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Effects of nutrients on *Microcystis* growth more easily forming bloom

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Abstract: Different nutrient media experimentally were N, P and Fe-limited conditions and a serial of diluted BG11 media. The cell change of morphology and life history, cell number, cell color and cell area of *Microcystis* were analyzed quantitatively. First, the effects of nitrogen, phosphorus and iron depletion were distinctively different. Phosphorus and iron depletion caused more special division cells, slowly growth increasing, the easier change of bigger cell area. Second, the nitrogen and iron depletion could make the color of alga from green to brown. Finally, according to the resource competition and Monod equation, *Microcystis* kinetics of phosphorus and iron were also examined. K_s and μ_{max} of phosphorus absorption were 0.0352 μmol/L, 0.493 d^{-1} respectively; iron absorption: 0.00323 μmol/L, 0.483 d^{-1}. In a word, some evidences of the *Microcystis* bloom dominance in certain nutrient conditions were indicated in the experiments. The dominances were determined as the reviving under the adverse circumstances through the special division, the various nitrogen resources, and the lower kinetics of phosphorus and iron than that of most of other algae. The conclusions provided the scientific basis for preventing and managing *Microcystis* bloom in freshwater.

Keywords: *Microcystis*; diluted BG11 media; nitrogen, phosphorus; iron; depletion; dynamics

Introduction

Cyanobacteria are probably the best-studied group of phytoplanktonic microorganisms, because of their success and ubiquity in freshwater systems(Bryant, 1994). For example, *Microcystis* bloom often occur in summer in eutrophic temperate lakes and cause various problems, such as reduced transparency, decreased biodiversity, the potential occurrence of oxygen depletion, odor and taste compounds, as well as production of toxins hazardous to animals and threaten human health(Turner, 1990; Martin, 2000). Besides the morphology of lakes, elevated temperatures, low light-energy requirements of *Microcystis* as the steering factor for bloom formation, modes of nutrient supply and nutrient competition between micro-algae are of decisive importance for *Microcystis* dominance. Rarely will a single factor be responsible for the mass appearance of the species; the reasons for such outbreaks still largely remain unclear in spite of considerable researches summarized above.

In the present study, characteristics of *Microcystis* cells and special reproductive style were conducted under different nutrient conditions. And the growth dynamics of *Microcystis* were measured with the concentration gradients of phosphorus and iron based on Tilman's mechanistic theory of resource competition. The overall aims were to get some evidences of the *Microcystis* dominance forming blooms in some conditions, to deepen our understanding of ecology and the resource competition energy of the species, to cut the cycle life of alga and control the nutrient resource as a potential management strategy for the prevention of the next *Microcystis* bloom.

1 Materials and methods

1.1 Cyanobacterial strain and culture condition

Algal strain used was *Microcystis aeruginosa* kindly provided by FACHB in Institute of Hydrobiology. The cells were grown axenically in liquid cultures at 26℃ and in the light: dark cycle 12 h: 12 h, using BG11 growth medium. Illumination was provided by cool-white fluorescent light at a continuous intensity of 2 mW/cm^2.

1.2 Culture preparation and counting of cells

N, P and Fe element deficient BG11 media were prepared by removing sodium nitrate, 2-potossium hydrogen phosphates and ferric ammonium citrate from BG11 medium respectively. Prior to use, the alga was incubated under N, P and Fe-limited conditions separately for 4 days. Then the alga was transferred into 1000 ml flasks containing 400 ml medium, the initial algal density was about 10^5 cells/ml.

After staining with Lugol's Iodine solution, the population of *Microcystis aeruginosa* cells was counted on a compound microscope (Nikon 400) using a hemocytometer. The cells were taken photos and the cell area was analyzed with software Image-Pro Plus (Media Cybernetics Inc.).

1.3 Experimental design

The whole experimental design I included the different nutrients (Table 1). The initial BG11 medium was diluted to 1/5, 1/10 and 1/20. And the other treated media were deprived of certain nutrient from BG11 medium, such as N, P and Fe designed as de-N, de-P and de-Fe ("de" means deficiency). According to design Ⅰ, the experimental design Ⅱ was carried out as Table 2.

In the experimental design I, the cell density and cell area were measured and the reproductive style was investigated. Growth dynamics of *Microcystis aeruginosa* competition for phosphorus and iron, half-saturated constants and the maximal specific growth rate, were calculated in design Ⅱ. Then its growth trends can be predicted by the modification of Monod equation in some concentration ranges of P and Fe.

Table 1 The experimental design I

Experimental condition	Control sample	1/5 BG11	1/10 BG11	1/20 BG11	de-Fe	de-N	de-P	Distilled water
Group	1	2	3	4	5	6	7	8

Table 2 The experimental design Ⅱ

Nutrient element	1	2	3	4	5
Fe, mol/L	No adding	1.23×10^{-8}	4.305×10^{-8}	8.61×10^{-8}	1.23×10^{-7}
P, mol/L	No adding	1.75×10^{-7}	6.125×10^{-7}	1.225×10^{-6}	1.75×10^{-6}

1.4 The Monod model and *R*-rule hypothesis

Under steady state conditions, we used the equation of Monod model

$$\mu = (\mu_{max} \cdot C_s)/(K_s + C_s) \rightarrow C_s/\mu = K_s/\mu_{max} + C_s/\mu_{max} \quad (1)$$

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to calculate the growth kinetics of the micro-alga. The specific growth rate, reported here as the percentage increase in biomass per day, was calculated by the equation

$$\mu(d^{-1}) = (\ln N_t - \ln N_0)/t. \quad (2)$$

Data were fitted to the treated Monod model.

Tilman provided the *R*-rule hypothesis as fundament of resource competition theory in 1982 (Tilman, 1982). A single species will deplete the external concentration of the limiting nutrient to the corresponding level at a given equilibrium turnover rate (reproduction rate = loss rate in chemostat culture given by the dilution rate):

$$R = D \cdot K_s/(\mu_{max} - D), \quad (3)$$

Where, μ is the per capita reproduction rate, d^{-1}; μ_{max} is the maximal growth rate, d^{-1}; K_s is the half-saturation constant, μmol/L; C_s is the concentration of the limiting nutrient, μmol/L; N_0 is the initial cell number; N_t is the cell number on the day t; R is the residual equilibrium nutrient concentration for a species, μmol/L; D is the dilution rate for a species.

2 Results and discussion

2.1 Effects of the nutrient treatments

The increase of the individual biomass, the proportion between special cells and total cells, the morphology change of the *Microcystis* cell, and the change of cell area were important indicators of *Microcystis* response to the different nutrient environments.

Confronted with the gradients of BG11 media and with the nitrogen, phosphorus and iron deprived from BG11 medium, the alga *Microcystis* showed the growth curves as Fig.1. 1/10·BG11 and 1/20·BG11 media presented negligible influence on the alga and the decrease of effect in the gradients was accompanied by an increase of nutrient concentration of BG11 medium. The results indicated that the reduced complete nutrient culture of *Microcystis* in laboratory might be better for alga growth in certain range of initial alga density. Because the cells of *Microcystis* in the typical BG11 medium become mature and divide into next generation sooner, at the same time cell decay rate rises. High density, conversely, can cause part of cultures to be low light and severe nutrient limitation at that moment. Therefore, complete BG11 medium, nutrient enrichment, is probably suitable for small amounts of inoculation with optimal physical growth condition. In the nitrogen, phosphorus and iron depleted media, the growth trends were clear. Similar effects were found in the cultures of 1/20 BG11 and nitrogen depletion medium (Fig. 1). However, in the two systems the numbers of *Microcystis* cells were larger than that of the phosphorus and iron deficient cultures after the early ten cultivating days. From the seventeenth day to the twenty-fifth day the biomass quantities in the four different treated flasks respectively were:

1/20·BG11 group, de-N group > de-P group > de-Fe group.

It was found that the solution of de-N became yellowish-brown during the incubated period, though its cell number rose like the complete nutrient cultures. There was also obviously color variety in the solution of de-Fe during the incubated period, and only slight color change was found in the solution of de-P.

On the aspect of producing special cell form, there was significant difference between the de-N, de-Fe and de-P media. Special form was that the materials in the cell membrane concentrated into one point that next came out of the membrane and then grew into vegetative cell. Nitrogen deficiency did not nearly induce abnormal cell division. On the contrary, the effect of iron scarcity was the greatest, in which medium special cells from abnormal division possessed over 35% of total cell after being incubated for five days, decreased gradually since the fifteenth day. There was the variant number of special cells in the phosphorus deficient medium (Fig.2, 3). The cell areas in the BG11, de-Fe, and de-P media were the greatest. The other media (Fig.4) were almost similar on the small cell size. There was a possibility that alga might accumulate reserves of nitrogen which would carry them through several successive divisions before a stress was imposed. And Cyanobacteria accumulated nitrogen in the form of granules of the polypeptide cyanophycin and might also use their nitrogenous photosynthetic pigments, phycobilins, as reserves (Simon, 1987). However, in the phosphorus and iron - deficient batch culture in the stationary phase the cycle of cell division changed even stopped at certain point.

In fact, these phenomena were led by the different mechanisms. Clearly, the elements of N, P and Fe in charge of algal cell are distinct. The macronutrients of N and P and the micronutrient Fe are indispensable for the alga *Microcystis*. N is incorporated in the most important structural and functional macromolecules (proteins, nucleic acids, and to a lesser extent polysaccharides). P is also part of fundamental building blocks that constitute nucleic acids, phospholipids and complex carbohydrates. Laboratory experiments also point out an essential role of iron in regulation of biochemical processes in phytoplankton organisms. This element participates in biosynthesis of a protoporphyrine precursor, δ-aminolevuline acid, therefore it takes part in chlorophyll formation (Alicja, 2004). It is also a constituent of the other photosynthetic electron transporter-feredoxine. As an element of nitrogenase it takes part in N_2 assimilation and nitrate reduction. Moreover, it is an activator of peroxidase and catalaes, enzymes protecting cells against harmful influence of peroxides (Kudo, 2000; Davey, 2001).

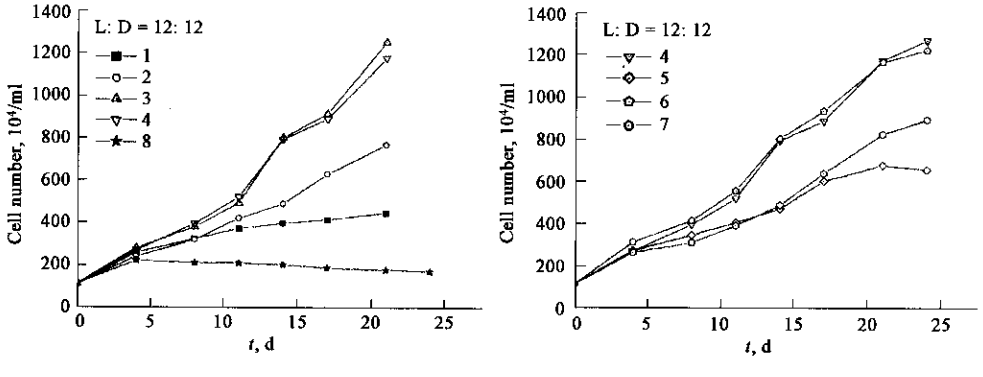

Fig.1 *Microcystis* growth curves in the gradients of BG11 medium and in the N, P and Fe deficient media

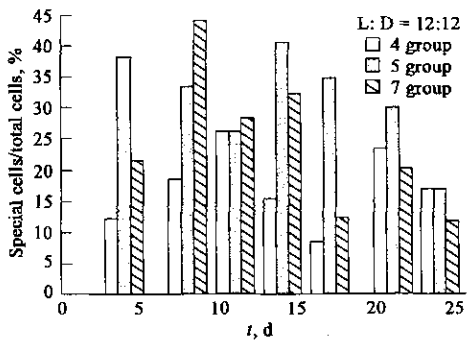

Fig.2 The proportions of the special cells and the total cells of the 4th, 5th and 7th group

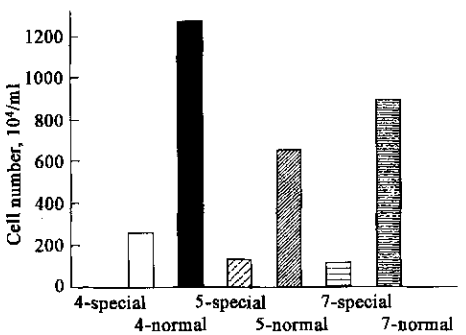

Fig.3 The numbers of special cells and normal cells of the 4th, 5th and 7th group on day 24

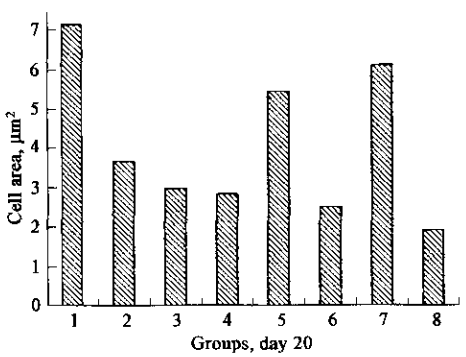

Fig.4 The cell areas of the eight groups on day 20

In general, cyanobacteria can use a wide variety of nitrogenous compounds to uptake and assimilate, which is not beneficial for controlling the N sources. Let alone that many kinds of cyanobacteria are N_2-fixing ones. In the stress of N limitation in the study the *Microcystis* must be able to sense its intracellular N nutritional status. This signal then must be transduced to enzymes in charge of the adaptive response, or the genome to induce or repress the synthesis of appropriate enzymes, and to form a series of varieties such as the reduction of cell area and the pigment losing with the growth rise. The so-called de-N was not meaning no-nitrate completely, since in the BG11 medium there were still citric acid, ferric ammonium citrate and EDTA that were nitrogenous compounds which could also fulfill *Microcystis* N requirements. Combined with the results in the experiments, it is possible that depriving simply of nitrogen to control or remove the *Microcystis* bloom is negative.

Since no comparable gaseous atmospheric cycle exists for phosphate, and since more or less all organisms need the same form of phosphorus, namely soluble orthophosphate (Reynolds, 1993; 1998), there is no compensatory mechanism for losses of this nutrient, for example, due to sedimentation. Accordingly, in most lakes the production of phytoplankton biomass can be limited by the amount of available phosphate. Vanishingly low Fe concentrations as a limiting factor in some regions can control phytoplankton abundance in certain areas of the open ocean (Li, 2003), and high concentration of Fe is also possibly an promoting factor forming alga bloom in fresh water column or red tide in some ocean areas (Kenneth, 1996). In this study, nutrient (N, P and Fe) deficiency caused series of varieties in the *Microcystis* cells. With the presented experiment phenomena more qualitative and quantitative data of physiology will be investigated to obtain the concrete effects of nitrate, phosphorus and iron on the alga *Microcystis*. Iron and phosphorus deficiencies with the bottleneck effects may be the potential limiting factors in many given natural environment. There is a potentially powerful direction to eliminate the cyanobacteria bloom.

2.2 Growth dynamics of *Microcystis* competition for phosphorus and iron

According to the above studies, this experiment of growth dynamics was made sequentially. Fig. 5 shows the results of the single-species Monod growth experiments for *Microcystis* under P and Fe limitation separately. The estimated maximal per capita growth rates of *Microcystis* on the P limited medium was appreciably greater than that under Fe limited condition, which were 0.493 d^{-1} and 0.483 d^{-1} respectively. The phosphorus and iron half-saturation constants for *Microcystis* were 0.0352 μmol/L, 0.00323 μmol/L, which were significantly different. Although the initial algal population densities of *Microcystis* may affect the growth rate and certain nutrient half-saturation constants to some extent, there was no experiment investigating its impact. Comparison among the parameters indicated that the result of competition was affected by the cultivating conditions including the limited-nutrient concentration range and the physical environment, independent of the initial algal population densities.

The per capita reproduction rate (μ) of an algal population is related to the ambient concentration of the limited nutrient (C_s) according to the Monod equation that has since become one of the most influential and widespread models to describe the growth behavior of nutrient-limited cultures under steady state and sometimes even non-steady state-conditions (Grover, 1991a). If several species are competing for a nutrient and if their loss rates are known, the outcome of competition can be predicted by calculating R for each species, and the species with the lowest R will win the competition. There were extensively tested with microalgae competing in laboratory microcosms near equilibrium (Hansen, 1980), in higher plants (Wedin, 1993) and in zooplankton communities (Rothhaupt, 1988). The effects of dilution rates and concentrations of phosphorus (Chen, 2000) and ammonium (Akira, 2000) on the competition in a chemostat between *Microcystis* and other algal organisms were also tested. However, no such study for comparison using the concentration of iron was found so far.

With the stated D resource competition theory and the Monod equation predict the species with the higher μ_{max}, the lower K_s and the lower R should be dominant (Nan, 2003). If the two species had almost the same R calculated from the K_s and μ_{max}, they should therefore coexist under nutrient-limited condition. There were the K_s and μ_{max} of P being 0.412 μmol/L and 1.098 d^{-1} respectively in the phosphorus concentration range of 0.136—16.950 μmol/L (Chen, 2000), and 0.017 mg/L and 1.143 d^{-1} in the range of 0.05—0.36 mg/L (Qu, 2002). However, the presented studies performed the two parameters of 0.0352 μmol/L and 0.493 d^{-1} of P which much lower than the

illustrational outcomes mentioned above. The iron K_s and μ_{max} of *Microcystis* were 0.00323 μmol/L and 0.483 d^{-1} that are also lower than most of other algae. So, the *Microcystis* Monod kinetics of P and Fe in the experiments further approved its competitive advantage in the natural environment, especially in the low concentration range. With the known data about the physical condition the Monod model may be the best predictor for the competition between *Microcystis* and other microorganisms.

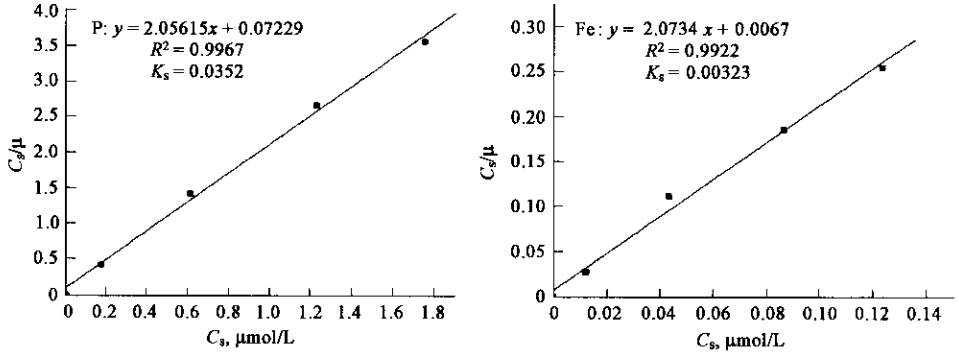

Fig.5 *Microcystis* Monod growth kinetic experiments performed under conditions of limiting phosphorus and iron element respectively

3 Conclusions

Some factors investigated in the study were the diluted gradients of complete BG11 and the element deficiency of nitrogen, phosphorus and iron. The morphology and life history of *Microcystis* in the different conditions were examined experimentally, and it was found that the special cell divisions took place in the complete BG11 medium and in the certain element, such as P and Fe deficient media, but not in the nitrogen deficient medium. The nitrogen deficiency influence on the *Microcystis* growth was different from those of the phosphorus and iron on the other aspects of the growth rate, the changes of the pigment of chlorophyll and cell area. Combined with the lower *Microcystis* Monod kinetics of P and Fe than that of other algae, *Microcystis* further approved its competitive dominant reasons in lakes and ponds, even overwintering to the next *Microcystis* bloom.

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