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**Research Article**

# Impact of low frequency ultrasound on pathogens in polluted potable water

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**Abstract:** The importance of potable water as a natural resource is vital for human health. Enterococcus faecalis, Salmonella typhimurium, Pseudomonas aeruginosa and Streptococcus faecalis are commonly linked to waterborne disease epidemics and so were chosen for this research. The aim of this study was to evaluate the impact of low frequency ultrasound on pathogens in polluted water hence 200 ml of bacterial suspensions were prepared at an optical density of 0.17 at λ 440nm and were subjected to sonication using a 20 kHz probe (13.88W/cm$^2$). Samples were taken at 0, 2, 5, 15 and 30 minutes, serially diluted and enumerated using the viable plate count technique. Results revealed that Enterococcus faecalis (Gram positive bacteria which has a tough peptidoglycan cell wall) demonstrated a "declumping" effect following 2 minutes sonication and also Pseudomonas aeruginosa, Salmonella typhimurium(Gram negative bacteria) and Streptococcus faecalis(Gram positive) demonstrated a large reduction in bacterial cell numbers with increasing sonication time. Gram negative bacterial cell walls were more susceptible to damage induced by sonication, and this was evident in this study than the gram positive pathogens It is widely accepted that the use of ultrasound in combination with conventional biocides (chlorine) will results in a synergistic effect. This is the focus and intention of this study as declumping renders bacterial cells more susceptible to conventional chemical treatments.
**Keywords:** bacteria, pathogens, potable water, low frequency, sonication.

## INTRODUCTION

The advancement and use of ultrasound and ultrasonic devices in the processing of materials from laboratory to industrial scale is of great importance. Increasingly, there is aparadigm shift among water professionals and industries to develop and explore new technologies for treatment of potable water that are green, safe, and cost-effective and reduce the impact of chemical residues on humans. Ultrasonic technology has been identified as a nonchemical method to inactivate pathogens and reduce the formation of bio films [1]. Thus, when used in conjunction with a reduced quantity of biocides such as chlorine, reasonable and effective results are achieved [2].

Potable water is water that is safe for human consumption. Lack of this vital resource globally [3, 4, 5] has led to increased outbreaks of waterborne diseases with 3.5 million deaths occurring every year from water related infections [6,7]. The quality of water used has a great impact on overall health and effective sanitation is vital. Many poor nations in the world have serious environmental problems and health issues due to the continued use of polluted water for food and other domestic needs. Consequently, improving access to safe water supplies and sanitation services will be an important step towards reducing the number of water borne infection episodes [8]. Given the dynamic and overwhelming public health implications of microbial waterborne disease outbreaks in our various communities; professionals and potable water providers often consider microbial contamination as the principal risk factor and threat to human health when considering drinking water quality [9]. Gastro-enteritis in humans is caused by pathogens which are associated with the following diseases, Salmonellosis, Hepatitis, Ameobiasis, Typhoid fever, Shigellosis, Cryptosporidiosis and Giardiasis. However, in the light of the above and huge amount of the cofounding evidence, it is suggested that the application of effective methods for the treatment of potable water will reduce the spread of water related epidemics globally, especially among vulnerable groups that are susceptible to water epidemic infection[10, 11]. The inefficiency of chemical treatments, and their associated health risks,

has driven research to explore alternative means of disinfecting water that are safe, effective and environmentally friendly [12, 13]. Ultrasonic systems for the disinfection of water offer a green alternative and a range of equipment is available from the laboratory to the industrial scale [14]. The term ultrasound is used to describe sound at frequencies that are too high for human hearing (>18 kHz).Much emphasis has been made on the vital role frequency plays in the use of ultrasound for water treatment and the differences between low and high frequency in aqueous solutions[15, 16, 17]. Ultrasonic applications in the medical field include diagnosis, treatment of cancer, gene transfer and assisting the movement of therapeutic drugs across membranes to targeted cells and organs [15, 18, 19]. In the field of food processing, and the extraction of essential compounds from medicinal plants, there has been much progress from laboratory to industrial applications as documented by Vinatoru [20]. Studies have shown that ultrasound can enhance the production and extraction of substances showing great potential in reducing costs and improvements in health and ecosystem [21, 22, 23, 24]. Thus, there is great potential for the use of ultrasound in industrial applications in science [25]. Many advances have been made in the use of ultrasonic technology in environmental protection, disinfection of potable water, treatment of waste effluents and air pollution control with reduced chemical effects on the environment [15]. Studies in the field of potable water disinfection have shown that microorganisms are rendered inactive, and cell walls are disrupted, due to the ultrasonic mediated generation of free radicals and the mechanical and physical effects of cavitations bubble collapse. Once the cell membrane is damaged, chemical oxidants can permeate cells and attack internal organelles promoting cell death. Studies have also shown that sonication of water enhances the deagglomeration of pathogens especially when they appear in clusters [1]. The synergistic effect of ultrasound and biocide treatments may provide an enhanced and robust disinfection for pathogens in water [26, 27, 1, 28].

The study of cavitational effects produced by an ultrasonic device on materials is collectively known as Sonochemistry [15]. The effects of ultrasound in potable water treatment stem from the production of acoustic cavitation which involves the formation, growth, and collapse of bubbles. Rapid collapse of these bubbles results in the generation of high temperatures and chemical radicals [29, 30]. Cavitation produces many biological, chemical and mechanical changes in liquids [31, 32]). This is the mechanism through which bacterial cells are rendered inactive and non-pathogenic when sonicated in water.

Evidence from the literature search revealed that there is much work already done on the effect of power ultrasound in material sciences and on bacterial pathogens [15]. The mode of action is through physical, mechanical and chemical effects. However, there is lack of data on the application of power ultrasound to bacterial inactivation for different strains of pathogens such as (Streptococcus faecalis (NCIMB775), Enterococcus sp. (ATCC51299), Pseudomonas aeruginosa (NCIMB8295), and Salmonella typhimurium (WT STRAIN) in potable water. Most of the studies done on bacteria are on waste water and sludge with limited data published on potable water hence the relevance of this study cannot be over emphasised.

## MATERIALS AND METHODS

### Materials Bacterial strains

The impact of ultrasound on bacteria viability was evaluated using Streptococcus faecalis, Enterococcus sp., Pseudomonas aeruginosa, and Salmonella typhimurium. Stock plate cultures on a nutrient agar plates were prepared by removing a loop of bacteria from a mother dish (obtained from the Microbiology Department, Coventry University).

### Preparation of bacterial suspensions

A stock suspension of bacteria was prepared by aseptically transferring a loop of bacteria from the mother dish into 100mls of sterile nutrient broth in a 250ml conical flask (13g/l, CM 001 Oxoid Ltd. England), and incubated in a shaking incubator (200 rpm) at 37$^{o}$C for 24 ±4 hours. 10ml of this overnight culture was aseptically transferred to 100ml of sterile nutrient broth. The resultant broth was placed in a shaking incubator (200 rpm) at 37$^{o}$C for 3 hours. The suspension was then subjected to a series of centrifugations at 3500 rpm for 15 minutes to obtain a bacterial cell pellet which was then washed three times using saline solution (9 g/L, code S13120163 Fisher scientific Ltd UK). Following washing, the cells were ready for use for the experiments. The pellet was suspended in 1ml of sterile saline and this concentrated suspension was then added drop wise to 1000ml of sterile saline. The bacterial samples were prepared by adjusting the turbidity/optical density of the suspensions to 0.17 ($\lambda$ 440 nm) which was equivalent to 1 x $10^{8}$ cfu/ml. During sonication 1ml of bacterial sample was removed at the following time intervals 0, 2, 5,15 and 30 minutes and aseptically added into 9ml of 0.9% sterile saline using a sterile micro-pipette. Samples were serially diluted in the following order, 1:10, 1:100, 1:1000, and 1:10000. 100 µl of each dilution was spread on nutrient agar (CM003, Oxoid Ltd. England) plates in triplicate and incubated at 37$^{0}$C for 24 hours.

### Calibration of bacterial suspensions

To ensure reproducibility between experiments, it was important to have approximately the same concentration of bacteria in each test suspension. The number of bacterial cells present in a given suspension was determined using the turbidity method. Optical density is the amount of light which is transmitted through a turbid solution and light is

deflected away due to the presence of bacteria in suspension. A CORNING colorimeter 253 was used which is an analogue sensitive instrument and covers the range of 400 – 700nm. Cell suspensions can be determined at any wavelength (λ) however, 440nm was chosen as this is ideal λ for the detection of bacterial cells in 0.9% sterile saline water.

**Calorimetry (heat energy input method)**

Calorimetry was used to measure the actual ultrasonic power and intensity entering the system. During sonication the temperature of a liquid increases. This is due to the collapse of cavitation bubbles during the rarefaction cycle of an ultrasonic wave which induces very high temperatures and atmospheric pressures (in excess of $5000^0$C and 200 atmospheres). An increase in the bulk temperature of a solution was observed during sonication, and this was measured using a thermocouple. The initial rate of temperature rise was calculated using a plot of temperature versus time and drawing a tangent at time = 0. The results can be expressed in overall power (W), intensity ($Wcm^{-2}$) or power density ($Wcm^{-3}$). Changing the area of the emitting face of the transducer alters intensity while changing the reaction volume alters the power density.

The temperature time reading were fitted into a polynomial curve of the form:

$$\mathbf{T = at^3 + bt^2 + ct + d} \qquad \textbf{Eqn.1}$$

Differentiation of equation X gives:

$$\mathbf{Dt/dt = 3at^2 + bt + c} \qquad \textbf{Eqn.2}$$

Where t = 0, dT/dt =c, and 'c' is $^0$C/sec or K/sec

The power (P) entering the ultrasonic system is defined by:

$$\mathbf{P = 'c' \times Cp \times m} \qquad \textbf{Eqn.3}$$

Where ($C_P$) is the specific heat capacity and (m) is the mass of water used in each system. The specific heat capacity of water is 4.19 kj $Kg^{-1}$.

The intensity can be calculated for each system by dividing the applied power by the volume used or area of the tip of sonication probe, which gives:

$$\mathbf{I = P/A\ (W/cm^2)\ or\ I = P/V\ (W/ml)} \qquad \textbf{Eqn. 4}$$

The probe system was calibrated by inserting the tip of the horn to a depth of approximately 0.5 cm in distilled water in the reaction vessel (glass beaker) used for each experiment.

**Determination of acoustic power with different ultrasonic equipment**

The acoustic power of the ultrasonic probe used in this study was evaluated prior to commencing each experiment. 200 ml of distilled water was placed in a 250 ml beaker with the ultrasonic probe (40% amplitude). Temperature readings were taken every 10 seconds for a continuous period of 3 minutes. This was carried out in triplicate and the average was used to determine the ultrasonic power.

**Sonication of bacterial suspensions in saline**

The use of low frequency ultrasound employed in this study was a 20 kHz probe. 200 ml bacterial suspension was placed in a reaction vessel (250 ml beaker) and sonicated by immersing a 20 kHz probe tip (amplitude 40%) directly into the suspension (Vibra cell CV600, Sonic and Material Inc. Danbury, CT, USA). Samples were taken after 0, 2, 5, 15 and 30 minutes and the bacterial populations were analysed by counting colony forming units (CFU's) using the Protocol Symbiosis software (Scientific Laboratory supplies USA) to enumerate the viable plate counting technique. All experiments were repeated in triplicate and the average was used to produce data and results.

The ultrasonic power entering the system was measured using calorimetry and was calculated as 13.88 $W/cm^2$ for the 20 kHz probe respectively. The temperature of the bacterial suspension was controlled at 18-23$^o$C by surrounding the reaction vessel with an ice bath in the case of 20 kHz probe.

**Statistical consideration.**

The use of Microsoft excel computer software were used to analyse the data with respect to calculating the average and standard deviation as well as percentage reduction of bacterial cells over time . Graphs were created and plotted as seen below in result section.

**RESULTS**

**Sonication of bacteria at 20 kHz Probe**

Inactivation results for 20 kHz ultrasonic probe are outlined below. Bacterial suspensions ($10^8$cell/ml in saline) of Pseudomonas aeruginosa, Salmonella typhimurium, Enterococcus faecalis and Streptococcus faecalis exhibited different degrees of susceptibility to sonication which most time are probably dependent on the type of ultrasonic device used and the amount of power entering the reaction medium. Pseudomonas aeruginosa showed very high reduction in bacterial cell numbers as the sonication time increased, resulting in 97% reduction within 30 minutes of sonication with 20 kHz(Fig.1a). Salmonella typhimurium also showed very high levels of bacteria cell reductions as the sonication time increased (Fig.1b).

E. faecalis(Fig. 1c)demonstrated a declumping effect during the first two minutes and an overall bacteria kill of 50% in CFU/ml as the sonication time increased to 30 minutes. Streptococcus faecalis also showed approximately 50% reduction in CFU/ml after 30 minutes of sonication of probe

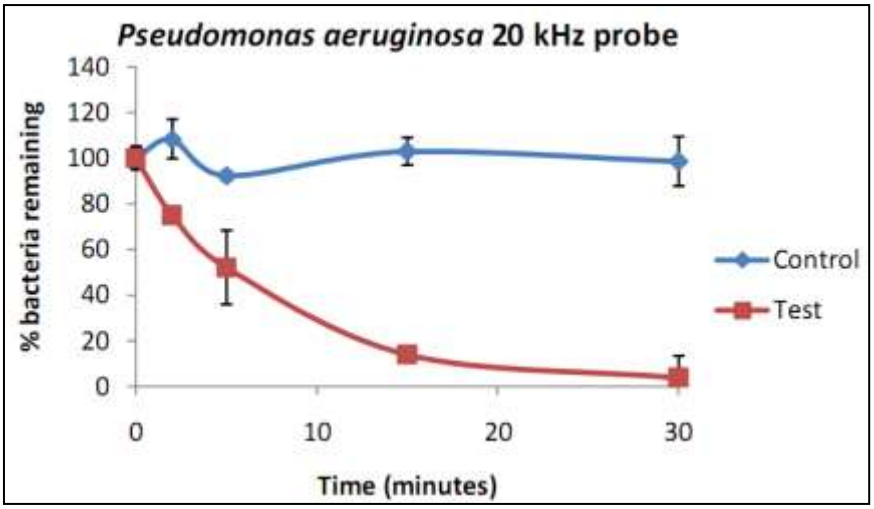


**Fig- 1(a): Pseudomonas aeruginosa (G-ve)**

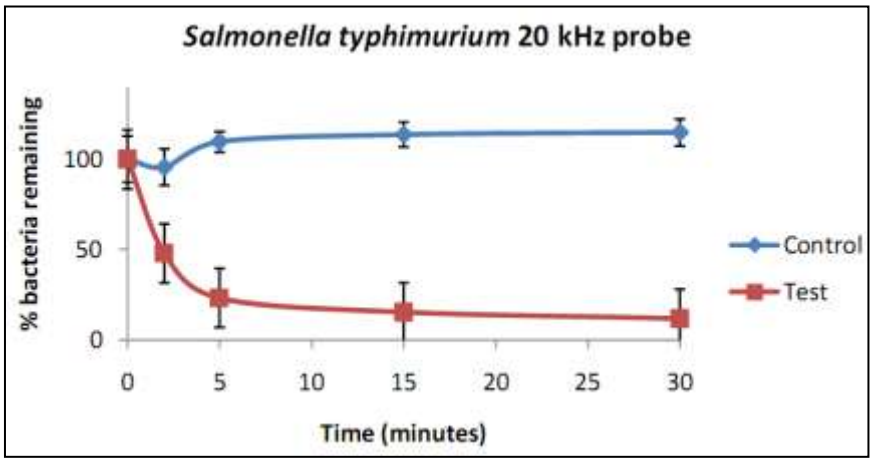


**Fig-1(b): Salmonella typhimurium(G-ve)**

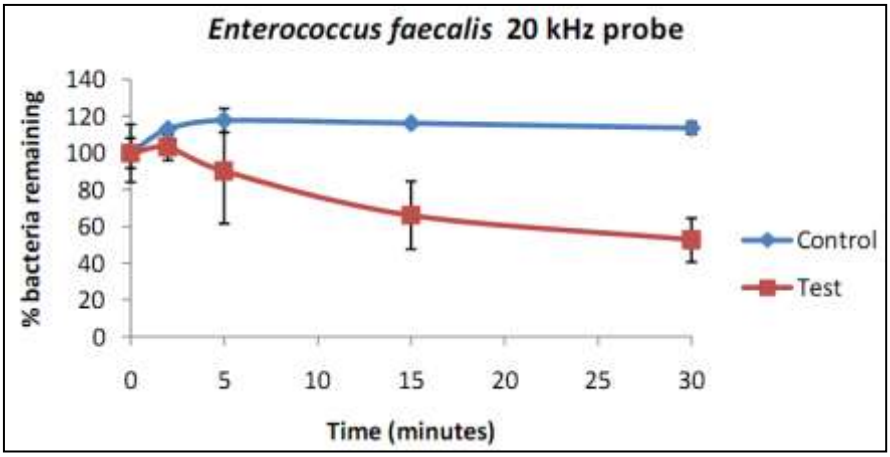


**Fig-1(c): Enterococcus faecalis (G+ve)**

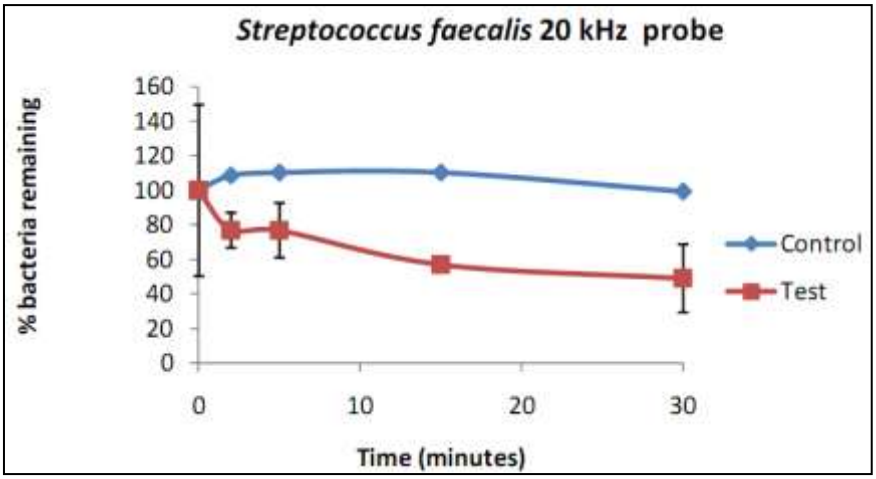


**Fig-1(d): Streptococcus faecalis (G+ve)**

**Fig-1: Sonication of 200 ml bacterial suspensions (a) P. aeruginosa, (b) S. typhymurium, (c) E. faecalis and (d) S.faecalis. -$10^8$ cfu/ml) using a 20 kHz probe (Sonic and Material Vibra cell VC 600), intensity 13.88W/$cm^2$ for 30 minutes.**

## DISCUSSION

In this study two Gram positive and two Gram negative bacteria were chosen due to their global importance and public health implication in water and food borne epidemics. The bacteria include Streptococcus faecalis (NCIMB775) and Enterococcus sp. (ATCC51299) which are Gram positive bacteria and Salmonella typhimurium (WT Strain) and Pseudomonas aeruginosa (NCIMB 8295) which are Gram negative bacterial pathogens.

The effect of low frequency sonication (20 kHz probe) on bacterial viability was evaluated using the viable plate count method. The viable plate count method is an effective and reliable microbiological technique that is scientifically designed and accepted globally to evaluate cell viability especially after inactivation processes. This involves culturing bacteria on a suitable growth media and incubating them for 18 – 48 hours which allows the bacterial cells to form colonies resulting in easy enumeration.

Sonication of bacterial suspension with the 20kHz ultrasonic probe resulted in a significant reduction in cell numbers for Pseudomonas aeruginosa, Salmonella typhimurium, Enterococus faecalis and Streptococcus faecalis(Figure a-d).However, different bacteria had different degrees of susceptibility to ultrasonic treatment which may be determined by the type of cell wall of the pathogen. Gram-positive bacteria possess tougher cell walls than the Gram negative bacteria and shows high degree of resistance to sonication. Differences in structural and chemical composition of bacterial cell walls could probably account for this. Gram positive bacterial cell walls are made up of N-acetylemuramic acid (NAM) and teichoic acids, thus this are interlaced with muramic acid to form peptidogylcan polymer which protects the cell from external environmental attack or aggression. Conversely, gram negative cell walls are made of lipo polysaccharide which is not as robust and protective like the cell wall of Gram positive bacteria that retains the Gentian violet stain after a thorough decolourisation with alcohol or acetone [33]. However, Joyce *et al*., [13] reported the same trend of result with20 kHz treatments on bacterial cell reductions and cell viability, which is in agreement with the present findings. High frequency power ultrasound is known to induce de-agglomeration effects on cells that are clustered together, but this does not necessarily lead to significant bacterial cell inactivation or reduction[34]. This could be potentially useful when ultrasound is used in conjunction with other methods of bacterial inactivation such chlorination as ultrasound seems to be more effective during a synergetic application of both technique.

Results for the 20 kHz probe treatment with Gram positive and Gram negative bacteria show significant effects on cell reductions over time. The acoustic power of the probe is delivered through the tip which generates high mechanical, physical and chemical effects when in contact with the reaction medium. This is due to the direct vibrational mechanism of the probe which delivers high acoustic energy into a stationary phase.

Susceptibility of pathogens to acoustic energy is affected by the structural shape and size of the bacteria. Bacterial cells with large surface areas are more likely to be inactivated faster than pathogens of smaller size. Additionally, the chemical composition of the medium in which the treatment is undertaken, distribution of acoustic energy and viscosity of the medium are very important factors that affect the sonochemical reaction in the medium [35].

It is strongly believed that the antimicrobial activity of power ultrasound on the bacterial cell walls is facilitated by the formation and collapse of micro-bubbles [13]which generates high pressures and localised temperatures[36].It is also reported that sonication leads to the formation of hydroxyl radicals which attacks bacterial cell walls [14]. Pyrolysis reactions and ultrasonic sheer forces are also factors that are responsible for the bactericidal effects of power ultrasound on pathogenic microorganisms in water [37]. One critical issue which has remained unresolved is the power consumption of ultrasonic devices. This will be a crucial factor in the application of this technology in developing nations where the issue of power infrastructure is a great challenge and has remains critical element in fast tracking the infrastructural development of those member countries.

## CONCLUSION

Pseudomonas aeruginosa, Salmonella typhimurium, and Streptococcus faecalis showed a significant reduction in bacterial numbers within two minutes treatment and as the sonication time increased, using a 20 kHz ultrasonic probe.

Enterococcus faecalis demonstrated a declumping effect in 2 minutes sonication with the 20 kHz probe which is normally observed with Gram positive cocci upon sonication [1].

It is widely accepted that the use of ultrasound in combination with conventional biocides(chlorine) will result in more effectiveness in bacteria inactivation. This is the focus of future study of the research as declumping renders bacterial cells more vulnerable to conventional disinfection techniques.

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