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Effects of nitrate on intracellular nitrite and growth of *Microcystis aeruginosa*

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Abstract Although nitrate is a macronutrient and can serve as good nitrogen source for many species of phytoplankton, high nitrate concentrations do not benefit the growth of phytoplankton. We hypothesise that algae cultured under high nitrate concentrations can accumulate intracellular nitrite, which is produced by nitrate reductase (NR) and can inhibit the growth of algae. To assess the validity of this hypothesis, *Microcystis aeruginosa* was grown under different nitrate concentrations from 3.57 to 21.43 mM in low CO_2 and high CO_2 conditions for 15 days. We observed that, with increasing nitrate concentrations, the intracellular nitrite concentrations of the alga increased and the growth rates and photosynthesis declined. When grown under high CO_2 conditions, *M. aeruginosa* showed lower intracellular nitrite concentrations and higher growth rates and P_m^{chla}, R_d^{chla}, α^{chla} than under low CO_2 conditions. These results suggest that the accumulation of intracellular nitrite could be the cause of inhibition of algal growth under high nitrate concentrations.

Keywords Growth characteristics · Photosynthesis · Intracellular nitrite · Nitrate reductase · Nitrite reductase · *Microcystis aeruginosa* · Cyanobacteria

Introduction

Under certain conditions, a combination of high nutrient load and warm, stable conditions, cyanobacteria can grow excessively and form blooms which cause a variety of water quality problems, including toxin production, odors, scums and unsafe drinking water (Paerl 1988; Benoufella et al. 1994). Nutrient concentrations play an important role in the growth of phytoplankton, and the nitrogen sources considered most important for the growth of phytoplankton are nitrate and ammonium (Viaroli et al. 1995; Herndon and Cochlan 2007). Nitrate occurs naturally from mineral sources and animal wastes, and anthropogenically as a by-product of agriculture and human wastes (Madison and Burnett 1985). In many parts of the world, nitrate may build up and, as a result, aquatic nitrate concentrations range from normal background level of below 0.71 mM to over 7.14 mM (Rouse et al. 1999), while in some marine aquaculture systems, nitrate concentrations can approach values of 35.71 mM (De Graaf 1964; Pierce et al. 1993).

Although many studies have reported that nitrate could affect photosynthesis, growth and cellular toxicity of phytoplankton and metal toxicity to phytoplankton (Bates et al. 1993; Rijstenbil et al. 1998; Menéndez 2005), there are few reports on the accumulation of intracellular nitrite under high nitrate concentrations. We know that, once inside the alga, nitrate is converted to ammonium (reduced nitrogen) in two successive steps catalysed by nitrate reductase (NR) and nitrite reductase (NiR) in the cytosol and chloroplast, respectively, before being incorporated into organic compounds. Generally, NR catalysing the reduction of nitrate to nitrite is the rate-limiting step for the conversion of nitrate to ammonium (Maldonado et al. 1996). However, nitrate can regulate NR activity which increases with increasing nitrate concentrations (Crawford 1995; Sivasankar and Oaks 1996), and when nitrite formed by NR is more than the nitrite reduced by NiR, the accumulation of intracellular nitrite will occur.

Nitrite ion has a negative influence upon phytoplankton (Abe et al. 2002; Yang et al. 2004). Sijbesma et al. (1996)

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has reported that nitrite ion acted as a protonophore, an uncoupler that increases the proton permeability of membranes by a shunting mechanism, inhibiting ATP synthesis and stimulating ATP hydrolysis. Almeida et al. (1995) proposed that nitrite could induce a lower efficiency of respiratory-chain-linked energy conservation, due to an increase of proton permeability by the nitrite ion, counteracting the proton pumping effect of ATP-ase.

In order to gain some insight on accumulation of intracellular nitrite in algae under high nitrate concentrations and the effects of increasing intracellular nitrite, this study examined the effects of different levels nitrate on the specific growth rate, NR activities, NiR activities, intracellular nitrite concentrations and photosynthesis of *Microcystis aeruginosa* under both low and high CO_2 conditions. Suzuki et al. (1995) and Hu and Zhang (2007) have reported that high CO_2 concentrations enhanced NiR activities, but had no effect on NR activities, which could decrease intracellular nitrite concentration. *Microcystis aeruginosa* is recognised as one of the most common bloom-forming cyanobacteria found in fresh waters (Colman 1989).

Materials and methods

Microcystis aeruginosa was obtained from the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). It was grown in 1,000-mL flasks containing 500 mL sterilised BG-11 medium in a plant growth chamber at 25°C, 40 μmol photons m^{-2} s^{-1} and a 12 h light:12 h dark cycle. $NaNO_3$ was added to the BG-11 medium (Saito et al. 2002) (N omitted) to gain three treatments (3.57, 10.71, 21.43 mM nitrate). The experiment was divided into a low CO_2 group and a high CO_2 group; in the low CO_2 group, three treatments were aerated with air at 100 mL min^{-1}, and in the high CO_2 group were aerated with 5% CO_2 air at 100 mL min^{-1}. Every treatment had three replicates. The initial cell density was about 5×10^4cells mL^{-1}.

Cell density Cell density was monitored daily with a phytoplankton-counting chamber (0.1 mL). Specific growth rates (μ) were calculated using the equation $\mu=\ln(X_t/X_0)/t$, where X_0 is the initial cell density, X_t is the cell density after t days.

Intracellular nitrite concentration The culture was centrifuged at 3,000 *g* for 10 min on day 10, and rinsed twice with a solution of 0.18 mM $NaHCO_3$. After centrifugation, the algal pellet was mixed with 10 mL 5% (w/v) trichloracetic acid, disintegrated ultrasonically for 10 min in an ice bath, and placed in a 45°C water bath for 30 min. The mixture was centrifuged and the nitrite concentrations of supernatant were determined by the N-1-naphthylethylenediamine method (Wei and Qi 2002). The intracellular nitrite concentrations of alga were calculated from the biomass of the alga and the nitrite contents of supernatant.

NR activities and NiR activities The culture was centrifuged at 3,000 *g* for 10 min on day 10, and proteins were extracted with a buffer containing 50 mM Hepes (pH 7.6), 10 mM $MgCl_2$, 5 μM flavin adenine dinucleotide (FAD), 1 μM leupeptine, 10 mM 2-mercaptoethanol and polyclar 50 g L^{-1}. NR activities were measured as described in Pigaglio et al. (1999). NiR activities were assayed as described by Lillo (1984). The NR activities were expressed as mg NO_2^-N formed per cell and NiR activities were expressed as mg NO_2^-N reduced per cell.

Photosynthetic activities The culture was centrifuged at 3,000 *g* for 10 min on day 10, and resuspended in BG-11 medium. Their photosynthetic activities were assayed by measuring the rate of O_2 evolution under different irradiances using a Clark-type O_2 electrode (Hansatech Instuments, UK). The temperature was kept at 25°C by a circulating water bath. Data were treated by non-linear fitting technique using model $P=P_m\times\tanh(\alpha\times I/P_m)+R_d$ (Henley 1993), where I = irradiance, and P = photo synthetic rate at certain irradiance, P_m = light-saturated photosynthesis, α = the initial slope at limiting irradiance, was calculated to assess the photosynthetic efficiency and R_d = dark respiration rate.

Statistical analysis All treatments were analysed using an analysis of variance (ANOVA). Results of all tests were considered significant at 95% confidence if $p < 0.05$ for a given *F* statistic test value.

Results

Growth of Microcystis aeruginosa

The effect of different nitrate concentrations on growth are shown in Fig. 1 and Table 1. The growth of *Microcystis aeruginosa* tended to be inhibited with nitrate concentrations increasing in both the high CO_2 group ($p=0.011$, two-way ANOVA) and the low CO_2 group ($p=0.009$, two-way ANOVA). When aerated with 5% CO_2 air, the specific growth rates and maximum cell densities of alga at 10.71 and 21.43 mM nitrate showed a significant increase in comparison with the low CO_2 group ($p=0.005$, two-way ANOVA). Maximum cell densities at 10.71 and 21.43 mM nitrate in the high CO_2 group were, respectively, approx. 1.32- and 1.34-fold that in low CO_2 group, and specific growth rates were approx. 1.05- and 1.06-fold, but these

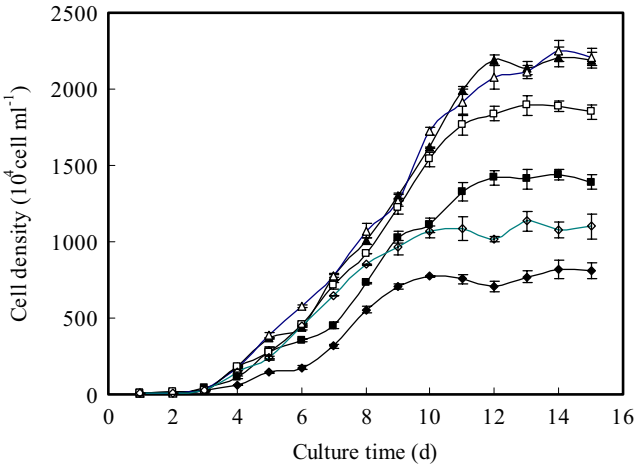

Fig. 1 The growth curves of *Microcystis aeruginosa* at different nitrate concentrations. 3.57 mM nitrate (▲), 10.71 mM nitrate (■), 21.43 mM nitrate (♦) in low CO_2 group; 3.57 mM nitrate (Δ),10.71 mM nitrate (□), 21.43 mM nitrate (◊) in high CO_2 group. *Error bars* denote the standard deviation of triplicate incubations

values did not markedly change when nitrate concentrations were 3.57 mM (p=0.92, one-way ANOVA)

NR activities and NiR activities

After 10 days cultivation, NR activities and NiR activities showed a significant increase with increasing nitrate concentrations in both the high CO_2 group (p=0.002, p=0.004, one-way ANOVA) and the low CO_2 group (p=0.008, p=0.002, one-way ANOVA) (Figs. 2 and 3). In the low CO_2 group, the NR activities at 10.71 and 21.43 mM nitrate were, respectively, 2.79- and 7.15-fold higher than at 3.57 mM nitrate, and the NiR activities at 10.71 and 21.43 mM nitrate were, respectively, 1.12- and 1.53-fold higher than at 3.57 mM nitrate. 5% CO_2 air significantly enhanced NiR activities (p=0.017, two-way ANOVA), compared to the low CO_2 group, the NiR activities at 3.57, 10.71 and 21.43 mM nitrate were increased, respectively by 115, 179 and 175%. However, high CO_2

Table 1 Maximum cell densities and specific growth rates of *Microcystis aeruginosa* grown under different nitrate concentrations

Nitrate (mM)		Specific growth rates (day^{-1})	Maximum cell densities (×10^4cells mL^{-1})
Aerated with air	3.57	0.43	2,210±110
	10.71	0.40	1,440±63
	21.43	0.36	820±56
Aerated with 5% CO_2 air	3.57	0.43	2,250±127
	10.71	0.42	1,895±70
	21.43	0.38	1,100±68

Data are means ± SD (n=3)

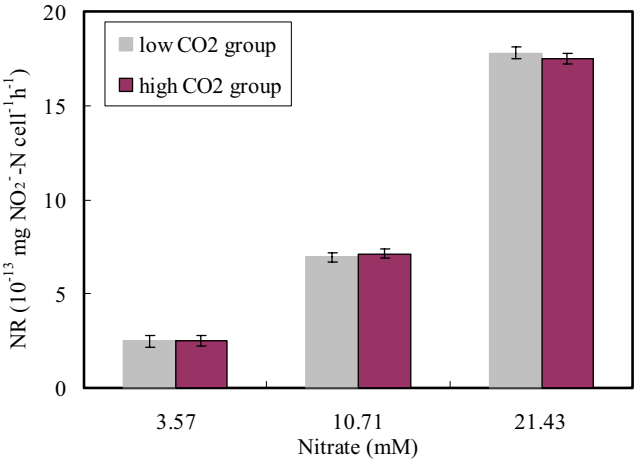

Fig. 2 The NR activities of *Microcystis aeruginosa* at different nitrate concentrations. *Error bars* denote the standard deviation of triplicate incubations

conditions (high CO_2 group) did not significantly influence NR activities compared to low CO_2 conditions (low CO_2 group) (p=0.717, two-way ANOVA).

Intracellular nitrite concentrations

On day 10, intracellular nitrite concentrations of *M. aeruginosa* increased with nitrate increasing concentrations in both the high CO_2 group (p=0.003, one-way ANOVA) and the low CO_2 group (p=0.004, one-way ANOVA) (Fig. 4). In the low CO_2 group, the intracellular nitrite concentrations at 10.71 and 21.43 mM nitrate were, respectively, 2.12- and 13.38-fold higher than at 3.57 mM nitrate. When aerated with 5% CO_2 air, the intracellular nitrite concentrations at 10.71 and 21.43 mM nitrate were, respectively, 0.81- and 0.72-fold that of the low CO_2 group, but no significant difference was

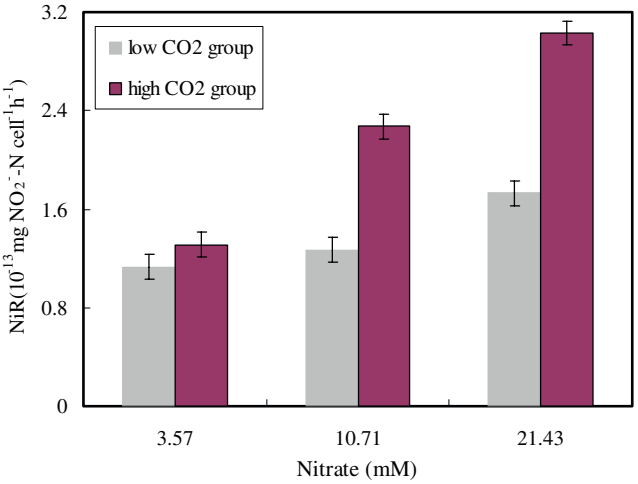

Fig. 3 The NiR activities of *Microcystis aeruginosa* at different nitrate concentrations. *Error bars* denote the standard deviation of triplicate incubations

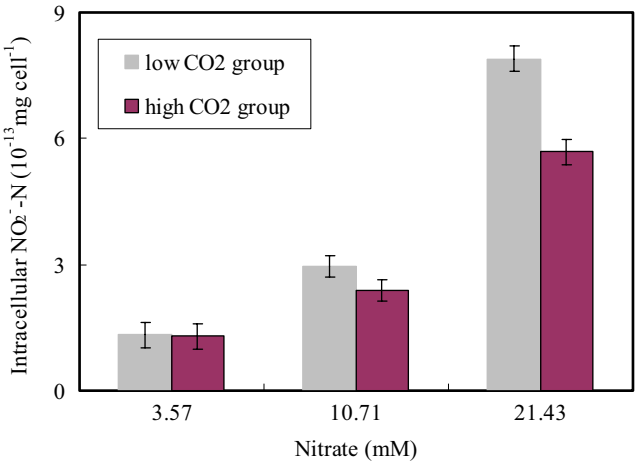

Fig. 4 The intracellular nitrite concentrations of *Microcystis aeruginosa* at different nitrate concentrations. *Error bars* denote the standard deviation of triplicate incubations

observed for alga at 3.57 mM nitrate ($p=0.68$, one-way ANOVA).

Photosynthesis

Increasing nitrate concentrations inhibitied the chlorophyll *a*-specific light-saturated photosynthetic rate $\left(P_m^{chla}\right)$, dark respiration rate $\left(R_d^{chla}\right)$ and photosynthetic efficiency (α^{chla}) in both high the CO_2 group ($p=0.009$, $p=0.012$, $p=0.032$, one-way ANOVA) and the low CO_2 group ($p=0.002$, $p=0.007$, $p=0.041$, one-way ANOVA) (Table 2). P_m^{chla}, R_d^{chla} and α^{chla} at 10.71 and 21.43 mM nitrate in the high CO_2 group showed a significant increase, compared to the low CO_2 group ($p=0.011$, $p=0.007$, $p=0.032$, two-way ANOVA). However, at 3.57 mM nitrate there was no significant difference in P_m^{chla}, R_d^{chla} and α^{chla} between the low and high CO_2 groups ($p=0.95$, $p=0.79$, $p=0.34$, one-way ANOVA).

Discussion

Nitrate is a macronutrient and can serve as a good nitrogen source for phytoplankton (Dortch, 1990; Syrett 1981). Menéndez (2005) reported that moderate levels of fertilizing nitrate could increase the maximum rate of net photosynthesis of the green alga *Chaetomorpha linum*. Touzet et al. (2007) also found that the dinoflagellate *Alexandrium minutum* showed higher maximum cell densities with increasing nitrate concentrations. Similarly, Leong et al. (2004) showed a positive correlation between the growth rates of *A. tamarense* and nitrate concentrations. However, Shi et al. (2005) observed that there was a negative effect of excess nitrate on the growth of *A. tamarense*, and Hwang and Lu (2000) also reported *A. minutum* exposed to excess nitrate had a low growth rate. These findings suggested that, although nitrate was a good nitrogen source, excess nitrate supply was harmful to phytoplankton and could depress growth, which is in agreement with the results of the present study.

As mentioned in the Introduction, nitrate is converted to ammonium in two successive steps catalysed by NR and NiR, and when nitrite formed by NR was more than nitrite reduced by NiR, the accumulation of intracellular nitrite of alga will appear. In the present study, *M. aeruginosa* grown under high nitrate concentrations showed a significant increase in NR activities, which is consistent with the results of Sivasankar and Oaks (1996) and Crawford (1995), where the activities of NR increased with nitrate increase. Although the NiR activities also increased with increasing nitrate concentrations, the extent of the NiR activitiy increases was less than that of NR activities, which caused an increase in intracellular nitrite concentrations under high nitrate concentrations. Compared to the low CO_2 group, at the high CO_2 condition (high CO_2 group), NiR activities were higher and NR activities remained constant, due to the high CO_2 condition enhancing NiR activities, but not NR activities (Suzuki et al. 1995; Hu and Zhang 2007). This resulted in a decrease in intracellular nitrite concentrations at the high CO_2 condition.

In general, excessive nitrate can be stored in cells without detrimental effect (Tylova-Munzarova et al. 2005), but nitrite is an inorganic monovalent anion which affects the process of photosynthesis quite significantly. It is known to inhibit photosynthetic electron transport (Spiller and Boger 1977; Loranger and Carpentier 1994), change intracellular pH and damage cell membranes of

Table 2 Photosynthetic parameters of *Microcystis aeruginosa* grown under different nitrate concentrations

Photosynthetic parameters		Nitrate (mM)		
		3.57	10.71	21.43
Aerated with air	P_m^{chla} *u*mol O_2 (mg chl *a* h)$^{-1}$	863±11.3	575±10.5	349±10.8
	α^{chla} *u*mol O_2 (mg chl *a* h)$^{-1}$ (*u*mol photons m^{-2} s^{-1})$^{-1}$	1.3±0.2	0.9±0.1	0.4±0.1
	R_d^{chla} *u*mol O_2 (mg chl *a* h)$^{-1}$	62.8± 4.1	36.7±5.1	15.4±3.2
Aerated with 5% CO_2 air	P_m^{chla} *u*mol O_2 (mg chl *a* h)$^{-1}$	872±13.2	729±9.3	517±11.2
	α^{chla} *u*mol O_2 (mg chl *a* h)$^{-1}$ (*u*mol photons m^{-2} s^{-1})$^{-1}$	1.3±0.1	1.1±0.1	0.6±0.1
	R_d^{chla} *u*mol O_2 (mg chl *a* h)$^{-1}$	64.5±3.9	52.8±4.2	29.7±5.2

These parameters were determined by fitting a three-parameter model, $P = P_m \times \tanh(\alpha \times I/P_m) + R_d$ (see "Materials and Methods") Data are means ± SD ($n=3$)

algae (Almeida et al. 1995; Sijbesma et al. 1996; Yang et al. 2004). In the present study, a significant increase was observed in intracellular nitrite concentrations under high nitrate concentrations. Therefore, it is possible that the increase of intracellular nitrite resulted in the decrease of growth and P_m^{chla}, R_d^{chla}, α^{chla} observed at high nitrate concentrations. Our data clearly support this conclusion. At 3.57 mM nitrate, the intracellular nitrite concentrations did not change significantly in the high and low CO_2 groups and, as a result, P_m^{chla}, R_d^{chla}, α^{chla} remained constant. However, at 10.71 and 21.43 mM nitrate, the intracellular nitrite concentrations were lower in the high CO_2 group than in the low CO_2 group, which resulted in an increase in P_m^{chla}, R_d^{chla}, α^{chla} in the high CO_2 group compared to the low CO_2 group.

The levels of nitrate concentrations used in the present study ranged from 3.57 to 21.43 mM. Much higher nitrate concentrations (from 7.14 to 35.71 mM) have been found in aquatic ecosystems which were strongly contaminated by agricultural and urban activities (Rouse et al. 1999; De Graaf 1964; Pierce et al. 1993). According to the present study, these high nitrate concentrations could become an inhibitor of growth of phytoplankton. Therefore, further research is required to investigate the actual effect of high nitrate concentrations on phytoplankton community composition and the occurrence of blooms in the field.

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