

Quantification of the ultrasound induced sedimentation of *Microcystis aeruginosa*.

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Abstract

It has been known for more than 40 years that vacuolate organisms can be induced to sediment with ultrasound. However, robust indicators are still needed to compare the efficacy of different treatments. A repeatable index is proposed that makes it possible to quantify the ultrasonic induced sedimentation. The procedure is used to monitor the long term sedimentation of *Microcystis aeruginosa* after sonication. Results reveal that the sedimentation process continues after gas vesicles have fully recovered, although at a slower rate.

Keywords: Cyanobacteria, sedimentation, gas vesicle, collapse, buoyancy.

1. Introduction

Microcystis aeruginosa is a phototropic bacterium that grows in nutrient-rich, slowly moving water. This species of cyanobacteria produces the potent

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1 hepatotoxin microcystin, that can be released into the water when its cell
2 wall breaks. Ingestion of water containing microcystin is known to cause
3 animal and human poisoning [1]. Several treatment methods are available to
4 control cyanobacterial populations, but economic and environmental factors
5 are still motivating the search for alternatives. In the last 12 years, ultrasonic
6 irradiation has been intensively studied as a potential pollutant-free method
7 to control cyanobacterial blooms.

8 *M. aeruginosa* cells contain gas filled pockets called gas vesicles. It has
9 been shown that gas vesicles can be collapsed by applying ultrasound [2].
10 Consequently, the cells lose buoyancy and move to lower levels in the water
11 column [3], having less exposure to sunlight and, potentially, less ability to
12 photosynthesise. The collapse of gas vesicles induced by ultrasound has been
13 observed by electron microscopy [2, 3, 4] and quantified by flow cytometric
14 measurements [5]. However, the study of the subsequent sedimentation has
15 attracted very little attention. To date, most of the reported data consists
16 of qualitative observations.

17 Previous studies on the effects of ultrasound have often focused on gross
18 general impacts upon cell activity, such as cell growth or photosynthetic
19 activity. But by doing so the study of ultrasound induced effects may have
20 been biased toward the region of high acoustic power. The gas vesicles of
21 *M. aeruginosa* can be collapsed with relatively low acoustic intensities, while
22 high power ultrasonic fields are required to cause significant damage to cell
23 division and the photosynthetic mechanisms.

1 Investigations involving the specific analysis of threshold effects and mech-
2 anisms of subtle changes to gas vesicles may lead to the development of a more
3 energy efficient control without the risk of gross cell damage and metabolite
4 release. The collapse of gas vesicles and loss of buoyancy would allow the
5 cells to sink without being lysed.

6 In this paper we show that the sedimentation induced by ultrasound is
7 a much slower process than previously assumed. Inconsistencies in quantify-
8 ing and reporting that sedimentation allow results to be easily mistaken as
9 an overall reduction in cell concentration. We believe that the experimen-
10 tal techniques used to quantify this phenomenon so far are subject to high
11 variability due to the sampling and culturing techniques. In consequence, we
12 propose an experimental method to quantify the ultrasound induced sedimen-
13 tation with greater accuracy. The technique was applied to systematically
14 measure the time span of the sedimentation process under standard illumi-
15 nation conditions and found that it continues after the gas vesicles have fully
16 recovered, however at lower velocities.

17 This result reinforce the original proposition by Lee et al. [3] that the
18 application of ultrasound to induce sedimentation could be applied as a cost-
19 effective method for controlling cyanobacterial populations.

20 **2. Prior work**

21 Lehman and Jost [2] were the first to use ultrasound to collapse the gas
22 vesicles that regulate the buoyancy of *M. aeruginosa*. However, at that time,

1 they did not consider ultrasound as a practical method to control cyanobacte-
2 rial blooms. It was not until 2001 that Lee et al. [3] proposed that ultrasonic
3 irradiation could be used to induce the sedimentation of cyanobacteria, and
4 showed that sedimentation was followed by a reduction in the photosynthetic
5 activity of the cells.

6 Since the work by Lee et al. [3], many researchers have investigated the
7 effect of ultrasound on cyanobacteria and a number of different effects have
8 been shown (see Table 1). Ultrasound induced sedimentation is qualitatively
9 observed in Refs. [3, 4, 6], in the form of photographs. An assessment of
10 the published experimental procedures used in this field indicates that scarce
11 quantitative data is available, as some authors report having measured cell
12 concentration at the top of containers after allowing the sonicated sample to
13 settle [7, 8, 9]. Variation in both sampling techniques and the calculation of
14 cell regrowth makes it difficult to distinguish whether the reported results de-
15 scribe the sedimentation process or an actual reduction in cell concentration
16 of the whole sample.

17 The absence of reliable information on the settling time and the sampling
18 depth leads to non-reproducible outcomes, but even with this information
19 the concentration gradient with depth makes the quantitative measurements
20 extremely sensitive to the depth at which the sample is taken. These small
21 variations in depth give rise to very different concentration values, and hence
22 measurements subjected to a large amount of uncertainty.

23 The collapse of gas vesicles is accepted as the primary cause for the loss

1 of buoyancy of vacuolate organisms [10]. Vesicle collapse has an immediate
2 effect on cyanobacterial suspensions, which visibly lose their optical charac-
3 teristics due to the disappearance of the light-scattering bodies [11]. This
4 effect can be observed within a few seconds after submitting the sample to
5 increased pressure, either with the application of ultrasound [2] or of static
6 pressure [10].

7 Once collapsed, gas vesicles appear as flat rectangular envelopes often
8 forming scrolls or folds [10]. Cells can produce new gas vesicles [2] that
9 originate as small bi-cones that grow to become spindled- or cylinder-shaped
10 [11]. Lee et al. [5] used flow cytometry measurements to show that, under
11 normal light conditions, gas vesicles seemed to be fully reformed after 24
12 hours. The likely consequence of this is that cells should recover their ability
13 to regulate buoyancy about the same time. However, the sedimentation
14 process has never been observed over time, and hence to date, we can only
15 assume the former hypothesis is correct.

Observed effect	Assessment method	Reference
Loss of buoyancy	Photography	Lee et al. [3], Jachlewski et al. [4], Mahvi and Dehghano [6]
Gas vesicle collapse	TEM	Lee et al. [3]
	SEM	Lehman and Jost [2]
	Flow cytometry	Lee et al. [5]
Growth inhibition	Cell counting	Lee et al. [3], Tang et al. [12], Ahn et al. [13],
	OD ₆₈₄	Zhang et al. [9, 8, 14]
	OD ₅₆₀	Hao et al. [15, 16]
Immediate reduction in cell concentration	Cell counting	Wu et al. [17]
	OD ₆₈₀	Wu et al. [17], Joyce et al. [18]
	OD ₆₈₄	Rajasekhar et al. [7], Zhang et al. [9], Ma et al. [19]
Reduction of cell integrity	Spectrofluoro	Wu et al. [17]
	Electrolyte measurement	Tang et al. [12]
	Differential Interference Microscopy	Hao et al. [15, 16]
	TEM	Jachlewski et al. [4]
Reduction of cell viability	Flow cytometer	Wu et al. [17]
Reduction of photosynthetic activity	Chl-a concentration	Lee et al. [3], Jachlewski et al. [4], Zhang et al. [9, 14], Ahn et al. [13], Hao et al. [15]
	Fluorescence spectroscopy	Hao et al. [15]
Toxin release	Microcystins concentration	Lee et al [3], Zhang et al. [9, 14], Ma et al. [19]

Table 1: Review of the effects of ultrasound on cyanobacteria as reported in literature. TEM: transmission electron microscopy, SEM: scanning electron microscopy, OD: optical density, Chl-a: chlorophyll-a.

1 **3. Materials and Methods**

2 *3.1. Culturing conditions*

3 *Microcystis aeruginosa* was obtained from the culture collection of the
4 Australian Water Quality Centre (SA Water, South Australia) and cultured
5 in ASM-1 media for 64 days in an incubator (Thermolyne Scientific, Aus-
6 tralia) at 20°C under 70 μ E/m²/s light intensity with a light/dark cycle
7 of 12/12 hours. A cell count was performed at the start of the experiment
8 giving a concentration of $13.3 \cdot 10^6$ cells/ml.

9 *3.2. Ultrasonic equipment*

A custom-made ultrasonic transducer (bath-type) was used to sonicate
the samples at 21.5 kHz. The acoustic power of the system was determined
by calorimetry [20]. A 600 ml volume of ultra-pure water was sonicated
for 10 minutes using continuous wave ultrasound and the temperature was
monitored with a set of three temperature probes of 0.05°C accuracy. The
temperature of the three probes was averaged and the gradient $\partial T/\partial t$ was
obtained by linear regression. The acoustic power W was obtained as

$$W = \frac{\partial T}{\partial t} C_p M, \quad (1)$$

10 where C_p is the heat capacity of water and M is the molar mass of the
11 water. The acoustic power of the system was 8.24 W. A 600 ml volume of
12 *M. aeruginosa* suspension was then exposed to 10 minutes of ultrasound and

1 the temperature was monitored to ensure it was kept within $22 \pm 1^\circ C$ with
2 external cooling.

3 *3.3. Sample preparation*

4 After sonication the suspension was shaken thoroughly and 39 samples of
5 1.2 ml were extracted and placed in polystyrene flow tubes for analysis using
6 a BD FACSCalibur[®] flow cytometer over a period of 30 hours. Another set
7 of 27 samples of 15 ml were extracted and placed in 15 mm diameter glass
8 tubes for measuring sedimentation over the next two weeks. A 10 ml sample
9 was extracted and placed on a Petri dish for monitoring the gas vesicle state
10 through a Live Cell Imaging (Nikon, Japan) optical microscope. A series of
11 control samples were also taken for comparison.

12 All the samples were left to settle inside an incubator under the same
13 conditions used for culturing. Destructive sampling was used to avoid inter-
14 fering with the sedimentation process. All measurements were made on three
15 replicates randomly placed inside the incubator.

16 *3.4. Monitoring of the gas vesicle state*

The 1.2 ml samples were analysed using flow cytometry which provided
information about cell granularity from the measurement of optical Side Scat-
ter (SSC). Following the method used in Ref. [3], the percentage of intact
gas vesicles was estimated from SSC values as

$$GV(\%) = \frac{SSC_s - SSC_p}{SSC_c - SSC_p} \cdot 100\%, \quad (2)$$

1 where the subscript s refers to a sonicated sample, c to a control sample
2 with intact gas vesicles, and p to a control sample whose gas vesicles were
3 collapsed in a pressure vessel (Brister & Co Pty Ltd.) by placing them under
4 900 kPa of hydrostatic pressure for 2 minutes. Equation (2) normalises the
5 SSC values of the sonicated sample s between the values of intact c and
6 collapsed p gas vesicles, providing a quantitative estimation of the state of
7 the gas vesicles.

8 Cell granularity was also observed with optical microscopy and moni-
9 tored with a Live Cell Imaging (Nikon, Japan) system facility at Adelaide
10 Microscopy facility.

11 *3.5. Monitoring of the sedimentation process*

12 The sedimentation process in the 15 ml samples was quantified as follows:

- 13 1. the 4 cm upper layer (7.5 ml) of the sample was extracted,
- 14 2. the extracted volume was shaken to ensure homogeneity,
- 15 3. 2 ml subsamples were extracted from the upper volume and cell concen-
16 tration was estimated using a Sedgewick-Rafter under a Nikon Eclipse
17 50i microscope at 60x magnification.

This procedure overcomes the potential variability associated with the con-
centration gradient across the sampling depth. The same process was applied
to the control sample. The concentration values of both samples were then

used to define the sedimentation index $S_{4 \text{ cm}}$ calculated as

$$S_{4 \text{ cm}} = \left(1 - \frac{C_{4 \text{ cm}}^s}{C_{4 \text{ cm}}^c} \right) \cdot 100\% \quad (3)$$

1 where $C_{4 \text{ cm}}$ denotes the concentration of the 4 cm upper volume, and the
2 superscripts s and c denote the sonicated and control samples. The sedi-
3 mentation index $S_{4 \text{ cm}}$ expresses the amount of biomass that has been moved
4 from the top 4 cm layer to lower layers.

5 Note that we express that index in distance from the surface (4 cm)
6 rather than in volume (7.5 ml). By doing so we make it possible to compare
7 results of different experiments giving a single number (4 cm), since sinking
8 rates are independent of the volume of the container. Otherwise, to produce
9 reproducible results we should not only report the extracted volume (7.5 ml)
10 but also the dimensions of the container. In addition, that index is more
11 readily extrapolated to an environmental scenario where volumes have little
12 practical value.

13 It must be also noted that using different depths will yield different values
14 of the sedimentation index and, hence, different descriptions of the same
15 effect. Therefore, only sedimentation indexes S_d using the same depth d
16 could be directly compared. The value for d should be chosen so that S_d
17 properly describes the sedimentation process that is being studied. If d is
18 too small, S_d may become quickly saturated. If d is too large, S_d will be of
19 no practical use.

1 Our choice of 4 cm correctly describes the sedimentation process during
2 the time span of the experiment and, in addition, it corresponded to half the
3 height of the volume in the glass test tubes. However, a $S_{4\text{ cm}}$ sedimentation
4 index may not be the right choice to study very intense sedimentation. Hence,
5 different species, treatments or environmental conditions may need of indexes
6 based on other d values.

7 **4. Results**

8 The SSC values of the sonicated sample is monitored during 30 hours
9 and the percentage of intact gas vesicles, shown in Figure 1, is computed
10 with Eq. (2). In agreement with previous results [5], Figure 1 shows that gas
11 vesicles are fully recovered after 24 hours.

12 This was also observed by light microscopy, as shown in Figure 2. The
13 time-lapse sequence of photomicrographs of sonicated cells shows a progres-
14 sive increase in cell granularity within the first 24 hours.

15 The experimental procedure described in Section 3.5 was used to monitor
16 the sedimentation process and the sedimentation index $S_{4\text{ cm}}$ was computed
17 with Eq. (3). Figure 3 shows the evolution of $S_{4\text{ cm}}$ over the two weeks after
18 irradiation. Surprisingly, it shows that sedimentation did not stop after 24
19 hours, but continued for one week with reduced intensity. After 14 days
20 a slight increase of cell concentration was observed demonstrating recovery
21 associated with growth, however cell numbers were still 82% less than in the
22 control sample.

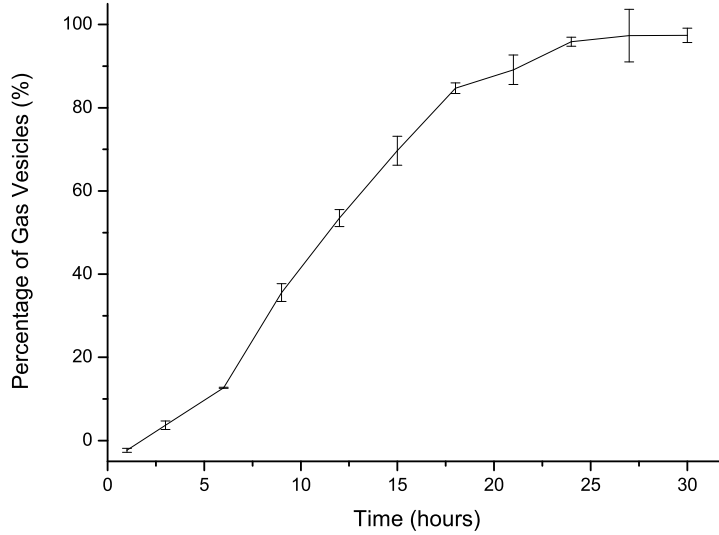


Figure 1: Reformation of the gas vesicles of a sonicated sample, estimated with Eq. (2).

1 5. Discussion

2 The apparent sedimentation ‘inertia’ shown in Figure 3 can be explained
 3 based upon the gas vesicle formation process.

4 Individual gas vesicles are brittle structures that, once collapsed, can not
 5 regain structural form and become then unusable to provide buoyancy. The
 6 cell will however assimilate the constituent proteins of collapsed gas vesicles
 7 and synthesise entirely new ones [2]. As previously mentioned, gas vesicles
 8 originate as small bi-cones that grow to become cylindrical structures [11] and
 9 it takes some time for the gas vesicle to grow from bi-cone to cylinder shape.
 10 In Ref. [2] a freeze-etching micrograph shows that 24 hours after sonication
 11 gas vesicles still present a bi-cone shape, which could indicate that, even

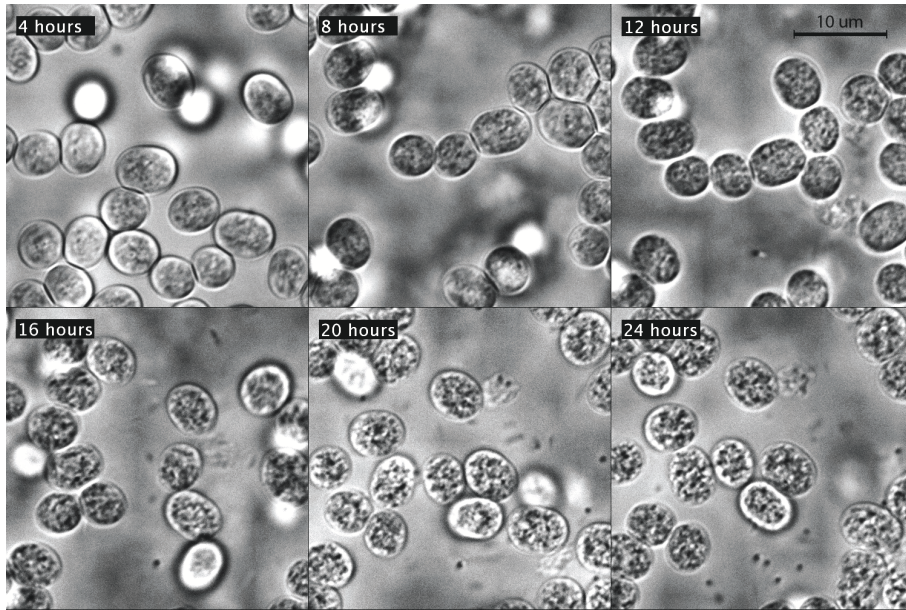


Figure 2: Reformation of gas vesicles observed through optical microscopy at 60x magnification. Live Cell Imaging System at Adelaide Microscopy facility.

1 though a large number of gas vesicles are formed within the first 24 hours,
2 their total volume does not provide sufficient buoyancy to completely stop
3 the sedimentation process. As a result the cells continue to sink for about
4 a week until the total gas volume compensates the higher density of the cell
5 body and finally halts the ongoing sedimentation process.

6 It must be noted that both the composition of culturing media and the
7 illumination conditions have important effects on cell buoyancy [21]. In
8 Ref. [5] it was observed that cells placed in the dark immediately after son-
9 ication could not reform their gas vesicles. Under this condition buoyancy
10 cannot be regained and sedimentation continued until cells had reached the
11 bottom. Under high photon irradiance, photosynthetic cyanobacteria accu-

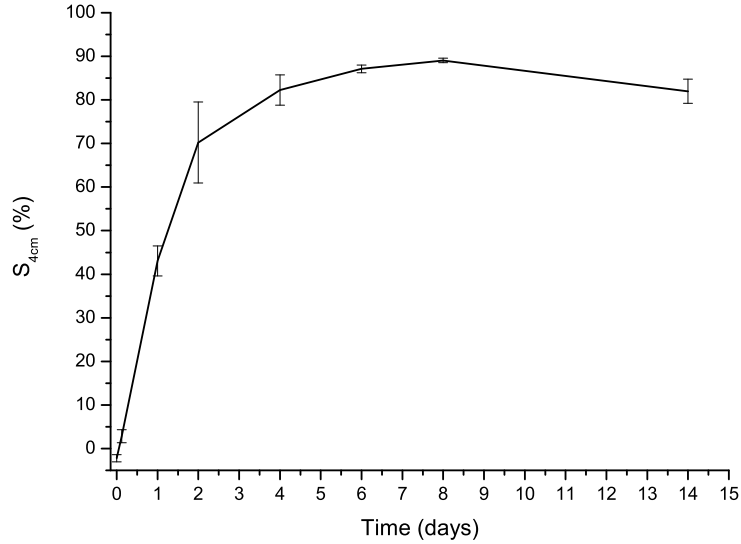


Figure 3: Monitoring of the sedimentation process over two weeks.

1 multate stores of carbohydrate, which is denser than water. That storage
 2 becomes a permanent ballast that could alter the buoyancy regulation mech-
 3 anisms, inhibiting cells tendency to float [22]. Nutrient enrichment can also
 4 affect the buoyancy regulation mechanism providing the cells with persistent
 5 positive buoyancy [23].

6 In this study, we used a *M. aeruginosa* cultured strain which exists as
 7 single or pairs of cells in culture. However, in the environment *M. aeruginosa*
 8 generally form colonies which are aggregations of several hundred cells. Such
 9 aggregation does not alter the average density of the population but reduces
 10 the frictional resistance that those organisms suffer when they move in the
 11 water columns [24]. As a consequence, colonies present higher flotation and

1 sinking rates, and that may increase the efficacy of the ultrasound induced
2 sedimentation process. Intra-colony spaces may also play a key role in the
3 buoyancy regulation of cyanobacterial organisms [25], and its removal by
4 means of ultrasound may provide even more drastic sinking rates. However,
5 very little is known about the dose-response curve when cells are in colonial
6 form.

7 It can be assumed that the efficacy of the ultrasound enhanced sedimen-
8 tation will vary for the different aforementioned scenarios, and therefore it is
9 not possible to extrapolate the results obtained to allow a comparison with
10 other cases.

11 **6. Conclusions**

12 The experimental procedure proposed in Section 3.5 demonstrates it is
13 possible to reliably quantify the subtle sedimentation process and solves the
14 reproducibility problem of previously reported assessment techniques. The
15 method facilitates the comparison of the efficacy of the ultrasonic treatment
16 under different scenarios, which assists in determining how ultrasound inter-
17 acts with the various mechanisms that regulate cell buoyancy.

18 By applying this method we have monitored, for the first time, the sedi-
19 mentation process over time. Results show, as expected, that sedimentation
20 rate is greatest within the first 24 hours. However, only about 50% of the
21 total sedimentation occurred within the initial 24 hours. Sedimentation did
22 however continue for the next 7 days at a reduced rate. After 14 days in-

1 cubation a slight recovery of cell numbers was observed, but the number of
2 cells in the top 4 cm layer was still 82% lower than in the control sample.

3 This finding disproves that ultrasound induced sedimentation is a short-
4 term effect and supports the original hypothesis by Lee et al. [3] of using
5 ultrasound to collapse the gas vesicles of cyanobacteria.

6 According to our results the sedimentation process is significant in the
7 first two days after irradiation, a time lapse that could be sufficient for the
8 cells to descend to a level where light intensity is not sufficient for reforming
9 the gas vesicles. In such a case sedimentation will be irreversible. This is
10 especially probable in the case of naturally occurring multi-cellular colonies,
11 since they will present higher sinking rates and, hence, a stronger sedimen-
12 tation ‘inertia’.

13 Further research is required to determine how efficient such a treatment
14 will be when applied under different conditions. Application of ultrasound
15 to cyanobacteria may be more effective at particular times in the diurnal
16 cycle. It has been shown that *M. aeruginosa* is most susceptible to ultra-
17 sonic irradiation immediately after cell division [13], late in the daylight
18 cycle. Gas vesicle reformation can be delayed with low photon irradiance,
19 and hence, sedimentation effects will have a longer lasting effect if cells are
20 sonicated in the evening. Different frequencies may produce different effects
21 in cyanobacterial organelles, and it has been suggested that there will be an
22 optimal frequency at which gas vesicles can be collapsed with minimal energy
23 [9, 12, 15]. Also the spatial pattern of the ultrasound field could have an im-

1 pact upon the efficacy of the system. These factors and other still unknown
2 interactions could be the key for the successful application of the ultrasonic
3 treatment for the control of cyanobacteria.

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