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Bubble stimulation efficiency of dinoflagellate bioluminescence

Grant B. Deane, M. Dale Stokes and Michael I. Latz*

ABSTRACT: Dinoflagellate bioluminescence, a common source of bioluminescence in coastal waters, is stimulated by flow agitation. Although bubbles are anecdotally known to be stimulatory, the process has never been experimentally investigated. This study quantified the flash response of the bioluminescent dinoflagellate *Lingulodinium polyedrum* to stimulation by bubbles rising through still seawater. Cells were stimulated by isolated bubbles of 0.3–3 mm radii rising at their terminal velocity, and also by bubble clouds containing bubbles of 0.06–10 mm radii for different air flow rates. Stimulation efficiency, the proportion of cells producing a flash within the volume of water swept out by a rising bubble, decreased with decreasing bubble radius for radii less than approximately 1 mm. Bubbles smaller than a critical radius in the range 0.275–0.325 mm did not stimulate a flash response. The fraction of cells stimulated by bubble clouds was proportional to the volume of air in the bubble cloud, with lower stimulation levels observed for clouds with smaller bubbles. An empirical model for bubble cloud stimulation based on the isolated bubble observations successfully reproduced the observed stimulation by bubble clouds for low air flow rates. High air flow rates stimulated more light emission than expected, presumably because of additional fluid shear stress associated with collective buoyancy effects generated by the high air fraction bubble cloud. These results are relevant to bioluminescence stimulation by bubbles in two-phase flows, such as in ship wakes, breaking waves, and sparged bioreactors. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: bubble; dinoflagellate; *Lingulodinium*; model

Introduction

Bioluminescence, the production of visible light by organisms, is ubiquitous in the world's oceans and found in all regions, at all depths. Dinoflagellates, the most common sources of bioluminescence in surface coastal waters (1,2), use light emission as an anti-predator strategy. Bioluminescence stimulation is also associated with high shear flows created in boundary layers around swimming animals (3–5), in ship wakes (2,6), and in breaking surface waves (2,7,8), leading to spectacular displays of bioluminescence during periods of high cell abundance (2,4,9).

The bioluminescence of dinoflagellates is stimulated by spatial gradients in fluid velocity rather than absolute flow velocity (10–12) and can thus serve as a reporter of local fluid shear stress, making it a unique tool for both field (6,13) and laboratory (7,14–16) flow visualization. Based on laboratory studies of bioluminescence stimulation in cylindrical Couette flow (10,17–19), a statistical model has been developed that predicts bioluminescence intensity as a function of fluid shear-stress level and cell concentration (20). This model has been used to infer levels of fluid shear stress and turbulence in the complex two-phase flows found in the crests of actively breaking waves, which are not easily measured with other means.

Study of the interaction between cells and bubbles has application to a number of subjects. The aerosolization of cells scavenged by bubbles entrained by breaking surf is thought to be part of the dispersal pathway of certain near-shore marine pathogens, such as cholera (21) and brevetoxin, a neurotoxin produced by the dinoflagellate *Karenia brevis*, which forms harmful blooms in the Gulf of Mexico (22). Bubble-induced aeration and mixing in bioreactors creates high stresses that can lyse cultured cells (23–26). In addition, the nighttime stimulation of bioluminescence by bubbles

created by divers and moving platforms such as surface ships and submerged vehicles has important military implications (6,27,28).

Early observations of bubble-stimulated bioluminescence indicated that isolated, ascending bubbles were highly stimulatory of dinoflagellate bioluminescence (Latz and Case, unpublished data). The objective of this study was to quantify bioluminescence stimulation by isolated bubbles and bubble clouds. Isolated bubble experiments determined the relationship between the intensity of bubble-stimulated bioluminescence and bubble size, in which equivalent diameters ranged from 0.3–3 mm, and identified a critical bubble diameter for stimulating bioluminescence. Bubble cloud experiments examined the interactions of bubbles in comparison with isolated bubble results.

Experimental

Organisms

The study was conducted with cultures of *Lingulodinium polyedrum* (Stein) Dodge 1989, a well studied dinoflagellate in terms of general biology (29) and flow responses (17–19,30,31). *L. polyedrum* is a coastal species of 35 µm in diameter (32) that is responsible for extensive blooms (33,34) with dramatic nighttime

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Abbreviations: CCD, charged coupled device; PMT, photomultiplier tube.

displays of bioluminescence (9). *L. polyedrum* was grown in half-strength f/2 medium (35) minus silicate at 20°C on a 12 h:12 h light:dark cycle. Only cultures in mid-exponential growth phase, i. e., 5–20 days after inoculation, were used for testing. Cell concentrations were determined from cell counts of replicate defined volumes (usually 10 µL) using a stereomicroscope.

Bubble apparatus

The bubble apparatus (Fig. 1) consisted of a bubble stimulation tank constructed out of clear acrylic with dimensions 15.2 × 15.2 cm square and 47.5 cm high and placed within a 61 cm diameter spherical light integrating chamber. The spherical chamber had ports for video imaging and light measurements. An air line terminating in a Swagelok fitting was plumbed through the base of the tank to supply air to the bubble injection system. The air injection rate was controlled using a needle valve placed between the laboratory air supply and the acrylic tank. Syringe needles of varying gauge were attached to the gas line terminus to create bubbles with different diameters. An ITC 1089D broad-band hydrophone (1–300 kHz) was placed within the stimulation tank approximately 1 cm to the side of the nozzle to record the acoustic emissions of individual bubbles as they were released (Fig. 2a).

Stimulated bioluminescence was imaged with a Roper Scientific Cascade:512B electron multiplication charged coupled device (CCD) camera system fitted with an f/0.95 Schneider Optics lens used at the widest aperture. The camera was positioned 75 cm from the test tank, providing a view of the entire tank volume through an 8.9 × 24 cm opening in the wall of the integrating chamber. Image analysis was limited to the region of the tank through which the bubbles rose, thereby eliminating light from other regions of the tank, such as spontaneous flashes and bioluminescence stimulated by bubbles bursting at the surface. Camera settings, image acquisition, and image analysis were controlled using MetaMorph software (Molecular Devices, LLC). Bioluminescence was also measured with an Electron Tubes model P10232

photomultiplier tube (PMT) detector fitted with a Vincent Associates electronic shutter (36). The PMT signal was recorded synchronously with the output from the 1089D hydrophone using custom code written with Labview software (National Instruments Corp.).

Toward the end of the light phase, when dinoflagellate bioluminescence is not mechanically stimutable (37), a sample from the culture was diluted with glass fibre filtered seawater to a final concentration of 100 cells mL⁻¹; for isolated bubbles ≤ 0.5 mm a higher concentration of 500 cells mL⁻¹ was used. The diluted culture was then gently added to the test chamber, which remained undisturbed until testing. The apparatus was further swathed in black cloth to minimize light leakage during data collection, which took place in a darkened room at the culture temperature of 20°C.

Bubble generation

Single, isolated bubbles were generated by releasing air through nozzles and generating small electrolysis bubbles with an electrode. Bubble clouds were created by forcing air through two grids of different spacing (0.135 mm and 1 mm), an air stone made of ceramic, and another made of wood.

Nozzle release of isolated bubbles. The nozzles released air bubbles with radii in the range 1–3 mm at a rate controlled by adjusting a needle valve. A typical data collection run was 100 sec in duration. Bubbles were released at a nominal rate of 1 every 2 sec, yielding 20–40 stimulation events per run. When a bubble detaches from a nozzle, it produces an exponentially decaying sinusoidal pulse of sound (38). The frequency of the sound pulse is inversely proportional to the radius of the bubble, according to the relationship (39):

$$f_0 \approx \frac{3.3}{R_b} \quad (1)$$

where f_0 is the frequency in Hz and R_b is the bubble radius in mm. The hydrophone recording was used to determine the bubble radius, in addition to timing their release and providing a total count of bubbles released.

During experiments, the air flow was adjusted so that individual bubbles were released every few seconds, as indicated by the acoustic pulse (Fig. 3a), which lasts for only a few tens of milliseconds, so the pulses show up as thin, vertical bands when displayed. The PMT record shows bursts of light synchronized with the bubble release. The terminal velocity of a contaminated 1.3 mm radius air bubble in water is approximately 21 cm sec⁻¹ and the depth of the water from nozzle to surface was approximately 13 cm. Neglecting the time taken for the bubble to reach its terminal velocity, the expected rise and stimulation time for cells is approximately 0.6 sec. Most of the stimulated bioluminescence occurred within 0.6 sec or so of the acoustic pulse (Fig. 3b). However, considerable numbers of flashes that occurred after the bubble had finished rising through the tank were associated with two additional processes. The first additional stimulation mechanism is spontaneous emission from cells near or on the bottom of the test tank. Spontaneous flashes are well known to occur in *L. polyedrum* (37,40,41). The second mechanism occurs when bubbles bursting at the surface stimulated bioluminescence; bubbles encountering the surface remained for a short period of time before bursting. These two mechanisms accounted for more than half of the stimulated cell emission, and, because of the nature of the light integrating chamber, could not be eliminated from the PMT recordings.

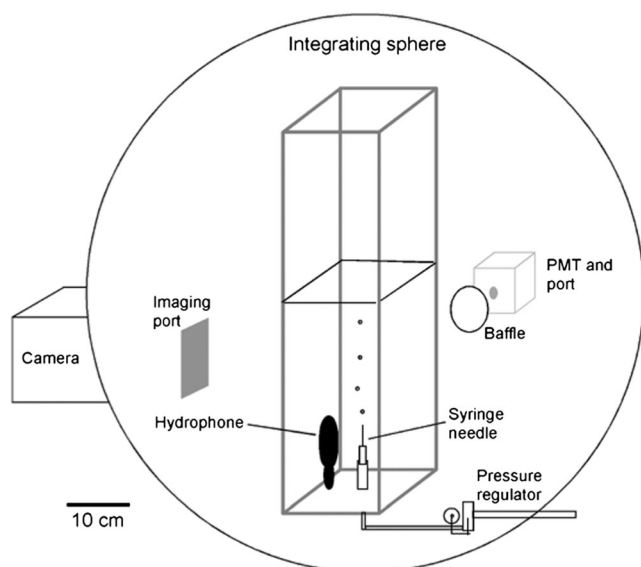


Figure 1. Schematic of the acrylic bubble stimulation tank showing the syringe needle bubble injector and hydrophone inside the integrating light collection chamber. An imaging camera views the tank through a port on one side of the integrating sphere, and a photomultiplier tube (PMT) is positioned behind a second port oriented at right angles to the imaging camera (behind the sphere as drawn in the schematic), viewing a baffle so that it measures integrated light intensity.

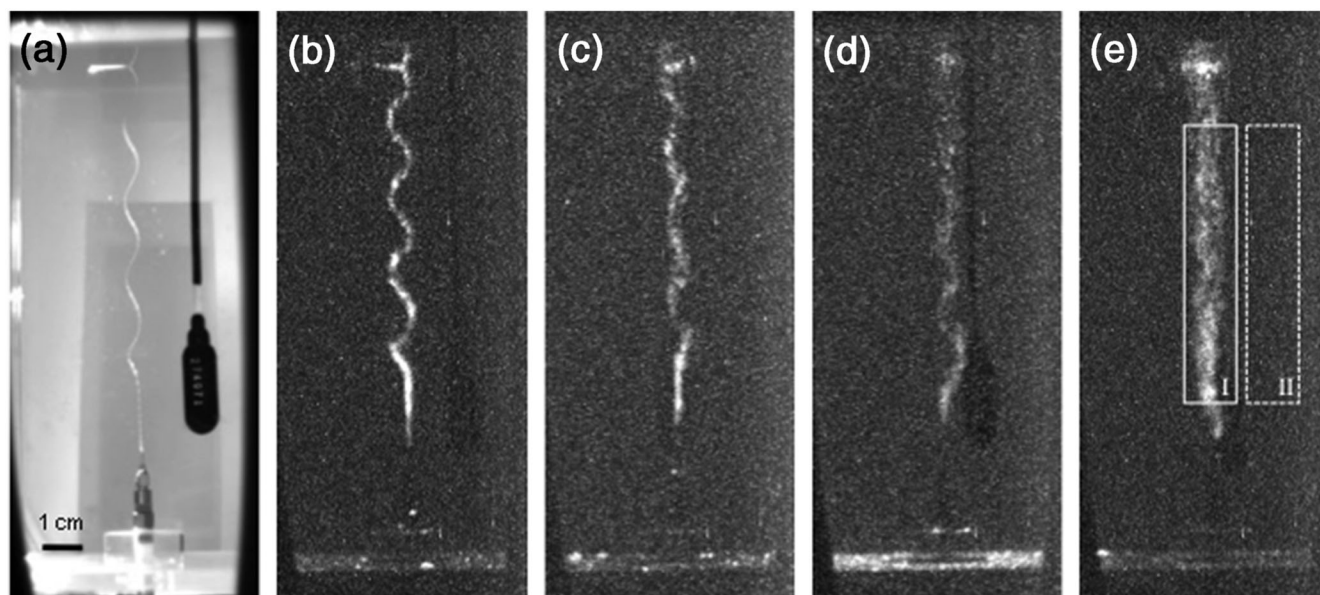


Figure 2. Side view of the acrylic bubble stimulation tank showing the results from a stream of isolated bubbles. (a) White light image showing the nozzle injection system and hydrophone. The composite image shows a stream of 1.8 mm radius bubbles formed by a 20 gauge needle and bursting at the surface. Panels (b–e) show a single 90 sec duration time exposure image of the bioluminescence of *Lingulodinium polyedrum*, at a concentration of 100 cells ml^{-1} , stimulated by isolated bubbles, and have a similar pixel grey scale range. (b) Bioluminescence stimulated by 39 bubbles of 1.3 mm radius. (c) Bioluminescence stimulated by 24 bubbles of 1.5 mm radius. (d) Bioluminescence stimulated by 26 bubbles of 1.9 mm radius. (e) Bioluminescence stimulated by 12 bubbles of 3.0 mm radius. For analysis of bioluminescence intensity, flashes within the solid, white rectangle labelled region I are associated with the rising bubbles. The dashed rectangle labelled II is a control region that is used to estimate the background light level. Contaminating light from cells flashing at the base of the tank and bursting bubbles at the water surface are thus eliminated from analysis.

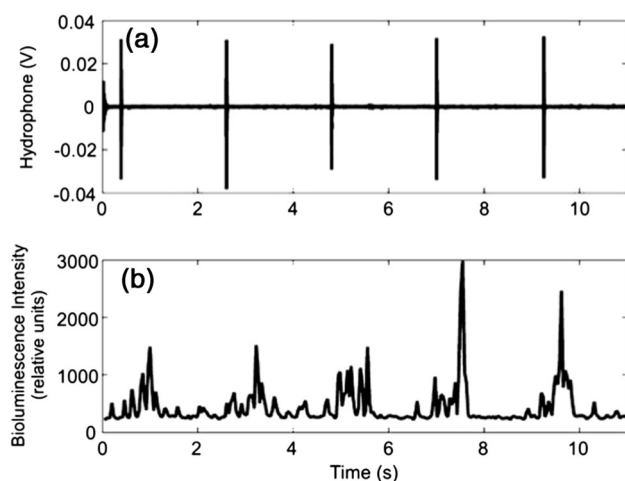


Figure 3. Representative record of synchronized time series of five bubble releases. (a) Hydrophone output. (b) PMT measurement of bioluminescence of *Lingulodinium polyedrum* stimulated by each rising bubble.

A second methodology involving imaging was developed to eliminate the contribution of bioluminescence from spontaneous flashes and bursting bubbles. The acrylic test tank was imaged by a low-light camera, providing a view that revealed the position of flashing cells. This approach allowed image analysis to be limited to a field of view that included the region of water through which bubbles rose, thereby eliminating spontaneous emission associated with the tank bottom and emission stimulated by bubbles bursting at the surface. Due to the high 16-bit pixel resolution and gain stability of the camera, images could be used as quantitative measurements of light emission using standard image processing techniques.

Images of bubble-induced bioluminescence (Fig. 2) were analyzed as follows. Single frame exposures of either 90 or 120 sec were translated into a rectangular matrix of pixel intensities p_{ij} , where i, j denote row and column index, respectively. A rectangular region of image (the solid rectangle annotated II in Fig. 2e) was selected to estimate the background counts from stray illumination and electronic noise from the camera. The background pixel intensity within the region was calculated using:

$$P_{bk} = \frac{1}{n_{bk}} \sum_{\text{Region II}} p_{ij} \quad (2)$$

where n_{bk} is the number of pixels in the background region. The total light output in the analysis region (the dashed rectangle annotated I in Fig. 2e) was then found by summing all the pixel values in the analysis region minus the average background:

$$P_{tot} = \sum_{\text{Region I}} (p_{ij} - P_{bk}) \quad (3)$$

The number of flashes contributing to P_{tot} can be estimated if the light output for a single flash, P_{sgl} , is known. This number was estimated by identifying a number of isolated flashes in the images and summing the light output (minus the background) for each one and then averaging. With an average P_{sgl} determined, the total flashes stimulated per bubble, f , is given by:

$$f = \frac{1}{n_B} \frac{P_{tot}}{P_{sgl}} \quad (4)$$

where n_B is the number of bubbles released during the exposure time, determined by counting the acoustic pulses in the synchronized hydrophone record.

Electrolysis production of isolated bubbles. As the nozzle release method did not provide a reliable stream of bubbles smaller than

a 1.3 mm radius, smaller bubbles were generated by electrolysis on glass-encased, platinum electrodes. The electrodes were fabricated by melting a glass capillary tube over fine platinum wire and then abrading the wire until it was flush with the glass tip. The generation of bubbles on the electrode tip depended sensitively on the microscopic topography of the tip end, but the fabrication of several electrodes provided one that reliably produced a stream of isolated bubbles. The selected electrode was attached to the syringe mount near the base of the acrylic tank (Fig. 4a) and used as a cathode to produce bubbles of hydrogen gas. A second electrode, encased in reticulated, small-cell foam, was placed at the top of the tank. Without the foam jacket, hypochlorous acid created by chlorine gas released by the anode diffused through the tank and stimulated considerable levels of bioluminescence due to the low pH, which directly activates the dinoflagellate light emitting chemical reaction (42,43). The electrolysis setup was successful in producing small bubbles (Fig. 4b).

Bubbles were generated by controlling the passage of current between the two electrodes with a custom-fabricated, charge-control system. This system controlled both the duration of the current bursts and their frequency. As the volume of gas produced at the electrode tip is directly proportional to the total charge passed between the electrodes, this method allowed for a precise control of bubble radius from 50 to 500 μm , and precise control of the bubble release rate. The electrode method has the disadvantage that a cap of magnesium and calcium electrolysis byproducts builds up over the electrode and eventually disrupts bubble generation. This undesirable effect was managed by pre-conditioning

the electrode with a large current burst. The continuous stream of bubbles generated in this way acted to dislodge the scale and clean the electrode. Once conditioned, the electrode would reliably generate a stream of roughly 100–200 bubbles before it needed reconditioning. Bubble release was checked at the beginning and end of all electrode experiments and any runs terminating in unreliable bubble release were discarded.

The electrode was calibrated by determining the relationship between charge flux and bubble size. The 500 μm radius bubble was sufficiently large to have its size determined from photographs taken through the side wall of the test tank. The smallest bubble was too small to be sized by imaging, so its radius was inferred by measuring its ascent velocity and assuming the bubble was not contaminated with surfactants, as the relationship between bubble size and terminal velocity in seawater is well established (44). Because the volume of hydrogen gas produced on the cathode scales directly with total charge, knowing the diameter of a single bubble and its associated charge flux is sufficient to determine the bubble size versus charge flux relationship for the entire range of bubbles produced. However, measuring the radii and associated charge fluxes of two bubbles at the extreme ends of the bubble size range provided redundant data and enabled us to cross-check the electrode performance across its working range. The theoretical estimates of the smallest bubble diameter, based on measurement of its associated charge flux and the charge flux, and size of the largest bubble were consistent with the radius inferred from the velocity measurement.

Bubble clouds. Two experiments were conducted to study the stimulation of cells by clouds of bubbles. The first made use of two mesh screens with different grid sizes (1 and 0.135 mm grids) placed across the outlet of a short segment of 4.2 cm diameter PVC pipe, which was mounted on the same air outlet at the base of the test tank that we used for the isolated bubble tests (Fig. 5a). A fixed volume of air was manually injected through the grids from a syringe connected to the base of the test tank. Air was injected at different rates to check the sensitivity of cell stimulation to variability in the flow rate between runs. Single frame exposures of bioluminescence, typically 10 sec in duration, were taken for air injection volumes of 10, 20, 40 or 60 ml (Fig. 5).

For measuring bubble size and abundance, bubble clouds were separately imaged at 250 frames sec^{-1} with a Kodak Motion Corder analyzer through the glass wall of an aquarium filled with salt water. Selected images were analyzed using the NIH Image program (<http://rsb.info.nih.gov/nih-image/>). Bubbles in the images were manually sized and counted by drawing ellipses around bubble edges and determining the equivalent bubble radii from the measured cross-sectional areas. Results from multiple frames were combined to produce the estimates of the bubble size distributions (Fig. 6). The 0.5-mm grid fragments a fixed volume of air into a larger number of smaller bubbles than the 1-mm grid.

Although the mesh experiments served to demonstrate that, in general, the stimulating capacity of a fixed volume of air decreases as the volume is broken up into smaller bubbles, they had the problem that occasional large bubbles would escape through the mesh without fragmentation. Because we wished to make a detailed comparison between the isolated bubble model developed in the section on 'Analysis of the air stone bubble cloud data' with the bubble cloud stimulation, and because the presence of very large bubbles lay outside of the range of single bubble sizes investigated, we performed additional bubble cloud experiments with air stones.

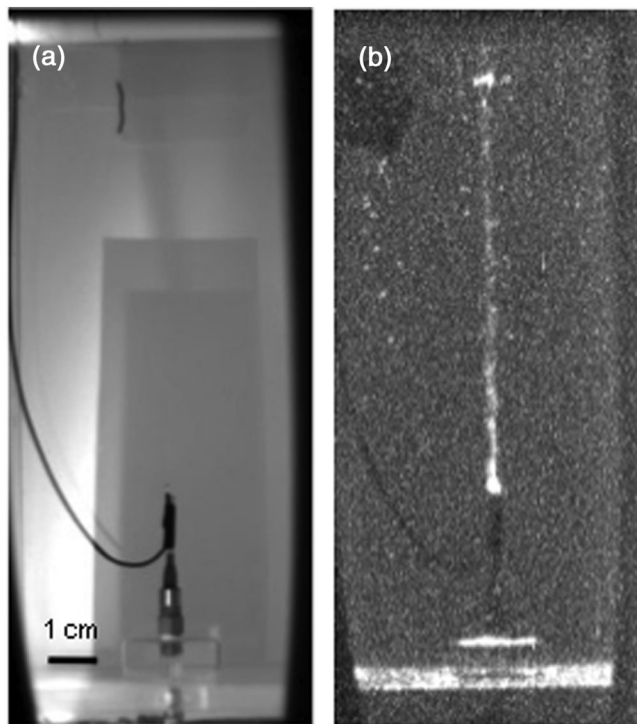


Figure 4. Bioluminescence of *Lingulodinium polyedrum* stimulated by small bubbles released from an electrode. (a) Image of the electrode assembly taken through the side wall of the stimulation tank. The electrode at the upper left was not encased in its foam jacket for the picture. (b) Single 90 sec time exposure image of bioluminescence stimulated by a stream of 0.5 mm radius bubbles released from the electrode. Due to the long exposure time, the bubble-stimulated bioluminescence appears as a light vertical streak directly above the electrode. The region of light at the electrode tip was apparently caused by microscopic spark discharges at the electrode end that were sufficiently bright to be imaged by the camera. This source of light was excluded from the subsequent image analysis.

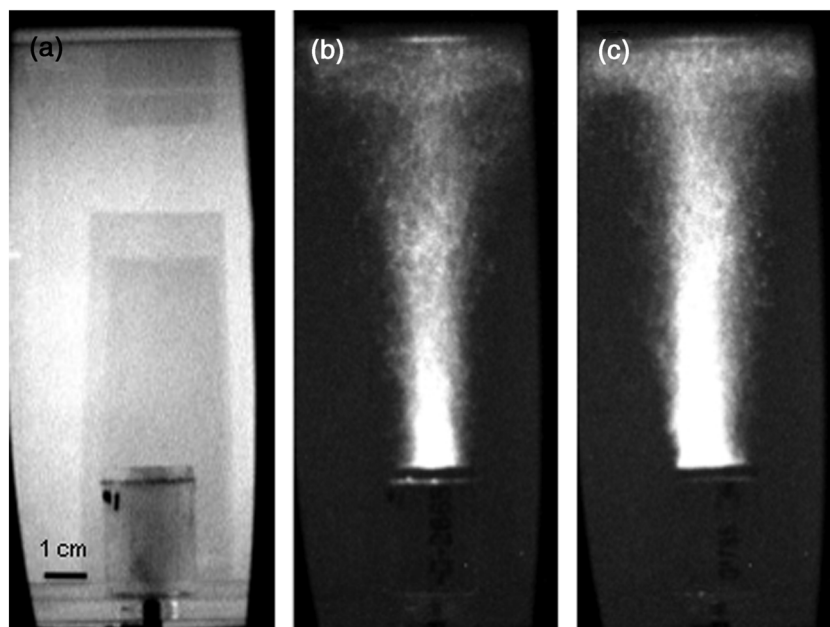


Figure 5. Bioluminescence of *Lingulodinium polyedrum* stimulated by a cloud of bubbles created by forcing 60 ml of air through a 1-mm sized mesh. The cell concentration was $100 \text{ cells ml}^{-1}$. (a) Single 30 msec image of the chamber showing the mesh holder positioned over the air orifice. (b) Single 10 sec time exposure image of bioluminescence stimulated by a 1-mm mesh. (c) Single 10 sec time exposure image of bioluminescence stimulated by a 0.135-mm mesh.

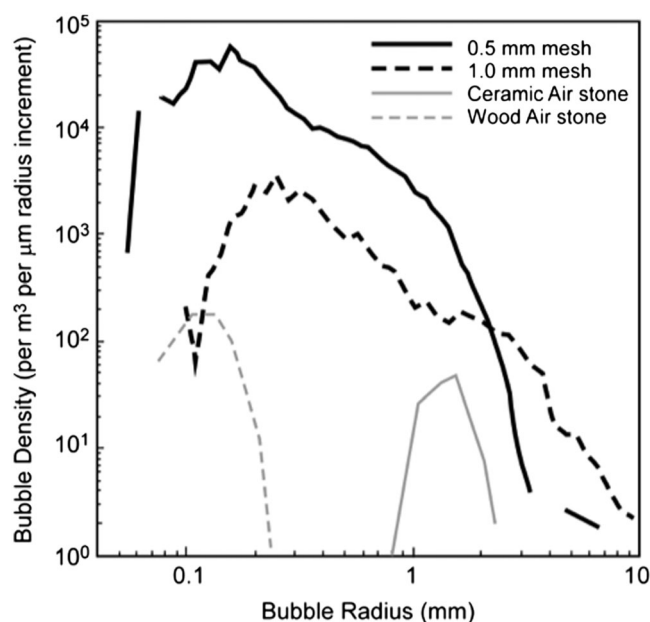


Figure 6. Bubble size distributions for clouds of bubbles. Bubbles were created by forcing air through a 0.5-mm mesh (solid black line) or 1-mm mesh (dashed black line). The smaller mesh resulted in a peak in the bubble distribution at about $150 \mu\text{m}$ radius and a rapid decrease in bubble density at around 1 mm radius. The larger mesh resulted in a peak at about $200 \mu\text{m}$ radius and a relatively larger number of bubbles greater than 2 mm radius. Bubbles were also created by air stones, which created bubbles within a narrow size range, with a peak of 1.5 mm radius for the ceramic air stone (solid grey line) and 0.14 mm radius for the wood air stone (dashed grey line). As volume air fraction was not measured for the air stone bubble clouds, the results were scaled to a 0.05% air fraction for the ceramic air stone and a $1.6 \times 10^{-5}\%$ air fraction for the wood air stone so that the size distributions could be displayed along with that of the mesh-created bubbles.

Two air stones were used to produce a well defined and controllable cloud of bubbles, labelled 'ceramic' and 'wood'. The gas pressure at the air stone inlet was varied and the resulting air flow rate

was determined by timing the interval required to fill a known volume within a graduated flask inverted above the air stone. Bubble size distributions were determined by analyzing photographic images following the same procedure used to determine the mesh bubble distributions. Experiments were conducted to measure the bioluminescence stimulation potential for both air stones as a function of air flow rate, as discussed in the section 'Analysis of the air stone bubble cloud data'.

The number of flashes per ml of air injected through the mesh at the base of the chamber was calculated by first dividing the total light stimulated by the volume of air injected during the 10 sec integration time, then dividing this number by the light emission from a single flash. Background levels were estimated from an image taken with no injected air.

Results

Isolated bubble stimulation

Bioluminescence stimulation was highly correlated with the size of isolated bubbles (Fig. 7). There is a critical bubble radius of 0.325 mm in that smaller bubbles were not observed to stimulate light emission greater than background levels. Bubbles larger than 1 mm radius were much more effective in stimulating bioluminescence than smaller bubbles.

Mesh bubble cloud stimulation

Single frame images of bubble cloud stimulation with an exposure time of 10 sec (Fig. 5) were analyzed in a similar manner as for individual bubbles, in which light emission due to flashes at the tank bottom or surface was excluded.

Total light increased with increasing air volume (Fig. 8a) based on the average image pixel value from bubble cloud stimulation for the 1-mm and 0.5-mm grids. The 0.5-mm grid created a bubble cloud that was less efficient at stimulating bioluminescence than

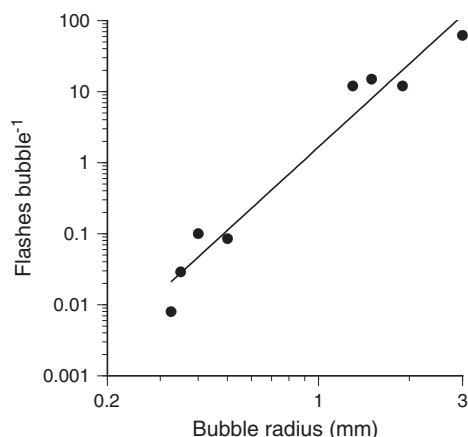


Figure 7. Bioluminescence of *Lingulodinium polyedrum* stimulated by individual bubbles. Bioluminescence response expressed as flashes per bubble was highly correlated with bubble radius, according to the log-log relationship $y = 1.70 * x^{3.89}$ ($R^2 = 0.96$).

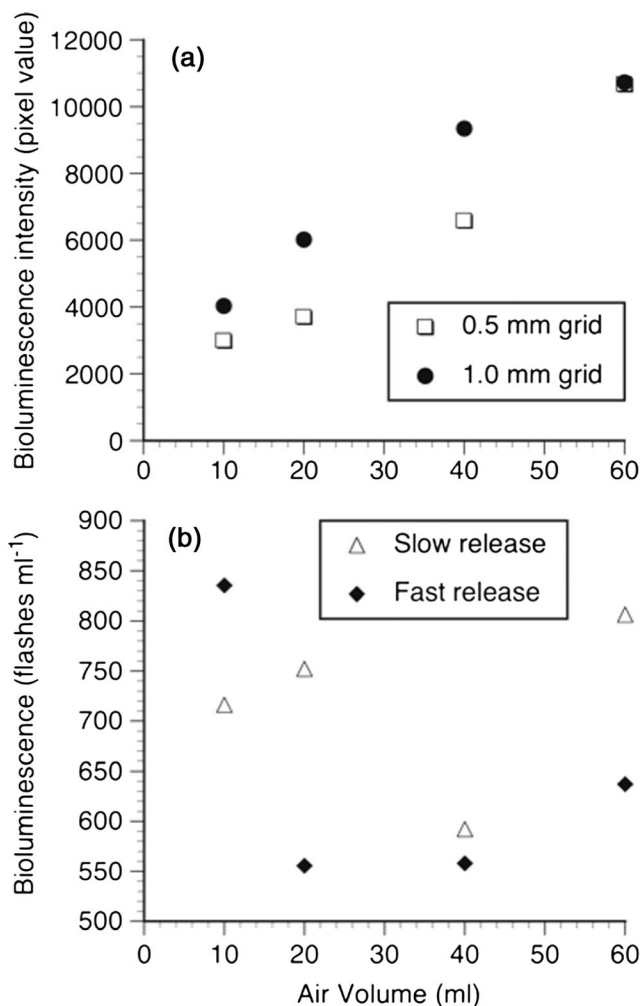


Figure 8. Bioluminescence of *Lingulodinium polyedrum* stimulated by bubble clouds. (a) Bioluminescence intensity, expressed as average pixel grey scale value, from images of bioluminescence of *Lingulodinium polyedrum* stimulated by a cloud of bubbles as a function of volume of air forced through a 0.5-mm mesh (squares) or a 1-mm mesh (circles). (b) Bioluminescence of *Lingulodinium polyedrum*, expressed as number of flashes mL^{-1} , stimulated by a cloud of bubbles as a function of the volume of air forced through a 0.5-mm mesh. Two relative speeds of air release were tested: slow (triangles) and fast (diamonds).

the 1-mm grid for the same volume of air injected. As discussed later, this result is a consequence of the tendency of the 0.5-mm grid to fragment a fixed volume of injected air into a larger number of smaller bubbles than the 1-mm grid, with the overall effect of reducing the stimulation capacity.

The number of flashes per ml of air injected was independent of both injection rate and total volume of air injected (Fig. 8b). The overall response rate was 680 ± 110 flashes per mL, reflecting a 16% variation.

Air stone bubble cloud stimulation

The bubble size distributions from the air stones were narrow compared with the mesh bubble clouds; the radius was 1.5 ± 0.3 mm for the ceramic air stone and 0.14 ± 0.03 mm for the wood air stone (mean with standard deviation) (Fig. 6). Thus the ceramic air stone produced bubbles that were slightly more than a factor of 10 larger in mean radius than those produced by the wood air stone.

Single frame images of air stone stimulation with an exposure time of 1 sec were analyzed in a similar manner as for individual bubbles, in which a test region was selected so that light emission due to flashes at the tank bottom or surface was excluded. Bioluminescence increased as a function of air flow rate, but was always approximately two times greater for the ceramic air stone, which produced larger bubbles (Fig. 9).

Analytical models for cell stimulation

This section details the development of a simple collision-based interpretation of the bioluminescence stimulated by isolated bubbles and a theoretical framework for understanding bubble cloud stimulation in terms of a probabilistic stimulation model developed by Deane and Stokes (20).

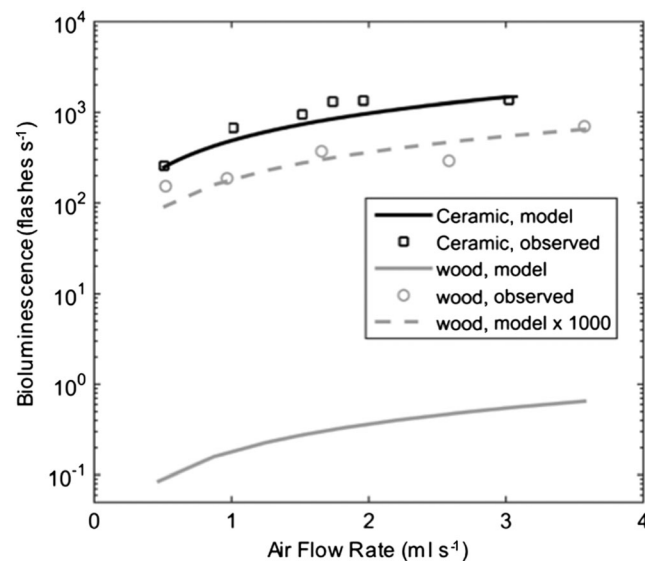


Figure 9. Observed and modelled bioluminescence of *Lingulodinium polyedrum* stimulated by bubbles from air stones as a function of air flow rate. Bioluminescence, expressed as flashes sec^{-1} , was stimulated by bubbles from the ceramic air stone with a mean bubble radius of 1.5 mm (open squares), and predicted based on the isolated bubble stimulation model (solid line). Bioluminescence was stimulated by bubbles from the wood air stone with a mean bubble radius of 0.14 mm (open circles) and predicted based on the model for isolated bubble stimulation (solid grey line). When the model prediction for the wood air stone is multiplied by 1000 (grey dashed line) it better fits the experimental data.

A simple model for single bubble stimulation. The single, isolated bubble stimulation model is based on the experimental finding that, when accounting for cell concentration and bubble swept volume, the number of flashes stimulated by an isolated bubble scales with the bubble cross-sectional area for bubbles larger than the critical bubble radius. One interpretation of this observation is that stimulated emission is the result of collisions between the bubble and cells encountered within the volume swept out as it rises, with each collision having a finite probability of resulting in a flash.

The model starts with flash probability, η , defined as the observed number of flashes stimulated per bubble divided by the number of cells encountered in the volume swept out by the bubble during its ascent from the nozzle to the surface. The rise trajectories of bubbles greater than approximately 1 mm radius can take a spiral path depending on water temperature and nozzle exit conditions. We have ignored this complication here and assume that all bubbles rise to the surface in a straight line path. With this assumption, a formula for the stimulation efficiency, defined as the fraction of bubbles encountered that flash, is given by:

$$\eta = \frac{f}{\pi a^2 h C} \quad (5)$$

where f is the number of cells stimulated to flash, a is the bubble radius, h is the distance from the end of the nozzle to the water surface and C is the cell concentration. Stimulation efficiency, calculated using eqn (5) and values for f obtained from Fig. 7 (Fig. 10a), for bubbles larger than 1 mm radius suggest that these bubbles stimulate a constant fraction of the cells they encounter (approximately 1 in 24 cells for *L. polyedrum*) during their ascent to the surface. For the three smaller bubbles in the size range 0.325–0.5 mm radius, the probability of stimulation tended to decrease with decreasing bubble size, the stimulation efficiency for the 0.5-mm radius bubble being an exception to this rule. Isolated bubbles smaller than 0.3 mm radius were not observed to stimulate bioluminescence in *L. polyedrum* at all for the cell concentrations studied.

From eqn (5), the number of cells stimulated by a bubble is given by:

$$f = (\pi a^2 h C) \eta \quad (6)$$

As the flash probability η is approximately constant for bubbles in the size range 1–3 mm radius, the total number of cells stimulated by a bubble in this size range scales with the cross-sectional area of the bubble, or bubble radius squared. The flash probability shows a very different dependence on bubble radius for smaller bubbles, decreasing rapidly with decreasing bubble radius.

The overall shape of the relationship between stimulation efficiency with bubble radius has features in common with bubble terminal rise velocity (Fig. 10b), based on the terminal velocity expression for contaminated seawater (45; eqn 2.11), as the high concentration of dinoflagellates in the test solution most likely resulted in the presence of surface-active exudates. Bubble terminal velocity is approximately constant for bubbles with radii in the range 1–3 mm, and scales with radius to the power of roughly 1.5 for bubbles smaller than a 1 mm radius.

Based on the roughly constant stimulation efficiency observed for bubbles in the 1–3 mm range, and the rapid decrease in stimulation efficiency for smaller bubbles, we explored a purely empirical description of bubble-stimulated bioluminescence in terms of

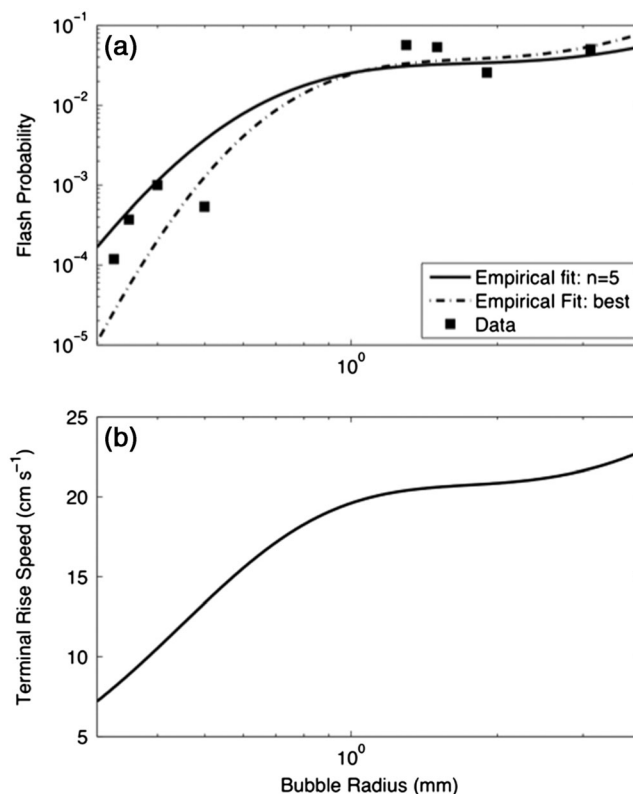


Figure 10. Probability of bioluminescence stimulation of *Lingulodinium polyedrum* by isolated bubbles. (a) Experimental data showing the probability that a cell encountering an ascending bubble will stimulate a flash as a function of bubble radius. The solid black and dash-dot black curves show two least-mean-squares empirical-model fits for flash probability as a function of bubble terminal velocity. The dash-dot line shows the best overall fit. The solid black line shows the best fit with the flash probability constrained to fixed power of terminal velocity (see text for details). (b) Bubble terminal rise speed as a function of bubble radius for contaminated bubbles.

bubble terminal velocity. The bubble-stimulated flash probability has been expressed as u_b raised to the power of η_{emp} and multiplied by a constant α_{emp} resulting in the empirical formula:

$$\eta_{emp} = \alpha_{emp} u_b^{\eta_{emp}} \quad (7)$$

Values for η_{emp} and α_{emp} were determined using a least-mean-squares analysis of the residues between the data in Fig. 7 and eqn 7, where the residue was calculated using:

$$R = \sum_{k=1}^8 \frac{(\eta_k - \eta_{emp,k})^2}{\eta_k} \quad (8)$$

where the index k runs from 1 to the total number of bubbles studied and $\eta_{emp,k}$ was calculated using eqn (7) and Fan and Tsuchiyas' formula (45) for contaminated bubble terminal rise speed given the k th bubble radius. The residue has been normalized by $1/\eta_k$ to increase the weight of the small probabilities encountered for the small bubbles studied. As η_k is dimensionless, the residue is also dimensionless.

Two analyses were performed (Fig. 10). The first employed a global, two-parameter search across values for η_{emp} and α_{emp} to find the minimum residue defined by eqn (8). This search yielded the values $\eta_{emp} = 7.704$ and $\alpha_{emp} = 6909$ for the best-fit curve (Fig. 10a, dashed line). A study of the behaviour of the residue R

showed that there is an ambiguity between η_{emp} and α_{emp} ; pairs of these parameters tended to give good fits to the data with α_{emp} increasing with increasing η_{emp} . This behaviour motivated a second analysis, which was to find the value of α_{emp} that minimized the residue for a fixed value $n_{emp} = 5$, which provided a better fit for the small-sized bubbles. This power law scaling was chosen to minimize the dependence of flash probability on terminal velocity, while keeping the minimum residue within a factor of 2 of the global minimum. The result of this analysis was a value of $\alpha_{emp} = 87.04$ for the empirical probability (Fig. 10a, solid line). Both curves give an adequate fit to the data for bubbles greater than 1 mm radius. The $\eta_{emp} = 5$ value gives a superior fit to the flash probabilities for small bubbles, if it is accepted that the flash probability observed for the 0.5-mm radius bubble is anomalously low.

Stimulation by clouds of bubbles. A model for cell stimulation by clouds of bubbles is accomplished by calculating the value of shear stress throughout a volume of pure fluid that would have the same stimulating capacity as the total collection of rising bubbles, neglecting bubble–bubble interaction effects. By neglecting bubble–bubble interaction effects, we assume that bubbles within a cloud have the same stimulating capacity as isolated bubbles. Thus, the potential for cells to be stimulated by buoyancy-driven shear instabilities at the edge of the bubble cloud, for example, is not accounted for. The motivation for this model development is to simplify the computational fluids dynamics problem of determining bioluminescence stimulation in two-phase flows. Once an equivalent model has been formulated, cell stimulation by bubbles and fluid shear stress can be treated in a uniform manner in fluid dynamic calculations of the stimulatory effects of air-entraining breaking waves, or ship wakes, for example.

The objective is to describe the stimulation of bioluminescence by bubbles in terms of the same theoretical construct presented by Deane and Stokes (20) for the stimulation of bioluminescence by fluid shear stress in a single phase flow. The theoretical formulation begins with the assumption that the probability P_0 of a cell flashing over some small time interval Δt is proportional to the product of the time interval with a parameter λ that depends on the cell species, and potentially the cell's history of prior stimulation:

$$P_0 = \lambda \Delta t \quad (9)$$

For a population of N cells, the mean number of cells that flash per second is:

$$\Theta = \lambda N \quad (10)$$

in which case λ is the mean flash rate of the population subjected to a specified fluid shear stress. Assuming that the N cells are distributed uniformly throughout a sample of volume V , then the number of cells that flash per second, per cubic metre of volume, Ξ , is given by:

$$\Xi = \lambda \frac{N}{V} = \lambda C \quad (11)$$

where C is the cell concentration in cells per cubic metre. Rearranging eqn (11) yields an expression for λ in terms of cell concentration and Ξ :

$$\lambda = \frac{\Xi}{C} \quad (12)$$

The objective is to compute a value of λ for a cloud of bubbles, assuming that the bubbles stimulate cells uniformly throughout

the volume V , and this computation can be done by determining the total number of cells that flash per second per cubic metre for a specified cell concentration, and then using eqn (12).

Consider a single bubble of radius a rising through the water column, which contains a concentration of C cells per cubic metre. The number of cells stimulated by the bubble, N_a , is given by:

$$N_a = \eta C V_a \quad (13)$$

where η is the stimulation efficiency of the bubble and V_a is the volume swept out by the bubble, which is $V_a = \pi a^2 h$, where h is the length of the bubble trajectory. The number of cells stimulated per second by the rising bubble, Θ_a , is just N_a divided by the bubble ascent time τ :

$$\Theta_a = \frac{N_a}{\tau} = \eta C \pi a^2 \frac{h}{\tau} = \eta C \pi a^2 u_a \quad (14)$$

where N_a is computed using eqn (13) and u_a is the bubble terminal velocity.

Equation (14) applies to a single bubble. Now suppose that the volume of water contains $n(a)$ bubbles per cubic metre, per metre radius increment. The number of bubbles per cubic metre in the radius range $a_0 - \Delta a/2 \leq a < a_0 + \Delta a/2$ is given by $n(a_0)\Delta a$, and the number of cells stimulated per second per cubic metre by these bubbles is given by the product of the cells stimulated per second by a single bubble multiplied by the number of bubbles within the radius increment:

$$\theta_a = \Theta_a n(a) \Delta a \quad (15)$$

The total number of cells flashing per second per cubic metre is the integral of θ_a over the range of bubble radii present:

$$\Xi_a = \int_{a_0}^{a_1} \Theta_a n(a) da = C \int_{a_0}^{a_1} \eta_a \pi a^2 u_a n(a) da \quad (16)$$

where a_0 , a_1 respectively, are the smallest and largest bubbles in the distribution, and eqn (14) has been used to substitute for Θ_a . Note that $n(a)$ is often encountered in the bubble literature with units of bubbles per cubic metre per micrometre radius increment, and with these units the right hand side of eqn (16) needs to be multiplied by 10^6 to make the calculation correct. From eqns (12) and (16), λ_a is given by:

$$\lambda_a = \pi \int_{a_0}^{a_1} \eta_a a^2 u_a n(a) da \quad (17)$$

where both η_a and u_a are functions of bubble radius and $\lambda_a \Delta t$ is the probability that any individual cell within the total population of cells interacting with the cloud of rising bubbles will be stimulated to flash in the time interval Δt . Equation (17) provides a means of computing a flash probability that is indistinguishable from the flash stimulating potential of fluid shear stress, and thus provides a simple means of comparing the light emission from bubble clouds with that of fluid turbulence, for example.

Calculation of the cell flash rate requires knowledge of all the terms in eqn (17) as a function of bubble radius: the bubble stimulation efficiency, the bubble rise velocity, and the bubble size distribution. Our current, best estimate for the stimulation efficiency is given by the velocity power law of eqn (7) with $n_{emp} = 5$ and $\alpha_{emp} = 87.04$.

Equation (17) has some deficiencies. An important assumption in its derivation is that collective buoyancy effects can be neglected. This assumption may be adequate when air fractions are low and bubbles are large (>0.7 mm radius), for which stimulation efficiencies are greater than 0.01 (Fig. 10a), but it breaks

down in the case of high air fraction clouds of small bubbles. In this case, the observed stimulation efficiencies of isolated bubbles are small (see Fig. 10a) but fluid turbulence within the bubble cloud due to collective buoyancy can still drive cells to flash. An example of this situation is described in section 'Analysis of the air stone bubble cloud data'.

Analysis of the air stone bubble cloud data

A model for the bioluminescence stimulated by the bubble clouds generated by the ceramic and wood air stones is formulated as follows. As the size distribution of bubbles injected by each air stone is relatively narrow, it is assumed that all bubbles are created with the mean bubble radius. This assumption yields reasonable results provided that the bubble stimulation efficiency is a slowly varying function over the range of bubble sizes produced by the air stone. In this case, the bubble size distribution is approximated by:

$$n(a) = N_m \delta(a - a_m); \quad (18)$$

$m = 1 \text{ for ceramic, } m = 2 \text{ for wood}$

where a_m and N_m respectively are the mean bubble radius and the number of bubbles per cubic metre generated by the ceramic or wood air stone. Substituting eqn (18) into eqn (17) yields:

$$\lambda_a = \pi N_m \eta_a a_m^2 u_a = \pi \bar{N}_m \eta_a a_m^2 h \quad (19)$$

where $\bar{N}_m$ is the number of bubbles per cubic metre released per second. The total number of cells predicted to be observed flashing per second is given by eqn (19) multiplied by the cell concentration and bubble cloud volume.

The bubble cloud volume was calculated using estimates of its width and height. The total number of bubbles released per second was derived by dividing the volume of air injected every second, Q , measured by the timed collection of air into an inverted container, by the volume of the mean bubble. The air flow rate Q was measured for a range of inlet gas pressures after the bioluminescence experiments, and these measurements were used to compute the gas flux from the inlet gas pressure, which was monitored during the bioluminescence trials. The gas flux divided by the mean bubble volume is converted into units of bubbles released per second per cubic metre by dividing through by the bubble cloud volume.

For model calculations for the ceramic and wood air stones (Fig. 9), the bubble rise velocity and stimulation efficiency were taken to be 20 cm sec^{-1} and 0.0279 , respectively, for the 1.5-mm bubble cloud, while for the 0.14-mm bubble cloud they were 2.5 cm sec^{-1} and 8.5×10^{-7} , respectively. The stimulation potential calculated for the ceramic air stone, which produced large bubbles with a relatively high stimulation efficiency, is in good agreement with the observations. This encouraging result indicates that the empirical characterization of the isolated bubble stimulation efficiency can be used to accurately predict stimulation from a cloud of bubbles, and that bubble–bubble interaction effects did not play a dominant role in this case.

In contrast, the predicted stimulation for the wood air stone is approximately three orders of magnitude less than the observed stimulation. The isolated bubble stimulation efficiency extrapolated down to a bubble of 0.14 mm radius is very small and, in addition, bubbles of this size lie below the observed critical bubble radius for stimulation of 0.32 mm . The model calculations predict that less than two flashes sec^{-1} were expected across the air flux range studied (for an air flux of 1 ml sec^{-1} , $119,000 \text{ bubbles sec}^{-1}$

were released from the wood air stone, which were expected to stimulate fewer than one flash sec^{-1}). The thousand-fold difference between the modelled and observed flash rates suggests that when a fixed flux of gas is broken into microbubbles, bubble–bubble interactions can be very important in stimulating bioluminescence, with fluid shear stress associated with collective buoyancy effects dominating the stimulating potential of single bubble–cell interactions.

The bubble–bubble interaction effects for the wood air stone cloud are consistent with casual observation of the bubble cloud produced by the wood air stone. It was visually apparent that the cloud was sufficiently dense for collective buoyancy effects to dominate its behaviour. Water entrained inside the plume gained vertical momentum, reducing the drag on individual bubbles within the plume and creating a region of enhanced fluid shear at the plume boundary. Small-scale flow vortices mixing bubble-free water with the plume were observed at the edges of the plume, resulting in a growth of the plume cross-section with height above the air stone. We speculate that the enhanced shear at the plume boundary, and turbulence within the plume, are responsible for the enhanced levels of stimulation observed. The net result is that the stimulation efficiency of the plume of small bubbles is only a factor of approximately three less than the larger bubbles, despite the fact that the stimulation potential of a small isolated bubble is a factor of approximately 33,000 less than the larger. The implication is that the stimulation potential of clouds of small bubbles is strongly dependent on the air fraction of the plume, but there are insufficient data in this study to make any quantitative statements.

Conclusions

This study presents a quantitative analysis of the potential of isolated bubbles rising at their terminal velocity and plumes of bubbles to stimulate a bioluminescence flash response from suspended cells of the dinoflagellate *Lingulodinium polyedrum*. Isolated bubble experiments have shown that bubbles with radii in the range $1\text{--}3 \text{ mm}$ stimulate an approximately constant fraction of the cells they encounter whereas the stimulation potential of smaller bubbles decreases rapidly with bubble radius. The similarity of the scaling between flash probability and bubble terminal velocity as a function of bubble radius motivated the study of an empirical model for flash stimulation based on terminal velocity. A least-mean-squares fit between the probability of stimulating a flash and bubble terminal velocity showed that the flash response scales with terminal velocity raised to the power of an index lying in the range $5\text{--}7$. Bubbles smaller than a 0.325 mm radius were not observed to stimulate a flash response, a phenomenon almost certainly related to the fact that *L. polyedrum* cells have a fluid shear stress threshold for flash stimulation (10,19).

The isolated bubble empirical flash model was applied to the stimulation of cells by a cloud of bubbles. Good agreement was observed between the modelled and measured flash response for a cloud of 1.5 mm radius bubbles, suggesting that the isolated bubble model can adequately predict the stimulation of a cloud of relatively large ($>1 \text{ mm}$ radius) bubbles. Very poor agreement was found when the model was applied to a cloud of 0.14-mm radius bubbles, with observed stimulation exceeding modelled stimulation by a factor of 1000. We attribute the high levels of stimulation observed relative to the model predictions to fluid shear stress driven by collective buoyancy effects in the microbubble plume.

This research has application to bubble–cell interactions beyond the field of bioluminescence, such as the study of airlift bioreactors, where clouds of air bubbles are used to maintain cells in suspension and promote mixing for mass transfer of gases and nutrients (23,46). According to Hua *et al.* (46): ‘it would be unlikely for either a supported or suspended cell to experience shear stresses due to rising bubbles much larger than perhaps 1 dyn cm^{-2} in aqueous media’. However, based on the data presented here it can be concluded that *L. polyedrum* cells interacting with a rising bubble greater than 1 mm radius are subjected to an effective fluid shear stress exceeding their threshold for stimulation, which is approximately 0.2 N m^{-2} ($\approx 2 \text{ dyn cm}^{-2}$). Sparged bioreactors are characterized by a range of bubble sizes depending on the type of sparger. For example, a ring sparger can create bubbles ranging from 2.5–5 mm in diameter, a G-4 disk with 5–10- μm orifices creates bubbles of 45–100 μm in diameter, and a G-1 disk with 100–200 μm orifices creates bubbles of 250–500 μm in diameter (47). The results of this study suggest that the effects of bubble–cell interactions in the bulk media would be minimized by using bubbles somewhat smaller than a 1 mm radius. Unfortunately the hydrodynamic conditions lead to cell damage due to cell bursting at the surface (48–51) and not cell–bubble interactions within the bulk fluid (26,52,53). Bubbles adhere to the rising bubble and experience large mechanical stresses when the bubble bursts at the air–water interface, thus any reduction in bubble–cell interactions in the bulk gained by reducing bubble size would have to be weighed against the damage caused by an increased number of smaller bubbles bursting at the surface.

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