Marine Pollution Bulletin 149 (2019) 110617

Contents lists available at ScienceDirect

Marine Pollution Bulletin

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

Transcriptomic responses of harmful dinoflagellate *Prorocentrum donghaiense* to nitrogen and light

Lin-jian Ou[a,1], Kai-xuan Huang[a,1], Jing-Jing Li[a], Wen-Yu Jing[a], Hong-Po Dong[b,*]

[a] Research Center of Harmful Algae and Marine Biology, Key Laboratory of Eutrophication and Red Tide Prevention of Guangdong Higher Education Institutes, Jinan University, Guangzhou, 510632, China

[b] State Key Laboratory of Estuarine and Coastal Research, East China Normal University, Shanghai, 200062, China

ARTICLE INFO

Keywords:
Transcriptional responses
Prorocentrum donghaiense
Dinoflagellate
Nitrogen
Light
Diel cycle

ABSTRACT

Prorocentrum donghaiense is a notoriously harmful bloom species in the coastal waters of China. The molecular processes by which *P. donghaiense* assimilates nitrogen (N) are poorly understood. Expression of 12 genes involved in N transport and assimilation in *P. donghaiense* was studied under different conditions of N availability, irradiance and diel cycle. We demonstrated that expression of 5 genes was regulated by N limitation and 7 genes regulated by different N sources or irradiances. The diel cycle had little effect on the regulation of the studied genes. The responses of the genes involved in the ornithine-urea cycle (OUC) were different from those found in model diatoms. Constitutive expression of genes related to N transport and assimilation may assist *P. donghaiense* efficient utilization of different N sources in varying marine environments. The OUC of *P. donghaiense* may play a minor role in the distribution and repackaging of carbon and N.

1. Introduction

Prorocentrum donghaiense Lu is one of the most well-known harmful algal bloom species in the coastal waters of China (Lu et al., 2005; Zhou et al., 2017b). Since the year 2000, large scale *P. donghaiense* blooms have been recorded annually during spring seasons in the Changjiang River Estuary and the adjacent East China Sea (ECS), with records of more than several thousand square kilometers and persisting for over 1 month (Lu et al., 2005; Chen et al., 2013; Liu et al., 2013). These notorious blooms severely affect regional marine ecosystems, marine fisheries and public health (Zhou and Zhu, 2006; Lu et al., 2011).

Eutrophication is considered one of the most important factors responsible for *P. donghaiense* blooms in the ECS (Zhou and Zhu, 2006; Glibert et al., 2012; Liu et al., 2015). Dissolved inorganic nitrogen (DIN) has sharply climbed from 5 μmol L^{-1} in 1959 to 25 μmol L^{-1} in 2010 as a result of large scale effluent of nitrate pouring into the ECS from the Changjiang River Estuary (Strokal et al., 2014; Zhou et al., 2017a). Consequently, the nutrient structure in the region has changed greatly (Glibert et al., 2012; Liu et al., 2015). Excess nitrate is believed to be responsible for the frequent *P. donghaiense* blooms in the ECS (Glibert et al., 2012; Zhou et al., 2017b).

Many studies have examined the physiological and ecological adaptations of *P. donghaiense* for nitrogen (N) utilization and have shown that it is able to utilize a variety of N sources for growth (Hu et al., 2012, 2014; Zhang, 2013). *P. donghaiense* exhibits similar uptake kinetics for nitrate, ammonium, and urea under either *N*-replete or *N*-depleted conditions, although the affinity for urea is higher than for nitrate or ammonium (Zhang, 2013; Hu et al., 2014). Urea contributes more than 20% of the total dissolved N pool in *P. donghaiense* blooms, so urea might be an important N source in these blooms (Glibert et al., 2012; Li et al., 2012). The N content in seawater determines the scale and duration of *P. donghaiense* blooms (Zhou and Zhu, 2006; Zhou et al., 2017a). These observations indicate that an understanding of the mechanisms underlying N metabolism in *P. donghaiense* is important to unravel factors underlying the occurrence of its blooms. However, the molecular mechanisms associated with nutrient metabolism in *P. donghaiense* and other dinoflagellates remain poorly understood due to lack of genomic data as well as complex genome organizations (Janouškovec et al., 2017). Recently, homologs of enzymes in the ornithine-urea cycle (OUC) have been found in transcriptomes of *Alexandrium tamarense* (Dagenais-Bellefeuille and Morse, 2013) and *Gambierdiscus caribaeus* (Price et al., 2016). Jing et al. (2017) investigated four genes involved in N transport and assimilation in *P. donghaiense* and noted their regulatory response to the bioavailability of urea. Meanwhile, Zhang et al. (2015) compared the protein profiles of *P. donghaiense* with differing N status and showed that it possessed a specific ability to regulate

* Corresponding author.
E-mail address: hpdong@sklec.ecnu.edu.cn (H.-P. Dong).
[1] These authors contribute equally.

https://doi.org/10.1016/j.marpolbul.2019.110617
Received 10 January 2019; Received in revised form 7 September 2019; Accepted 19 September 2019
Available online 16 October 2019
0025-326X/

intracellular N metabolism when suffering severe N stress.

P. donghaiense blooms often occur in turbid estuary waters (Zhou and Zhu, 2006; Lu et al., 2011) and therefore it is possible that, besides high nutrient availability, capacity for acclimation to a broad range of irradiance is an important characteristic of this alga (Xu et al., 2010; Hu et al., 2014, 2016). The molecular response of *P. donghaiense* to the mixed effects of N availability and irradiance are poorly understood. Based on transcriptomic data from *P. donghaiense* with differing N status in a previous study (Li et al., 2018), the present study measures six genes related to N transport and assimilation, and six genes related to the OUC under different conditions of N availability, irradiance and diel cycle using quantitative reverse transcription PCR (qRT-PCR). The aim of this study was to investigate the transcriptional regulation of N assimilation and metabolism in *P. donghaiense* in response to N availability and irradiance.

2. Materials and methods

2.1. Culture and experimental design

P. donghaiense (strain: MEL203) was isolated from the Pearl River Estuary, China in 2009, and was maintained in the Algal Collection, Research Center of Harmful Algae and Marine Biology, Jinan University, China. Before formal experiments, the cultures were twice re-inoculated into Aquil artificial seawater medium (enriched with f/2) and were maintained at 21 ± 1 °C under a 12: 12 h light: dark cycle with irradiation of ~100 μmol photon m^{-2} s^{-1}. Penicillin G and streptomycin sulfate were added with final concentrations of 3 and 5 g L^{-1}, respectively, to eliminate bacterial contamination 48 h before each inoculation. The cultures were checked for bacterial contamination at regular intervals under an epifluorescence microscope (Olympus X61, Tokyo, Japan) at 100 × magnification using 4′, 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, US state) stain. About 60 L of *P. donghaiense* during the exponential phase were centrifuged at 260 × *g* for 5 min and the pellets were transferred to fresh artificial f/2 seawater medium. The initial cell densities of *P. donghaiense* were ~2.0 × 10^4 cells mL^{-1}. All treatments were set up in triplicate.

Experiment I investigated high (~880 μmol L^{-1}) and low (~60 μmol L^{-1}) concentrations of NO_3^- in 4.2 L cultures (referred to as 'High-N' and 'Low-N' treatments, respectively). Samples for cell counts, *in vivo* fluorescence, photosynthetic yield of photosystem II (*Fv/Fm*) and NO_3^- concentration analysis were taken on alternate days at 9:00 a.m. Samples for total RNA extraction were taken on days 0, 7, 12 and 15 based on the growth curves of *P. donghaiense* and NO_3^- concentrations in media. Samples for Chlorophyll *a* (Chl *a*) were only taken on later period of days 12 and 15.

Experiment II set out to investigate the effect of various N sources, where NO_3^-, NH_4^+, and urea with final N concentrations of ~880 μmol L^{-1} were added separately into three treatments (referred to as 'NO_3^-', 'NH_4^+' or 'urea' treatments, respectively). *P. donghaiense* in these treatments were again acclimated to grow under light irradiation of ~50 or ~300 μmol photons m^{-2} s^{-1} (referred to as 'Low-light' or 'High-light' treatments, respectively). Samples for N concentrations, *in vivo* fluorescence and *Fv/Fm*, were taken every day, or every few days, at 9:00 a.m. Samples for total RNA extractions were harvested at 9:00 a.m. and 21:00 p.m. on day 11 based on the *in vivo* fluorescence curve for *P. donghaiense*.

2.2. Cell growth and nutrient analysis

Samples for cell counts were fixed in 2% acid Lugol's solution and were then placed in a Palmer-Maloney counting chamber (0.1 mL), and at least 500 cells were counted using a light microscope (Olympus CKX41, Tokyo, Japan). For Chl *a* analysis, 15–20 mL culture samples were filtered through GF/F filters. The Chl *a* was extracted using 90% acetone in the dark for 1 day and were measured using a Turner 720 Fluorometer (California, USA) according to the method of Jespersen and Christoffersen (1987). The *in vivo* fluorescence of 2 mL culture samples were measured directly using the Fluorometer. 1 mL of samples for *Fv/Fm* were monitored with a Phyto-PAM Phytoplankton Analyzer after dark adaption of 5 min (Walz, Effeltrich, Germany).

30 mL culture samples were filtered through pre-combusted GF/F filters (450 °C, 2 h) and filtrates were used for nutrient analysis. Concentrations of NO_3^- and NH_4^+ were analyzed according to the methods of Valderrama (1995). Urea concentrations were measured according to Revilla et al. (2005).

2.3. RNA extraction

About 10^6 cells of *P. donghaiense* were harvested by centrifugation at 5000 × *g* for 5 min and were stored at −80 °C. Total RNA was extracted from frozen cells using total RNA extraction kit (Magen, Shanghai, China) according to the manufacturer's instruction. RNase-free DNAase (Takara, Tokyo, Japan) was used to remove residual DNA contamination in RNA. The extracted RNA was dissolved in RNase-free deionized water and the concentration and purity was measured using a spectrophotometer (Agilent Technologies, California, USA) at wavelengths of 260 and 280 nm. The RNA concentration used in this study was 400–500 ng μL^{-1} and the ratio of $OD_{260/280}$ was 1.9–2.0.

2.4. Sequence assembly, gene choice and quantitative reverse transcription PCR (qRT-PCR)

High-quality reads from transcriptomic sequencing were assembled by Trinity software (https://trinitysoft.net/about-trinity-software) (Grabherr, 2011; Li et al., 2018). The candidate coding regions of the assembled reads were analyzed by TransDecoder (https://omictools.com/transdecoder-tool). The functional annotation of unigenes and open reading frames were achieved using Trinotate (https://omictools.com/trinotate-tool). Twelve genes involved in N transport and assimilation and the OUC were selected, and their sequences were provided in Table S1. Primers were devised and verified using Primer-BLAST and listed in Table 1.

To assay the efficiency of the specific primer sets, qRT-PCR was

Table 1
Primer sequences used for qRT-PCR.

Genes	Primer sequenced (5′-3′)
Nitrogen assimilation-related	
Nitrate reductase (NAR)	NAR-F AGCCTTCTGTGCCTCGAAC
	NAR-R GCTGTGCTTCTCCAACTCG
Nitrite reductase (NIR)	NIR-F GTACTTCACGCACTCGTCGTC
	NIR-R CACCACCAAGGGCTACAACT
Nitrate transporter (NART)	NRT-F CCAGAACTGGTTCACGACGA
	NRT-R AACTTGGACCTGCGAACACA
Ammonium transporter (AMT)	AMT-F TTGAATGCGAGGAAGCCAGT
	AMT-R ATTTCGCGTAAGGTGTGGGT
Glutamine synthetase II (GSII)	GSII-F TTGATTGCTTCCGGTGTCGT
	GSII-R GACGCCGTGGCTGATATGTA
Glutamine synthetase I (GSI)	GSI-F AATGCGATGCACTGACCAGA
	GSI-R CCAACGTCGAGAGAGGTTCC
Urea cycle related	
Urease (URE)	URE-F AGGTGATGTCTTCGCGGATG
	URE-R AGACTGCTCTGACGCTGAAG
Carbamoyl-phosphate synthase (CPS)	CPS-F GTCTCGTGGCTAAGCTCCTC
	CPS-R CTGTGTCTCGTGCGTGAAGA
Arginase (ARG)	ARG-F AGAAGGGCCAGCTCTTGTTC
	ARG-R CCGGCATCCCGTGATAATGA
Argininosuccinate synthase (ARGS)	ARGS-F CGGTCACAATCCTCCCAACA
	ARGS-R CGATATCAGCGACGTGGTCA
Argininosuccinate lyase (ARGL)	ARGL-F TCGAAGGTGAACAGGCCTTC
	ARGL-R GCTGTCTTCGTTTGAGTCGC
Ornithine aminotransferase (ORNA)	ORNA-F ACGTCGTTCCTTCAGTGCAA
	ORNA-R ATTCACCCCTCCTCTCGTCA

performed using each primer set and a dilution series of cDNA from 10^1 to 10^5. Standard curves of all the primer sets showed efficiencies between 90%–110%, with $R^2 > 0.99$. qRT-PCR was performed using a BioRad Realplex Mastercycler (BioRad, California, USA) according to standard methods, as previously described (Guenin et al., 2009).

Cycling parameters were 95 °C for 30 s, 40 cycles of 95 °C for 10 s, 58 °C for 30 s, and melt-curve analysis starting at 60 °C and ending at 95 °C. A 25 μL reaction system contained 0.5 μL of cDNA, 12.5 μL of SYBR Premix Ex *Taq*II master mix (Takara), 1 μL of Forward Primer (10 μmol L^{-1}), 1 μL of Reverse Primer (10 μmol L^{-1}) and 10 μL of sterile Milli-Q water. Hypoxanthine phosphoribosy transferase (HPRT) was used as the endogenous housekeeping gene. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method after normalization to the housekeeping gene (Siaut et al., 2007). Three biological replicates were performed. In experiment I, cells grown on High-N on day 0 were set as the expression control while in experiment II, cells grown on NO_3^- and Low-light at daytime were set as the expression control. The relative expression of genes in each control was regarded as 1.

2.5. Statistical analysis

All statistical analysis was performed with SPSS 19.0 software. A one way ANOVA and Tukey's HSD post hoc test were used to analyze the difference among treatments. A p-value < 0.05 was considered significant, and $p < 0.01$ highly significant. Prior to the analysis, data were tested for normality and homogeneity of variance. Log 10 or square-root transformation of the data were performed prior to any statistical test, when necessary.

3. Results

3.1. Experiment I: Effect of N concentration on N assimilation and the OUC in P. donghaiense

The concentrations of NO_3^- in High-N decreased slowly from 882.0 to 663.6 μmol L^{-1} during the whole experiment, while those in Low-N decreased sharply from the beginning and were lower than 1.0 μmol L^{-1} after day 5 (Fig. S1). Cell densities in High-N increased gradually from the beginning to the end of the experiment. In contrast, cells in Low-N stopped growing after day 3 (Fig. 1). The variation of *in vivo* fluorescence showed similar trends with that of cell densities. Chl *a* per cell showed no difference between treatments on day 12 ($p > 0.05$), but Chl *a* per cell in High-N was about 30 times of that in Low-N on day 15 ($p < 0.01$) (Fig. S2). *Fv/Fm* varied between 0.57 and 0.68 and showed no difference between treatments during the whole experiment ($p > 0.05$) (Fig. S3).

Compared with those in High-N, the expression levels of ammonium transporter (AMT) and glutamine synthetase II (GSII) genes were up-regulated 2 to 4 times ($p < 0.01$) whereas that of nitrate transporter (NART) gene was down-regulated ~3.5 times in Low-N ($p < 0.01$) (Fig. 2). In addition, the expression levels of nitrate reductase (NAR) and glutamine synthetase I (GSI) genes in Low-N were down-regulated slightly after day 12 compared with those in High-N ($p < 0.05$). No difference was observed in the expression levels of nitrite reductase (NIR) between the two treatments ($p > 0.05$).

Genes related to the OUC was also analyzed. The expression levels of arginase (ARG) and carbamoyl-phosphate synthase (CPS) in High-N was up-regulated by 3–4 times and 1.6 times respectively, compared to those in Low-N after day 7 ($p < 0.01$) (Fig. 3). But, the gene expression levels of urease (URE), ornithine aminotransferase (ORNA), argininosuccinate synthase (ARGS), and argininosuccinate lyase (ARGL) in High-N didn't change much compared with those in Low-N ($p > 0.05$).

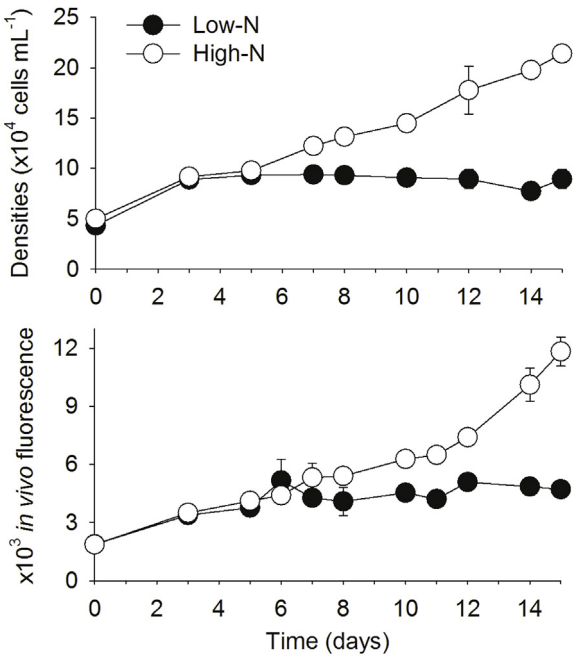

Fig. 1. Variations of densities and *in vivo* fluorescence of *Prorocentrum donghaiense* over time, under high and low nitrogen (N). Treatments were supplemented with ~880 μmol L^{-1} NO_3^- (High-N) and ~60 μmol L^{-1} NO_3^- (Low-N).

3.2. Experiment II: Combined effects of N source and irradiance on N acclimation and the OUC in P. donghaiense over a diel cycle

The concentrations of different N sources in all treatments were higher than 200 μmol L^{-1} at the end (data not shown here), suggesting that N was not limiting during the whole experiment. The *in vivo* fluorescence was similar between High- and Low-light (Fig. 4). However, the *in vivo* fluorescence on urea was significantly higher than that on NO_3^- or NH_4^+ during days 3–6 in High-light ($p < 0.01$), and also so on days 7 and 11 in Low-light ($p < 0.05$). *Fv/Fm* varied from 0.48 to 0.70 and values in Low-light were significantly higher than those in High-light ($p < 0.05$) (Table 2) (Fig. S4).

The expression levels of the AMT gene in NH_4^+ and urea supplements were up-regulated significantly than that on NO_3^- during both day and night ($p < 0.05$) (Fig. 5). AMT gene expression didn't change much between High- and Low-light ($p > 0.05$). In contrast, NART gene expression on NH_4^+ and urea were down-regulated by about twice compared with that on NO_3^- ($p < 0.05$), and this trend was strengthened in Low-light and night. Interestingly, no difference was observed in gene expression of NAR or NIR grown on different N sources irrespective of light intensities or the diel cycle ($p > 0.05$). During day-time, the expression level of GSI on NO_3^- was up-regulated by about 3 times compared with that on NH_4^+ in both light treatments ($p < 0.01$) but it showed no difference with that on urea. However, at night, the level of GSI on NO_3^- was up-regulated compared to that on NH_4^+ or urea only in High-light ($p < 0.05$). The regulation of GSII gene was dependent on both N source and light intensity. The expression level of GSII on NH_4^+ or urea were down-regulated more than twice compared with that on NO_3^- in Low-light during the day time ($p < 0.01$), whereas at night its expression on NH_4^+ or urea were down-regulated by about 2–8 times than that on NO_3^- in both High- and Low-light ($p < 0.05$).

Among the six genes related to the OUC, only three were modulated by the N source and/or light level (Fig. 6). URE, ARGS and ARGL did not change significantly among N sources, light intensities or diel phase ($p > 0.05$). During the day, the expression level of ORNA gene on NO_3^- were up-regulated more than 5 times compared with that on

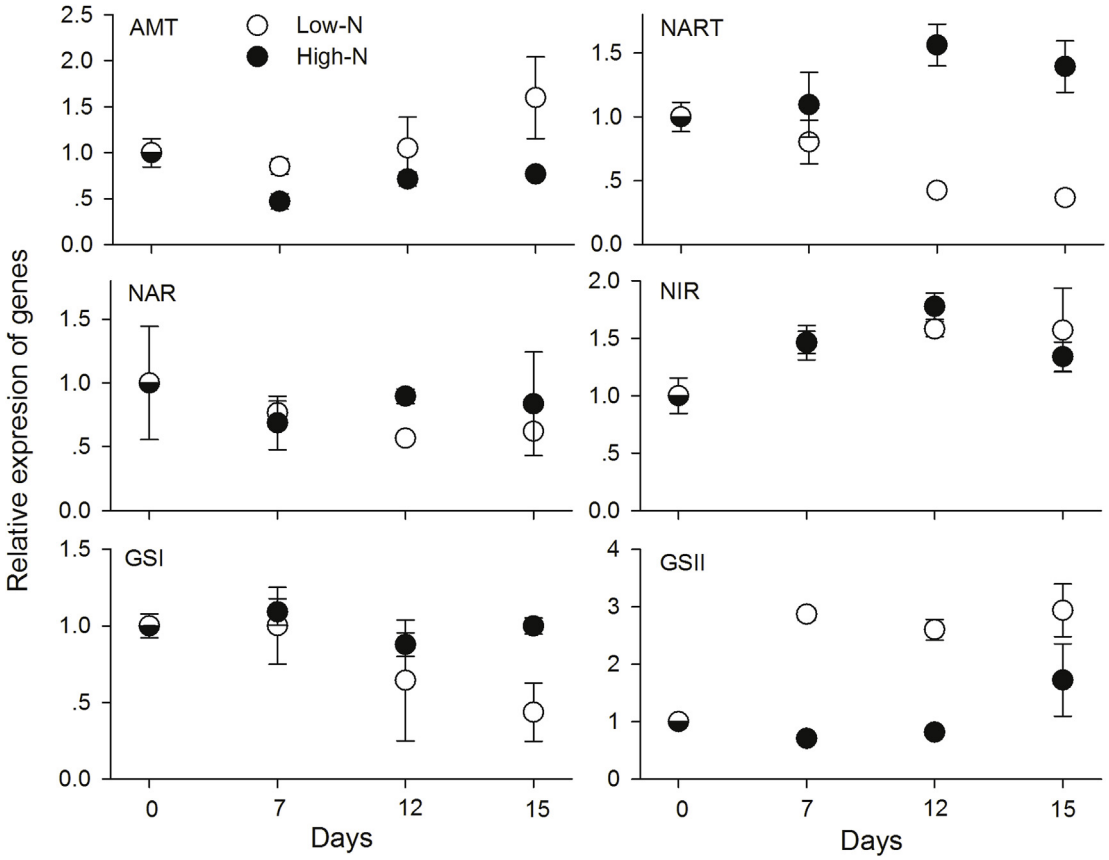

Fig. 2. Relative expressions of nitrogen (N) assimilation related genes on days 0, 7, 12 and 15. Treatments were supplemented with ~880 μmol L^{-1} NO_3^- (High-N) and ~60 μmol L^{-1} NO_3^- (Low-N). Ammonium transporter (AMT), nitrate transporter (NART), nitrate reductase (NAR), nitrite reductase (NIR), glutamine synthetase I (GSI) and glutamine synthetase II (GSII). The expression of all samples is normalized against High-N on day 0 (to the value of 1).

NH_4^+ in High-light ($p < 0.05$) whereas no differences were observed in ORNA gene expression on different N sources in Low-light ($p > 0.05$). However, at night, the expression level of ORNA gene on NH_4^+ or urea were down-regulated about 4–5 times in High-light ($p < 0.05$) whereas its expression on NH_4^+ was up-regulated 12 times compared with that on NO_3^- in Low-light ($p < 0.05$). The expression level of ARG gene on NH_4^+ or urea was down-regulated about 3 times compared with that on NO_3^- in Low-light during the day ($p < 0.05$). When comparing diel phase, in Low-light, the expression level of CPS gene on urea during the day was significantly higher than that on urea at night. In order to present changes of gene expression more clearly under different conditions, a schematic diagram was provided in Fig. 7 showing the pathways of N assimilation and OUC.

4. Discussion

As in most dinoflagellates, *P. donghaiense* stopped growing when N was limited. The Chl *a* contents of *N*-depleted cells were much lower than those in *N*-replete cells, which suggests that during N depletion, Chl *a* was degraded and its N was recycled to other metabolic processes. However, cell densities and *Fv/Fm* did not change after the N concentration in media was depleted for many days. This result contrasts with that observed in most other phytoplankton, in particular diatoms (Hockin et al., 2012; Dong et al., 2013, 2015). Jing et al. (2017) also found no significant differences in *Fv/Fm* in *P. donghaiense* between *N*-replete and *N*-limited conditions in an 8-day experiment. These results suggest that, in contrast to diatoms, the dinoflagellate *P. donghaiense* might preserve its high photochemical efficiency even when N is severely depleted; this characteristic might favor the maintenance of a long bloom period. Furthermore, the *in vivo* fluorescence was similar between the two light treatments, and the *Fv/Fm* in Low-light (50 μmol photons m^{-2} s^{-1}) was even higher than that in High-light (300 μmol photons m^{-2} s^{-1}), which suggests that *P. donghaiense* acclimates well to low light conditions. Liu et al. (2011) suggested that *P. donghaiense* is able to acclimate to different light conditions and observed the highest growth rates at 70 μmol photons m^{-2} s^{-1}. Xu et al. (2010) found that *P. donghaiense* was able to grow even when the irradiance was as low as 2 μmol photons m^{-2} s^{-1}. Together, these observations help to explain why *P. donghaiense* blooms may occur during cloudy and rainy weather, as well as in turbid waters, and last more than 1 month after the nutrients in seawater are depleted.

Hu et al. (2014) found that the uptake dynamics of nitrate, ammonium and urea in *P. donghaiense* under *N*-limited and *N*-replete conditions were similar. Glibert et al. (2012) suggested that *P. donghaiense* does not exhibit high affinity for any N sources but its capacity for affinity regulation is high. These physiological observations suggest that the N transporters of *P. donghaiense* are not acutely responsive to variations in N. *P. donghaiense* stopped growing after day 3 in Low-N, and the N status of *P. donghaiense* varied from N stress (day 5) to N limitation (day 12). The expression of AMT and GSIIs in *N*-depleted cells were up-regulated relative to those in *N*-replete cells after day 7. This is consistent with observations in *N*-starved *Karenia brevis* (Morey et al., 2011). Our results were consistent with increased expression of the AMT gene observed in *N*-starved diatoms *Phaeodactylum tricornutum* and *Cylindrotheca* sp., and haptophyte *Isochrysis galbana* (Hildebrand, 2005; Kang et al., 2007). Thus, AMT is induced by N stress in different phytoplankton phyla. Glutamine synthetase (GS) catalyzes the condensation of glutamate with ammonia to yield glutamine. The expressions of GSI and GSII showed different responses to N stress in this study. Expression of different types of GS seems to vary to *N*-starvation

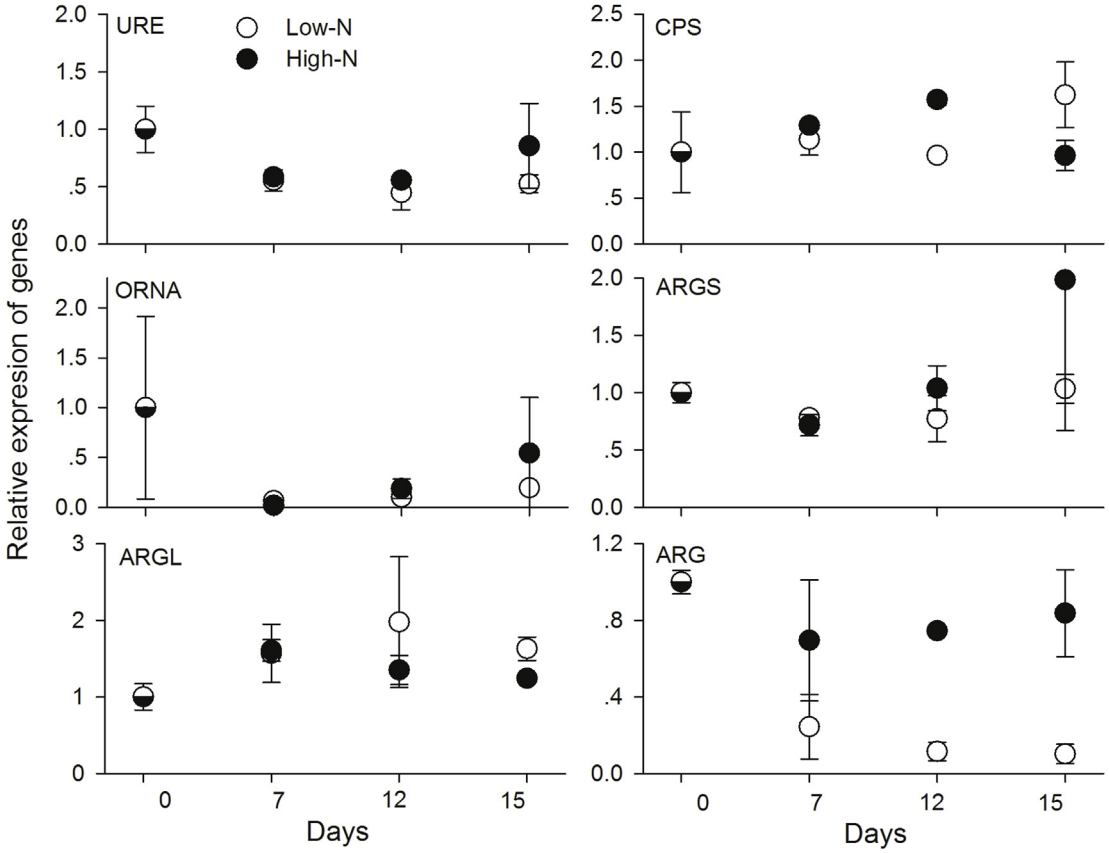

Fig. 3. Relative expression of ornithine-urea cycle related genes on days 0, 7, 12 and 15. Treatments were supplemented with ~880 μmol L^{-1} NO_3^- (High-N) and ~60 μmol L^{-1} NO_3^- (Low-N). Urease (URE), carbamoyl-phosphate synthase (CPS), ornithine aminotransferase (ORNA), argininosuccinate synthase (ARGS), argininosuccinate lyase (ARGL) and arginase (ARG). The expression of all samples is normalized against High-N on day 0 (to the value of 1).

in microalgae. In *K. brevis,* only GSIII gene expression was up-regulated, while GSI and II did not respond (Morey et al., 2011). In contrast, GSII transcription was reduced in *N*-starved diatom *Skeletonema costatum* (Takabayashi et al., 2005). It is likely that different GS types have different roles in intracellular N utilization. Surprisingly, NART gene expression was down-regulated in the *N*-limited condition in this study. Jing et al. (2017) reported that NART transcription in *P. donghaiense* was strongly up-regulated under limitating conditions of either nitrate or urea. NART expression was also observed to increase in *N*-depleted *K. brevis, I. galbana,* and *Cylindrotheca* sp. cells (Poulsen and Kroger, 2005; Morey et al., 2011). Two possible explanations could be suggested. We speculated that *P. donghaiense* might have more than two

Table 2
Fv/Fm of *Prorocentrum donghaiense* in treatments containing different nitrogen (N) sources (NO_3^-, NH_4^+ or urea) supplemented to ~880 μmol N L^{-1} and under different light irradiation (High-light, ~300 μmol photons m^{-2} s^{-1}; Low-light, ~50 μmol photons m^{-2} s^{-1}).

Treatments	Nitrogen added		
	NO_3^-	NH_4^+	Urea
High-light	0.52 ± 0.03	0.48 ± 0.02	0.48 ± 0.01
Low-light	0.69 ± 0.01	0.55 ± 0.01	0.67 ± 0.01

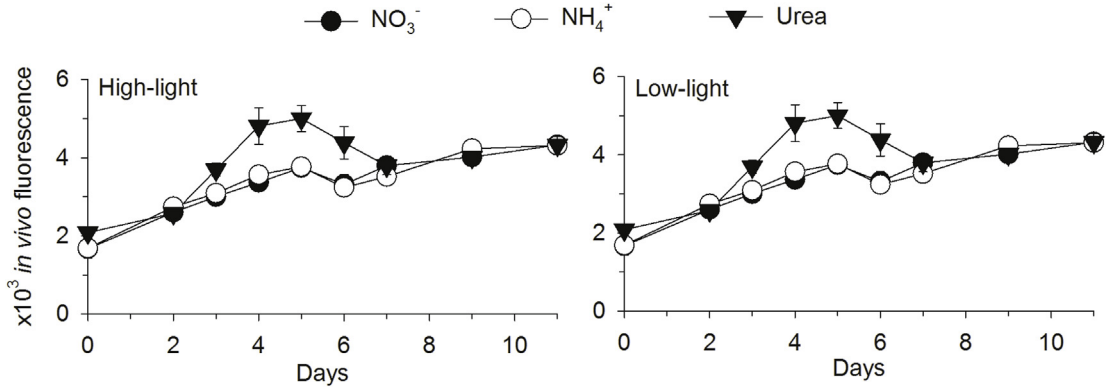

Fig. 4. Variations of *in vivo* fluorescence of *Prorocentrum donghaiense* with time supplemented with various nitrogen (N) sources and under high and low light. N sources supplement with ~880 μmol N L^{-1} of NO_3^-, NH_4^+ or urea, exposed to different light irradiation (Low-light, ~50 μmol photons m^{-2} s^{-1}; High-light, ~300 μmol photons m^{-2}·s^{-1}).

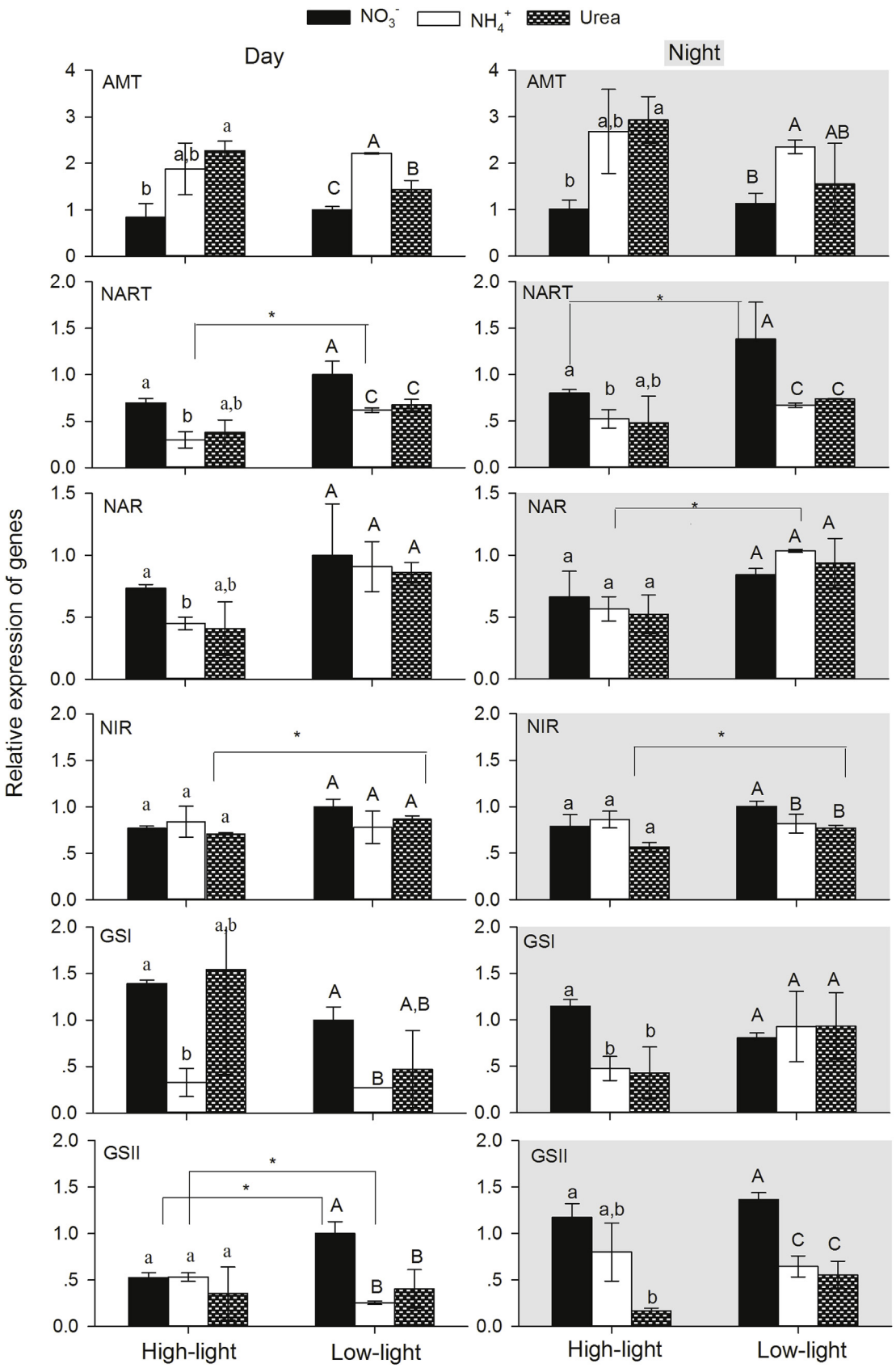

Fig. 5. Relative expression of genes involved in nitrogen (N) assimilation in the treatments of *Prorocentrum donghaiense* supplemented with various N sources and under high and low light. N sources supplement with ~880 μmol N L^{-1} of NO_3^-, NH_4^+ or urea, exposed to different light irradiation (Low-light, ~50 μmol photons m^{-2} s^{-1}; High-light, ~300 μmol photons m^{-2} s^{-1}). The left panel shows day samples (9:00 a.m.) and the right panel night samples (21:00 p.m.). The expression of all samples is normalized against NO_3^- in the daytime at Low-light (to the value of 1). Significant differences ($p < 0.05$) among different N sources in High- and Low-light are shown with different lowercase and uppercase letter labels respectively. Significant differences between High- and Low-light in the same N source are shown with asterisk.

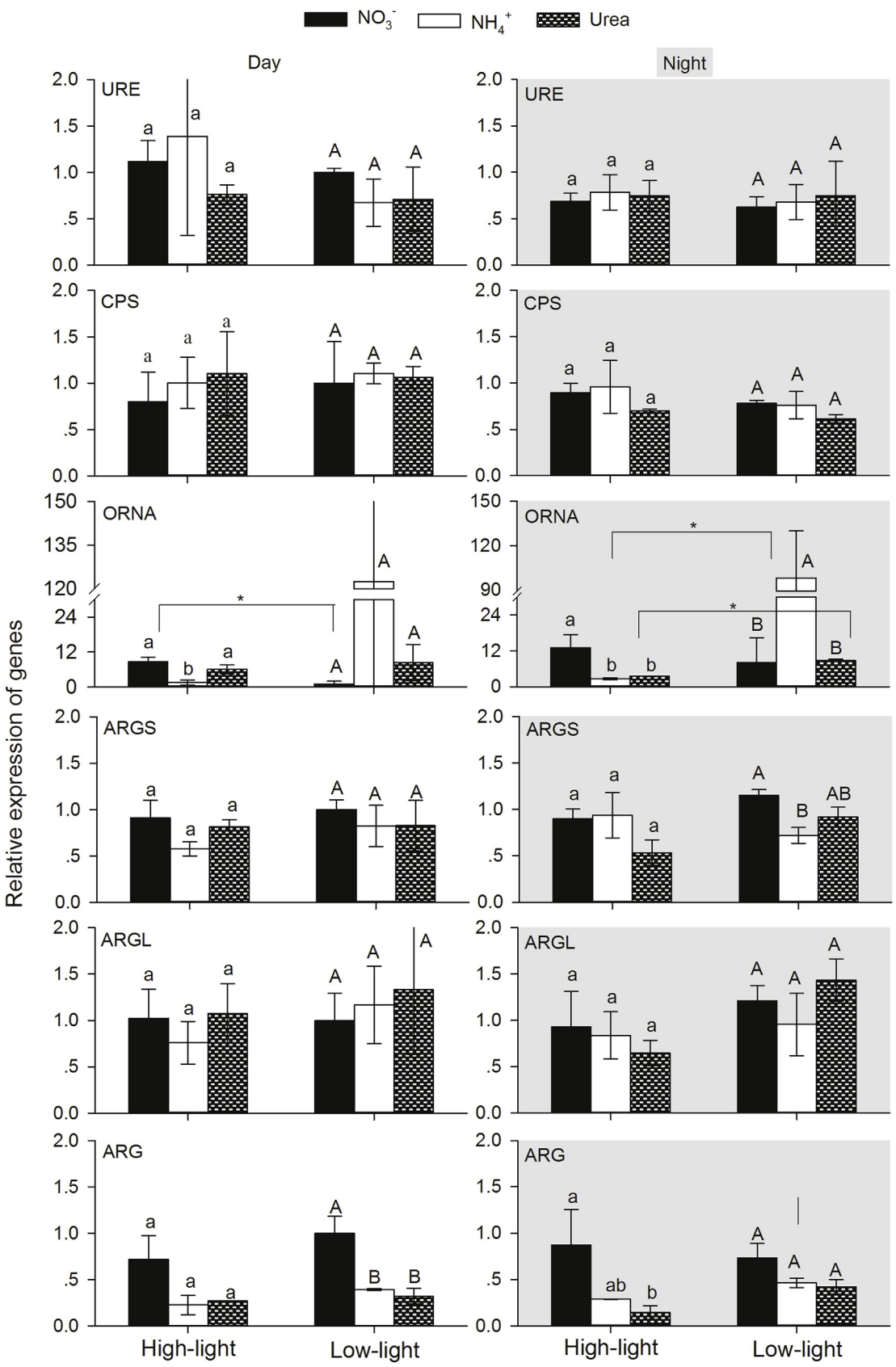

Fig. 6. Relative expression of genes involved in the OUC in the treatments of *Prorocentrum donghaiense* supplemented with various nitrogen (N) sources and under high and low light. N sources supplement with ~880 μmol N L^{-1} of NO_3^-, NH_4^+ or urea, exposed to different light irradiation (Low-light, ~50 μmol photons m^{-2} s^{-1}; High-light, ~300 μmol photons m^{-2} s^{-1}). The left panel shows day samples (9:00 a.m.) and the right panel night samples (21:00 p.m.). The expression of all samples is normalized against NO_3^- in the daytime at Low-light (to the value of 1). Significant differences ($p < 0.05$) among different N sources in High- and Low-light are shown with different lowercase and uppercase letter labels respectively. Significant differences between High- and Low-light in the same N source are shown with asterisk.

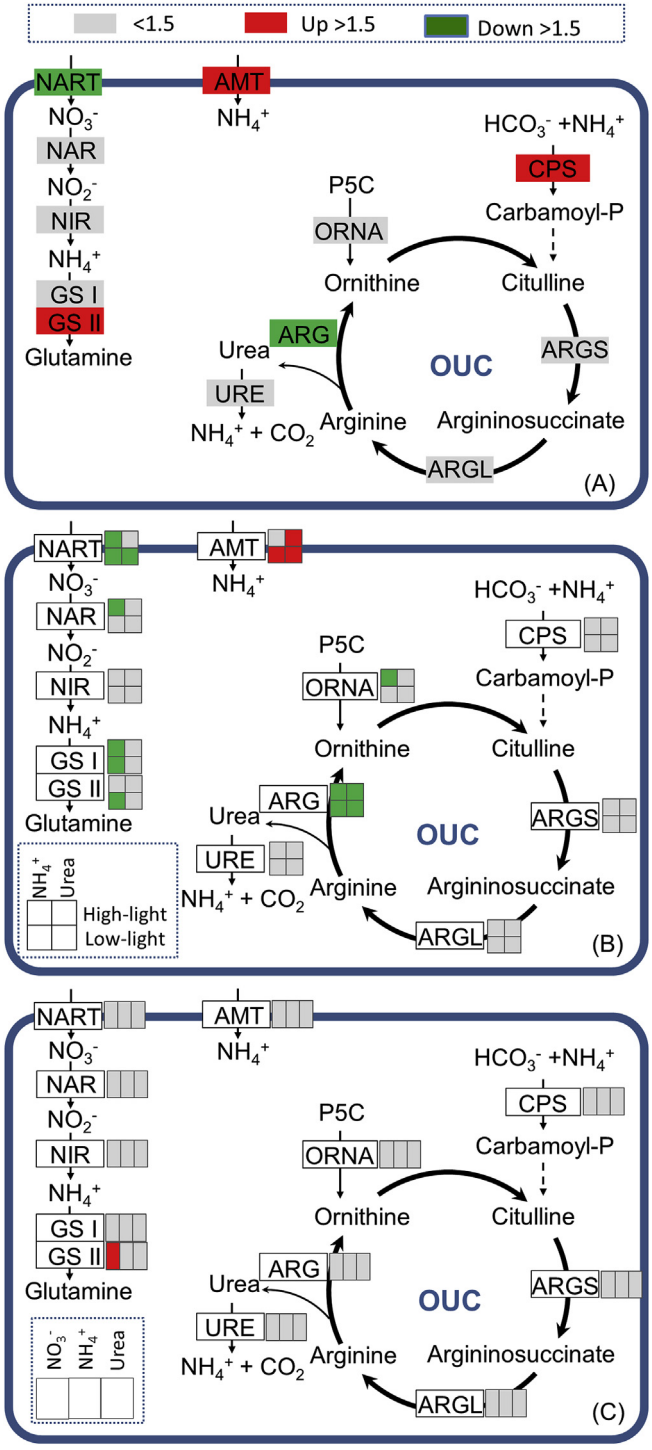

Fig. 7. Schematic of the nitrogen (N) assimilation and ornithine-urea cycle (OUC) related pathway in *Prorocentrum donghaiense* responding to different N concentrations and sources, light and diel cycles. Color filling of gene name box depicts the fold change of expression levels of the gene based on qRT-PCR data; Panel (A) indicates changes of genes in *N*-limited condition compared with that in *N*-replete condition; Panel (B) indicates changes of genes grown on NH_4^+ or urea when compared with those grown on NO_3^- in both high-light (~300 μmol photons m^{-2} s^{-1}) and low-light conditions (~50 μmol photons m^{-2} s^{-1}) during the daytime (9:00 a.m.); Panel (C) indicates changes of genes at night (21:00 p.m.) when compared with those during the day (9:00 a.m.) grown on different N sources (NO_3^-, NH_4^+ or urea). AMT, ammonium transporter; NART, nitrate transporter; NAR, nitrate reductase; NIR, nitrite reductase; GSI, glutamine synthesis I; GSII, glutamine synthesis II; URE, urease; CPS, carbamoyl-phosphate synthase; ORNA, ornithine aminotransferase; ARGS, argininosuccinate synthase; ARGL, argininosuccinate lyase; and ARG, arginase. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

types of NARTs. Their opposite responses to N stress could help *P. donghaiense* to better adapt to environmental changes. Accordingly, *P. donghaiense* might alleviate its N requirement by down-regulating expression of redundant transporters under long-term N depletion. A second explanation could be that specific regulation pathways related to NO_3^- assimilation may be present under different N status, or in different strains. It is assumed that extreme N depletion leads to suppression of NART, NAR and NIR expression (Zhang et al., 2015).

Globally, urea concentrations in coastal waters have increased sharply in recent decades (Glibert et al., 2006, 2016). Urea is considered an important N source for phytoplankton, which in some cases might benefit particular species that are able to outcompete others in phytoplankton communities (Glibert et al., 2017). Most phytoplankton use urease to produce CO_2 and NH_4^+ from urea, and then incorporate NH_4^+ into amino acids. In our transcriptomic data, several gene sequences were annotated as urease genes, but they did not all change under N limitation, and were not regulated by the N source, light intensity or diel cycle. We infer that multiple urease genes are present in *P. donghaiense,* some constitutive and others inducible. The notion was also supported by our previous study that considerable urease activity in *P. donghaiense* was observed in all treatments augmented with various N sources but the urease activity increased markedly when urea was added (Huang, 2015). In a previous study, a urease gene in *P. donghaiense* showed a remarkable up-regulation in expression level under N limitation (Jing et al., 2017). It is likely that it was an inducible urease isoform. However, the constitutive expression of urease genes would ensure that *P. donghaiense* always has sufficient urease activity to process urea.

The OUC has been shown to play critical roles in both N metabolism and energy balance in diatom cells (Allen et al., 2011; Bender et al., 2012). The diatom OUC serves as a hub to redistribute inorganic C and N to the tricarboxylic acid and glutamine synthetase/glutamate synthase cycles (Allen et al., 2011). Homologs of all enzymes in the urea cycle have been found in transcriptomes of dinoflagellates, *A. tamarense* (Dagenais-Bellefeuille and Morse, 2013) and *G. caribaeus* (Price et al., 2016), suggesting that dinoflagellates possess a complete OUC pathway. However, the roles of the urea cycle in dinoflagellates are largely unknown. In High-N in the present study, the expression of most genes related to the OUC did not change, with the exception of CPS and ARG. In Low-N, the expression of CPS and ARG genes were down-regulated significantly when N became limited. The expression of CPS gene increased in *N*-depleted *Thalassiosira pseudonana* (Mock et al., 2008; Hockin et al., 2012). The expression levels of URE, ORNA and ARG were up-regulated significantly in *N*-limited *T. pseudonana* and *P. tricornutum* (Levitan et al., 2014). These results suggest that the role of the OUC in N metabolism differs between dinoflagellates and diatoms.

The specific growth rate of *P. donghaiense* grown on reduced NH_4^+ or urea is significantly greater than those grown on NO_3^- (Hu et al., 2014; Huang, 2015). The affinity of *Prorocentrum* species for NH_4^+ and urea is much higher than that for NO_3^- (Fan et al., 2003; Hu et al., 2014). However, no significant differences were observed in cell abundance, POC, or PON for *P. donghaiense* or *P. minimum* among those grown on different N sources (Ou et al., 2014; Huang, 2015). In the present study, the AMT gene expression level was up-regulated on NH_4^+ or urea relative to that on NO_3^- and the expression of NART gene was up-regulated on NO_3^-, irrespective of the light condition or diel phase. This suggests that the expression of AMT and NART are regulated by different N sources. In contrast, the expression of NAR and NIR were constitutive and did not change among different N sources,

light conditions or diel phases. Although NH_4^+ can be incorporated directly into amino acids in cells, low expression of GSI and GSII in cells grown on NH_4^+ may limit NH_4^+ assimilation. These features have been observed in other microalgae (Wase et al., 2014).

For diatoms under light, the expression level of the CPS gene in cells grown on either NO_3^- or urea is up-regulated compared to those on NH_4^+ (Bender et al., 2012). This suggests that exogenous NH_4^+ does not influence the OUC in diatom cells. It is generally considered that low expression levels of CPS in diatom cells grown on NH_4^+ reflects the relatively low flux of NH_4^+ from the cytosol to the plastid (Bender et al., 2012). Here, CPS gene expression was unchanged by different N sources, suggesting that different pathways of N assimilation to those of diatoms are present in dinoflagellates, or at least in *P. donghaiense*.

In this study, most of the genes related to N assimilation and the OUC did not respond to the diel cycle, except for GSII and CPS. The gene expression of GSII in cells grown in High-light with NO_3^- was up-regulated at night when compared to expression during the day. It is likely that when cells were grown on NO_3^-, a high flux of NH_4^+ produced in the plastid is transported to the mitochondria for glutamine biosynthesis. Thus, GSII may be localized within mitochondria in *P. donghaiense*. The gene expression of CPS was up-regulated in urea-grown cells during the day when compared to expression at night. We did not identify the subcellular localization of the CPS. In diatoms, pgCPSII is localized to the cytosol and is affected by the diel cycle, with greater expression in the dark. In contrast, unCPS is localized to mitochondria and is transcribed throughout the diel cycle (Bender et al., 2012). We hypothesize that the CPS of *P. donghaiense* is an unCPS. When grown on urea, cells transport NH_4^+ to the plastid during the day to produce glutamine, with subsequent transport to the mitochondria to fuel CPS activity in the light. Overall, the diel cycle did not affect most of the genes related to N assimilation and the OUC. Compared with other phytoplankton, this may provide a competitive advantage for *P. donghaiense* contributing to its capacity to bloom in a range of marine environments.

In conclusion, in this study, we examined the effects of variations of N concentrations and sources, and irradiances on N assimilation and the OUC in *P. donghaiense*. Long-term N limitation reduced Chl *a* content but did not affect photosynthetic efficiency. In fact, low light even led to an elevated photosynthetic efficiency. *P. donghaiense* showed similar capacities in utilizing different N sources for growth. *P. donghaiense* down-regulated the expression of NART and ARG genes, and up-regulated the expression of AMT, GSII and CPS genes under N limitation. The exposure to different N sources and irradiances also regulate the expression of some studied genes. However, it seemed that the studied genes scarcely respond to the diel cycle. When compared with model diatoms, the transcriptional response of most genes related to N assimilation and the OUC in *P. donghaiense* were very different. The regulatory factors underlying this response still need further study. There may be multiple copies for these genes in *P. donghaiense*, where other responsive isoforms were not considered in our targets for this study. In any case, the constitutive expression of these genes give *P. donghaiense* an advantage to efficiently utilize various N sources in dynamic marine environments and contributes to the occurrence and maintenance of *P. donghaiense* blooms.

Acknowledgments

HPD, LJO and KXH designed the study. JJL, LJO and KXH carried out experiments. LJO, JJL and WYJ analyzed the data. HPD, LJO and KXH wrote the manuscript. LJO and HPD funded this study. This work was supported by the National Key R&D Program of China (No. 2017YFC1404300) and the National Natural Science Foundation of China (No. 41176087 and 41776121).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2019.110617.

Declarations of interest

None.

References

Allen, A.E., Dupont, C.L., Oborník, M., Horák, A., Nunes-Nesi, A., McCrow, J.P., Zheng, H., Johnson, D.A., Hu, H., Fernie, A.R., Bowler, C., 2011. Evolution and metabolic significance of the urea cycle in photosynthetic diatoms. Nature 473, 203–207.
Bender, S.J., Parker, M.S., Armbrust, E.V., 2012. Coupled effects of light and nitrogen source on the urea cycle and nitrogen metabolism over a diel cycle in the marine diatom *Thalassiosira pseudonana*. Protist 163, 232–251.
Chen, G.F., Ma, C.S., Zhang, C.Y., Zhou, J., Wang, Y.Y., Wang, G.C., Zhang, B.Y., Xu, Z., Lu, D.D., 2013. A rapid and sensitive method for field detection of *Prorocentrum donghaiense*, using reverse transcription-coupled loop-mediated isothermal amplification. Harmful Algae 29, 31–39.
Dagenais-Bellefeuille, S., Morse, D., 2013. Putting the N in dinoflagellates. Front. Microbiol. 4, 369.
Dong, H.P., Williams, E.W., Wang, D.Z., Xie, Z.X., Hsia, R., Jenck, A., Halden, R., Li, J., Chen, F., Place, A.R., 2013. Responses of *Nannochloropsis oceanica* IMET1 to long-term nitrogen starvation and recovery. Plant Physiol. 162, 1110–1126.
Dong, Y.L., Jiang, T., Xia, W., Dong, H.P., Lu, S.H., Cui, L., 2015. Light harvesting proteins regulate non-photochemical fluorescence quenching in the marine diatom *Thalassiosira pseudonana*. Algal Res 12, 300–307.
Fan, C.L., Glibert, P.M., Alexander, J., Lomas, M.W., 2003. Characterization of urease activity in three marine phytoplankton species, *Aureococcus anophagefferens, Prorocentrum minimum*, and *Thalassiosira weissflogii*. Mar. Biol. 142, 949–958.
Glibert, P.M., Burford, M.A., 2017. Globally changing nutrient loads and harmful algal blooms: recent advances, new paradigms, and continuing challenges. Oceanography 30, 58–69.
Glibert, P.M., Harrison, J., Heil, C., Seitzinger, S., 2006. Escalating worldwide use of urea – a global change contributing to coastal eutrophication. Biogeochemistry 77, 441–463.
Glibert, P.M., Burkholder, J.M., Kana, T., 2012. Recent insights about relationships between nutrient availability, forms, and stoichiometry, and the distribution, ecophysiology, and food web effects of pelagic and benthic *Prorocentrum* species. Harmful Algae 14, 231–259.
Glibert, P.M., Wikerson, F.P., Dugdale, R.C., Raven, J.A., Dupont, C.L., Leavitt, P.R., Parker, A.E., Burkholder, J.M., Kana, T.M., 2016. Pluses and minuses of ammonium and nitrate and assimilation by phytoplankton and implications for productivity and community composition, with emphasis on nitrogen-enriched conditions. Limnol. Oceanogr. 61, 165–197.
Grabherr, M.G., 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat. Biotechnol. 29, 644–652.
Guenin, S., Mauriat, M., Pelloux, J., Wuytswinkel, O.V., Bellini, C., Gutierrez, L., 2009. Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references. J. Exp. Bot. 60, 487–493.
Hildebrand, M., 2005. Cloning and functional characterization of ammonium transporters from the marine diatom *Cylindrotheca fusiformis*, (Bacillariophyceae). J. Phycol. 41, 105–113.
Hockin, N.L., Mock, T., Mulholland, F., Kopriva, S., Malin, G., 2012. The response of diatom central carbon metabolism to nitrogen starvation is different from that of green algae and higher plants. Plant Physiol. 158, 299–312.
Hu, Z.X., Mulholland, M.R., Duan, S.S., Xu, N., 2012. Effects of nitrogen supply and its composition on the growth of *Prorocentrum donghaiense*. Harmful Algae 13, 72–82.
Hu, Z.X., Duan, S.S., Xu, N., Mulholland, M.R., 2014. Growth and nitrogen uptake kinetics in cultured *Prorocentrum donghaiense*. PLoS One 9, e94030.
Hu, Z.X., Mulholland, M.R., Xu, N., Duan, S.S., 2016. Effects of temperature, irradiance and pCO_2 on the growth and nitrogen utilization of *Prorocentrum donghaiense*. Aquat. Microb. Ecol. 77, 155–166.
Huang, X.Y., 2015. The Study on Physiological Characteristics of Urease Activities of Five Phytoplankton. Master thesis. Jinan University, Guangzhou, China (in Chinese with English abstract).
Janouškovec, J., Gavelis, G.S., Burki, F., Dinh, D., Bachvaroff, T.R., Gornik, S.G., Bright, K.J., Imanian, B., Strom, S.L., Delwiche, C.F., Waller, R.F., Fensome, R.A., Leander, B.S., Rohwer, F.L., Saldarriaga, J.F., 2017. Major transitions in dinoflagellate evolution unveiled by phytotranscriptomics. Proc. Natl. Acad. Sci. U.S.A. 114, E171–E180.
Jespersen, A.M., Christoffersen, K., 1987. Measurements of chlorophyll-a from phytoplankton using ethanol as extraction solvent. Arch. Hydrobiol. 109, 445–454.
Jing, X.L., Lin, S.J., Zhang, H., Koerting, C., Yu, Z.G., 2017. Utilization of urea and expression profiles of related genes in the dinoflagellate *Prorocentrum donghaiense*. PLoS One 12, e0187837.
Kang, L.K., Hwang, S.P.L., Gong, G.C., Lin, H.J., Chen, P.C., Chang, J., 2007. Influences of nitrogen deficiency on the transcript levels of ammonium transporter, nitrate transporter and glutamine synthetase genes in *Isochrysis galbana* (Isochrysidales, Haptophyta). Phycologia 46, 521–533.

Levitan, O., Dinamarca, J., Zelzion, E., Lun, D.S., Guerra, L.T., Kim, M.K., Kim, J., Van Mooy, B.A., Bhattacharya, D., Falkowski, P.G., 2014. Remodeling of intermediate metabolism in the diatom *Phaeodactylum tricornutum* under nitrogen stress. Proc. Natl. Acad. Sci. U.S.A. 112, 412–417.

Li, J., Glibert, P.M., Alexander, J.A., Molina, M.E., 2012. Growth and competition of several harmful dinoflagellates under different nutrient and light conditions. Harmful Algae 13, 112–125.

Li, J.J., Xia, W., Dong, H.P., Ou, L.J., 2018. Transcriptome data of *Prorocentrum donghaiense* Lu under nitrogen and phosphorus limitation. Data in Brief 18, 799–802.

Liu, S.X., Yu, Z.G., Yao, P., Zheng, Y., Li, D., 2011. Effects of irradiance on pigment signatures of harmful algae during growth process. Acta Oceanol. Sin. 30, 46–57.

Liu, L.S., Zhuo, J., Zheng, B.H., Cai, W.Q., Lin, K.X., Tang, J.L., 2013. Temporal and spatial distribution of red tide outbreaks in the Yangtze River Estuary and adjacent waters, China. Mar. Pollut. Bull. 72, 213–221.

Liu, Y., Chen, T.T., Song, S.Q., Li, C.W., 2015. Effects of nitrogenous nutrition on growth and nitrogen assimilation enzymes of dinoflagellate *Akashiwo sanguine*. Harmful Algae 50, 99–106.

Lu, D.D., Goebel, J., Qi, Y.Z., Zou, J.Z., Han, X.T., Gao, Y.H., Li, Y.G., 2005. Morphological and genetic study of *Prorocentrum donghaiense* Lu from the East China Sea, and comparison with some related *Prorocentrum* species. Harmful Algae 4, 493–505.

Lu, D.D., Wang, H.X., Huang, H.Y., Xia, P., Dai, X.F., Göbel, J., Jeong, H.J., 2011. Morphological and genetic comparison of two strains of a *Prorocentrum* species isolated from Zhejiang coastal water of China and Masan Bay of Korea. Chin. J. Oceanol. Limnol. 29, 832–839.

Mock, T., Samanta, M.P., Iverson, V., Berthiaume, C., Robison, M., Holtermann, K., Durkin, C., BonDurant, S.S., Richmond, K., Rodesch, M., Kallas, T., Huttlin, E.L., Cerrina, F., Sussman, M.R., Armbrust, E.V., 2008. From the cover: whole-genome expression profiling of the marine diatom *Thalassiosira pseudonana* identifies genes involved in silicon bioprocesses. Proc. Natl. Acad. Sci. U.S.A. 105, 1579–1584.

Morey, J.S., Monroe, E.A., Kinney, A.L., Beal, M., Johson, J.G., Hitchcock, G.L., Van Dolah, F.M., 2011. Transcriptomic response of the red tide dinoflagellate, *Karenia brevis*, to nitrogen and phosphorus depletion and addition. BMC Genomics 12, 346.

Ou, L.J., Lundgren, V., Lu, S.H., Granéli, E., 2014. The effect of riverine dissolved organic matter and other nitrogen forms on the growth and physiology of the dinoflagellate *Prorocentrum minimum* (Pavillard) Schiller. J. Sea Res. 85, 499–507.

Poulsen, N., Kröger, N., 2005. A new molecular tool for transgenic diatoms. FEBS J. 272, 3413–3423.

Price, D.C., Farinholt, N., Gates, C., Shumaker, A., Wagner, N.E., Bienfang, P., Bhattacharya, D., 2016. Analysis of *Gambierdiscus* transcriptome data supports ancient origins of mixotrophic pathways in dinoflagellates. Environ. Microbiol. 18, 4501–4510.

Revilla, M., Alexander, J., Glibert, P.M., 2005. Urea analysis in coastal waters: comparison of enzymatic and direct methods. Limnol Oceanogr. Methods 3, 290–299.

Siaut, M., Heijde, M., Mangogna, M., Montsant, A., Coesel, S., Allen, A., Manfredonia, A., Falciatore, A., Bowler, C., 2007. Molecular toolbox for studying diatom biology in *Phaeodactylum tricornutum*. Gene 406, 23–35.

Strokal, M., Yang, H., Zhang, Y.C., Kroeze, C., Li, L.L., Luan, S.J., Wang, H.Z., Yang, S.S., Zhang, Y.S., 2014. Increasing eutrophication in the coastal seas of China from 1970 to 2050. Mar. Pollut. Bull. 85, 123–140.

Takabayashi, M., Wilkerson, F.P., Robertson, D., 2005. Response of glutamine synthetase gene transcription and enzyme activity to external nitrogen sources in the diatom *Skeletonema costatum*, (Bacillariophyceae). J. Phycol. 41, 84–94.

Valderrama, J.C., 1995. Methods of nutrient analysis, p 251-568. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae. UNESCO, Paris.

Wase, N., Black, P.N., Stanley, B.A., DiRusso, C.C., 2014. Integrated quantitative analysis of nitrogen stress response in *Chlamydomonas reinhardtii* using metabolite and protein profiling. J. Proteome Res. 13, 1373–1396.

Xu, N., Duan, S.S., Li, A.F., Zhang, C.W., Cai, Z.P., Hu, Z.X., 2010. Effect of temperature, salinity and irradiance on the growth of the harmful dinoflagellate *Prorocentrum donghaiense* Lu. Harmful Algae 9, 13–17.

Zhang, Y., 2013. Comparative Study on the Ecophysiological Responses of Different Groups of Phytoplankton to Urea. Master Thesis. Jinan University, Guangzhou, China (in Chinese with English abstract).

Zhang, Y.J., Zhang, S.F., He, Z.P., Lin, L., Wang, D.Z., 2015. Proteomic analysis provides new insights into the adaptive response of a dinoflagellate *Prorocentrum donghaiense* to changing ambient nitrogen. Plant Cell Environ. 38, 2128–2142.

Zhou, M.J., Zhu, M.Y., 2006. Progress of the project "Ecology and Oceanography" of harmful algal blooms in China". Adv. Earth Sci. 21, 673–679 (in Chinese with English abstract).

Zhou, Y.P., Zhang, Y.M., Li, F.F., Tan, L.J., Wang, J.T., 2017a. Nutrients structure changes impact the competition and succession between diatom and dinoflagellate in the East China Sea. Sci. Total Environ. 574, 499–508.

Zhou, Z.X., Yu, R.C., Zhou, M.J., 2017b. Seasonal succession of microbial blooms from diatoms to dinoflagellates in the East China Sea: a numerical simulation study. Ecol. Model. 360, 150–162.