Note

Effect of sonication on live and fixed cells of *Microcystis aeruginosa*

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Abstract: Sonication has been used in many studies that require observation of the cells of *Microcystis aeruginosa*. This study investigates the extent to which sonication (42 kHz, 150 W) destroys live and Lugol-fixed cells of *M. aeruginosa*, using two strains (NIES-1090 and NIES-1157). Live cells were easily destroyed by sonication regardless of growth stage. Sonication for 50 s caused up to 32.8% (NIES-1090) and 40.8% (NIES-1157) cell losses, and extended sonication (250 s) destroyed more cells. In contrast, sonication did not cause loss of cells that had been fixed with Lugol's iodine solution. Interestingly, sonication often resulted in slightly higher counts of Lugol-fixed cells under a microscope. Sedimentation of Lugol-fixed cells is enhanced after sonication owing to the collapse of gas vacuoles and this appeared to facilitate reliable microscopic observations.

Key words: cell destruction, fixation, Lugol's iodine solution, *Microcystis aeruginosa*, sonication

Microcystis aeruginosa Kützinger (Kützinger), a freshwater cyanobacterium with diverse morphological variation, forms blooms in a wide range of latitudes (Reynolds et al. 1981, Zohary & Roberts 1990, Yunes et al. 1996, Jacoby et al. 2000). Owing to its ability to produce toxins that are potentially harmful to higher organisms, including humans (Carmichael 2001), its bloom formation has attracted a great deal of attention. *M. aeruginosa* normally exists in nature in the form of colonies of several tens to hundreds of cells (Reynolds et al. 1981, Yamamoto & Nakahara 2009), making the process of disaggregating colonies essential in ecophysiological studies of this cyanobacterium.

Several methods for disaggregating *Microcystis* colonies have been proposed. Reynolds & Jaworskii (1978) compared alkaline hydrolysis and sonication and showed that the latter was more effective. Humphries & Widjaja (1979) demonstrated that heating at 80°C for 5–10 min, followed by agitation, more completely and safely dispersed *Microcystis* colonies than did sonication. Box (1981) re-examined the heating method and found that 10 min of heating was occasionally insufficient but 15 min of heating worked consistently. Similarly, Joung et al. (2006) compared four methods (vortexing, sonication, TiO₂ treatment and boiling) and concluded that boiling was most effective. The effectiveness of high-speed blending (Zohary & Pais-Madeira 1987) and grinding (Bernard et al. 2004) in disaggregating *Microcystis* colonies

has also been reported. Nevertheless, owing to the simplicity of the procedure and the immediacy of its results, sonication has become a commonly used method in studies that require observation of the cells of *M. aeruginosa* (e.g. Kurmayer et al. 2003, Tsujimura 2003, Ihle et al. 2005, Imai et al. 2009, Yamamoto & Nakahara 2009).

It should be noted that sonication is only effective when its duration is appropriate: a shorter period of sonication does not sufficiently disaggregate colonies while prolonged sonication can destroy the cells (Reynolds & Jaworski 1978, Humphries & Widjaja 1979, Rajasekhar et al. 2012). However, older cells tend to be more easily damaged by sonication (Reynolds & Jaworski 1978), implying that the influence of sonication on *M. aeruginosa* varies with its growth stage. Furthermore, although a fixative such as Lugol's iodine solution is often added to water samples, less attention has been paid to the impact of sonication on fixed cyanobacterial cells. The aim of this study was to examine the extent to which sonication destroys live and fixed cells of *M. aeruginosa* at different growth stages.

Non-axenic *M. aeruginosa* strains NIES-1090 and NIES-1157 were supplied by the Microbial Culture Collection, the National Institute for Environmental Studies (Ibaraki, Japan). These strains had completely lost the ability to form colonies and existed in unicellular form. Their stock cultures were maintained in modified C medium (Yamamoto & Shiah 2010) under the standard conditions of 24°C, a 12 : 12 h light-dark cycle and a light intensity of 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

The *M. aeruginosa* strains were precultured for seven days

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in glass test tubes (16×150 mm) that contained 8 mL of modified C medium. Then, 0.2 mL aliquots were added to 2 L culture bottles that contained 1.4 L of modified C medium, and incubated for 42 days under the standard conditions with continuous stirring (150 rpm) in a stream of filtered air (0.22 μm pore filter, 720 mL min⁻¹). Fifteen milliliter samples were taken from each bottle every seven days. The sample water was distributed in volumes of 1 mL to 15 glass test tubes (13×100 mm) and six of them were immediately fixed with Lugol's iodine solution (1% final concentration) to investigate whether the impact of sonication differed between fixed and non-fixed cells, as described below.

Sonication was conducted by using an ultrasonic cleaner Bransonic 5510R-DTH (Branson Ultrasonic Corporation, Danbury, CT, USA) with a frequency of 42 kHz and an output power of 135 W. Depth of water in the ultrasonic cleaner was approximately 6 cm; this condition effectively dispersed *Microcystis* colonies at the center of the machine. A preliminary test on two colony-forming strains of *M. aeruginosa* that were sourced from different water bodies in northern Taiwan revealed that relatively large Lugol-fixed colonies were occasionally present following exposure to ultrasound for 40 s, whereas no colonies existed after sonication for 50 s or longer (data not shown). Therefore, 50 s was regarded as the minimum duration of sonication required to disperse completely

the cyanobacterial colonies into their constituent single cells, constricted cells and paired cells (Yamamoto & Shiah 2010).

The aforementioned non-fixed cells in three groups of triplicate test tubes were exposed to ultrasound for different times (0, 50 and 250 s) before being fixed with Lugol's iodine solution at a final concentration of 1%. The non-sonicated group was used as controls. The six Lugol-fixed samples in two groups of triplicate test tubes were exposed to ultrasound for either 50 or 250 s, 72 hours after fixation.

An aliquot of 30 μL of a sample was put on a Lovins microslide field finder (Gurley Precision Instruments, Troy, NY), and the number of *M. aeruginosa* cells that settled on the slide was counted under an Axio Observer A1 inverted microscope (Carl Zeiss, Göttingen, Germany) at a magnification of 200×. When necessary, water samples were diluted with 1% Lugol's iodine solution prior to counting. Duplicate measurements were averaged to determine the cell density. The growth was evaluated using the formula $(\ln N_t - \ln N_{t-7})/7$, where t is the day of sampling ($t=7, 14, 21, 28, 35$ and 42) and N_t is the cell density on day t . The highest calculated value was adopted as the specific growth rate (d⁻¹). The difference between the cell densities in the treated sample and the control was evaluated by performing a two-tailed t -test. Values of $p < 0.05$ were considered significant.

Both strains grew steadily until day 21 and then reached

Fig. 1. Measurements of cell density of *Microcystis aeruginosa* (a, c) and ratio of cell density of sonicated samples to that of the controls (b, d). Closed circles, control; open circles, live cells sonicated for 50 s; closed triangles, live cells sonicated for 250 s; open triangles, fixed cells sonicated for 50 s; closed squares, fixed cells sonicated for 250 s. Error bars show SD ($n=3$). Asterisks indicate values significantly different from the control (two-tailed t -test, $p < 0.05$).

Table 1. Specific growth rates (d^{-1}) of *Microcystis aeruginosa*. 50s-L, live cells sonicated for 50 s; 250s-L, live cells sonicated for 250 s; 50s-F, fixed cells sonicated for 50 s; 250s-F, fixed cells sonicated for 250 s.

Treatment	Strains	
	NIES-1090	NIES-1157
Control	0.778	0.573
50s-L	0.721	0.549
250s-L	0.796	0.501
50s-F	0.773	0.580
250s-F	0.781	0.575

the stationary phase (Fig. 1). The cell densities of the strains NIES-1090 and NIES-1157 reached 4.72×10^7 cells mL^{-1} and 1.46×10^7 cells mL^{-1} , respectively, at the end of the experiment. Sonication caused a slight difference (either an increase or a decrease) in the specific growth rate from that of the control, whereas fixation with Lugol's iodine solution prior to sonication did not influence the specific growth rate (Table 1).

Sonication reduced the cell densities of the non-fixed samples of both strains, except when performed for 50 s on day 7 on the strain NIES-1090. Sonication for 50 s on day 14 destroyed 32.8% of the NIES-1090 cells, whereas the same treatment did not cause such a severe cell loss from day 21 to day 42. The loss of cells of the strain NIES-1157 upon 50 s sonication was less than 18.5% until day 21, whereas it increased to 40.8% on day 28. Extended sonication destroyed more cells. From 51.6% to 63.9% of cells of the strain NIES-1090 were destroyed by extended sonication. The loss of cells of the strain NIES-1157 fluctuated between 29.1% and 60.0% with no clear pattern. No significant cell loss was observed in Lugol-fixed samples; rather, sonication often marginally increased the cell density of Lugol-fixed samples.

When sonication is performed for the purpose of microscopic observation of *M. aeruginosa* cells, the duration of sonication should be minimized, for a maximum of 1 min (Reynolds & Jaworski 1978). This study showed that even 50 s of sonication destroyed a considerable number of non-fixed cells, suggesting that sonication inevitably destroys live *M. aeruginosa* cells. Reynolds & Jaworski (1978) reported that sonication more easily destroys cells in later growth stages. However, in the present study, sonication-caused cell loss depended less on the growth stage.

We showed that sonication had no significant negative impact on Lugol-fixed cells. Furthermore, it is noteworthy that fixation with Lugol's iodine solution prior to sonication tended to yield higher cell densities than the control. In general, the addition of Lugol's iodine solution effectively precipitates planktonic organisms, whereas a small portion of *M. aeruginosa* cells may retain buoyancy after fixation owing to the presence of gas vacuoles. Such cells appear to be easily overlooked during microscopic observation. However, sonication collapses gas vacuoles (Lee et al. 2000, Tsujimura 2003) and thereby facilitates the sedimentation of cells, explaining

why the cell densities of Lugol-fixed samples were higher after sonication than before. How the cells that were fixed with Lugol's iodine solution acquired tolerance to ultrasonic waves remains unexplained.

The susceptibility of *M. aeruginosa* to alkaline hydrolysis, sonication and heating varies among strains, so selecting the optimal method for disaggregating colonies of the target strain is essential (Box 1981). The heating technique is reportedly better than alkaline hydrolysis and sonication, but it does not always work well, especially on Lugol-fixed compact colonies (Box 1981, Bernard et al. 2004). This study showed that Lugol-fixed cells were not destroyed by sonication regardless of their growth stage and/or strain. Given that compact *Microcystis* colonies can also be eventually dispersed into their constituent cells by extended sonication, fixation with Lugol's iodine solution followed by sonication appears to be applicable to all strains to disaggregate colonies without causing cell loss.

In summary, exposure of live *M. aeruginosa* cells to ultrasonic waves involves cell destruction, and the cell loss increases with the duration of sonication. The addition of Lugol's iodine solution per se does not settle all of the cells and so the counting of cells by microscopy tends to lead to underestimation due to oversight of floating cells. However, *M. aeruginosa* cells acquire tolerance to ultrasonic waves upon fixation with Lugol's iodine solution and all of the Lugol-fixed cells settle effectively after sonication, enabling their reliable microscopic observation.

References

- Bernard C, Monis P, Baker P (2004) Disaggregation of colonies of *Microcystis* (cyanobacteria): efficiency of two techniques assessed using an image analysis system. *J Appl Phycol* 16: 117–125.
- Box JD (1981) Enumeration of cell concentrations in suspensions of colonial freshwater microalgae, with particular reference to *Microcystis aeruginosa*. *Br Phycol J* 16: 153–164.
- Carmichael WW, Azevedo SMFO, An JS, Molica RJR, Jochimsen EM, Lau S, Rinehart KL, Shaw GR, Eaglesham GK (2001) Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins. *Environ Health Perspect* 109: 663–668.
- Humphries SE, Widjaja F (1979) A simple method for separating cells of *Microcystis aeruginosa* for counting. *Br Phycol J* 14: 313–316.
- Ihle T, Jähnichen S, Benndorf J (2005) Wax and wane of *Microcystis* (Cyanophyceae) and microcystins in lake sediments: a case study in Quitzdorf Reservoir. *J Phycol* 41: 479–488.
- Imai H, Chang K, Kusaba M, Nakano S (2009) Temperature-dependent dominance of *Microcystis* (Cyanophyceae) species: *M. aeruginosa* and *M. wesenbergii*. *J Plankton Res* 31: 171–178.
- Jacoby JM, Collier DC, Welch EB, Hardy FJ, Crayton M (2000) Environmental factors associated with a toxic bloom of *Microcystis aeruginosa*. *Can J Fish Aquat Sci* 57: 231–240.
- Joung SH, Kim CJ, Ahn CY, Jang KY, Boo SM, Oh HM (2006) Simple method for a cell count of the colonial cyanobacterium, *Microcystis* sp. *J Microbiol* 44: 562–565.
- Kurmayer R, Christiansen G, Chorus I (2003) The abundance of microcystin-producing genotypes correlates positively with colony size in *Microcystis* sp. and determines its microcystin net production in Lake Wannsee. *Appl Environ Microbiol* 69: 787–795.
- Lee TJ, Nakano K, Matsumura M (2000) A new method for the rapid evaluation of gas vacuoles regeneration and viability of cyanobacteria

- by flow cytometry. *Biotechnol Lett* 22: 1833–1838.
- Rajasekhar P, Fan L, Nguyen T, Roddick FA (2012) Impact of sonication at 20 kHz on *Microcystis aeruginosa*, *Anabaena circinalis* and *Chlorella* sp. *Water Res* 46: 1473–1481.
- Reynolds CS, Jaworski GHM (1978) Enumeration of natural *Microcystis* populations. *Br Phycol J* 13: 269–277.
- Reynolds CS, Jaworski GHM, Cmiech HA, Leedale GF (1981) On the annual cycle of the blue-green alga *Microcystis aeruginosa* Kütz. Emend. Elenkin. *Philos Trans R Soc Lond B* 293: 419–477.
- Tsujimura S (2003) Application of the frequency of dividing cells technique to estimate the *in situ* growth rate of *Microcystis* (Cyanobacteria). *Freshw Biol* 48: 2009–2024.
- Yamamoto Y, Nakahara H (2009) Seasonal variations in the morphology of bloom-forming cyanobacteria in a eutrophic pond. *Limnology* 10: 185–193.
- Yamamoto Y, Shiah FK (2010) Relationship between cell growth and frequency of dividing cells of *Microcystis aeruginosa*. *Plankton Benthos Res.* 5: 131–135.
- Yunes JS, Salomon PS, Matthiensen A, Beattie KA, Raggett SL, Codd GA (1996) Toxic blooms of cyanobacteria in the Patos Lagoon Estuary, southern Brazil. *J Aquat Ecosyst Health* 5: 223–229.
- Zohary T, Pais-Madeira AM (1987) Counting natural populations of *Microcystis aeruginosa*: a simple method for colony disruption into single cells and its effect on cell counts of other species. *J Limnol Soc Sth Afr* 13: 75–77.
- Zohary T, Robarts RD (1990) Hyperscums and the population dynamics of *Microcystis aeruginosa*. *J Plankton Res* 12: 423–432.