scale bars represent 1 cm.

1.41 is as follows: Firstly open photo files of *U. prolifera* in the software, and then use the Analyze/Set Scale dialog to define the spatial scale; afterwards, use the segmented line tool to create a line selection that outlines the branch contour; finally, use the Analyze/Measure command to measure the length of the branch contour. In terms of branch numbers per cm primary axis, obtain the length of the primary axis firstly using the method mentioned above, count the branches on the primary axis and divide the total number by the length of the primary axis to get branch numbers per cm primary axis. The mean value from three repeated operations was taken as one valid value for branch length or number to reduce the error in the operations.

2.3. Measurement of growth

The growth of *U. prolifera* was determined by weighing fresh thalli at the end of the experiment. The thalli of *U. prolifera* were blotted gently with tissue paper to remove water on the surface of the thalli before weighing. The blue tissue paper was used to absorb the surface water of the thalli. It was visible (the color of blue tissue paper changed) if the blue tissue paper absorbed water and it was considered that all surface water was removed when the color of the tissue paper did not change. The relative growth rate (RGR) was calculated by the formula: RGR (%) = $[\ln(W2/W1)]/t \times 100$, where $W2$ is the final weight, $W1$ is the initial weight, and t is the number of culture days.

2.4. Measurement of net photosynthetic rate

Photosynthetic oxygen evolution was measured by using a Clark-type oxygen electrode (YSI model 5300A). Approximately 0.01 g fresh weight (FW) of *U. prolifera* were transferred to a cuvette containing 2 ml of fresh seawater. The salinity and temperature of the fresh seawater was the same as that in which *U. prolifera* was cultured. The light intensity during the measurement was 240 μmol photons m^{-2} s^{-1}. The change of the oxygen content in the seawater within 2 min was recorded. The net photosynthetic rate was presented as μmol O_2 g^{-1} FW h^{-1}.

2.5. Determination of photosynthetic pigments

Approximately 0.03 g FW of *U. prolifera* was extracted with 10 ml of absolute methanol at 4 °C for 24 h in darkness. The sample was not grinded as it has demonstrated that the duration of 24 h is enough to extract the pigments completely by comparing the ungrinded data to grinded data in a preliminary experiment. Concentrations of chlorophyll *a*, chlorophyll *b*, and carotenoids were estimated according to Wellburn (1994).

2.6. Assay of nitrate reductase (NR) activity

NR activity was assayed as described by Corzo and Niell (1991) with some modifications. After the four weeks culture under different conditions, approximately 0.2 g of fresh thalli of *U. prolifera* were transferred to a tube containing 10 ml of fresh medium with a phosphoric acid buffer (pH 7.5) and 0.2 mM $NaNO_3$. The fresh medium was bubbled with pure nitrogen gas for 2 min to remove the oxygen and then kept in the dark for 1 h incubation. Afterwards, 1 ml of reaction solution was mixed with 3 N HCl, 2 ml of 1% (w/v) sulfanilamide, and 2 ml of 0.01% (w/v) N-(1-naphthyl)-ethylenediamine. After 30 min incubation, the amount of nitrite produced was determined by measuring the absorbance at 540 nm with a UV/Visible Spectrophotometer (Ultrospec 3300 pro, GE, U.S.). NR activity was presented as mmol NO_2^- g^{-1} FW h^{-1}.

2.7. Assay of soluble protein

The soluble protein was measured according to Bradford (1976). Approximately 0.2 g thalli were ground in a mortar with 10 ml phosphoric acid buffer (pH 7.0) and centrifuged (5000 × *g*, 15 min, 4 °C). One ml supernatants were mixed with 5 ml 0.8 M Coomassie blue dye G-250 solution. After incubation at 20 °C for 15 min, the absorbance at 595 nm was recorded using a UV/Visible Spectrophotometer (Ultrospec 3300 pro, GE, U.S.). The soluble protein content was calculated based on a standard curve of bovine serum albumin (Sigma, U.S.).

2.8. Statistical analysis

Results were expressed as means of replicates ± standard deviation. Data were analyzed using the software SPSS v.21. 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, $P > 0.05$). Two-way analysis of variance (ANOVA) was conducted to assess the effects of temperature and salinity on number and length of branches, relative growth rate, net photosynthesis rate, chlorophyll *a*, chlorophyll *b*, carotenoids, NR activity, and soluble protein of *U. prolifera*. Tukey HSD was conducted for post hoc investigation. A confidence interval of 95% was set for all tests.

3. Results

When *U. prolifera* was cultured under various temperature and salinity conditions, contrasting morphology was observed (Fig. 1). *U. prolifera* grown under lower temperature and salinity conditions were in an aggregated form while they were well-dispersed at higher temperature and salinity. The details were uncovered via further analysis of Image J. The lower salinity induced more branches in thalli of *U. prolifera* (Fig. 2A and Table 1). For instance, the number of branches was 91 ± 5 cm^{-1} under LTLS, it decreased to 60 ± 4 cm^{-1} under LTMS and further to 35 ± 4 cm^{-1} under LTHS (Tukey HSD, $P < 0.05$). A similar trend was shown at the higher temperature. The lower temperature also led to more branches (Fig. 2A and Table 1). Furthermore, salinity and temperature had an interactive effect on the number of branches (Table 1), which indicates the inducible effects of lower temperature were different at various salinities. For instance, the lower temperature resulted in an increase of 223.81% in branch number at LS whilst they were 331.33% and 417.50% at MS and HS respectively.

In regard to branch length, both temperature and salinity had main effects on it and higher temperature and salinity dramatically increased the branch length (Fig. 2B and Table 1). The branch length was 0.16 ± 0.08 cm (LS), 0.47 ± 0.12 cm (MS), 0.62 ± 0.12 cm (HS) at low temperature and they increased to 4.23 ± 0.79 cm (LS), 7.70 ± 1.12 cm (MS), 12.13 ± 2.01 cm (HS) at higher temperature. Temperature and salinity had an interactive effect (Table 1). Higher temperature increased the branch length by 2439.99% at LS, 1549.99% at MS and at 1867.57% at HS, which indicates the highest positive effect of temperature occurred at LS.

Table 1
Two-way analysis of variance for the effects of temperature and salinity on branch number and length of *U. prolifera*. Temperature*Salinity means the interactive effect of temperature and salinity, df means degree of freedom, *F* means the value of *F* statistic, and Sig. means *P*-value.

Source	Branch number			Branch length		
	df	*F*	Sig.	df	*F*	Sig.
Temperature	1	928.823	<0.001	1	523.713	<0.001
Salinity	2	227.167	<0.001	2	52.772	<0.001
Temperature*Salinity	2	45.493	<0.001	2	42.189	<0.001
Error	12			12		

Table 2
Two-way analysis of variance for the effects of temperature and salinity on relative growth rate (RGR) and net photosynthetic rate of *U. prolifera*. Temperature*Salinity means the interactive effect of temperature and salinity, df means degree of freedom, *F* means the value of *F* statistic, and Sig. means *P*-value.

Source	RGR			Net photosynthetic rate		
	df	*F*	Sig.	df	*F*	Sig.
Temperature	1	5.513	0.037	1	0.670	0.429
Salinity	2	1.381	0.289	2	8.817	0.004
Temperature*Salinity	2	2.097	0.166	2	0.702	0.515
Error	12			12		

At the higher temperature, there was a significant increase in the RGR of *U. prolifera* (Fig. 3A and Table 2). The average RGR at LT was 17.04 ± 2.22% and it rose to 21.09 ± 5.17% at HT. Salinity did not affect the RGR. There was no an interactive effect between temperature and salinity. Contrary to growth, the net photosynthetic rate was affected by salinity rather than temperature (Fig. 3B and Table 2). Post hoc Tukey HSD comparison ($P = 0.05$) showed that the difference of net photosynthetic rate between LS (207.82 ± 37.68 μmol O_2 g^{-1} FW h^{-1}) and MS (170.24 ± 38.79 μmol O_2 g^{-1} FW h^{-1}) or MS and HS (124.16 ± 20.93 μmol O_2 g^{-1} FW h^{-1}) was not significant but the net photosynthetic rate at LS was higher than that at HS. Temperature and salinity did not have an interactive effect on the net photosynthetic rate.

Both temperature and salinity had main effects on chlorophyll *a* (Fig. 4A and Table 3). The higher temperature reduced the content of chlorophyll *a* (0.53 ± 0.11 mg g^{-1} FW) compared with the lower temperature (0.99 ± 0.30 mg g^{-1} FW). Higher salinity also decreased the content of chlorophyll *a*. Post hoc Tukey HSD comparison

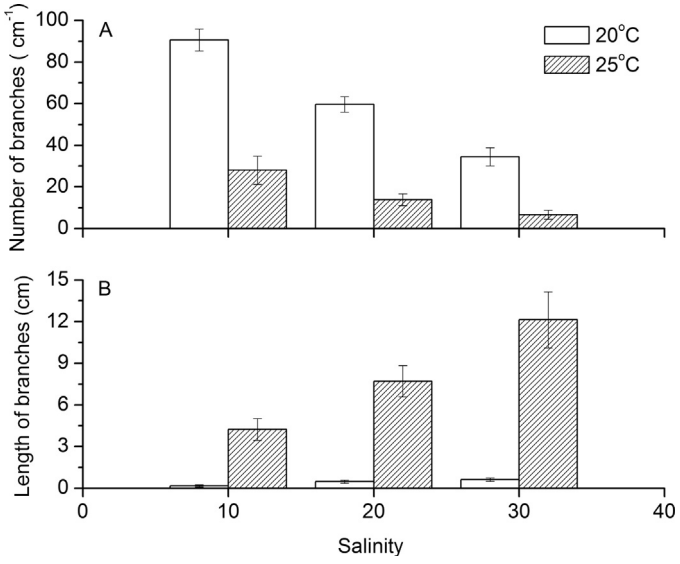

Fig. 2. Number (A) and length (B) of branches in *U. prolifera* grown at various temperature and salinity conditions. The error bars indicate the standard deviations ($n = 3$).

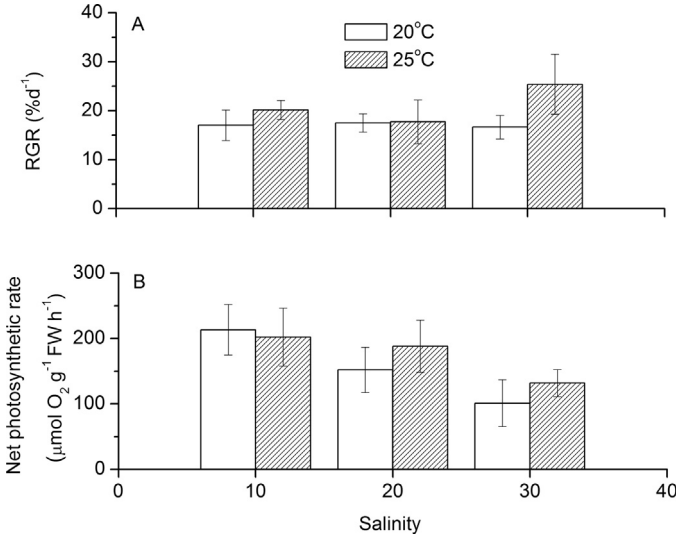

Fig. 3. RGR (A) and net photosynthetic rate (B) in *U. prolifera* grown at various temperature and salinity conditions. The error bars indicate the standard deviations ($n = 3$).

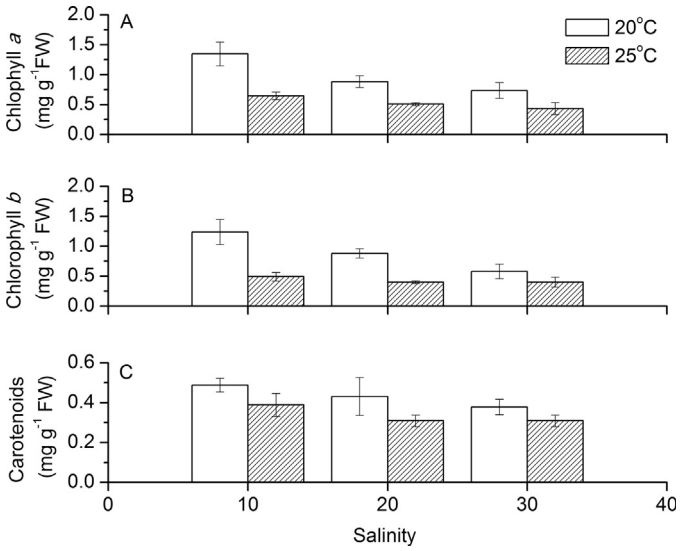

Fig. 4. Chlorophyll *a* (A), chlorophyll *b* (B), and carotenoids (C) in *U. prolifera* grown at various temperature and salinity conditions. The error bars indicate the standard deviations ($n = 3$).

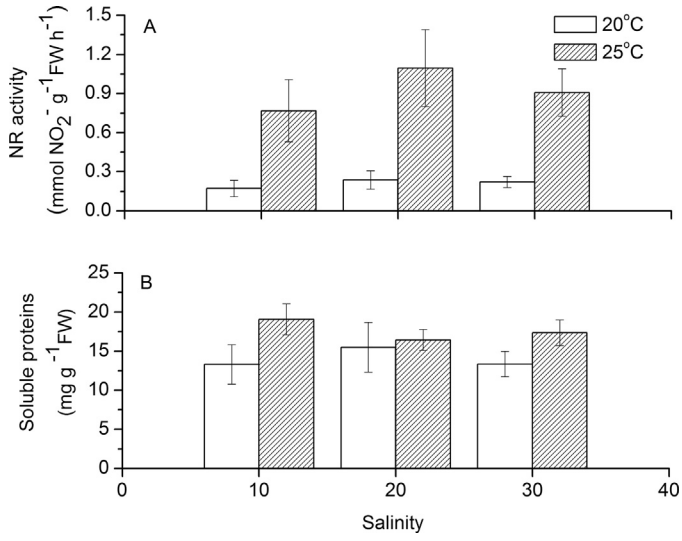

Fig. 5. NR activity (A) and content of soluble protein (B) in *U. prolifera* grown at various temperature and salinity conditions. The error bars indicate the standard deviations ($n = 3$).

($P = 0.05$) showed that the content of chlorophyll *a* at LS (1.00 ± 0.41 mg g^{-1} FW) was significantly higher than that at MS (0.69 ± 0.22 mg g^{-1} FW) and HS (0.58 ± 0.20 mg g^{-1} FW) but the difference between MS and HS was insignificant. Temperature and salinity showed some interaction in their effects on the content of chlorophyll *a* (Table 3). The higher temperature decreased the content of chlorophyll *a* by 52.07% at LS and it was 42.51% at MS and 40.99% HS, which indicates that the highest negative effect of temperature occurred at LS. The pattern for chlorophyll *b* was similar to that of chlorophyll *a* in terms of the effects of temperature and salinity (Fig. 4B and Table 3). Increased temperature reduced the content of carotenoids (Fig. 4C and Table 3). The carotenoids content was 0.43 ± 0.07 mg g^{-1} FW at LT and 0.32 ± 0.07 mg g^{-1} FW at HT. Salinity also decreased the carotenoids content (Fig. 4C and Table 3). Tukey HSD comparison ($P = 0.05$) showed that the content of carotenoids (0.44 ± 0.07 mg g^{-1}) at LS was significantly higher than that (0.32 ± 0.07 mg g^{-1}) at HS. The difference in the carotenoids content between LS and MS (0.37 ± 0.09 mg g^{-1}) or MS and HS was not significant. There was no interactive effect between temperature and salinity.

The NR activity of *U. prolifera* fluctuated between 1.10 ± 0.18 and 0.17 ± 0.06 mmol NO_2^- g^{-1} FW h^{-1} (Fig. 5A). The higher temperature dramatically increased the NR activity (Table 4). The NR activity at the higher temperature was 0.77 ± 0.24 mmol NO_2^- g^{-1} FW h^{-1} (LS), 0.91 ± 0.29 mmol NO_2^- g^{-1} FW h^{-1} (MS), and 1.10 ± 0.18 mmol NO_2^- g^{-1} FW h^{-1} (HS) while they were only 0.17 ± 0.06 mmol NO_2^- g^{-1} FW h^{-1} (LS), 0.24 ± 0.07 mmol NO_2^- g^{-1} FW h^{-1} (MS), and 0.22 ± 0.04 mmol NO_2^- g^{-1} FW h^{-1} (HS) at the lower temperature. Salinity did not have a main effect on NR activity. No interactive effect between temperature and salinity was detected. The content of soluble protein ranged between 13.31 ± 2.52 and 19.06 ± 1.97 mg g^{-1} FW (Fig. 5B). The higher temperature increased the content of soluble protein from 14.05 ± 2.44 to 17.61 ± 1.85 mg g^{-1} FW (Fig. 5B and Table 4). Similar to NR activity, salinity did not affect soluble protein. There was no interactive effect between temperature and salinity.

4. Discussion

4.1. Morphology and growth

Contrasting morphological forms were found when *U. prolifera* was cultured under various temperature and salinity conditions.

Table 3
Two-way analysis of variance for the effects of temperature and salinity on chlorophyll *a*, chlorophyll *b*, and carotenoids of *U. prolifera*. Temperature*Salinity means the interactive effect of temperature and salinity, df means degree of freedom, *F* means the value of *F* statistic, and Sig. means *P*-value.

Source	Chlorophyll *a*			Chlorophyll *b*			Carotenoids		
	df	*F*	Sig.	df	*F*	Sig.	df	*F*	Sig.
Temperature	1	70.571	<0.001	1	75.856	<0.001	1	20.351	<0.001
Salinity	2	20.243	<0.001	2	16.584	<0.001	2	7.658	0.007
Temperature*Salinity	2	5.048	0.026	2	9.445	0.003	2	0.109	0.898
Error	12			12			12		

Table 4
Two-way analysis of variance for the effects of temperature and salinity on nitrate reductase (NR) activity and soluble protein content of *U. prolifera*. Temperature*Salinity means the interactive effect of temperature and salinity, df means degree of freedom, *F* means the value of *F* statistic, and Sig. means *P*-value.

Source	NR activity			Soluble protein content		
	df	*F*	Sig.	df	*F*	Sig.
Temperature	1	73.274	<0.001	1	12.489	0.004
Salinity	2	1.723	0.220	2	0.243	0.788
Temperature*Salinity	2	1.003	0.396	2	1.946	0.185
Error	12			12		

Ulva has been considered a genus with morphological plasticity (Wichard, 2015; Wichard et al., 2015). Previous studies focused on the dominant effects of bacteria on the normal development of *Ulva* morphology. In addition to that, it has been found that abiotic factors can also affect the morphology of *Ulva*. Messyasz and Rybak (2011) investigated the effects of environmental factors on the development of *Ulva* sp. and suggested that the degree of branching of *Ulva* sp. did not depend on sodium chloride concentration but was positively correlated with water temperature. However, the significance of morphological variation in *Ulva*'s acclimation to changes of environmental factors has not been investigated. The present study demonstrated that *U. Prolifera* tended to have fewer but longer branches under conditions of higher temperature and salinity. What is the reason behind that? Moderately high temperature and salinity commonly increase the growth of *Ulva* (Mohsen et al., 1973; Choi et al., 2010; Mantri et al., 2011). The higher temperature did enhance the growth of *U. prolifera* in the present study. The increased growth would lead to shading. The fewer but longer branches could reduce shading and obtain more space for light and nutrient uptake to maintain a relatively high growth rate.

On the other hand, lower temperature and salinity resulted in more but shorter branches. A possible reason for this might be that the slower growth rate at lower temperature and salinity had reduced requirements of light and nutrients and therefore the fewer and longer branches were not needed physiologically. In addition, hyposaline usually decreases the growth of *Ulva* (Martins et al., 1999; Choi et al., 2010; Luo and Liu, 2011; Mantri et al., 2011). However, salinity did not affect the growth of *U. prolifera* in the present study. The lower salinity induced more braches and thalli were in an aggregated form, which increased the density of thalli and thus led to a more stable micro-environment around *Ulva* thalli. This stable micro-environment may protect *Ulva* from the osmotic pressure generated by lower salinity and thus offset the negative effects of lower salinity on growth. The mute effect of salinity with a range of 12.0–30.4 on growth was also reported in *U. prolifera* and *U. intestinalis* in five field surveys in the Yellow Sea (Keesing et al., 2016). In addition, *U. prolifera* was reported to have higher tolerance to desiccation compared with *U. intestinalis* in a field investigation (Keesing et al., 2016). The morphological plasticity of *U. prolifera* displayed in the present study may help us to understand its high tolerance to hyposaline and desiccation and why it can outcompete other *Ulva* species during the development of green tides.

4.2. Photosynthesis and pigments

One unusual finding in the present study is that lower salinity increased the net photosynthetic rate of *U. prolifera* while higher temperature did not. Previous studies have shown that lower salinity can reduce the photosynthetic rate of *Ulva* (Zavodnik, 1975; Liu et al., 2001; Luo and Liu, 2011). The reason for the inconsistency between the present and previous findings could be due to the photosynthetic pigment content. In the present study lower salinity induced more pigment synthesis, including chlorophyll *a*, chlorophyll *b*, and carotenoids. These three kinds of pigment can serve as light-harvesting antennae, and chlorophyll *a* can also be the primary electron donor of the electron transport chain in both photosystems I and II. The increase of these pigments would result in an enhanced photosynthetic rate (Falkowski and Raven, 2013). The reason that lower salinity and temperature induced more pigment could be attributed to the morphological variation of *U. prolifera*. More branches led to more shading. Low light levels commonly induce more pigments in algae (Raven and Geider, 2003; Larkum et al., 2012). Another odd phenomenon is that the higher net photosynthetic rate at lower salinity did not result in increased growth. Growth rate is usually strongly and positively correlated with net photosynthetic rate in plants (Shipley, 2002, 2006). One possible reason that can explain the unconventionality in the present study is that part of the energy generated from photosynthesis might be used to confront the osmotic pressure from lower salinity—morphological variation did not completely offset the negative effects of osmotic pressure—and thus the ATP flowing to carbon fixation and other organic compound synthesis was reduced. Consequently, increased growth did not occur.

In terms of temperature, the photosynthetic rate of *Ulva* usually increases with temperature (Henley, 1992; Kim et al., 2011; Eun Ju, 2016). However, the higher temperature did not increase the net photosynthetic rate of *U. prolifera* in the present study. As shown in the Results section, the higher temperature reduced the pigments content of *U. prolifera*. The mute effect of temperature on photosynthetic rate may be a compromise between enhanced activities of photosynthesis related enzymes and reduced pigment content at higher temperature.

4.3. NR activity and soluble protein

NR activity increased with salinity (from 29 to 37) in seagrasses *Zostera marina* (Touchette and Burkholder, 2001). The increase in NR activity was considered to be related to a Na^+-dependent NO_3^- transport system. Higher salinity could promote the uptake of NO_3^- through the Na^+-dependent NO_3^- transport system, resulting in higher cellular NO_3^- concentration and thus higher NR activity within the leaf (Touchette, 2007). Meanwhile, the addition of K^+, Mg^{2+}, and Ca^{2+} also increased the NR activity in microalgae *Chlorella fusca* (Shafea, 2003). The effect of salinity on the NR activity in *Ulva* has not been documented. In situ an NR activity assay showed that the highest NR activity in *Gelidium* sp. was at the site with a salinity of 20 and lowest at the site with a salinity of 10 (Eun Ju, 2016). The previous studies suggest NR activity would increase with salinity within a certain range. However, the lower salinity did not reduce the NR activity of *U. prolifera* in the present study. Despite the species difference, the possible reason is that *U. prolifera* cultured at lower salinity had a higher net photosynthesis rate which could supply more ATP for the synthesis of NR. NR activity is largely regulated by synthesis of the NR (Berges, 1997). The higher NR content might offset the effect of low salinity on NR activity. Salinity did not affect the content of soluble protein in *U. prolifera* in the present study. The soluble protein is highly related to NR activity in *Ulva* species (Gordillo et al., 2001). Our study displays that *U. prolifera* could maintain a relatively stable NR activity and thus nitrogen assimilation at various salinity conditions by morphological, pigment, and then photosynthesis adjustment.

Gao et al. (2000) examined the temperature dependence of NR activity in several marine phytoplankton species and vascular plants, and found that similar temperature response curves were observed among the chromophytic phytoplankton (*Skeletonema costatum*, *Skeletonema tropicum*, *Thalassiosira Antarctica*, and *Phaeocystis Antarctica*), with optimal temperatures for NR activity being in the range 10–20 °C. In contrast, the optimal temperatures for NR activity in green alga *Dunaliella tertiolecta* and vascular plants (*Cucurbita maxima* and *Zea mays*) were 25–30 °C. These findings indicate dramatic species difference in the optimal temperature for NR activity. The in situ NR activity in *Ulva* sp. increased with temperature (13.7–32.5 °C), suggesting a temperature optimum around 32 °C for NR in *Ulva* sp. Our finding also demonstrated that the RNA at the higher temperature (25 °C) was higher than that at the lower temperature (20 °C). Metabolic theory predicts that the metabolic rates of organisms generally rise exponentially with temperature within a certain range

(Iken, 2012), leading to higher rates for most physiological processes. The higher temperature promoted synthesis of soluble protein in the present study. This is consistent with Mohsen et al. (1973)'s finding in which the protein content of *U. fasciata* also increased with culture temperature (15–25 °C).

5. Conclusions

U. prolifera can acclimatize itself in the laboratory to the changes of temperature and salinity via morphological variation. It produced more but shorter branches at lower salinity to protect itself from osmotic pressure and fewer but longer branches at the higher temperature to obtain more light and nutrients. This can assist in understanding the dominance of *U. prolifera* during the development of green tides. In addition, the branching of thalli has been regarded as the key morphological character to identify *Ulva* species, although it was doubted by some researchers (De Silva and Burrows, 1973; Leskinen et al., 2004). Our findings further confirm that branches cannot be regarded as the key morphological character to identify *Ulva* species.

Acknowledgments

This study was supported by the Chinese National Natural Science Foundation (No. 41476097), the Natural Science Foundation of Jiangsu Province (No. BK20161295), the Jiangsu key Laboratory of Marine Biotechnology (No. 2012HS013) and Priority Academic Program Development of Jiangsu Higher Education Institutions. We sincerely thank Bill Gibson for his contribution to the writing improvement of this paper.[CG]

References

Berges, J., 1997. Miniview: algal nitrate reductases. Eur. J. Phycol. 32 (1), 3–8.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72 (1–2), 248–254.
Charlier, R.H., Morand, P., Finkl, C.W., 2008. How Brittany and Florida coasts cope with green tides. Int. J. Environ. Stud. 65 (2), 191–208.
Chen, B., Zou, D., Jiang, H., 2015. Elevated CO_2 exacerbates competition for growth and photosynthesis between *Gracilaria lemaneiformis* and *Ulva lactuca*. Aquaculture 443, 49–55.
Choi, T.S., Kang, E.J., Kim, J.h., Kim, K.Y., 2010. Effect of salinity on growth and nutrient uptake of *Ulva pertusa* (Chlorophyta) from an eelgrass bed. Algae 25 (1), 17–26.
Corzo, A., Niell, F., 1991. Determination of nitrate reductase activity in *Ulva rigida* C. Agardh by the in situ method. J. Exp. Mar. Biol. Ecol. 146 (2), 181–191.
De Silva, M., Burrows, E.M., 1973. An experimental assessment of the status of the species *Enteromorpha Intestinalis* (L.) Link and *Enteromorpha Compressa* (L.) Grev. J. Mar. Biol. Assoc. UK 53 (4), 895–904.
Eun Ju, K., 2016. Effects of future climate conditions on photosynthesis and biochemical component of *Ulva pertusa* (Chlorophyta). Algae 31 (1), 49–59.
Falkowski, P.G., Raven, J.A., 2013. Aquatic Photosynthesis. Princeton University Press.
Fletcher, R.L., 1996. The occurrence of "green tides"—a review. In: Schramm, W., Nienhuis, P.H. (Eds.), Marine Benthic Vegetation. Springer, Berlin, pp. 7–43.
Gao, S., Chen, X., Yi, Q., Wang, G., Pan, G., Lin, A., Peng, G., 2010. A strategy for the proliferation of *Ulva prolifera*, main causative species of green tides, with formation of sporangia by fragmentation. PLoS ONE 5 (1), e8571.
Gao, Y., Smith, G.J., Alberte, R.S., 2000. Temperature dependence of nitrate reductase activity in marine phytoplankton: biochemical analysis and ecological implications. J. Phycol. 36 (2), 304–313.
Gao, Z., Xu, D., Meng, C., Zhang, X., Wang, Y., Li, D., Zou, J., Zhuang, Z., Ye, N., 2014. The green tide-forming macroalga *Ulva linza* outcompetes the red macroalga *Gracilaria lemaneiformis* via allelopathy and fast nutrients uptake. Aquat. Ecol. 48 (1), 53–62.
Gordillo, F.J.L., Niell, F.X., Figueroa, F.L., 2001. Non-photosynthetic enhancement of growth by high CO_2 level in the nitrophilic seaweed *Ulva rigida* C. Agardh (Chlorophyta). Planta 213 (1), 64–70.
Henley, W.J., 1992. Growth and photosynthesis of *Ulva rotundata* (Chlorophyta) as a function of temperature and square wave irradiance in indoor culture. J. Phycol. 28 (5), 625–634.
Hu, C., Li, D., Chen, C., Ge, J., Muller-Karger, F.E., Liu, J., Yu, F., He, M., 2010. On the recurrent *Ulva prolifera* blooms in the Yellow Sea and East China Sea. J. Geophys. Res. 115 (C5), 640–646.
Hu, C., He, M., 2008. Origin and offshore extent of floating algae in Olympic sailing area. Eos Trans. Am. Geophys. Union 89 (33), 302–303.
Iken, K., 2012. Grazers on benthic seaweeds. In: Wiencke, C., Bischof, K. (Eds.), Seaweed Biology. Springer, Berlin, pp. 157–175.
Jin, Q., Dong, S., 2003. Comparative studies on the allelopathic effects of two different strains of *Ulva pertusa* on Heterosigma akashiwo and Alexandrium tamarense. J. Exp. Mar. Biol. Ecol. 293 (1), 41–55.
Johnson, D.A., Welsh, B.L., 1985. Detrimental effects of *Ulva lactuca* (L.) exudates and low oxygen on estuarine crab larvae. J. Exp. Mar. Biol. Ecol. 86 (1), 73–83.
Keesing, J.K., Liu, D., Fearns, P., Garcia, R., 2011. Inter- and intra-annual patterns of *Ulva prolifera* green tides in the Yellow Sea during 2007–2009, their origin and relationship to the expansion of coastal seaweed aquaculture in China. Mar. Pollut. Bull. 62 (6), 1169–1182.
Keesing, J.K., Liu, D., Shi, Y., Wang, Y., 2016. Abiotic factors influencing biomass accumulation of green tide causing *Ulva* spp. on *Pyropia* culture rafts in the Yellow Sea, China. Mar. Pollut. Bull. 105 (1), 88–97.
Kim, J.H., Kang, E.J., Park, M.G., Lee, B.G., Kim, K.Y., 2011. Effects of temperature and irradiance on photosynthesis and growth of a green-tide-forming species (*Ulva linza*) in the Yellow Sea. J. Appl. Phycol. 23 (3), 421–432.
Larkum, A., Douglas, S., Raven, J.A., 2012. Photosynthesis in Algae. Springer Science & Business Media, Dordrecht.
Leskinen, E., Alström-Rapaport, C., Pamilo, P., 2004. Phylogeographical structure, distribution and genetic variation of the green algae *Ulva intestinalis* and *U. compressa* (Chlorophyta) in the Baltic Sea area. Mol. Ecol. 13 (8), 2257–2265.
Lin, A., Shen, S., Wang, J., Yan, B., 2008. Reproduction diversity of *Enteromorpha prolifera*. J. Integr. Plant Biol. 50 (5), 622–629.
Liu, C.F., Zhang, Z.Y., Lei, Y.Z., 2001. Effects of salinity, light and nutrients on photosynthesis of sterile *Ulva pertusa*. Acta Ecol. Sin. 21 (5), 795–798.
Liu, D., Keesing, J.K., He, P., Wang, Z., Shi, Y., Wang, Y., 2013. The world's largest macroalgal bloom in the Yellow Sea, China: formation and implications. Estuar. Coast. Shelf Sci. 129, 2–10.
Liu, D., Keesing, J.K., Xing, Q., Shi, P., 2009. World's largest macroalgal bloom caused by expansion of seaweed aquaculture in China. Mar. Pollut. Bull. 58 (6), 888–895.
Liu, F., Pang, S., Zhao, X., Hu, C., 2012. Quantitative, molecular and growth analyses of *Ulva* microscopic propagules in the coastal sediment of Jiangsu province where green tides initially occurred. Mar. Environ. Res. 74, 56–63.
Lu, K., Lin, W., Liu, J., 2008. The characteristics of nutrient removal and inhibitory effect of *Ulva clathrata* on Vibrio anguillarum 65. J. Appl. Phycol. 20 (6), 1061–1068.
Luo, M.B., Liu, F., 2011. Salinity-induced oxidative stress and regulation of antioxidant defense system in the marine macroalga *Ulva prolifera*. J. Exp. Mar. Biol. Ecol. 409 (1), 223–228.
Luo, M.B., Liu, F., Xu, Z.L., 2012. Growth and nutrient uptake capacity of two co-occurring species, *Ulva prolifera* and *Ulva linza*. Aquat. Bot. 100, 18–24.
Magre, E.J., 1974. *Ulva lactuca* L. negatively affects *Balanus balanoides* (L.) (Cirripedia Thoracica) in tidepools. Crustaceana 27 (3), 231–234.
Mantri, V.A., Singh, R.P., Bijo, A.J., Kumari, P., Reddy, C.R.K., Jha, B., 2011. Differential response of varying salinity and temperature on zoospore induction, regeneration and daily growth rate in *Ulva fasciata* (Chlorophyta, Ulvales). J. Appl. Phycol. 23 (2), 243–250.
Martins, I., Oliveira, J.M., Flindt, M.R., Marques, J.C., 1999. The effect of salinity on the growth rate of the macroalgae *Enteromorpha intestinalis* (Chlorophyta) in the Mondego estuary (west Portugal). Acta Oecol. 20 (4), 259–265.
Matsuo, Y., Imagawa, H., Nishizawa, M., Shizuri, Y., 2005. Isolation of an algal morphogenesis inducer from a marine bacterium. Science 307 (5715), 1598.
Messyasz, B., Rybak, A., 2011. Abiotic factors affecting the development of *Ulva* sp. (Ulvophyceae; Chlorophyta) in freshwater ecosystems. Aquat. Ecol. 45 (1), 75–87.
Mohsen, A.F., Nasr, A.H., Metwalli, A.M., 1973. Effect of temperature variations on growth, reproduction, amino acid synthesis, fat and sugar content in *Ulva fasciata* delile plants. Hydrobiologia 42 (4), 451–460.
Nelson, T.A., Gregg, B.C., 2013. Determination of EC50 for normal oyster larval development in extracts from bloom-forming green seaweeds. Nautilus 127 (4), 156–159.
Nelson, T.A., Lee, D.J., Smith, B.C., 2003. Are "green tides" harmful algal blooms? Toxic properties of water-soluble extracts from two bloom-forming macroalgae, *Ulva fenestrata* and *Ulvaria obscura* (Ulvophyceae). J. Phycol. 39 (5), 874–879.
Raven, J.A., Geider, R.J., 2003. Adaptation, acclimation and regulation in algal photosynthesis. In: Larkum, A., Douglas, S., Raven, J.A. (Eds.), Photosynthesis in Algae. Springer, Dordrecht, pp. 385–412.
Rosenberg, G., Ramus, J., 1981. Ecological growth strategies in the seaweeds *Gracilaria foliifera* (Rhodophyceae) and *Ulva* sp. (Chlorophyceae): the rate and timing of growth. Bot. Mar. 24 (11), 583–590.
Scanlan, C., Foden, J., Wells, E., Best, M., 2007. The monitoring of opportunistic macroalgal blooms for the water framework directive. Mar. Pollut. Bull. 55 (1), 162–171.
Shafea, A., 2003. Growth and nitrate reductase activity of *Chlorella fusca* cells as affected by long term salinity. Biol. Plant. 46 (3), 423–427.
Shipley, B., 2002. Trade-offs between net assimilation rate and specific leaf area in determining relative growth rate: relationship with daily irradiance. Funct. Ecol. 16 (5), 682–689.
Shipley, B., 2006. Net assimilation rate, specific leaf area and leaf mass ratio: which is most closely correlated with relative growth rate? A meta-analysis. Funct. Ecol. 20 (4), 565–574.

Sieburth, J.M., 1964. Antibacterial substances produced by marine algae. Dev. Ind. Microbiol. 5, 124–134.
Smetacek, V., Zingone, A., 2013. Green and golden seaweed tides on the rise. Nature 504 (7478), 84–88.
Tang, Y.Z., Gobler, C.J., 2011. The green macroalga, *Ulva lactuca*, inhibits the growth of seven common harmful algal bloom species via allelopathy. Harmful Algae 10 (5), 480–488.
Touchette, B.W., 2007. Seagrass–salinity interactions: physiological mechanisms used by submersed marine angiosperms for a life at sea. J. Exp. Mar. Biol. Ecol. 350 (1), 194–215.
Touchette, B.W., Burkholder, J., 2001. Nitrate reductase activity in a submersed marine angiosperm: controlling influences of environmental and physiological factors. Plant Physiol. Biochem. 39 (7), 583–593.
Van Alstyne, K.L., Nelson, T.A., Ridgway, R.L., 2015. Environmental chemistry and chemical ecology of "green tide" seaweed blooms. Integr. Comp. Biol., icv035.
Wang, X.H., Li, L., Bao, X., Zhao, L.D., 2009. Economic cost of an algae bloom cleanup in China's 2008 Olympic sailing venue. Eos Trans. Am. Geophys. Union 90 (28), 238–239.
Wellburn, A.R., 1994. The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. J. Plant Physiol. 144 (3), 307–313.
Wichard, T., 2015. Exploring bacteria-induced growth and morphogenesis in the green macroalga order Ulvales (Chlorophyta). Front. Plant Sci. 6 , http://dx.doi.org/10.3389/fpls.2015.00086.
Wichard, T., Charrier, B., Mineur, F., Bothwell, J.H., De Clerck, O., Coates, J.C., 2015. The green seaweed *Ulva*: a model system to study morphogenesis. Front. Plant Sci. 6 , http://dx.doi.org/10.3389/fpls.2015.00072.
Xu, D., Li, F., Gao, Z., Wang, D., Zhang, X., Ye, N., Zhuang, Z., 2013. Facilitative interactions between the green-tide macroalga *Monostroma arctium* and the red macroalga *Porphyra yezoensis*. J. Exp. Mar. Biol. Ecol. 444, 8–15.
Xu, J., Fan, X., Zhang, X., Xu, D., Mou, S., Cao, S., Zheng, Z., Miao, J., Ye, N., 2012. Evidence of coexistence of C3 and C4 photosynthetic pathways in a green-tide-forming alga, *Ulva prolifera*. PLoS ONE 7 (5), e37438.
Ye, N.H., Zhang, X.W., Mao, Y.Z., Liang, C.W., Xu, D., Zou, J., Zhuang, Z.M., Wang, Q.Y., 2011. 'Green tides' are overwhelming the coastline of our blue planet: taking the world's largest example. Ecol. Res. 26 (3), 477–485.
Ye, N.H., Zhang, X.W., Mao, Y.Z., Zhuang, Z.M., Wang, Q., 2008. Life history of *Enteromorpha prolifera* under laboratory conditions. J. Fish. Sci. China 15 (5), 853–859.
Yuanzi, H., Liang, H., Hailong, W., Jianheng, Z., Jianjun, C., Xiwen, H., Kefeng, Y., Honghua, S., Peimin, H., Dewen, D., 2014. Abundance and distribution of *Ulva* microscopic propagules associated with a green tide in the southern coast of the Yellow Sea. Harmful Algae 39, 357–364.
Zavodnik, N., 1975. Effects of temperature and salinity variations on photosynthesis of some littoral seaweeds of the North Adriatic Sea. Bot. Mar. 18 (4), 245–250.
Zhang, J.H., Huo, Y.Z., Zhang, Z.L., Yu, K.F., He, Q., Zhang, L.H., Yang, L.L., Xu, R., He, P.M., 2013. Variations of morphology and photosynthetic performances of *Ulva prolifera* during the whole green tide blooming process in the Yellow Sea. Mar. Environ. Res. 92, 35–42.
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. Limnol. Oceanogr. 56 (1), 233–242.