

Reduction of *Legionella* spp. in Water and in Soil by a Citrus Plant Extract Vapor

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Legionnaires' disease is a severe form of pneumonia caused by *Legionella* spp., organisms often isolated from environmental sources, including soil and water. *Legionella* spp. are capable of replicating intracellularly within free-living protozoa, and once this has occurred, *Legionella* is particularly resistant to disinfectants. Citrus essential oil (EO) vapors are effective antimicrobials against a range of microorganisms, with reductions of 5 log cells ml⁻¹ on a variety of surfaces. The aim of this investigation was to assess the efficacy of a citrus EO vapor against *Legionella* spp. in water and in soil systems. Reductions of viable cells of *Legionella pneumophila*, *Legionella longbeachae*, *Legionella bozemanii*, and an intra-amoebal culture of *Legionella pneumophila* (water system only) were assessed in soil and in water after exposure to a citrus EO vapor at concentrations ranging from 3.75 mg/liter air to 15g/liter air. Antimicrobial efficacy via different delivery systems (passive and active sintering of the vapor) was determined in water, and gas chromatography-mass spectrometry (GC-MS) analysis of the antimicrobial components (linalool, citral, and β -pinene) was conducted. There was up to a 5-log cells ml⁻¹ reduction in *Legionella* spp. in soil after exposure to the citrus EO vapors (15 mg/liter air). The most susceptible strain in water was *L. pneumophila*, with a 4-log cells ml⁻¹ reduction after 24 h via sintering (15 g/liter air). Sintering the vapor through water increased the presence of the antimicrobial components, with a 61% increase of linalool. Therefore, the appropriate method of delivery of an antimicrobial citrus EO vapor may go some way in controlling *Legionella* spp. from environmental sources.

In 2011, 4,897 cases of Legionnaires' disease were reported by European Union member states, Norway, and Iceland, with six countries (France, Italy, Spain, Germany, Netherlands, and the United Kingdom) contributing to 83% of all the cases (1). In the same year, 239 cases were reported by the National Surveillance Scheme in England and Wales, with the number of cases steadily increasing since the mid-1990s, when on average, between 110 and 160 cases per annum were recorded (2).

Legionnaires' disease is a severe form of pneumonia (2, 3) caused by the Gram-negative, aerobic rod *Legionella* spp. It affects mainly the elderly and immunocompromised people and is more generally reported in men (3, 4). *Legionella pneumophila* is the predominant human-pathogenic strain and is responsible for about 90% of all human infections by *Legionella* spp. (5, 6). Sixteen serotypes of *L. pneumophila* exist, but serotype 1 is the most important clinically (5). An international collaborative survey showed that 84.2% of all isolates in patients with community-acquired Legionnaires' disease were serotype 1 (7). However, *Legionella longbeachae* and *Legionella bozemanii* were also isolated at rates of 3.9% and 2.4%, respectively (7). However, incidence rates vary from country to country and also from source to source. For example, *L. longbeachae* is the most commonly isolated species from patients in Australia (8), accounting for about 30% of *Legionella* isolates from Australia and New Zealand. A recent study by Currie et al. (9) in the United Kingdom has shown that 15 of 24 compost samples were positive for *Legionella* spp., with *L. longbeachae* being the most commonly isolated. In one study, *L. longbeachae* was found in the sputum of a patient who had been in contact with potting soil (6), and it was suggested that aerosol-aided spread and evaporation of water in the potting soil were the possible routes of transmission (6, 10). *Legionella* has also been isolated from waste management facilities dealing with unwashed solid articles, probably via exposure to soil (11).

The natural habitat of *Legionella* is freshwater, such as lakes and

ivers (3), where it grows planktonically or in biofilms, with an optimum temperature range for growth and survival between 30°C and 40°C. However, it can enter man-made water systems and survive, thus creating a potential source for infection. Previous studies have isolated the bacterium from drinking water systems, cooling towers of air conditioning units, whirlpools, spas, fountains, ice machines, vegetable misters, dental devices, and shower heads (12). Infection in humans occurs via inhalation of an aerosolized form of *Legionella* spp. from a contaminated source or via aspiration of contaminated water, which can occur within milliseconds (5, 6, 10). There are no specific standards in the United Kingdom for acceptable levels of *Legionella* spp. in water; however, there is a statutory requirement that the owners of buildings that have equipment predisposed to harboring *Legionella* spp. must ensure that the equipment is maintained to prevent the growth and spread of the organism (13).

Legionella spp. are also capable of invading and replicating intracellularly within free-living protozoa (3, 12). *Acanthamoeba polyphaga* is the most common host of *Legionella* spp. in natural environments (3). Free-living amoebae are capable of forming cysts, which confer resistance to extreme temperatures, desiccation, and disinfection (14) and also provide protection to the intracellular *Legionella* cells, hence making them more able to survive similarly unfavorable conditions. Furthermore, several studies have shown that *L. pneumophila* exhibits a higher stress resistance and is more

Received 18 April 2014 Accepted 17 July 2014

Published ahead of print 25 July 2014

Editor: M. V. Yates

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doi:10.1128/AEM.01275-14

invasive and virulent after it has replicated within a protozoan cell than under other conditions (3, 12, 15). It has been suggested that *Legionella* cells invade and grow within human macrophages in a way similar to invasion and growth within protozoan cells (16).

Commonly, chemical disinfectants or biocides are used to prevent microbial contamination and growth of prospective pathogenic microorganisms in man-made aquatic sites (14). However, they are effective only in high concentrations, which tend to be harmful to humans, and thus the use of natural alternatives to these chemicals may reduce the risk of toxicity. Citrus essential oils (EOs) were first noted for their antimicrobial effect in 1949 by Piacentini (17). In contrast to chemical disinfectants, citrus EOs are generally recognized as safe (GRAS) and therefore are acceptable for use in food and water systems.

In recent studies, a range of pathogenic bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and both vancomycin-susceptible and vancomycin-resistant strains of *Enterococcus faecium* and *Enterococcus faecalis*, have been shown to be susceptible to the vaporized form of the unique blend of the citrus essential oils at a concentration of 15 mg/liter air (18, 19). Furthermore, the citrus EO vapor used in this study has been shown to be effective against the food-borne pathogens *Listeria monocytogenes*, *Bacillus cereus*, *Escherichia coli* O157, and *Campylobacter jejuni* (20, 21). However, to date, the studies on the antimicrobial nature of the citrus EO vapor have tested its effectiveness only on surfaces such as stainless steel and other food contact surfaces and not against microorganisms in liquid systems because of the hydrophobic nature of its components. In addition, the use of EOs in water is not very effective because their vapors consist mainly of phenolic compounds, which have poor solubility, resulting in reduced antimicrobial activity. Water also reduces volatility, as compounds with hydroxyl groups may be more solvated and remain in the water phase (22). Previous studies using a bioautography method followed by atmospheric pressure chemical ionization mass spectrometry (APCI-MS) and solid-phase microextraction gas chromatography (SPME GC)-MS have shown that there is a favorable release of the active compounds (linalool, citral, and β -pinene) from the citrus EO vapor, which facilitates the antimicrobial activity (23).

The aim of this study was to investigate the effectiveness of the antimicrobial citrus EO vapor against *Legionella* spp. in soil and to establish if sintering is effective as an active delivery system against *Legionella* spp. and intra-amoebal *L. pneumophila* in water.

MATERIALS AND METHODS

All investigations were carried out in duplicate on at least three separate occasions.

Microorganisms and culturing methods. *Legionella pneumophila* (ATCC 33152), *Legionella longbeachae* (ATCC-33462), and *Legionella bozemanii* (ATCC 33217) were grown on a *Legionella* charcoal-yeast extract (CYE) agar base (CM0655) supplemented with *Legionella* buffered CYE (BCYE) growth supplement (SR0110C) at 37°C for 48 h. *Acanthamoeba polyphaga* (CCAP 1501/14) was cultured using peptone yeast glucose (PYG) medium (10 g proteose-peptone, 0.5 g yeast extract, 0.1 M glucose, 25 ml Page's amoebal saline solution 1 [PAS 1], 25 ml PAS 2, 450 ml water) adjusted to pH 6.5 with KOH PAS solutions (24). Aliquots of 1 ml of *A. polyphaga* cultures were suspended in 5 ml PYG medium in tissue culture flasks. The protozoan cultures were incubated for 3 days at 35°C.

Preparation of *Acanthamoeba polyphaga* for coculture experiments. PYG broth (22 ml) was inoculated with 2 ml of a 3-day *A. polyphaga* culture. The culture flasks were incubated horizontally for 3 days at room temperature.

After incubation, the flasks were shaken to remove the protozoa from

their surface, and the sample was centrifuged at $400 \times g$ (Hettich Rotanta 460 S; Hettich, Tuttlingen, Germany) for 6 min at room temperature. The pellet was then washed twice in 20 ml of PAS and resuspended in 15 ml of amoebal saline. Cell counts were obtained using a hemocytometer (Thoma, 0.1 mm, 1/400 mm²; Hawksley, London, United Kingdom). Coculturing required a final concentration of 10^5 cells ml⁻¹.

Intra-amoebal culture of *Legionella pneumophila*. A suspension of 10 ml *A. polyphaga* (10^5 cells ml⁻¹) was mixed with a suspension of 10 ml *L. pneumophila* (10^2 cells ml⁻¹) in a tissue culture flask, and the mixture was incubated at 35°C for 10 days. The sample was then centrifuged at $400 \times g$ for 6 min at room temperature to remove the protozoa. The supernatant was subsequently centrifuged at $2,080 \times g$ for 15 min at room temperature and discarded. The pellet was washed twice with 20 ml PAS. The resulting suspension (10^5 cells ml⁻¹) was then mixed with 20 ml of a fresh 3-day-old culture of *A. polyphaga* (10^5 cells ml⁻¹) and incubated again at 35°C for 3 days. The wash steps were then repeated. The final pellet was resuspended in 20 ml PAS, with a final concentration of *Legionella* of 10^8 cells ml⁻¹.

Citrus EO vapor and vapor components. The citrus EO blend consisted of orange (*Citrus sinensis*) and bergamot (*Citrus bergamia*) essential oils (Belmay, Northampton, United Kingdom) in a 1:1 (vol/vol) ratio. Limonene (97%, catalog no. 183164), linalool (97%, number W26, 350-8), citral (95%, number C8, 300-7), and β -pinene (99%, number 402753) were purchased from Sigma-Aldrich Co. Ltd. (Dorset, United Kingdom).

Assessment of a citrus EO vapor and its components against *Legionella* spp. in soil. Potting soil (Miracle Gro potting mix; Scotts, United Kingdom) was sterilized, and 1.5 g was placed in a petri dish in a 1,000-ml beaker. The soil was inoculated with 400- μ l suspensions of either *L. pneumophila*, *L. longbeachae*, or *L. bozemanii*. Filter papers (Whatman disks, 2 cm) were impregnated with the EO mix to give final concentrations of either 3.75 mg/liter air, 7.5 mg/liter air, or 15 mg/liter air and sealed with parafilm (FIL1026; Scientific Laboratory Supplies, United Kingdom). The beakers were incubated for 24 h at 37°C. The soil was then placed in 30 ml of maximum recovery diluent (MRD), vortexed for 2 min, spread plated onto CYE agar, and incubated for 48 h at 37°C, and counts were obtained. Controls were inoculated soil samples not exposed to the citrus EO vapor or components.

Survival of *Legionella* spp. in water after exposure to the citrus EO vapor. (i) **Passive exposure.** Cells of either *L. pneumophila*, *L. longbeachae*, or *L. bozemanii* or of *L. pneumophila* that had been passaged through *A. polyphaga* were inoculated into sterile water in 1-liter beakers to give a final concentration of 10^7 cells ml⁻¹. Filter papers impregnated with the citrus oil to give final concentrations of 3.75 mg/liter air, 7.5 mg/liter air, 15 mg/liter air, 150 mg/liter air, or 15 g/liter air in the atmosphere were placed in the beaker, which was sealed and incubated at room temperature for 24 h. After exposure, 100- μ l samples were spread plated on CYE agar and incubated at 37°C for 48 h, and colonies were counted. Controls were water samples not exposed to the citrus EO vapor.

(ii) **Active exposure.** A cylinder filled with compressed air was connected to a 500-ml vacuum flask (headspace, 590 ml), containing either 150 mg/liter air, 820.5 mg/liter air, or 1,500 mg/liter air of citrus EO vapor. A sinter (10- μ m pores; Sigma-Aldrich, United Kingdom) running from the vacuum flask was then placed into a 100-ml conical flask containing 100 ml water and *L. pneumophila* at a final concentration of 10^6 cells ml⁻¹ (Fig. 1). The sinter forces the air containing the EO through micrometer-sized pores into the water, creating small bubbles that continuously move through the water sample. The citrus EO vapor was left to equilibrate in the vacuum flask for 15 min before the airflow (0.225 liter/min) and the heating plate (30°C) were switched on. Samples were removed at 0, 1, 2, 4, 6, and 24 h after starting the airflow, spread plated onto a CYE agar plate in triplicate, and incubated at 37°C for 48 h, and colonies were counted.

The investigations were repeated using either *L. longbeachae*, *L. bozemanii*, or an intra-amoebal culture of *L. pneumophila* and the concentration of citrus EO vapor shown to be the most effective against *L. pneumophila*. Controls were cells exposed to pure airflow.

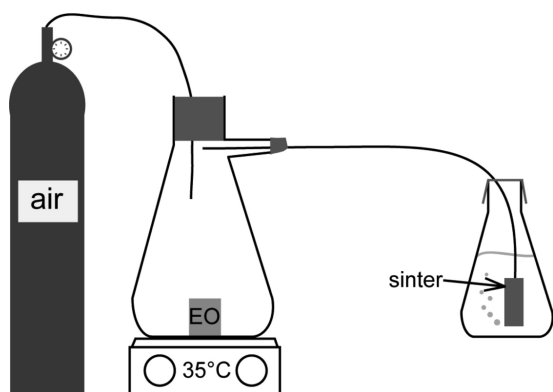


FIG 1 Schematic diagram of the setup of active exposure of *Legionella* spp. to the vapor of a citrus EO.

GC-MS analysis. To quantify the active antimicrobial components (linalool, citral, and β -pinene) in the passive and active exposures, the same apparatus as the one described above was used, but without microorganisms added to the water. Additionally, the initial amount of citrus EO was increased 10-fold (15 g/liter) to enable detection. GC-MS analysis was undertaken on the citrus EO vapor in water or in ethanol without any further sample preparation. Experiments were performed for 24 h in the case of water and for 4 h in the case of ethanol due to volatility limitations (Table 1). All experiments were performed in duplicate.

The GC-MS analyses were performed using a Bruker 450GC and 300-MS SQ mass spectrometer operated in electron ionization (EI) mode at 70 eV. A sample volume of 1 μ l with a split ratio of 10:1 was injected at an inlet temperature of 250°C. The carrier gas was helium and maintained at a constant flow rate of 1.0 ml min⁻¹. The gas chromatograph was equipped with a FactorFour VF-5MS capillary column (30-m long, 0.25-mm inner diameter [ID]) with 0.25- μ m film thickness. The temperature of the column was held at 40°C for 2 min, ramped to 70°C at 10°C min⁻¹, held for 5 min, ramped to 150°C at 5°C min⁻¹, held for 1 min, and then ramped to 200°C at 10°C min⁻¹.

The MS ion source temperature was 180°C. Quantitative analysis was carried out using the selected-ion monitoring (SIM) mode at 70 eV. For each compound, the most abundant ions were selected from its spectrum. The chosen ions for SIM were 69, 84, and 152 for citral, 93, 69, 79, and 136 for β -pinene, and 71, 93, and 154 for linalool. The limits of quantification for all three antimicrobial agents were 1.0 mg/liter.

TABLE 1 Chemical properties of antimicrobial components in the orange and bergamot EO blend^a

Name	Structure	Molar mass (g/mol)	Solubility in water (mg/liter)	Partition coefficient (log P _{OW})
β -Pinene		136.23	Insoluble	5.4 at 25°C
Linalool		154.25	1,589 at 25°C	2.97 at 23.5°C
Citral		152.23	590 at 25°C	3.0 at 25°C

^a Data from reference 22. P_{OW}, octanol-water partition coefficient.

TABLE 2 Reduction of *Legionella* spp. in soil when exposed to a citrus EO vapor for 24 h^a

Citrus EO vapor concn (mg/liter)	Mean log reduction \pm SE		
	<i>L. longbeachae</i>	<i>L. bozemanii</i>	<i>L. pneumophila</i>
0 (control)	1.2 \pm 0.2	1.05 \pm 0.28	0.56 \pm 0.22
3.75	1.47 \pm 0.18	1.42 \pm 0.32	0.55 \pm 0.23
7.5	1.65 \pm 0.16	1.71 \pm 0.25	0.64 \pm 0.24
15	1.53 \pm 0.22	7.88 \pm 0	0.7 \pm 0.41

^a n = 3.

RESULTS

The citrus EO vapor at concentrations as low as 3.75 mg/liter air reduced *Legionella* spp. by 0.5 to 1.5 log cells ml⁻¹ in soil; however, when this concentration was increased to that of 15 mg/liter air, which had previously been shown to be effective against a range of different microorganisms on surfaces, up to an 8-log cells ml⁻¹ reduction was observed against *L. bozemanii* compared to 1.53 log cells ml⁻¹ and 0.7 log cells ml⁻¹ log₁₀ for *L. longbeachae* and *L. pneumophila*, respectively (Table 2).

When water inoculated with *Legionella* cells was passively subjected to the citrus EO vapor, no reductions in counts were observed at 3.75 mg/liter air, 7.5 mg/liter air, 15 mg/liter air, or 150 mg/liter air. However, when subjected to 15 g/liter air, a 2-log cells ml⁻¹ reduction occurred for *L. longbeachae*, although the other strains were unaffected (results not shown).

Actively sintering the citrus EO vapor into water inoculated with *L. pneumophila* resulted in reductions over 24 h of 1.5 log cells ml⁻¹ and 4.5 log cells ml⁻¹ ($P \leq 0.05$) for 150 mg/liter air and 1,500 mg/liter air, respectively (Fig. 2). These concentrations are 10- to 100-fold higher than that previously shown (15 mg/liter air) to reduce microorganisms on surfaces such as stainless steel (19).

A reduction in cell numbers of *Legionella* spp. in water treated with a citrus EO vapor (15 g/liter) through a sintering system was observed at 2 h of exposure with reductions of between 1 and 2 log cells ml⁻¹. *L. pneumophila* was the most susceptible species, with a 4-log cells ml⁻¹ ($P \leq 0.05$) reduction in cell numbers at 24 h, while *L. bozemanii* and *L. longbeachae* were reduced by 2.8 log cells ml⁻¹

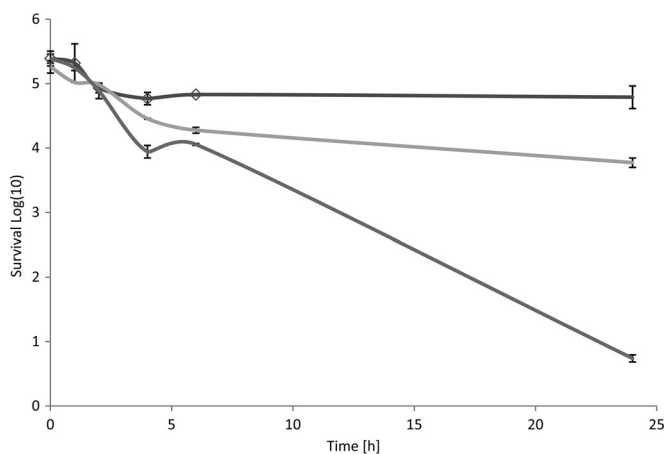


FIG 2 Mean survival of *L. pneumophila* when exposed to an antimicrobial citrus EO vapor in water via a sintering system. Dark grey line, control (exposed to air only); light grey line, 150 mg EO vapor/liter air; medium grey line, 15 g EO vapor/liter air.

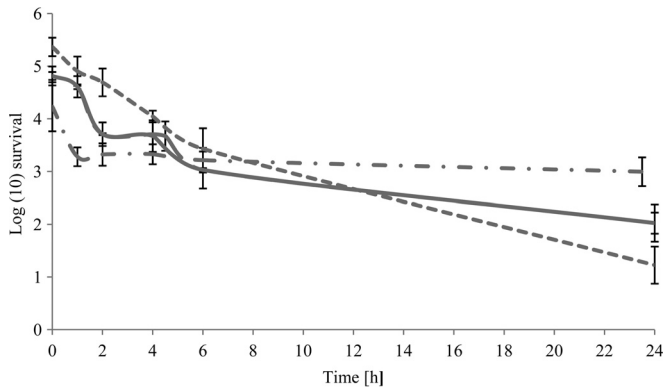


FIG 3 Mean survival of *Legionella* spp. when exposed to an antimicrobial citrus EO vapor (15 g/liter air) in water via a sintering system. Dashed line, *L. pneumophila*; continuous line, *L. longbeachae*; long-dashed line, *L. bozemanii* (mostly overlapping the *L. longbeachae* line); dash-and-dot line, cocultured *L. pneumophila*.

and 2.2 log cells ml⁻¹, respectively. However, the vapor had only a minimal effect on the cocultured *L. pneumophila*, with a 1.24-log cells ml⁻¹ reduction in cell numbers over 24 h (Fig. 3). There was no significant difference ($P \leq 0.05$) between the active and passive systems of delivery of the citrus EO vapor against *L. longbeachae*, for which a 2-log cells ml⁻¹ reduction was observed in both systems.

Only linalool was detected in water when 15 g/liter citrus EO vapor was passed through either passively or via the sintering system (active diffusion), which can be attributed to the relatively high water solubility of linalool (Table 1). As shown in Fig. 4, there was no significant difference in the linalool content of water between the passive and active systems, with concentrations of 24.9 mg/liter and 26.5 mg/liter linalool, respectively, after 45 min of exposure. From 1 h onwards, a significant difference ($P \leq 0.05$) in the concentration of linalool in the water was noted. After 24 h of exposure, the linalool concentrations were 35.43 mg/liter and 57.17 mg/liter in the passive and active systems, respectively, making the linalool content in the active system 61% greater than that in the passive system.

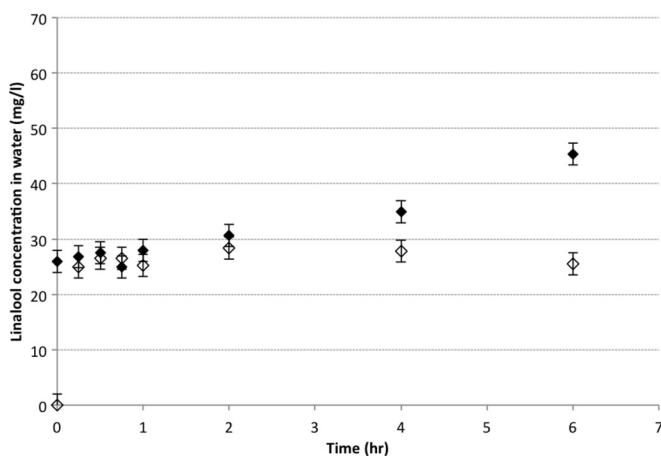


FIG 4 Linalool content in 100 ml water exposed to 15 g/liter antimicrobial citrus oil vapor in passive and active modes. Symbols: \diamond , linalool passive; \blacklozenge , linalool active.

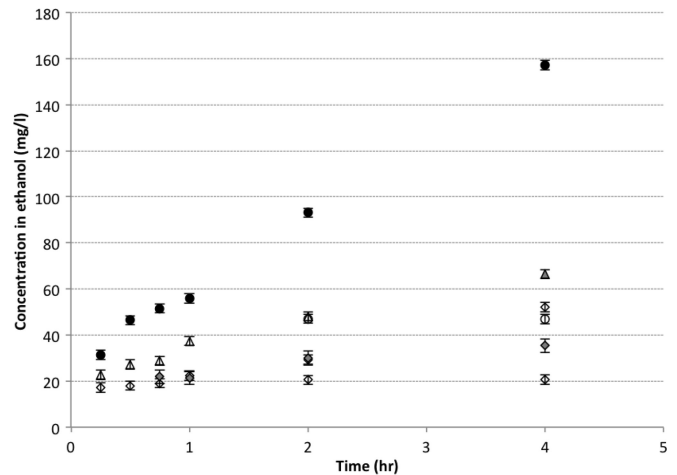


FIG 5 Antimicrobial agents in 100 ml ethanol exposed to 15 g/liter antimicrobial citrus oil vapor in passive and active modes. Symbols: \triangle , β -pinene passive; \blacktriangle , β -pinene active; \diamond , linalool passive; \blacklozenge , linalool active; \circ , citral passive; \bullet , citral active.

When ethanol was the solute, no linalool was detected within the first 30 min in solution when the citrus EO vapor was diffused either passively or by active sintering. After 4 h, the linalool content was 46% higher in the active system (Fig. 5). This trend of a higher linalool concentration in solution in the active system is similar to that observed when water was used as the solute, and this is also the case for both citral and β -pinene. However, citral showed the highest difference with up to 2.35-fold greater concentration in the active system after 4 h.

DISCUSSION

Overall, the citrus antimicrobial vapor was active against *Legionella* spp. However, the extent of its efficacy was dependent on strain and substrate. In soil, the vapor was most effective against *L. bozemanii* at 15 mg/liter air (Table 2) with a 7.88-log cells ml⁻¹ reduction, and there was no significant difference in reductions between the controls and 15 mg/liter air of citrus EO vapor against *L. longbeachae* and *L. pneumophila*, demonstrating the vapor to have strain-specific activity. *L. longbeachae* is the most isolated *Legionella* sp. from potting soil in Australia (58%), with the rates of isolation of *L. pneumophila* being 13.3% (10). Against both *L. longbeachae* and *L. bozemanii*, the most effective concentration of the citrus EO vapor was 15 mg/liter air. Similar results have been previously reported for *Enterococcus* survival on a range of surfaces, including lettuce, cucumber, and stainless steel, with reductions of up to 5 log cells ml⁻¹ (19, 25).

The use of essential oils (EOs) as antimicrobials in water-based environments has not been explored in depth, which is probably due to the lipophilic nature of the EOs and their relative insolubility in water. Traditionally, when assessing EO MICs, an agar dilution method is usually chosen over a broth dilution method for this very reason (6). However, improvements in methodologies for the determination of the antimicrobial efficacy of EOs in broth cultures with the use of emulsifiers have been made, making the assessment of EOs in aqueous solutions more effective (26). The use of a sintering system to force the vapors of EOs through water eliminates the need for other emulsifying agents such as ethanol and Tween 80, thus increasing its potential use within

equipment predisposed to harboring *Legionella* spp., such as air conditioning units.

The use of the vapors of EOs rather than EOs *per se* allows for single components to be targeted and analyzed for their solubility and antimicrobial efficacy in water. Previous studies have shown that linalool, citral, and β -pinene are the main antimicrobial components of the citrus EO vapor as determined by a bioautography method (23).

Figures 1 to 4 demonstrate that the use of a sintering system, forcing the vapors components through the water, gives a greater reduction in *Legionella* spp. than does natural diffusion of the components. The consequent reduction in cell numbers increased from zero in the passive system to 4.5 log₁₀ (Fig. 2) in the active system against *L. pneumophila* at a citrus EO vapor concentration of 15 g/liter air. However, the concentration of the vapor needed to reduce the *Legionella* counts in water had to increase 100-fold, from the 15-mg/liter air concentration observed to be active in a soil system and other surfaces to 15 g/liter air when being sintered into water. There is limited published research on the effect of EOs in water environments. The use of buffered yeast extract broth with Tween as an emulsifier has been shown to be a suitable medium to assess tea tree EO activity against *Legionella* spp. (26), and Chang et al. (27) assessed the use of cinnamon oil in hot-spring water at a range of pHs with ethanol being used as an emulsifying agent. Minimum bacterial concentrations (MBCs) against *L. pneumophila* ranged from 400 to 1,200 mg μl^{-1} with a contact time of 10 min and from 400 to 750 $\mu\text{g ml}^{-1}$ with a contact time of 60 min.

The way in which the components are being passed through the water may also be crucial to the antimicrobial efficacy of the citrus EO vapor. Linalool, which has a higher solubility in water (1,589 mg/liter at 25°C) when sintered, is trapped within the water, thus continuing to have an antimicrobial effect on the *Legionella* cells after 2 h of exposure (Fig. 4), resulting in an accumulation effect; this in part may explain the 61% difference in the concentrations of linalool between the passive and active systems at 24 h. However, both citral and β -pinene have a lower solubility in water (590 mg/liter at 25°C and no solubility, respectively) and are not retained within the water when sintered, as shown by the amount of citral and β -pinene in the water at any given time as they pass through the water before they evaporate (Fig. 5). This suggests that the antimicrobial effect of citral and β -pinene may be based on a collision process between the compounds and the *Legionella* cells as they pass through the water. The increase in concentration of the antimicrobial compounds is noted from 2 h onwards (Fig. 3 and 4), corresponding to a reduction in the *Legionella* cells in water from the same time point (Fig. 3). The increase of the antimicrobial compounds after 2 h is also noted in the vapor release intensity of headspace with a 0.5-log cells ml^{-1} increase for linalool and β -pinene and 2-log cells ml^{-1} increase for citral (23).

Since there are no acceptable levels of *Legionella* spp. specified by the Health and Safety Executive (HSE) in the United Kingdom, the maintenance of equipment that is predisposed to harboring *Legionella* spp. is the responsibility of the manager of the site/equipment; therefore, a range of different disinfectants and physical treatments are used, including chlorine, monochloramine, UV, and heat, all of which have drawbacks, including the need for rinsing, expensive equipment, and high running costs and are often not effective against intercellular *L. pneumophila*. The use of citrus antimicrobial vapor that is sintered through an enclosed

water system such as air conditioning units and cooling towers may be a natural alternative, which the FDA has deemed GRAS under the general provisions of essential oils, oleoresins (solvent free), and natural extractives (28). The components identified to be antimicrobial (linalool, citral, and β -pinene) are widely found in plants, including fruits and herbs, and are often used within the food and fragrance industries. Linalool has no recommended threshold limit value (TLV) or biological exposure index (BEI), and citral and β -pinene are listed by the International Fragrance Association (IFRA) as being commonly found in fragrances safe for use (29–31).

In conclusion, this citrus EO vapor may be a potential solution to controlling *Legionella* spp. from environmental sources such as soil and water. The novel delivery system using sinters to force hydrophobic compounds through water could allow for the use of EO-based products in new arenas.

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