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Effects of Iron on Growth, Pigment Content, Photosystem II Efficiency, and Siderophores Production of *Microcystis aeruginosa* and *Microcystis wesenbergii*

Wei Xing,[1,2,3] Wen-min Huang,[1,2] Dun-hai Li,[1] Yong-ding Liu[1]

[1]State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, 430072, People's Republic of China
[2]Graduate School of Chinese Academy of Sciences, Beijing, 100039, People's Republic of China
[3]Wuhan Botanical Garden, Chinese Academy of Sciences, Wuhan, 430074, People's Republic of China

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Abstract. Changes in growth, photosynthetic pigments, and photosystem II (PS II) photochemical efficiency as well as production of siderophores of *Microcystis aeruginosa* and *Microcystis wesenbergii* were determined in this experiment. Results showed growths of *M. aeruginosa* and *M. wesenbergii*, measured by means of optical density at 665 nm, were severely inhibited under an iron-limited condition, whereas they thrived under an iron-replete condition. The contents of chlorophyll-*a*, carotenoid, phycocyanin, and allophycocyanin under an iron-limited condition were lower than those under an iron-replete condition, and they all reached maximal contents on day 4 under the iron-limited condition. PS II photochemical efficiencies (maximal PS II quantum yield), saturating light levels (I_k) and maximal electron transport rates (ETR_{max}) of *M. aeruginosa* and *M. wesenbergii* declined sharply under the iron-limited condition. The PS II photochemical efficiency and ETR_{max} of *M. aeruginosa* rose , whereas in the strain of *M. wesenbergii*, they declined gradually under the iron-replete condition. In addition, I_k of *M. aeruginosa* and *M. wesenbergii* under the iron-replete condition did not change obviously. Siderophore production of *M. aeruginosa* was higher than that of *M. wesenbergii* under the iron-limited condition. It was concluded that *M. aeruginosa* requires higher iron concentration for physiological and biochemical processes compared with *M. wesenbergii*, but its tolerance against too high a concentration of iron is weaker than *M. wesenbergii*.

Iron is an essential trace element for biological requirements of photoplankton. It can be involved in chlorophyll and phycobilin pigment biosynthesis, in many components of photosynthetic (PS I and PS II) and electron transport systems, and in nitrate assimilation as an enzyme cofactor (nitrate reductase and nitrite reductase) [4]. Since Martin and Fitzwater [10] presented their findings in the subarctic North Pacific Ocean, more and more studies have been conducted on the effects of iron limitation on the physiological and biochemical processes of phytoplankton. In recent years, a large amount of reports demonstrated that iron limitation inhibits photosystem II (PS II) photochemistry, the amount of photo-oxidizable reaction center pigment of photosystem I (PS I) (P700), and the partial reaction rates associated with PS II and PS I, respectively [15]. Concomitantly, a large decrease in the amount of phycocyanin (PC) and chlorophyll-*a* (Chl. *a*) is accompanied by structural alterations of the thylakoid membranes and phycobilisomes, and the number of iron-containing proteins within the photosynthetic apparatus is reduced [6]. In addition, ferredoxin is replaced by flavodoxin. Compared with iron limitation, only a few experiments have been done under an iron-replete condition, and results revealed that iron-replete algae have higher productivity and metabolism [6, 18].

Under an iron-limited condition, most prokaryotic cells and certain fungi and plants secrete siderophores

Correspondence to: Yong-ding Liu; *email:* liuyd@ihb.ac.cn

[12]. Siderophores are organic Fe^{3+}/metal-chelating molecules that serve to solubilize and scanvenge Fe^{3+} from the environment. The Fe^{3+}-siderophore complex is subsequently imported into the cell [2].

In spite of the intensive research over the years, most of the studies on the effects of iron limitation in cyanobacteria have been done on cells that were clear in a stage of iron starvation, in which most cellular functions are severely hampered. However, detailed information concerning the effects of iron on changes in growth, photosynthetic pigments, PS II efficiency, and siderophore production of *Microcystis* is very limited. In order to provide detailed information, we carried out this experiment using *Microcystis aeruginosa* and *Microcystis wesenbergii*, strains isolated from Lake Dianchi, China.

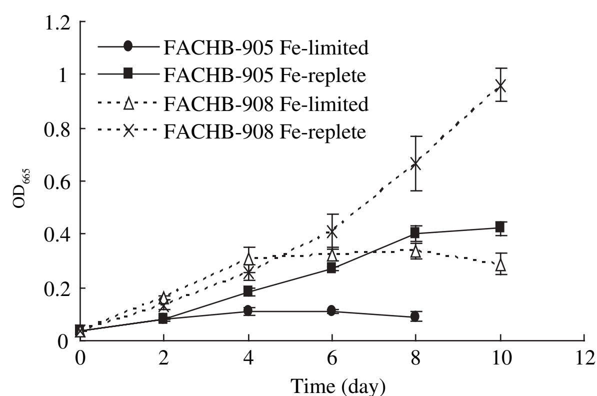

Fig. 1. Growth curves for *M. aeruginosa* (FACHB-905) and *M. wesenbergii* (FACHB-908) under iron-limited and iron-replete conditions. Data are means ± SD of three replicates and error bars are not visible when they are smaller than the symbols.

Materials and Methods

Culture and Medium. *Microcystis aeruginosa* and *M. wesenbergii* were obtained from the Freshwater Algae Culture Collection of Institute of Hydrobiology (FACHB), Chinese Academy of Sciences, and their number marks were FACHB-905 and FACHB-908. The two strains were isolated from shallow, eutrophic Lake Dianchi, located in subtrophical Yungui plateau, southwestern China, and they often dominate in phytoplankton and form blooms in summer and autumn year after year.

Microcystis aeruginosa and *M. wesenbergii* were cultured in BG-11 medium with the pH value adjusted to 8.0, from which ferric ammonium citrate was omitted. All macronutrient stocks were treated with Chelex-100 (Bio-Rad, Cat. No. 142-2832) to remove trace metal contaminants. Iron was added separately to cultures in a solution of $FeCl_3$. Iron-replete cultures were provided 100 μM Fe^{3+}, whereas iron-limited cultures were provided 0.01 μM Fe^{3+} at the beginning of this study. The $FeCl_3$ solution was filter-sterilized (0.22 μm).

Iron starvation was induced by transferring cells into medium that lacked iron. *M. aeruginosa* and *M. wesenbergii* were inoculated into media loaded by triple acid-washed bottles when the biomass was enough. All cultures were maintained at 25°C and were provided illumination at a proton flux density of 30 μmol quanta/m^2/s under 24-h light.

Determination of Growth. Growths of *M. aeruginosa* and *M. wesenbergii* were determined by optical density at 665 nm using a spectrophotometer (Ultrospec 3000, England).

Determination of Pigment Content. The precedure was carried out in darkness at 4°C. Whole-cell spectra were taken using 1-cm quartz cuvettes in a Ultrospec 3000 spectrophotometer (Pharmacia Biotech., England).

A 5-mL cell suspension was centrifuged at 8000*g* (Jouan BR 4i, France) for 10 min. The supernatant was decanted and the pellet was resuspened in 95% ethanol. Then the resuspended cells were placed in a mortar and grounded, and extract was kept overnight in the dark at 4°C. Afterward, the sample was centrifuged for 10 min at 8000*g*. The supernatant was collected and read at 665, 649, and 470 nm for Chl. *a* and carotenoid content. The contents of Chl. *a* and carotenoid were calculated using the following equations [8]:

$$\text{Chlorophyll}-a = 13.95A_{665} - 6.88A_{649},$$
$$\text{Carotenoid} = (1000A_{470} - 2.05\ \text{Chl.}\,a)/245$$

The determination of phycocyanin (PC) and allophycocyanin (APC) was followed the method of Myers and Kratz [11]. A 5-mL cell suspension was transferred to a centrifuge tube and the cells were broken by prolonged sonication (Brandson Digital Sonifier, Mexico). The solution was centrifuged for 15 min at 7000*g* (Jouan BR 4i, France) and absorbencies were determined at 615, 652, and 562 nm. The contents of PC and APC were calculated using the following equations:

$$\text{Phycocyanin} = (A_{615} - 0.474A_{652})/5.34,$$
$$\text{Allophycocyanin} = (A_{652} - 0.208A_{615})/5.09.$$

Fluorescence Measurements. Chlorophyll fluorescence measurements were conducted using the PHYTO-PAM phytoplankton analyzer (Heinz Walz, Effeltrich, Germany). The maximum photochemical efficiency of PS II (maximal PS II quantum yield), maximal relative electron transport rates through PS II (ETR_{max}), and saturating light levels (I_k) of *M. aeruginosa* and *M. wesenbergii* were obtained in Light Curve-windows and Report-windows of PHYTO-PAM [1].

Siderophore Detection. A 1.0-mL aliquot of supernatants of liquid cultures of *M. aeruginosa* and *M. wesenbergii* were mixed with 1.0 mL CAS (chrome azurol S) assay solution prepared according to Schwyn and Neilands [14]. Also, according to Machuca and Milagres [9], a reference was prepared with BG-11 that lacked added iron but was uninoculated. Absorbencies of the sample (s) and reference (r) at 630 nm were measured after 1 h of incubation at room temperature. The percentage of iron-binding compounds of the siderophore type was calculated by subtracting the sample absorbance values from the reference. Siderophore units are defined as $(A_r - A_s/A_r) \times 100 = \%$ siderophore units. Percentages of siderophore units less than 10 were considered to be negative.

Results

Growth Characteristics. Figure 1 illustrates the growth changes of *M. aeruginosa* and *M. wesenbergii* under iron-limited and iron-replete conditions. The optical density at 665 nm (OD_{665}) values of *M.*

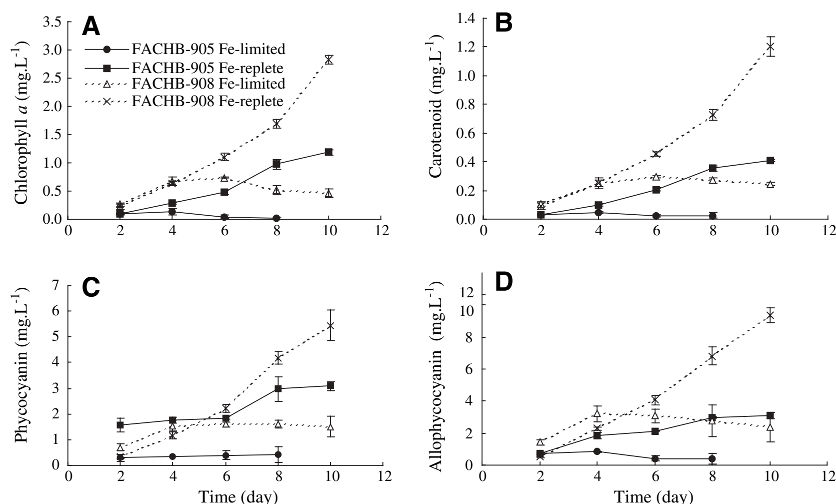

Fig. 2. Changes in chlorophyll-*a* (a), carotenoid (b), phycocyanin (c), and allophycocyanin (d) contents of *M. aeruginosa* (FACHB-905) and *M. wesenbergii* (FACHB-908) against culture time (days) under iron-limited and iron-replete conditions. Data are means ± SD of three replicates and error bars are not visible when they are smaller than the symbols.

aeruginosa and *M. wesenbergii* under iron-limited condition reached maximal values on day 4 synchronously, then declined slowly. At the same time, under the iron-replete condition, the OD_{665} rose during culture period, and on day 10, the OD_{665} value of *M. wesenbergii* was 2.5 times than that of *M. aeruginosa*.

Photosynthetic Pigments Contents. Iron-altered Chl. *a*, carotenoid, PC, and APC contents of *M. aeruginosa* and *M. wesenbergii* are presented in Fig. 2. All of these pigments increased under iron-replete conditions and decreased under-limited conditions on day 6. The contents of PC and APC of *M. aeruginosa* changed similarly under the iron-limited condition with those under the iron-replete condition; in contrast to *M. aeruginosa*, *M. wesenbergii* changed remarkably. The contents of PC and APC were higher than those of Chl. *a* and carotenoid on each day during the culture period. The ratios Chl. *a*/carotenoid of *M. aeruginosa* decreased 70% and that of *M. wesenbergii* decreased 28% under the iron-limited condition; at the same time, the ratios Chl. *a*/carotenoid of *M. aeruginosa* and *M. wesenbergii* under the iron-replete condition had no obvious variations. The ratios Chl. *a*/PC of *M. aeruginosa* and *M. wesenbergii* decreased, except that *M. aeruginosa* cultured under the iron-replete condition increased during the culture period.

Analysis of Chlorophyll *a* Fluorescence. Figure 3 illustrates PS II photochemical efficiencies (maximal PS II quantum yield), saturating light levels (I_k), and maximal electron transport rates (ETR_{max}) of *M. aeruginosa* and *M. wesenbergii* under iron-limited and iron-replete conditions. The three parameters declined sharply under the iron-limited condition, and maximal PS II quantum yield and ETR_{max} of *M. aeruginosa* rose , whereas in *M. wesenbergii*, they declined gradually under the iron-replete condition. In addition, I_k of *M. aeruginosa* and *M. wesenbergii* under the iron-replete condition had no obvious changes.

Siderophores Production. Iron limitation can induce the production of siderophores in both *M. aeruginosa* and *M. wesenbergii*. Siderophore production of *M. aeruginosa* (50% siderophore units) was higher than that of *M. wesenbergii* (30% siderophore units) under the iron-limited condition.

Discussion

According to Martin's iron hypothesis [10], seeding the ocean's high-nutrient, low-chlorophyll (HNLC) areas with iron should make marine phytoplankton multiply dramatically; that is, in those regions, iron has limited the phytoplankton productivity and metabolism, and iron supply could promote their reproduction. Some experiments demonstrated that iron limitation might affect phytoplankton in two independent ways: reduced rate processes (photosynthesis) and/or biomass yield [3, 16, 17]. Our results were in agreement with their reports.

The values of OD_{665} were based on Chl. *a* content that measured at 665 nm and 649 nm. Chl. *a* was influenced by iron limitation and iron enrichment. Although Chl. *a* itself does not contain iron, there are both direct and indirect requirements for iron by enzymes involved in the Chl. *a* biosynthetic pathway. For example, iron in coproporphyrinogen oxidase can catalyze protoporphyrin and turn it into protochlorophyllide and might be affected directly by iron starvation. In addition, precursor production such as δ-aminolevulinic acid requires NADPH and organic acids from the Krebs

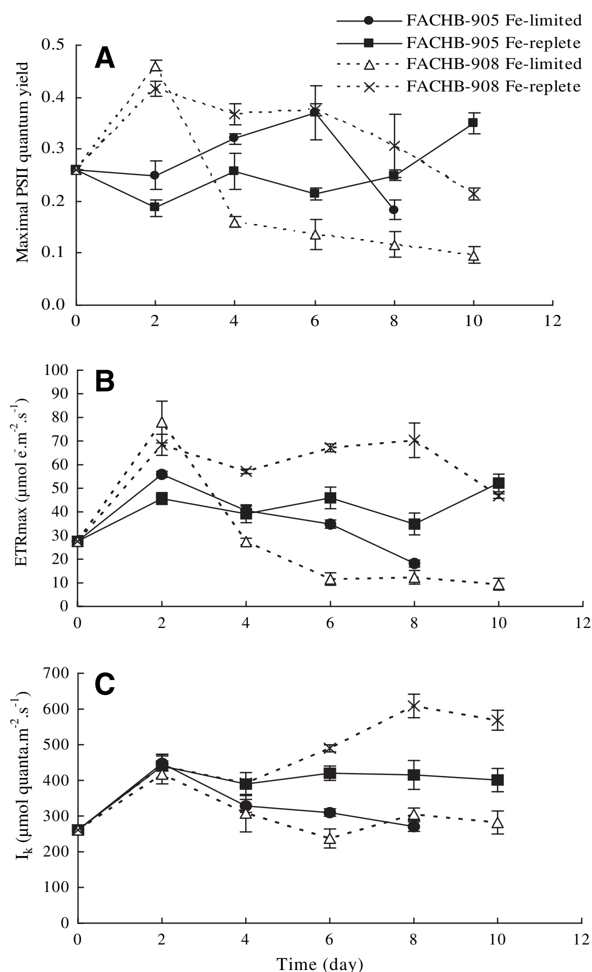

Fig. 3. Changes in maximal PS II quantum yield (a), maximal electron transport rates (b), and saturating light levels (c) of *M. aeruginosa* (FACHB-905) and *M. wesenbergii* (FACHB-908) against culture time (days) under iron-limited and iron-replete conditions. Data are means ± SD of three replicates and error bars are not visible when they are smaller than the symbols.

cycle and could be iron-regulated aconitase and Fe-S protein [19]. A reduction in iron availability might simply reduce the cellular abundance or activity of these enzymes and thus reduce the Chl. *a* synthesis rate. Under the iron-limited condition, the contents of PC and APC decreased due to the decline of Chl. *a*. In order to protect the PS II apparatus from intense light, these light-harvesting pigments contents, including carotenoid, became lower and lower. Kudo et al. [6] reported that iron-limited algae had lower pigment concentrations.

Results showed that the ratio Chl. *a*/carotenoid under the iron-limited condition decreased gradually. There are two potential reasons: One is that oxidant stress induced the production of carotenoid; the other is that Chl. *a* and carotenoid declined after day 4, but the decreasing amplitude of carotenoid was smaller than that of Chl. *a*. At the same time and under the same condition, the ratio Chl. *a*/PC increased first and then decreased. The result was a protective measure of PS II. In an earlier period, the contents of Chl. *a* and light-harvesting pigments were low after inoculation; thus, PS II photochemical efficiency was also measured at a low level, but in the late period, PS II was damaged by iron limitation so that all photopigments declined and the decreasing amplitude of phycocyanin was smaller than that of Chl. *a*. Sandström et al. [13] reported that the well-known drop in phycobilism content was not due to an increased degradation, but to a decreased rate of synthesis.

The cyanobacterial photosynthetic system is unique by its large outer antenna pigments that transfer the absorbed energy to the reaction center. Therefore, the reduction of photosynthetic pigments concentration would reduce the photosynthesis by decreasing light absorption. Davey and Geider [3] found that about 40% of the decline of cell-specific photosynthesis in *Chaetoceros muelleri* (Bacillariophyceae) could be accounted for by the decline of pigment content. Results also showed that iron limitation could reduce the maximal PS II quantum yield and ETR_{max}, which indicate that an important portion of the PS II reaction center was damaged. Iron limitation might lead to reduction of the electron transport chain. I_k is often used as an index of the photoacclimation state of phytoplankton. High values of I_k are associated with growth at high irradiance and indicate a relatively greater capacity for light-saturated photosynthesis relative to the rate of light absorption. Our results indicate that I_k was reduced by iron limitation. This might have resulted from the reduction of photopigments and photochemical efficiency.

Under the iron-replete condition, pigments and photochemical efficiency of *M. aeruginosa* and *M. wesenbergii* were promoted, indicating that 100 μM Fe_3^+ did not inhibit the growth and photosynthesis of *M. aeruginosa* and *M. wesenbergii*. In our experiment, the two strains inoculated in 1000 μM Fe_3^+ die quickly within 1 h (data not presented), indicating that the high Fe_3^+ concentration inhibited their growth and reproduction.

Lammers and Sanders-Loehr [7] provided evidence that some strains of *Anabaena* could produce schizokinen (a siderophore). Consistent with this view, Wilhelm and Trick [17] found that cyanobacteria can produce siderophores and acquire iron via a siderophore-based system. Imai et al. [5] also demonstrated that *M. aeruginosa* could produce hydroxamate-type siderophores. Results of this study show that the two strains produced siderophores and the amount of siderophores of *M. aeruginosa* was more than those of *M. wesenbergii*, as *M. aeruginosa* requires a higher iron concentration than *M. wesenbergii*.

In conclusion, iron can affect photopigments, photochemical efficiency and siderophore production. A too low or too high concentration of iron is able to inhibit the metabolism of *Microcystis.* Thus, we can use these results to control and eliminate water blooms of *Microcystis.*

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