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# Effects of temperature, irradiance and $p\text{CO}_2$ on the growth and nitrogen utilization of *Prorocentrum donghaiense*

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**ABSTRACT:** Environmental factors such as temperature, irradiance, and nitrogen (N) supply affect the growth of *Prorocentrum donghaiense*, but the interactive effects of these physical factors and the effects of atmospheric  $\text{CO}_2$  ( $p\text{CO}_2$ ) on growth and N uptake have not been examined. We compared growth kinetics of *P. donghaiense* grown on 4 different N substrates (nitrate [ $\text{NO}_3^-$ ], ammonium [ $\text{NH}_4^+$ ], urea, and glutamic acid [glu]) with respect to temperature, irradiance, and  $p\text{CO}_2$ . Temperature (15 to 30°C) had a positive effect on growth (max. growth rates: 0.17 to 0.65  $\text{d}^{-1}$ ; optimal temperature: 25 to 30°C); maximum specific growth rates ( $\mu_{\text{max}}$ ) declined when cultures were grown at 30°C. *P. donghaiense* grew well on all 4 N sources, under irradiances ranging from 10 to 180  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ .  $\mu_{\text{max}}$  ( $2.0 \pm 0.1 \text{ d}^{-1}$ ) was observed in cultures growing with  $\text{NH}_4^+$  as the sole N source in the highest irradiance treatment (180  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ). These rates were significantly higher than those measured in cultures grown on  $\text{NO}_3^-$ , urea, and glu (all  $\sim 1.4 \text{ d}^{-1}$ ). Half-saturation constants ( $K_s$ ) ranged from  $66.5 \pm 4.6$  to  $99.4 \pm 6.7 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$  for cultures grown with glu or  $\text{NH}_4^+$ , respectively, as the sole source of N. Both growth and N uptake rates were higher in cultures grown under elevated  $p\text{CO}_2$ . Our results suggest that *P. donghaiense* exhibits flexible adaptation for N utilization under broad environmental conditions (temperature, irradiance,  $p\text{CO}_2$ ), which may play an important role in the formation and duration of *P. donghaiense* blooms.

**KEY WORDS:** *Prorocentrum donghaiense* · Temperature · Irradiance ·  $\text{CO}_2$  · Nitrogen · Growth rate · Nitrogen uptake

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## INTRODUCTION

Harmful algal blooms (HABs) are a worldwide ecological problem that have become more frequent, extensive, and severe in recent years (Wang & Wu 2009, Anderson et al. 2012, Dia et al. 2014, Glibert 2014, Song et al. 2016). HABs most often occur in coastal waters, where they exert serious economic

impacts on marine fisheries and aquaculture, and threaten public health and aquatic ecosystems (Heisler et al. 2008). HABs commonly occur in the coastal areas of China, including the South China, East China, and Bohai seas, and their frequency, intensity, and duration have increased dramatically in the last 2 decades (Wang & Wu 2009, Zhou 2010, Lu et al. 2014, Song et al. 2016). Since 2000, blooms

of *Prorocentrum donghaiense*, a photosynthetic eukaryote, have occurred every year in the Changjiang River Estuary and coastal waters adjacent to Zhejiang province, causing serious economic impacts (Li et al. 2009, Zhou 2010, Lin et al. 2014). While blooms of *P. donghaiense* have been studied since they were first recorded in early 2000s (Lu & Goebel 2001, Lu et al. 2005, Wang & Wu 2009, Zhou 2010, Lin et al. 2014), the identity of factors promoting the initiation and persistence of these blooms remains elusive.

Growth of marine phytoplankton is inherently complex, and there are generally predictable but nonlinear relationships between their growth and environmental parameters such as temperature, irradiance, and nutrient concentrations (Harrison & Platt 1986, Litchman & Klausmeier 2008, Boyd et al. 2013, Edwards et al. 2015a,b). HABs have most often been linked to nutrient over-enrichment and consequent eutrophication of coastal waters (Heisler et al. 2008). Both the form of dissolved nitrogen (N) and its concentration in coastal waters are thought to be important in the formation and development of HABs and phytoplankton blooms (Dugdale et al. 2007). Increased nutrient loads (i.e. N) from the Changjiang (Yangtze) River have led to increased HAB events, including *P. donghaiense* blooms (Li et al. 2014). Over the progression of a *P. donghaiense* bloom, concentrations of dissolved inorganic and organic N can vary from several  $\mu\text{M}$  to more than  $50 \mu\text{mol N l}^{-1}$  (Li et al. 2010). Previous studies have examined the growth kinetics of *P. donghaiense* on different N sources and determined that *P. donghaiense* has high affinities for and exhibits maximum growth rates under a diverse suite of N compounds (Hu et al. 2012, 2014).

Responses of marine phytoplankton to temperature are species-specific and occasionally strain-specific, depending on the geographic region in which they are isolated (Thomas et al. 2012, Boyd et al. 2013). Temperature is an important regulator of morphology, growth rate, resource allocation, and metabolism in phytoplankton (Lomas & Glibert 1999, Persson et al. 2013, Toseland et al. 2013), and can also affect toxin production by some harmful algae (Band-Schmidt et al. 2014, Thorel et al. 2014). Blooms of *P. donghaiense* typically occur in late April or early May at temperatures of 15 to 26°C (Li 2009, Li et al. 2010, Lu et al. 2005, 2014), and monitoring data has confirmed that temperature is a key factor affecting the initiation and development of *P. donghaiense* blooms (Li 2009, Zhu et al. 2009). Blooms of this species usually last several weeks to more than a month, over which time surface water temperatures gener-

ally increase. Even though *P. donghaiense* can efficiently utilize different N compounds (Hu et al. 2012), even when present at low concentrations (Hu et al. 2014), the activity of enzymes involved in nutrient utilization varies with temperature. Water temperatures in the Changjiang River Estuary and adjacent waters varies from 15 to 26°C during the period when blooms commonly occur (Li 2009, Li et al. 2010, Lu et al. 2005, 2014). Previous culture studies have been conducted at temperatures between 20 and 25°C (Hu et al. 2012, Wang et al. 2013), but the ability of *P. donghaiense* to grow on different N sources at the range of temperatures experienced in the environment has not yet been fully investigated.

Irradiance is another major factor controlling growth of autotrophic phytoplankton because it is essential for photosynthesis. In highly turbid areas such as East China Sea (Sun et al. 2008), light availability can limit photosynthesis. The intensity of photosynthetically active radiation (PAR) can be estimated from the depth of the mixed layer and turbidity (Diehl et al. 2002). In spring, the surface PAR of the East China Sea has been estimated to be  $\sim 200 \text{ W m}^{-2}$  (Sun et al. 2008), with light intensity attenuating rapidly with depth. *P. donghaiense* blooms mainly appear near the surface, but in some cases the bloom depth can extend to 10 m. The maximum recorded cell density during a bloom was  $36 \times 10^7 \text{ cells l}^{-1}$ , which gave the water a brownish discoloration (Lu et al. 2005). Because algal cells are also particles that contribute to turbidity, during dense surface blooms light can rapidly attenuate with depth and limit the ability of photosynthetic cells to grow and take up N.

While not generally thought to limit algal growth, during dense blooms dissolved inorganic carbon (DIC) can be rapidly drawn down, thereby raising the pH and limiting photosynthesis (Boyd & Hutchins 2012). Atmospheric  $\text{CO}_2$  concentrations are expected to double by the end of this century (IPCC 2013), and elevated  $p\text{CO}_2$  increases growth rates of cyanobacteria and some other phytoplankton (Wu et al. 2014), perhaps by alleviating carbon (C) limitation of photosynthesis (Hutchins et al. 2007). Elevated  $p\text{CO}_2$  also results in higher DIC concentrations in seawater, and this alters the inorganic carbon buffer system and decreases pH in oligotrophic waters where photosynthetic drawdown of DIC is lower. In nutrient-enriched eutrophic coastal systems, however, increasing the supply of DIC may alleviate C limitation of algal growth, provided light or some other element does not limit their growth. Higher supplies of DIC could also impact marine phytoplankton growth, element ratios, photosynthetic rates, nutrient utilization,

toxin production, and community structure (Burkhardt et al. 1999, Hutchins et al. 2013, Li & Campbell 2013, Tatters et al. 2013, Errera et al. 2014, Celis-Plá et al. 2015, Johnson et al. 2015).

Environmental factors such as temperature and irradiance affect the growth of *P. donghaiense* in culture experiments and in the field (Deng et al. 2009, Zhu et al. 2009, Xu et al. 2010), but the interactive effects of these physical factors and N availability have not been examined. A diverse suite of N compounds are available to phytoplankton in natural seawater that can potentially support their growth. These include inorganic N compounds (nitrate [NO<sub>3</sub><sup>-</sup>], nitrite [NO<sub>2</sub><sup>-</sup>], and ammonium [NH<sub>4</sub><sup>+</sup>]) and organic N compounds (including urea and amino acids) (Bronk 2002, Mulholland & Lomas 2008, Mulholland & Lee 2009). Many enzymes facilitating the uptake of these compounds are sensitive to environmental factors such as temperature and irradiance (Gao et al. 2000). In previous studies, we examined the growth kinetics of *P. donghaiense* on different N sources and determined that *P. donghaiense* had high affinities to, and experienced maximum growth rates under a diverse suite of N compounds (Hu et al. 2012, 2014). Here, we examined the growth and N uptake responses of cultured populations of *P. donghaiense* to changes in temperature, irradiance, and pCO<sub>2</sub> concentration, in order to better understand how physical factors affect growth and N (NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup>, urea, and amino acid) uptake.

## MATERIALS AND METHODS

### Alga and culture conditions

*Prorocentrum donghaiense* was isolated from the coastal waters of Zhoushan, Zhejiang province, China, and obtained from the Research Center of Hydrobiology, Jinan University, Guangzhou, China. Cultures were grown in artificial seawater enriched with sterile, silicate-free medium supplied with f/2 trace metals and vitamins (Guillard 1975). Concentrations of N in the culture media were 50 μmol N l<sup>-1</sup> (supplied as NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup>, urea, or glutamic acid [glu]), and phosphate concentrations were 3.12 μmol P l<sup>-1</sup> (as phosphate, PO<sub>4</sub><sup>3-</sup>). A series of experiments were conducted wherein temperature, irradiance, or pCO<sub>2</sub> were varied to examine their individual effects on *P. donghaiense* growth rates. Growth responses of *P. donghaiense* with respect to temperature and irradiance were examined in cultures grown on 4 different N sources (NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup>, urea, and glu), while

growth responses of *P. donghaiense* with respect to pCO<sub>2</sub> were measured only in cultures containing NO<sub>3</sub><sup>-</sup> as the sole N source. Experiments in which temperature and irradiance were the experimental variables were conducted in an incubator maintained at present day pCO<sub>2</sub> and either temperature or irradiance was varied; temperature treatments were 15, 20, 23, 25, or 30°C and irradiance treatments were 10, 20, 50, 80, 120, and 180 μmol quanta m<sup>-2</sup> s<sup>-1</sup>. In all experiments, light was supplied on a 12 h light:12 h dark cycle. For temperature treatments, cultures were maintained at a constant irradiance (60 μmol quanta m<sup>-2</sup> s<sup>-1</sup>) and for irradiance treatments, cultures were maintained at a constant temperature (23°C). For pCO<sub>2</sub> experiments, cultures were maintained in an environmental room at constant temperature (23°C) and irradiance (60 μmol quanta m<sup>-2</sup> s<sup>-1</sup>), and N was supplied as NO<sub>3</sub><sup>-</sup> (50 μmol N l<sup>-1</sup>). To estimate growth rates, *in vivo* fluorescence was monitored at the same time each day using a Turner Designs AU-10 fluorometer.

### Experimental design

**Growth response to temperature.** For each nutrient treatment (NO<sub>3</sub><sup>-</sup> [data from Boyd et al. 2013], NH<sub>4</sub><sup>+</sup>, urea or glu), growth curves were constructed from batch cultures grown at 15, 20, 23, 25, and 30°C. For each nutrient and temperature treatment, cultures were grown in triplicate, capped 50 ml Pyrex test tubes containing 35 ml of medium. Cultures were maintained under the treatment growth conditions for more than 5 generations prior to the start of growth experiments to ensure cells were acclimated to treatment conditions (temperature and N source combination). On the first (the day of culture inoculation) and last days (the day of culture just going to stationary stage) of the experiments, samples were collected to measure cell abundance and chlorophyll *a* (chl *a*).

**Growth response to irradiance.** Using neutral density screening and by adjusting the distance between cool white fluorescent lamps, cultures growing on 50 μmol l<sup>-1</sup> NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup>, urea or glu as the sole N source were grown at irradiances of 10, 20, 50, 80, 120 or 180 μmol quanta m<sup>-2</sup> s<sup>-1</sup>, supplied on a 12 h light:12 h dark cycle. Cultures were maintained under each treatment condition (irradiance and N source combination) for more than 5 generations prior to the start of experiments in order to ensure cells were acclimated prior to the start of the experiments. For each treatment, cultures were grown in

triplicate, capped, 50 ml Pyrex test tubes containing 35 ml of medium. On the first (the day of culture inoculation) and last days (the day of culture just going to stationary stage) of the experiments, samples were collected to measure cell abundance, and chl *a*.

**Growth response to  $p\text{CO}_2$ .** For experiments examining the effects of  $p\text{CO}_2$  on growth of *P. donghaiense*, triplicate cultures were equilibrated to 190 (glacial maximum  $p\text{CO}_2$ ), ~380 (present day), 500 (predicted 2050  $p\text{CO}_2$ ), or 750 (predicted 2100  $p\text{CO}_2$ ) ppm  $p\text{CO}_2$ . Present day  $p\text{CO}_2$  was maintained with filtered air from the room (0.2  $\mu\text{m}$  filtered) using an air pump. The other 3  $\text{CO}_2$  concentrations were commercially prepared certified standard  $\text{CO}_2$  gas mixtures (Gilmore Liquid Air Company). As with previously described experiments, cultures were acclimated to different  $p\text{CO}_2$  levels for more than 5 generations prior to the start of experiments. *In vivo* fluorescence, cell density, chl *a*, particulate nitrogen and carbon (PN and PC), and pH were monitored daily, and nutrient concentrations ( $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , urea, dissolved free amino acids [DFAAs], and  $\text{PO}_4^{3-}$ ) were measured every other day over the course of experiments.

On the 9<sup>th</sup> day of each  $p\text{CO}_2$  experimental treatment, N uptake rates were measured using stable isotopes as tracers. Uptake experiments were initiated by adding 100 nmol  $\text{N l}^{-1}$  of highly enriched (96 to 99%)  $^{15}\text{N}$ -labeled  $\text{NO}_3^-$  or  $\text{NH}_4^+$ , or dually labeled ( $^{15}\text{N}$  and  $^{13}\text{C}$ ) urea or algal amino acid mixture to culture aliquots placed in 30 ml acid-washed bottles. Incubations were terminated after 30 to 60 min by gentle filtration through pre-combusted (450°C for 2 h) GF/F (nominal pore size ~0.7  $\mu\text{m}$ ) filters. Filters were then frozen in sterile polypropylene cryovials until analysis.

### Analytical methods

**Measurement of chl *a*, cell density, PN/PC and nutrient concentrations.** Chl *a* concentrations were measured fluorometrically after extraction in 90% acetone (Welschmeyer 1994). Cells were enumerated using a Sedgwick-Rafter counting chamber and a Zeiss inverted microscope or a FACSCalibur flow cytometer (Becton Dickinson Instruments). PC and PN concentrations and isotopic ratios were analyzed on a Europa Scientific 20-20 isotope ratio mass spectrometer equipped with an automated N and C analyzer. Dissolved  $\text{NO}_3^-$  and urea concentrations were measured on an Astoria Pacific autoanalyzer using standard colorimetric methods according to the

manufacturer's specifications (Parsons et al. 1984).  $\text{NH}_4^+$  was determined using the manual phenol hypochlorite method (Solorzano 1969). Concentrations of DFAAs were measured by high performance liquid chromatography (HPLC) (Cowie & Hedges 1992).

**Calculation of specific growth rates.** Specific growth rates ( $\mu$ ;  $\text{d}^{-1}$ ) were calculated using a least squares fit to a straight line after logarithmic transformation of *in vivo* fluorescence data, as described by Guillard (1973):

$$\mu = \frac{\ln N_1 - \ln N_0}{T_1 - T_0} \quad (1)$$

where  $N_1$  and  $N_0$  are the *in vivo* fluorescence at time  $T_1$  and  $T_0$ , respectively, during the linear portion of exponential phase growth.

The relationship between growth rate and irradiance was described using the equation modified from Lederman & Tett (1981):

$$\mu = \frac{\mu_{\max}(I - I_0)}{I + K_s - 2I_0} \quad (2)$$

where  $\mu_{\max}$  is the maximum specific growth rate ( $\text{d}^{-1}$ ),  $I$  is the irradiance ( $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ ),  $I_0$  is the compensation irradiance ( $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ ) and  $K_s$  is the irradiance at  $\mu_{\max}/2$  (i.e. half-saturation light intensity).

**Calculation of N uptake rates.** N uptake rates were calculated using the following equation (Mulholland & Lee 2009):

$$^{15}\text{N uptake (V)} = \frac{(\text{atom\% PN})_{\text{final}} - (\text{atom\% PN})_{\text{initial}}}{(\text{atom\% N source pool} - \text{atom\% PN})_{\text{initial}} \times \text{time}} \quad (3)$$

where the source pool was the dissolved N pool that was enriched.

**Statistical analysis.** All statistical tests were conducted using Microsoft® Excel 2007 and SPSS 13.0 with the level of significance set at  $\alpha = 0.05$ . Differences in maximum *in vivo* fluorescence, specific growth rates and N uptake rates were compared using a 1-way ANOVA.

## RESULTS

### Growth response of *P. donghaiense* to temperature

Temperature had a positive effect on the growth of *Prorocentrum donghaiense* when  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , urea or glu were supplied as the sole N source.  $\text{NO}_3^-$  results were obtained from Boyd et al. (2013), who

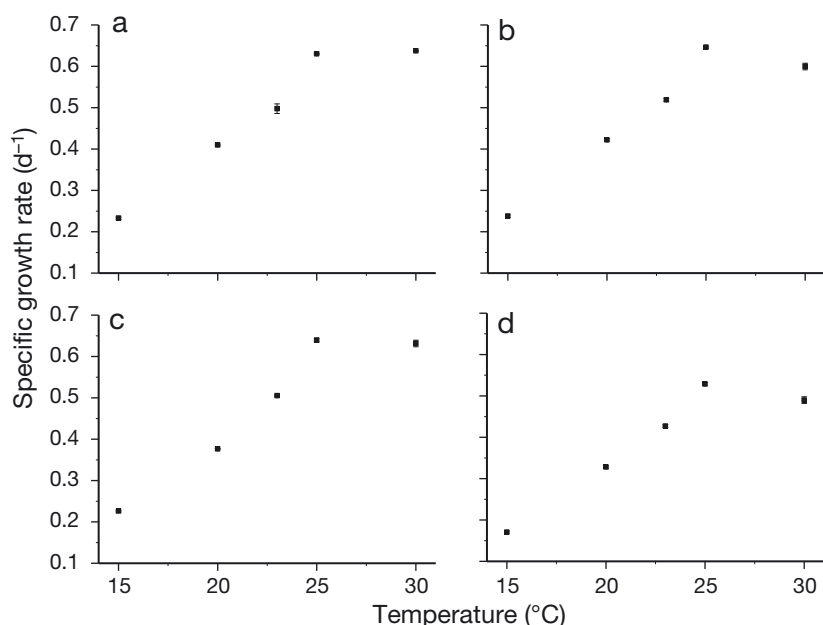


Fig. 1. Specific growth rates of *Prorocentrum donghaiense* grown on (a) NO<sub>3</sub><sup>-</sup> (redrawn from Boyd et al. 2013), (b) NH<sub>4</sub><sup>+</sup>, (c) urea, and (d) glutamic acid as a function of temperature. Values are means ± SD of triplicate samples

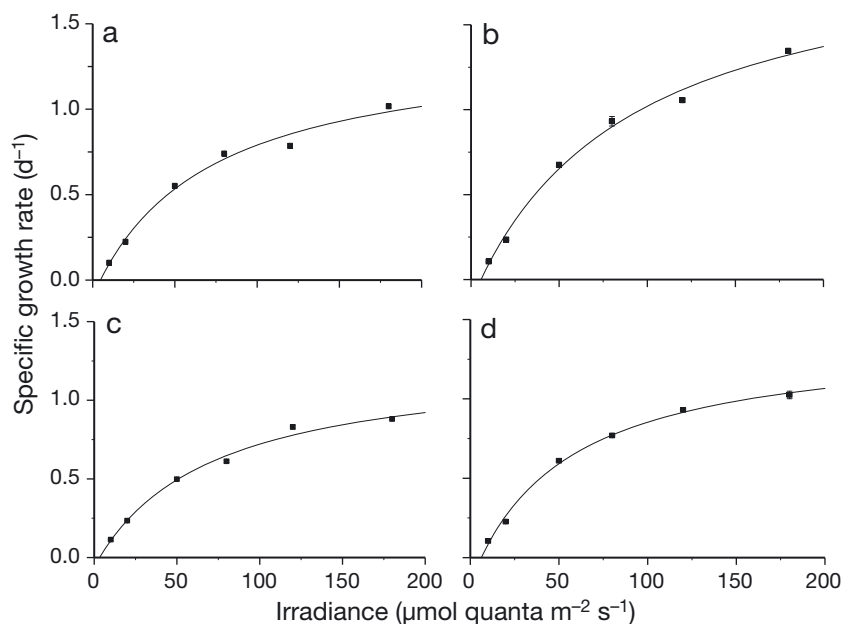


Fig. 2. Specific growth rates of *Prorocentrum donghaiense* grown on (a) NO<sub>3</sub><sup>-</sup>, (b) NH<sub>4</sub><sup>+</sup>, (c) urea, and (d) glutamic acid as a function of irradiance. Solid lines are fitted iteratively to the data according to the Monod equation. Values are means ± SD of triplicate samples

addressed the temperature versus growth responses of phytoplankton from polar to tropical waters. Here, we redraw the NO<sub>3</sub><sup>-</sup> data to compare with the growth response of *P. donghaiense* grown on NH<sub>4</sub><sup>+</sup>, urea, and glu. Specific growth rates ranged from 0.17 to 0.65 d<sup>-1</sup> (Fig. 1); maximum specific growth rates were

observed in cultures of *P. donghaiense* grown at 25°C (Fig. 1). The specific growth rates for cultures grown on NO<sub>3</sub><sup>-</sup> at 25 and 30°C were not significantly different from each other (Fig. 1). Specific growth rates of cultures supplied with NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup> and urea as the sole source of N were significantly higher than those supplied with glu as an N source at all temperatures tested (15 to 30°C) ( $p < 0.01$ ; Fig. 1).

### Growth response of *P. donghaiense* to irradiance

Growth curves for *P. donghaiense* with respect to irradiance were fit using maximum specific growth rates measured in cultures growing at irradiances between 10 and 180 μmol quanta m<sup>-2</sup> s<sup>-1</sup> for each of the 4 N compounds (NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup>, urea and glu) as a sole source of N (Fig. 2). The average specific growth rates of *P. donghaiense* cultured using the 4 different N compounds were not significantly different from each other, and averaged ~0.1 d<sup>-1</sup> in cultures maintained at the lowest irradiance (10 μmol quanta m<sup>-2</sup> s<sup>-1</sup>) (Fig. 2). However, at the highest irradiance tested (180 μmol quanta m<sup>-2</sup> s<sup>-1</sup>), the average specific growth rates ranged from 0.9 d<sup>-1</sup> in cultures grown on urea to 1.4 d<sup>-1</sup> in cultures growing with NH<sub>4</sub><sup>+</sup> as the sole N source (Fig. 2). We calculated μ<sub>max</sub> for *P. donghaiense* grown on the 4 different N sources to range from 1.3 ± 0.0 to 2.0 ± 0.1 d<sup>-1</sup>. The highest μ<sub>max</sub> were found in cultures grown with NH<sub>4</sub><sup>+</sup> as the sole source of N (2.0 ± 0.1 d<sup>-1</sup>) and these were significantly higher than those calculated for cultures grown on NO<sub>3</sub><sup>-</sup> (1.4 ± 0.0 d<sup>-1</sup>), glu (1.4 ± 0.1 d<sup>-1</sup>) or urea (1.3 ± 0.0 d<sup>-1</sup>) (Table 1). The calculated I<sub>0</sub> ranged from 2.8 ± 0.3 to 6.2 ± 0.1 μmol quanta m<sup>-2</sup> s<sup>-1</sup> (Table 1). The K<sub>s</sub> for cultures grown on NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup>, urea, and glu were 78.0 ± 3.5, 99.4 ± 6.7, 79.7 ± 1.4 and 66.5 ± 4.6 μmol quanta m<sup>-2</sup> s<sup>-1</sup>, respectively (Table 1).

Table 1. Mean ( $\pm$ SD;  $n = 3$ ) growth parameters as a function of irradiance for *Prorocentrum donghaiense* growing with  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , urea, or glutamic acid (glu) as the sole source of nitrogen in the culture medium. The maximum specific growth rate ( $\mu_{\text{max}}$ ;  $\text{d}^{-1}$ ), compensation irradiance ( $I_0$ ;  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) and half-saturation light intensity ( $K_s$ ;  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) for each nitrogen compound was calculated according to the equation modified from Lederman & Tett (1981)

|                 | $\mu_{\text{max}}$ | $I_0$         | $K_s$          | $R^2$ |
|-----------------|--------------------|---------------|----------------|-------|
| $\text{NH}_4^+$ | $2.0 \pm 0.1$      | $5.7 \pm 0.4$ | $99.4 \pm 6.7$ | 0.99  |
| $\text{NO}_3^-$ | $1.4 \pm 0.0$      | $4.7 \pm 0.1$ | $78.0 \pm 3.5$ | 0.98  |
| Glu             | $1.4 \pm 0.1$      | $6.2 \pm 0.1$ | $66.5 \pm 4.6$ | 0.99  |
| Urea            | $1.3 \pm 0.0$      | $2.8 \pm 0.3$ | $79.7 \pm 1.4$ | 0.99  |

### Growth response of *P. donghaiense* to $p\text{CO}_2$

*P. donghaiense* growth rates positively responded to elevated  $p\text{CO}_2$  (Fig. 3) but data were insufficient to perform a kinetic analysis. Cultures of *P. donghaiense* maintained at 500 and 750 ppm  $p\text{CO}_2$ , had significantly higher specific growth rates than those grown using air mixtures containing 190 or 380 ppm  $p\text{CO}_2$  (Fig. 3;  $p < 0.05$ ). There was no significant difference in growth rates between cultures grown at 190 and 380 or 500 and 750 ppm  $p\text{CO}_2$  (Fig. 3).

Although all N compounds tested were taken up,  $\text{NH}_4^+$  and DFAA were taken up at higher rates than urea and  $\text{NO}_3^-$  in all cultures regardless of the  $p\text{CO}_2$  treatment (Fig. 4). Furthermore, rates of  $\text{NO}_3^-$  and urea uptake were not significantly different in the 4  $p\text{CO}_2$  treatments (Fig. 4;  $p > 0.05$ ). In contrast,  $\text{NH}_4^+$  uptake rates were significantly higher in cultures grown at 750 ppm  $p\text{CO}_2$  relative to those grown under the other  $p\text{CO}_2$  treatments, although rates were also high in the 190 ppm  $p\text{CO}_2$  treatment (Fig. 4;  $p < 0.05$ ). DFAA uptake rates were significantly higher in cultures grown under 750 ppm  $p\text{CO}_2$  than in those grown at 190 or 380 ppm  $p\text{CO}_2$  (Fig. 4;  $p < 0.05$ ).

### DISCUSSION

Environmental factors including temperature, irradiance, N availability, and  $p\text{CO}_2$  are important controls for the growth of phytoplankton (Thomas et al. 2012, Boyd & Hutchins 2012). In previous studies, we exam-

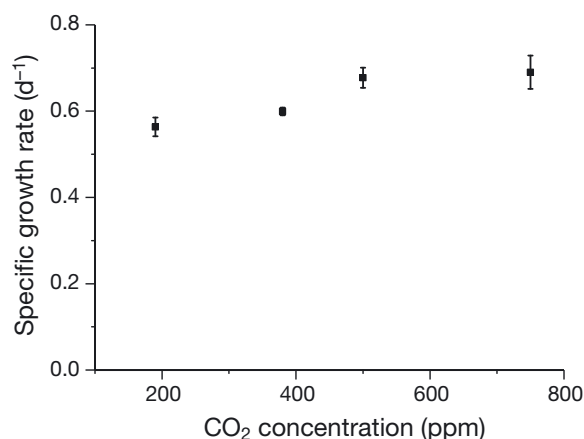


Fig. 3. Specific growth rates of *Prorocentrum donghaiense* as a function of  $\text{CO}_2$ . Values are means  $\pm$  SD of triplicate samples

ined the role of N availability and uptake kinetics on the growth of *Prorocentrum donghaiense* (Hu et al. 2012, 2014). In this study, we found that temperature, irradiance, and  $p\text{CO}_2$  all had significant impacts on *P. donghaiense* growth, and that growth kinetics varied depending on the N source.

### Temperature

Temperature affects growth, morphology, and rates of enzyme activity, and thereby affect rates of nutrient utilization and the seasonal and geographical distribution of phytoplankton, as well as toxins production by some harmful algae (Lomas & Glibert 1999, Levitan et al. 2010, Thomas et al. 2012, Boyd et al. 2013, Mousing et al. 2014). Until recently, most

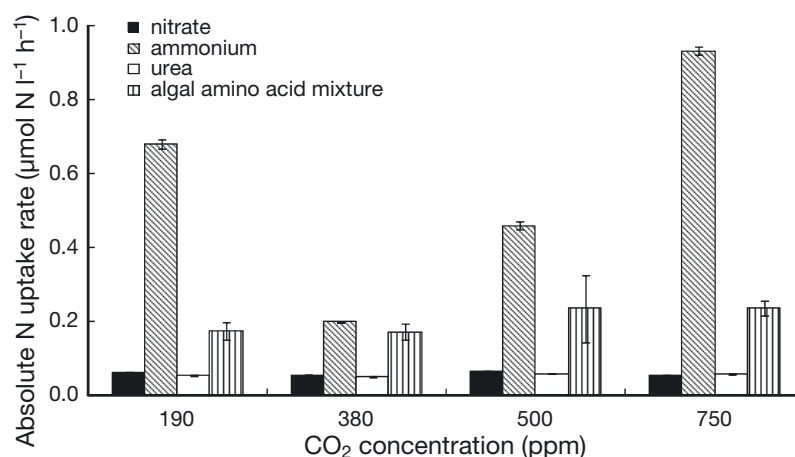


Fig. 4. Nitrogen ( $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , urea, and algal amino acid mixture) uptake rates of *Prorocentrum donghaiense* grown on different concentrations of  $\text{CO}_2$ . Values are means  $\pm$  SD of triplicate samples

studies examining the effects of temperature on the growth rates of phytoplankton have used cultures wherein  $\text{NO}_3^-$  was supplied as the sole N source (e.g. Xu et al. 2010, Laabir et al. 2011). However, there are many other forms of N available in natural seawater, including  $\text{NO}_2^-$ ,  $\text{NH}_4^+$ , urea, and amino acids (Bronk 2002). In this study, we examined growth of *P. donghaiense* under a wider range of temperatures (15 to 30°C), representative of those during which blooms occur. The maximum specific growth rates were obtained at 25°C in cultures growing on all N sources tested ( $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , urea and glu), however, the maximum specific growth rate was lower for cultures grown on glu relative to those observed in cultures growing on the other 3 N substrates (Boyd et al. 2013) (Fig. 1).

Previous culture studies suggested that *P. donghaiense* grows optimally at ~22°C (Chen et al. 2005), however, Xu et al. (2010) found that *P. donghaiense* grew fastest at higher temperatures (27°C). In the field, blooms of *P. donghaiense* typically occur in late April or early May in the Changjiang River Estuary, when water temperatures are generally between 15 and 26°C (Li 2009, Li et al. 2010, Lu et al. 2005, 2014). Monitoring data suggests that temperature is a key factor affecting the initiation and development of *P. donghaiense* blooms (Li 2009, Zhu et al. 2009). In our culture experiments, *P. donghaiense* exhibited the maximum specific growth rates at 25°C when supplied with  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , urea, and glu (Boyd et al. 2013) (Fig. 1), which is comparable with the other culture and field studies.

However, in most environments, recycled N compounds, not  $\text{NO}_3^-$ , are the dominant N source satisfying the N demand of resident microbes (Mulholland & Lomas 2008), particularly at high water temperatures. We found that temperature and N sources had interactive effects on *P. donghaiense*. The highest specific growth rates occurred when *P. donghaiense* was grown on  $\text{NH}_4^+$  at temperatures ranging from 15 to 25°C, while cultures grown on glu always had lower specific growth rates than cultures grown on other N sources at the temperatures tested. The difference could be N transporters and enzyme activity. Many enzymes are involved in the assimilation of different N species (Lomas 2004, Berges & Mulholland 2008). Temperature affects enzymes associated with N metabolism differently, and the relative responses of these enzymes to changes in temperature may also contribute to differences in N utilization by phytoplankton with respect to temperature (Gao et al. 2000). For example, temperature affects urease activity in *P. dong-*

*haiense*, with the highest urease activity observed at 25°C (Cai et al. 2016). In addition, growth phase, temperature, irradiance, and nitrogen source all impact the amino acid oxidase (AO) activity of *P. donghaiense*, which plays an important role in N utilization (Liu et al. 2013).

Once initiated, blooms of this species usually last several weeks to more than a month in the estuary and adjacent waters (Li 2009, Li et al. 2010, Lu et al. 2005, 2014). *P. donghaiense* can efficiently utilize many N compounds (Hu et al. 2012), even when present at low concentrations (Hu et al. 2014). The ability to grow under a wide range of temperatures and use a broad spectrum of N compounds is likely an important factor affecting the success of *P. donghaiense* in an environment where temperatures warm seasonally and concentrations of N species ( $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , urea and DFAA) vary (Wang & Huang 2003, Li 2009, Li et al. 2009). This metabolic flexibility may offer *P. donghaiense* an advantage in a highly variable estuarine environment.

### Irradiance

In addition to temperature, irradiance is one of the most important physical factors impacting the growth and photosynthetic efficiency of phytoplankton. Both irradiance and duration affect the efficiency of photosynthesis and the overall photosynthetic rate. In previous culture studies examining the effects of irradiance on *P. donghaiense* growth rates,  $\text{NO}_3^-$  was used as the sole N source (Chen et al. 2005, Xu et al. 2010). However, in the Changjiang River Estuary, there are many forms of bioavailable N, including  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , urea, and DFAAs, and their relative abundance changes over the course of blooms (Li et al. 2009, 2010). Therefore, to better understand the interaction between irradiance and N sources on the growth of *P. donghaiense*, we examined growth responses of this organism to irradiances ranging from 10 to 180  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ , a realistic range of irradiance during dense blooms, in cultures grown on 4 different N substrates ( $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , urea and glu). We found that *P. donghaiense* grew on all 4 N sources at each irradiance tested, and that growth rates increased as irradiance increased (Fig. 2). The  $\mu_{\text{max}}$  and  $K_s$  values for irradiance for *P. donghaiense* grown on  $\text{NH}_4^+$  were significantly higher ( $p < 0.05$ ) than those measured in cultures grown on  $\text{NO}_3^-$ , urea or glu as the sole source of N (Table 1, Fig. 2).

Most autotrophic phytoplankton cannot survive, or grow very slowly at low irradiances, but growth may



Table 2. Summary of compensation irradiance ( $I_0$ ;  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) and  $K_s$  ( $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) for different dinoflagellate species

| Species   | $I_0$          | $K_s$          | Reference                  |
|---|----------------|----------------|----------------------------|
| <i>Akashiwo sanguinea</i>                                   | 14.4           | 114            | Matsubara et al. (2007)    |
| <i>Karenia mikimotoi</i>                                    | 0.7            | 110            | Yamaguchi & Honjo (1989)   |
| <i>Prorocentrum donghaiense</i> (grown on $\text{NH}_4^+$ ) | $5.7 \pm 0.4$  | $99.4 \pm 6.7$ | This study                 |
| <i>Alexandrium tamarensense</i>                             | 76             | 90             | Yamamoto & Tarutani (1997) |
| <i>Cochlodinium polykrikoides</i>                           | 10.4           | 90             | Kim et al. (2004)          |
| <i>P. donghaiense</i> (grown on urea)                       | $2.8 \pm 0.3$  | $79.7 \pm 1.4$ | This study                 |
| <i>P. donghaiense</i> (grown on $\text{NO}_3^-$ )           | $4.7 \pm 0.1$  | $78.0 \pm 3.5$ | This study                 |
| <i>Gyrodinium instriatum</i>                                | 10.6           | 70             | Nagasoe et al. (2006)      |
| <i>P. donghaiense</i> (grown on glutamic acid)              | $6.2 \pm 0.1$  | $66.5 \pm 4.6$ | This study                 |
| <i>P. donghaiense</i>                                       | 0.1            | 30             | Xu et al. (2010)           |
| <i>Gymnodinium catenatum</i>                                | 10             | 16.8           | Yamamoto et al. (2002)     |
| <i>Alexandrium fundyense</i>                                | 15.0           | –              | Etheridge & Roesler (2005) |
| <i>Alexandrium minutum</i> (grown at 20°C)                  | $10.6 \pm 5.6$ | –              | Lim et al. (2006)          |
| <i>A. minutum</i> (grown at 25°C)                           | $10.0 \pm 1.1$ | –              | Lim et al. (2006)          |
| <i>Alexandrium tamiyavanichii</i> (grown at 20°C)           | $10.3 \pm 3.3$ | –              | Lim et al. (2006)          |
| <i>A. tamiyavanichii</i> (grown at 25°C)                    | $9.9 \pm 1.6$  | –              | Lim et al. (2006)          |

also be inhibited at high irradiances (MacIntyre et al. 2002, Dubinsky & Stambler 2009). We observed low growth rates for *P. donghaiense* under low light conditions but did not observe growth inhibition at the highest irradiance tested, which was about 20% of the average spring surface PAR estimated for the East China Sea (Sun et al. 2008) (Fig. 2). The values of  $I_0$  and  $K_s$  determined for *P. donghaiense* in this study were within the ranges of those observed for other dinoflagellate species (Table 2). For example, *Karenia mikimotoi* had lower  $I_0$  than *P. donghaiense*, but higher  $K_s$  (Yamaguchi & Honjo 1989), while *Alexandrium tamarensense* had higher  $I_0$  but similar values of  $K_s$  (Yamamoto & Tarutani 1997). Growth temperatures (20 versus 25°C) were shown to affect  $I_0$  values for *A. minutum* and *A. tamiyavanichii* (Lim et al. 2006).

Sun et al. (2008) found that the optimal growth irradiance for *P. donghaiense* was  $174 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ , which is similar to the highest irradiance used in the present study. In contrast, Xu et al. (2010) showed that *P. donghaiense* grew at high rates at light intensities as low as  $2 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$  and that irradiance was saturating at just 30 but not yet inhibited at  $230 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . Liu et al. (2011) observed maximum specific growth rates for *P. donghaiense* at a light intensity of  $70 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ , but reported that growth rates were inhibited at irradiances higher than  $150 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . In highly turbid areas such as the East China Sea (Sun et al. 2008), light availability can limit photosynthesis. The intensity of PAR can be estimated from the depth of mixed

layer and turbidity (Diehl et al. 2002). In spring, the surface PAR in the East China Sea was estimated to be  $\sim 914 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$  (Sun et al. 2008), with light intensity attenuating rapidly with depth. *P. donghaiense* blooms are observed mainly near the surface (upper 10 m) and the maximum recorded cell density was  $36 \times 10^7 \text{ cells l}^{-1}$  (Lu et al. 2005). Because algal cells are also particles that contribute to turbidity and light scattering and attenuation, during dense surface blooms the euphotic depth can become shallow, and the light reaching cells is likely to be substantially less than that measured at the surface. While light may be abundant as blooms initiate and cell densities are still low, as *P. donghaiense* abundances increase (reaching densities as high as  $36 \times 10^7 \text{ cells l}^{-1}$ ; Lu et al. 2005) light may become rapidly attenuated, causing self-shading. The ability of *P. donghaiense* to grow at low irradiances ( $10$  to  $20 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ), is likely important, because dense blooms often last more than a month. All the results from field and laboratory are comparable with ours, which suggests that *P. donghaiense* can easily adjust to widely fluctuating irradiance levels.

Irradiance can affect the cell size of phytoplankton, its nutrient quota, and also nutrient preference and uptake (Thompson et al. 1989, Chang & Page 1995, Fan & Glibert 2005, Herndon & Cochlan 2007). In this study, we found that irradiance affected the N preference of *P. donghaiense*. At lower irradiance ( $< 20 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ), *P. donghaiense* had similar growth rates when grown on the 4 N species tested, but when the irradiance

increased, the growth rates varied in different N sources. At the highest irradiance ( $180 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ ), the average specific growth rates ranged from  $0.9 \text{ d}^{-1}$  in cultures grown on urea, to  $1.4 \text{ d}^{-1}$  in cultures growing with  $\text{NH}_4^+$  as the sole N source (Fig. 2);  $\mu_{\text{max}}$  for *P. donghaiense* grown on the 4 different N sources ranged from  $1.3 \pm 0.0$  to  $2.0 \pm 0.1 \text{ d}^{-1}$  (Table 1). Our results are comparable with other phytoplankton species. Chang & Page (1995) found that light affected cell size of *Heterosigma carterae*, and also had more influence on cultures grown with  $\text{NO}_3^-$  than cultures grown with  $\text{NH}_4^+$  or urea. In *Thalassiosira pseudonana* culture experiments at lower irradiance ( $\leq 29 \mu\text{E m}^{-2} \text{ s}^{-1}$ ),  $\text{NO}_3^-$ -grown cells had equal growth rates, N quotas, chl *a*, and equal or greater carbon quotas compared to  $\text{NH}_4^+$ -grown cells, but at higher irradiance, growth rates were lower for cells growing on  $\text{NO}_3^-$  compared to cells growing on  $\text{NH}_4^+$  (Thompson et al. 1989)—results similar to ours. At lower irradiance, growth rates of *T. pseudonana* grown on  $\text{NO}_3^-$  and  $\text{NH}_4^+$  were not significantly different, which suggests that  $\text{NO}_3^-$ -grown cells may make other physiological adjustments to compensate for their greater need for reductant. Conversely, at higher irradiance, culture of this organism grown on  $\text{NO}_3^-$  had lower carbon and N quotas, implying that the higher energy requirements of  $\text{NO}_3^-$ -grown cells (for N reduction) are compensated for by a reduction of C quota, N quota, chl *a*, and growth rate (Thompson et al. 1989), and suggesting that phytoplankton can adapt by changing different parameters at specific irradiance levels in order to maintain a high growth rate. We did not measure the same parameters as Thompson et al. (1989), but from our *in vivo* fluorescence data, we found cultures grown on  $\text{NH}_4^+$  had higher fluorescence than cultures grown on  $\text{NO}_3^-$  at higher irradiance ( $>80 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ ), hence, our result might be partially explained in a similar way as for *T. pseudonana*. In another similar work, the growth rate of *Heterosigma akashiwo* was found to be slightly higher in cultures grown on  $\text{NH}_4^+$  ( $0.89 \text{ d}^{-1}$ ) compared to those grown on  $\text{NO}_3^-$  or urea at saturating irradiance ( $110 \mu\text{E m}^{-2} \text{ s}^{-1}$ ), but at sub-saturating irradiance ( $40 \mu\text{E m}^{-2} \text{ s}^{-1}$ ), both cultures supplied with urea and  $\text{NH}_4^+$  grew faster than cultures supplied with  $\text{NO}_3^-$  (Herndon & Cochlan 2007). Besides the culture experiments, field studies also demonstrate that irradiance has an important role in N uptake and preference (Hu & Smith 1998, Kudela & Cochlan 2000, Fan & Glibert

2005). Previous studies and our results clearly demonstrated that irradiance and N source have interactive effects on N preference and uptake. Adaptation to varying irradiance and an ability to utilize an array of N species may contribute to the ability of *P. donghaiense* to form dense blooms in highly turbid waters.

## $\text{CO}_2$

$\text{CO}_2$  concentrations in the atmosphere have risen from 280 ppm during the Industrial Revolution to 400 ppm in 2015, and are still rising at an accelerating rate. It is expected that  $p\text{CO}_2$  will double by the end of this century (IPCC 2013). The increased  $\text{CO}_2$  concentration in the atmosphere has led to a series of environmental problems, including ocean acidification. Increasing  $p\text{CO}_2$  may also influence phytoplankton growth rates, element ratios, photosynthetic rates, nutrient utilization, toxin production, and community structure (Burkhardt et al. 1999, Fu et al. 2007, Hutchins et al. 2013, Li & Campbell 2013, Tatters et al. 2013, Errera et al. 2014). While an increase in HABs has long been linked with nutrient over-enrichment (Heisler et al. 2008), the effects of climate warming and increased  $p\text{CO}_2$  are not as well understood for most taxa (Moore et al. 2008, Paerl & Huisman 2009, Hallegraeff 2010, O'Neil et al. 2012, Paerl & Paul 2012).

We found that *P. donghaiense* growth rates increased by  $>10\%$ , and  $\text{NH}_4^+$  uptake rates increased by about 57 to 79% in cultures maintained at elevated  $p\text{CO}_2$  (500 to 750 ppm) relative to those grown at present-day  $p\text{CO}_2$  (Figs. 3 & 4). This suggests that increases in  $p\text{CO}_2$  may positively affect the growth of *P. donghaiense*, and this could affect the magnitude and duration of blooms. Higher atmospheric  $\text{CO}_2$  concentrations and elevated  $p\text{CO}_2$  may offset some of the DIC drawdown. Elevated  $p\text{CO}_2$  increases growth rates of many cyanobacteria and some other phytoplankton (Fu et al. 2007, 2012, Wu et al. 2014), likely by alleviating C limitation of photosynthesis (Hutchins et al. 2007). Elevated  $p\text{CO}_2$  also results in higher DIC concentrations in seawater, and this alters the inorganic carbon buffer system and decreases pH in oligotrophic waters where photosynthetic drawdown of DIC is relatively low. In contrast, in nutrient-enriched coastal systems, increasing the supply of DIC may alleviate C limitation of algal growth, provided light or some other element does not limit their growth.

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