Ann. Limnol. - Int. J. Lim. 2020, 56, 28

https://doi.org/10.1051/limn/2020026

Available online at:
www.limnology-journal.org

Research Article

Impacts of different extracellular polysaccharides on colony formation and buoyancy of *Microcystis aeruginosa*

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Received: 27 September 2020; Accepted: 24 October 2020

Abstract – On the surface of *Microcystis* cells, there is a carbohydrate called extracellular polysaccharides (EPS) playing a significant role in the colony formation of *Microcystis.* EPS consists of tightly cell-bound EPS (TB-EPS), and both of these substances are considered to be strongly related to the colony formation and buoyancy of *Microcystis.* In this study, *Microcystis aeruginosa* (strain: NIES-843) was used to examine the effects of EPS, TB-EPS, and divalent metal cations such as calcium and magnesium on the buoyancy and colony formation of *M. aeruginosa* NIES-843. Under various light conditions, the addition of TB-EPS into the culture medium induced *M. aeruginosa* NIES-843 to obtain high buoyancy at concentrations of Ca^{2+} and Mg^{2+} concentrations of 10 mg/L and 30 mg/L, respectively. Under the absence of light, the addition of EPS could lead *M. aeruginosa* to form a colony and obtain buoyancy, and the addition of TB-EPS could not significantly change the buoyancy of *M. aeruginosa* NIES-843. The colony size analysis showed that at the same cationic concentration, the addition of TB-EPS could induce *M. aeruginosa* to form the largest colony and present strong buoyancy. This study suggested that temperature and illumination are conducive to colony formation and present higher buoyancy of *M. aeruginosa.*

Keywords: *Microcystis* / buoyancy / colony formation / extracellular polysaccharides / tightly cell-bound extracellular polysaccharides

1 Introduction

Microcystis bloom outbreaks often occur in eutrophic lakes in summer and have been found in many regions of the world (Otten *et al.*, 2012; Park *et al.*, 1993). The toxin microcystin produced by *Microcystis* has caused great harm to the survival of animals and plants in water (Nishiwaki *et al.*, 1992). *Microcystis* overwinters at the lake bottom in winter after their growth season (Brunberg and Blomqvist, 2002; Preston *et al.*, 1980). In the spring, *Microcystis* slowly rises to the water column with the increase of temperature and light intensity. During the summer, *Microcystis* floats to the water surface and multiplies. *Microcystis* gradually sinks to the bottom of the water as the temperature and light intensity decrease in autumn. The movement of *Microcystis* in lakes is reciprocating in water as an annual cycle (Sigee, 2005; Walsby, 1994). Two factors of *Microcystis* buoyancy have been proposed based on the previous studies; gas vesicle and extracellular polysaccharides (EPS). *Microcystis* has a particular structure called gas vesicle and can obtain buoyancy by synthesizing gas vesicles (Walsby, 1994). Under laboratory conditions, *Microcystis aeruginosa* NIES-843 synthesized gas vesicles and floated in a test tube, while none of the gas vesicles were detected at the bottom of the test tube (Wei *et al.*, 2018).

It was reported that the buoyancy of *Microcystis* is also strictly related to colony formation (Wei *et al.*, 2019). Wild *Microcystis* strain presents strong buoyancy, and the large size of the colony can be observed with a microscope (Liu *et al.*, 2018; Wei *et al.*, 2019). On the surface of *Microcystis* cells, there is a carbohydrate called extracellular polysaccharides (EPS) that play a significant role in the colony formation of *Microcystis* (Sato *et al.*, 2017; Zhao *et al.*, 2011). EPS contains polysaccharides, proteins, and DNA (De Philippis and Vincenzini, 1998; Flemming and Wingender, 2010). Carboxyl groups in polysaccharides can combine with divalent metal ions by the ionic attractive force, which induces *M. aeruginosa* colony formation in culture medium (Hahn *et al.*, 2004; Sato *et al.*, 2017; Wang *et al.*, 2011). *M. aeruginosa* could combine with calcium or cadmium ions to form a colony (Bi *et al.*, 2016; Pradhan and Rai, 2001; Wang *et al.*, 2011; Zhao *et al.*, 2011). With the EPS supplement, *M. aeruginosa* (strain: PCC7005) obtain buoyancy and form a bloom (Dervaux *et al.*, 2015). The combination of cationic ions and EPS addition could make

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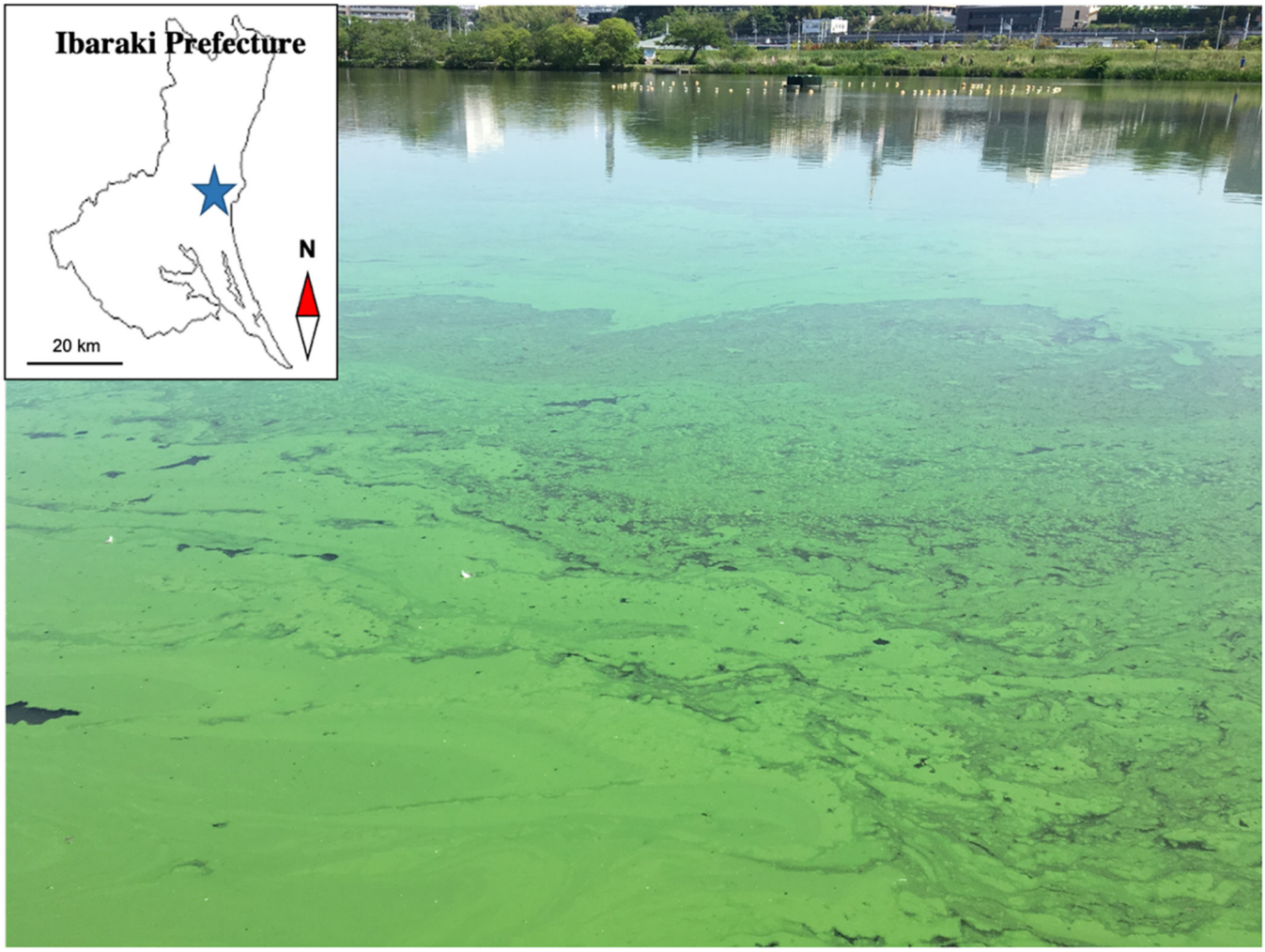

Fig. 1. Wild *Microcystis* floating in the surface of Lake Senba.

M. aeruginosa NIES-843 form a similar colony as wild *M. aeruginosa* and possessed buoyancy in a measuring cylinder (Wei *et al.*, 2019).

EPS consists of bound EPS (bEPS) and soluble EPS (sEPS) (Wingender *et al.*, 1999). bEPS can be divided into loosely cell-bound EPS (LB-EPS) and tightly cell-bound EPS (TB-EPS) (Basuvaraj *et al.*, 2015; Qu *et al.*, 2012). TB-EPS is one of the main components of *M. aeruginosa* EPS. Chemical analysis shows that the protein content of TB-EPS exceeds that of EPS, and more enormous amounts of carboxy groups were observed in TB-EPS (Qu *et al.*, 2012). Colony formation induced by TB-EPS is more efficient than EPS in wild *Microcystis* (Omori *et al.*, 2019; Sakurai *et al.*, 2019). The relationship between two types of bound EPS and *M. aeruginosa* colony formation has been investigated, and TB-EPS could influence the formation of *M. aeruginosa* aggregates, and LB-EPS contributed to the development from aggregates to mucilaginous colonies (Tan *et al.*, 2019). However, environmental factors such as light and temperature on the effect of EPS and TB-EPS are unknown.

In this study, from the perspective of colony size, *M. aeruginosa* NIES-843 and wild *Microcystis* were cultivated to examine the relationship between colony formation and buoyancy. Based on the experimental data, the efficiency of EPS and TB-EPS to colony formation was investigated. Furthermore, the illumination and temperature conditions on the *M. aeruginosa* NIES-843 colony formation and buoyancy were discussed.

2 Materials and methods

2.1 Collection of test algae sample and culture conditions

The wild *Microcystis* strain was collected from Lake Senba, where *Microcystis* blooms often occur in summer (36°22′N; 140°27′E), Ibaraki, Japan, on September 2019 (Fig. 1). The collection of the sample was carried out at a depth of 5 cm from the water surface. The samples were brought to the laboratory and kept at 4 °C in the dark condition until they were used for microscopic observation and EPS isolation.

M. aeruginosa NIES-843 obtained from National Institute for Environmental Studies (NIES), Japan. This strain was cultured in 500 mL of modified Wright's Cryptophytes (WC) medium in 1 L Erlenmeyer flasks at 25 °C for about 14 days under 4500 lx continuous illumination. The WC medium

consisted of a mixture of $CaCl_2$ (36.76 mg), $MgSO_4 \cdot 7H_2O$ (36.97 mg), $NaHCO_3$ (12.60 mg), K_2HPO_4 (8.71 mg), $NaNO_3$ (85.01 mg), $Na_2 \cdot EDTA$ (4.36 mg), $FeCl_3 \cdot 6H_2O$ (3.15 mg), $CuSO_4 \cdot 5H_2O$ (0.01 mg), $ZnSO_4 \cdot 7H_2O$ (0.022 mg), $CoCl_2 \cdot 6H_2O$ (0.01 mg), $MnCl_2 \cdot 4H_2O$ (0.18 mg), $Na_2MoO_4 \cdot 2H_2O$ (0.006 mg), H_3BO_3 (1.0 mg), thiamin · HCl (0.1 mg), biotin (0.005 mg), Vitamin B_{12} (0.005 mg), ferric citrate (3 mg), citric acid (3 mg), 500 mg tris-(hydroxymethyl)-aminomethane (Tris buffer) in 1 L of distilled water (Guillard and Lorenzen, 1972). The pH of the medium was adjusted to 8.0 ± 0.1 by using 0.5 M HCl. The WC media used in the experiment were sterilized by autoclaving at 115 kPa for 20 min at 121 °C. Inoculation and sampling of the culture medium were conducted in a clean bench to minimize bacterial contamination.

2.2 Isolation of EPS and TB-EPS

The EPS extraction protocol was referred to as the previous studies (Amemiya and Nakayama, 1984; Nishikawa and Kuriyama, 1974; Sato *et al.*, 2017). Under the room temperature condition, 0.25 M sodium hydroxide (NaOH) and 2% (w/v) ethylenediaminetetraacetic acid ($Na_2EDTA \cdot 2H_2O$) were added to the wild *Microcystis* sample. After stirring the solution well, the sample was allowed to be left for 1 hour to dissolve the EPS from *Microcystis* cells. The sample solution was centrifuged at 3000 rpm for 15 minutes, and the supernatant was collected. The solution was further filtered with GF/C filter (Whatman, UK) to remove impurities, and ethanol was added into the filtrate to give a final concentration of 60% (v/v), and it was allowed to precipitate the EPS in the solution. Then, the mixture was stored at -20 °C for 16 hours. After that, the solution was centrifuged at 3000 rpm for 15 min, and the precipitate was collected. The collected sample was freeze-dried at −0.1 MPa, and the dried sample was ground with a mortar and a pestle.

The TB-EPS extraction protocol was referred to as the previous studies (Sakurai *et al.*, 2019; Xu *et al.*, 2013). Sodium chloride (NaCl) was added to the wild *Microcystis* sample to concentrate of 0.05% (w/v). The supernatant of the sample was then removed by centrifugation at 3000 rpm for 15 min. After that, distilled water was added into the *Microcystis* residue and filled to the same original volume. NaCl (0.05% (w/v)) was added into the solution and heated at 60 °C for 30 min. After the sample was cooled to room temperature, it was centrifuged at 3000 rpm for 15 min, and the supernatant was collected. The sample was filtrated by GF/C filter (Whatman, UK) and mixed with 1.5 times the amount of ice-cold ethanol to precipitate EPS. The subsequent procedure was the same as mentioned above, and TB-EPS was obtained. The powdered EPS and TB-EPS sample were stored in a desiccator until use.

2.3 Colony formation and buoyancy experiment of *M. aeruginosa* NIES-843

M. aeruginosa NIES-843 was precultured for 14 days. The cations (Ca^{2+} and Mg^{2+}) concentration were analyzed by atomic absorption spectrometer (novAA 300, Analytik Jena AG, Jena, Germany). Then, 50 mL of the sample was poured into a graduated cylinder, and then calcium chloride ($CaCl_2$), magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), EPS, and TB-EPS were added. Four experimental groups were set up in the experiment, "control", "$Ca^{2+} + Mg^{2+}$ group", "EPS added group", and "TB-EPS added group". The concentration of EPS and TB-EPS was 200 mg/L in EPS and TB-EPS added group. The control medium was prepared without any addition of Ca^{2+}, Mg^{2+}, EPS, or TB-EPS. In the previous study, the influences of cationic ions and extracellular polysaccharides (EPS) on colony formation of *Microcystis* buoyancy were investigated (Sakurai *et al.*, 2019; Wei *et al.*, 2019). With different proportional addition of magnesium and calcium concentration, *M. aeruginosa* exhibited the strongest buoyancy at the mass ratio of 3 (Wei *et al.*, 2019). Therefore, the mass ratio of Ca^{2+} and Mg^{2+} was kept constant at 3 in all experiments. The Ca^{2+} concentration (w/v) used in the buoyancy experiments was controlled from 0 mg/L to 50 mg/L, while Mg^{2+} (w/v) was from 0 mg/L to 150 mg/L.

The prepared medium was cultivated at 25 °C for 24 hours at 10,000 lx and 0 lx in the light-controlled experiment. The prepared medium was cultivated at 25 °C and 30 °C for 24 hours at 10,000 lx in the temperature-controlled experiment. The experiment was conducted in triplicate ($n = 3$), and the results were expressed as the mean value ± standard deviation (SD).

2.4 Microscopic observation and measurement of colony size

The microscope (Eclipse E100, Nikon, Japan) was used to observe the colonial morphology of *M. aeruginosa*. In this experiment, more than three cells in the aggregation were regarded as a colony. Optical microscopic images were taken with a digital camera system (AM-4023X, AnMo Electronics Corp, Taiwan). Since the *M. aeruginosa* NIES-843 colony always presents irregular morphologies, the following method was used to measure and calculate the diameter. The length and width of colonies were measured directly from the longest axis (length, μm) and the shortest axis (width, μm, aligned perpendicular to the longest axis) (Li *et al.*, 2014). The following equation calculated the diameter (μm) of the colony.

$$Diameter = \sqrt{length \times width}$$

The measurement of colony size was conducted in triplicate ($n = 3$), and the results were expressed as the mean value ± SD.

2.5 Evaluation of the buoyant ability of *Microcystis*

The ability of buoyancy can be reflected by calculating the buoyancy rate (Wang *et al.*, 2011). Wild *Microcystis* was cultivated for 24 hours and exhibited strong buoyancy floating to the upper 10 mL of the water surface, as mentioned in detail below (Fig. 2). From these trends, wild *Microcystis* in the upper 10 mL layer (V_1, mL) of the graduated cylinder was considered to possess strong buoyant ability, and the cell density was recorded as C_1 (cells/mL). On the other hand, wild *Microcystis* in the lower 40 mL (V_2, mL) of the medium was supposed to present weak buoyant ability, and the density of

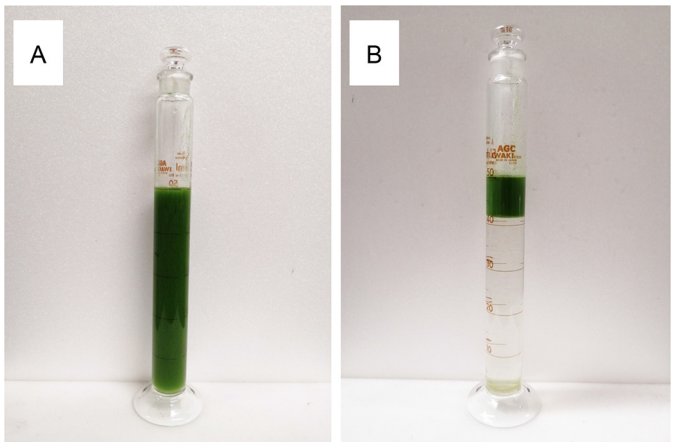

Fig. 2. Wild *Microcystis* strain cultured in a cylinder for (A) 0 hour and (B) 24 hours.

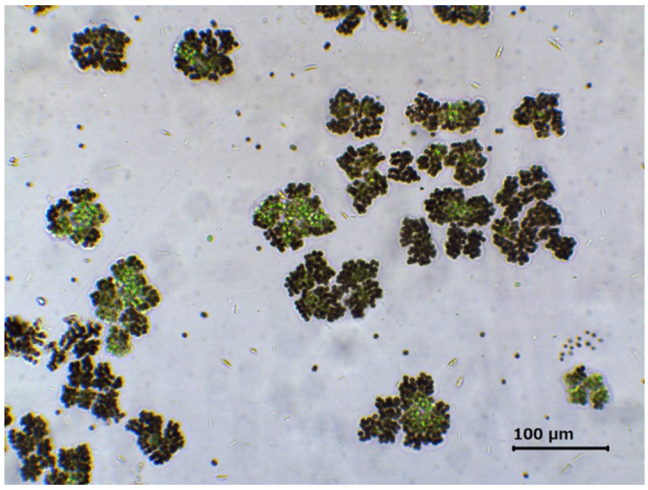

Fig. 3. Morphology of *Microcystis* wild strain.

this layer was recorded as C_2 (cells/mL). To evaluate buoyancy of *M. aeruginosa*, the relative buoyancy (RB_{20},%) was calculated from the cell density (C_1, C_2) and the solution volume (V_1, V_2) by the following equation (Wang *et al.*, 2011).

$$RB_{20}(\%) = \frac{C_1 V_1}{C_1 V_1 + C_2 V_2} \times 100$$

When *M. aeruginosa* was uniformly distributed in the graduated cylinder, the RB value was calculated to be 20%.

3 Results and discussion

3.1 Buoyancy of wild *Microcystis*

The sample of wild *Microcystis* obtained from Lake Senba was cultivated for 24 h in a cylinder (Fig. 2A). Wild *Microcystis* floated to the water surface after 24 hours of cultivation and presented strong buoyancy (Fig. 2B). The relative buoyancy value of wild *Microcystis* was calculated to be nearly 100%. Microscopic observation showed that wild *Microcystis* formed a large colony, indicating that the colony size was about 100 μm (Fig. 3).

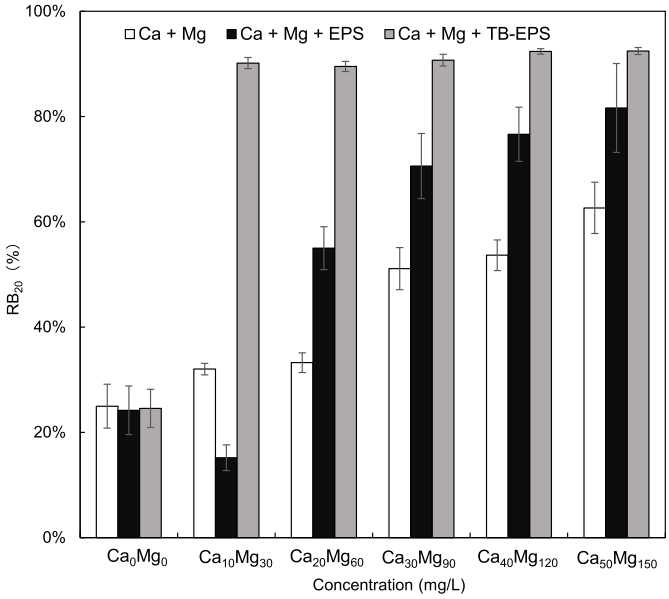

Fig. 4. Relative buoyancy of *M. aeruginosa* NIES-843 under light conditions.

3.2 Buoyancy regulation of *M. aeruginosa* NIES-843 under the light-limited condition

Under the light condition, the buoyancy of *M. aeruginosa* NIES-843 increased in all groups except control. In the TB-EPS added group, *M. aeruginosa* NIES-843 presented high buoyancy when concentrations of Ca^{2+} and Mg^{2+} were more than 10 mg/L and 30 mg/L, respectively. In the EPS added group, the RB value of *M. aeruginosa* NIES-843 was close to 80%, with the Ca^{2+} and Mg^{2+} concentrations of 50 mg/L and 150 mg/L, respectively. At the same concentration of Ca^{2+} and Mg^{2+}, the buoyancy of EPS added group buoyancy was lower than the TB-EPS added groups (Fig. 4).

Under the dark condition, the buoyancy of *M. aeruginosa* NIES-843 increased in the EPS added group. In the EPS added group, the RB value of *M. aeruginosa* NIES-843 was close to 75%, with the Ca^{2+} and Mg^{2+} concentrations of 50 mg/L and 150 mg/L, respectively (Fig. 5). In the TB-EPS added group, the buoyancy of *M. aeruginosa* NIES-843 changed slightly, and RB values were nearly 30% with the Ca^{2+} and Mg^{2+} concentrations of 50 mg/L and 150 mg/L, respectively. The buoyancy of *M. aeruginosa* NIES-843 did not change obviously with the addition of $Ca^{2+} + Mg^{2+}$ (Fig. 5). *M. aeruginosa* NIES-843 presented higher buoyancy in light conditions than dark conditions under the same concentration (Figs. 4 and 5). In the northern rivers of Lake Taihu (China), where *Microcystis* blooms often occur, the Ca^{2+} and Mg^{2+} concentrations were reported to be about 58 mg/L and 15 mg/L, respectively (Ye *et al.*, 2010). The cationic ion concentrations in our study are same level in the field study.

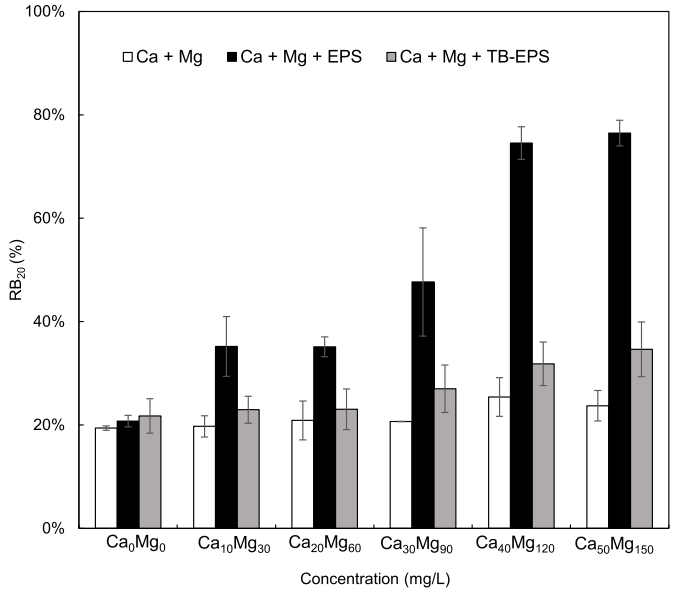

Fig. 5. Relative buoyancy of *M. aeruginosa* NIES-843 under dark conditions.

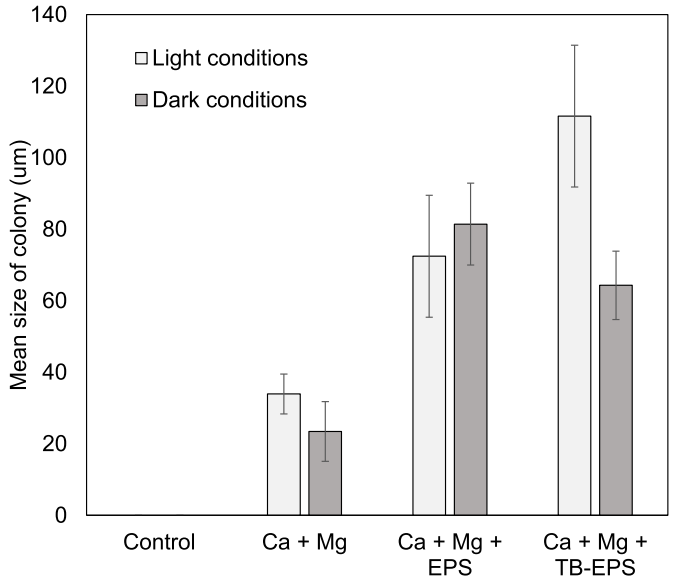

Fig. 6. Colony size of *M. aeruginosa* NIES-843 under light conditions and dark conditions.

The TB-EPS addition can change the buoyancy more efficiently than EPS addition in the case of lighting conditions. The colony size analysis showed that the colony size in Ca^{2+}+ Mg^{2+} added group was about 35 μm, while that of the EPS added group was about 70 μm. The largest colony size was about 110 μm observed in the TB-EPS added group, which was similar to the wild *Microcystis* strain is about 100 μm (Fig. 6). With the same concentration of addition, TB-EPS can induce *M. aeruginosa* NIES-843 to form a more massive colony than EPS. TB-EPS contains more carboxyl groups than EPS (Omori *et al.*, 2019). In conclusion, *M. aeruginosa* can combine with more cationic ions such as

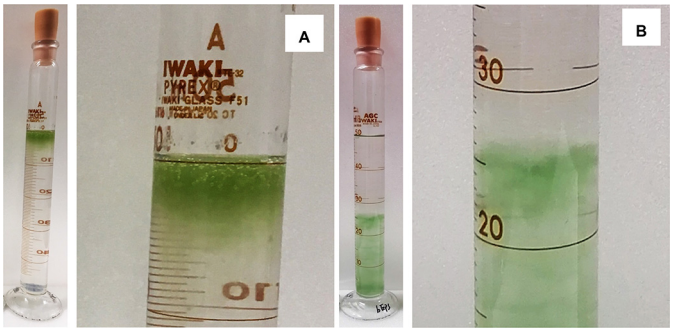

Fig. 7. *M. aeruginosa* NIES-843 in the surface layers of a cylinder and bubbles can be confirmed: (A) light condition, Mg^{2+} 90 mg/L, Ca^{2+} 30 mg/L, and TB-EPS 200 mg/L (B) dark condition, Mg^{2+} 90 mg/L, Ca^{2+} 30 mg/L, and TB-EPS 200 mg/L.

Ca^{2} and Mg^{2+} and obtain higher buoyancy with TB-EPS addition.

Under dark conditions, the colony induced by the Ca^{2+} + Mg^{2+} added group and TB-EPS added group was smaller than under light conditions. The largest colony size was observed about 80 μm in the EPS added group (Fig. 6). *M. aeruginosa* cannot obtain energy without photosynthesis (Takamura *et al.*, 1985). EPS is mainly composed of polysaccharides, and the carboxyl groups combined with Ca^{2} and Mg^{2+} do not require energy, while TB-EPS is mainly composed of protein (Basuvaraj *et al.*, 2015; Liu *et al.*, 2010). Protein hydrolysis requires energy, and some of the carboxyl groups exist as peptide bonds in protein molecules. These carboxyl groups need hydrolysis to combinate with cationic ions (Liu *et al.*, 2008; Mimmack *et al.*, 1989). Therefore, the addition of TB-EPS cannot change the buoyancy of *M. aeruginosa* NIES-843 in the absence of light. In the previous study, the addition of powdered TB-EPS sample and the adjustment of cationic ion concentrations were promoted effective the colony formation and enlarged the colony size of wild *Microcystis* (Sakurai *et al.*, 2019). Consequently, *Microcystis* buoyancy was enhanced by enlarging the colony size, indicating that the control of EPS and cationic ion concentrations would be one of the options for the removal of *Microcystis* blooms from the viewpoint of cost-effective, low-energy, environmentally-friendly.

M. aeruginosa NIES-843 induces higher buoyancy than dark conditions even at the same concentration of addition, indicating that light affects the buoyancy of *M. aeruginosa* NIES-843 (Fig. 7). In the present study, *M. aeruginosa* NIES-843 generated many bubbles in the graduated cylinder with TB-EPS under the light condition (Fig. 7A). Under the dark condition, no bubbles were observed in the graduated cylinder, and the buoyancy of *M. aeruginosa* NIES-843 was lower than the light condition (Fig. 7B). EPS enhanced the photosynthesis of *M. aeruginosa*, *M. aeruginosa* generated many bubbles via photosynthesis after the addition of EPS (Dervaux *et al.*, 2015; Wei *et al.*, 2019). These bubbles have a particular effect on buoyancy reconfirmed in this experiment. In this study, the concentration of calcium and magnesium greatly enhanced the colony formation of *M. aeruginosa* NIES-843. Compared with the chemical composition in WC (Guillard and Lorenzen, 1972) and BG-11 (Waterbury and Stanier, 1981) medium,

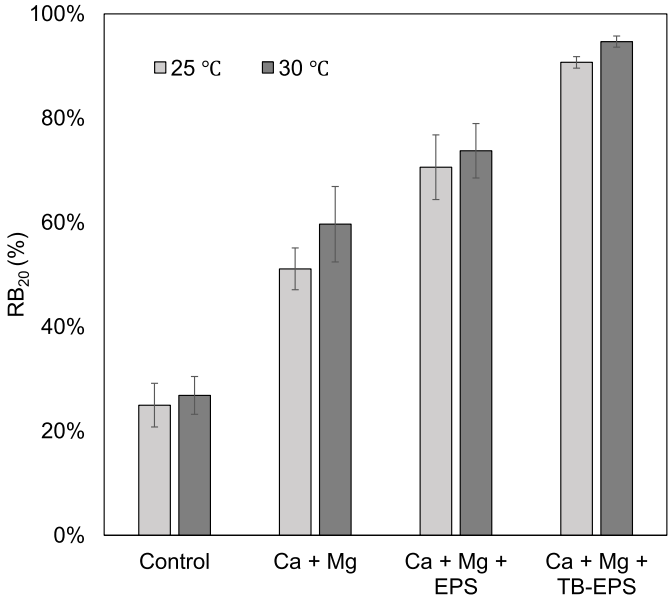

Fig. 8. Relative buoyancy of *M. aeruginosa* NIES-843 under the condition of 25 °C and 30 °C.

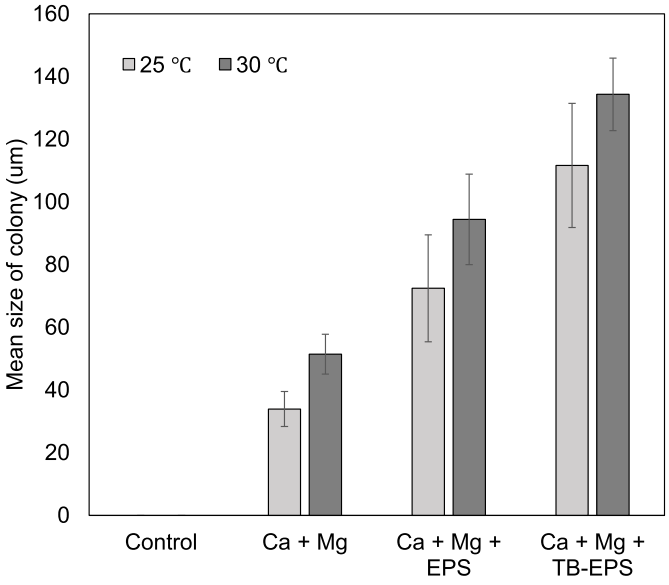

Fig. 9. Colony size of *M. aeruginosa* NIES-843 under the condition of 25 °C and 30 °C.

their concentrations are not significantly different. Therefore, the same experiment could be carried out in BG-11 medium or other media.

3.3 Buoyancy regulation of *M. aeruginosa* NIES-843 under the temperature controlled condition

The specified concentration conditions (Ca^{2+} and Mg^{2+} of 30 mg/L and 90 mg/L, EPS and TB-EPS of 200 mg/L, respectively) were set in the experiment to confirm temperature effect on buoyancy and colony formation. In the control group, the buoyancy of *M. aeruginosa* NIES-843 did not change significantly under different temperature conditions. With the temperature increased from 20 °C to 30 °C, the buoyancy of the $Ca^{2+} + Mg^{2+}$ added group increased from 50% to 53%, the EPS added group increases from 70% to 73%, and the TB-EPS added group increased from 90% to 94% (Fig. 8). The colony size of $Ca^{2+} + Mg^{2+}$ added group increased to 51 μm, the colony size of the EPS addition group increased to 94 μm, and the colony size of the TP-EPS added group increased to 134 μm (Fig. 9). Under the same concentration conditions, the increase in temperature is conducive to colony formation and presents higher buoyancy of *M. aeruginosa* NIES-843.

Microcystis wesenbergii and *Microcystis ichthyoblabe* cultivated under strong light intensity and warmer temperatures might enhance the growth of surface *Microcystis* directly through increasing the colony size (Duan *et al.*, 2018). Under high-temperature conditions, *M. aeruginosa* NIES-843 failed to form a colony in the control group, indicating the temperature has different effects on different types of *Microcystis* strains (Fig. 8). The buoyancy of *M. aeruginosa* AK1 increased with high temperature, but the gas vesicle volume showed no significant change during the transient state. The high temperature helps *M. aeruginosa* consume carbohydrates in cells and lead to the changes in polysaccharide ballast (Kromkamp *et al.*, 1988). *M. aeruginosa* NIES-843 can obtain buoyancy by synthesizing gas vesicles. The buoyancy of *M. aeruginosa* NIES-843 did not change obviously when the temperature rises without addition, suggesting that the changes in the number of gas vesicles do not affect the buoyancy of *M. aeruginosa* NIES-843 (Fig. 8). The number of *Microcystis* colonies decrease as a decrease in water temperature under the natural environment. Wild *Microcystis* strain isolated from Lake Nieuwe Meer in Holland, the percentage of total colonies that were sinking increased a few days to 100% after the temperature shifted from 20 °C to 10.5 °C (Visser *et al.*, 1995). Lake Taihu is the third-largest freshwater lake in China, and seasonal *Microcystis* blooms have regularly formed in the lake for the past three decades. With global warming, *Microcystis* growing season has advanced by approximately 20 days over the last two decades (Deng *et al.*, 2014; Otten and Paerl, 2011). Therefore, monitoring the lakes' temperature where *Microcystis* bloom often occurs is incredibly essential to protect the water environment.

4 Conclusions

In this study, we induced *M. aeruginosa* NIES-843 to form colony by using EPS and TB-EPS extracted from *Microcystis* blooms. The change of the *M. aeruginosa* buoyancy was examined, and principal conclusions were summarized as follows:

- The light influenced the colony formation and buoyancy of *M. aeruginosa* NIES-843. Under light conditions, the addition of TB-EPS colud induce *M. aeruginosa* to obtain high buoyancy at low Ca^{2+} and Mg^{2+} concentration.
- The colony size analysis showed that at the same cationic concentration, the addition of TB-EPS could induce *M. aeruginosa* to form the largest colony and present strong buoyancy.

– The increase in temperature is conducive to the colony formation of *M. aeruginosa* and presents higher buoyancy.

Funding

The present work was supported in part by JFE 21st Century Foundation and JSPS KAKENHI Grant Number JP18K04404.

Acknowledgments. The authors would like to extend deep gratitude to Prof. Dr. Fumio Imazeki, Safety and Health Organization of Chiba University, for his fruitful and helpful discussion.

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Cite this article as: Wei K, Amano Y, Machida M. 2020. Impacts of different extracellular polysaccharides on colony formation and buoyancy of *Microcystis aeruginosa*. *Ann. Limnol. - Int. J. Lim.* 56: 28