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# The increasing aluminum content affects the growth, cellular chlorophyll *a* and oxidation stress of cyanobacteria *Synechococcus* sp. WH7803

by

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## Abstract

Effects of marine aluminum (Al) on phytoplankton are controversial, making it important to elucidate the mechanisms underlying Al effects. This study was aimed at identifying the effects of Al on the growth, chlorophyll *a* (chl *a*) content and the antioxidant mechanism of cyanobacteria *Synechococcus* sp. WH7803. The growth rate increased from 0.33 to 0.52 $d^{-1}$ in media with the increasing Al concentration from 0.2 (control) to 20 µmol $l^{-1}$ and almost saturated to 0.44 $d^{-1}$ at ~ 0.5 µmol Al $l^{-1}$. The higher growth resulted in the higher biomass in both stationary and decay phases in the conditions of higher Al content. Chl *a* per cell reached 10.19 µg $cell^{-1}$ in the exponential phase at 20 µmol Al $l^{-1}$, approximately 1.6 and 3.1 times higher than those in stationary and decay phases, respectively, and chl *a* per cell showed a similar pattern as a growth rate when plotted with Al content. Al addition increased the cellular methane dicarboxylic aldehyde (MDA) content in the exponential phase and decreased the superoxide dismutase (SOD) activity in the decay phase. In particular, our results indicated a positive relationship between chl *a* per cell and the growth rate, suggesting the stimulation of increasing Al on the growth of *Synechococcus* is related to the enhancement of cellular chl *a* content.

**Key words:** Aluminum, *Synechococcus*, growth, cellular chl *a* content, oxidative stress

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## Introduction

Aluminum (Al) plays an important role in marine ecosystems due to its inhibition or promotion effects on phytoplankton (Vrieling et al. 1999, Saçan et al. 2007), although its concentration is very low as it is a particle-reactive element, easily removed from seawater (Minakawa & Watanabe 1998, Koshikawa et al. 2002, Li et al. 2013). Naturally, marine dissolved Al is mainly derived from atmospheric precipitations and river run-offs, and its concentration is thus increasing as a result of increasing severe sandstorms and acidic precipitations caused by atmospheric pollution (Han et al. 2008, Ren et al. 2011). For example, the concentration of dissolved Al ranged respectively from 48.62 to 852 nmol $l^{-1}$ and from 82.4 to 470 nmol $l^{-1}$ in the surface water of the Pearl River Estuary and the northern South China Sea; it is much higher than 0.1 nmol $l^{-1}$ in the Pacific Ocean (Orians & Bruland 1986, Measures et al. 2005, Wang & Tan 2013).

Moreover, there is a growing concern about the influence of the increasing Al levels on the organisms in natural habitats, particularly on forests or aquatic communities (Stoffyn 1979, Kochian 1995, Hajiboland et al. 2013). A wide range of toxic effects of Al was detected in terrestrial biota (Yamamoto et al. 2002), such as the negative effects on animals as a potent neurotoxin (Wang et al. 1997) and on plants growth through triggering intracellular reactive oxygen species (ROS) production, mitochondrial dysfunction and ATP depletion (Kochian 1995, Pan et al. 2001). Gensemer and Playle (1999) also reported toxic effects of Al on algae under acidic conditions of pH ~6.0. The effects of Al on marine organisms were also observed to be species-specific, e.g. Vrieling et al. (1999) found that the increased Al positively affected the growth and final cell density of pennate *Navicula salinarum*, but not of centric species T*halassiosira weissflogii*; while Saçan (2007) found that Al negatively affected the growth of diatom *Minutocellus polymorphus* and green alga *Dunaliella tertiolecta*. However, a limited Al effect was observed for the growth of diatom *Skeletonema costatum* (Stoffyn 1979); and no significant effects were detected on the brown macroalga *Hormosira banksii*, sea urchin embryo *Heliocidaris tuberculata* and 2 juvenile fish species of Lates calcarifer and Acanthochromis polyacanthus, even at an extremely high Al level (37 µmol $l^{-1}$) (Golding 2014).

The aerosol particles of dust storms carried about 7 times more of Al compared to normal weather conditions (Chung et al. 2011), which could be one of the causes of the increased marine primary production in oligotrophic open oceans after the dust storms (Jickells 1995, Herut et al. 2005). Al could positively affect the phytoplankton growth through influencing their absorption of nutrients and Al could also be incorporated into diatom frustules (Stoffyn 1979, Gehlen et al. 2002, Koning et al. 2007); it is thus likely involved in the marine biogeochemical cycles by affecting the phytoplankton growth. However, the mechanism associated with Al impact on phytoplankton physiology is poorly understood.

Cyanobacteria *Synechococcus*, accounting for up to 20% of the primary production, plays an important role in sustaining the marine microbial food webs (Waterbury et al. 1986, Sharma et al. 2011); and it is also considered to be a useful genus for microbial ecology research due to its simple structure, abundant genotype and ecotype (Glover 1985). In this study, we showed the effects of Al addition on the growth, cellular chlorophyll *a*, superoxide dismutase (SOD) and methane dicarboxylic aldehyde (MDA) contents of *Synechococcus* sp. WH7803, with the objective to elucidate the physiological mechanisms underlying the effects of Al on cyanobacteria.

## Materials and methods

### Culture and sampling protocol

The cyanobacteria *Synechococcus* sp. WH7803 was obtained from the Center for Collections of Marine Algae (CCMA), Xiamen University (Xiamen, Fujian, China) and cultured in f/2 medium at 24°C with a light-dark cycle of 12:12 h. Light intensity of 25 µmol $m^{-2}$ $s^{-1}$ was supplied with fluorescent tubes in an incubator; and it was measured in the culture bottles filled with seawater using a quantum meter (Apogee MQ-200 PAR Meter, USA).

Before the Al addition experiments, the *Synechococcus* culture was inoculated into a 2 l polycarbonate bottle (Nalgene, USA) with the volume fraction of 10% (v/v) and incubated for 3 days to make algal growth reach the exponential phase with the cell density of approximate $1.0 \times 10^6$ cells $ml^{-1}$. Then, $AlCl_3$ was added to duplicate 2 liter

cultures at final concentrations of 0, 0.5, 5 and 20 μmol $l^{-1}$ $Al^{3+}$. The cultures were then incubated for 16 days under the condition as described above. On day 0, 1, 2, 3, 4, 8, 12 and 16, we took aliquots of culture from each replicate to determine the environmental factors: Al, nutrient concentration and pH level, as well as the biological factors: cell abundance, chlorophyll *a* (chl *a*), cellular superoxide dismutase (SOD) and methane dicarboxylic aldehyde (MDA) as described below.

### Determinations of environmental factors

To measure the concentration of dissolved Al in the medium, the 20 ml culture was filtrated through a cellulose acetate membrane filter (diameter 47 mm; pore size 0.45 μm) that was soaked for 24 h in hydrochloric acid (pH = 2) and thoroughly washed with Milli-Q water until a neutral pH was obtained. The concentration of dissolved Al in the filtration was determined according to Ren et al. (2001).

To measure the nutrient concentration, the 20 ml culture was filtrated through a cellulose acetate membrane filter (diameter 47 mm; pore size 0.45 μm). The content of $NO_3$ + $NO_2$ and $PO_4$ in the filtration was measured with a nutrient-autoanalyzer (Quickchem 8500, Lachat Instruments, USA). This equipment has detection limits of 0.014 and 0.005 μmol $l^{-1}$ for $NO_3$+$NO_2$ and $PO_4$, respectively. It was calibrated regularly with the support of the manufacturer against CSK standard solutions, and was calibrated before and during the measurements of each of the eight samples. Analytical errors were less than 10% (Li et al. 2012).

### Measurements of biological factors

To measure cell abundance, duplicate 1 ml cultures were fixed with filtered formaldehyde (final concentration of 1%) and stored at -20°C for later analysis. Cell abundance was determined using a flow cytometry (FACS Calibur, Becton Dickinson, USA); and the specific growth rate ($\mu$) was calculated as:

$$\mu = \frac{(\ln N_2 - \ln N_1)}{T_2 - T_1}$$

wherein $T$ represents sampling time and $N_1$ and $N_2$ represent a mean value of duplicate cell samples at $T_1$ and $T_2$, respectively.

Chl *a* concentration was determined by filtrating 10 ml culture on a Whatman GF/F filter (47 mm in diameter) and filter membranes were wrapped in aluminum foil and stored at -20°C for later extraction. Following the extraction protocol of dipping the filter in 90% acetone (v/v) for 24 h in darkness, chl *a* concentration was measured using a Turner-Design fluorometer (10AU, USA ) according to Parsons et al. (1984); and chl *a* per cell was calculated by dividing the total chl *a* content per ml culture by a cell number per ml culture.

To assess the antioxidant protection mechanism, the 60 mL culture was filtrated on a cellulose acetate membrane filter (diameter 47 mm; pore size 0.45 μm) and the filters were stored at -20°C for later determinations of SOD and MDA. The cells on filters were re-suspended in 1.3 ml of PBS buffer (phosphate buffered saline, pH 7.2) together with lysozyme enzyme (2 mg $ml^{-1}$), and ultrasonicated under 400 W for 6 min (pulverization time: 3 s; interval time: 3 s) in the ice bath to protect bioactive substances. The extraction was then centrifuged at 12,000 r $min^{-1}$ for 5 min at 4°C. SOD activity and MDA content in supernatant were measured with kits (Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer's instructions (Kong et al. 2011). The SOD activity and MDA content per cells were calculated by dividing the total amount by cell density.

### Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the significant differences ($p<0.05$) between the treatments. The correlation between variables was established using a Kendall's t-test with 95% confidence band. The speciation of Al and their proportions in total Al at different pH levels were calculated using the isual MINTEQ model (USA).

## Results

Both reference and Al-treated cultures grew at the exponential phase within the initial three days after Al addition (Fig. 1A), and then to the stationary phase from day 3 to day 12 (For the convenience of

the following calculations, we assumed day 12 was still in the stationary phase although algae started to die in the reference). Thereafter, algal cells entered into the decay phase after 12 days of cultivation (Fig. 1A).

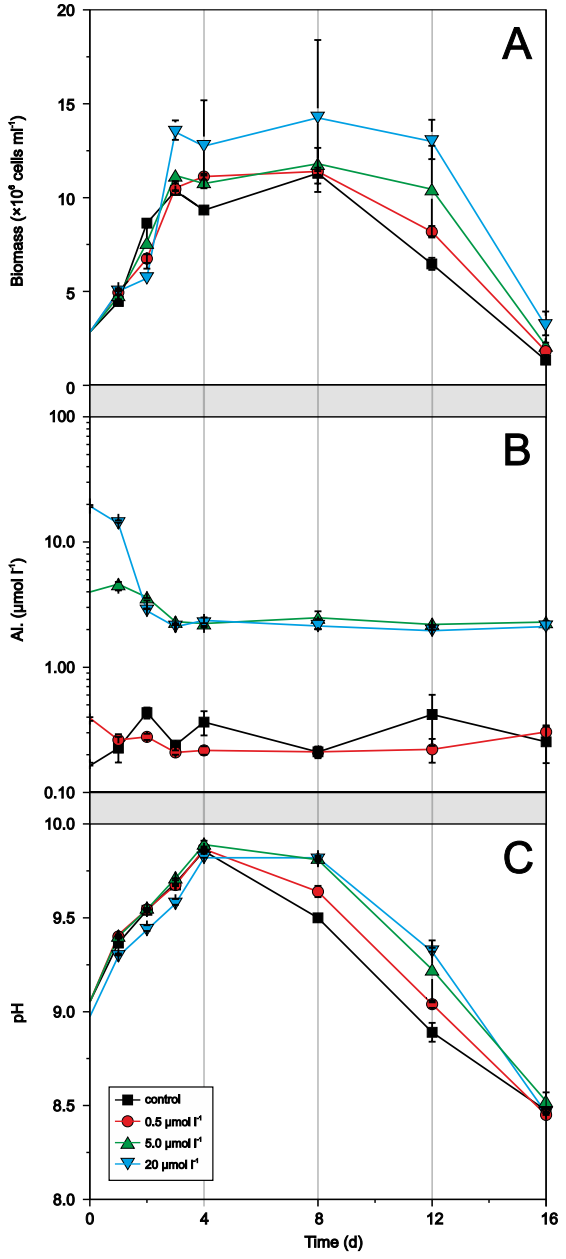


**Figure 1**

Changes in *Synechococcus* biomass (A, ×10⁶ cells ml⁻¹), aluminum concentration (B, Al, μmol l⁻¹) and pH (C) in the algal cultures exposed to different Al levels. The error bars represent half of the range of the duplicate culture.

The Al levels in all treatment cultures decreased within the initial three days, and then remained relatively constant until the end of the experiments (Fig. 1B). The ambient pH of both reference and Al-treated cultures increased from 9.0 to 9.9 with the increasing cell abundance from day 0 to day 4, with no significant differences among the treatments ($p>0.05$) (Fig. 1A,C). The pH value decreased to 8.5 at day 16; during the period of days 4 to 16, however, the pH level was significantly higher in Al-treated cultures than the reference (Fig. 1C).

Figure 2 showed the changes in the specific growth rate in the exponential phase and biomass in stationary and decay phases with increasing Al

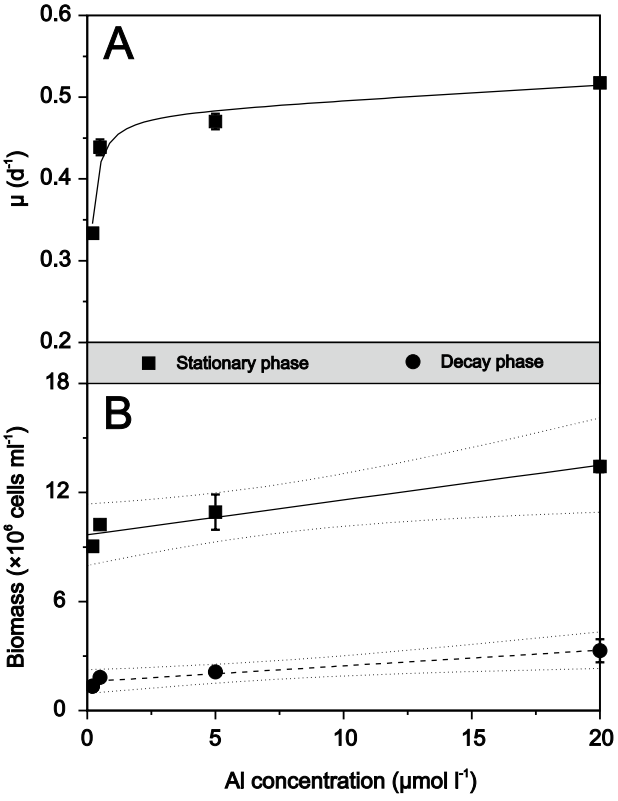


**Figure 2**

Specific growth rate (A, $d^{-1}$) in the exponential phase and mean biomass (B, $\times 10^6$ cells $ml^{-1}$) in stationary and decay phases of the cultures as a function of pre-treated Al levels (μmol $l^{-1}$). The equation of $\mu = [Al]/(a[Al]^2 + b[Al] + c)$ was used to fit the growth rate vs. Al concentration; while solid and dash lines represent the best fit lines with 95% confidence intervals (dotted lines). The error bars represent half of the range of the duplicate culture.

concentration. The growth rate sharply increased from 0.33 to 0.44 $d^{-1}$ with Al concentration increasing from 0.2 (control) to 0.5 µmol $l^{-1}$, then increased gradually to 0.52 $d^{-1}$ at the Al concentration of 20 µmol $l^{-1}$ (Fig. 2A). Consistent with the growth rate, the biomass of culture (Fig. 2B) displayed a significant increase with the increasing Al concentration, either in the stationary phase or in the decay phase ($p<0.05$).

Similar to the growth rate, Al addition increased the cellular chl *a* content, especially in exponential and decay phases; however, the chl *a* per cell appeared to decrease with increasing Al levels in the stationary phase (Fig. 3A). In particular, chl *a* per cell was significantly higher in the exponential phase compared to the decay phase ($p<0.05$), and intermediate in the stationary phase. Especially

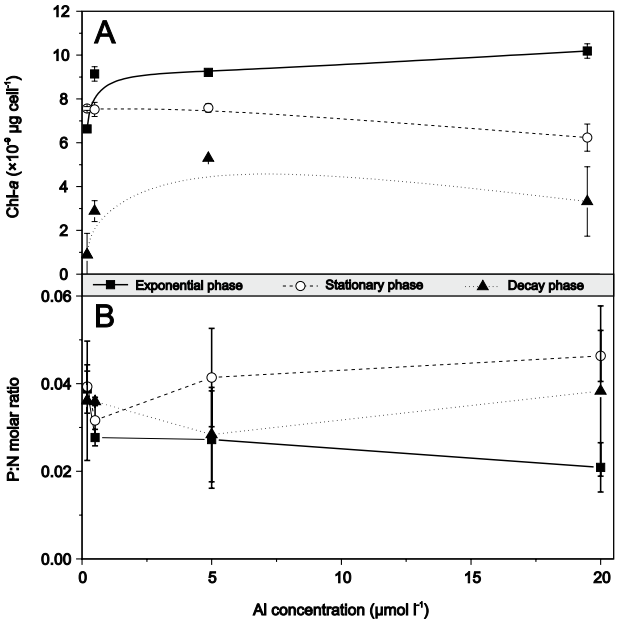


**Figure 3**

Cellular chlorophyll *a* (Chl *a*) content (A, ×$10^{-9}$ µg $cell^{-1}$) and P:N (B, molar ratio) of the cultures in exponential, stationary and decay phases as a function of pre-treated Al concentration (µmol $l^{-1}$). The error bars represent half of the range of the duplicate culture.

at the high Al level (20 µmol $l^{-1}$), the cellular chl *a* content reached 10.19 µg $cell^{-1}$ in the exponential phase, higher compared to stationary (6.24 µg $cell^{-1}$) and decay (3.32 µg $cell^{-1}$) phases. Cellular P:N (molar ratio), derived from the decreasing of medium nutrients, showed an increase trend with increasing Al levels in stationary phase, and cellular P:N of algae in stationary phase was higher than that in exponential phase especially in higher Al condition (i.e., >0.5 µmol $l^{-1}$) (Fig. 3B).

Cellular SOD activity showed significantly higher values in decay than exponential and stationary phases (Fig. 4A). Moreover, the SOD activity sharply decreased from 8.19 to 5.79 × $10^{-6}$ U $cell^{-1}$ in the decay phase with Al content increasing from 0.2 (control)

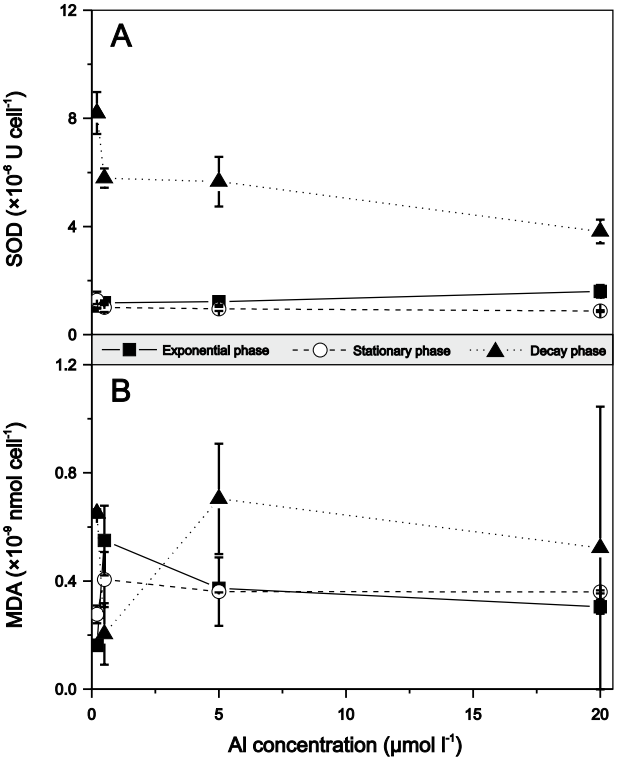


**Figure 4**

Cellular SOD activity (A, ×$10^{-6}$ U $cell^{-1}$) and MDA content (B, ×$10^{-9}$ nmol $cell^{-1}$) of the cultures in exponential, stationary and decay phases as a function of pre-treated Al concentration (µmol $l^{-1}$). The error bars represent half of the range of the duplicate culture.

to 0.5 µmol $l^{-1}$, then decreased gradually to 3.81 × $10^{-6}$ U $cell^{-1}$ of 20 µmol Al $l^{-1}$ condition; however, no significant changes were observed in exponential and stationary phases, or among different Al treatments (Fig. 4A). Cellular MDA content was enhanced by Al addition in exponential and stationary phases, but not in the decay phase; and no significant changes were detected for higher Al content (Fig. 4B).

## Discussion

Al, as one of the vital oceanic trace metals, is of general importance to marine ecosystems because it modifies the physiology of phytoplankton. Our results showed that Al addition could enhance the growth and cellular chl *a* content of cyanobacteria *Synechococcus*, and change the nutrients (N, P) uptake and antioxidant protection systems within cells. In particular, we found that the growth rate of *Synechococcus* was almost saturated when Al increased from 0.2 (control) to 0.5 µmol $l^{-1}$, indicating that the increasing content of oceanic Al from atmospheric precipitations or river run-offs could induce in future a rapid growth of marine phytoplankton.

The higher growth rate of *Synechococcus* was induced by Al addition (Fig. 2A), which was consistent with previous studies that indicated a stimulatory effect of Al on the growth of *Navicula salinarum* (Vrieling et al. 1999). In the light-limited conditions, phytoplankton cells often synthesize and accumulate more pigments, including chl *a* to harvest more light energy and maintain their growth, resulting the higher chl *a* per cell under light-limited condition than that under high light condition (Li & Campbell 2013). In the oceans, *Synechococcus* occurs mainly at the bottom of the euphotic zone for available nutrients where light intensity is usually very low (Iturriaga & Mitchell 1986), whilst the light intensity that saturates its growth is ~ 100 µmol photons $m^{-2}$ $s^{-1}$ (Li et al. unpublished data). The growth light of 25 µmol photons $m^{-2}$ $s^{-1}$ in this study is close to the bottom level of euphotic zone but much lower than the saturated light, therefore the cells were growing under light limited status. Al addition increased the cellular chl *a* content (Fig. 3A), enhancing the capacity of light harvesting and photosynthesis and, finally promoting the growth of phytoplankton as indicated by a positive correlation of the growth rate and chl *a* per cell (Fig. 5). On the other hand, faster growing phytoplankton cells often need more nitrogen to synthesize more cellular enzyme that can efficiently transform the obtained inorganic substances from environments to their own cellular organic matter (Xie et al. 2014), additionally indicated by the lower P:N molar ratio in the exponential phase compared to that of stationary and decay phases (Fig. 3B).

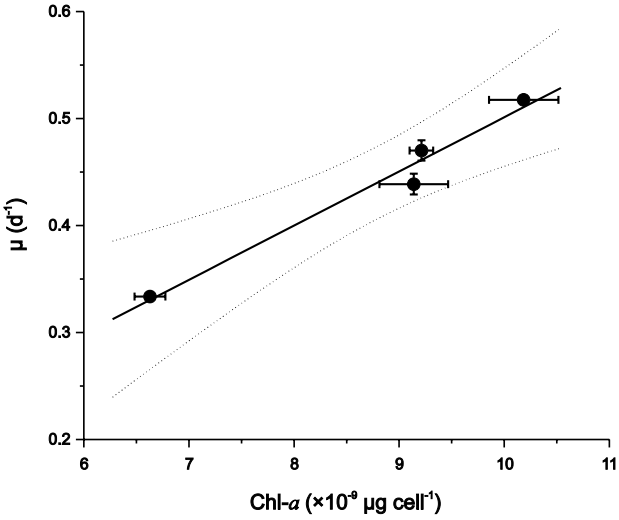


**Figure 5**

Specific growth rate (µ, $d^{-1}$) as a function of cellular chl *a* content (×$10^{-9}$ µg $cell^{-1}$) in the exponential phase. Solid line represents the best fit line with 95% confidence intervals (dotted lines). The error bars represent half of the range of the duplicate culture.

Cellular superoxide dismutase (SOD) activity indicates the capacity of scavenging harmful oxygen free radicals in cells; while methane dicarboxylic aldehyde (MDA), one of the products of membrane damage, indicates the extent of cell damages. Both cellular SOD activity and MDA content were used to evaluate the oxidative stress of algal cells (Qian et al. 2011, Kuran & Gill 2014). Although Al addition slightly increased the SOD activity in the exponential phase (Fig. 4A) to acclimate to the new environment, the synthesis of SOD might not be sufficient for scavenging all the oxygen free radicals in cells, leading to a higher MDA content with increasing Al levels (Fig. 6B). As biomass increased along with the growth, the nutrient environments became stressful, resulting in higher cellular SOD activity together with higher MDA content in the decay phase (Fig. 6). Simultaneously, the cellular SOD activity in the decay phase significantly decreased after Al addition ($p<0.05$), which may result from a better suitability of algal cells to the nutrient limitation, indicated by a lower MDA content at e.g. 0.5 µmol Al $l^{-1}$ condition compared to the reference.

Dissolved Al speciation in the medium were composed of noncolloidal neutral hydroxide ($Al(OH)_3$) and aluminate ($Al(OH)_4^-$) within the range of pH in this study (Millero et al. 2009, Golding et al.

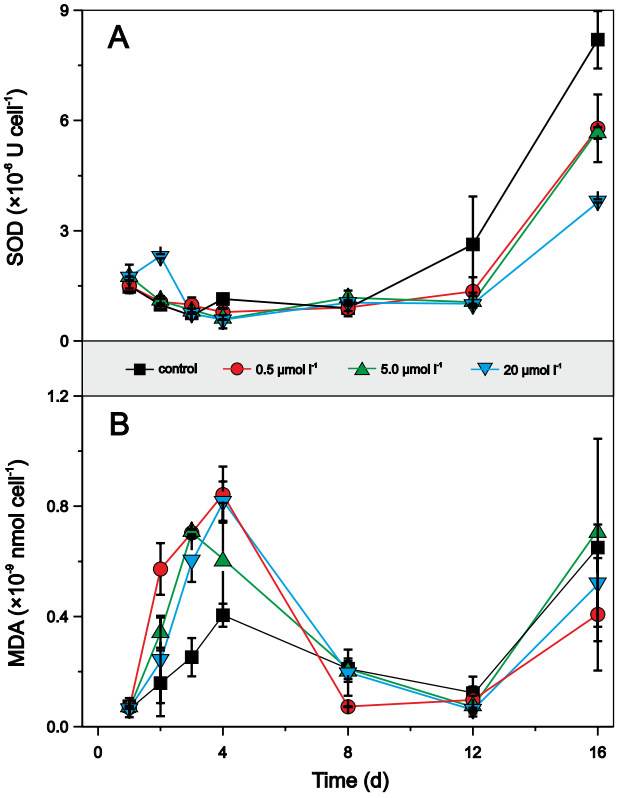


**Figure 6**

Changes in cellular SOD activity (A, $\times 10^{-6}$ U cell$^{-1}$) and MDA content (B, $\times 10^{-9}$ nmol cell$^{-1}$) of the cultures exposed to different Al levels. The error bars represent half of the range of the duplicate culture.

2014). The pH value (8.5-9.9) in our culture appeared to be a little bit higher, which might be caused by an addition of nutrients and trace metals; however, its variation resulted in limited changes of $Al(OH)_3$ and $Al(OH)_4^-$ content (Table 1), indicated that the pH changes due to algae growth have less effects on Al speciation and Al could not affect the algae growth through complexation formed with P in alkaline environment. The cells can absorb or/and adsorb the dissolved $Al^{3+}$ and release $OH^-$ to the medium (Measures & Edmond 1988, Moran & Moore 1991, Ren et al. 2011), which could increase the medium pH value during their growth (Fig. 1C). A significant pH decrease from the stationary to decay phase (Fig. 1C) might be caused by dying cells as indicated by a biomass decrease (Fig. 1A), however the pH level decreased slowly with the increasing of Al content.

**Table 1**

The speciation of Al and its percent of total concentration in different pH at 24°C

| | Al treatment | | | | | |
|---|---|---|---|---|---|---|
| | 0.5 μmol l⁻¹ | | 5 μmol l⁻¹ | | 20 μmol l⁻¹ | |
| pH | 8.5 | 9.9 | 8.5 | 9.9 | 8.5 | 9.9 |
| $Al(OH)_{3(aqueous)}$ | 0.48% | 0.02% | 0.42% | 0.02% | 0.47% | 0.02% |
| $Al(OH)_4^-$ | 99.51% | 99.98% | 99.58% | 99.98% | 99.53% | 99.98% |

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