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Benefits and costs of the grazer-induced colony formation in *Microcystis aeruginosa*

Zhen Yang[1,2], Fanxiang Kong[1]*, Zhou Yang[3], Min Zhang[1], Yang Yu[1] and Shanqin Qian[1,2]

[1] State Key Laboratory of Lake Science Environment, Nanjing Institute of Geography and Limnology, Chinese Academy of Sciences, 73 East Beijing Road, 210008 Nanjing, China
[2] Graduate School of the Chinese Academy of Sciences, 100039 Beijing, China
[3] Jiangsu Key Laboratory for Biodiversity and Biotechnology, School of Biological Sciences, Nanjing Normal University, 1 Wenyuan Road, 210046 Nanjing, China

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Abstract – Colonial *Microcystis aeruginosa* were obtained when the unicellular algae were exposed to flagellate *Ochromonas* sp. filtrate. To investigate the benefit of this morphological change, flagellates were added into cultures of unicellular and colonial *M. aeruginosa*, respectively. The clearance rates of flagellates on algae were markedly decreased when they were cultivated with induced colonial *M. aeruginosa*. This result indicated that colony formation in *M. aeruginosa* was a predator-induced defense, which could reduce predation risk from flagellate. The increased content of soluble extracellular polysaccharide (sEPS) and bound extracellular polysaccharide (bEPS) may play an important role in adhering *M. aeruginosa* cells together to form colonies. The decrease of ΦPS II and the increase of sinking rates of induced colonial *M. aeruginosa* showed that the costs of grazed-induced colony formation in *M. aeruginosa* may reflect in the photosystem II efficiency, and in the sinking rates.

Key words: Morphological plasticity / *Ochromonas* sp. / clearance rate / sinking rate / polysaccharide

Introduction

In aquatic systems, morphological anti-predator reaction is an important defense for plankton to reduce predation risk, which has not only been found in zooplankters (Marinone and Zaragese, 1991; Wicklow, 1997; Tollrian and Dodson, 1999), but also in algae. Hessen and van Donk (1993) discovered the grazing from *Daphnia* could induce colony formation in green alga *Scenedesmus subspicatus*. As most grazers are size selective, this enlarged-volume defense reaction could reduce their predation risk from small *Daphnia magna* (Hessen and van Donk, 1993) and *Dahpnia cucullata* (Lürling and van Donk, 1996).

According to defense theory, preys should pay some costs for their anti-grazing defense strategy (Mole, 1994; Agrawal, 1998). The grazing-induced colony formation in alga is no exception, because otherwise the defensive form would be norm (Dodson, 1989). Lürling and van Donk (2000) suggested colony formation would involve two potential costs: (1) reduced nutrient and light harvesting expressed in lower growth rates, and (2) enhanced sinking. However, no clear effect on growth rates have been observed in pervious studies on induced colony formation in *Scenedesmus* sp. (Hessen and van Donk, 1993; Lampert *et al*., 1994; Lürling and van Donk, 1996). Colony formation also did not lead to any decline at PS II efficiency of algae. Only the higher settling velocities of colonial population was demonstrated, which has been considered as the cost for their enlarged-volume defense reaction, because higher settling velocities would enhance their chance to sink to deeper water layers and result in reduced growth as light and temperature may be lower (Lürling and van Donk, 2000).

Microcystis aeruginosa, one of the most common species in blooms of cyanobacteria (Reynolds and Walsby, 1975; Valério *et al*., 2008), often forms large colonial aggregates with tens of thousands algae cell under natural conditions. But after they were isolated from the field and cultivated in axenic cultures in the laboratory for some generations, the colonial algae disaggregate and develop into unicellular algae (Reynolds *et al*., 1981; Bolch and Blackburn, 1996). The absence of biotic factor may be an important reason for *M. aeruginosa* remaining unicellular form in axenic cultures. A previous study in our laboratory showed that filtered lake water with

*Corresponding author: fxkong@niglas.ac.cn

abundant zooplankton could induce colony formation in *M. aeruginosa*, whereas *M. aeruginosa* populations in the control and the treatment of filtered lake water with few zooplankton were still strongly dominated by unicellular and paired cells and no colony was formed (Yang *et al.*, 2005). *Microcystis aeruginosa* would release microcystin to resist the grazing pressure from cladocerans (Jungmann, 1992; Jang *et al.*, 2003; Lürling, 2003), so the colony formation in *M. aeruginosa* could not be induced by cladocerans (Fulton and Paerl, 1987; Hessen and van Donk, 1993). Correspondingly, we observed colonies when *M. aeruginosa* was cultivated with the flagellate *Ochromonas* sp. (Yang *et al.*, 2006). In further studies, we have demonstrated that flagellate filtrate has the ability to induce colony formation in *M. aeruginosa*, which suggested infochemicals released from flagellate fed with *M. aeruginosa* may be a trigger for colony formation in *M. aeruginosa* (Yang *et al.*, 2009). Since most colonies induced by filtered flagellate culture in our previous experiments were made of few cells, we wish to determine whether these small colonies could resist the grazing of the flagellate *Ochromonas* sp. or not. In addition, it is worth to study if the costs of this morphological antipredator defense in *M. aeruginosa* are expressed in lower growth rates and involve effects upon their PS II efficiency and sinking rate.

Materials and methods

Algae and flagellate

The cyanobacteria *Microcystis aeruginosa* (Kütz.) (PCC 7806, 4.61 ± 0.32 μm), were obtained from Institute of Hydrobiology, the Chinese Academy of Sciences, and grown in a BG-11 medium in batch culture in 250-mL flasks (Rippka *et al.*, 1979). The flagellate *Ochromonas* sp. (8.3 ± 0.80 μm) used in the experiment was isolated from the water of Lake Taihu, cultivated in culture prepared from 1000 mL distilled water with 20 seeds of wheat after autoclaved sterilization. The algae and flagellate cultures were grown at 25 °C under fluorescence light at an intensity of 40 μmol quanta.m^{-2}.s^{-1} (PAR) with a light:dark period of 12:12 h. The algae in its late exponential growth phase were used in the experiment.

Colony induction experiment

In order to produce inducible cultures, 5 mL of flagellates *Ochromonas* sp. with a concentration at 1.4×10^5 individuals.mL^{-1} were appended into 95 mL of *M. aeruginosa* (3.5×10^7 cell.mL^{-1}). After 48 h of grazing, the filtered cultures of flagellate were obtained by passing them through 0.10 μm membrane filters (Milipore, USA). Inoculums of the exponentially growing unicellular *M. aeruginosa* (1.74×10^6 cell.mL^{-1}) were transferred to three replicate 250 mL Erlenmeyer flasks containing medium with 20% filtered cultures of flagellates (treatments) and blank BG-11 medium (controls), respectively. Batches were incubated at 25 °C under fluorescence light at an intensity of 40 μmol quanta.m^{-2}.s^{-1} (PAR) with a light:dark period of 12:12 h for five days. Samples were daily taken and we measured the growth rates on cell number by using a hemacytometer in a microscope. The mean numbers of cells per unit and the mean proportion of different cells (unicells, two-celled, and colonies) were computed at the 5th day of the experiment after counting at least 800 units.

Defense benefit

To determine if colony formation in *M. aeruginosa* brings defense value to to the algae or not, flagellates (40 000 individuals.mL^{-1}) were added into the inducible colonial (4.23×10^6 cell.mL^{-1}) and unicellular (4.08×10^6 cell.mL^{-1}) *M. aeruginosa* cultures to observe their grazing on the induced colonial algae. Changes in the densities of algae and flagellates were determined after 12, 24, 36, and 48 h. The clearance rates (CR, μL.ind.$^{-1}$.h^{-1}) were calculated using the equation of Peters (1984). All treatments and controls were run in triplicate under the conditions as described above.

Pigment assay

The contents of chlorophyll *a* and phycobiliprotein in algae were determined at the 5th day after exposure to the filtrate, using the methods developed by Yan *et al.* (2004). For chlorophyll *a*, samples were subject to filtration with GF/C glass microfiber filters (Whatman, UK) and extracted with 90% acetone and measured on the spectrofluorophotometer (Shimadzu, RF-5301PC, Japan) at scan speed of 60 nm.min^{-1} with a wavelength difference $\Delta\lambda = 258$ nm, band pass of 5 nm, response time of 2 s, and low PM gain. Phycocyanin measurement was extracted using 0.05 M pH 7.0 Tris buffer and determined on the spectrofluorophotometer at an excitation wavelength of 620 nm and an emission wavelength of 647 nm.

Polysaccharide content assay

The content of soluble extracellular polysaccharide (sEPS), bound extracellular polysaccharide (bEPS) and intracellular polysaccharide (IPS) were quantified spectrophotometrically by the anthrone method (Herbert *et al.*, 1971) using glucose as standard. Samples (10 mL) were centrifuged at 27 500$\times g$ for 15 min. The supernatants were filtrated through 0.45 μm membrane filters. After dialyze of the filtrate against deionized water to remove the interference of the presence of ions in BG-11 cultures, they were used to assay the content of sEPS. The pellets were resuspended in 10 mL distilled water in centrifuge tubes, and the pH was adjusted to 10. The centrifugation tubes were incubated in 45 °C water for 4 h to extract a polysaccharide fraction that is more tightly associated

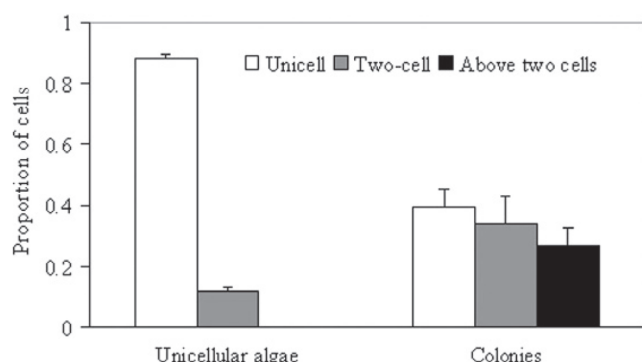

Fig. 1. Composition of the populations of unicellular and inducible colonial algae. Error bars represent 1 SD ($n = 3$).

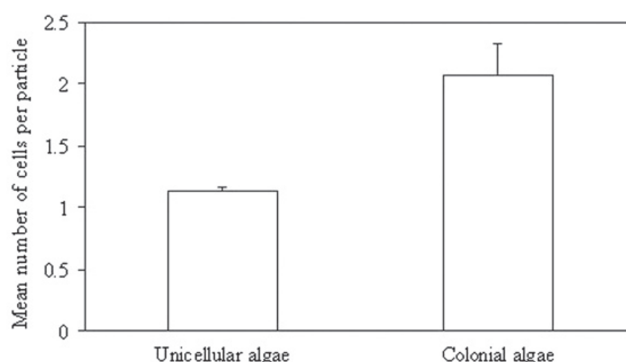

Fig. 2. Mean number of *M. aeruginosa* cells per particle in unicellular and inducible colonial algae. Error bars represent 1 SD ($n = 3$).

with the surface of *M. aeruginosa* cells. Subsequently, the cell suspensions were centrifuged at 27 500× g for 15 min. The supernatants were filtered and dialyzed as described above and were used to assay the content of bEPS. The pellets were resuspended in 10 mL distilled water. After sonicating and water bath heating at 85 °C for 1 h, they were treated with 1 mL 85% trichloracetic and centrifuged at 27 500× g for 15 min to remove the fragments and proteins. The supernatants were filtrated and dialyzed as described above and used to assay the content of intracellular polysaccharide IPS. The polysaccharide content in each supernatant dialyzed was determined using glucose solutions for calibration.

PS II efficiency and sinking rate assay

The effective quantum yield of PS II (ΦPS II) was determined daily using a PHYTO-PAM fluorometer (Walz, Germany) with an actinic light intensity of 364 µmol quanta.m^{-2}.s^{-1} according to the equation: ΦPS II = (Fm' – F)/Fm', where F and Fm' are the light-adapted actual and maximum fluorescence, respectively (Genty *et al.*, 1989). The sinking rate was determined according the SETCOL procedure (Bienfang, 1981). Three 100 mL replicates of the controls and treatments of *M. aeruginosa* cultures after colony induction experiment were respectively poured into a 100 mL glass measuring cylinder and placed in the dark in a climate-controlled cabinet at 25 °C. The algal densities of the bottom (C_{sed}), suspension (C_{sus}, sampling site up 10 cm above the bottom) were determined after 24 h. The sinking rate (V_{SED}) of *M. aeruginosa* was calculated according to the equation:

$$V_{SED} = (C_{sed} - C_{sus}) \times C_0^{-1} \times h \times t^{-1}$$

where the C_0 is the initial algal density before the sinking experiment, and the h and t are the height between the sampling sites and the elapsed time, respectively.

Statistical analyses

All data were presented as mean ± SD. Differences between the unicellular and colonial algae were analyzed

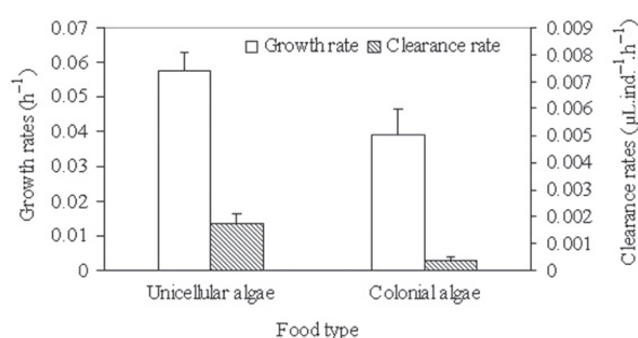

Fig. 3. Growth rates and clearance rates on *M. aeruginosa* of flagellates cultivated with unicellular and colonial algae in grazing experiments. Error bars represent 1 SD ($n = 3$).

by one-way analysis of variance (ANOVA) after testing for homoscedasticity of the data, and the level of statistical significance was set at $P < 0.05$.

Results

Microcystis aeruginosa in treatments and controls expressed good growth with mean growth rates of 0.177 (± 0.026) d^{-1} and 0.17 (± 0.019) d^{-1}, respectively. No significant difference on the algal growth rates was detected between them. After exposure to the 20% flagellates filtrate for five days, the proportion of unicells reduced to 39.6 ± 1.65% from 88.1 ± 1.57% and the colonies increased from 0 to 26.5 ± 6.42% (Fig. 1). The mean number of algae cells per unit significantly increased from 1.135 (± 0.02) to 2.073 (± 0.253) ($F_{1,4} = 41.024$, $P < 0.05$) (Fig. 2).

Colony formation in *M. aeruginosa* could reduce their predation risk from flagellates. The clearance rates on induced colonial *M. aeruginosa* were significantly lower ($F_{1,4} = 33.17$, $P < 0.05$) than those on unicellular algae (Fig. 3). Meanwhile, as the size of food enlarged, the growth rates of flagellates in the induced colonial *M. aeruginosa* cultures were significantly lower ($F_{1,4} =$ 13.39, $P < 0.05$) than those in unicellular algae cultures.

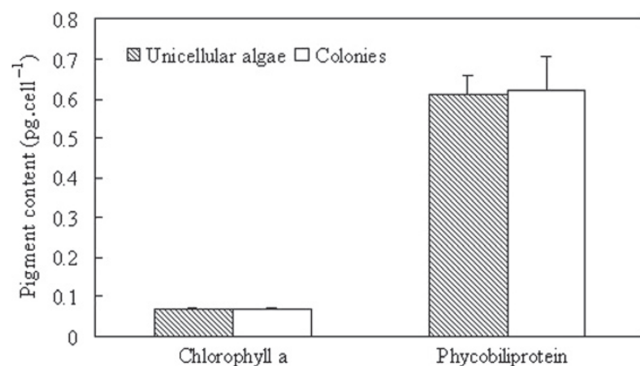

Fig. 4. Chlorophyll *a* and phycobiliprotein content of *M. aeruginosa* in the unicellular and inducible colonial algae. Error bars represent 1 SD ($n = 3$).

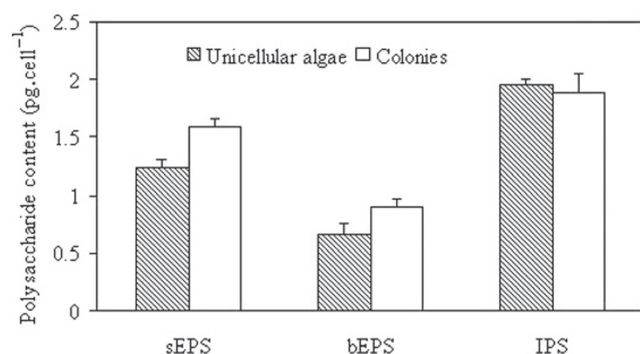

Fig. 5. Polysaccharide content of *M. aeruginosa* in the unicellular and inducible colonial algae. Error bars represent 1 SD ($n = 3$). sEPS, soluble extracellular polysaccharide; bEPS, bound extracellular polysaccharide; IPS, intracellular polysaccharide.

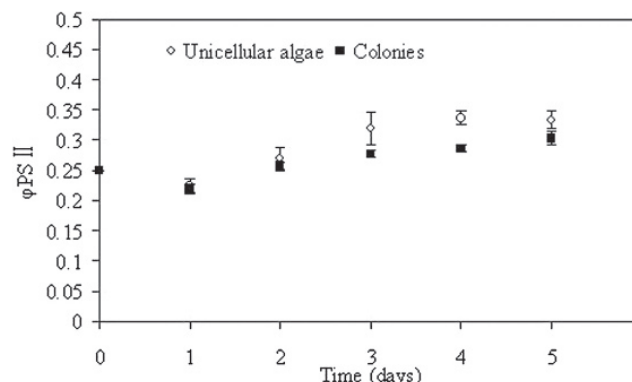

Fig. 6. Changes in the effective quantum yield of PS II (ΦPS II) of *M. aeruginosa* in unicellular and inducible colonial algae. Error bars represent 1 SD ($n = 3$).

Neither chlorophyll *a* nor phycobiliprotein showed significant difference between unicellular and colonial algae (Fig. 4). Correspondingly, the content of sEPS and bEPS in induced colonial *M. aeruginosa* were significantly higher (sEPS: $F_{1,4} = 37.786$, $P < 0.05$, bEPS: $F_{1,4} = 11.25$, $P < 0.05$) than those in unicellular algae cells (Fig. 5). However, no significantly difference in the IPS was found between induced colonial and unicellular algae.

The effective quantum yield of PS II (ΦPS II) in both the controls and treatments increased slowly during the experiment. However, induced colonial *M. aeruginosa* had a lower ΦPS II than that of unicellular algae from the second day and significant difference occurred from the third day (Fig. 6). Measured sinking rates differed significantly ($F_{1,4} = 89.75$, $P < 0.05$) between colonial and unicellular algae with mean rates of 0.008 ($\pm$0.004) m.d^{-1} for unicellular and 0.056 ($\pm$0.008) m.d^{-1} for colonial *M. aeruginosa*.

Discussion

Infochemicals released by herbivorous zooplankton have been reported to be responsible for morphological changes in some species of algae (Hessen and van Donk, 1993; Tang, 2003; van Holthoon *et al.*, 2003). In our study, a number of unicellular algae formed colonies after exposure to 20% flagellate filtrate for five days. This suggests that infochemicals released from flagellates may trigger the morphological response of *M. aeruginosa*. Infochemicals are produced and released into the culture after the grazing of flagellates on *M. aeruginosa*. *Microcystis aeruginosa* coud thus perceive strong grazing pressure from the flagellates, and then could form colonies to resist grazing when they were exposed to the flagellate filtrate.

The colonies induced by flagellate filtrate consisted of a mixture of unicells and different colonies (two- to dozen-celled). The volume of algae was enlarged after they formed colony. We found that the largest inducible colony could reach over 16 µm in diameter, which led to a significant decrease in the clearance rates of flagellates because the size of prey had a significant effect on their maximum ingestion rates (Zhang and Watanabe, 1996). Releasing microcystin is a defense strategy of *M. aeruginosa* when they face the grazing pressure from cladocerans (Jungmann, 1992; Jang *et al.*, 2003; Lürling, 2003). However, flagellates have the ability to degrade microcystin and the toxin could not serve as a growth depressor for this organism (Ou *et al.*, 2005). Therefore, *M. aeruginosa* could evolve other defense strategy to have the upper hand in aquatic systems. Our results indicate that colony formation in *M. aeruginosa* could be an effective strategy to face the grazing of flagellate.

The effects of EPS on the stickiness of cells surfaces and its contribution to cell aggregation has been demonstrated in some algae (De Philippis and Vincenzini, 1998; van Rijssel *et al.*, 2000; Pajdak-Stós *et al.*, 2001; Thornton, 2002). In our study, the content of sEPS and bEPS in induced colonial *M. aeruginosa* were significantly higher than in unicellular algae. This result is consistent with previous ones, when *M. aeruginosa* was directly exposed to flagellates (Yang *et al.*, 2008). The infochemicals released from the flagellates may be a main stimulator for the increased production of EPS in *M. aeruginosa*, which sticks algae cells together to form colonies.

Prey should pay some costs for colony formation as an anti-grazing defense strategy (Mole, 1994; Agrawal, 1998).

However, the costs associated with colony formation is not clear or at least not reflected in growth rates, as no decrease in growth rates of treatments relative to controls were observed (Hessen and van Donk, 1993; Lampert *et al.*, 1994; Lürling and van Donk, 1996). Also, we did not observed significant difference in growth rates between the two morphotypes in our study.

The good correlation between the Photosystem II electron flow and the rate of C-fixation in algae has been demonstrated (Kolber and Falkowski, 1993; Geel *et al.*, 1997). This correlation suggests that algal growth and PS II electron flow are correlated as well (Hofstraat *et al.*, 1994). Therefore, the change of efficiency of Photosystem II (ΦPS II) electron transport in *M. aeruginosa* could be used as a sensitive method to estimate the costs of induced colonies in our experiments. We found that the ΦPS II of inducible colonial *M. aeruginosa* were significantly lower than those of unicellular algae. Moreover, the content of chlorophyll *a* and phycobiliprotein between the two morphotypes were similar. This result indicates that the decreased of ΦPS II may be a metabolic cost of colonial *M. aeruginosa* in the defense strategy.

Sinking to deeper water layers could imply reduced growth as light and temperature may be lower. Thus, higher sinking rates could also be considered as a cost of grazer-induced colony formation (Reynolds, 1984; Lürling and van Donk, 2000).

Gas-vacuolate cyanobacteria such as *M. aeruginosa* have the ability to regulate their buoyancy in response to light and nutrition (Reynolds *et al.*, 1987; Oliver, 1994; Brookes and Ganf, 2001). When cultivated in the laboratory with equal light and nutrition conditions, *M. aeruginosa* do not need to regulate buoyancy by gas vesicle to obtain more light or nutrition. Therefore, the diameter of particle becomes a main factor for the sinking rates of algal cell according the Stokes' Law. This could explain that colonial *M. aeruginosa* with an increased volume exhibit higher sinking rates than unicells in our experiments. Consequently, the cost of colony formation in *M. aeruginosa* also reflects in their higher sinking rates.

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