DOI: 10.1007/s11099-017-0689-0 PHOTOSYNTHETICA 55 (4): 623-629, 2017

Physiological responses of a green algae (*Ulva prolifera*) exposed to simulated acid rain and decreased salinity

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Abstract

In order to evaluate the combined effects of simulated acid rain (SAR) and salinity on the physiological responses of macroalgae, *Ulva prolifera* was cultured under three salinity treatments (5, 10, 25 ‰) and at different pH, *i.e.*, at pH 4.4 (C), pH 4.4(F), where the pH of the culture increased from 4.4 to approximately 7.8 during the cultivation period, or in absence of SAR at pH 8.2(C), at 100 μmol(photon) m^{-2} s^{-1} and 20°C. Compared to 25‰ salinity, Relative growth rate (RGR) of *U. prolifera* was enhanced by 10‰ salinity, but decreased by 5‰ salinity. No significant differences in RGR were observed between the pH 8.2(C) and pH 4.4(F) treatments, but the chlorophyll *a* content was reduced by SAR. Negative effects of SAR on the photosynthesis were observed, especially under low salinity treatments. Based on the results, we suggested that the *U. prolifera* showed a tolerance to a wide range of salinity in contrast to the low pH induced by acid rain.

Additional key words: chlorophyll fluorescence, growth, hyposalinity, photosynthetic O_2 evolution.

Introduction

Acid rain, the precipitation with pH values lower than 5.60, which is caused mainly by dissolution of SO_2 and nitrogen oxides in the atmosphere, is one of the foremost examples of regional air pollution (Charlson and Rodhe 1982; Felten *et al.* 2013). The southern and southwestern parts of China show particularly high acid depositions (Wang *et al.* 2009a), especially at Zhejiang Province. Both Ningbo (Ding *et al.* 2012) and Jinhua (Zhang *et al.* 2007) received large quantities of acidic inputs, the pH of acid rain was below 4.74 and 4.12 (Luo and Li 2011), especially in the Xiangshan Bay. Frequency of acid rain increased to 98% and the ecosystems have been damaged in some areas. Some effort has been devoted to study the impacts of acid rain on terrestrial plants and soil traits of forests in China (Wang *et al.* 2009b, Chen *et al.* 2012, Ramlall *et al.* 2015). Unfortunately, only a few studies have focused on the impact of acid rain on marine macroalgae, except the one focused on a decline in biodiversity at intertidal zones of Wenzhou induced by acid rain (Gao *et al.* 2016). But to our knowledge, the combined effects of acid rain and salinity, which changes of the global ocean surface (Durack *et al.* 2012), are particularly lacking.

Received 23 January 2016, *accepted* 7 October 2016, *published as online-first* 8 February 2017.
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Abbreviations: Chl – chlorophyll; EPB – epibrassinolide; F_v'/F_m' – effective quantum yield of PSII photochemical efficiency; F_v/F_m – maximal quantum yield of PSII photochemical efficiency; FM – fresh mass; pH 8.2(C) – the treatment without simulated acid rain, the pH was kept at about 8.2; pH 4.4(C) – in the presence of simulated acid rain, the pH of the culture was kept at about 4.4; pH 4.4(F) – in the presence of simulated acid rain, the pH of the culture increased from 4.4 to approximately 7.8 during the cultivation period; P_G – gross photosynthetic rate; P_N – net photosynthetic rate; R_D – dark respiration rate; RGR – relative growth rate; SAR – simulated acid rain; S5 – the treatment with salinity of 5‰; S10 – the treatment with salinity of 10‰; S25 – the treatment with salinity of 25‰.
Acknowledgments: This study was supported by the Natural Science Foundation of Zhejiang Province (LQ15D060002), the National Natural Science Foundation (41606129), the Natural Science Foundation of Anhui Province (1508085QC67), PhD research startup foundation of Ningbo Univ. (F01259144702), Ningbo University "Fisheries" Priority Subject Open Fund of Zhejiang Province (xkzsc1516) and was also sponsored by K.C.Wong Magna Fund in Ningbo University.
Author contributions: On the basis of an original idea from Y.H. Li, the concept of this paper was developed in discussion between all authors. Y.H. Li, D. Wang, X.T. Xu, and X.X. Gao performed the experiments. All the authors contributed to data analysis and discussion and the writing of the paper.

Macroalgae, living in the mid-intertidal to upper subtidal zones, experience variable and diurnal daily exposure and endure long periods of changeable salinity, light, temperature, and desiccation with the evaporative water loss during emersion at low tide (Davison *et al.* 1996, Lee *et al.* 2003). Salinity fluctuation in marine environments, especially in coastal ecosystem, is an abiotic factor with deleterious effects on aquatic organisms. Responses of macroalgae to salinity have been studied in *Sargassum fusiforme* (Xie *et al.* 2016), *Pterocladiella capillacea* (Schmidt *et al.* 2015), and other *Ulva* species (Lartigue *et al.* 2003, Xia *et al.* 2004, Yamochi 2013, Mantri *et al.* 2011), as well as in *Oedogonium* (freshwater green macroalgae) (Lawton *et al.* 2015), showing the species-specific responses to salinity. For instance, low salinity enhanced the zoospore induction, regeneration, and growth of *U. fasciata* (Mantri *et al.* 2011), but reduced the rate of photosynthesis of *U. pertusa* (Yamochi 2013) as well as lowered net oxygen production, which was observed during rapid fluctuations in salinity (Lartigue *et al.* 2003). Moreover, the effects of ocean acidification (lower pH induced by elevated CO_2) on *Ulva* species have been also evaluated. They were mediated by the irradiance level (Xu and Gao 2012, Rautenberger *et al.* 2015), but the lower pH induced by acid rain or by elevated CO_2 showed different effects on the biodiversity in the intertidal zone (Gao *et al.* 2016). However, it is fundamentally unknown how the acid rain mediates effects of salinity changes on macroalgae. *Ulva prolifera* is distributed in the intertidal zones and is known by its tolerance to a wide range of salinity, water temperature, as well as rapid proliferation (Gao *et al.* 2010) and has received more attention, because *U. prolifera* caused the world largest green tide. Additionally, from an economic perspective, *U. prolifera* is sustainable source for the food, bioremediation, integrated aquaculture systems, and potential biofuel production (Bolton *et al.* 2009). In this study, *U. prolifera* was subjected to three salinity stress treatments with static (termed as pH 8.2C and pH 4.4C) or fluctuating pH (termed as pH F). The growth rate, photosynthetic properties, dark respiration, and antioxidant activity after treatments were investigated in order to find out effects of simulated acid rain (SAR) on the physiological performance of *U. prolifera* under hyposaline conditions. We examined occurrence of synergetic or antithetical or no interactive effects between salinity and SAR and reasons for such a performance.

Materials and methods

Plants and culture conditions: *Ulva prolifera*, provided by Xiangshan Xuwen Seaweed Development Co., Ltd. (Xiangshan, Zhejiang province), was collected from the intertidal zone of the eastern gulf, Xiangshan, Ningbo, China (29.5°N, 121.7°E). The thalli were rinsed gently in sterile sea water and cleaned thoroughly in order to remove the epiphytes, attached sediment, and small grazers. The thalli were cultured in filtered (0.22 μm) sea water (salinity of 25‰) collected from the coastal region of Ningbo, China (22.9°N, 121.7°E), enriched with Provasoli medium (Provasoli 1968) at 15°C and PAR of 100 μmol(photon) m^{-2} s^{-1}, with a light/dark period of 12/12 h and aeration with ambient air (400 mL min^{-1}). Then, the 24-epibrassinolide (EPB, 0.2 mg L^{-1}) was added in order to induce gametogenesis and zoosporogenesis and release.

Gametes and zoospores were collected and grown at 20°C and irradiance of 100 μmol(photon) m^{-2} s^{-1} and aerated with air (400 mL min^{-1}). Then thalli, *ca* 10 cm long, were used in the experiment.

Experimental design: The gradients of salinity were set as 5 (S5), 10 (S10), and 25‰ (S25) by diluting sterilized natural sea water with distilled water. Salinity was measured with a visual hand-held refractometer (*Index Instruments*, Ramsey, UK). Prior further experiments, all media were aerated with air for 24 h. During the cultivation periods, increasing algal biomass was constantly taken out and the medium was renewed every 24 h at 13:00 h in order to maintain the pH stable, thereafter termed as pH 8.2(C) in order to distinguish it from the SAR treatments.

For the SAR treatments, the pH was set at 4.4 (Luo and Li 2011). The mixed solution was prepared using H_2SO_4 (1 mol L^{-1}) and HNO_3 (1 mol L^{-1}) in a molar ratio of 2:1 for SO_4^{2-}: NO_3^-. Additionally, two types of changes in SAR conditions were tested: (*1*) pH 4.4(C): the culture medium was renewed at 13:00 h during the cultivation period; (*2*) pH 4.4(F): the medium was not renewed and the increased algal biomass was not removed causing the pH increase from 4.4 to approximately 7.8 during the cultivation period. The pH was measured at the same time (about 13:00 h) every day with a pH meter (*Mettler Toledo*, China), which was calibrated daily with a standard National Bureau of Standards (NBS) buffer (*Hanna*, China) and the variables of pH were shown in the following text table:

The pH values during the culture conditions for different pH treatments.

Conditions	Culture time [h]	pH 4.4	pH 8.2
Everyday change of the media [pH constant; pH (C)]	0	4.34	8.19
	24	4.41 ± 0.04	8.21 ± 0.03
Continuous culture for 3 d [pH fluctuating; pH (F)]	0	4.39	-
	72	7.76 ± 0.03	-

Approximately 0.2 g of fresh mass (FM) of thalli were cultivated in 500 mL of culture medium (aerated with

ambient air of 400 mL min^{-1}) in each treatment at 20°C and 100 μmol(photon)m^{-2} s^{-1}. Each treatment was carried out with three replicates. After 3 d (72 h), the growth and physiological responses were determined.

Growth and chlorophyll (Chl) content: The relative growth rate (RGR) was estimated in the thalli grown under salinity and SAR treatments as follows:

$$RGR\ (\%\ \text{per day}) = 100 \times (\ln W_t - \ln W_{t-1}) / \Delta t$$

where W_t and W_{t-1} are the fresh masses (FM) at time t and t – 1.

Approximately 0.02 g of thalli FM was extracted in 5 ml of absolute methanol overnight at 4°C, then the absorption spectrum of the supernatant was obtained by scanning the sample from 280 to 750 nm with a scanning spectrophotometer (*UV6100A*, *Yuanxi Instrument Co. Ltd*, Shanghai, China). The content of Chl *a*, Chl *b,* and carotenoids (Car) were calculated according to Wellburn (1994).

***In vivo* Chl fluorescence**: A pulse amplitude modulated fluorometer (*Water-PAM*, *Walz*, Germany) was used to determine the maximal (F_v/F_m) and effective (F_v'/F_m') quantum yield of PSII photochemical efficiency, with the former measured after 15-min dark adaptation and the latter measured during the light period with the actinic light of about 100 μmol(photon) m^{-2} s^{-1} (the same as growth irradiance, Zou and Gao 2014).

Dark respiration and photosynthetic oxygen evolution: After 3-d (72 h) incubation under the salinity and pH combinations, both the photosynthesis and dark respiration were measured at about 12:00–15:00 h using a Clark-type oxygen electrode (*Hansatech*, England) at 20°C, which was controlled by a cooling circulator (*Jinghong*, Shanghai, China). Approximately 0.02 g of thalli FM were introduced into the chamber containing 2 mL of fresh medium corresponding to the treatment combination. Dark respiration rate (R_D) was obtained by determining dark O_2 consumption under dark conditions. The net photosynthetic rate (P_N) was measured under growth irradiance of 100 μmol(photon) m^{-2} s^{-1}. The gross photosynthetic rate (P_G) were the sum of the P_N and the R_D. P_N, R_D, and P_G were all expressed in μmol(O_2) g^{-1}(FM) h^{-1}.

Superoxide dismutase (SOD) activity and soluble sugars content: Approximately 0.1 g of FM was sampled to determine the SOD (EC 1.15.1.1) activity by using nitroblue tetrazolium (NBT) method (Li 2000). Each sample was ground in a mortar (precooled to –20°C) with 2 ml of 50 mmol L^{-1} phosphate buffer (pH 7.8) to a homogenate. This procedure was carried out on ice, then the homogenate was centrifuged (12,000 × *g*) for 20 min. Supernatants were removed and transferred to clean 2-mL centrifuge tubes, and stored at –4°C until analyzed. Supernatant (50 μL) was transferred to test tubes along with 3 mL of mixed solution with 2.7 mL of 14.5 mmol L^{-1} methionine, 10 μL of 30 μmol L^{-1} EDTA-Na_2, 90 μL of 50 mmol L^{-1} phosphate buffer (pH 7.8), 100 μL of 2.25 mmol L^{-1} NBT, and 100 μL of 60 mmol L^{-1} vitamin B12. The solution was allowed to react for 20 min at 20°C and irradiance of 100 μmol(photon) m^{-2} s^{-1}. Then the absorbance of the reaction mixture was measured at 560 nm (*UV6100A*, *Yuanxi Instrument Co. Ltd*, Shanghai, China). The SOD activity was calculated as follows:

$$\text{SOD activity [U } g^{-1}\text{(FM)]} = [(A_{CK} - A_E) \times V] / (0.5\ A_{CK} \times W \times V_t),$$

where the A_{CK} and A_E represent the absorbance of control and samples, respectively, V and V_t represent the volume of phosphate buffer (2 mL) and supernatant (50 μL), respectively, used in the reaction. W represents the FM.

The content of soluble sugar was determined by anthracene – ketone – sulphuric acid colorimetric method (Fairbairn 1953). Water extract (1 mL) of *U. prolifera* was added to 3 mL of anthrone reagent (2 g L^{-1}). The mixture was heated at 100°C for 10 min, and its absorbance at 620 nm was read after cooling to room temperature. A calibration curve with glucose was used as a standard ($y = 0.3145 \times OD_{620} - 0.0285$; $R^2 = 0.997$) and the content was expressed as mg g^{-1}(FM).

Data analysis: *Origin 7.0* (*Origin Lab Corp*., Northampton, MA, USA) and *SPSS 18.0* were used for data processing and statistical analysis. Two-way analysis of variance (*ANOVA*) was used to analyze differences between different treatments with 95% confidence level. All data were expressed as means ± SD ($n = 3$).

Results

Growth and Chl content: In the absence of SAR, pH 8.2(C), *U. prolifera* grown under moderate hyposaline (S10) conditions showed the highest growth rate, which was 39% higher than that under S25. The S5 treatment significantly reduced the growth rate, compared to the S25. RGR was also significantly reduced under SAR treatments, especially at pH 4.4(C). Additionally, the negative effect of SAR was accelerated by low salinity. However, compared to pH 4.4(C), the growth rates were significantly enhanced under pH 4.4(F), showing the salinity-mediated negative effects of SAR, which was also affected by duration of acid rain (Fig. 1).

No significant effect of salinity on the Chl *a* content was observed under different salinity treatments, independent of pH treatments with 900 μg g^{-1}(FM) for the pH 8.2(C) treatment. But the content of Chl *a* was significantly reduced by SAR (40–50%). Compared to pH 4.4(C), the Chl *a* content showed a slight increase under pH 4.4(F),

especially under S10 conditions (Fig. 2).

Compared to pH 8.2(C), the ratio of Chl *a/b* decreased under pH 4.4(C), but it was enhanced under pH 4.4(F). The Chl/Car ratio showed the lowest values under pH 4.4(F). There was no significant difference between salinity treatments (Table 1).

Chl fluorescence parameters: For F_v/F_m, there were no significant differences between salinity treatments, independently on pH conditions, but the presence of SAR significantly reduced its values. For F_v'/F_m', a similar trend was observed, as well as the salinity-mediated the effects of SAR, with lower value under hyposaline (S5) and pH 4.4(C) treatment. Compared to pH 4.4(C), both F_v/F_m and F_v'/F_m' showed high values, especially for the former one, but still lower than the values under the pH 8.2(C) treatment (Table 2).

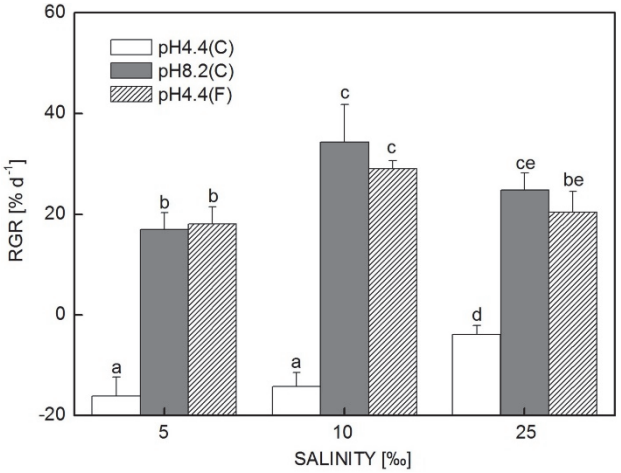

Fig. 1. The relative growth rate (RGR) of *Ulva prolifera* grown under different treatments. *Different letters* indicate significant differences (p<0.05) between different treatments.

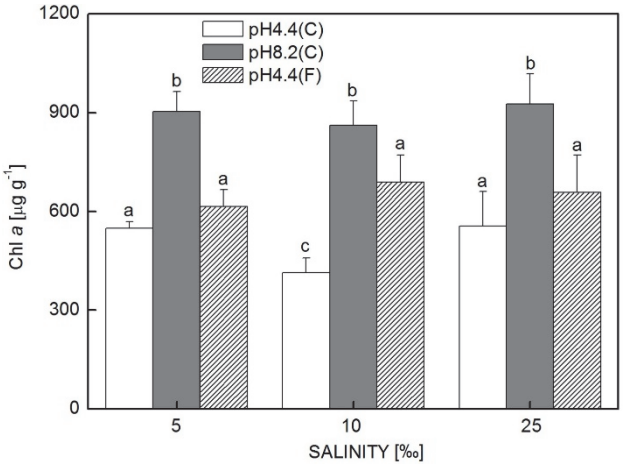

Fig. 2. Chlorophyll (Chl) *a* content of *Ulva prolifera* grown under different treatments. *Different letters* indicate significant differences (p<0.05) between different treatments.

Dark respiration and photosynthetic rate: R_D and P_N showed the highest values under S25 and pH 8.2(C) treatments, with 25 or 145 μmol(O_2) h^{-1} g^{-1}(FM), respectively. The R_D decreased at low salinity (S5, S10) and SAR treatment. Compared to pH 8.2(C) treatments, lower R_D were observed under pH 4.4(F) treatments, but the values were higher in thalli grown under pH 4.4(C), especially under S10 condition (Fig. 3*A*).

Table 1. The ratio between chlorophyll (Chl) *a* to Chl *b* and Chl *a* to cartenoids (Car) of *Ulva prolifera* grown under different treatments. *Different letters* indicate significant difference between different treatments (p<0.05).

pH	Salinity	Chl *a*/*b*	Chl *a*/Car
pH 4.4(C)	5	1.15 ± 0.07^a	5.12 ± 1.00^a
	10	1.14 ± 0.04^a	2.94 ± 0.63^b
	25	1.10 ± 0.02^a	4.58 ± 1.68^a
pH 8.2(C)	5	1.42 ± 0.01^b	3.80 ± 1.33^a
	10	1.30 ± 0.04^{bc}	4.19 ± 1.26^a
	25	1.24 ± 0.01^a	4.54 ± 1.12^a
pH 4.4(F)	5	1.55 ± 0.02^d	2.88 ± 0.99^b
	10	1.57 ± 0.05^d	2.93 ± 0.74^b
	25	1.35 ± 0.10^c	3.59 ± 1.00^a

Table 2. F_v/F_m and F_v'/F_m' of *Ulva prolifera* grown under different treatments. *Different letters* indicated significant difference between different treatments (p<0.05).

pH	Salinity	F_v/F_m	F_v'/F_m'
pH 4.4(C)	5	0.39 ± 0.05^a	0.15 ± 0.01^a
	10	0.40 ± 0.08^a	0.16 ± 0.04^a
	25	0.46 ± 0.06^a	0.27 ± 0.09^{bc}
pH 8.2(C)	5	0.79 ± 0.01^b	0.71 ± 0.01^d
	10	0.79 ± 0.02^b	0.70 ± 0.03^d
	25	0.80 ± 0.05^b	0.66 ± 0.01^e
pH 4.4(F)	5	0.70 ± 0.01^c	0.29 ± 0.02^b
	10	0.72 ± 0.03^c	0.33 ± 0.02^b
	25	0.69 ± 0.03^c	0.23 ± 0.03^c

Similar trends were observed for P_N and P_G, with more significant negative effects of SAR, especially for low salinity treatments, with 86% of P_N declined under S10 or S5 conditions. Significant enhancement was observed under pH 4.4(F), compared to pH 4.4(C) treatments, but no effects of salinity was observed (Fig. 3*B*,*C*).

SOD activity: Compared to S25, the SOD activity decreased by low salinity under pH 8.2(C), but increased by low salinity under the pH 4.4 treatment. Under S5 treatment, pH 4.4(F)-grown algae showed the highest values, but under S10 conditions, no significant differences were found between pH 4.4(F) and pH 8.2(C) treatments (Fig. 4).

Soluble sugars: The content of soluble sugars was reduced by low salinity under all pH conditions, showing the highest values under S25 treatments. The presence of SAR, especially under the constant pH conditions, significantly increased the soluble sugar content without any dependence on salinity treatments (Fig. 5).

Discussion

The present study showed different physiological responses of *U. prolifera* to SAR in association with different salinities, which was influenced by its original history (light, salinity, temperature, *etc.*). The effects of hyposalinity on marine macroalgae differed from species to species and the tolerance was dependent mostly on their grown conditions and acclimation potentials (Karsten 2007). Fluctuations in salinity have a deleterious effect on marine aquatic organisms by affecting reproductive patterns (Steen 2004, Mantri *et al.* 2011), photosynthesis (Yamochi 2013), and growth rates (Mantri *et al.* 2011, Chen *et al.* 2015). In this study, under the control pH treatment, pH 8.2(C), the growth of *U. prolifera* was enhanced by medium hyposalinity (S10), but was significantly reduced by S5 (Fig. 1), showing the limited tolerance to hyposalinity. Compared to S10 and S25 treatments, lower salinity at S5 significantly reduced the photosynthetic and dark respiration rates, increased the energy requirements due to energy demanding osmotic adjustments (Touchette 2007), declined cellular ion content (Simon *et al.* 1999), and reduced thalli cells viability (Chang *et al.* 1999). Additionally, under the low salinity (S5), products of photosynthesis might be inefficient to be utilized for growth and to balance the osmotic pressure caused by declined salinity, as it was shown in *U. fasciata* (Chen *et al.* 2015) and *Griffithsia monilis* (Bisson and Kirst 1979). Actually, there was no difference in soluble sugar contents between salinity treatments. R_D, P_N, and P_G were reduced by low salinity, but the F_v'/F_m' showed high values under hyposalinity treatments. All this indicated that there were different effects of salinity on carbon-fixation reaction and dark-

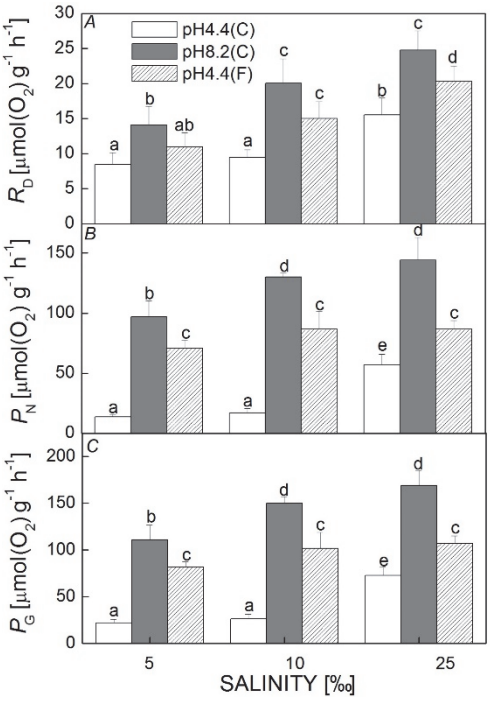

Fig. 3. Dark respiration rate (R_D, *A*), net (P_N, *B*) and gross (P_G, *C*) photosynthetic rates of *Ulva prolifera* grown under different treatments. *Different letters* indicated significant difference between different treatments (p<0.05).

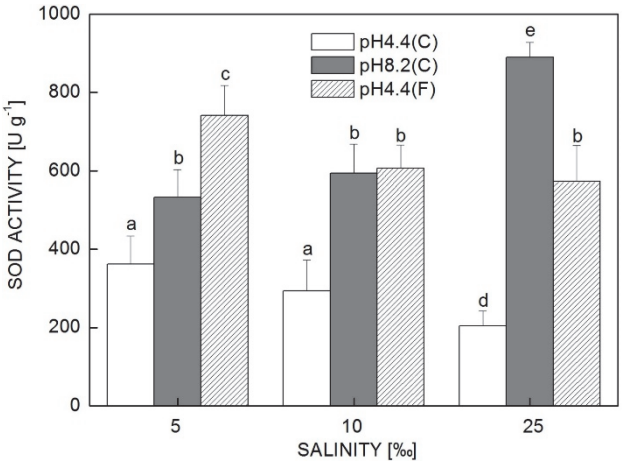

Fig. 4. The superoxide dismutase (SOD) activity of *Ulva prolifera* grown under different treatments. *Different letters* indicated significant difference between different treatments (p<0.05).

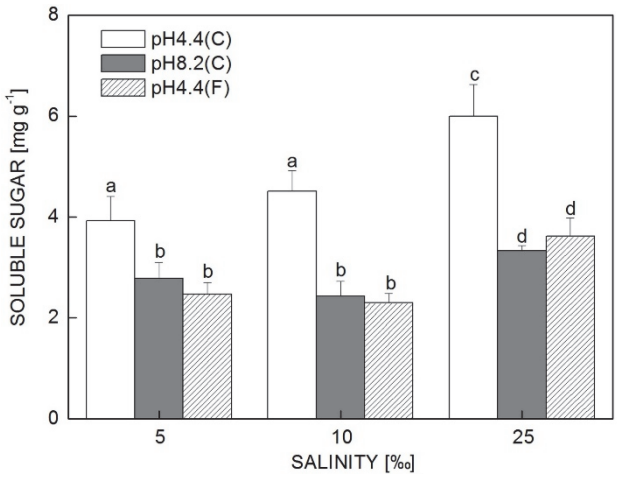

Fig. 5. The soluble sugar content of *Ulva prolifera* grown under different treatments. *Different letters* indicated significant difference between different treatments (p<0.05).

respiration chain. Furthermore, the tolerance of macroalgae to difference in salinity might be species-specific (Mantri *et al.* 2011), and further work are needed.

In terrestrial plants, the stress caused by acid rain can destroy the cell membrane, inhibit the respiration, photosynthesis, and stomatal conductance, and disorder the metabolism of lipids, glucose, and amino acids, as well as the signal transduction (Liu *et al.* 2014). Acidified waters induced by acid rains, are characterized by low pH, low alkalinity, low conductivity, and high aluminum concentration (Gensemer *et al.* 1999, Felten *et al.* 2013), affect the membrane electrochemical potential and enzyme activity (Milligan *et al.* 2009, Wu and Gao 2009), and also change the species richness and community structure (Raut *et al.* 2012, Gao *et al.* 2016). The pigment content of phytoplankton was also reduced by acid rains (Manny *et al.* 1987), which was in accordance with our study (Fig. 2). It should be noted that there was no significant difference in the content of Chl *a* at the pH 4.4(C) and pH 4.4(F), but the higher P_N was observed after the pH 4.4(F) treatment (Fig. 3*B*). The higher SOD activity, especially under the low salinity treatments, might be the reason (Fig. 4). There were no significant effects of salinity on F_v/F_m and F_v'/F_m', but these values were reduced by low pH, suggesting the thalli were experiencing osmotic stress, which also was confirmed by the high soluble sugar content (Fig. 5). Moreover, Rautenberger *et al.* (2015) pointed that both light and low pH had no effect on the F_v/F_m of *U. rigida*, although the F_v/F_m of *U. prolifera* was significantly reduced by low pH in our study, showing the differences between low pH induced by elevated CO_2 or acid rain (Gao *et al.* 2016). The magnitude of the pH changes might be another reason. Additionally, the membrane electrochemical potential and enzyme activities were also influenced by the extracellular pH changes (Milligan *et al.* 2009). Compared to PSII, PSI showed higher tolerance and faster recovery rate to the osmotic stress induced by the sorbitol or by desiccation (Gao *et al.* 2011, Gao *et al.* 2014). However, how they are affected by the acid rain, it needs to be studied yet.

It is well known that soluble sugar can function as an agent to maintain osmotic pressure protecting cells from stress, as an osmoprotector, stabilizing proteins and membranes (Bohnert *et al.* 1995). In our study, no significant effects of salinity or pH changes on the soluble sugar content was observed under pH 8.2(C) and pH 4.4(F) treatments, showing the greater tolerance to salinity (Gao *et al.* 2010) and pH fluctuation. Under the pH 4.4(C) treatment, thalli showed the highest soluble sugar content, especially under S25 treatment, assigning a defense mechanism of rearrangement of biosynthetic metabolic pathways against SAR stress. Under the pH 4.4(C) treatment, the growth and photosynthesis were suppressed, especially under hyposalinity treatments; therefore the starch degradation was the main reason for the increase in the soluble sugar content.

In summary, physiological responses of *U. prolifera* to salinity and simulated acid rain (low pH) were found to be linked to enzyme activity (*e.g.*, SOD activity), respiration and photosynthetic processes. Our study was the first attempt to elucidate the effects of simulated acid rain on the macroalgae grown at different salinity levels. However, further investigations in order to evaluate the simulated acid rain-mediated long-term acclimation of *U. prolifera* to hypo- or hypersalinity stresses are needed.

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