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SHORT COMMUNICATION

Effects of nitrogen and phosphate enrichment on the activity of nitrate reductase of *Ulva prolifera* in coastal zone

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Abstract As one of the main species causing "green tides", *Ulva prolifera* always inhabits in estuarine areas with changes in salinity and nutrients. Reduced salinity may affect directly or indirectly the processes of uptake and assimilation of nitrate, in which the nitrate reductase (NR) activity play the crucial roles. In this experiment, we investigated the different effects of enriched nitrogen and phosphate on NR activity of *Ulva prolifera* at salinity 30, 15, and 5 psu. The results showed that when salinity being lowered NR activity decreased under no enrichment (CT) or PO_4^{3-} enrichment condition. NO_3^- or combination with PO_4^{3-} could significantly enhance NR activity at three salinities, among which the highest value occurred at 15 psu. Enrichment of NH_4^+ significantly decreased NR activity at 30 and 15 psu, but not at 5 psu. The results suggested NR of *Ulva prolifera* could be triggered by NO_3^-, especially at middle salinity, and keep low when exposed under hyposaline or NH_4^+ enrichment for long term to rapidly respond to pulse of NO_3^- in estuarine areas.

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Introduction

Nitrate often exist in eutrophic brackish waters is one of the main nitrogen sources, and its uptake and assimilation by microalgae and macroalgae are enhanced by a series of enzymes, particular nitrate reductase (NR) (Berges and Hageman 1997). Generally the activity of NR could be influenced by both internal (Kaiser and Huber 2001) and external factors, such as salinity, temperature, light and circadian rhythm (Lopes et al. 1997), and it seems that N availability and salinity rather than illumination changes determine the pattern of nitrate reduction (Lartigue and Sherman 2006); however, in situ NR activity in *U. Olivascens* was dependent on UV radiation (Viñegla et al. 2006), and the algae exposed to periods of dryness and direct solar radiation exhibited higher NR activities (Huovinen et al. 2007).

The activity of NR in several seaweeds can be enhanced by nitrate but reduced by ammonium (Solomonson and Barber 1990; Lartigue and Sherman 2005), and when nitrate and ammonium co-exist the net expression of NR may change according to the ratio of two nutrients or species differences (Lartigue and Sherman 2006). Besides, addition of certain small molecules (especially phosphate, EDTA and AMP) can activate NR (Agüera et al. 1999; Mackintosh and Meek 2001).

In every spring and early summer since 2008, "green tides" mainly consisted of *Ulva prolifera* persistently flourish along the coastal regions of norther Yellow Sea, Jiangsu province of China (Han et al. 2013), then floated northward until landed on seashore of Shandong province

and resulted in around 20 million wet tons of algae biomass, which negatively affected the coastal ecosystems after decay (Zhang et al. 2014). As a cosmopolitan species, *U. prolifera* could inhabit in the inshores and estuaries as well as mixed brackish and fresh water, which contained enriched nitrate, ammonium and phosphate from agriculture and animal aquaculture (Pang et al. 2010; Lv et al. 2015). This algae consisted of filamentous shoots with monostromatic hollow cylinders and had higher ratio of surface to volume (S/V), that endow them with high nutrient uptake rates and the potentiality to substantially increase their biomass when exposed to excessive nutrient (nitrogen and phosphorus).

Besides negative effects of salt stress on coastal plant or macro-algae are evaluated by the changes in biomass and photosynthesis, or induction of antioxidant enzymes (Yan et al. 2013; Luo and Liu 2011; Tang et al. 2015), but the change of NR activity in macro-algae under both salt stress and eutrophication has less studied. So it is essential to understand how the reduced salinity and different patterns of eutrophication influence the nitrate assimilation of *Ulva prolifera* inhabiting there, especially the activity of NR. In this experiment, by means of simulating the condition of eutrophic estuarine or coastal areas, we investigated how enrichment treatments affected the activity of NR in *Ulva prolifera* at lowered salinity. The result will help to partially explain why it could flourish and cause "green tides" in these areas, be of value to green feed production and coastal wetlands management.

Method and material

Macroalgal collection and cultivation

U. prolifera was collected in June 2013 from the "green tides" on the sea surface of southern Yellow Sea, the samples were left to acclimate in a temperature-controlled culture room (20 °C, 100 μmol photons/m^2 s) for at least 2 weeks under natural seawater with salinity of 33 collected from the coast of Lianyungang, China, their asexually reproducing shoots (3–5 cm) were used as experimental material.

Experimental set-up

Approximately 0.1 g (fresh weight) of *Ulva* thalli was placed in a 0.5 L round glass bottle, kept at 20 ± 0.5 °C with continuous aeration and at an irradiance of 100 μmol photons/m^{-2} s^{-1} (light: darkness = 12 h: 12 h) in GXZ-260C temperature controlled incubator (Ningbo Jiangnan, China). After being pre-cultured in artificial sea water (ASW, containing about 6.0 μM nitrate and 0.5 μM phosphate) at salinity 30 for 7 days, *Ulva* thalli were exposed to three salinities (30, 15, and 5 psu) with different enrichment treatments shown as Table 1, three replicates were made for each experiment, and the activity of NR was measured after 3 days exposure.

Table 1 Enrichment by phosphate, nitrate and ammonium or their combination

$Pi\text{-}PO_4^{-3}$	N (300 μM)			
	0	$NaNO_3$	NH_4Cl	NH_4NO_3
0 μM	CT	NO_3^-	NH_4^+	NH_4NO_3
60 μM	P	P + NO_3^-	P + NH_4^+	P + NH_4NO_3

Determination of the activity of nitrate reductase (NR)

NR activity in situ was assayed at the middle of the light phase of 3rd day after treatment, the assay was modified from Lartigue and Sherman (2002). Nearly 0.1 g FW of *Ulva* thalli was cut into pieces, placed into a 10 mL phosphorus buffer solution (0.2 M, pH 8.2; instead of artificial seawater at 20 psu used by Lartigue and Sherman (2002)) containing 30 mM KNO_3 and 2.25 % *n*-propanol, then nitrogen gas was pumped into the tube for 2 min to expel air before covering with seal film. The assay tubes were incubated in 20 °C water bath for 1 h in the dark. The production of nitrite (NO_2^-) was then quantified by photometry and NR activity expressed as μg NO_2^{-1} g dw^{-1} h^{-1}.

Analysis of data

All experiments were performed in triplicate with independent replication of data ($n = 3$), the results were analyzed by using Origin 7.5, One-way ANOVA and the *t* test were employed to compare the differences in the activity of NR among various treatments, two-way ANOVA was employed to examine the interaction between different factors.

Results

The long-term effect (3 days exposure) of nitrate, ammonium or phosphate at three salinities (30, 15, and 5 psu) on NR activity were evaluated (Fig. 1a–c). Results of One-way AVONA showed that nitrogen and (or) phosphate enrichment could significantly change the NR activity of *U. prolifera* at three salinities, it seemed that the influence of enrichment increased when salinity decreased (Table 2).

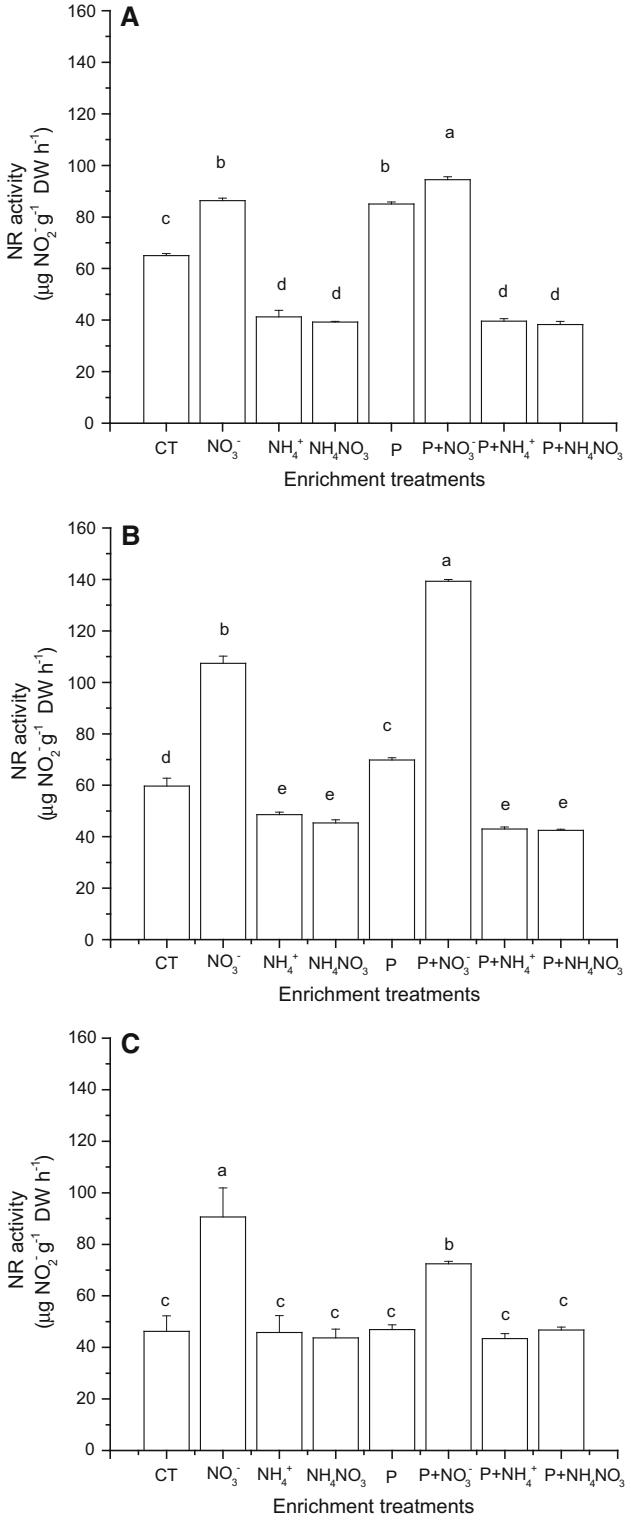

Fig. 1 NR activity in *Ulva prolifera* under different enrichment treatments at salinity (psu) 30 (**a**), 15 (**b**), and 5 (**c**). *Bars* are mean values ± SE ($n = 3$). *Letters* denote treatments under the same salinity that are significantly different (Tukey's test, $P < 0.05$)

Table 2 One-way ANOVAs: NR activity of *U. prolifera* in relation to nutrient enrichment at salinity 30, 15, or 5

Salinity (psu)	DoF	*F*	*P*
30	7	378.19	<0.001
15	7	476.25	<0.001
5	7	536.6	<0.001

Table 3 Two-way ANOVAs: NR activity of *U. prolifera* in relation to phosphate and nitrate at salinity 30, 15, or 5

Salinity (psu)	Source of variation	DoF	*F*	*P*
30	Phosphate	1	223.51	<0.001
	Nitrate	1	267.97	<0.001
	Nitrate × phosphate	1	39.52	<0.001
15	Phosphate	1	94.43	<0.001
	Nitrate	1	733.61	<0.001
	Nitrate × phosphate	1	25.27	<0.001
5	Phosphate	1	116.61	<0.001
	Nitrate	1	1851.83	<0.001
	Nitrate × phosphate	1	135.82	<0.001

When compared with no nitrogen or phosphate supplement (CT), the addition of nitrate (300 μM) as sole nitrogen source significantly increased NR activity at 30 psu ($P < 0.01$), and as much as around twice at 15 or 5 psu. Meanwhile enrichment of phosphate (60 μM) could be significantly raised NR activity at 30 and 15 psu ($P < 0.05$), but had no effect on NR activity at 5 psu ($P > 0.05$). Furthermore under combination enrichment of nitrate (300 μM) and phosphate (60 μM), NR activity increased to higher value when compared with treatments only enriched by nitrate or phosphate at 30 and 15 psu, but decreased to lower value at 5 psu, there existed interaction between nitrate and phosphate at three salinities (Table 3).

NH_4^+ enrichment significantly lowered NR activity when compared with CT at 30 and 15 psu, whether or not there existed NO_3^- or PO_4^{3-}, and no significant differences was observed among these NH_4^+ enrichment treatments at the same salinity ($P > 0.05$); however, ammonium enrichment had no significant effect on NR activity at 5 psu ($P > 0.05$).

Under the same enrichment *Ulva prolifera* expressed different NR activities at three salinities and interaction existed between salinity and nutrient (Table 4). Under CT or PO_4^{3-} enrichment, NR activity of *Ulva prolifera* decreased along with salinity being reduced ($P < 0.05$), the maximum value occurred at 30 psu. On the other hand,

Table 4 Two-way ANOVAs: NR activity of *U. prolifera* in relation to salinity and nutrients (nitrate, ammonium, or phosphate)

Source of variation	DoF	*F*	*P*
Salinity	1	620.01	<0.001
Nitrate	2	32.98	<0.001
Nitrate × salinity	2	29.78	<0.001
Salinity	2	12.54	0.00115
Ammonium	1	66.73	<0.001
Ammonium × salinity	2	21.89	<0.001
Salinity	2	206.78	<0.001
Phosphate	1	79.16	<0.001
Salinity × Phosphate	2	23.28	<0.001

reduced salinity could significantly enhanced NR activity under NO_3^- enrichment ($P < 0.05$), and the highest value of NR activity occurred at 15 psu. Under NH_4^+ treatments, reduced salinity could elevate value of NR activity when compared with 30 psu ($P < 0.05$), and no differences was found between 5 and 15 psu ($P > 0.05$).

Discussions

Effects of reduced salinity on the activity of NR

In this study we found that without nitrogen enrichment NR activity of *U. prolifera* was positively correlated with salinity, and reach the lowest value at 5 psu. Because lowered salinity can cause oxidative stress in *Ulva* (Luo and Liu 2011), and depress the photosynthesis of *Ulva* (Lartigue et al. 2003) or even growth (Martins et al. 1999), that would decrease the content of NADPH and ATP necessary for activating NR in turn.

On the other hand, under hypotonic conditions algal cells may pump monomeric metabolite ions across cell membranes to decrease internal osmotic pressure, (Dickson et al. 1982; Lobban and Harrison 1994), in this processes DIP (phosphate) or DIN (nitrate), essential for maintaining NR activity, could leak from inside (Fig. 1).

Effects of phosphate on the activity of NR

The stimulative effect of PO_4^{3-} only occurred at 30 and 15 psu, that might because PO_4^{3-} could be efficiently absorbed by *U. prolifera* at 30 and 15 psu but not at 5 psu (unpublished data), which might be due to osmosis regulation (Dickson et al. 1982; Lobban and Harrison 1994). Luxury uptake of PO_4^{3-} resulted in high concentration of phosphate inside algae cells and increasing ATP synthesis (Agüera et al. 1999), as well as promoting the transformation of NADH to NADPH (Ahmad and Abdin 1999). P-starved cells could attain much higher nutrient uptake rate and formed PolyP as internal P pool (Nishikawa et al. 2006), which could also act as a substitute for ATP in the sugar metabolism (Phillips et al. 1993). In that, by increasing the concentration of NADPH and ATP or its substitute inside the cells, PO_4^{3-} enrichment could indirectly enhance NR activity. Large amount of phosphate has been released from the soils (Lv et al. 2015), that might trigger much fast assimilation of nitrate in *U. Prolifera* and help this algae dominate the coastal waters.

Effects of nitrate and ammonium

Many experiments have demonstrated that NH_4^+ and NO_3^- were critical inorganic ions which influence NR activity of algae (Lartigue and Sherman 2005, 2006). and NR activity in *Ulva rigida* increased linearly from low values in algae incubated at 0 μM NO_3^- to high values in tissue incubated at 50 μM NO_3^- (Cabello-Pasini et al. 2011). In this study, *Ulva prolifera* expressed higher NR activity when exposed to NO_3^- enrichment at salinity from 5 to 30 psu than CT or NH_4^+ enrichment. This was consistent with the results of Teichberg et al. (2007) who found *Ulva lactuca* being continuously exposed to high nitrate supply seemed be better predisposed to take advantage of excess nitrate through increased NRA and N assimilation than that enriched by high ammonia supply.

NO_3^- showed stronger effect on NR when salinity lowered, that could be due to much more nitrate being absorbed at reduced salinity (unpublished date). In situ survey in estuarine areas, Lartigue and Sherman (2006) also found that NR activity of *E. kylinii* was higher in waters with high concentration of NO_3^- and even very low salinity than that with low concentration of NO_3^- and high salinity. Although lowered salinity have negative effect on NR activity, it seemed to play positive role under nitrate enrichment in activating NR of macro-algae inhabiting in brackish waters.

NR activity of algae can be affected by NH_4^+ in several ways, including inhibition of the transport of nitrates (Cohen and Fong 2004), toxicity due to changes in pH, or negative feedback regulation of nitrate reductase, or direct influence through enzyme degradation and post-translation regulation (Flynn 1991), experiments showed that even low concentration of NH_4^+ could inhibit NR activity of *Gracilaria lemaneiformis* (Chow and Oliveira 2008). In this study, although there co-existed the same molal NO_3^- NH_4^+ enrichment resulted in significant reduction of NR activity at 30 and 15 psu, which was in accordant with the results of survey in the Mobile Bay estuary conducted by Lartigue and Sherman (2006), who found that when the concentration of NH_4^+ higher or similar to that of NO_3^-, NR activity of *Enteromorpha lingulata* decreased, whatever the salinity was about 30 or 15 psu. For NH_4^+ enrichments but had no further

influence on NR at 5 psu, it seemed that there existed same response mechanism of NR in *U. Prolifera* when exposed to either low salinity or NH_4^+, maybe by temporarily modifying to iso-form instead of degradation (Lopes et al. 1997; Lartigue and Sherman 2005), so that when NH_4^+ was depleted or higher salinity returned, NR in *Ulva* could recover its normal form and express high activity as soon as possible to assimilate available NO_3^-.

Conclusions

Results of this experiment provide novel data as to how reduction in salinity and enrichment of nitrogen and (or) phosphorus affect NR activity in long term. At reduced salinity (15 and 5 psu), the activity of NR in *U. Prolifera* was inhibited when there is no enrichment, but enhanced when nitrate enrichment occurred, it suggests that the algae tends to adopt "r-competition strategy" for timely occurrence of nitrate, which may be essential for dealing with changes in salinity and depleting pulse of nitrate from runoff at the same time (Ding et al. 2009). In addition the relatively low but stable activity of NR in *Ulva prolifera* could enable it to endure long-term stress such as lower salinity or higher concentration of NH_4^+, and ensure the enzyme rapidly recover if NH_4^+ is deficiency and NO_3^- available (Fig. 1). So that by depleting pulse nutrients from runoff, *Ulva prolifera* can occupy most niches in brackish environments such as estuaries, adapt to changing salinity, obtain higher growth rates in diluted seawater (Lin et al. 2011), and even reproduce at salinity (Alström-Rapaport et al. 2010). If nutrients supply continued for a long term in estuarine areas, great growth rate of *U. prolifera* might result in huge amount of biomass and even cause "green tides" under certain physical conditions (Liu et al. 2013).

Author contribution statement MZ: performed the experiments and wrote the manuscript. ZPL: designed the experiment. HBS: analyzed data and revised the paper and supported the experiment. YJ: participated the experiment.

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