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Acoustic vibration can enhance biofilm formation

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Abstract

This paper explores the use of low-frequency-low-amplitude acoustic vibration on biofilm formation. Biofilm growth is thought to be governed by a diverse range of environmental stresses and much effort has gone into researching the effects of environmental factors including; nutrient availability, pH and temperature on the growth of biofilms. Many biofilm-forming organisms have evolved to thrive in mechanically challenging environments, for example soil, yet the effects of the physical environment on biofilm formation has been largely ignored. Exposure of Pseudomonas aeruginosa to vibration at 100, 800 and 1600Hz for 48 hours, resulted in a significant increase in biofilm formation compared to the control, with the greatest growth seen at 800Hz vibration. The results also show that this increase in biofilm formation is accompanied with an increase in P. aeruginosa cell number. Acoustic vibration was also found to regulate the spatial distribution of biofilm formation in a frequency-dependent manner. Exposure of Staphylococcus aureus to acoustic vibration also resulted in enhanced biofilm formation with the greatest level of biofilm being formed following 48hours exposure at 1600Hz. These results show that acoustic vibration can be used to control biofilm formation and therefore presents a novel and potentially cost effective means to manipulate the development and yield of biofilms in a range of important industrial and medical processes.

1. Introduction

Cells, by their very nature, have evolved to respond to external mechanical and physical cues and it is now known that there is a complex interplay between the physical extracellular microenvironment and cellular function (DuFont et al. 2011; Jamney & Miller 2011; Miller and Davidson 2013). Cells sense their physical surroundings by converting mechanical forces and distortions into biochemical signals, via the activation of diverse intracellular signalling pathways, through a process known as mechanotransduction (Ingber et al. 2006). Very little is known about mechanotransduction, however work on eukaryotic cells is helping to unravel the complexities of this process. For example, it is known that stretch-sensitive ion channels (Martinac 2004) and an architectural control of mechanotransduction, through a mechanochemical coupling between the cell surface and nucleus (Wang et al. 2009), are key regulators of this process.

Recent work in this area has seen some workers manipulate important cellular behaviours, such as stem cell differentiation, using low-frequency-low-amplitude mechanical stimulation (Kim et al. 2012; Kulkarni et al. 2013; Wang et al. 2013). In contrast to eukaryotic cells, very little has been done to investigate the response of prokaryotic cells to external physical stimuli. This is quite surprising given that many prokaryotic organisms have evolved to thrive in physically challenging environments such as soil. Recent work has shown that mechanical stimulation at infrasound frequency (<20Hz), can be used to either stimulate or inhibit the growth of Escherichia coli in a frequency-dependent manner, although the mechanisms behind this are unknown (Martirosyan & Ayrapetyan 2014). Such work offers great potential into the possibility of manipulating and controlling microbial communities, using physical cues. One such area which may benefit from microbial manipulation through physical stimulation is biofilm formation.

The ability to exist in biofilms, communities of adherent cells held together in a self-produced matrix of extracellular polymeric substances (EPS), is a characteristic of a range of medically and industrially relevant bacteria and yeast species. Opportunistic human pathogens such as Pseudomonas aeruginosa and Staphylococcus aureus can form biofilms during infection, in wound sites or on inorganic materials such as catheters and stents, giving the cells increased protection from antibiotics and modulating their virulence (Savage et al. 2013, Drenkard 2003). The formation of biofilms can also be detrimental in a number of industrial processes (Torres et al. 2011), where damage to equipment or contamination of products via the actions of bacterial biofilms can incur significant financial costs which, combined with the effects of biofilms in healthcare, are believed to total billions of Euros per year in the E.U. alone (Stavridou and Forzi, 2011). The formation of biofilms is thought to be governed by a diverse number of factors, including nutritional availability (Lim et al. 2004), osmolarity (Karatan and Watnick, 2009), self-generated quorum sensing signals (De Kievit, 2009) and the chemistry and topography of the host surface (MacKintosh et al. 2006). Much work has gone into trying to disrupt or prevent biofilm formation, some of which has focussed on using ultrasound (Hazan et al. 2006). However, to date very little has been done to manipulate the formation of biofilms using low-frequency-low-amplitude acoustic vibration. The present research aimed to demonstrate the effects of low-frequency-low-amplitude acoustic vibration on the formation of P. aeruginosa biofilms. For the first time, it is shown that P. aeruginosa biofilm formation can be significantly enhanced through acoustic vibration, and that this is associated with an increase in cell number and a frequency-dependent spatial distribution of biofilm formation on the attachment surface. This work therefore offers a means to manipulate P. aeruginosa biofilm development and may offer potential solutions to promote or prevent biofilm growth in industrial processes.

2. Methods

Comment [M1]: Need to add methods for *S. aureus* and latex beads.

2.1 Development & Calibration of Vibration System

For mechanical stimulation of bacteria a speaker-based device was developed using a 0.2W super-thin, Mylar speaker (45mm) and an Arduino board programmed to generate a sinusoidal acoustic waveform. This system, shown in figure 1, enables acoustic vibrations to be applied to the underside of a culture dish, at frequencies ranging between 100-1600Hz. In order to confirm that the system did deliver accurate vibrational frequencies a laser vibrometer (Polytec Ltd) was used. This also allowed the measurement of displacement of amplitude (µm) at a given frequency. Briefly, the laser was projected onto the inside bottom surface of a 35mm petri dish which was placed on top of the speaker. The speaker was then set to vibrate at set frequencies (100, 200, 400, 800 and 1600Hz). The laser vibrometer can then determine vibration frequency and amplitude, by measuring the displacement of the laser spot on the dish.

2.2 Bacterial Cell Culture

Bacteria were maintained on nutrient (P.aeruginosa (PAO1)) or brain heart infusion (BHI) (S.aureus (S-235)) agar (Oxoid, UK) at 37^{o}C. A single colony was taken and added to 10ml of nutrient (P.aeruginosa) or BHI (S.aureus) broth (Oxoid, UK), and incubated statically for 24 hours at 37^{o}C. A 1ml aliquot of this culture was added to 9ml of nutrient broth and incubated for 3 hours at 37^{o}C to ensure the culture was in log phase of growth; the bacterial culture was adjusted to OD_{600} with nutrient broth, and this suspension was used in further experiments.

2.3 The effects of vibration on P. aeruginosa cell density and biofilm formation

Cell culture dishes containing 2ml of nutrient or BHI broth were inoculated with 20µl of P. aeruginosa or S.aureus suspension (OD_{600} 0.1). To apply mechanical stimulation a cell culture dish was rested upon the Mylar speaker, which was placed inside the static incubator (37^{o}C) and vibration at a frequency of either 100, 800 or 1600Hz was continually applied for 48 hours. A control dish containing the cell suspension was also placed in the incubator, away from the speaker to ensure these cells received no vibration. After 48 hours both planktonic and biofilm cell number of the mechanically stimulated and control cells were quantified manually through the use of a Hawksley bacterial counting chamber. The crystal violet assay (method adapted from O'Toole 2011) was also carried out in order to determine any differences in biofilm production between those cells receiving vibration and the control cells. Experiments at each frequency were repeated in triplicate.

2.4 Crystal violet biofilm assay

Cell culture dishes (35mm) containing P. aeruginosa or S.aureus culture were removed from the 37°C incubator after 48 hours in order to be stained. Bacterial culture was removed from the culture dishes, and the absorbance at 600nm was determined via spectrophotometer (BMG Labtech, Germany). The dishes were dipped sequentially in three reservoirs of distilled water in order to remove residual culture material and non-adherent cells, and then dried against a paper towel in order to remove any water. A 2ml volume of 0.1% crystal violet solution was added to the dishes, which were incubated at room temperature for 10 minutes. The crystal violet solution was washed off by repeatedly submerging the dishes into reservoirs of distilled water, which was then shaken out, and the dishes were held in an incubator at 37^{o}C until dry. Dishes could then be examined for qualitative analysis through imaging with either a standard digital camera or a Nikon Eclipse T5100 microscope, equipped with a SPOT idea camera and SPOT software (v.5.1), Diagnostic Instruments Inc.

To destain the biofilm, a 2ml aliquot of 30% acetic acid was then applied to each dish, and drawn over the stained areas using a pipette in order to draw up any pigment. Next, 1ml of the destaining solution was removed and the absorbance of the crystal violet present at 550nm was determined using a spectrophotometer. A 30% solution of acetic acid was used as the blank solution, in order to give a quantitative measurement of the biofilm. Statistical analysis was carried out using an unpaired, two-tailed Student's t-test at 95% confidence limit (Winter 2013).

2.5 Latex beads assay

200µl of 2µm diameter latex beads in aqueous suspension (Sigma) were suspended in 2ml BHI broth in a 35mm petri dish and subjected to acoustic vibration as above at 100Hz for 48h, then photographed.

3. Results & Discussion

3.1 Vibration System & Calibration

To assess the effects of vibration upon P. aeruginosa biofilm formation, a speaker-based system was developed (figure 1 -left) to deliver low-frequency-low-amplitude acoustic vibration, via a sinusoidal waveform, to cell culture dishes. A laser vibrometer was used to calibrate the vibration frequency and amplitude. As can be seen from figure 1 (right) there was a steady decay in displacement amplitude (µm) of the bottom inner surface of the dish, as frequency increased. For example, amplitude of displacement was found to be approximately $9x10^{-6}$m at 100Hz vibration and $0.1x10^{-6}$ m at 1600Hz vibration, respectively. Below 100Hz and above 1600Hz the system was unstable (in terms of frequency) as laser vibrometry recorded multiple harmonics outside of this frequency range. Therefore, frequencies between 100 and 1600Hz were used so as to accurately deliver stable, low-frequency-low-amplitude vibrations to bacterial cultures in a continuous manner.

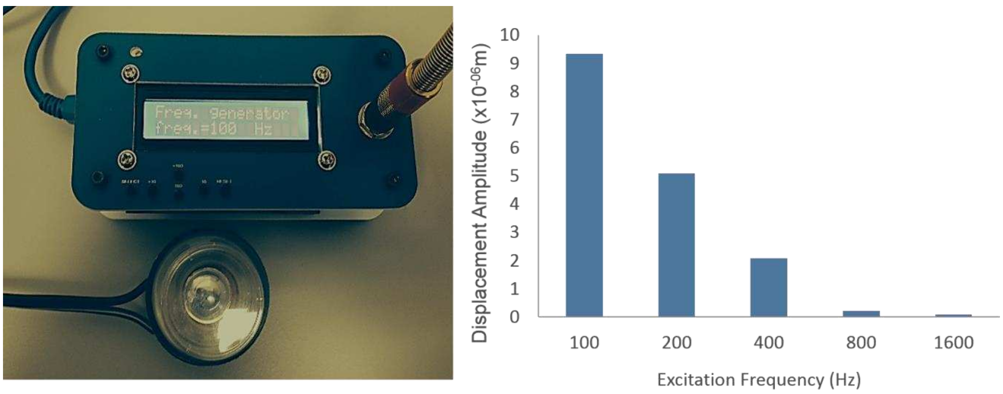


Figure 1: Speaker-based device with 35mm cell culture dish (left) and calibration of frequency versus amplitude of displacement of the inside bottom surface of a 35mm cell culture dish (right).

3.2 The effects of acoustic vibration on P. aeruginosa biofilm formation

Given that the system was stable between 100-1600Hz vibration frequencies of 100, 800 and 1600Hz were chosen to apply continuous vibration for 48 hours. A time period of 48 hours was chosen as P. aeruginosa has been shown to form a mature biofilm over this period when cultured at 37^{o}C (Zenga et al. 2012). After 48 hours of vibration, crystal violet staining was carried out to quantify biofilm formation. Biofilm formation was significantly enhanced in the vibrated cultures compared to the control (no vibration) (figure 2). Vibration at 100Hz resulted in an increase in biofilm formation by a factor of 0.3, whereas vibration at 800 and 1600Hz significantly increased P. aeruginosa biofilm formation by a factor of 2.8 and 2.6 respectively ($p<0.05$).

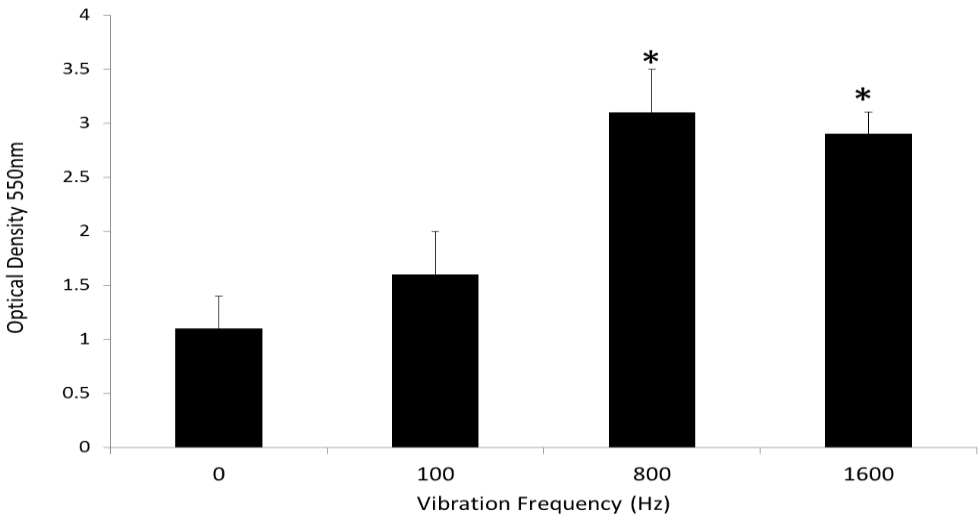


Figure 2 Optical density of crystal violet stain (as an indicator of P. aeruginosa biofilm formation) versus vibration frequency (Hz). Error bars represent standard deviation n=3 (*$p<0.05$).

In order to determine if the increase in biofilm formation was due to an increase in cell number, a cell count was conducted following acoustic vibration. For this purpose only 800Hz was chosen, as crystal violet staining had found this frequency to produce the greatest level of biofilm formation. As can be seen from figure 3 there was found to be no difference in the planktonic cell number between the control and 800Hz sample. However, within the biofilm there was found to be an increase in cell number compared to the control (approximately 2.6-fold more than the control, respectively, $p<0.05$). This result supports the crystal violet staining results and shows that the increased biofilm formation is due to an increase in cell number.

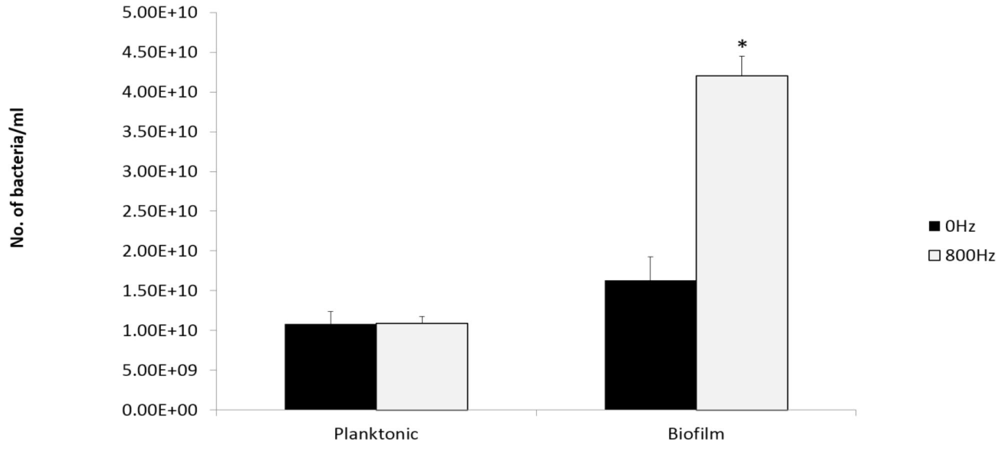


Figure 3 P. aeruginosa planktonic and biofilm cell number versus vibration frequency (Hz). Error bars represent standard deviation n=3 (*$p<0.05$).

Interestingly, for the vibrated cultures, the biofilm was often observed to form in a concentric ring pattern, radiating out from the centre of the dish towards its periphery, with the biofilm rings appearing to increase in density the further away from the centre of the dish. This can be seen from figure 4 where; (A) shows the unstained control biofilm after 48 hours of growth (B) shows the unstained biofilm formed after 48 hours exposure to acoustic vibration at 100Hz and (C) shows the crystal violet stained biofilm formed following exposure to 100Hz vibration for 48 hours. There is a clear difference between the biofilm growth-pattern for the control culture, compared to the vibrated cultures. This was evident at all frequencies, but was more prominent in those cultures that received vibration at 100Hz, possibly due to the larger amplitude.

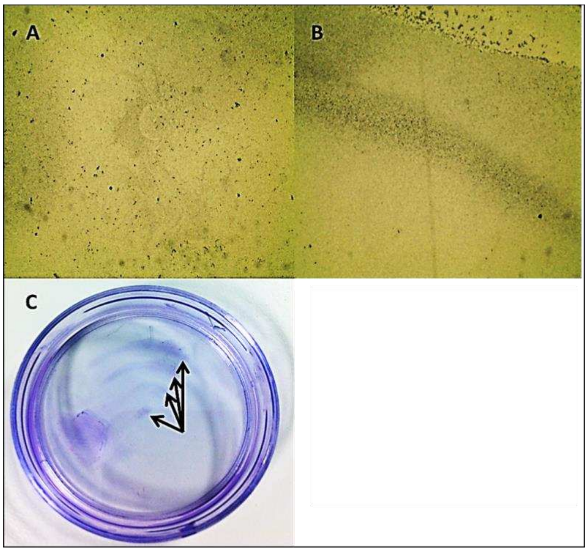


Figure 4 Microscope images showing biofilm formation after 48 hours: (A) random biofilm formation of control sample (x40), (B) biofilm formation in concentric rings following 100Hz vibration (x 40 -unstained) and (C) Photomicrograph of biofilm formation following 100Hz (stained)

It is conceivable that this arrangement of biofilm formation is due, in part, to a physical mechanism. For example, a standing wave, as generated by a speaker, has both nodes (points of no displacement) and antinodes (points that undergo the maximum displacement during each vibrational cycle of the standing wave) as shown in figure 5a. Given that the speaker was coupled to the dish, it is possible that the acoustic vibration would cause the bottom of the dish to also vibrate as a standing wave, thus providing static areas and areas of changes in amplitude, either of which may act to attract or trap the bacteria. In order to investigate this further, experiments where repeated using 2µm diameter latex beads suspended in medium while vibrating at 100Hz for 48 hours. It was found that acoustic vibration of the latex beads also produced a concentric-ring pattern (Figure 5b). However, these beads where dispersed when the dish was moved, unlike the biofilm which did not move and was clearly adhered to the surface of the dish. This shows that biofilm formation can be controlled using acoustic vibration. It is possible that the cells are being trapped/forced between the vibrational nodes of either the acoustic waveform or through the deformation of the cell culture dish generated by the acoustic wave.

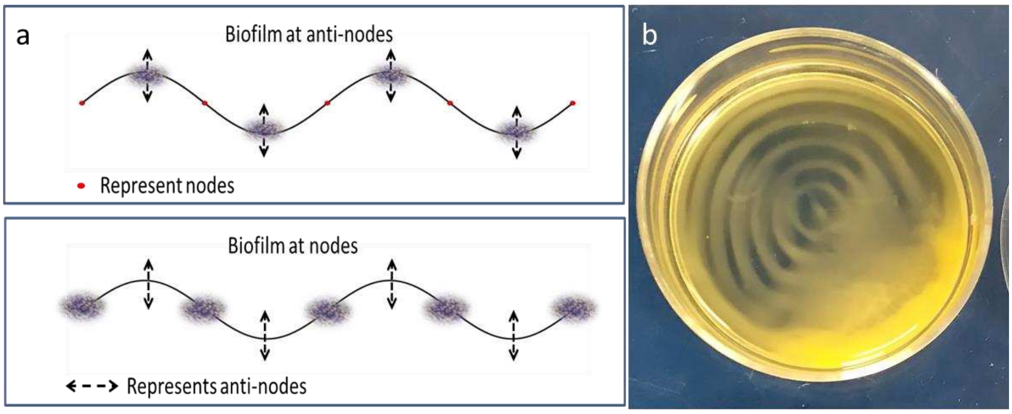


Figure 5 (a) Schematic diagram highlighting how biofilm may have formed in a concentric ring pattern due to vibration of the cell culture dish in a sinusoidal wave pattern causing biofilm growth at anti-nodes (top) or nodes (bottom) and (b) Photomicrograph showing concentric ring formed from 2μm latex beads following acoustic vibration at 100Hz for 48hrs.

Even when obvious concentric rings were not observed (mainly at frequencies >100Hz), the pattern of biofilm formation of the vibrated cultures was still different to the non-vibrated cultures and was observed to radiate out from the centre of the dish (which contained the least dense biofilm) becoming progressively more dense towards the edges of the dish. Upon closer inspection, using phase contrast microscopy, it was observed that the vibration enhanced biofilm radiated out from the centre of the speaker in a striated pattern (figure 6).

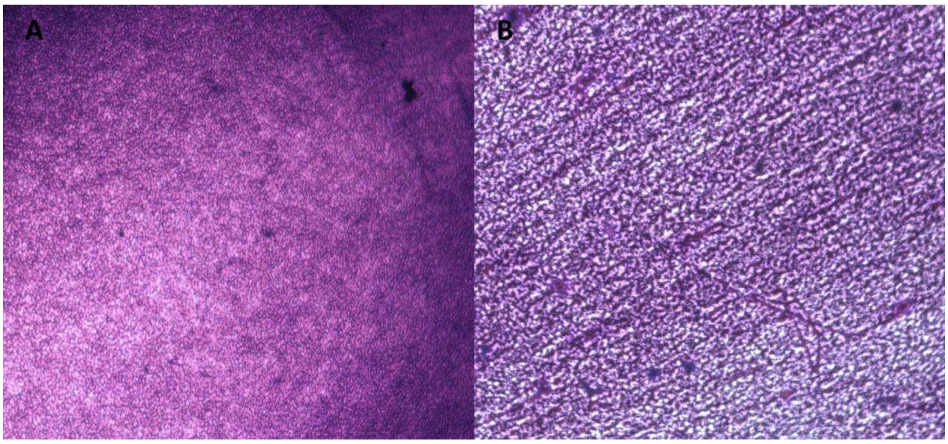
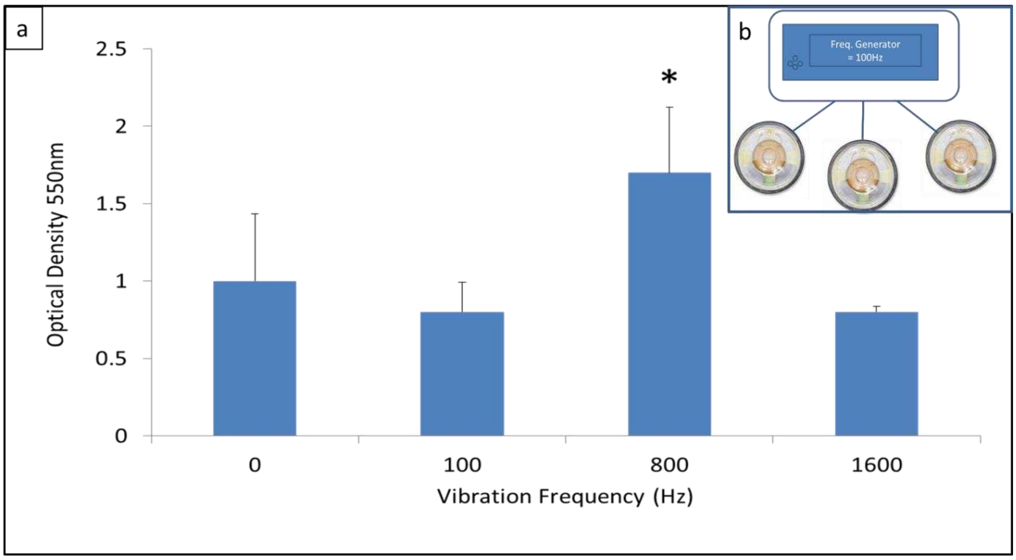


Figure 6 Phase contrast images (x100 mag) of crystal violet stained P. aeruginosa biofilm following exposure to continuous acoustic vibration at 800Hz for 48hrs. Left - non-vibrated culture. Right – vibrated culture, highlighting a striated arrangement of biofilm formation.

Given that the extent of biofilm formation of the vibrated cultures was found to be frequency dependent, the effects of reducing the amplitude of displacement on biofilm formation was examined. To reduce the amplitude in a controlled manner, more speakers were added to the system. This resulted in a proportional reduction in power and thus amplitude of each speaker. For example, adding 1 extra speaker would reduce the amplitude by half. For this study a total of 3 speakers were added, which resulted in individual speaker amplitudes being reduced to one third of the original amplitude (see schematic of system with 3 speakers figure 7b). Following vibration for 48 hours at 100, 800 and 1600Hz with reduced amplitude it was found that at only 800Hz vibration was biofilm formation significantly greater than the control (figure 7a). This suggests that amplitude, as well as frequency of vibration, plays an important role in P. aeruginosa biofilm formation.



3.3 Does acoustic vibration enhance biofilm formation in other bacterial species?

The results presented above are novel and interesting and raise the question of whether the observed phenomena are specific to P. aeruginosa. In order to address this, the effect of acoustic vibration on Staphylococcus aureus biofilm formation was investigated. Following 48hours vibration at 100Hz, crystal violet staining was carried out. It was found that biofilm formation was enhanced in all vibrated cultures compared to the control, with biofilm increasing as vibration frequency increased (Figure 8). Vibration at 100Hz resulted in an

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increase in biofilm formation by approximately a factor of 3, whereas vibration at 800 and 1600Hz increased S. aureus biofilm by a factor of 6.7 and 7.7, respectively.

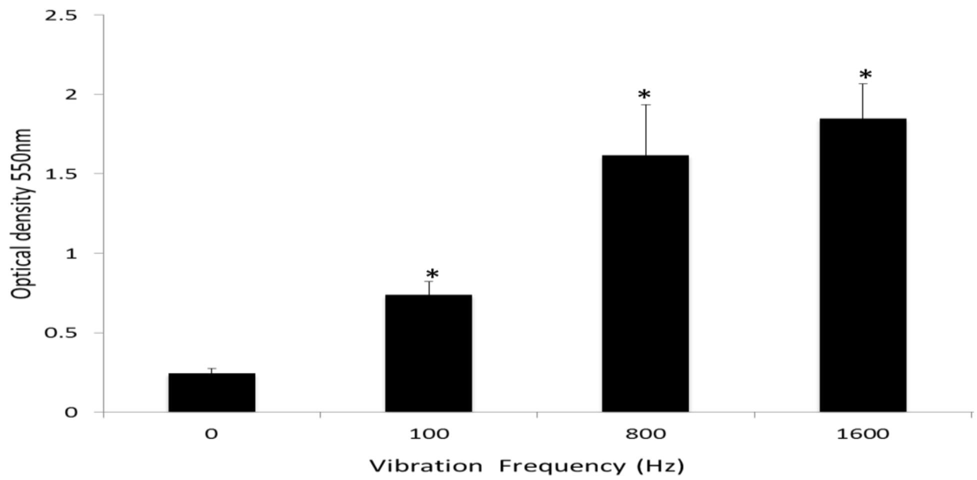

Figure 8 Optical density of crystal violet stain (as an indicator of S. Aureus biofilm formation) versus vibration frequency (Hz). Error bars represent standard deviation n=3 (*$p<0.05$).

These results suggest that the response of bacterial species to acoustic vibration may be a common one. The exact mechanisms underlying the enhanced cell growth and biofilm formation are, at present, unclear. However, it is thought that mechanotransduction may play a key role. Most microbes appear to possess members of one or both families of bacterial mechanosensitive channels, MscS and MscL. These mechanosensitive channels are thought to sense tension in the membrane bilayer and act to control turgor pressure within the cell, thus preventing cell rupture (Booth and Blount, 2012). It is thought that this is achieved through the mechanosensitive channels opening in response mechanical signals thus allowing the passage of solutes across the cell wall. It is therefore possible that the force generated by the acoustic stimulation may have activated Mscs within the bacterial cells and that this has contributed to the increase in cell growth. In a recent study by Gu et al it was shown that acoustic sound delivered to E.coli k-12 via a speaker system, resulted in an increased biomass, faster cell growth and an increase in average length of E.coli cells when compared to the control group. Moreover, it was also found that sound exposure promoted intracellular RNA and protein synthesis. The authors suggest that the E.coli cells may sense the acoustic stimuli via Mscs and convert the physical stimuli into biological signals through the influx of solutes (e.g. Ca^{2+}) into the cell (Gut et al., 2016). Another possible explanation is that the bacteria are being 'pushed' together by the acoustic wave or deformation of the dish (i.e. as seen in the ring formations and highlighted in figure 5) and that this may affect the quorum sensing of the population, as the increase in cell density/proximity in the early stages, from being physically moved closer together, would have a knock-on effect of increasing QS signalling and biofilm/EPS production. Similarly, it is also possible that the waves of nutrient broth are helping to distribute the QS signalling molecules to a wider audience of bacteria. Clearly this is speculation at this stage and more work is needed to understand the mechanisms behind the observed phenomena reported in the present study. However, it would seem that both physical and biological mechanisms are responsible for the biofilm distribution/formation and enhanced cell growth.

The effect of sound on biofouling has also been reported for non-bacterial species. For example, recent work has shown that the acoustic noise emitted through a ship's hull, in port, increases the settlement, growth and spatial distribution of non-bacterial biofouling organisms, (McDonald et al. 2014; Stanley et al. 2014) thus further highlighting the link between acoustic stimulation and biofouling. Much work and financial burden has been devoted to reducing biofilm formation. However, the work presented here raises questions as to whether biofouling/biofilm formation could be prevented and/or promoted by controlling the vibration/acoustic noise. Such question can only be answered if more fundamental work is done in this area, so as to develop a new understanding of the mechanobiology of microorganisms and biofilms. Such investigations may provide us with a novel approach to manipulate and exploit the use of biofilms for a range of industrial, medical and environmental applications.

Comment [M4]: Do we need this?

Comment [JS5]: I'm not sure we do actually, although it is further evidence of the importance of acoustic stimulation it now seems a bit out of place. I don't think the paper would lose anything if it wasn't included.

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