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Beneficial effects of aluminum enrichment on nitrogen-fixing cyanobacteria in the South China Sea

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ABSTRACT

Few studies focus on the effects of aluminum (Al) on marine nitrogen-fixing cyanobacteria, which play important roles in the ocean nitrogen cycling. To examine the effects of Al on the nitrogen-fixing cyanobacteria, bioassay experiments in the oligotrophic South China Sea (SCS) and culture of *Crocosphaera watsonii* in the laboratory were conducted. Field data showed that 200 nM Al stimulated the growth and the nitrogenase gene expression of *Trichodesmium* and unicellular diazotrophic cyanobacterium group A, and the nitrogen fixation rates of the whole community. Laboratory experiments demonstrated that Al stimulated the growth and nitrogen fixation of *C. watsonii* under phosphorus limited conditions. Both field and laboratory results indicated that Al could stimulate the growth of diazotrophs and nitrogen fixation in oligotrophic oceans such as the SCS, which is likely related to the utilization of phosphorus, implying that Al plays an important role in the ocean nitrogen and carbon cycles by influencing nitrogen fixation.

1. Introduction

Aluminum (Al) is widespread in the environment. Atmospheric dust input has been recognized as a very important source of Al in the upper layer of open oceans (Duce et al., 1991; Measures and Vink, 2000; Kramer et al., 2004). The concentrations of dissolved Al ranged from 0.1 nM in the surface waters of the Pacific Ocean to 657 nM in the Arabian Sea (Measures et al., 2005; Narvekar and Singbal, 1993). Previous studies have demonstrated that dissolved Al can be removed from seawater through absorption by marine phytoplankton (Gehlen et al., 2002), and adsorption onto inorganic or organic particles (Measures and Vink, 2000). Although Al is present in every organism, no biological function of Al has been found yet (Exley and Mold, 2015). It is widely accepted that Al can be toxic to different phytoplankton in acidic waters (Gensemer and Playle, 1999). Just a few studies indicated that the influence of Al on marine phytoplankton may depend on the Al concentration, phytoplankton species, and nutrient status in seawater (Stoffyn, 1979; Vrieling et al., 1999; Saçan and Balcioğlu, 2006; Golding et al., 2015; Shi et al., 2015; Zhou et al., 2016; Gillmore et al., 2016). However, our understanding of the effects of Al on nitrogen-fixing cyanobacteria in seawater is very limited.

Nitrogen-fixing cyanobacteria play important roles in the nitrogen cycle and potentially influence the efficiency of the biological carbon pump, by providing new nitrogen and supporting primary production (Karl et al., 2002). The South China Sea (SCS), which is located in the subtropical and tropical western Pacific Ocean, is one of the largest marginal seas on Earth. Its central gyre is warm, permanently stratified and oligotrophic, suggesting that the environment is favorable for nitrogen fixation (Wu et al., 2003). Cyanobacteria *Trichodesmium* spp. are commonly found in the SCS (Chen et al., 2003; Moisander et al., 2008). Recent studies revealed that unicellular diazotrophic cyanobacteria were also abundant and could contribute significant nitrogen fixation in the SCS (Shiozaki et al., 2014; Chen et al., 2014). Nitrogen fixation plays an important role in the contribution of nitrogen to primary production in the open waters of the SCS (Grosse et al., 2010; Liu et al., 2016).

It has been reported that nitrogen fixation could be limited by phosphorus and iron availability in oligotrophic oceans (Mills et al., 2004; Sañudo-Wilhelmy et al., 2001). A few recent studies have demonstrated that iron input from atmospheric dust has a large impact on nitrogen-fixing cyanobacteria (Langlois et al., 2012). The factors limiting nitrogen fixation in the SCS are still not clear. A previous study

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suggested that nitrogen fixation in the SCS could be limited by available iron because of the lack of iron-binding organic ligands (Wu et al., 2003), and a recent study showed that addition of iron decreased the expression of the iron limitation-induced gene *idiA* in *Trichodesmium* spp. (Hong et al., 2017), whereas another study indicated that nitrogen fixation was unlikely limited by iron in the northwestern SCS (Zhang et al., 2015).

Al is usually used as a tracer of atmospheric input of dust and iron, and its direct effects on nitrogen fixation and nitrogen-fixing cyanobacteria in the ocean are often unnoticed and unexplored, although there are studies show that *Trichodesmium* abundance and nitrogen fixation rate were more positively correlated with dissolved Al than dissolved iron in the Atlantic Ocean (Moore et al., 2009), and the concentration of total nitrogen-fixing nitrogenase gene (*nifH*) in the North Atlantic Ocean was five times more related to dissolved Al than dissolved iron (Rijkenberg et al., 2011). Moreover, high content of Al in *Trichodesmium*, and a *Trichodesmium* gene involved in resistance to Al toxicity had also been reported (da Cunha et al., 2009; Tovar-Sanchez et al., 2006). By adding high concentrations of Al (2 μM and 20 μM) in the natural seawater of the SCS, enhanced growth of *Trichodesmium* and increased nitrogen fixation rates were indeed observed in a recent study (Zhou et al., 2017). Nevertheless, will environmentally relevant low concentrations of Al still beneficially influence nitrogen fixation and nitrogen-fixing cyanobacteria, and why and how Al enrichment stimulates the *Trichodesmium* growth and nitrogen fixation are unknown.

Dissolved Al concentration in the SCS is relatively high (from 48 to 852 nM and from 82 to 470 nM in the surface waters of the Pearl River Estuary and the northern SCS, respectively) (Liu et al., 2017; Wang et al., 2014). We hypothesize that environmentally relevant low levels of Al could still be beneficial to the growth of nitrogen-fixing cyanobacteria and nitrogen fixation. We also hypothesize that the beneficial effects of Al on nitrogen-fixing cyanobacteria might be related to phosphorus utilization by the cyanobacteria, as one recent study showed that enhanced utilization of dissolved organic phosphorus likely accounted for the beneficial effects of Al on a marine diatom in a phosphorus-depleted medium (Zhou et al., 2016).

To examine the hypotheses, we evaluated the responses of the *nifH* abundance and *nifH* expression of nitrogen-fixing cyanobacteria, and nitrogen fixation to low levels of Al enrichment in the oligotrophic SCS, and axenic cultures of *C. watsonii* WH0003 (one of the main models of marine unicellular diazotroph) in phosphorus-depleted Aquil* media with or without Al were grown in the laboratory.

2. Materials and methods

2.1. Bioassay experiments in the field

The field study was carried out during three cruises in August 2012, December 2012 and August 2015 in the SCS (Fig. 1, Table 1). Duplicate surface seawater was collected at windward sides on the ship bow using a polypropylene bucket tied together with a nylon rope (rinsed three times with Milli-Q water after soaked in 2.5 mol L^{-1} HCl solution for a week) and was pre-filtered with 200 μm nylon mesh to remove grazers larger than 200 μm, and then discharged into acid-cleaned and seawater-rinsed 2 L polycarbonate Nalgene bottles. $AlCl_3$ (99.999% trace metals basis, Sigma-Aldrich, USA) in stock solutions was added to cultures to achieve nominal final concentrations of 200 nM and 2000 nM, corresponding to the high Al level in natural seawater and the maximum Al level in the upper end of the estuary, respectively (Measures et al., 2005; Narvekar and Singbal, 1993; Zhang et al., 1999). Potential contamination of iron and other metals in the Al stock solution was excluded by preparing the stock in a 100-class clean room and testing the stock solution using the inductively coupled plasma mass spectrometer (Agilent 7700) (Zhou et al., 2017). Therefore, any trace amount of inadvertent addition of other metals into the experimental bottles was considered to be the same for each bottle and minimal. Bottles were then placed in incubators on the deck shaded to 20% of incident surface irradiance with neutral density black net, and water temperature was maintained with constant flowing surface seawater.

Nitrogen fixation rates of the whole community of plankton larger than 0.7 μm were measured at two oligotrophic stations (X28 and X31). Water samples for extracting DNA (at KJ31) and RNA (at Y308) were collected at the time points of 48 h and 96 h of the two short-term (4 days) bioassays. Since RNA transcripts vary by orders of magnitude on a diel cycle, and *Trichodesmium* and unicellular diazotrophic cyanobacterium group A (UCYN-A) express the *nifH* during the day (Zehr et al., 2007), we sampled at the same time-points of the daytime (Table 1). Although the incubation period was only 48/96 h in the field study, it was enough for the cells to respond at the DNA replication level and for growth to occur (Langlois et al., 2012). In order to determine whether the response of the *nifH* expression to Al varies under the conditions of exposing nitrogen-fixing cyanobacteria to sufficient trace metals (including iron) and long-term Al, we also conducted a longer-term (8 days) semi-continuous incubation by diluting cultures every three days to achieve a target Al and nutrient condition according to the f/200 recipe (https://ncma.bigelow.org/algal-recipes) without nitrogen nutrient at station Y104. RNA samples were collected on the 6th and 8th days of the experiment. Since there is one copy of *nifH* per genome in UCYN-A and *Trichodesmium* spp., *nifH* abundance can be used as an rough estimate of cellular abundance, and could be quantified by using the quantitative polymerase chain reaction (qPCR) (Moisander et al., 2010). It was reported that the diel cycle of *nifH* gene expression of cyanobacteria in the lab is similar to that of natural populations, and cell-specific nitrogen fixation rate generally agrees with the level of *nifH* expression of each group of diazotrophs (Zehr et al., 2007). This genetic approach provides a comprehensive portrait of nitrogen fixation in natural assemblages of microbes (Zehr and Turner, 2001).

2.2. Laboratory culture experiments

Axenic culture of *C. watsonii* WH0003 was grown in modified Aquil* medium (Sunda et al., 2005) without silicate or nitrogen in 0.5 L polycarbonate bottles, at 28 °C and under an illumination of 120 μmol photons m^{-2} s^{-1} with a light:dark cycle of 12:12 h. Oligotrophic surface seawater collected from the SCS was used to prepare the medium. The seawater was 0.22-μm filtered and sterilized by microwave, while stock solutions of phosphate, vitamins and trace metals were sterilized by filtration. For the experimental media, two phosphate concentrations of 10 μM and 0.1 μM were set to make up the phosphorus-replete and phosphorus-depleted conditions, respectively. $AlCl_3$ in similar stock solutions used in the field study was added to the media to achieve nominal final Al concentrations of 0, 0.2, 2 and 20 μM (which is approximate to the solubility of Al in seawater (Angel et al., 2016). Four replicates were set for each treatment. *C. watsonii* cells in exponential growth phase were transferred to the experimental media to reach an initial cell density of 20×10^3 cells mL^{-1}, and cell abundance in each treatment was monitored by using a flow cytometry (Accuri C6, Becton Dickinson, USA). All the experimental cultures were incubated with a semi-continuous culturing method by diluting the cultures every 3 days with new experimental media to keep the algae growth in the exponential phase. Eight times of dilution were finished before the final measurement of the algal growth rate and nitrogen fixation rate for each treatment. After the last dilution, the algal growth rate was measured during the exponential growth phase, and the nitrogen fixation rate was estimated on the second day after the dilution.

2.3. Determination of picophytoplankton abundance, and nutrient and chlorophyll a (Chl a) concentrations

To measure the influence of Al enrichment on non-diazotrophic pico-size cyanobacteria (i.e. *Prochlorococcus* and *Synechococcus*),