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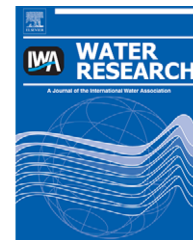

Evaluation of power ultrasound for disinfection of both *Legionella pneumophila* and its environmental host *Acanthamoeba castellanii*

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ABSTRACT

The objectives of this study were to (1) examine the effect of power ultrasound on the viability of both *Legionella pneumophila* and *Acanthamoeba castellanii* trophozoites and cysts, (2) investigate if intracellular *Legionella* replication in trophozoites positively affects bacterial resistance to ultrasound and (3) study if *Legionella* renders viable but non-culturable (VBNC) due to ultrasound treatments. Using laboratory scale experiments, micro-organisms were exposed for various time periods to power ultrasound at a frequency of 36 kHz and an ultrasound power setting of 50 and 100%. Due to a fast destruction, trophozoite hosts were not able to protect intracellular *Legionella* from eradication by ultrasound, in contrast to cysts. No significant effects of ultrasound on cyst viability could be detected and power settings of 100% for 30 min only made intracellular *Legionella* concentrations decrease with 1.3 log units. Due to intracellular replication of *Legionella* in trophozoites, ultrasound no longer affected bacterial viability. Concerning the VBNC state, ultrasound treatments using a power setting of 50% partly induced *Legionella* (±7%) to transform into VBNC bacteria, in contrast to power settings of 100%. Promising results obtained in this study indicate the possible application of power ultrasound in the control of both *Legionella* and *Acanthamoeba* concentrations in anthropogenic water systems.

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1. Introduction

Legionella pneumophila is one of the most widespread human pathogenic *Legionella* species, accounting for more than 90% of all reported cases of Legionnaires' disease outbreaks (Yu et al., 2002). Infection occurs upon inhalation of aerosolized droplets (<5 μm), generated by a number of contaminated anthropogenic water systems like air conditioning systems, respiratory therapy equipment and whirlpools containing numerous infectious bacteria (Atlas, 1999). During the last decade the percentage of elderly and immune compromised persons significantly increased, leading to a higher number of people particularly susceptible to *Legionella* infection (Steinert et al., 2002).

In a recent study we proved *Acanthamoeba castellanii* to play a crucial role in the increase and spread of *L. pneumophila* in anthropogenic aquatic systems (Declerck et al., in press). Biofilm associated and planktonic (free-living) *L. pneumophila* only

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increased after intense intracellular replication in the *A. castellanii* host. Similar results have been reported previously by Kuiper et al. (2004) and Murga et al. (2001) for *Hartmanella vermiformis*. According to the environmental conditions, *Acanthamoeba* spp. occur as actively feeding and dividing trophozoites or as dormant cysts (Marciano-Cabral and Cabral, 2003). Cysts are double-walled and consist of an ecto- and endocyst, making them resistant to adverse temperature conditions, desiccation and disinfection (Turner et al., 2000; Lloyd et al., 2001). In that way, the amoeba host does not only provide the required nutrients to allow intracellular *Legionella* replication (trophozoites), but also offers a shelter against harsh environmental conditions (cysts) (Borella et al., 2005). Moreover, amoeba-grown *L. pneumophila* show a dramatic increase in their resistance to harsh environmental conditions such as fluctuations in temperature, osmolarity, pH and oxidizing agents (Barker and Brown, 1995). Due to the crucial role of amoebae in the replication and persistence of *Legionella* in anthropogenic water systems, despite repeated control measures like chlorination, there is an urgent need to find a way to destroy both the infected amoeba host and intracellular *Legionella*.

Ultrasound at lower frequencies (20–100 kHz), which is referred to as "power ultrasound", is known to induce mortality in bacteria (Piyasena et al., 2003). During the sonication process, longitudinal waves are generated, thereby creating regions of alternating compression and expansion (Sala et al., 1995). These regions of pressure change produce cavitation bubbles which on collapse weaken microbial cells via a number of physical (high local temperature and pressure changes), mechanical (microjets) and chemical (free radicals) processes (Ahn et al., 2003; Joyce et al., 2003).

The aim of this study was to investigate if power ultrasound is able to destroy infected *A. castellanii* trophozoites and cysts. Direct consequences of ultrasound effects on intracellular *Legionella* were also studied. As trophozoites allow intracellular *Legionella* replication, the impact of intracellular replication on the resistance of *Legionella* to ultrasound treatments was investigated. Additionally, it was studied if *Legionella* renders viable but non-culturable (VBNC) after ultrasound treatments as they do for chlorine (Garcia et al., 2007). VBNC *Legionella* are still metabolically active but incapable of cell division. This makes them undetectable by the standard culture method (Giglio et al., 2005), which automatically results in an underestimation of *Legionella* concentrations in suspected water samples.

2. Material and methods

2.1. Microorganisms and culture conditions

L. pneumophila Philadelphia-1 (American type culture collection (ATCC) 33152) was used in this study. Bacteria were cultured by standard procedures on buffered charcoal yeast extract agar (BCYE agar pH 6.9, ATCC 1099), supplemented with sterile ferric acid and L-cystein, at a temperature of 37 °C.

A. castellanii (ATCC 30234) was maintained in 25 cm^3 tissue culture flasks containing 10 ml axenic culture medium, i.e., proteose yeast glucose broth (PYG broth pH 6.5, ATCC 712), at 30 °C.

2.2. Ultrasound treatments

Ultrasound treatments were performed using a DG-100 probe Disintegrator (Alpha, Belgium) at a fixed operating frequency of 36 kHz and ultrasound power settings of 50 (Ups 50%) and 100% (Ups 100%). Exposure periods ranged from 0 to 30 min (see Section 2.3). The specific power or intensity (I), transferred from the ultrasound probe into the suspension, is calculated as the ratio PV^{-1} and expressed in kW L^{-1} (Foladori et al., 2007). The intensity of the DG-100 system was determined using calorimetric measurements (Mason et al., 1992). In the next step, the specific energy (E_s) was calculated using the following formula: $E_s = I \times t$ (duration of the ultrasound treatment) and expressed as kJ L^{-1}. At an Ups 50 and 100%, I ranged between 0–0.064 kW L^{-1} and 0–0.191 kW L^{-1}, respectively. Consequently, E_s ranged between 0–114 kJ L^{-1} and 0–343 kJ L^{-1}. All ultrasound treatments were carried out in open to the atmosphere cylindrical glass recipients (100 ml), each containing different types of cell suspensions (50 ml) (described in Section 2.3). These recipients were mounted in a cooling water bath ($V_{tot} = 3000$ ml), keeping the temperature of the samples stable at 20 °C during all treatments. Ultrasonic energy was transmitted into the cell suspensions through the 5 mm tip of the titanium-made probe and the tip was always submerged for 7 cm into the cell suspension. All treatments were performed in a biosafety cabinet to avoid the likelihood of airborne *L. pneumophila* contamination.

2.3. Experimental set-up

The effect of power ultrasound on the microbial viability was tested using different types of cell suspensions: (1) uninfected or infected *A. castellanii* trophozoites at time zero (T_0), (2) uninfected or infected *A. castellanii* cysts, (3) intracellular *Legionella* in infected *A. castellanii* trophozoites at T_0 and cysts, (4) *Legionella* after intracellular replication in trophozoites at time 24 (T_{24}) and 48 h (T_{48}) and (5) free-living *L. pneumophila*.

Uninfected and infected *A. castellanii* trophozoites (both amoebae and intracellular *Legionella*, cell suspensions no. 1 and 3) were ultrasonically treated for 0 (control), 1 and 5 min. In case of uninfected and infected cysts (both the amoeba host and intracellular *L. pneumophila*, cell suspensions no. 2 and 3) and free-living *Legionella* (cell suspension no. 5), microorganisms were ultrasonically treated for 0, 1, 5, 15 and 30 min. Intracellular replicated *Legionella* (cell suspension no. 4) were treated for 15 min.

The effect of ultrasound on the viability of *L. pneumophila* and *A. castellanii* was evaluated using viable plate counts (Mason et al., 2003) and trypan blue stainings (Garcia et al., 2007), respectively. Additionally, fluorescent *in situ* hybridization (FISH) was used to monitor the intracellular presence and replication of *Legionella* in *A. castellanii* (Declerck et al., 2005) as well as the possible bacterial release after ultrasound treatment of the amoeba host. Fluorescein diacetate stainings (FDA) were used to evaluate if ultrasound caused *Legionella* to transform to the VBNC status.

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