Buoyancy regulation of *Microcystis flos-aquae* during phosphorus-limited and nitrogen-limited growth

ZHAOSHENG CHU1, XIANGCAN JIN1*, BO YANG2 AND QINGRU ZENG2

1STATE ENVIRONMENTAL PROTECTION KEY LABORATORY FOR LAKE POLLUTION CONTROL/RESEARCH CENTER FOR LAKE ECOLOGY AND ENVIRONMENTS, CHINESE RESEARCH ACADEMY OF ENVIRONMENTAL SCIENCES (CREAES), BEIJING 100012, CHINA AND 2DEPARTMENT OF ENVIRONMENTAL TECHNOLOGY, HUNAN AGRICULTURAL UNIVERSITY, HUNAN 410128, CHINA

*CORRESPONDING AUTHOR: jinxc@craes.org.cn

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The dominance of gas-vacuolate cyanobacteria is often attributed to their buoyancy and to their ability to regulate buoyancy in response to environmental conditions. Changes in absolute gas vesicles volume, carbohydrate content, protein content and colony buoyancy of *Microcystis flos-aquae* were investigated during nitrogen-limited, phosphorus-limited and nutrient-replete growth. When nutrient-replete, *M. flos-aquae* cells consistently had excess gas vesicles, which provided sufficient buoyancy that the influence of daily carbohydrate changes on cells upon floatation was negligible. However, during nitrogen-limited growth, gas vesicle volume per cell decreased significantly with nitrogen exhaustion. The maximum decrease of gas vesicle volume was up to 84–88%. At the same time, cellular carbohydrate content had an accumulation trend. The decrease of gas vesicle buoyancy together with the daily increase in carbohydrate are suggested to explain the daily changes in the cell floatation. During phosphorus-limited growth, gas vesicle volume per cell decreased slightly (maximum to 22–32%), and they still provided sufficient buoyancy that most cells kept floating even though there were significant daily carbohydrate changes. Since nitrogen limitation caused more significant buoyancy loss than phosphorus limitation did, surface water blooms may disappear or appear frequently in nitrogen limited water bodies while they may persist a longer time in phosphorus limited water bodies. The quantitative analysis in buoyancy change by gas vesicles, carbohydrate and protein suggested that long-term buoyancy regulation was mainly determined by changes of gas vesicle volume whereas short-term buoyancy regulation was mainly determined by carbohydrate accumulation and consumption. Both long-term and short-term buoyancy regulation were influenced by cell nutrient status. Furthermore, gas vesicle volume per cell and protein content changed in the same way in both nitrogen-limited and phosphorus-limited growth, which implied that the decrease of gas vesicles were associated with controls of total protein synthesis.

INTRODUCTION

Cyanobacterial blooms often cause significant problems, such as unpleasant odors, water deoxygenation and clogging of water supply systems (Reynolds and Walsby, 1975). Furthermore, some cyanobacteria produce toxins, which cause great harm to aquatic organisms, domestic animals and humans (Sivonen, 1996).

The dominance of gas-vacuolate cyanobacteria is often attributed to their buoyancy and to their ability to regulate buoyancy in response to environmental conditions (Reynolds et al., 1987; Walsby, 1994; Oliver and Ganf, 2000). Buoyancy regulation provide cyanobacteria with several advantages, including decrease in sedimentation loss (Reynolds and Walsby 1975), overcoming the
separation of light and atmosphere CO₂ in the surface and nutrient in the depth of stratified water (Ganf and Oliver, 1982; Klemer, 1996).

Cyanobacterial buoyancy depends on the extent to which the lift provided by gas vesicles counteracts cell ballasts, such as carbohydrate (density \( \sim 1550 \text{ kg m}^{-3} \)) and protein (density \( \sim 1300 \text{ kg m}^{-3} \)) (Oliver and Walsby, 1984). Three mechanisms of buoyancy regulation have been proposed: change in cell ballasts, production and dilution of gas vesicles and irreversible collapse of gas vesicles (Walsby, 1994). Gas vesicles of Microcystis cannot be collapsed by turgor pressure due to collapse of gas vesicles (Walsby, 1994). Three mechanisms of buoyancy regulation have been proposed: change in cell ballasts, production and dilution of gas vesicles and irreversible collapse of gas vesicles (Walsby, 1994). Gas vesicles of Microcystis cannot be collapsed by turgor pressure due to their narrow width, which generates greater strength (Thomas and Walsby 1985, Walsby 1994). Consequently, buoyancy regulation of Microcystis results from the former two mechanisms.

Several studies proposed that buoyancy changes in Microcystis are regulated by carbohydrate accumulation and consumption in response to light (Kromkamp and Mur, 1984; Wallace and Hamilton, 1999; Wallace and Hamilton, 2000). Cell concentration (after gas vesicles collapsed) showed a linear relationship with carbohydrate content (or carbohydrate to protein ratio). This mechanism was often used to explain the daily vertical migrations of Microcystis.

Konopka et al. (Konopka et al., 1987) studied buoyancy change by Microcystis cells in phosphorus-limited conditions. Their results suggested that buoyancy increases in the dark were correlated with a decrease in carbohydrate content and there was an increase in gas vesicle content in the light. Brookes and Ganf (2001) further found that Microcystis displayed certain buoyancy variability in response to light, which was dependent upon the previous nutrient and light history of the cells.

Environmental factors such as light (Deacon and Walsby, 1990) and nutrients are important in gas vesicles production (Klemer, 1978, 1982, 1991, 1996; Kromkamp et al., 1989; Brookes and Ganf, 2001). Nitrogen and phosphorus limitation resulted in gas vesicle content decreases, which may cause buoyancy loss, and cells may regain buoyancy in response to nitrogen or phosphorus re-supply (Klemer, 1982; Kromkamp et al., 1989). Continuous carbon limitation restrained gas vesicles synthesis due to energy shortage (Klemer, 1996; Brookes and Ganf, 2001). However, these studies did not give a quantitative buoyancy contribution of gas vesicles, as they measured the presence of vesicles by light scattering method. Use of a compression tube (Walsby, 1982; Walsby et al., 1992) provides a method for direct measurement of gas vesicle volume.

The compression tube has been used in several studies for investigating changes in gas vesicles and buoyancy in cyanobacteria (Oliver et al., 1985; Thomas and Walsby, 1985; Utkilen et al., 1985; Verspagen et al., 2004). The first quantitative analysis of what caused the buoyancy change was made by Oliver et al. (Oliver and Walsby, 1984), drawing up a balance sheet of the ballasts contributed by each of the major cell component. However, quantitative studies on buoyancy regulation and the knowledge about relative importance of carbohydrate regulation and gas vesicles regulation during different nutrient-limited growth process remain limited (Walsby et al., 1992).

*Microcystis flos-aquae* is a gas-vacuolate cyanobacterium which often forms blooms in eutrophic lakes. This study provides a quantitative study on the buoyancy regulation of *M. flos-aquae* during nitrogen-limited and phosphorus-limited growth by analysis of buoyancy contributions of gas vesicle volume, carbohydrate and protein content.

**METHOD**

Algae and cultivation experiments. *Microcystis flos-aquae* (Wittr.) Kirch (colony form) was isolated from Lake Taihu in 2005 and was maintained at 20°C under a 12 h:12 h light: dark cycle in M11 medium, which consists of 100 mg NaNO₃, 10 mg K₂HPO₄, 75 mg MgSO₄·7H₂O, 40 mg CaCl₂·2H₂O, 20 mg Na₂CO₃, 6 mg Fe·citrate and 1 mg Na₂EDTA·2H₂O in 1 L deionized water. It was not axenic, but the biomass of bacteria was lower than 2% during the cultivation period. Bacterial biomass was estimated by counting under a fluorescence microscope after acridine orange staining.

Cultivation experiments were conducted in Pyrex bottles in duplicates (15 L culture medium), which were autoclaved for 30 min and then aerated with sterilized air through a 0.2 μm filter. When the culture medium was cooled and saturated with oxygen, *M. flos-aquae* was inoculated and incubated at 28°C under 12 h:12 h light: dark cycle. The irradiance was 25 μmol m⁻² s⁻¹ (white fluorescent tube) and the inoculation cell concentration is 5 × 10⁴ cell mL⁻¹. Nitrogen or phosphorus was decreased to one-tenth of those in M11 medium to create the nitrogen-limited or phosphorus-limited growth, respectively. Cultures in M11 medium were set as the control. To ensure nitrogen and phosphorus in control experiments were replete, the growth experiments in the former 14 days were set as the control, in which the decreases of dissolved nitrogen or phosphorus was less than half of initial concentrations.

About 100 mL culture medium was sampled just before the end of the light phase and the dark phase to determine the population cell concentration, gas vesicle volume, total nitrogen (TN), total phosphorus (TP),...
dissolve nitrogen (DN), carbohydrate and protein content. The sampled culture medium was centrifuged at 500 G for 30 min, and then the clear medium in the middle was siphoned to concentrate the algae. The maximum pressure estimated in the centrifuge tube was 25 kPa, which was unable to break down the gas vesicles in M. flos-aquae.

Determination of the volume of gas vesicle and cell components. Gas vesicle volume was determined by capillary compression tube (Walsby et al., 1992). The collapse pressure for gas vesicles was 0.8 MPa. Carbohydrate content was determined with the anthrone reagent employing D-glucose as standard, and protein content was determined using Folin-Ciocalteu method with bovine serum albumin as standard (Hebbert et al., 1971).

Population cell concentration analysis. After measurement of gas vesicle volume, the colonies of algae were dispersed by ultrasonication for 30 s in ultrasonic cleaner (20 w), and then diluted to count the cells number using haemacytometer (TIT A, Japan). The counting procedure was carried out three times for each sample. At least 500 cells were counted each time.

Net buoyancy calculation. Net ballast was calculated according to Oliver and Walsby (1984) as the mass of the carbohydrate, protein and gas vesicles minus the mass of water they displaced [equation (1)].

\[
M_C - M_W = \left( \frac{M_C}{\rho_C} \right) \times (\rho_C - \rho_W)
\]

where \(M_C\) is the mass of ballasts, including gas vesicles, carbohydrate and protein; \(M_W\) is the mass of water they displaced; and \(\rho_C\) and \(\rho_W\) is the density of ballasts and water, respectively. The density of water (20°C), carbohydrate, protein and gas vesicle gas is 998, 1550, 1330 and 1 kg m\(^{-3}\), respectively. Only major cell components, e.g. carbohydrate and protein were considered here to calculated total ballast as they generally accounted for 80% or more dry mass of colony (Oliver et al., 1985) and have a relative high density. Net buoyancy was calculated by the sum of the ballast masses multiplied by \(-1\).

RESULTS

The growth characteristics. As shown in Fig. 1, the growth curves of M. flos-aquae in nutrient-limited growth (0–20 days) consisted of exponential growth phase and stationary phase whereas those of nutrient-replete growth (0–14 days) only had exponential growth phase. The exponential growth phase could be divided into two sub-phases, e.g. early exponential growth phase, in which the log of biomass increased linearly with the cultivation time, and the later exponential phase, in which the biomass increased linearly with the cultivation time. In the early exponential phase (0–4 days), the biomass of M. flos-aquae in nutrient-limited growth was approximately equal to that in nutrient-replete growth, but during the following phase, the biomass in nutrient-limited growth was lower than that in nutrient-replete growth and the biomass in nitrogen-limited growth was the lowest.

Nitrogen cell quota (\(Q_N\)) and phosphorus cell quota (\(Q_P\)). It can be seen from Fig. 2 that cells could store excess nutrients at early exponential phase in nutrient-limited growth. But after then, cell quota of limited nutrient decreased and constrained the algal growth at the later exponential phase. When reached the stationary phase, cell quota of limited nutrient decreased to the lowest (\(Q_{N_{\text{min}}}, Q_{P_{\text{min}}}\)). \(Q_{P_{\text{min}}}\) in phosphorus-limited growth only accounted for 15–20% of the quota in nutrient-replete growth (controls). \(Q_{N_{\text{min}}}\) in nitrogen-limited growth accounted for about 60% of the quota in nutrient-replete growth.

Gas vesicle volume per cell. Comparison with nutrient-replete growth, gas vesicle volume per cell in nutrient-limited growth decreased with nitrogen or phosphorus consumption (Fig. 3); moreover, the decrease in nitrogen-limited growth was greater than that in phosphorus growth. When \(Q_N\) and \(Q_P\) decreased to the lowest level in nitrogen-limited and phosphorus-limited cultures, the decrease of gas vesicle volume per cell was about 84–88% and 22–32%, respectively. In nutrient-replete growth, gas vesicle volume per cell remained almost constant in the first 14 days. However, in nitrogen-limited growth, gas vesicle volume per cell decreased quickly in the later exponential growth phase and slightly decreased in the stationary phase; when in phosphorus-limited growth, gas vesicle volume per cell decreased...
only decreased slowly in the later exponential growth phase while had no change in the early exponential growth phase and stationary phase.

**Protein content per cell.** The change of protein content per cell in all cultures was similar to the change of gas vesicle volume per cell (Fig. 4). During nutrient-replete growth, protein content remained almost constant, but during nutrient-limited growth, protein content decreased with nutrient consumption and the decrease in nitrogen-limited growth was greater than that in phosphorus-limited growth.

**Carbohydrate content per cell (CCPC).** Carbohydrate content per cell varied daily in all cultures (Fig. 5). Carbohydrate accumulated in the light time and consumed at dark time, and the extent of change was greater in the exponential phase than that in the stationary phase in nutrient limited growth.

Carbohydrate content changed differently with the different nutrient consumption. During the later exponential phase, carbohydrate content increased rapidly during nitrogen-limited growth, whereas it changed only slightly during phosphorus-limited growth.

**Net buoyancy and the ratio of floating colonies.** Net buoyancy was calculated by the integrated influence of gas vesicles, carbohydrate and protein (Fig. 6). Gas vesicles provided much excess buoyancy, which almost eliminated the influence of daily carbohydrate changes on the net buoyancy in nutrient-replete and phosphorus-limited cultures. However, during nitrogen-limited growth, net buoyancy became negative when $Q_N$ decreased to the lowest, and the main reason for this was decrease of gas vesicle volume. What is more, the accumulation of carbohydrate plays an important role on the loss of buoyancy; during phosphorus-limited growth, although the net buoyancy decreased in the later exponential phase, gas vesicles still provide sufficient buoyancy to cells even when $Q_P$ decreased to the lowest level.
The ratio of floating colonies during the cultivation process agreed well with net buoyancy calculated from gas vesicles, proteins and carbohydrates (Fig. 7). All colonies were floating during nutrient-replete growth and phosphorus-limited growth. Significant portion of colonies (90%), which were initially buoyant, lost buoyancy when $Q_N$ decreased to the lowest in nitrogen-limited growth, and the day 50% colonies lost their buoyancy was near the day that the net buoyancy became negative.

**DISCUSSION**

Gas vesicle volume per cell in *M. flos-aquae* showed little change when nutrients, i.e. nitrogen and phosphorus, were replete; however, it decreased when nitrogen limitation or phosphorus limitation occurred. The results were consistent with previous studies on several cyanobacteria, such as *Microcystis, Anabaena, Aphanizomenon* and *Oscillatoria* spp. (Klemer, 1982; Kromkamp et al., 1989; Oliver, 1994; Brookes et al., 1999; Brookes and Ganf, 2001), in which gas vesicles were measured by light scattering method and expressed as relative gas vesicle (RGV) volume. However, RGV volume did not give the value of the absolute gas vesicle volume and the buoyancy contributions could therefore not be calculated. Gas vesicle volumes were determined here by pressure capillary tube during the time-course in nutrient-limited growth. The results showed that the decrease in gas vesicle volume per cell in nitrogen-limitation was much greater than that in phosphorus limitation. The calculated buoyancy (12–18 pg cell$^{-1}$) provided by gas vesicles in nutrient-replete condition was much in excess and all the colonies were floating. But when $Q_N$ decreased to the lowest level in nitrogen-limited cultures, gas vesicle volume per cell decreased to 12% of the initial content and more than 90% of the cells lost buoyancy, and when $Q_P$ decreased to the lowest level in phosphorus-limited cultures, only less than half of gas vesicle volume per cell was lost and almost all the cells were still floating.

The influence of nitrogen limitation on gas vesicle content was greater than that of phosphorus limitation suggesting nitrogen is more important than phosphorus in gas vesicle protein synthesis. Furthermore, the change of protein content were similar to that of gas vesicle volume per cell in both of the nitrogen- and phosphorus-limited growth implying gas vesicle production was associated most closely with total protein synthesis.

Several studies (Kromkamp and Mur, 1984; Visser et al., 1997; Wallace and Hamilton, 1999, 2000; Porat et al., 2001) proposed that the diurnal vertical migration
of some cyanobacteria, such as *Microcystis*, could be explained by carbohydrate accumulation and utilization because their gas vesicles cannot be collapse by turgor pressure. Brookes and Ganf (Brookes and Ganf, 2001) showed that *M. aeruginosa* displays a range of variability in buoyancy in response to light, which is dependent upon previous nutrient and light history of the cells. But the relative importance of gas vesicles and carbohydrate on cell buoyancy was not compared.

The results here also suggested cell nutrient conditions might influence carbohydrate accumulation and utilization. However, cell buoyancy calculated from gas vesicles, carbohydrate and protein suggested that the importance of daily carbohydrate changes was dependent upon on gas vesicle content, which was determined by cell nutrient status. When cell nutrient was replete, gas vesicles provided excess buoyancy. Although daily carbohydrate change was significant, its influence on cell floating may be negligible. When algal growth was limited by nutrients, gas vesicle content per cell was diluted by cell division and the role of daily carbohydrate change became more important.

As gas vesicles in phosphorus limitation still provided excess buoyancy, the influence of carbohydrate changes probably could not alter cells floating. But in nitrogen-limited growth, gas vesicles decreased greatly and both gas vesicle dilution and daily carbohydrate change play an important role on buoyancy regulation. The integrated influence of gas vesicles, carbohydrate and protein suggested that long-term buoyancy regulation was mainly determined by gas vesicle production and dilution, whereas short-term buoyancy regulation was determined by carbohydrate accumulation and consumption; both of these processes are influenced by nutrient status.

In stratified lakes, since nutrient limitation often occurs in the surface water layers, gas vesicle dilution and carbohydrate accumulation may result in buoyancy loss and causing cells to migrate to deeper water. When cells fulfilled the nutrient requirements, gas vesicle production and carbohydrate consumption result in their migrating to the water surface. As nitrogen limitation causes a much greater decrease in gas vesicle volume per cell and more significant carbohydrate accumulation compared with phosphorus limitation, surface water blooms may disappear and reappear frequently in nitrogen limited water body while surface blooms may persist a longer time in phosphorus limited water bodies.

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