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An alternative method to improve the settleability of gas-vacuolated cyanobacteria by collapsing gas vesicles

Helayaye Damitha Lakmali Abeynayaka, Takashi Asaeda, Kyoko Tanaka, Kimie Atsuzawa, Yasuko Kaneko, Hidenori Nishda and Seiichi Inada

ABSTRACT

This study estimated the ability of pressurization to collapse gas vesicles and thereby enhance the settleability of fresh water cyanobacteria. Settling velocities of *Pseudanabaena galeata* and *Microcystis aeruginosa* were measured at 11 different pressure values from 0 to 0.5 MPa. The morphological variations that occurred in the gas vacuoles according to the applied pressure were investigated using transmission electron microscopy images. The settleability of both cyanobacteria species increased statistically significantly ($P = 0.000$) with increasing pressure, whereas the gas-vacuolated area of both species decreased significantly ($P = 0.000$) with the magnitude of the applied pressure. The removal ability of cyanobacterial cells from the water layer increased with high pressure treatment. The maximum removal efficiency observed of *P. galeata* and *M. aeruginosa* cells relative to the control culture were 82% and 95%, respectively, at the maximum tested pressure value of 0.5 MPa.

Key words | cyanobacteria, gas vacuoles, high pressure, *Microcystis aeruginosa*, *Pseudanabaena galeata*, settling velocity

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INTRODUCTION

Cyanobacterial blooms in surface water resources such as lakes, reservoirs and water treatment plants can be problematic for conventional water purification processes. Such blooms cause poor taste and odor in treated water (Kakimoto *et al.* 2014) and are a threat to human health (Otten & Paerl 2015). Several on-site treatment methods, such as ultrasonication (Rajasekhar *et al.* 2012; Rodriguez-Molares *et al.* 2014), de-stratification by artificial mixing (Asaeda & Imberger 1993; Visser *et al.* 1996), pressurization (Sutcliffe & Jones 1992; Clarke & Walsby 1996; Porat *et al.* 1999) and nutrient limitation (Paerl *et al.* 2011) were tested in natural water resources to control cyanobacteria growth. In water treatment plants, ozonation (Coral *et al.* 2013), filtration (Westrick *et al.* 2010), chlorination (Zamyadi *et al.* 2012), conventional sedimentation followed by coagulation and flocculation (Ghernaout *et al.* 2010) and dissolved air floatation (Teixeira *et al.* 2010) were studied to remove cyanobacteria for drinking water purification purposes, yet practical applications of most of these methods are difficult in natural water resources due to their associated adverse environmental and economic factors. In particular, the sedimentation process is impractical due to the large amounts of coagulant required to settle the buoyant cyanobacterial cells. Moreover, this buoyancy of cells causes more complications in subsequent treatment processes.

Buoyancy regulation of cyanobacteria depends on three mechanisms. These are: (a) change of cell ballast such as carbohydrate (density $\sim$1,550 kgm^{-3}) and protein (density $\sim$1,300 kgm^{-3}) in cells (Oliver & Walsby 1984); (b) lift provided by the production of gas vesicles; and (c) change in cell density by dilution and irreversible collapse of gas vesicles (Walsby 1994). A gas vacuole, which is the combination of one or several gas vesicles in cytoplasm, plays an important role in the buoyancy regulation of cyanobacterial cells (Kalff 2002). However,

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in the context of settling, destruction of gas vesicles by external force could cause a loss of buoyancy of cyanobacterial cells due to the increment of the density compared to water (Walsby 1972). This acute variation of buoyancy depends only on gas vacuole reduction during the acute pressure increment because the variation of cell ballast within such a short time period can be ignored. Basic experiments, such as the classic 'Hammer, Cork and Bottle', demonstrated the effect of pressure on gas vesicle destruction of cyanobacteria (Klebahn 1895). When gas vesicles are exposed to moderate pressures, they immediately collapse irreversibly. Destruction of the gas vesicles of *Microcystis aeruginosa* by external physical forces, such as ultra-sonic forces (Hao *et al.* 2004; Tang *et al.* 2004; Zhang *et al.* 2006; Rajasekhar *et al.* 2012; Jachlewski *et al.* 2013; Rodriguez-Molares *et al.* 2014), were studied widely and were found to lead to destructive effects, such as cell lysis (Wu *et al.* 2011). Other than ultra-sonication, the generation of momentary high pressure by explosion was suggested as an energy source to collapse gas vesicles of cyanobacteria in lakes and reservoirs (Walsby 1970, 1972; Menday & Buck 1972). As a pre-treatment to prevent cyanobacteria entering water treatment plants, a process of sedimentation followed by hydrostatic pressure exposure has been evaluated (Clarke & Walsby 1996). In this process cyanobacteria were subjected to a pressure head by circulating lake water through a deep concentric pipe. Hydrostatic pressure generated by this way effectively removed gas vesicles and increased the sedimentation of cyanobacteria (Clarke & Walsby 1996). Although the efficiency and environmental feasibility of external pressure application to destroy the gas vesicles for the settling of cyanobacterial cells is higher (Clarke & Walsby 1996; Porat *et al.* 1999), very few researchers reported the findings.

When they are exposed to natural disturbances in artificially de-stratified lakes, cyanobacterial cells migrate from the surface water to the bottom relatively quickly. It is widely known that the deterioration of a light climate decreases the amount of cyanobacteria (Asaeda & Imberger 1993; Imteaz & Asaeda 2000). Another possibility is that the increasing hydrostatic pressure, which has an impact on the gas vesicles of the cyanobacterial cells, causes cells to settle. Applying a pressure to destroy gas vesicles and allowing them to settle could be a feasible mechanism for controlling excessive amounts of cyanobacterial cells in water. In addition, there is a possible mechanism of settling cyanobacterial cells in the de-stratification treatment of lakes. However, the increment in hydrostatic pressure via circulation in natural lakes is on the order of 0.5 MPa; such a high pressure is rather difficult to achieve in laboratory experimental conditions. The prime objective of this study was to analyze and compare the effect of a large pressure increment on buoyancy loss related to the gas vacuole morphological changes of the filamentous cyanobacterium *Pseudanabaena galeata*, an odorous compound releaser and the bloom-forming species *Microcystis aeruginosa*, a potential intoxicator. Furthermore, the pressure application method was evaluated as a cell removal process by examining the cell removal efficiency.

MATERIALS AND METHODS

Cultures and incubation

P. galeata and *M. aeruginosa* (National Institute for Environmental Studies (NIES), Japan) were grown in an autoclaved BG11 medium (Rippka 1979) at 20 °C under 12L:12D (12 hours of light and 12 hours of darkness per day; the white light intensity was 20 μmol m^{-2} s^{-1}). The cultures were maintained at these incubation conditions for more than 14 days.

Settling velocity measurement under pressurization

To obtain the settling velocity of pressurized cyanobacteria cells, incubated *P. galeata* and *M. aeruginosa* cells were separately subjected to high pressure using compressed helium gas. A thin transparent polyurethane tube with an inner diameter of 0.2 mm (MISUMI, Japan) was used as a pressure vessel, due to the advantage of transparency and tolerance to high pressure values (up to 0.8 MPa). Figure 1 displays the experimental set-up for recording the settling velocity of pressurized samples. Immediately after the application of pressure, vertical movements of cyanobacteria inside the polyurethane tube were observed under a light microscope using an oil immersion objective lens (100 × N.A. 1.25). The light microscope was supported with a Nikon digital camera DXM 120 °C at the highest resolution (4,116 × 3,072 pixels). The settling velocities of cells subjected to high pressure values of 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4,

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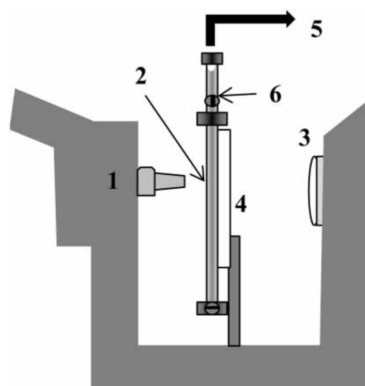

1. **Light microscope**
2. **Culture filled transparent tube**
3. **Light source**
4. **Glass slide**
5. **To compressed He gas cylinder**
6. **Control valve**

Figure 1 | Schematic drawing of the settling velocity meter used in this study.

0.45 and 0.5 MPa were measured. The applied pressures were controlled at constant rates by a pressure regulator (MISUMI, Japan) before and during the settling velocity experiment. The time taken by the cyanobacterial cells to settle 0.1 mm was measured using a stopwatch with a minimum value of 0.1 s. The light microscope eyepiece scale was calibrated with a stage micrometer (SPI Supplies, Japan) to determine the settling depth and settling velocity, where

$$\text{Settling velocity } (\mu\text{m s}^{-1}) = \frac{\text{Settling depth } (100\ \mu\text{m})}{\text{Time taken (s)}} \quad (1)$$

TEM analysis

Conventional electron microscopy images of the pressure-treated cyanobacterial cells were obtained using the same methodology as Seki *et al.* (2014). The collected cells were fixed in 2% glutaraldehyde in 0.05 M potassium phosphate buffer (pH 7.0) for 2 h at room temperature and at 4 °C (inside refrigerator) overnight. After rinsing in the buffer, the cells were post-fixed in 2% OsO_4 in 0.05 M potassium phosphate buffer (pH 7.0) for 2 h at room temperature. Then, the cells were dehydrated in an acetone series and then embedded in Spurr's resin. Ultra-thin sections (ca. 90 nm thick, as estimated from the silver–gold interference color) were cut with a diamond knife on a Sorvall MT -2B microtome (Sorvall, Norwalk, CT, USA). After staining with uranyl acetate for 5 min and lead citrate for 1 min, sections were observed using a Hitachi H 7500 transmission electron microscope (TEM) (Hitachi, Tokyo, Japan) at an accelerating voltage of 100 kV (Seki *et al.* 2014).

The TEM images were analyzed using the ImageJ software to obtain the cell area and the remaining gas vacuole area in the cells. Subsequently, the gas vacuole area per cell area ratio was calculated.

Cell removal efficiency

After different pressure applications, pressure-treated cell suspensions were allowed to settle in settling chambers. After 24 hours, 3 mL of water sample was removed from the top water layer of each settling chamber. Cell suspensions were fixed by adding formaldehyde solution to reach a final formaldehyde concentration of 12% (v/v) in a clean tube and kept at room temperature for 2 hours to complete the fixation. Then the samples were stored at 4 °C until cell counting. Numbers of *Microcystis sp.* and *P. galeata* cells in 1 mL were determined by using a haemocytometer with chamber depth 0.02 mm (SLGC, Japan). The fixed cell suspension was loaded into the counting chambers in the haemocytometer carefully. After 2 min, cells in side chambers were counted by observation through a light microscope supported with a Nikon digital camera DXM 1200C and set at the highest resolution (4,116 × 3,072 pixels). Samples for cell counting were triplicated and one sample was counted six times. The number of cells per mL was calculated from the known volume of the counting chamber and the dilution factor. The cell removal efficiency was calculated by using the following equation:

$$\text{Cell removal efficiency (\%)} = \left\{ \frac{\left[\left(\begin{array}{c} \text{cell concentration} \\ \text{of} \\ \text{treated sample (cells/mL)} \end{array} \right) \left(\begin{array}{c} \text{cell concentration} \\ \text{of} \\ \text{control (cells/mL)} \end{array} \right) \right]}{\begin{array}{c} \text{cell concentration} \\ \text{of} \\ \text{control (cells/mL)} \end{array}} \right\} \times 100 \quad (2)$$

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Statistical analysis

Statistical analysis was performed using SPSS 16.0 software for all obtained data.

RESULTS AND DISCUSSION

Effects of pressure on settling velocity and gas vacuole morphology

Figure 2 shows the settling velocities of cells that were exposed to external pressures. The settling velocities of *P. galeata* and *M. aeruginosa* cells were found to gradually increase with the applied external pressure increment. At atmospheric pressure (0 MPa), the average settling velocity of the cells was −0.07 (±0.57) μms^{-1} and −56.8 (±5.01) μms^{-1} for *P. galeata* and *M. aeruginosa*, respectively, indicating that the majority of the cells had positive buoyancy. The sudden imposition of 0.05 MPa pressure altered the floatation of the cells to sinking status. The gradual increment of pressure from 0.0 to 0.1, 0.2, 0.3, 0.4, and 0.5 MPa increased the settling velocity of *P. galeata* cells to 5.6 ± 2.09, 13.6 ± 3.29, 22.5 ± 6.09, 27.7 ± 3.54, and 42.9 ± 3.52 μms^{-1}, respectively, whereas the same pressure increment increased the settling velocity of *M. aeruginosa* cells to 8.4 ± 0.58, 16.4 ± 0.76, 26.7 ± 5.44, 57.7 ± 1.66 and 91.3 ± 3.83 μms^{-1}, respectively. The maximum settling velocity was achieved at the pressure of 0.5 MPa for both species. The variations in the settling velocity of both species' cells with applied pressure were found to be significant (analysis of variance (ANOVA), $P = 0.000$).

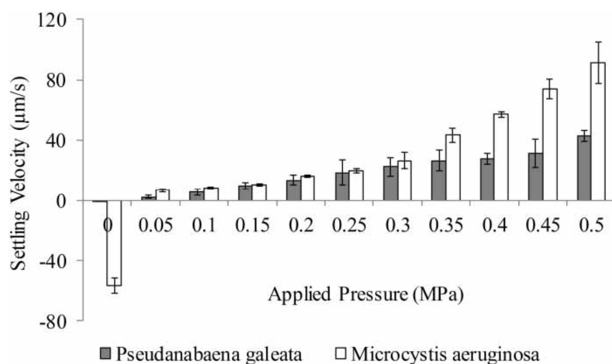

Figure 2 | Increase in settling velocity with applied external pressure for *P. galeata* and *M. aeruginosa*.

According to Stokes's law (Reynolds *et al.* 1987), the settling velocity of cyanobacterial cells in a particular medium depends on the size and density of the cells. The density variation of the cells is the governing factor of the settling velocity of a particular cell in a given medium. Under atmospheric conditions, cells could either float or sink, depending on the combination of the gas vacuole and the ballast conditions (Kalff 2002). In the initial stage, incubated *M. aeruginosa* and *P. galeata* cultures had a negative settling velocity, thus indicating the dominant effect of the gas vesicles in the cells. Ibelings *et al.* (1991) reported that the presence of the gas vacuoles lowered the density of the cyanobacteria below that of the water (988 $kg\,m^{-3}$ for freshwater at 20 °C). According to observations in the present study, with a larger amount of gas vesicles in the cell, greater reductions in density could be observed. The higher floatability of *M. aeruginosa* cells than *P. galeata* was caused by density reduction from the higher amount of gas-vacuolated area in the cells.

Under high-pressure treatment, the density increment via the collapse of gas vesicles had a positive effect on the settling of cyanobacterial cells. Previous studies have reported the same phenomena for the settling ability increment of cyanobacteria using ultrasonic and pressurization treatments, i.e., the collapse of cyanobacterial gas vesicles, resulting in the cells losing buoyancy and moving to deeper levels in the water column (Porat *et al.* 1999; Rodriguez-Molares *et al.* 2014). However, quantitative measurements of the cyanobacterial settling velocity for different experimental conditions are rarely found in the literature. Nakamura *et al.* (1993) reported that the settling velocity of *Microcystis* cells measured with a similar apparatus to this study were in the range of 3.0 to 3.5 μms^{-1} after 0.5 MPa pressure treatment. Measurements performed by Walsby & Holland (2006) based on suspensions of 15 mm polystyrene microspheres with a density of 1,050 $kg\,m^{-3}$ indicated that the mean velocity was 6.28 μms^{-1}, i.e., a value within 1.5% of the calculated theoretical value of 6.36 μms^{-1} using Stokes's equation (Reynolds *et al.* 1987). In the present study, we quantified both the gas vacuole reduction and the settling velocity increment with respect to the applied high pressure. In the present study, the measured settling velocities were in the ranges of −0.07 to 42.9 μms^{-1} and −56.8 to 91.3 μms^{-1} for *P. galeata* and

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M. aeruginosa, respectively. Our results were in reasonable agreement with the results obtained by Nakamura *et al.* (1993) and Walsby & Holland (2006). The floatability gained due to gas vacuoles of cyanobacteria drastically changed to settleability once they collapsed. Thus, the settling velocity increase was much higher in *M. aeruginosa* than in *P. galeata* after collapse of the gas vacuoles. The above observations in the settling velocity experiment are discussed in the following sections with support of TEM images. Figures 3 and 4 show the TEM images of gas vesicles in *P. galeata* and *M. aeruginosa* cells, respectively, under (a) control conditions and after exposure to pressures of (b) 0.3 and (c) 0.5 MPa.

The TEM observations shown in Figures 3 and 4 supported the presence of sufficient gas vesicles in the initial cell cultures. Although there are no previously reported studies on the buoyancy and gas vesicle morphology of *P. galeata*, the presented observations of this study for the initial control cell culture may be considered as a suitable gas vesicle state for cell flotation (Figure 3(a)). *M. aeruginosa* is a well-known bloom-forming cyanobacterium with ample gas vacuoles. Figure 4(a) shows the initial gas vesicle morphology of *M. aeruginosa*. These results suitably explain the observed behavior of the cells. The highlighted observations from the TEM images with increased pressure were (i) a reduction of the gas-vacuolated area with increased pressure and (ii) a reduction of the cell area with increased pressure (Figure 3(b) and 3(c), Figure 4(b) and 4(c)). Both of these factors affected the settling velocity variation induced by the density increment.

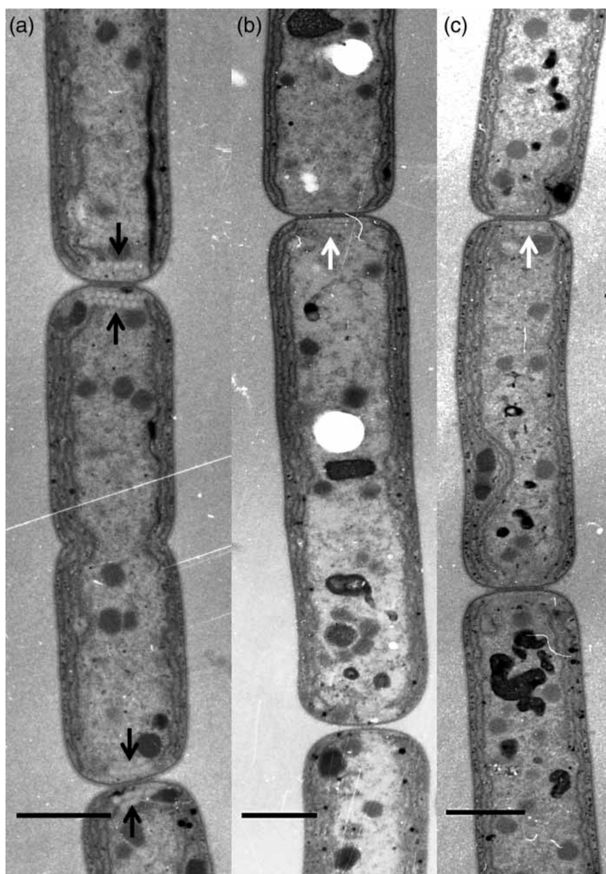

Figure 3 | Ultra-structure of *P. galeata* cells in (a) control and after pressure treatments with (b) 0.3 and (c) 0.5 MPa. Black arrows point at gas-vacuolated areas and white arrows point at collapsed gas vacuoles and residuals. Scale bars represent 1 μm.

As shown in Figure 5(a), the average cell area was reduced with increasing pressure. With pressures of 0, 0.3 and 0.5 MPa, the average cell area was 24.78 ± 5.05, 23.58 ± 2.96 and 20.28 ± 0.86 μm^2, respectively, for *M. aeruginosa* and was 10.65 ± 1.61, 8.38 ± 1.52 and 6.61 ± 0.16 μm^2, respectively, for *P. galeata*. The ratio of the gas-vacuolated area per cell area was altered at the applied pressures of 0 and 0.3 MPa as 0.46 ± 0.05 and 0.09 ± 0.02, respectively, and with a pressure of 0.5 MPa, the gas vacuoles were completely destroyed for *M. aeruginosa*. For *P. galeata*, the ratio was 0.06 ± 0.01, 0.01 ± 0.008 and 0.0001 at 0, 0.3 and 0.5 MPa, respectively. Critical pressure of gas vesicle collapse depends on the gas vesicle morphology in different species (Hayes & Walsby 1986; Walsby & Bleything 1988). The high-pressure requirement for complete destruction of gas vesicles differs between different culture conditions. For example collapse of all gas vesicles by high-pressure application in filamentous cyanobacterium *Aphanizomenon ovalisporum* has required 0.6 MPa (Porat *et al.* 1999). In the treatment suggested by Clarke & Walsby (1996), the recommended preferred depth to pump water to achieve complete collapse of gas vacuoles was 60 m (0.6 MPa). Yet in the present experiment both filamentous *P. galeata* and uni-algal *M. aeruginosa* achieved almost complete gas vacuole disappearance at 0.5 MPa.

The correlations of the applied pressure with the settling velocity, the remaining gas-vacuolated area and the cell area are shown in Table 1. According to the Pearson's correlation analysis, for *P. galeata*, the applied pressure had a strong negative correlation with variations in both the cell area

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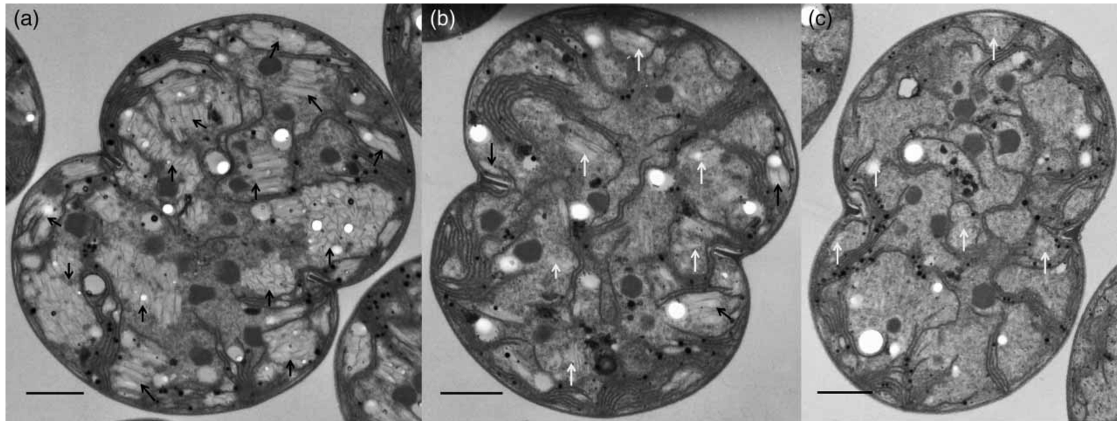

Figure 4 | Ultra-structure of *M. aeruginosa* cells in (a) control and after pressure treatments with (b) 0.3 and (c) 0.5 MPa. Black arrows point at gas-vacuolated areas and white arrows point at collapsed gas vacuoles and residuals. Scale bars represent 1 μm.

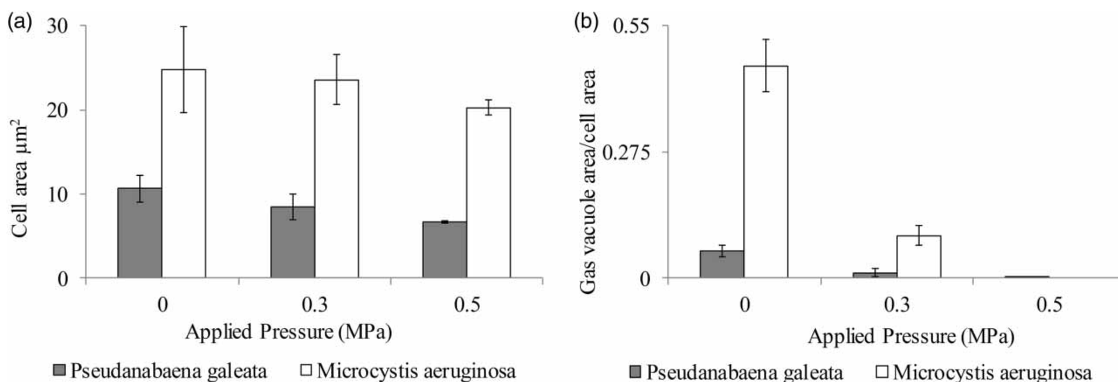

Figure 5 | The variation of (a) cell area and (b) gas vacuole area per cell area of *P. galeata* and *M. aeruginosa* with applied pressure.

Table 1 | Pearson's correlation analysis

Species	Relationship	Pearson's correlation
P. galeata	Applied pressure × Cell area	*−0.789**
	Applied pressure × Gas vacuole area	*−0.936***
	Applied pressure × Settling velocity	*0.924***
M. aeruginosa	Applied pressure × Cell area	*−0.453*
	Applied pressure × Gas vacuole area	*−0.964***
	Applied pressure × Settling velocity	*0.929***

*Correlation is significant at the 0.05 level (two-tailed).

**Correlation is significant at the 0.01 level (two-tailed).

and the gas-vacuolated area. However, for *M. aeruginosa*, the applied pressure had a strong negative correlation with only the gas-vacuolated area variation. For both species, the applied pressure had a strong positive correlation with the settling velocity. Previous studies have shown the relationship between settling enhancement of cyanobacteria by pressure application (Dinsdale & Walsby 1972; Oliver & Walsby 1984; Nakamura *et al.* 1993). In this study the increment of settling velocity due to external pressure could be attributed to the destruction of gas vesicles for gas vacuolated cyanobacteria.

For cyanobacteria with an ample amount of gas-filled volume, such as *M. aeruginosa*, the destruction of gas vacuoles governs the settling ability, and the deformation of the cell volume by external pressure is comparatively negligible.

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However, for cyanobacteria species with a lower gas-vacuolated volume, such as *P. galeata*, the buoyancy regulation depends on not only the volume reduction of gas vacuoles but also the shrinking of the whole cell volume by the applied pressure. This hypothesis was supported by the findings of this study, which showed that the settling velocities of *P. galeata* cells and *M. aeruginosa* cells are inversely proportional to the gas vacuole area per unit cell volume and the applied pressure, respectively.

The average settling velocity of cells is shown in Figure 6(a) and 6(b) as a function of either the ratio of gas vacuole area and cell area or the applied pressures, respectively. The settling velocity has a negative correlation with the fraction of gas vacuoles in the cell, with the reduction rate being higher for *M. aeruginosa* than for *P. galeata*, whereas it has a positive correlation with the magnitude of the applied pressure. The increasing rate with respect to pressure was higher for *M. aeruginosa* than it was for *P galeata*.

Cell removal efficiency

The efficiency of the removal rate of cells is presented in Figure 7 as a function of applied pressure. The removal rate substantially increased with the magnitude of the applied pressure for both species. The removal rate was only slightly higher for *M. aeruginosa* than it was for *P. galeata*.

After 24 h, the removal rates of *P. galeata* and *M. aeruginosa* cells were 14% and 0%, respectively, in the control cultures at atmospheric pressure. Application of pressure has enhanced the cell removal efficiency significantly. In the pressure-treated cultures at 0.3 MPa, the removal efficiencies were of 61% and 81% for *P. galeata* and *M. aeruginosa*, respectively. In pressure-treated cultures at 0.5 MPa the removal efficiencies were 82% and 95%, respectively. A previous study with gas-vacuolated filamentous cyanobacterium *Arthrospira platensis* showed a turbidity reduction rate of 65% and 80% with the application of 2.5 and 0.6 MPa hydrostatic pressures, respectively. This difference is attributed to the gas vacuole volume in natural conditions and the different laboratory conditions (Jachlewski *et al.* 2013). The morphology and strength of the gas vesicles are induced by several environmental factors, such as the nutrient level (Klemer *et al.* 1982; Chu *et al.* 2007), in natural conditions. However, hydrostatic pressure has a clear effect on the disruption of gas vacuoles in *P. galeata* and *M. aeruginosa*. Previous research has suggested a preferable apparatus for the application of high pressure on cyanobacterial cells in lakes and reservoirs, i.e., a steel-lined bore-hole with an optimum depth of 60 m (Clarke & Walsby 1996) and the hydrostatic pressure generated by down-pipes with maximum pressure head of 2.5 MPa (Porat *et al.* 1999). According to the present study, cells do not necessarily need to shift to that elevation for the destruction of cyanobacterial gas vesicles to occur; the application of 0.3–0.5 MPa of pressure is sufficient because those cells achieve irreversible settling. Naturally feasible cyanobacteria downwelling methods, such as artificial air plume de-stratification and pressurization pumping of epilimnion waters to deep layers, are topics of further research. Because the previous approaches used to collapse gas vesicles via ultra-sonication have been observed not to be permanent, with the reformation and full recovery of the gas vesicles damaged occurring over time (Rodriguez-Molares *et al.* 2014), the prolonged effect of hydrostatic pressure on gas vesicle reformation

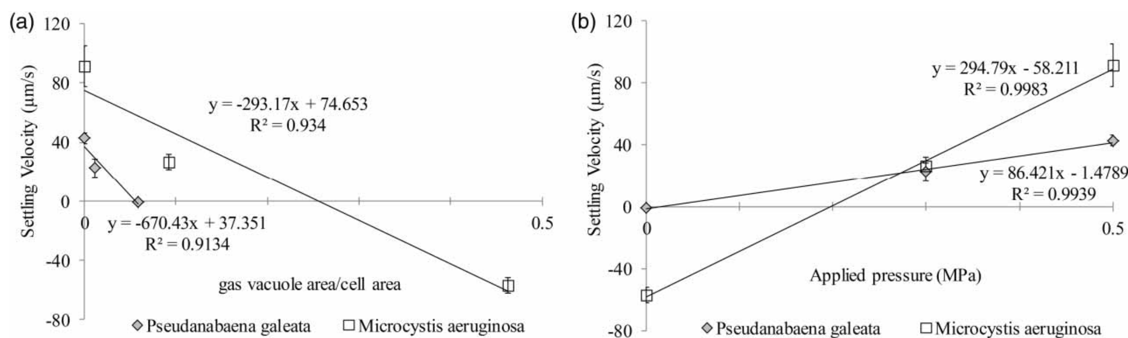

Figure 6 | Relationship between the settling velocity and (a) the gas vacuole area/cell area ratio and (b) the applied pressure.

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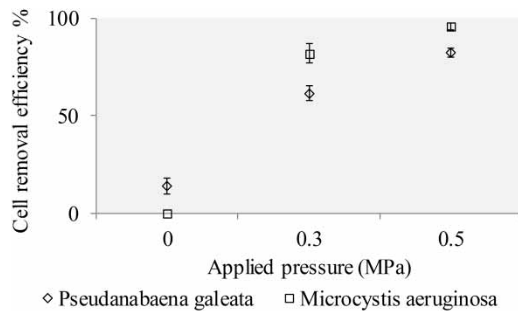

Figure 7 | Cyanobacterial cell removal efficiency, with the applied pressure error bars denoting the standard deviation.

requires further study to prevent the resuspension of settled cyanobacterial cells.

CONCLUSIONS

The loss of gas vesicles due to applied pressures in this study is sufficient to eliminate the buoyancy of both types of cyanobacterial species cells. The settleability of both cyanobacterial species significantly ($P = 0.000$) increased with increasing pressure, whereas the gas vacuolated area of both species significantly ($P = 0.000$) decreased with the magnitude of the applied pressure. The removal efficiencies of *P. galeata* and *M. aeruginosa* cells were 82% and 95%, respectively, at the hydrostatic pressure value of 0.5 MPa. Findings of this study indicated the capability of pressurization treatment to collapse gas vacuoles and increase the settleability of cyanobacteria. Hence, pressurization could be potentially applied to reduce the accumulation of cyanobacteria in water treatment plants and in the epilimnion layer of lakes (probably by artificial mixing, such as by bubble plume or using pressurized pumping of the top water layer to the deep layers of the water body). However, to investigate cell growth under deep water conditions, application of prolonged hydrostatic pressure and dark conditions is recommended.

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