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Enhanced utilization of organic phosphorus in a marine diatom *Thalassiosira weissflogii*: A possible mechanism for aluminum effect under P limitation

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ABSTRACT

Although many studies have reported the aluminum (Al) impacts on freshwater organisms and terrestrial plants in acidic and neutral pH media, little information is available on the effects of Al on organisms in the alkaline seawater. In this study, the Al effects on marine phytoplankton were investigated by growing the axenic diatom *Thalassiosira weissflogii* in seawater media amended with varied nutrient levels. Under phosphorus (P) limited conditions, Al enrichment resulted in an enhanced diatom growth and higher biomass accumulation, as well as the maintenance of high diatom biomass during the stationary phase. The diatom displayed higher cellular alkaline phosphatase activity (APA) under Al-enriched and P-limited conditions, which was responsible for the increased uptake of dissolved organic phosphorus (DOP). Lower dissolved APA was observed in the Al-enriched culture. In contrast, Al addition did not change the phosphate speciation in seawater or the diatom uptake of dissolved inorganic P (DIP) at low concentration, but instead made the diatoms uncompetitive under high DIP conditions. The results strongly indicated that Al treatment increased the proportion of the diatom cellular APA and their utilization efficiency of DOP, which may partly account for the beneficial effects of Al on the diatom under P-limited conditions. It is thus likely that Al may influence the ocean carbon cycling by promoting the phytoplankton utilization of DOP.

1. Introduction

Aluminum (Al) is the most abundant metallic element in the earth's crust, and is ubiquitous in the environment. Although Al has been regarded as a toxic element to a number of organisms including terrestrial plants (Osaki et al., 1997), freshwater biota and even humans (Macdonald and Martin, 1988; Gensemer and Playle, 1999; Yokel, 2000), the enhanced growth of terrestrial plants induced by Al has also been reported (reviewed in Foy, 1984). In contrast to these earlier substantial reports on the Al effects on biota in the acidic and neutral pH media, little is known regarding the effects of Al on organisms in alkaline seawater.

In alkaline seawater, Al hydroxides including $Al(OH)^0_3$ and $Al(OH)^-_4$ are the predominant forms of dissolved Al. Speciation of Al in alkaline seawater is greatly different from those in acidic and neutral pH freshwater media (Macdonald and Martin, 1988). Recent studies showed that the phytoplankton cellular accumulation of Al could not be solely explained by the free Al speciation when pH was higher than 6.5 (Crémazy et al., 2013a, 2013b). These results then led to speculation that phytoplankton could not only take up Al^{3+} but also the Al hydroxides. Clearly, the distinct Al speciation in alkaline seawater suggested that its influence on marine organisms may be different from those observed in freshwater organisms.

Earlier studies on Al and marine biota interaction mainly focused on the scavenging effects of biological activity on the distribution of Al in seawater, but seldom dealt with the Al influences on marine organisms. Many studies found that marine plankton could take up and/or absorb Al, and scavenge dissolved Al in seawater (e.g. Bostrom et al., 1974; Mackenzie et al., 1978; Moran and Moore, 1988; Saçan and Balcioglu, 2001; Ren et al., 2011; Dammshäuser et al., 2013; Li et al., 2013). Over fifty years ago, Menzel et al. (1963) found that the addition of Al to water collected from the Sargasso Sea had a stimulatory effect on the productivity of phytoplankton (particularly diatoms) in the water, if nitrate and phosphate were simultaneously added, and sufficient silicate was present. Stoffyn (1979) reported that Al had a stimulatory effect on the growth of a marine diatom *Skeletonema costatum*. Later, Vrieling et al. (1999) showed that Al enrichment increased the growth of a pennate diatom *Navicula salinarum* but not of a centric species *Thalassiosira weissflogii*. Saçan et al. (2007) reported that Al had a stimulatory effect at low concentration (500 μg l^{-1}) and an inhibitory effect at high concentration (>4000 μg l^{-1}) on the growth of *Dunaliella*

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tertiolecta. Other studies documented the incorporation of Al in dead and living diatom frustule (Gehlen et al., 2002; Koning et al., 2007). A most recent study reported the high tolerance to Al by 11 marine organisms (including four phytoplankton species) representing 6 taxonomic groups (Golding et al., 2015). All these findings implied that Al was likely involved in the marine biogeochemical cycles by affecting marine phytoplankton growth, but very few studies examined the Al effects on marine phytoplankton growth and the underlying mechanisms of such effect, if any.

Nutrient utilization is one possible mechanism for the beneficial effects of Al on plant growth. The possible roles of Al in the enhanced plant growth including the increased iron availability, promotion of phosphorus (P) uptake, and protection against copper, manganese and P toxicity were first reported for different plant genotypes and growth media (reviewed in Foy, 1984). Then, alleviation of H^+ toxicity (Kinraide, 1993) and increase of nitrogen, P and potassium uptake (Osaki et al., 1997) by plant roots were considered to be the general mechanisms for the stimulatory effects of Al on terrestrial plant growth in acidic media. Whether or not Al affects phytoplankton utilization of nutrients like P in alkaline seawater is unknown.

In the present study, the diatom growth under different Al enrichments and P-limited conditions was firstly quantified, and then the P (both inorganic P and organic P) utilization under Al-enriched conditions was measured. The present study provided evidence that the sustained diatom growth by Al was likely mediated by the availability of nutrients such as DOP.

2. Materials and methods

In order to examine the Al effects on marine phytoplankton growth and understand the underlying mechanisms, axenic cultures of a marine diatom *T. weissflogii* (CCMP1336) were grown in seawater media with varied nutrient levels (including low nutrient, and P-deficient) and with/without Al amendment, and the growth of the cells was monitored. Furthermore, the Al influences on DIP speciation and diatom utilization of DIP and DOP in seawater were examined. A range of Al concentrations (40 nM, 200 nM, 2 μM and 20 μM) were tested in this study. These four concentrations corresponded to the levels in ocean surface impacted by high dust deposition (40 nM), the upper range of dissolved Al in natural seawater (200 nM), the level in riverine waters of the estuary (2 μM), and the level approximate to the solubility of Al in seawater (20 μM), respectively (de Jong et al., 2007; Brown and Bruland, 2009; Golding et al., 2015). Golding et al. (2015) estimated that Al at levels of 24 μg/l (~0.89 μM) were safe for 95% of marine organisms. The Al salt used for amendment had a purity of 99.999% based on trace metals analysis (Sigma-Aldrich 563,919); therefore any possibility of other metal addition into the seawater medium was considered to be minimal if existent.

2.1. Seawater media and diatom incubation

Several incubation experiments were firstly conducted in media using seawater collected from an oligotrophic basin in the South China Sea, and aged seawater collected from the coast off eastern Hong Kong. For the first experiment, the oligotrophic seawater with low levels of dissolved inorganic nutrients (0.15 μM nitrate plus nitrite and 3.7 μM silicate) was spiked with sufficient silicate (42 μM), and was then enriched with 1) Al (Al, 2 μM $AlCl_3$), 2) nitrate and phosphate (NP, 1 μM $NaNO_3$ and 0.1 μM NaH_2PO_4), 3) nitrate, phosphate and Al (NPA, 1 μM $NaNO_3$, 0.1 μM NaH_2PO_4 and 2 μM $AlCl_3$), and 4) nothing as control. The diatom *T. weissflogii* in exponential phase was incubated for three days in the oligotrophic seawater medium spiked with 42 μM of silicate. The treated *T. weissflogii* was inoculated to experimental bottles to reach an initial cell density of 4.6×10^3 cells ml^{-1}.

For the other experiments, the aged seawater (with 0.37 μM nitrate plus nitrite, 4.3 μM silicate and non-detectable phosphate) was enriched according to modified f/2 recipes (https://ncma.bigelow.org/node/79) including the f/200 recipe, and the f/20 recipe with modified phosphate and/or silicate concentrations. Two different concentrations of phosphate (0.36 μM and 3.62 μM corresponding to the f/200 and f/20 recipes, respectively) were added into the media to achieve the P-deficient and -sufficient conditions, respectively. Silicate (106 μM) was added into the media to achieve the f/20 medium with modified silicate. $AlCl_3$ (20 μM) was added to the medium for the treatment but not for the control.

For all the above experiments, diatom cells were grown in 500-ml polycarbonate bottles. Three replicates were prepared for each treatment. The bottles were incubated under a constant temperature of 24 °C, with a light:dark cycle of 14 h:10 h and light intensity of 128 μmol photons m^{-2} s^{-1}. All the cultures were mixed gently (without disrupting the cells) twice a day. At the beginning of the cultures the pH in the media was adjusted to 8.1.

Cell abundance and/or chlorophyll *a* (Chl *a*) were monitored during the incubation. Diatom cell abundance was quantified by the microscope or by using a Becton Dickinson FACSCalibur cytometer with a 488-nm laser (with yellow-green fluorescent beads 10 μm in diameter, Polysciences, Inc. as the internal standard). The red fluorescence signal (FL3) was used as the indicator for cellular chlorophyll, and the side scatter signal (SSC) was used as the indicator for cell size (Tzur et al., 2011). To determine the Chl *a*, 5 ml of the diatom cells was filtered on Whatman GF/F filter. Chlorophyll *a* was extracted by dipping the filters into 90% acetone at −20 °C in darkness for 24 h, and analyzed by fluorometry using a Turner Designs 10-AU Fluorometer (Parsons et al., 1984).

2.2. Diatom growth under P-limited and P-sufficient conditions in Aquil medium

The influences of varied concentrations of Al on diatom growth were further tested under P-limited conditions in modified Aquil medium with only 0.2 μM phosphate. Chemicals and protocols for the preparation of the medium suggested by Sunda et al. (2005) were followed. Specifically, natural seawater collected from the oligotrophic basin of the South China Sea was used for the Aquil medium. All the stock solutions of macronutrients, vitamins, trace metals and Al were prepared in a 100-class clean room. The macronutrients were Chelex-treated in the 100-class clean room.

In this experiment, a range of Al addition, including 40 nM, 200 nM, 2 μM and 20 μM, was used. Three replicates were prepared for each treatment. All the bottles were set overnight before the inoculation of *T. weissflogii* cells in exponential phase to reach an initial density of 900 cells ml^{-1}. The diatom was cultured under a continuous light regime with a light density of 120 μmol proton m^{-2} s^{-1} at 20 °C. Diatom cell abundance and cell volume in the media was measured by a Beckman Coulter Counter (Z2). In vivo Chl *a* (fluorescence) was measured by using the Tuner Designs Trilogy Fluorometer. The pH was measured by using the Thymol-blue method (Zhang and Byrne, 1996). All the sampling was conducted in an aseptic, dust-free 100-class clean Laminar flow cabinet.

The diatom growth rates on the basis of cell abundance, cell volume and in vivo Chl *a* were calculated both in the exponential phase and in the early stationary phase (from the end of the exponential phase to the start of the decay phase).

To examine the influences of Al on diatom growth under P-sufficient conditions, *T. weissflogii* cells in the exponential phase was inoculated in the Aquil medium with 10 μM phosphate and varied amounts of Al to set the control treatment and the Al-enriched ones with final Al concentrations of 40 nM and 200 nM, respectively. Three replicates were prepared for each treatment. The diatom was cultured and monitored under the same conditions as described above.

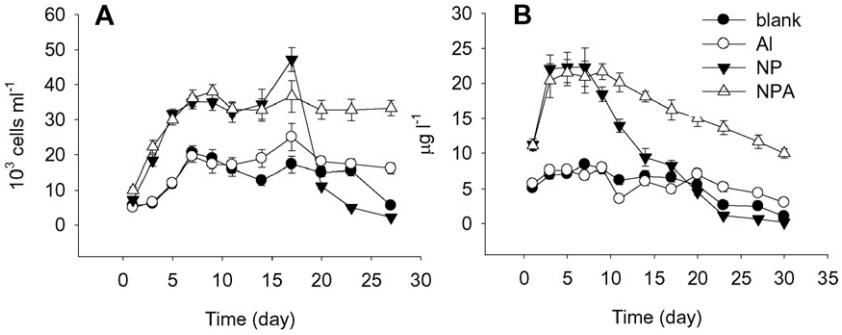

Fig. 1. Effects of Al enrichment on *Thalassiosira weissflogii* growth in low nutrient medium. A, cell abundance; B, chlorophyll *a*; Al, adding 2 μM $AlCl_3$; NP, adding 1 μM $NaNO_3$ and 0.1 μM NaH_2PO_4; NPA, adding 1 μM $NaNO_3$, 0.1 μM NaH_2PO_4 and 2 μM $AlCl_3$. Data are mean ± SD (n = 3).

2.3. Determination of dissolved Al and phosphate speciation in seawater medium

Dissolved Al during the cultures in P-deficient f/20 medium was measured by using the Al-lumogallion complex method according to Ren et al. (2001). Specifically, algal suspension was filtered through a polycarbonate filter with a pore size of 0.22 μm, the filtrate was used for the Al measurement according to the following procedure: 1) adding 5 μl o-phenanthroline solution (16 mM), and 30 μl Be^{2+} solution (50 mM) to the 10 ml samples, then mixing them thoroughly; 2) adding 100 μl sodium acetate buffer solution (pH = 5.0; prepared by dissolving 32.0 g sodium acetate and 12 ml acetic acid in deionized water, and then diluted to 100 ml), and thoroughly mixing; 3) adding 100 μl lumogallion (LMG) solution (0.02% m/v LMG), and thoroughly mixing; 4) heating the samples at 80 °C for 90 min; 5) adding 100 μl Triton X-100 solution (1% v/v); 6) after 30 min reaction, the fluorescence was measured at a 500-nm excitation/570-nm emission with a Wallac Victor 3 multilabel counter (Perkin Elmer). Dissolved Al concentration on the basis of the fluorescence was calculated according to the standard curve.

To examine the possible phosphate precipitation after the enrichment of $AlCl_3$ in seawater media, ^{33}P labeled phosphate was added to the targeted media (with varied levels of unlabeled phosphate (0, 0.36 and 3.62 μM)) at a final concentration of 6.0×10^5 Bq l^{-1}, and mixed thoroughly with the medium. Four levels of Al (0, 0.2, 2 and 20 μM) were added to the media. Then 1 ml of the ^{33}P labeled medium on the time points of 0, 2 h and/or 4 h were centrifugally filtered by centrifugal spin filters with a pore size of 0.2 μm. The initial labeled medium (0.5 ml) and the filtrate were dipped in 5 ml scintillation cocktail, and the samples were measured by the Wallac WinSpectral 1414 liquid scintillation counter. The proportion of the dissolved phosphate was calculated as the ratio of ^{33}P in the filtrate to that in the same volume of unfiltered medium.

Aluminum influence on phosphate speciation in seawater media was also examined by using the chemical equilibrium computer model Visual MINTEQ maintained by Jon Petter Gustafsson (http://vminteq.lwr.kth.se/). Ocean water composition described by Sunda et al. (2005) was used for the model computation. For the computation, the pH was fixed at 7.9 to 10.0, and temperature was at 24 °C.

2.4. Determination of alkaline phosphatase activity (APA)

Cellular APA of *T weissflogii* cells and dissolved APA were measured on the fourth and sixth day in the stationary phase of the incubation (when the diatoms started to be nutrient-limited) in the P-deficient f/20 medium, and cellular APA was measured on the sixth day in the stationary phase of the incubation in P-sufficient f/20 medium. The APA was measured using the 4-methylumbelliferyl phosphate disodium salt (MUP) as the DOP analog (Duhamel et al., 2010; Sun et al., 2012). For cellular APA, two to five milliliters of algal suspension was filtered on the 1-μm-porosity polycarbonate filter (25 mm in diameter). The filter was immersed in 1.5 ml AP buffer (50 ml 0.5 M $CaCl_2$, 100 ml 0.5 M Tris at pH 8.4, 800 ml autoclaved seawater, and 0.5 mM MUP) and then incubated at 25 °C under vibration. Then 100 μl APA mixture was added into a centrifuge tube with 400 μl Na_2CO_3 solution (0.2 M) at 15 min, 30 min, 45 min, and 60 min. After centrifuging at 12,000 rpm for 2 min, 200 μl supernatant was added in a 96-well plate. The time-dependent changes in the fluorescent product hydrolyzed from MUP by alkaline phosphatase were measured at a 360-nm excitation/460-nm emission with a Wallac Victor 3 multilabel counter (Perkin Elmer). For dissolved APA, 0.5 ml of 0.22-μm-pore-size-filtered algal suspension was added into 1 ml AP buffer (1 mM MUP), and the following procedures were the same as for the cellular APA. The APA was determined as the linear increase in fluorescence with time. Cellular APA was calculated by normalizing the APA to cell number, and dissolved APA was also expressed on a cell abundance basis. Bulk APA was calculated as the sum of the cellular and dissolved APA.

Bulk and cellular APA on the 5.2th day in the stationary phase of the incubation in P-deficient Aquil medium was measured by spectrophotometry with ρ-nitro-phenylphosphate (ρ-NPP) as the DOP analog (Xu et al., 2006), which was more convenient for large sample size than MUP as the DOP analog. For the bulk APA, 50 μl of 20 mM ρ-NPP

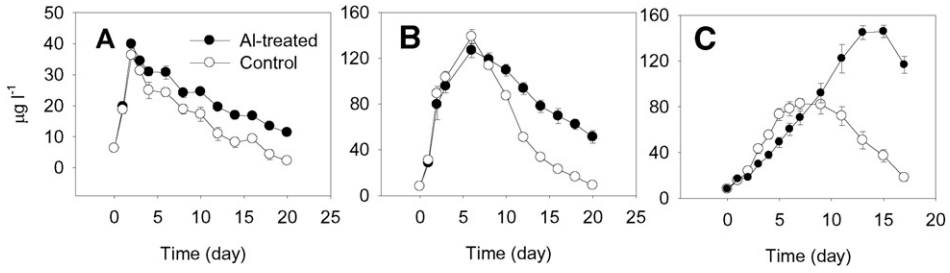

Fig. 2. Effects of Al enrichment on *Thalassiosira weissflogii* in media with varied nutrient levels. Cell growth was quantified as Chl *a*. A, f/200 medium; B, f/20 medium; C, f/20 medium with modified silicate of 106 μM. Data are mean ± SD (n = 3).

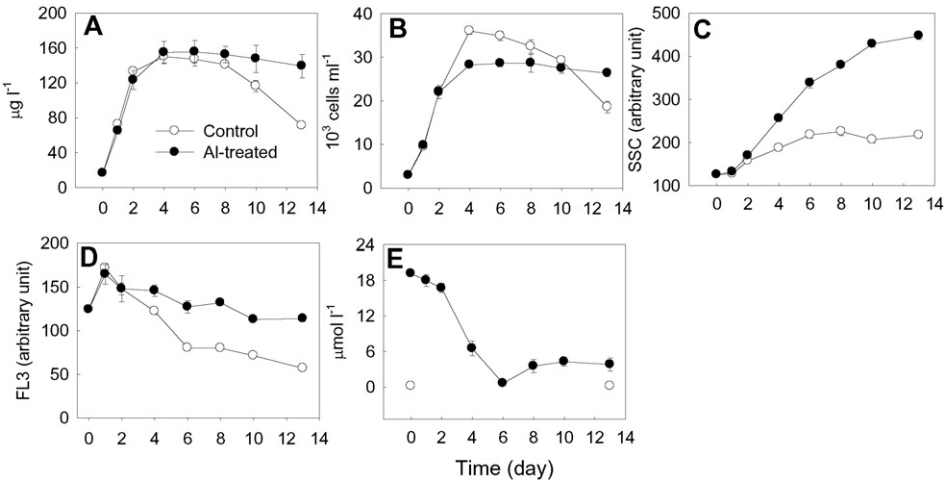

Fig. 3. Effects of Al enrichment on *Thalassiosira weissflogii* in P-deficient f/20 medium. A, chlorophyll *a*; B, cell abundance; C, SSC, side scatter signal indicates cell size; D, FL3, cellular red fluorescence excited by a 488-nm laser, indicating cellular chlorophyll content; and E, dissolved Al. Data are mean ± SD (n = 3).

(in 1 M Tris buffer at pH 8.2) was added into 1 ml algal suspension in a 1.5-ml centrifuge tube, and was thoroughly mixed with the algal suspension. 900 μl of the mixture was transferred into a quartz colorimetric utensil. The absorbance at 405 nm was measured continuously for two to five minutes using a UV–VIS spectrophotometer. The APA was computed from the linear regression of absorbance versus time and then normalized to cell number. For the cellular APA, two milliliters of algal suspension was filtered on the 1-μm-porosity polycarbonate filter (25 mm in diameter). Then the filter was immersed in 1 ml seawater medium spiked with 50 μl of 20 mM ρ-NPP in a 1.5-ml centrifuge tube. The mixture was thoroughly mixed using a vortex mixer, and the following procedures were the same as for the bulk APA.

2.5. Dissolved organic phosphorus (DOP) uptake by diatoms

^{33}P labeled DOP was prepared by incubating *T. weissflogii* for six days in the f/20 medium spiked with ^{33}P labeled phosphate (27.9 Ci mg^{-1}) to reach a final concentration of 4.85×10^5 Bq l^{-1}. After six-day incubation, *T. weissflogii* cells were collected by centrifugation, resuspended into aged seawater and disrupted by sonication. Then, the treated algal suspension was passed through the 0.22-μm filter, and the filtrate was used as the ^{33}P labeled DOP. The radiochemical concentration of the labeled DOP was measured as 4.11×10^5 Bq l^{-1}. Assuming that 4.85×10^5 Bq equaled to 3.62 μmol P, the specific activity of the ^{33}P-DOP was calculated as 421 Ci g^{-1} or 13 Ci mmol^{-1}.

To examine the effects of Al treatment on diatom uptake of DOP, *T. weissflogii* cells grown in both the P-deficient and -sufficient f/20 media with/without Al enrichment were collected on the sixth day of the incubations. The cells were centrifuged and resuspended in modified f/20 medium (without phosphate). ^{33}P labeled DOP was added to the medium with a final concentration of 4.11×10^4 Bq l^{-1}. Subsamples of a 10-ml algal suspension were collected on polycarbonate filters (with a pore size of 1 μm) at the time points of 15, 30, 45 and 60 min. The filter was washed (Orchard et al., 2010) and then dipped in a 5 ml scintillation cocktail, and the sample was measured using the Wallac WinSpectral 1414 liquid scintillation counter. Total DOP uptake rate was determined as the linear increase in cellular P with time. Cellular DOP uptake rate was calculated by normalizing the total DOP uptake rate to cell number.

2.6. Dissolved inorganic phosphorus (DIP) uptake by diatoms

To examine the effects of Al treatment on diatom uptake of DIP, *T. weissflogii* cells grown in the P-sufficient f/20 medium with/without Al enrichment were collected on the sixth day of the incubations for measuring the diatom uptake of DIP. The cells were centrifuged and resuspended in the modified f/20 medium without phosphate. ^{33}P labeled phosphate were added to the medium with a final concentration of 3.63×10^5 Bq l^{-1}. Unlabeled phosphate was simultaneously added to prepare the low (0.36 μM) and high (3.62 μM) P conditions. The diatom uptake rates of DIP under the low and high P conditions were measured. Specifically, 5–10 ml diatom cells were sampled on the time points of 20, 40, 60 and 80 or 90 min. 2.5 mM NaH_2PO_4 was added to stop the uptake of ^{33}P labeled phosphate before the filtration of the sample on polycarbonate filters (with a pore size of 1 μm). The filter was dipped in 5 ml scintillation cocktail, and the sample was measured by the Wallac WinSpectral 1414 liquid scintillation counter. Total DIP uptake rate was determined as the linear increase in cellular P with

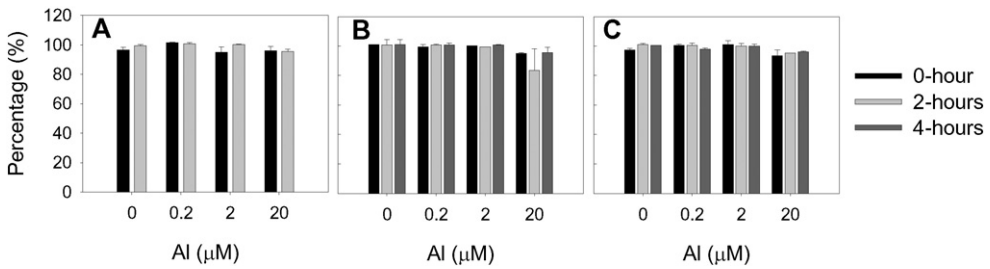

Fig. 4. Effects of Al enrichment on the size-fractionation of phosphate in seawater. The column represents the proportion of the dissolved form (<0.2 μm) after the addition of Al. A, aged seawater without adding of phosphate; B, aged seawater spiked with a low level of phosphate (0.36 μM); C, aged seawater spiked with a high level of phosphate (3.62 μM). Data are mean ± SD (n = 3).

Table 1
Comparison of phosphate speciation in f/20 seawater medium with/without Al enrichment. The results were computed by the chemical equilibrium computer model Visual MINTEQ maintained by Jon Petter Gustafsson. The calculation pH and temperature were fixed at 8.1 and 24 °C, respectively. Data in the table represents % of total concentration. aq in the bracket indicate the species in aqueous form.

Species name	f/20 medium enriched with 20 μM Al	f/20 medium without Al enrichment
HPO_4^{2-}	22.0	22.0
$H_2PO_4^-$	1.11	1.11
$MgPO_4^-$	0.19	0.19
$MgHPO_4$ (aq)	32.7	32.7
$CaHPO_4$ (aq)	5.03	5.03
$CaPO_4^-$	2.55	2.55
$CaH_2PO_4^+$	0.05	0.05
$SrHPO_4$ (aq)	0.02	0.02
$NaHPO_4^-$	30.9	30.9
$KHPO_4^-$	0.43	0.43
Na_2HPO_4 (aq)	4.54	4.54
$Na_2PO_4^-$	0.02	0.02
NaH_2PO_4 (aq)	0.43	0.43

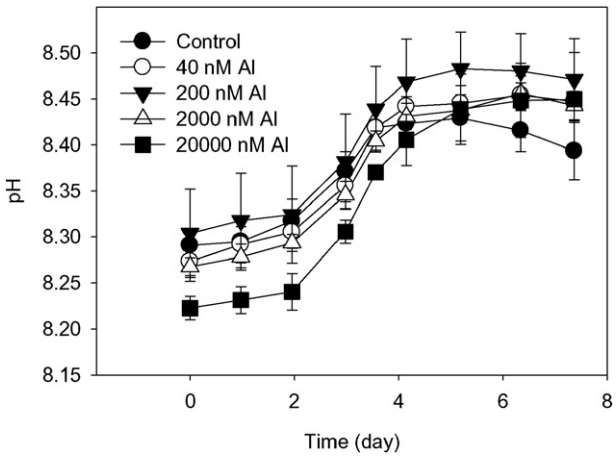

Fig. 6. pH change in the cultures of *Thalassiosira weissflogii* in P-deficient Aquil medium spiked with varied Al concentrations. Data are mean ± SD (n = 3).

time. Cellular DIP uptake rate was calculated by normalizing the total DIP uptake rate to cell number.

2.7. Data analysis

Comparisons of mean values between treatments were conducted by using t-test or one-way ANOVA. To compare the uptake rates indicated by the slopes of regression lines between treatments, ANCOVA was conducted. Comparison of the mean ratio values of cellular DOP uptake rate to cellular APA between treatments was conducted using the Mann–Whitney test. All the statistical analyses were done by using the SPSS 17.0.

3. Results

3.1. Diatom growth in low nutrient medium and modified f/2 media

By growing *T. weissflogii* in oligotrophic seawater spiked with sufficient silicate (42 μM), and/or a low level of nutrients (1 μM nitrate and 0.1 μM phosphate), there was no difference in the diatom growth rate in the exponential phase between the Al-enriched and the controlled treatments. Nevertheless, high diatom abundance persisted after the cell abundance reached a peak in the Al-enriched treatments (Fig. 1). In contrast, the diatom populations collapsed immediately after the abundance reached a peak in the controlled treatments.

By growing the diatom in modified f/2 media including the f/200 medium (Fig. 2A), the f/20 medium (Fig. 2B), and the f/20 (Fig. 2C) medium with modified silicate (106 μM), similar higher diatom biomass on the basis of Chl *a* were observed in the Al-enriched treatments than the controlled ones when the cultures came to the stationary phases. The growth difference between the Al-enriched and the controlled treatments varied in different media. In the f/200 medium (with low initial phosphate of 0.36 μM and ultimately P-limited as the molar ratio of nitrate to phosphate in the medium was higher than 24), there was no difference in the diatom growth rate between the Al-enriched and the controlled treatments in the exponential phase. Nevertheless, diatom in the Al-enriched treatment reached a higher peak biomass and persisted higher biomass after the biomass peak. In contrast, in the f/20 medium (with high initial phosphate of 3.62 μM and ultimately P- and/or silicate limited as the molar ratios of nitrate to phosphate and silicate were higher than 16 and 1, respectively), slightly lower biomass peak but significantly higher biomass persistence after the peak were observed in the Al-enriched treatment (Fig. 2B). In the f/20 medium (with high initial phosphate of 3.62 μM

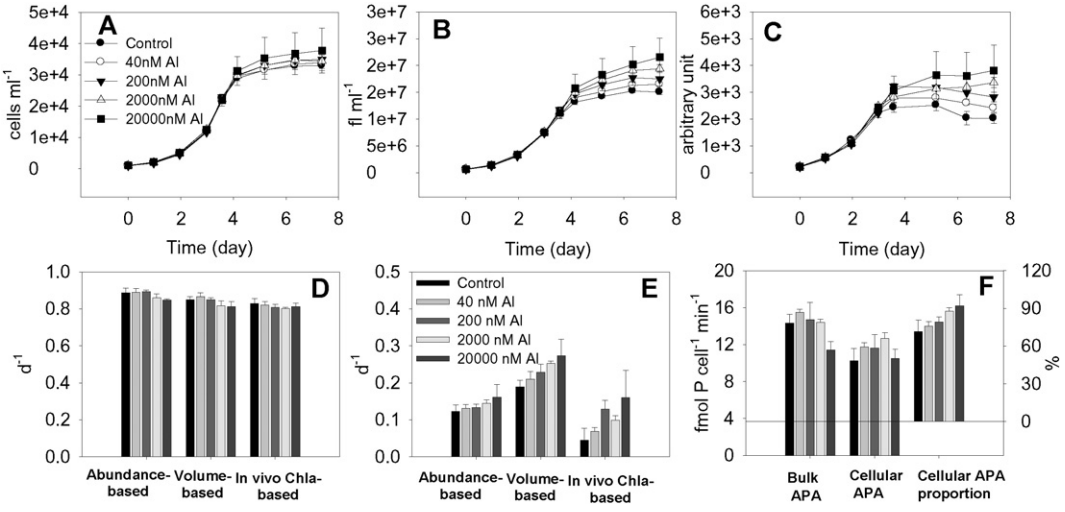

Fig. 5. Effects of Al enrichment on the growth of *Thalassiosira weissflogii* and alkaline phosphatase activity (APA) in the P-deficient Aquil medium. A, cell abundance; B, cell volume; C, in vivo chlorophyll *a*; D, growth rate in the exponential phase; E, growth rate in the early stationary phase (from the end of the exponential phase when the growth rate starts to slow down to the start of the decay phase when the growth rate starts to be approximate to zero or negative); and F, APA. Data are mean ± SD (n = 3).

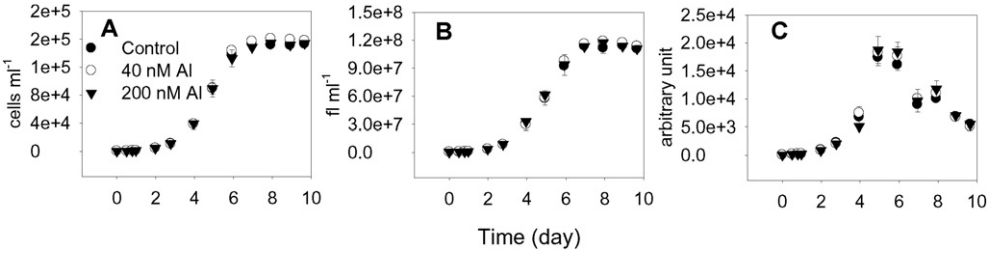

Fig. 7. Effects of Al enrichment on *Thalassiosira weissflogii* growth in P-sufficient Aquil medium. A, cell abundance; B, cell volume; C, in vivo chlorophyll *a*; Data are mean ± SD (n = 3).

and ultimately P-limited) with modified high silicate, lower growth rate in the exponential phase but significantly higher peak diatom biomass and higher biomass persistence after the peak were observed in the Al-enriched treatment (Fig. 2C).

3.2. Diatom growth in the P-deficient f/20 medium

Similar maintenance of high diatom biomass was indeed observed in the P-deficient f/20 medium. By growing *T. weissflogii* in P-deficient f/20 medium, prolonged stationary phases in the Al-enriched treatments was also observed (Fig. 3). Higher diatom biomass in terms of Chl *a* (Fig. 3A) and cell abundance (Fig. 3B) persisted when the biomass reached a peak in the Al-enriched treatments than the controls. Furthermore, diatoms in the Al-enriched treatment had larger sizes and higher cellular chlorophyll content as indicated by the higher side scatter (SSS) and red fluoresces (FL3) signals, respectively (Fig. 3C, D). This resulted in a higher peak of diatom biomass but relatively lower final diatom abundance in the Al-enriched treatment than the controlled one (Fig. 3A, B). In the medium containing the diatom cells, most (19.2 μM) of the nominally enriched Al (20 μM) was in the dissolved form at the beginning of the incubation. With increasing diatom biomass, dissolved Al in the medium decreased quickly to a minimum level (Fig. 3E). In addition, Al enrichment did not result in phosphate precipitate in seawater. More than 96% of phosphate was in the dissolved form even in treatments spiked with a high concentration of Al (20 μM) (Fig. 4). Based on the chemical equilibrium computer model Visual MINTEQ, Al enrichment did not influence phosphate speciation in alkaline seawater in the pH range of 7.9–10.0 (Table 1).

3.3. Diatom growth and APA in P-deficient Aquil medium

Enhanced growth rate, higher final biomass of *T. weissflogii* were observed by growing the diatom in P-deficient Aquil media spiked with Al (Fig. 5). Compared to the control, Al enrichment in levels from 40 nM to 20 μM all increased the final diatom biomass in terms of cell abundance, cell volume and in vivo Chl *a* (Fig. 5A, B, C). In addition, the more Al added in the media, the higher peak diatom biomass occurred. Al treatment did not impact the diatom growth in the exponential phase (when the growth was not nutrient-limited) (Fig. 5D), while the diatom growth rates in the early stationary phase (when the growth was limited by low P) were relatively higher (one-way ANOVA, $p < 0.05$) in treatments spiked with higher concentration of Al (Fig. 5E). In the early stationary phase, there was no significant difference (t-test, $p > 0.1$) in bulk APA in different treatments except that the bulk APA in the treatment with 20 μM Al was lower (t-test, $p < 0.05$) than that in the control one, but higher proportions of cellular APA (one-way ANOVA, $p < 0.05$) were found in treatments with higher concentrations of Al. The higher proportion of cellular APA was consistent with the higher growth rates in the early stationary phase (one-way ANOVA, $p < 0.05$), and the higher peak diatom biomass in treatments enriched with higher concentration of Al.

Adding Al in the levels of 40 nM–2 μM did not significantly influence the initial pH of the media, while adding 20 μM Al draw down the initial pH of only 0.07 unit. With increasing diatom biomass, the pH in all the treatments rose up. Consistent with the higher diatom biomass after the exponential phase in the Al-enriched treatments (Fig. 5A–C), the pH in the Al-enriched treatments (>0.017 pH unit per day) also rose up faster than that in the control (0.007 pH unit per day). Changes of

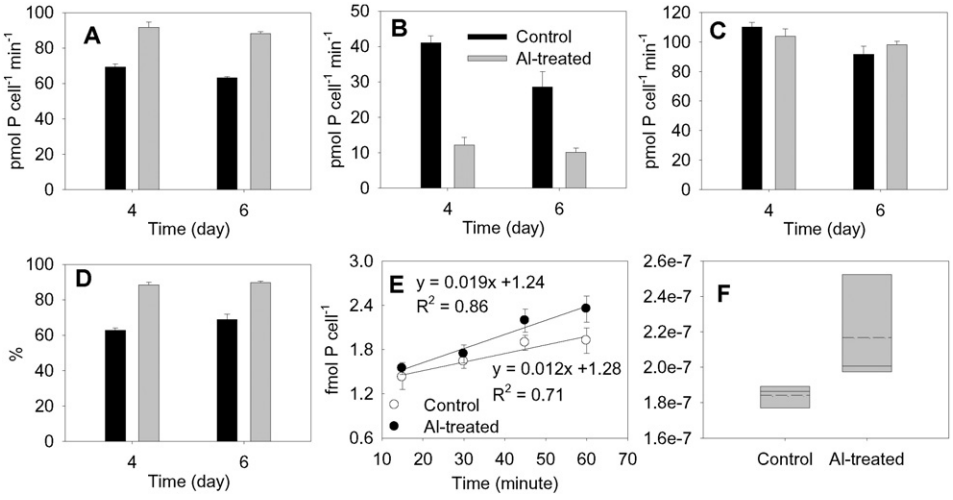

Fig. 8. Alkaline phosphatase activity (APA) in the stationary phase of the incubation in P-deficient f/20 medium and the diatom uptake of dissolved organic phosphorus (DOP). A, cellular APA; B, dissolved APA; C, bulk APA (the sum of cellular APA and dissolved APA); D, the proportion of cellular APA to bulk APA; E, diatom uptake of dissolved organic phosphorus; F, the ratio of cellular DOP uptake rate to cellular APA, the dash and the solid lines show the mean and the 5th/95th percentile, respectively. Data are mean ± SD (n = 3).

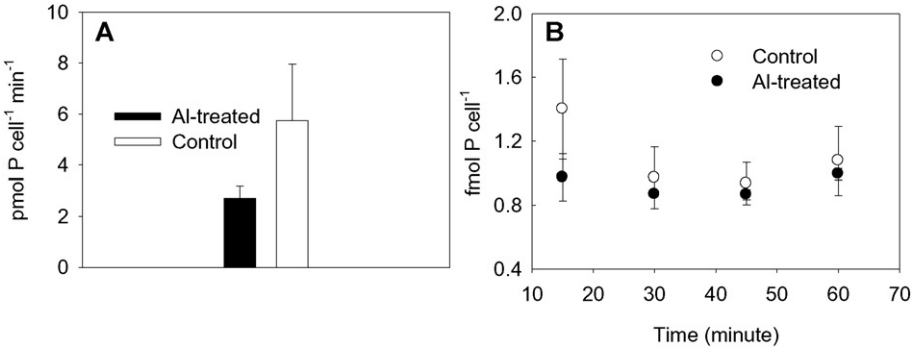

Fig. 9. Cellular alkaline phosphatase activity and uptake of dissolved organic phosphorus of *Thalassiosira weissflogii* cultured in P-sufficient medium. A, cellular alkaline phosphatase activity; B, the diatom uptake of dissolved organic phosphorus. Data are mean ± SD (n = 3).

pH were consistent with the higher diatom growth rates in the early stationary phase (Fig. 6).

In contrast, by growing *T. weissflogii* in the P-sufficient (with 10 μM phosphate) Aquil media, no difference in the diatom growth was found among the cultures spiked with varied levels of Al (Fig. 7).

3.4. APA and diatom uptake of P under P-limited conditions

Aluminum treatment enhanced the diatom utilization of DOP under P-limited conditions. By growing *T. weissflogii* in P-deficient f/20 medium, the diatom growth was supposed to be limited by P at the end of the exponential phase. High APA in the early stationary phase (on the fourth and sixth day) was indeed observed for both the Al-enriched and the controlled treatments (Fig. 8). Cellular APA of the Al-treated cell (89.9 pmol P $cell^{-1}$ min^{-1}) was higher than that of the controlled one (66.1 pmol P $cell^{-1}$ min^{-1}) (Fig. 8A), but dissolved APA was lower in the Al-enriched treatment than the control one (Fig. 8B). As a result, there was no significant difference in the bulk APA between the Al-enriched and the control treatments (Fig. 8C), but higher proportion of cellular APA in the Al-enriched treatment (Fig. 8D). Al-treated *T. weissflogii* cells with higher cellular APA also had a significantly higher (ANCOVA, $p < 0.05$) uptake rate (0.019 ± 0.002 fmol P $cell^{-1}$ min^{-1}) of the ^{33}P labeled DOP than the control one (0.012 ± 0.002 fmol P $cell^{-1}$ min^{-1}) (Fig. 8E). In addition, significantly higher (Mann–Whitney test, $p = 0.05$) ratio of cellular DOP uptake rate to cellular APA was observed in the Al-treated cells (2.17×10^{-7}) than the controlled ones (1.84×10^{-7}) (Fig. 8F).

In contrast, the cellular APA in the stationary phase of cultures in P-sufficient f/20 medium was apparently low (<6 pmol P $cell^{-1}$ min^{-1}) for both the Al-treated and controlled cells (Fig. 9A). Meanwhile, the DOP uptake rates for both the Al-treated and controlled diatom cells were not measurable (Fig. 9B).

The Al effects on the diatom uptake of DIP varied in different DIP concentrations, showing that Al-treatment did not influence the diatom competitiveness for low levels of DIP, but made the diatom uncompetitive under high DIP conditions. The results showed that there was no significant difference in the uptake rate of DIP between the Al-treated (0.100 ± 0.007 fmol P $cell^{-1}$ min^{-1}) and the controlled diatom cells (0.087 ± 0.008 fmol P $cell^{-1}$ min^{-1}) (ANCOVA, $p > 0.1$) in the low DIP (0.36 μM) medium (Fig. 9A). Nevertheless, the uptake rate of DIP was significantly lower for the Al-treated diatom cell (0.23 ± 0.06 fmol P $cell^{-1}$ min^{-1}) than the controlled one (0.58 ± 0.07 fmol P $cell^{-1}$ min^{-1}) (ANCOVA, $p < .01$) in the high DIP (3.62 μM) medium (Fig. 10B).

4. Discussion

The tested Al concentrations (from 40 nM to 20 μM) in the present study were below the solubility in seawater, and the diatom growth responses to the tested Al concentrations were basically consistent. Golding et al. (2015) reported that the dissolved forms of Al dominated the speciation below 500 μg total Al l^{-1}. With increasing total Al concentration, the particulate Al hydroxide was also present and became increasingly dominant at total concentrations >1000 μg l^{-1}. They suggested that particulate Al contributed to toxicity in the diatom *Minutocellus polymorphus*, and only particulate Al accounted for Al toxicity to the green flagellate alga *Tetraselmis* sp. Therefore, high concentrations of Al (>20 μM, including particulate Al, but was unlikely to be found in natural seawater) may have different impacts on the diatom growth.

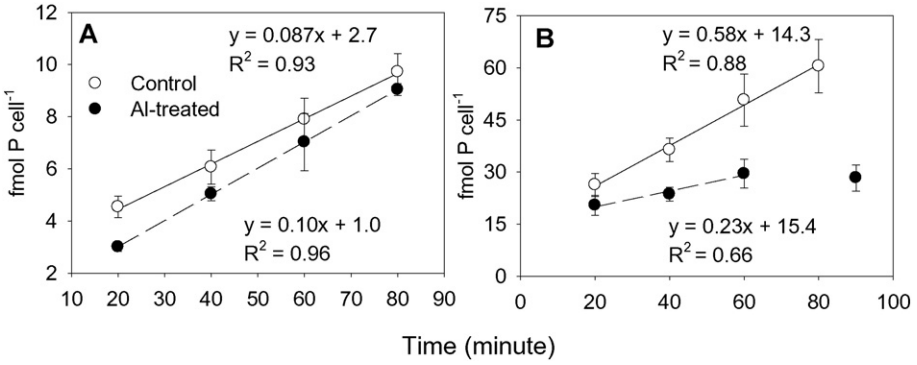

Fig. 10. Effects of Al enrichment on *Thalassiosira weissflogii* uptake of dissolved inorganic phosphorus. A, medium with a low concentration of phosphate (0.36 μM); B, medium with a high concentration of phosphate (3.62 μM). The regression lines (solid lines for the controlled and dotted lines for the Al-treated) and corresponding equations are shown. R, determination coefficient. Data are mean ± SD (n = 3).

4.1. Enhanced DOP utilization with increasing cellular APA

By growing the diatom *T. weissflogii* in P-deficient Aquil media with varied concentrations of Al, it was confirmed that the enhanced growth under P-limited conditions and higher diatom biomass accumulation and maintenance after the biomass reached a peak were the specific responses to Al enrichment. The present results also showed that the enhanced utilization of DOP likely explained at least part of the sustaining diatom growth.

Mechanisms such as nutrient utilization have been proposed for the toxicity of Al on freshwater phytoplankton and the beneficial effects of Al on plant growth. Previous studies suggested that high amount of Al in acidified freshwater could lead to flocculation of phosphate by forming the insoluble $AlPO_4$, which then decreased the growth of phytoplankton (Pettersson et al., 1985, 1988). Nevertheless, increasing phosphorus uptake was proposed as one mechanism for the beneficial effects of Al on plant (e.g. tea tree) growth (Foy, 1984; Osaki et al., 1997). In the present study, treatment led to neither significant precipitation of phosphate (Fig. 4), nor change of phosphate speciation in seawater (Table 1), even though Al treatment could change the phosphate speciation in weak acidic freshwater based on calculation by the chemical equilibrium model Visual MINTEQ (data not shown). In fact, nearly all the nominally spiked high concentration of Al (20 μM) was in the dissolved form even in the medium inoculated with diatom cells (Fig. 3E). Besides, Al treatment did not change the diatom uptake rate of DIP when the DIP concentration was low as those in the P-deficient media (Fig. 10A), but made the diatom uncompetitive at the high DIP concentration same to that of the f/20 medium. These results suggested that Al enrichment may lead to at least part of the lower diatom peak biomass in Al-enriched treatments in f/20 medium (Fig. 2B), and lower diatom growth rates in the exponential phase in Al-enriched treatments in f/20 medium with modified silicate (Fig. 2C), but did not significantly influence the diatom availability for low levels of DIP (Figs. 1, 2A, 3 and 5).

Instead, the beneficial effects of Al on diatoms (i.e. enhanced growth in the early stationary phase and higher peak diatom biomass, which were consistent with the higher cellular APA and its proportion, Figs. 5E, F and 8A, D) were more apparent under P-limited conditions (Figs. 2, 3A and 5A, B, C), but disappeared in the P-sufficient Aquil medium (Fig. 7). Evidences of the increasingly higher proportion of cellular APA, growth rates in the early stationary phase, as well as peak diatom biomass in treatments enriched with higher concentration of Al in P-deficient Aquil media (Fig. 5), all indicated that the beneficial effects of Al on the diatom were related to the enhanced utilization of DOP mediated by APA.

The DOP uptake experiments (Figs. 8E, F and 9) demonstrated that the increased uptake rate of DOP by the Al-treated diatom was related to the higher cellular APA under P-limited conditions (Fig. 8). In addition, the increasingly higher proportions of cellular APA in treatments with higher concentrations of Al (Figs. 5F and 8D) indicated that the increased proportion of cellular APA could make the Al-treated diatom cells utilize DOP with higher efficiency. Firstly, cellular AP associated with cell surface might be more effective than the dissolved AP in ambient media for the cells to take up the enzyme hydrolyzed P. Secondly, the significantly higher ratio of cellular DOP uptake rate to cellular APA by the Al-treated cells (2.17×10^{-7}) than the controlled ones (1.84×10^{-7}) (Fig. 8F) indicated that per unit cellular APA in the Al-treated cells could bring about more DOP uptake, i.e. the Al-treated cells could use AP more efficiently to utilize DOP. As a result, Al treatment made the cells to obtain the same amount of DOP by synthesizing less AP, or acquire more DOP by synthesizing the same amount of AP. Such enhanced utilization of DOP could account for at least part of the enhanced growth and higher accumulation of diatom biomass in the early stationary phase (Fig. 5), and the maintenance of high diatom biomass after the stationary phase in the Al-enriched treatments (Figs. 1, 2 and 3). However, in addition to the AP, other enzymes including phosphodiesterase are also theoretically related to organic phosphorus utilization (Yamaguchi et al., 2014). Therefore, further studies on the role of other enzymes related to organic phosphorus utilization in the Al effects on marine diatom growth under P-limited conditions are clearly needed.

4.2. Possible reasons for the increased cellular APA

The possible roles of Al in the increased cellular APA are not exactly known. There was no difference in the bulk APA among the controlled and the Al-enriched treatments. Instead, the cellular APA increased after Al enrichment, therefore Al treatment likely led to higher proportion of AP in the cellular pool. Dissolved Al in the medium decreased with diatom growth (Fig. 3E), suggesting that Al in the medium was taken up and/or adsorbed by the diatom. The cell-associated Al may adsorb more the exocrine AP in cellular form. Secondly, the cell surface-associated Al could be the important adsorption sites for nutrients like DOP (Bostrom et al., 1974; Ho et al., 2007, 2009), and the adsorption could result in a conditioning of the cell external milieu through which DOP as the substrate of AP became more accessible (Swallow et al., 1978). Therefore, the Al effects on diatom growth may be strongly dependent on the cell surface-associated Al and nutrients coexisting in the environment.

Mechanisms related to the possible physiological roles of Al could not be excluded for the Al-associated sustaining diatom growth, although no definite physiological function of Al has been found (Ganrot, 1986; Nayak, 2002). Increased antioxidant defense (resulting in delayed aging), Chl *a* content in leaves and photosynthesis rate have been proposed as possible reasons for the stimulatory effects of Al on the growth of tea plants (Ghanati et al., 2005; Hajiboland et al., 2013). A most recent study showed that Al treatment increased the cellular Chl *a* content and changed the oxidant stress of a cyanobacteria *Synechococcus* sp. WH7803 (Shi et al., 2015). Maintenance of relatively higher cellular chlorophyll content in the decay phase (Figs. 3D and 5C), indicating suspected delayed aging of diatom cells in the Al-enriched treatments, was also observed in the present study. These observations implied that Al may engage in some physiological processes such as photosynthesis, which may contribute to the Al-associated sustaining growth of marine phytoplankton. Nevertheless, such speculation clearly needs further experimental studies.

4.3. Implications of the beneficial effects of Al

The present study showed that Al treatment, even at low levels occurring in natural seawater (40 nM and 200 nM), led to the enhanced diatom growths under P-limited conditions, as well as higher accumulation and maintenance of diatom biomass. Such Al-associated sustaining phytoplankton growth was likely mediated by the availability of nutrients such as DOP. If the beneficial effects of Al on marine phytoplankton occurred as a result of natural Al enrichment through river runoff and/or atmospheric dust deposition, it is likely that Al may play an unrecognized and important role in marine and global carbon cycles. The potential roles of Al in the marine and global carbon cycles may be significant in the changing world oceans, since the high concentrations of nitrate but relatively low phosphate in river water may lead to P limitation in the estuary and its adjacent coastal seawaters (Xu et al., 2008). Nitrogen fixation and the enhanced deposition of reactive nitrogen from anthropogenic emissions over extensive oligotrophic open oceans may potentially lead to P being the ultimate limiting nutrients (Tyrrell, 1999; Sanudo-Wilhelmy et al., 2001; Kim et al., 2014). To confirm such hypothesis, growth responses of other marine phytoplankton species at low levels of Al similar to realistic ocean concentrations should be examined both in the laboratory and field.

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