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

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

Previous studies on the effects of ultrasound have often focused on gross general impacts upon cell activity, such as cell growth or photosynthetic activity. But by doing so the study of ultrasound induced effects may have been biased toward the region of high acoustic power. The gas vesicles of *M. aeruginosa* can be collapsed with relatively low acoustic intensities, while high power ultrasonic fields are required to cause significant damage to cell division and the photosynthetic mechanisms.
Investigations involving the specific analysis of threshold effects and mechanisms of subtle changes to gas vesicles may lead to the development of a more energy efficient control without the risk of gross cell damage and metabolite release. The collapse of gas vesicles and loss of buoyancy would allow the cells to sink without being lysed.

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

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

2. Prior work

Lehman and Jost [2] were the first to use ultrasound to collapse the gas vesicles that regulate the buoyancy of *M. aeruginosa*. However, at that time,
they did not consider ultrasound as a practical method to control cyanobacte-
rial blooms. It was not until 2001 that Lee et al. [3] proposed that ultrasonic
irradiation could be used to induce the sedimentation of cyanobacteria, and
showed that sedimentation was followed by a reduction in the photosynthetic
activity of the cells.

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

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

The collapse of gas vesicles is accepted as the primary cause for the loss
of buoyancy of vacuolate organisms [10]. Vesicle collapse has an immediate effect on cyanobacterial suspensions, which visibly lose their optical characteristics due to the disappearance of the light-scattering bodies [11]. This effect can be observed within a few seconds after submitting the sample to increased pressure, either with the application of ultrasound [2] or of static pressure [10].

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

3.1. Culturing conditions

*M. aeruginosa* was obtained from the culture collection of the Australian Water Quality Centre (SA Water, South Australia) and cultured in ASM-1 media for 64 days in an incubator (Thermolyne Scientific, Australia) at 20°C under 70 μE/m²/s light intensity with a light/dark cycle of 12/12 hours. A cell count was performed at the start of the experiment giving a concentration of 13.3 · 10⁶ cells/ml.

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 ∂T/∂t was obtained by linear regression. The acoustic power *W* was obtained as

\[
W = \frac{\partial T}{\partial t} C_p M, \tag{1}
\]

where *C*ₚ is the heat capacity of water and *M* is the molar mass of the water. The acoustic power of the system was 8.24 W. A 600 ml volume of *M. aeruginosa* suspension was then exposed to 10 minutes of ultrasound and
the temperature was monitored to ensure it was kept within 22 ± 1°C with external cooling.

3.3. Sample preparation

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

All the samples were left to settle inside an incubator under the same conditions used for culturing. Destructive sampling was used to avoid interfering with the sedimentation process. All measurements were made on three replicates randomly placed inside the incubator.

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 Scatter (SSC). Following the method used in Ref. [3], the percentage of intact gas vesicles was estimated from SSC values as

$$GV(\%) = \frac{SSC_s - SSC_p}{SSC_c - SSC_p} \times 100\%, \quad (2)$$
where the subscript \(s\) refers to a sonicated sample, \(c\) to a control sample with intact gas vesicles, and \(p\) to a control sample whose gas vesicles were collapsed in a pressure vessel (Brister & Co Pty Ltd.) by placing them under 900 kPa of hydrostatic pressure for 2 minutes. Equation (2) normalises the SSC values of the sonicated sample \(s\) between the values of intact \(c\) and collapsed \(p\) gas vesicles, providing a quantitative estimation of the state of the gas vesicles.

Cell granularity was also observed with optical microscopy and monitored with a Live Cell Imaging (Nikon, Japan) system facility at Adelaide Microscopy facility.

### 3.5. Monitoring of the sedimentation process

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

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

This procedure overcomes the potential variability associated with the concentration 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

$$S_{4\text{ cm}} = \left(1 - \frac{C_{s4\text{ cm}}}{C_{c4\text{ cm}}}\right) \cdot 100\%$$ (3)

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

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

It must be also noted that using different depths will yield different values of the sedimentation index and, hence, different descriptions of the same effect. Therefore, only sedimentation indexes $S_d$ using the same depth $d$ could be directly compared. The value for $d$ should be chosen so that $S_d$ properly describes the sedimentation process that is being studied. If $d$ is too small, $S_d$ may become quickly saturated. If $d$ is too large, $S_d$ will be of no practical use.
Our choice of 4 cm correctly describes the sedimentation process during the time span of the experiment and, in addition, it corresponded to half the height of the volume in the glass test tubes. However, a $S_{4 \text{ cm}}$ sedimentation index may not be the right choice to study very intense sedimentation. Hence, different species, treatments or environmental conditions may need of indexes based on other $d$ values.

4. Results

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

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

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

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

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

Figure 1: Reformation of the gas vesicles of a sonicated sample, estimated with Eq. (2).
though a large number of gas vesicles are formed within the first 24 hours, their total volume does not provide sufficient buoyancy to completely stop the sedimentation process. As a result the cells continue to sink for about a week until the total gas volume compensates the higher density of the cell body and finally halts the ongoing sedimentation process.

It must be noted that both the composition of culturing media and the illumination conditions have important effects on cell buoyancy [21]. In Ref. [5] it was observed that cells placed in the dark immediately after sonication could not reform their gas vesicles. Under this condition buoyancy cannot be regained and sedimentation continued until cells had reached the bottom. Under high photon irradiance, photosynthetic cyanobacteria accu-
mulate stores of carbohydrate, which is denser than water. That storage becomes a permanent ballast that could alter the buoyancy regulation mechanisms, inhibiting cells tendency to float [22]. Nutrient enrichment can also affect the buoyancy regulation mechanism providing the cells with persistent positive buoyancy [23].

In this study, we used a *M. aeruginosa* cultured strain which exists as single or pairs of cells in culture. However, in the environment *M. aeruginosa* generally form colonies which are aggregations of several hundred cells. Such aggregation does not alter the average density of the population but reduces the frictional resistance that those organisms suffer when they move in the water columns [24]. As a consequence, colonies present higher flotation and
sinking rates, and that may increase the efficacy of the ultrasound induced sedimentation process. Intra-colony spaces may also play a key role in the buoyancy regulation of cyanobacterial organisms [25], and its removal by means of ultrasound may provide even more drastic sinking rates. However, very little is known about the dose-response curve when cells are in colonial form.

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

6. Conclusions

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

By applying this method we have monitored, for the first time, the sedimentation process over time. Results show, as expected, that sedimentation rate is greatest within the first 24 hours. However, only about 50% of the total sedimentation occurred within the initial 24 hours. Sedimentation did however continue for the next 7 days at a reduced rate. After 14 days in-
cubation a slight recovery of cell numbers was observed, but the number of
cells in the top 4 cm layer was still 82% lower than in the control sample.

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

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

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

Acknowledgement

This work has been funded by the ARC project LP100200366 “Ultrasound for the control of Cyanobacteria”, with the collaboration of Water Quality Research Australia, Melbourne Water Corporation, Water Corporation of Western Australia, South Australian Water Corporation and United Water International Pty Ltd.

We would like to thank Judy Blackbeard for the useful comments she provided.


