Hydrobiologia (2007) 581:167–176
DOI 10.1007/s10750-006-0517-1

EUTROPHICATION IN LAKES

Intracellular phosphorus metabolism and growth of *Microcystis aeruginosa* in dark/light cycles under various redox potential difference conditions

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Abstract Phosphorus metabolism and growth of *M. aeruginosa* were studied under three different conditions of diel fluctuation in redox potential. Redox potential in the culture increased in light and decreased in dark in all treatments except one, when cysteine was added in darkness. Total phosphorus content in *M. aeruginosa* decreased in darkness and increased in light during exponential growth but increased continuously in the stationary phase. Conversely, polyphosphate (PolyP) accumulated in darkness but was lost in the light. Low redox potential in darkness promoted PolyP accumulation. Polyglucose and soluble orthophosphate may provide energy and phosphorus, respectively, for PolyP synthesis. PolyP was important to *M. aeruginosa* survival during poor growth conditions. If the redox potential difference in the dark/light cycle was large, *M. aeruginosa* initially grew faster, but soon lost viability.

Keywords *Microcystis aeruginosa* · Growth · Phosphorus metabolism · Dark/light cycle · Redox potential difference · Lake Taihu

Guest editors: B. Qin, Z. Liu & K. Havens
Eutrophication of shallow lakes with special reference to Lake Taihu, China

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Introduction

In Lake Taihu, *Microcystis aeruginosa* has formed dense blooms in summer since the 1980s (Chen et al., 2003). Due to oxygen-producing photosynthesis, *Microcystis* usually are associated with aerobic environments, and, consequently, physiological characterization has focused on aerobic metabolism. However, this characterization discounts the possibility that they are found in environments that are permanently anoxic or become anoxic in the dark (Stal & Moezelaar, 1997).

Microcystis is colonial and possesses gas vacuoles, hollow proteinaceous bodies that provide cell buoyancy. In Lake Taihu, when the water column is stable, colonies accumulate at the surface and form surface blooms. Wind may concentrate colonies into dense ''scums'' on the leeward shore, forming microbial mats, which may experience periodic anoxia. During daytime, oxygenic photosynthesis leads to oxygen supersaturation. In the

dark, cyanobacteria continue to respire, and oxygen diffusion into the mat usually is insufficient to prevent anoxia (Revsbech et al., 1983).

Microbial mats exhibit diel oxygen fluctuations due to cyanobacterial metabolism. In light, these organisms grow photoautotrophically, while *Microcystis* switches to chemotrophic metabolism in dark, at the expense of endogenous glycogen accumulated in light (Heyer & Krumbein, 1991). However, a cohesive, overall pattern of dark cyanobacteria metabolism has not emerged (Richardson & Castenholz, 1987).

Phosphorus is a major growth-limiting nutrient in aquatic systems, and, unlike nitrogen, there is no large atmospheric source that is biologically available. All organisms require phosphorus for growth and metabolism (Bostrom et al., 1988; Currie & Kalff, 1984; Howarth, 1988). Phosphorus in microalgae is contained in internal phosphorus pools of polyphosphate (PolyP), soluble orthophosphorus (Pi), structural and other organic phosphorus (John & Flynn, 2000). Cyanobacteria store inorganic phosphate as PolyP granules (Bental et al., 1991). However, little is known about phosphorus regulation in cyanobacteria (Lawrence et al., 1998). Previous studies have found that phosphorus metabolism of *M. aeruginosa* may inhibit continuous anaerobic dark conditions, which are unfavorable for *M. aeruginosa* growth (Shi et al., 2003). *M. aeruginosa* retaining viability under dark anaerobic/light aerobic cycles also may relate to phosphorus metabolism. However, to our knowledge, no studies on phosphorus metabolism of *M. aeruginosa* under dark/light cycle with high redox potential differences have been conducted. Here, we studied phosphorus metabolism and *M. aeruginosa* growth under diel fluctuations in redox potential.

Materials and methods

Organism and cultivation

Axenic *M. aeruginosa* cultures were obtained from the Institute of Hydrobiology of the Chinese Academy of Sciences and grown in batch culture in modified MA medium where inorganic phosphorus was replaced with β-Na_2glycerophosphate (Oh et al., 2000). Media were adjusted to pH 8.6 with 2 mol l^{-1} NaOH and incubated at 25°C under 40 μE m^{-2} s^{-1} light intensity provided by fluorescent lamps with a 14:10 h light/dark cycle. *M. aeruginosa* density was counted on a microscope with a 0.1 mm^3 counter, and about 300 cells were counted. Cell numbers were converted to dry weight using a 1.32×10^{-7} mg dry weight per cell (Johnston et al., 1994).

Experimental methods

Studies were conducted with *M. aeruginosa* cells harvested during exponential growth. Cells were washed twice and resuspended in the medium described above (MA). Aliquots were transferred to serum bottles with screw caps and a butyl rubber inlayer. Bottles were wrapped in black cloth and 0, 0.01, and 0.04% (m/v) cysteine (reducing agent) were introduced through serum caps with a syringe. After one day, all bottles were unwrapped and serum caps were opened. Then, *M. aeruginosa* was incubated under illumination intensity of 40 μE m^{-2} s^{-1} for another day. The cycle then was repeated. Therefore, days 1, 3, 5 and 7 had air-tight dark cultivation periods with different concentrations of cysteine, while days 2, 4, 6 and 8 were open light cultivation. All experiments were carried out with triplicate bottles. Cell density, polyglucose, PolyP and soluble orthophosphate content in *M. aeruginosa*, as well as phosphate concentration in the water, were assayed daily. A microelectrode was used to monitor initial and final redox potential.

Analysis

M. aeruginosa cells were harvested by centrifugation for physiological assay. Samples were digested by autoclaving in 10% persulphate for 30 min at 121°C for total phosphorus measurement (Grillo & Gibson, 1979). Orthophosphate in *M. aeruginosa* was extracted with 10% cold trichloroacetic acid. For PolyP, 40 ml of culture was centrifuged and digested with 1 ml alkaline hypochlorite reagent (5.4%) for 45 min at 25°C. Residue after centrifugation was washed twice, then extracted twice with distilled water. PolyP was precipitated with ethanol and measured after hydrolysis to orthophosphate at

100°C (Harold, 1963; Rao et al., 1985). Later, all phosphorus forms were converted to orthophosphate, and all samples were measured spectrophotometrically with an automated ascorbic acid reduction method (Greenberg, 1985). All phosphorus forms were expressed in milligrams of phosphorus per unit dry weight (DW) for *M. aeruginosa*. Polyglucose was extracted from cells, harvested from 20 ml culture with 1 ml 10% trichloroacetic acid, precipitated by adding three volumes of alcohol, and determined using anthrone reagent (Roe & Dailey, 1966).

Results

Redox potential decreased from 252 to 52 mV with 0.01% cysteine and to –50 mV with 0.04% cysteine at the beginning of the experiment (Fig. 1). Redox potential increased in light and declined in darkness in all treatments except the first dark period. Average redox potential differences were 67 mV, 83.5 mV and 276 mV, respectively, when 0, 0.01 and 0.04% cysteine were added in darkness.

M. aeruginosa cell density increased in all treatments, and high redox potential differences in the diel cycle enhanced *M. aeruginosa* growth, with maximum cell density reaching 7.5×10^6 cell ml^{-1} on the 7th day and 7.8×10^6 cell ml^{-1} on the 5th day when adding 0.01% and 0.04% cysteine in darkness, respectively (Fig. 2). Compared with the control, *M. aeruginosa* had a short growth period and lost viability after reaching maximum cell density.

Phosphate concentration in the aqueous phase increased in darkness and declined in light in all treatments initially, but this pattern ceased on the 5th day, when cysteine concentrations were 0.01% and 0.04% in the dark (Fig. 3). However, total phosphorus in *M. aeruginosa* increased (Fig. 4). There were no significant differences observed among the three treatments ($P > 0.05$), so enlarging the redox difference in the dark/light cycle did not have an obvious effect on phosphorous metabolism.

Soluble orthophosphate in *M. aeruginosa* declined initially, with increasing steepness at high cysteine concentrations, then increased in light and decreased in darkness (Fig. 5). Polyglucose

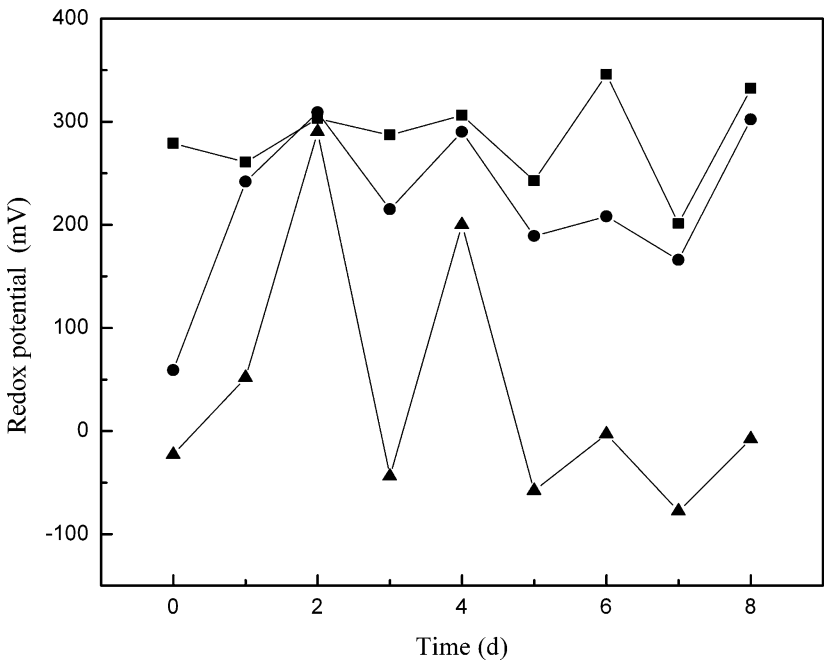

Fig. 1 Redox potential under different treatments. ■, Control; ●, 0.01% of cysteine in darkness; ▲, 0.04% of cysteine in darkness

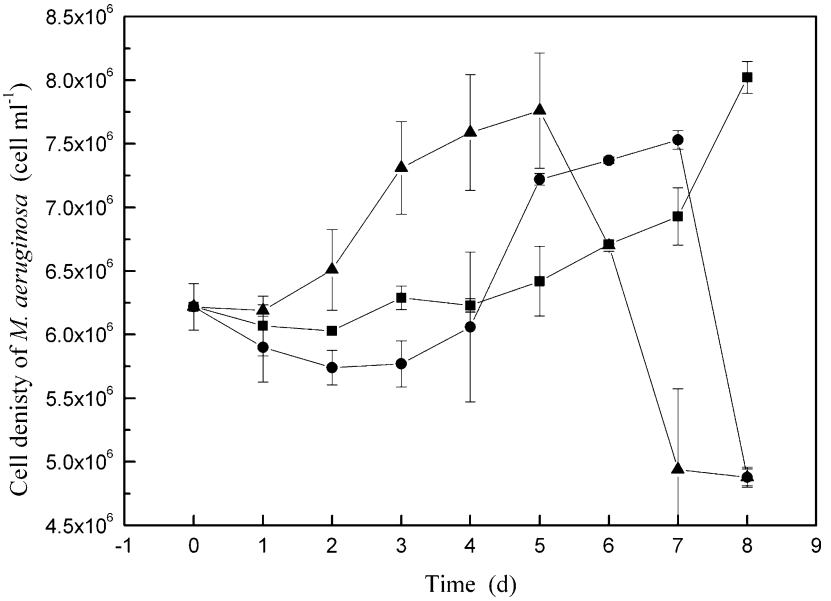

Fig. 2 Growth of *M. aeruginosa* under all the treatments. ■, Control; ●, 0.01% of cysteine in darkness; ▲, 0.04% of cysteine in darkness. Error bars represent standard derivation

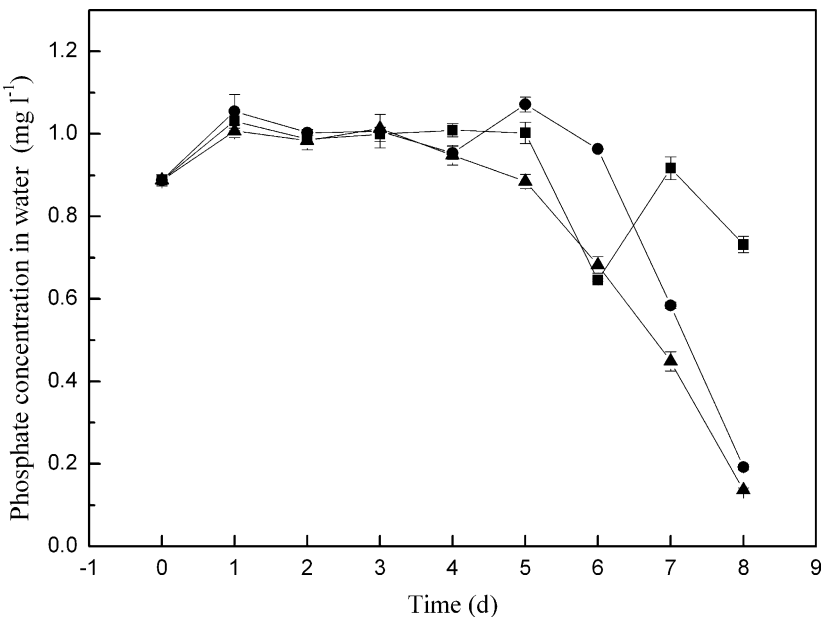

Fig. 3 Variation of phosphate concentration in water. ■, Control; ●, 0.01% of cysteine in darkness; ▲, 0.04% of cysteine in darkness. Error bars represent standard derivation

showed a similar pattern as soluble orthophosphate content (Fig. 6). Adding reducing agent promoted PolyP accumulation in *M. aeruginosa*, with PolyP reaching 0.038 and 0.043 mg g^{-1} DW on the 3rd day when cysteine concentrations were 0.01% and 0.04%, respectively. PolyP decreased in the light and increased in the dark (Fig. 7).

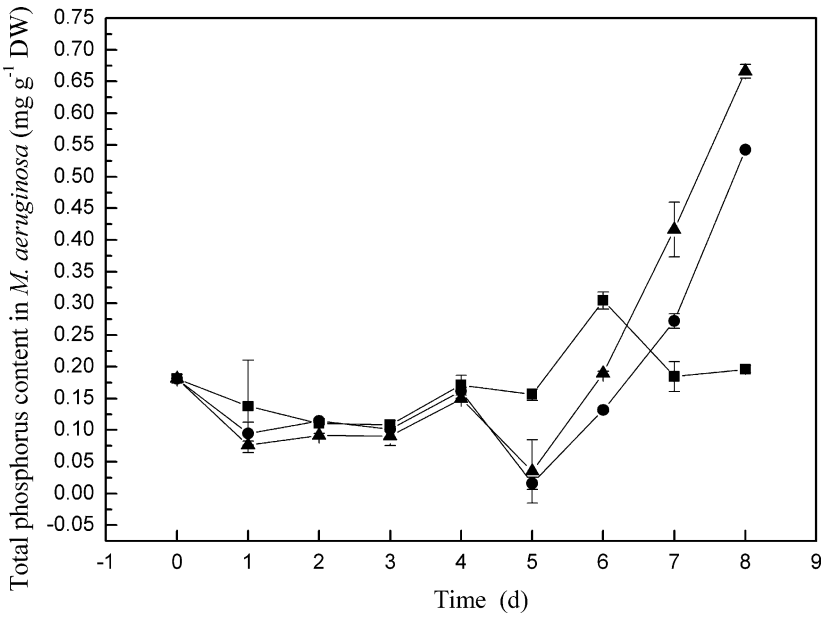

Fig. 4 Total phosphorus content in *M. aeruginosa*. ■, Control; ●, 0.01% of cysteine in darkness; ▲, 0.04% of cysteine in darkness. Error bars represent standard derivation

Discussion

Phosphorus metabolism of *M. aeruginosa* under dark/light cycles with different redox potential difference

M. aeruginosa does not form microbial mats or dense scums, which could cause anoxia, in the laboratory, so phosphorus metabolism of *M. aeruginosa* generally is studied under aerobic cultivation conditions. Phosphate uptake rate is considered a function of ambient phosphate and cell quota, and Droop's equation describes the relationship between cell quota and population growth rate (Fuhs et al., 1972). Phosphorus in *M. aeruginosa* decreased in darkness, increased in light, and increased during the stationary phase when reducing agent was added in darkness (Fig. 4). Thus, *M. aeruginosa* may release phosphorus in darkness and absorb phosphorus in light.

M. aeruginosa may synthesize PolyP when cells are added to fresh medium. PolyP in *M. aeruginosa* increased if redox potential was low. After a short period of growth, PolyP in *M. aeruginosa* increased in darkness and declined in the light (Fig. 7), opposite the trend of TP and polyglucose (Fig. 4). Synthesis of PolyP may help maintain phosphorous balance in the cell. PolyP accumulation coinciding with decreased polyglucose and soluble orthophosphate shows that these compounds may provide energy and phosphorus for PolyP synthesis. *M. aeruginosa* may accumulate polyglucose during daylight and consume it at night, and previous research showed that polyglucose fermentation can provide enough energy for PolyP synthesis (Shi et al., 2003). An experiment with yeast also indicated that endogenous substrate is adequate to supply energy for PolyP synthesis (Schuddemat et al., 1989).

Inorganic polyphosphates are polymers of orthophosphate (Pi) with a phosphoanhydride bond, which has free energy close to that of ATP. The exact physiological function of PolyP remains uncertain, although various biological functions have been demonstrated, including as a reservoir of energy and phosphate, a chelator of divalent cations, a capsule material, and a ''channelling'' agent in the phenomenon of bacterial transformation (Magrath & Quinn, 2000; Keasling & Hupf, 1996; Kornberg, 1995). In general, PolyP concentration is low during exponential growth

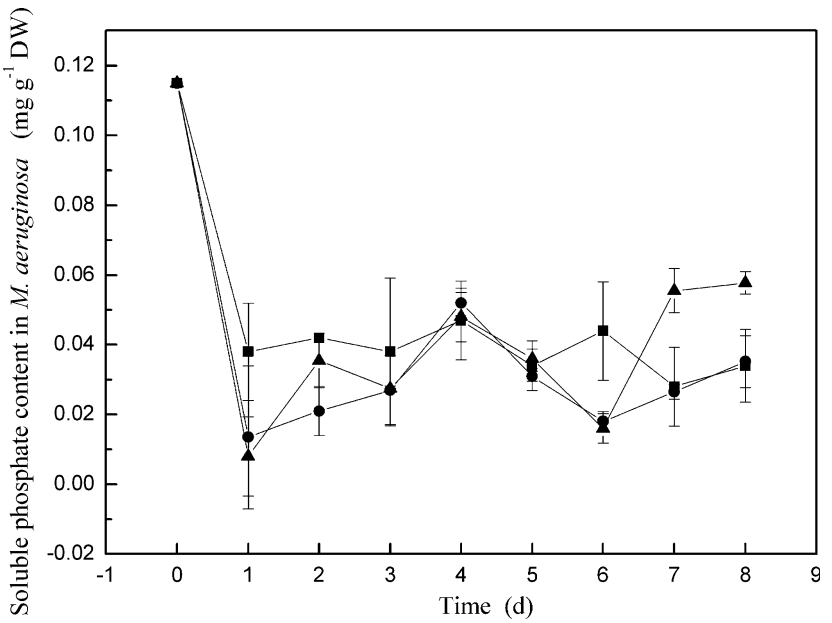

Fig. 5 Soluble phosphate content in *M. aeruginosa* under all the treatments. ■, Control; ●, 0.01% of cysteine in darkness; ▲, 0.04% of cysteine in darkness. Error bars represent standard derivation

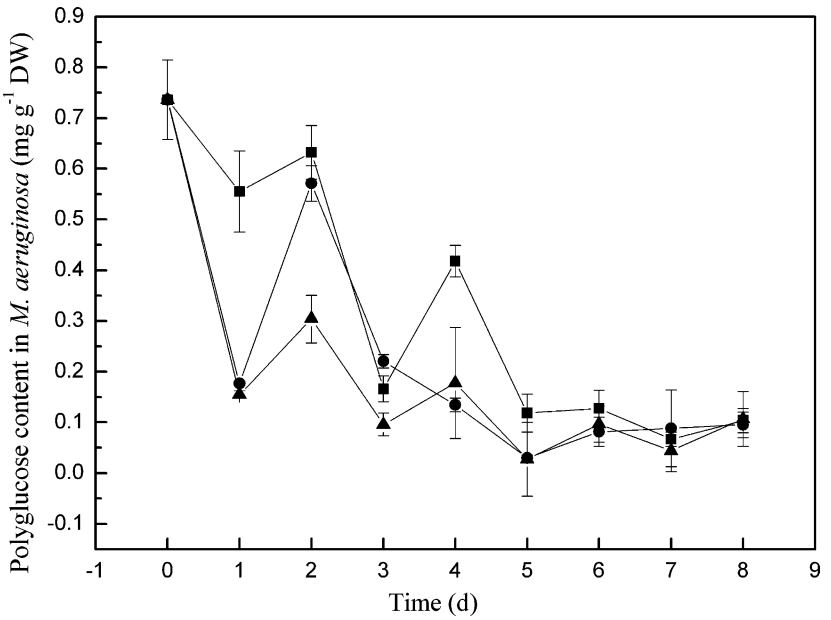

Fig. 6 Polyglucose content in *M. aeruginosa* under all the treatments. ■, Control; ●, 0.01% of cysteine in darkness; ▲, 0.04% of cysteine in darkness. Error bars represent standard derivation

but may increase when the stationary phase begins or when growth is arrested due to nutrient imbalance (Noegel & Gotschlich, 1983). Pi released and accumulated by the cells would be sequestered as PolyP to maintain Pi and H^+ concentration (Harold, 1963). Thus, rapid

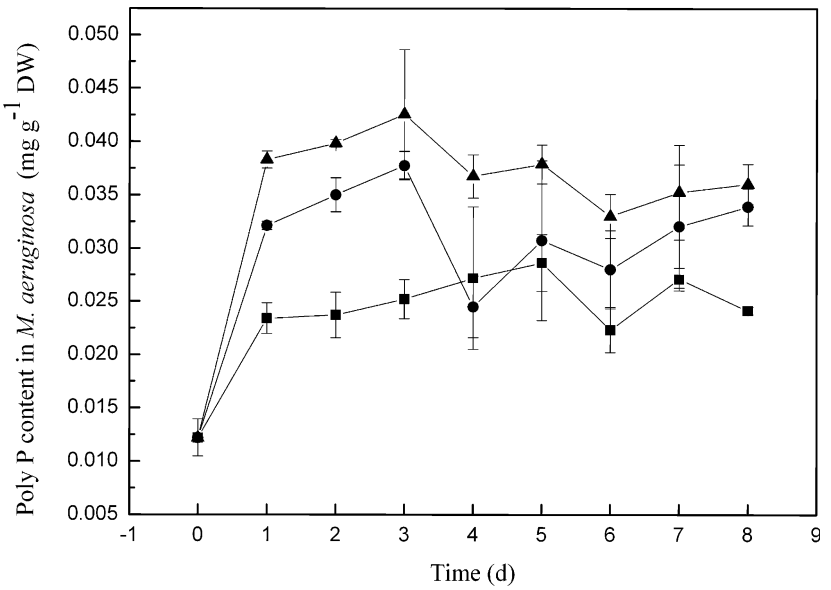

Fig. 7 PolyP content in *M. aeruginosa* under all the treatments. ■,Control; ●, 0.01% of cysteine in darkness; ▲, 0.04% of cysteine in darkness. Error bars represent standard derivation

orthophosphate release may stimulate PolyP synthesis. PolyP is involved in controlling Pi and serves, in effect, as a metabolic buffer.

Prokaryotes are composed of cytoplasm, cell surface, periplasm and plasma membrane. PolyP granules can be found in all of these, and different locations of PolyP may play different roles. Electron microscopy indicated that PolyP granules in *M. aerugnosa* were located in the cytoplasm (Shi et al., 2003). PolyP granules may be used for phosphorus and energy storage in our experiments (Kulaev et al., 1999).

M. aeruginosa growth in the dark/light cycle under different redox potential difference

Some cyanobacterial strains can survive in dark, reducing conditions. *Oscillatoria terebriformis*, a cyanobacterium, moved each night (Richardson & Castenholz, 1987). *O. chalybea* blooms are correlated with lowered partial pressure of O_2 accompanied by increased sulfide at increasing depth in lakes (Leventer & Eren, 1970). The presence of *O. redekei* in the lower strata of some lakes was attributed to reducing conditions of sulfide (Tash, 1967). Thus, *Oscillatoria* can survive in dark, reducing conditions, and some *Oscillatoria* species are dependent on lowered redox potential.

M. aeruginosa can endure low redox potential in darkness, which stimulated slight growth and reduced mortality in darkness (Shi, 2003). Large redox potential difference may promote *M. aeruginosa* growth initially, then cause cell death (Fig. 2). When adding 0.01% and 0.04% cysteine, average redox potential differences between light and dark were 83 mV and 276 mV, respectively. In this case, *M. aeruginosa* lost viability at days 5 and 7, respectively. In previous research, we found that large cell density in culture may result in high diel redox potential difference, and, when initial cell density reached 1.5×10^7, 6×10^7 and 1×10^8 cell ml^{-1}, average diel redox potential differences were 65, 330, and 450 mV, respectively (Fig. 8). During the 18-day experiment, *M. aeruginosa* grew and had no evident mortality (data not shown). Since cysteine had no other chemical effect besides lowing redox potential (Weller et al., 1975), we assumed that, only when *M. aeruginosa* reached a certain cell density, could it survive low redox potential. *M. aeruginsoa* seemed to thrive to

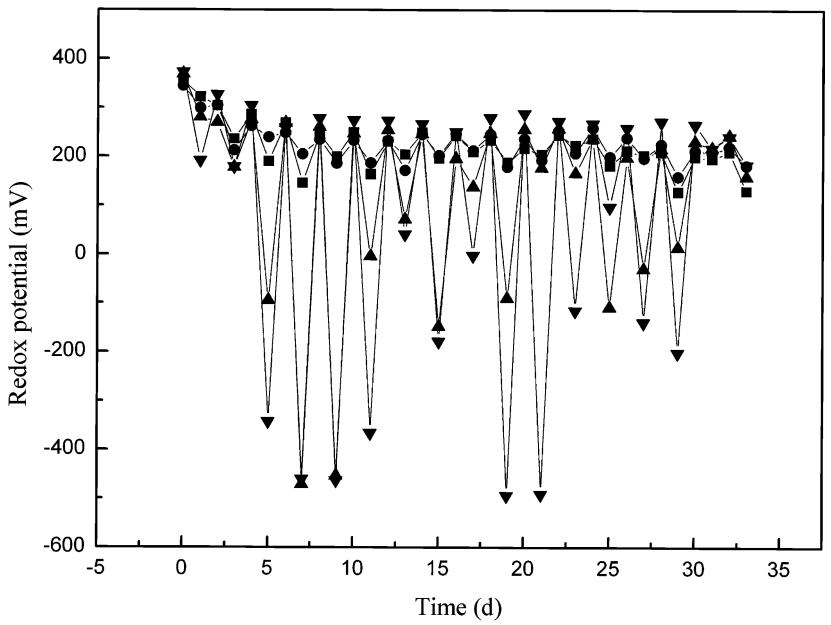

Fig. 8 Redox potential of the medium with different cell density of *M. aeruginosa*. ■, 3×10^6 cell ml^{-1}; ●, 1.5×10^7 cell ml^{-1}; ▲, 6×10^7 cell ml^{-1}; ▼, 1×10^8 cell ml^{-1}

resist the large diel redox potential difference since redox potential increased in the fist darkness period when the reducing agent was added, but could not hold back the designed trend. In our experiment, cell density of 6.2×10^6 cell ml^{-1} was too low to endure 83 and 276 mV diel redox potential difference. In Lake Taihu, large diel redox potential differences are observed in microbial mats or scums, and, in that case, cell density is high enough to adapt to such circumstances. Thus, large diel rodox potential difference may promote algae growth, but shows no negative effect.

In autumn, microbial mats of *Microcystis* sink to the sediment. *M. aeruginosa* colonies remain on the sediment as vegetative cells. These winter colonies are viable and constitute an inoculum for the following year, some reentering the water column in spring (Preston et al., 1980). Redox potential of sediment in Lake Taihu changes with depth from about -100 to + 100 mV (Fig. 9). Sediment suspension often occurs under wind and waves in large shallow lakes. Redox potential at the sediment surface increases at this time and decreases during calm periods. Therefore, *M. aeruginosa* in the sediment regularly experiences variation in redox potential.

Microcystis was a small proportion of biomass in non-stratified enclosures but was dominant in diurnally and continuously stratified enclosures (Nakano et al., 2001). Although thermal stratification was required for *Microcystis* growth, it is unclear why it was important. We hypothesize that stratification may lead to high diel redox potential difference, which is more favorable for *Microcystis* than other species, and, therefore, it is dominant in eutrophic environments.

Anoxia was vital for survival of *O. terebriformi* during dark periods, because fast degradation of glycogen under aerobic conditions would result in exhaustion of energy reserves within a few hours (Moezelaar et al., 1996). However, PolyP accumulation under low redox potential in dark may be important to *M. aeruginosa* for survival in unfavorable conditions. Therefore, the ability to accumulate PolyP in the dark and negative redox potential may be an advantage in low light, organically rich, and low-redox habitats. Finally, the phenomenon reported here may be important in understanding mechanisms of phosphorous

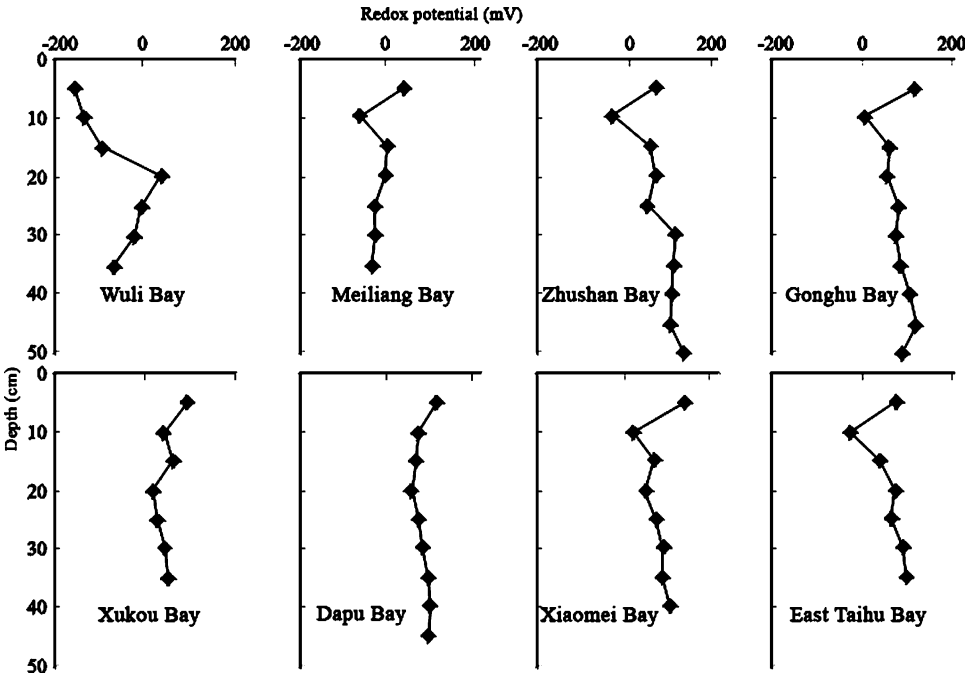

Fig. 9 Redox potential in sediment of main region of Lake Taihu. Data from (Zhu et al., 2004)

metabolism in *Microcystis* during high diel redox potential difference and explain why mats and *Microcystis* in sediment can remain viable for several months in Lake Taihu.

Acknowledgements This research was funded by Chinese Academy of Sciences (KZCX2-403), "973" Project (2002CB412307) and National Natural Science Foundation of China (No. 40371102, 40501078).

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