Water Science & Technology

Water Science & Technology Vol 87 No 10, 2457 doi: 10.2166/wst.2023.076

Evaluation of a novel porous antimicrobial media for industrial and HVAC water biocontrol

William James Blanford * and Gregory D. O'Mullan

School of Earth and Environmental Science, Queens College, CUNY, 6530 Kissena Boulevard, D202 SB, Flushing, NY 11367, USA
*Corresponding author. E-mail: william.blanford@qc.cuny.edu; blanfordwj@gmail.com

WJB, 0000-0001-7487-362X; GDO, 0000-0003-0590-7704

ABSTRACT

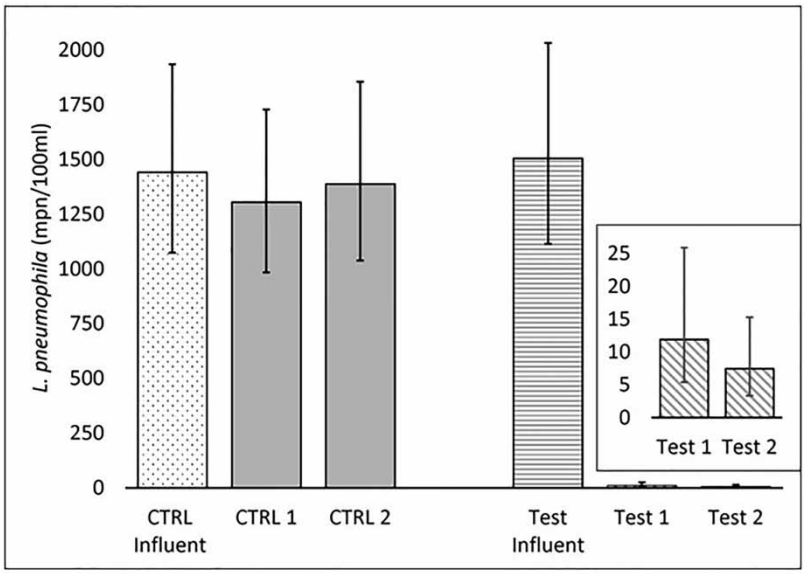
A novel treatment method, consisting of pea-gravel with a marine coating supplemented with alkyldimethylbenzylammonium chloride (ADBAC or benzalkonium chloride), has been examined for its antimicrobial performance and coating stability in aqueous environments. Initial column studies examining the porous media's ability to reduce bacterial loads in heating, ventilation, and air conditioning (HVAC) water found average reductions of 94% from pre-flush levels (10^6 colony forming unit (CFU)/mL) when assessed with R2A spread plates and 83% reductions with SimPlates. There was no observed statistical difference between the average of pre- and post-flush waters from four tests of the media without ADBAC. Taxonomic identification, by 16S rRNA gene sequencing, of colonies drawn from pre- and post-ABDAC R2A plates showed similarities with taxa observed in high frequency from prior cultivation-independent surveys of other cooling tower systems. With this proof of concept, two versions of the media were evaluated for potential coating components released during aqueous exposure. Neither released measurable volatile organic compounds (VOC) components, but one did release bisphenol A and ABDAC compounds. Subsequent column tests of the more durable coating were conducted using cultures of interest in industrial water and demonstrated significant reductions in neutralized post-column *Enterococcus faecalis* samples and near complete loss of *Legionella pneumophila* in non-neutralized fluids, but lower reductions in *Pseudomonas aeruginosa*.

Key words: antimicrobial, benzalkonium chloride, cooling tower, HVAC, *Legionella*, water treatment

HIGHLIGHTS

- Development of antimicrobial treatment based on benzalkonium chloride-amended marine-coated porous media.
- Minimal release of ABDAC and coating chemical components from media.
- Demonstrated performance in broad reduction bacterial levels in HVAC waters.
- Significant reductions in targeted bacteria including *Legionella pneumophila*.
- Greater reduction in bacteria non-neutralized solutions.

GRAPHICAL ABSTRACT



Results of water treatment for *Legionella pneumophila* in column studies of ABDAC-amended coated porous media (tests) and ABDAC-free coated porous media (CTRLs).

INTRODUCTION

Many industrial activities, ranging from electro-coating of automobiles (Semmens *et al.* 2013) to oil and gas extraction (Estrada & Bhamidimarri 2016) or maintenance of building air temperatures (Chua *et al.* 2013), commonly require significant quantities of high-quality process water. These activities result in the production of large quantities of wastewater that is often treated on-site and can require substantial operations oversight. Failure to provide reliable industrial water treatment results in increased maintenance costs, excess water usage, and escalating risks from the disruption of system performance or exposures to contaminated water (Caicedo *et al.* 2019).

Microbial releases associated with cooling towers have received increasing public health and management attention (Carducci *et al.* 2010; Fitzhenry *et al.* 2017; Llewellyn *et al.* 2017). During the operation of cooling tower systems, particles carried in ambient outdoor air become entrained in the circulating water, resulting in microbial enrichment and the need for control measures resulting in more favorable conditions permissive to growth for some microbes. Bacterial contamination of these cooling towers can be problematic for multiple reasons. Bacteria can form biofilms which may foul heat exchange surfaces and delivery pipes, lessening the performance of the unit (Bott 1992). Pathogenic organisms, including *Legionella pneumophila*, the causative agent of Legionnaires' disease and one of the primary microbial concerns in cooling towers (Cunha *et al.* 2016), can grow in these anthropogenic water systems creating a significant risk to public health when aerosolized (Breiman 1996; Cunha & Cunha 2017). Outbreaks of Legionnaires' disease linked to aerosols from cooling towers in major urban environments have expanded public awareness of the importance of industrial water biocontrol and regulations in some areas about cooling tower maintenance and monitoring practices (Chamberlain *et al.* 2017). Biocontrol challenges have a long history of study in drinking water (Szewzyk *et al.* 2000) and in the last two decades have received increasing research interest in the subfield of industrial cooling tower waters (Nguyen *et al.* 2006; Garcia & Pelaz 2008; Carducci *et al.* 2010; Walser *et al.* 2014; Cunha & Cunha 2017; Iervolino *et al.* 2017). Managing microbial growth in these systems is essential to risk management. It can also lead to lower rates of system water replacement which in turn can result in decreased water and biocide consumption and lower operational costs.

Many disinfection procedures, often involving the dosing of biocide solutions (e.g., sodium hypochlorite, hydrogen peroxide, silver salts, quaternary ammonium salts), have been utilized to control microbial growth in industrial waters (Bartram *et al.* 2007), including cooling towers (Garcia & Pelaz 2008). However, these approaches can require frequent

monitoring of biocide levels, and the oxidant nature of many of these chemicals can contribute to safety concerns with their handling, to corrosion to the system, and to the formation of toxic disinfection byproducts (Hua & Reckhow 2007; Giovanardi *et al.* 2020) when added in large doses. There is also increasing awareness that not all biocontrol strategies have similar efficiency toward all microbes and the differences in microbial structure, physiology, and ecology can influence the general efficacy of biocides. For example, in drinking water treatment, it is well known that *Cryptosporidium parvum* oocysts are highly resistant to chlorine (Korich *et al.* 1990; EPA 2020). Therefore, filtration and/or settling, commonly applied in addition to disinfection, has been recommended for the treatment of drinking water from surface water systems or groundwater under the direct influence of surface water to avoid *Cryptosporidium* outbreaks (EPA 2020). Similarly, the association of *Legionella pneumophila* with protozoan host species (Abu Kwaik *et al.* 1998) can provide protection from harsh environmental conditions, including some disinfection procedures (Caicedo *et al.* 2019). Because the efficiency of treatment for a given biocontrol strategy may vary for differing microbial taxa some users prefer to use more than one control strategy and it is helpful to evaluate new technologies using both natural diverse communities and select cultured species representing taxa of interest in industrial water treatment.

Alkylated quaternary ammonium compounds, which are commonly referred to as 'quats', are widely used as disinfectants in hospital settings (Merianos 2001; McDonnell 2020) and are known to be effective against a wide range of bacteria, including *L. pneumophila* (Miller *et al.* 1981; Kim *et al.* 2002; Garcia & Pelaz 2008), several species of *Enterococcus* including *E. faecium* (Suchomel *et al.* 2019) and *Pseudomonas aeruginosa* (Thomas *et al.* 2005). Quats are typically utilized in solution (e.g., for disinfecting the water in a tank) or by spraying on to a surface during routine maintenance (e.g., scrubbing of tank walls or food preparation areas). In applications related to the present work, they have been immobilized on to activated carbon powder (Liang *et al.* 2022) for water treatment and into dental sealers to improve antimicrobial and antibiofilm properties (Arias-Moliz *et al.* 2015). Immobilization may have added benefits due to their bactericidal action being attributed to a wide range of mechanisms including denaturation of cell proteins, disruption of the cell membrane, and inactivation of energy-producing enzymes (Sykes 1972; Petrocci 1983; Merianos 2001). It is also possible to embed the quats within surface coatings (Botequim *et al.* 2012; Imani *et al.* 2020), as accomplished in the porous treatment media evaluated in this study.

The objectives of this study were to evaluate (1) proof of concept: the potential for a porous media whose coating contains a common antimicrobial chemical agent (alkylated quaternary ammonium compounds) to reduce total bacterial concentrations from a diverse natural assemblage in HVAC process water as it passes through a treatment column; (2) treatment media stability analysis: evaluate the potential stability and release of chemical components of the biocide-amended coatings; and (3) performance testing against bacteria of concern: the performance of the porous media to reduce bacterial concentrations of targeted microbial cultures which are of general interest as a fecal indicator bacteria (*Enterococcus faecalis)* (Leskinen *et al.* 2012) and of specific interest in industrial water or cooling tower operations (*Pseudomonas aeruginosa* and *Legionella pneumophila*) (Breiman 1996; Liu *et al.* 2009; Cunha & Cunha 2017). These tests were intended to provide preliminary validation of the porous treatment media for use in industrial water, to evaluate initial coating formulations, and to inform potential applications that may be well suited for subsequent study and development.

METHODS

Proof of concept – antimicrobial treatment column experiment

In this component of the investigation, coated porous media containing the ABDAC biocide was placed into four replicate polyvinyl chloride (PVC) columns and for a control four more columns were prepared with the same coated media, but without the antimicrobial agent. The columns were schedule 40 PVC pipes (ASTM D1785), each 5 feet long with an internal diameter of 2.047 inches that are vertically oriented with a cap at the bottom. After twenty 0.125-inch holes were drilled in the bottom cap, a similar volume of media was filled in the columns (54.0 ($\pm$0.5) inches). The design and permeability of the system were such that gravity-fed water at a rate of approximately 3.8 L/min resulted in a fully saturated bed and steady flow rate through the column. The subject biocide-treated porous media (four columns) consisted of pea-gravel with a diameter of less than 0.5 inches that was treated with a modified marine coating amended with a common quaternary ammonia disinfectant. The marine coating was a two-part epoxy from Interlux (a division of AkzoNobel) whose base is Y2002E Interprotect White, and the curing agent is Interprotect Y2001E. The base and curing agent were combined at a

mass ratio of 79 and 21%. To that mixture was added the antimicrobial agent (MAQUAT MC1412-80), consisting of 80% active ingredients (alkyl (40% C_{12}, 50% C_{14}, 10% C_{16})-dimethylbenzylammonium chloride (ADBAC)) sourced from Pilot Chemical. The ABDAC represented 18% by mass of the coating mixture prior to the application to the pea-gravel. ADBAC is a class of quaternary ammonium chemicals that are commonly employed in mixtures of different alkyl chain lengths (typically C_{10}–C_{18}) that, due to their antimicrobial properties against viruses, bacteria, and fungi, have broad and diverse applications (Pereira & Tagkopoulos 2019). The high affinity for biological membranes of the cationic surfactant structure of the alkylated quaternary ammonium has been shown to act as an antimicrobial agent primarily through cellular lysis (Inacio *et al.* 2016; Barros *et al.* 2022). In addition, alkylated quats in the form of surface chemically modified cellulose fiber (Zhou *et al.* 2003), polymeric beads (Hu *et al.* 2005), nanoparticles (Kesler Shvero *et al.* 2013), and poly(methyl methacrylate) (Aly Saad Aly *et al.* 2016) have been found to exhibit antimicrobial properties. While alkylated quats have been instrumental in addressing the SARS-CoV2 outbreak (Schrank *et al.* 2020), affixing them to materials rather than freely dispersing them in waters is of interest in addressing this pathogen (Imani *et al.* 2020) where electrostatic forces are thought to be at work. It has the further potential attractive result of lessening the introduction level of these biocides into the environment, which is of rising concern (Harrison *et al.* 2020; Hora *et al.* 2020), and potentially increasing the cost-effectiveness of their use in water treatment.

For the initial proof-of-concept tests, four control columns were constructed employing the same experimental setup but with a marine coating lacking the antimicrobial agent. Therefore, the only difference between treated columns and control columns was the presence of the antimicrobial agent (ADBAC). This quaternary ammonium compound was immobilized within the coating of the porous media with the intention of physically lysing cells upon contact with the porous media surfaces. In the case of this study, the porous media (treated and controlled) was flushed with greater than 1,000 L of tap water prior to passing cooling tower water through the columns. This action was intended to pre-condition the media and to evaluate coating durability prior to the experimental test. The columns were then exposed to cooling tower water collected from an operating industrial cooling tower basin in the northeastern United States. The cooling tower water was collected in eight replicate plastic 4-L jugs within 24 h prior to the column test and was stored at room temperature in the absence of direct sunlight exposure during transport to the laboratory and prior to testing.

The experiment was designed with three sample categories (each with four replicates) plus a sterile water blank sample. The categories were: (1) 'Pre' which consisted of cooling tower water transferred from storage jugs directly into sampling bottles without exposure to any column or porous media; (2) 'Treated' which consisted of 1 L of the cooling tower water passed through the antimicrobial treated porous media columns at a flow rate of approximately 4 L/min, such that contact of the test water with the column was approximately 15 s and the sample bottles were filled as the water exited the column; and (3) 'control' which consisted of 1 L of the cooling tower water passed through columns filled with coated gravel containing no antimicrobial agent at a flow rate of approximately 4 L/min and the sample bottles were filled as the water exited the column. Our comparisons were designed to examine the relative reductions in control versus pre; treated versus pre; and treated versus control to evaluate the microbial reduction that can be attributed to the antimicrobial porous media in our columns. It was hypothesized that there would be significant bacterial reductions observed when comparing the 'treated' versus 'pre' and 'treated' versus 'control' experimental units, but no significant difference was observed when comparing the 'control' versus 'pre' samples.

Water used for microbial enumeration was collected in two sterile bottle types for each sample: (1) a 50 mL sterile tube for in-house (Queens College) laboratory analyses; and (2) a 250 mL sterile bottle for analysis at an external laboratory, EMSL Analytical Inc. (Chain of Custody #062,005,276 for this project). In the case of EMSL, samples from only three of the four replicates for each treatment were processed due to project constraints, while the Queens College laboratory sampled all four replicates from each treatment. In addition, a blank, sterile water control was processed in parallel to the cooling tower water samples to control for contamination in sample handling, in addition to laboratory sterile dilution water method controls to ensure the laboratory reagents and media were not contaminated during sample processing or reagent preparation. The blank controls and sterile dilution water controls had no growth in any assay and are therefore not included in the experimental figures presented below.

Proof on concept – bacterial enumeration and taxonomic identification of HVAC waters

Following completion of the column experiment, the collected samples were immediately processed by the Queens College laboratory (50 mL bottles), and parallel samples (250 mL bottles) were delivered to EMSL within 4 h. Bacterial enumerations

(as described below) were initiated within 6 h of initial column exposure, following commonly accepted procedures and holding times. The cooling towers where the water originated were well maintained and unlikely to contain significant levels of *Legionella* but were expected to have a measurable total bacterial level. As a result, bacterial tests focused on broader heterotrophic bacterial assays that are also of interest in HVAC maintenance and are similar to common assays (e.g., less quantitative dip slides) that are broadly used for functional and risk assessment in cooling towers. At Queens College, heterotrophic bacteria were quantified using spread plates incubated at 28 °C for 3 days on R2A media (Difco) as in Young *et al.* (2013). The EMSL samples were processed using a SimPlate (Idexx) for heterotrophic bacterial estimation (method SM9215E; Standard Methods Committee of the American Public Health Association, American Water Works Association, and Water Environment Federation, 2022) and incubated at 35 °C for 2 days. Therefore, if the pattern of the results is well correlated across two laboratories employing similar but not identical methods of assessing heterotrophic bacterial concentrations, a high level of confidence would exist in the common findings.

After incubation and enumeration of bacteria on R2A spread plates, a small number of colonies were randomly selected, picked with a sterile pipette tip, PCR amplified for 16S rRNA genes, and sequenced at Eton Bioscience (Union, NJ) for taxonomic identification following the methods of Young *et al.* (2013) and Montero *et al.* (2016). That effort resulted in 34 sequences comprised of 20 from R2A plates incubated with cooling tower water that had passed through columns filled with the antimicrobial porous media and 14 from R2A plates incubated with pre-column cooling tower water. These Sanger sequences, consisting of 500–700 base pairs of high-quality sequence data, were then analyzed using the Bayesian rRNA Classifier tool of the Ribosomal Database Project (Wang *et al.* 2007) to determine bacterial taxonomic associations with the sampled R2A colonies. Upon the acceptance of the manuscript, DNA sequences will be submitted to GenBank, and accession numbers will be released.

Treatment media stability – potential release coating components into water

The compatibility of coatings with the chosen quats mixture was a focus of initial screening. In prior rudimentary exploration, numerous coatings were evaluated in largely qualitative tests based on visual analysis of whether the addition of the quat resulted in denaturing of the coating liquid, excessive foaming upon light agitation, or an apparently poor ability to evenly coat and adhere to the chosen target porous media (pea-gravel). The Interprotect coating discussed above (Y2002E Interprotect White) showed promising compatibility and antimicrobial performance with the pea-gravel, but like many marine coatings, there is a relatively strong organic chemical odor likely indicating a significant release of VOCs during application. To address this issue, the investigators also investigated a related low-VOC version of the Interlux coating (Y2000VOC Gray). This two-part epoxy from Interlux uses the same curing agent and as marketed does have a noticeably lower release of VOC vapors during mixing, but more importantly, it is vital to evaluate the potential stability of the quat-coatings when exposed to an aqueous system.

For this evaluation of the potential release of coating components, the two marine coatings were mixed in ratios of 79/21 base to curing agent by mass. ABDAC versions of these were then diluted with MC1412-80 to the point that this addition represented 15% of the pre-application coating mass. The composition of the marine coatings was obtained from the supplier. While Interlux does specify the ingredients, their individual levels are given in fairly wide ranges for proprietary reasons. Table S1 in the supplemental materials lists the range of mass factions for the coating and quat suppliers. Based on that information, rough estimates of composition were calculated. Comparisons of the two Interlux marine coatings show the main difference lies with the inclusion *p*-chloro-α,α,α-trifluorotoluene and potentially a greater amount of ethylbenzene in the low-VOC epoxy.

Coated material was supplied to Applied Technical Services (ATS) to evaluate the stability of the ABDAC-amended commercial epoxy coatings in aqueous systems. The laboratories at ATS are experienced in testing the stability of automotive coatings. They were specifically supplied with cured unwashed samples of quat and non-quat-containing coatings of the Interprotect coated pea-gravel at the ratios described above. For this project, they randomly selected 15 mL aliquots of each material. These were washed with 1 L of water, placed on a paper towel, and dried for 30 min. They were then placed in a 40 mL vial with 15 mL of DI water. Each sample was extracted at 20 °C for 24 h. Analysis for VOCs was performed using gas chromatography/mass spectrometry (GC/MS) following EPA Method 5021A (USEPA 2014). The specific VOCs which were assessed included ethanol, isopropanol, butanol, xylenes, 1,2,4-trimethylbenzene, ethylbenzene, and triethylenetetramine. The solutions were also analyzed for levels of bisphenol A by liquid chromatography tandem mass spectrometry

(LC/MS/MS) following ATS internal method (367 Rev. 1) and for levels of the type of quat used, benzalkonium chloride, by LC/MS/MS following EPA Method 83121B (USEPA 2007). This process was performed in triplicate for each material.

Performance testing against bacteria of concern – quat-amended low-VOC column studies

ABDAC-amended low-VOC-coated pea-gravel was provided to the Water & Energy Sustainable Technology Center (WEST) at the University of Arizona to evaluate its ability to reduce bacterial loads in water streams. The media was formulated in the same manner as examined by ATS for component release. The first set of studies examined the ability of the media to reduce *Enterococcus faecalis* (ATCC#19433) and *Pseudomonas aeruginosa* (ATCC#15442). The test of treatment performance of the media again each bacterium initially consisted of preparing three sets of columns with media, conditioning the media with flushing with bacteria-free solution, then flushing with a solution of a single bacteria where effluent levels at the end of the flush are compared with those of the influent. Specifically, quat-containing test media was placed to bed depths of 35.0 inches in three schedule 40 PVC columns whose inner diameter is 2.047 inches. In a similar manner, three identical columns were prepared with quat-free media to function as a control. Due to project constraints, these control columns were filled with slightly less media resulting in bed thicknesses of 33.0 inches. All six columns were individually pre-flushed with 300 L of dechlorinated tap water at flow rates of 3.4 L/min. The levels of chlorine were diminished through prior passage through activated carbon (Water-Tec of Tucson, Inc., CFT-CB10-10, 10-inch filter with 10 μm pore size, 100% coconut carbon).

At the conclusion of the pre-flush, 19 L of the bacterial solution was flushed through the columns. This involved preparation of a 190-litre stock solution of a single bacteria in dechlorinated water. The experimental system involved 24-inches of PVC (2-inch ID Schedule 40) connecting the influent tank to a peristaltic pump (Cole Parmer) followed by a 25-inch section of PVC to a flowmeter assembly. That consisted of an electronic digital meter (Great Plains Industries, Inc, Model #A109GMN02SNA1) with a 12-inch-long section of 2-inch PVC tubing located before and a 13-inch-long section after to enable accurate measurement conditions. From the flow meter assembly fluid passed through 36-inches of 0.5-inch internal diameter clear vinyl tubing (Everbilt, #HKP001-PVC009) to the column. The columns were oriented vertically with an induced upward flow to ensure fully saturated conditions within the columns. Effluent from the columns passed through a 106-inch section of the vinyl tubing to a second carboy where it could be sampled. The same set of control and test columns was used for both *E. faecalis* and *P. aeruginosa* testing. The pre-flushing dry mass of media placed in the columns and the water-saturated mass after the completion of the studies were measured. The average and standard deviation of those are shown in Table S1 of the Supplemental Information along with the computed porosities and media bulk densities.

Bacterial levels of the influent solution and effluent were assessed using reagent most probable number (MPN) techniques. In this study, three replicate 100 mL samples were collected from the influent solution and three from the column effluent at the 19-L flush point. Those were directly analyzed. A second set of three 90 mL samples of effluent water was also collected and partially diluted with 10 mL of Dey/Engle (D/E) neutralizing broth (Fisher). D/E broth is considered a broad-spectrum neutralizer because it contains several common chemical agents that have been found to be effectively neutralizing an array of biocides (Eissa & Eissa 2016). The use of D/E agar as a means of neutralization has been found to be successful against an array of biocides including ADBAC when assessing their performance against common bacteria including *P. aeruginosa* (Dey & Engley 1994; Liguori *et al.* 2009), *E. faecalis* (Liguori *et al.* 2009), and *L. pneumophila* (Rusin *et al.* 2003).

E. faecalis levels were determined using Enterolert (IDEXX) (ASTM 2000) and *P. aeruginosa* using Pseudalert (Sartory *et al.* 2015) both employing the Quanti-Tray 2000 analytical option. After incubation, the number of positive wells in the trays is converted into an MPN concentration using the IDEXX software. That software also reports the lower and upper 95% confidence intervals (CI) for each sample. Enterolert was thought to be suitable for this assessment given its successful application to enumerate enterococci in recreational waters (Budnick *et al.* 1996), drinking and bathing waters (Eckner 1998), and urban runoff-contaminated marine waters (Colford *et al.* 2012). Likewise, Pseudalert was chosen due to its record of evaluating *P. aeruginosa* in a variety of environmental water samples (Ngwa *et al.* 2017) and drinking waters (Bedard *et al.* 2014).

Due to greater care needed in handling solutions of *Legionella pneumophila*, testing was performed in a class-2 biosafety hood (Fisher Scientific 1300 Series A2 (Model #1387)) which necessitated minor changes in methods. Specifically, shorter columns (2.047-inches diameter by 11-inches long) were employed, and studies were performed in duplicate rather than in triplicate columns. The columns were filled with 675 g of media resulting in 8.5–9 inches bed thicknesses in the columns (Table S1). These tests employed the same pump and flow meter assembly, but otherwise only used vinyl tubing to convey

solutions. Specifically, there was a 45-inch section of tubing between the influent carboy and the pump, followed by a 25-inch length to the meter assembly, then a 46-inch section to the column inlet, and an 82-inch section from the column to the second carboy. Different tubing was used for the control and test media columns.

In the *Legionella* studies, the pre-flush and bacterial solutions flushed through the columns were also 300 and 19 L. The flow rate for the *Legionella* studies was slightly greater at 3.8 litres per minute (L/min). The use of the biosafety hood necessitated that the control and quat columns be tested on separate solutions. *Legionella pneumophila* (ATCC#33152) was used in these studies and its level in solution was determined using another IDEXX MPN method, with Legiolert media. That method is widely used in the testing of potable water systems (Sartory *et al.* 2017; LeChevallier 2019) and non-potable including cooling towers (Rech *et al.* 2018; Barrette 2019). Due to project constraints, deactivated samples were not prepared and due to exposure to *L. pneumophila*, the saturated columns could not be taken from the hood for measurement of post-saturated mass.

Statistical analysis

Statistical evaluation of the experimental data was performed using the software Prism (Ver 6, GraphPad Software Inc.). Pearson correlation and linear regression were used to determine the level of statistical correlation of R2A plate count and SimPlate bacterial abundances in paired column samples. ANOVA with Tukey's post hoc tests was used to compare the significance of differences among and between treatments in column study bacterial concentrations using an alpha of 0.05.

RESULTS AND DISCUSSION

Proof of concept – antimicrobial treatment column experiment

The bacterial abundances of R2A plate counts and SimPlates in paired samples were found to be positively and linearly correlated ($p < 0.01$, $R^2 = 0.916$) (SimPlate $= 2.265 \times$ R2A $+ 1.866 \times 10^5$) across the examined experimental treatments. The initial (pre-column) concentrations of heterotrophic bacteria measured on R2A spread plates averaged 8.7×10^5 CFU/mL ($\pm 1.3 \times 10^5$, $n = 4$) (Figure 1(a)) and on SimPlates averaged 2.1×10^6 ($\pm 5.5 \times 10^5$, $n = 3$) (Figure 1(b)). Thus, there is a similar magnitude in the heterotrophic plate counts (HPC) concentrations by the two methods (6.32 (R2A) and 5.94 (SimPlate)) and there exists an appropriate initial abundance of assessable bacteria in the cooling water to conduct a meaningful evaluation of the efficacy of the biocontrol measure.

While cultivation-based enumeration techniques have known limitations in their ability to capture the full concentration or diversity of microbes (Amann *et al.* 1995), these techniques characterize a subset of the community that is known to be viable, an attribute that can be useful in demonstrating reductions in microbial loads due to the employed biocontrol measures. Heterotrophic plate counts, on R2A media, are a standard method for assessing bacterial levels in water systems, including cooling towers, but are often substituted for other less labor-intensive approaches in the field. When previously tested in parallel for cooling tower bacterial monitoring, Mueller *et al.* (2009) reported that the bacterial concentration measured by R2A

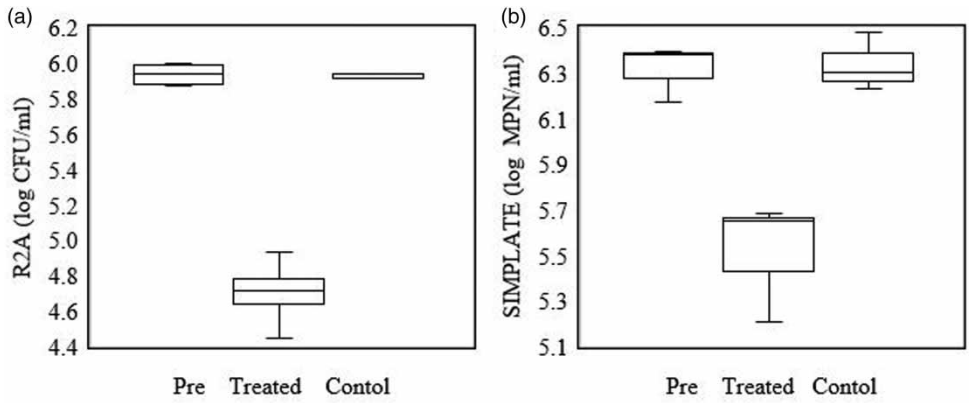


Figure 1 | Heterotrophic bacterial levels as assessed by R2A plate counts (Figure 1(a)) and SimPlate (Figure 1(b)) in HVAC test waters prior to flushing through columns (Pre) and after flushing through coated porous media whose coating contains ADBAC antimicrobial agent (Treated) and after that the same media, but whose coating does not contain biocide (Control).

plate counts was similar to values and correlated in the trends among sample populations to other common analytical methods including alternate plate count media, dip slides, and adenosine triphosphate bioluminescence. Their finding suggests that any relative trends that might be shown by assessing heterotrophic bacteria loads on R2A media plate should be able to provide a substantive evaluation of the impacts of the employed form of biocontrol. As such, heterotrophic plate count methods, including the SimPlate and R2A, spread plate techniques are utilized in this study to characterize a relatively broad diversity of bacteria from cooling water samples.

Bacterial levels from R2A plate counts were found to differ significantly among the employed treatments in these column experiments ($F(2,9) = 100.7$, $p > 0.01$) with no significant difference found between the 'pre' and the 'control' ($p = 0.99$). However, significant differences were found between the 'pre' and the 'treated' ($p < 0.01$) and between the 'control' and the 'treated' ($p < 0.01$) (Figure 1(a)). Similarly, assessable bacterial levels reported by SimPlate assays were found to differ significantly among the examined treatments in these column experiments ($F(2,6) = 12.48$, $p < 0.01$). No significant difference was found between the 'pre' and the 'control' ($p = 0.97$), but significant differences were again found between 'pre' and 'treated' ($p = 0.013$), as well as between the 'control' and the 'treated' ($p = 0.010$) (Figure 1(b)). The reduction in culturable bacterial following the brief (approximately 15 s) exposure involved in this test to the antimicrobial agent held in the porous media of the treated test columns (comparing 'pre' versus 'treated') averaged a 94% (±21%) reduction for R2A and an 83% (±35%) average reduction on SimPlates. Thus, the average of the two assessment methods in this test found a 1-log reduction.

This experiment employed relatively large diameter (<0.5 inches) gravel and a short-duration exposure (15 s) in a single-pass column configuration. The level of reduction in this study would likely have been greater, if these parameters were altered to values that would promote increased contact between the bacterial laden advecting fluids and the media-bound biocides. However, the specifics of an optimized configuration would depend on the characteristics of the system to be treated and the desired level of performance. For example, smaller media diameter, lower porosity, greater bed depth, and recirculation may be requisite in a cooling tower or an industrial water side stream filtration system, while the optimization for a passive urban stormwater filtration system (e.g., end-of-pipe treatment) may require a more permeable even though less microbial reducing design.

The intended mechanism of bacterial reduction for this porous media is cell lysis on contact with the treated media surface and is assumed to be the primary mechanism due to how the experiment was designed which reduces the potential for elution of the antimicrobial agent from the media coating as a secondary mechanism of biocontrol. The volume of tap water passed through each column prior to the test greatly exceeded the volume of pores in the column (>1,000 L, which is over 700 pore-volumes), so it is assumed that contact with the surface of the porous media is the primary mechanism, but it is possible that some elution of the antimicrobial agent from the media coating may also contribute to the observed bacterial reduction.

The taxonomic identification, utilizing 16S rRNA sequencing, of a small number of colonies from the R2A spread plates, pre- and post-column, demonstrates a relatively wide diversity of Gram-negative bacteria (Figure 2) and more importantly include taxa that have previously been demonstrated to be abundant in cooling tower systems. Alpha, Beta, and Gamma Proteobacteria have been determined to be the most common classes of bacteria in prior molecular genetic microbial surveys of

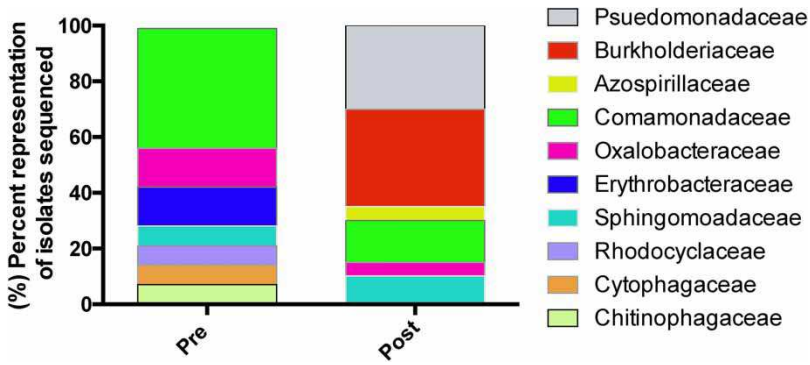


Figure 2 | Taxonomy of 34 colonies (14 pre and 20 post) from HVAC R2A spread plates based on 16S rRNA sequences evaluated in RDP classifier. The relative percent representation of these taxa (*y*-axis) in pre- and post-column (*x*-axis) isolates is shown.

Table 1 | Taxonomy of 34 colonies (14 pre and 20 post) based on 16S rRNA sequences evaluated in RDP classifier

					14 Pre isolates		20 Post isolates:	
Phylum	**Class**	**Family**	**Genus**	**Gram**	**Num. Found**	**%**	**Num. Found**	**%**
Bacteroidetes	Chitinophagia	*Chitinophagaceae*	*Sediminibacterium*	–	1	7	0	0
	Cytophaga	*Cytophagaceae*	Unclassified *Cytophagaceae*	–	1	7	0	0
Proteobacteria	Alphaproteobacteria	*Rhodobacteraceae*	Unclassified *Rhodobacteraceae*	–	1	7	0	0
		Sphingomonadaceae	*Sphingorhabdus*	–	1	7	2	10
		Erythrobacteraceae	*Novosphingobium*	–	2	14	0	0
		Azospirillaceae	*Skermanella*	–	0	0	1	5
	Betaproteobacteria	*Oxalobacteraceae*	*Noviherbaspirillum*	–	2	14	1	5
		Comamonadaceae	*Acidovorax*	–	6	43	2	10
			Delftia	–	0	0	1	5
		Burkholderiaceae	*Cupriavidus*	–	0	0	7	35
	Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	–	0	0	6	30

The percent representation of these taxa in pre- and post-column isolates is shown.

cooling towers (Wang *et al.* 2013; Llewellyn *et al.* 2017; Pereira *et al.* 2017; Tsao *et al.* 2019), as well as many other artificial freshwater distribution systems (Tokajian *et al.* 2005; Berry *et al.* 2006; Vaz-Moreira *et al.* 2017). All of the taxonomic families identified in our colony isolates (Table 1), with the exception of *Azospirillaceae* are among the taxa found at high frequency in prior cooling tower DNA-based community surveys (Pereira *et al.* 2017; Tsao *et al.* 2019). For example, *Comamonadaceae, Pseudomonadaceae, Burkholderiaceae, Sphingomonadaceae*, and *Rhodobacteraceae* were among the most abundant families shared among the cooling towers in Tsao *et al.* (2019). *Chitinophagaceae* were found to be highly persistent in some towers by the same study (Tsao *et al.* 2019) and many of those surveyed by Pereira *et al.* (2017). *Sediminibacterium, Acidovorax, Sphingomonas*, and *Novosphingobium* were among the most abundant genera found in the cooling towers (Pereira *et al.* 2017; Tsao *et al.* 2019). *Acidovorax, Pseudomonas,* and *Cupriavidus*, were also commonly found genera in cooling towers (Tsao *et al.* 2019) and have been determined to thrive, at least transiently in protists which is an apparent strategy often found to be associated with cooling towers microbes (Tsao *et al.* 2019). These findings suggest that while the cultivation-based tools used to assess bacterial abundance are known to culture only a subset of total cells, the assays used in this study were able to enumerate taxa thought to be important in these systems based on prior studies.

It is known that disinfection processes can influence the long-term microbial community composition in process water systems (Roeder *et al.* 2010; Wang *et al.* 2013; Baron *et al.* 2015). Therefore, it is also vital to evaluate alterations in the community composition and the potential for treatment-resistant 'persister' taxa that may occur following new biocontrol interventions (Paduano *et al.* 2020). While the number of colonies examined in this study and experimental design of single-pass columns are not adequate to provide a robust evaluation of the potential for persister taxa, it may be worth noting that *Pseudomonas* and *Cupriavidus* were found in much higher relative abundance in post (30 and 35%, respectively) vs pre (both taxa undetected in the sequenced colonies) column samples, perhaps suggesting relative enrichment or lower removal efficiency of these taxa. More importantly, these data do suggest that significant reductions are possible even in high-diversity, natural, assemblages, not just with selected pure cultures that may not represent environmental samples in real-world applications. However, despite this initial proof-of-concept result with diverse assemblages these initial tests were purposefully designed to be followed by an examination of cultures including taxa of particular interest in industrial water, as discussed below.

The commonly used methods of controlling microbial loads in cooling tower systems often involve exposure to toxic chemicals (e.g., oxidant biocides) and the resulting toxic disinfection byproducts, energy-intensive methods (e.g., UV and sand filters), or water-inefficient methods (e.g., frequent disposal and replacement of system waters). The energy, water, and cost inefficiencies as well as hazards associated with these methods result in a need for innovative and higher-performing treatment technologies. The examined technology in this study is held within a relatively high-permeability media which means that in practice a fraction of the energy may be consumed compared to other biocontrol methods (e.g., sand filtration).

While the results of the present study are positive, future studies will need to evaluate a wider range of conditions and demonstrate the ability of the media to have lasting and sustained performance. This needs to be further demonstrated at laboratory, pilot, and full-scale levels. Because this media may not be the only biocontrol measure used in some industrial systems or cooling towers, its compatibility with other technologies (e.g., oxidants, sand filters), as well as the design and operation of various cooling systems (e.g., hydraulics, air handlers), should be assessed. If it is to be used in parallel with common oxidant-based biocides, then the extended exposure to high concentrations of these chemicals needs to be assessed. In other applications, it might be deployed in the absence of other biocides (e.g., end-of-pipe stormwater treatment) but the coatings' durability will need to be more thoroughly evaluated under relevant environmental conditions. For example, the potential for leaching of ADBAC biocides into effluent waters needs to be examined both as a secondary biocontrol mechanism and as a concern for receiving waters. However, if ADBAC is found to be leached from the coating, then for many applications where current practices include release of biocides into system waters then such an attribute is not disqualifying and may even be advantageous.

Treatment media stability – examination of potential release coating components into water

The methods of the water extraction study were designed as a means for roughly evaluating the stability of the quat laced marine coatings and to screen which if any, might be suitable for further analysis of their antimicrobial efficacy. None of the test samples had the targeted VOCs found above the detection level (10 mg/kg). As seen in Table 2, bisphenol A was only detected above detection levels (100 μg/kg) in the quat-containing Y2002E coating (avg. 263μg/kg $\pm 0.064\%$, $n = 3$). The three chain lengths of benzalkonium chlorides (C_{12}, C_{14}, and C_{16}) in the Pilot Chemical formulation were detected in both Interlux coatings. The average mg/kg levels C_{12}, C_{14}, and C_{16} ABDAC in three analyses of the Y2002E-based coating were 449 ($\pm 11.2\%$), 794 ($\pm 3.5\%$), and 1272 ($\pm 26.8\%$). The Y2000VOC releases were 33 ($\pm 43.9\%$), 19 ($\pm 29.8\%$), and 0.109 ($\pm 72.5\%$). These are significantly lower (92.7%, 97.6%, and 99.9%).

Table 2 | Bisphenol A and benzalkonium chlorides in water extraction tests

		Y2002E		Y2000VOC	
Analyte	**Sample**	**Quat**	**w/o Quat**	**Quat**	**w/o Quat**
Bisphenol A	#1	277	N.D.	N.D.	N.D.
(μg/kg)	#2	267	N.D.	N.D.	N.D.
(Detection Level 100)	#3	244	N.D.	N.D.	N.D.
	Avg.	263	N.D.	N.D.	N.D.
	C.O.V.	0.064	NA	NA	NA
Benzalkonium Chl. (C12) (C12)	#1	399	N.D.	22.0	<0.2
(mg/kg)	#2	449	N.D.	49.2	N.D.
(Detection Level 0.2)	#3	500	N.D.	27.0	N.D.
	Avg.	449	N.D.	33	N.D.
	C.O.V.	0.112	NA	0.439	NA
Benzalkonium Chl. (C14)	#1	777	N.D.	14.7	N.D.
(mg/kg)	#2	780	N.D.	25.1	N.D.
(Detection Level 0.08)	#3	826	N.D.	16.0	N.D.
	Avg.	794	N.D.	19	N.D.
	C.O.V.	0.035	NA	0.298	NA
Benzalkonium Chl. (C16)	#1	1,359	N.D.	0.070	N.D.
(mg/kg)	#2	896	N.D.	0.200	N.D.
(Detection Level 0.018)	#3	1,560	N.D.	0.057	N.D.
	Avg.	1272	N.D.	0.109	N.D.
	C.O.V.	0.268	NA	0.725	NA

C.O.V. is an abbreviation for the statistical term coefficient of variation. N.D., not detected.

In an assessment of biocides for application to address *Legionella* in cooling towers (Garcia & Pelaz 2008), ABDAC and seven other organic chemical agents were evaluated for their performance against *L. pneumophila* using European Committee Standards (Standardization 1999). They examined concentrations from 2 to 8,192 ppm of the biocide versus the same strain of *L. pneumophila* as this work to determine the minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) (Standardization 1999). While they did not specify the alkyl chain information on the ABDAC used in their assessment, they found MIC_{50} and MBC for their ABDAC of 8 and 32 ppm. That level of performance ranked third best among the biocides examined. They found the greatest level of strains killed near 2,000 ppm with decreased performance above that. It should be cautioned that concerns associated with worker safety, system performance and life expectancy, and costs were not considered in this study, and thus biocide selection is multifaceted. With that being stated, these findings show that Y2002E likely releases enough ABDAC to contribute to bacterial loss and Y2000VOC releases may exhibit inhibitory and even bactericidal conditions. Because the levels of quats being released from the low-VOC epoxy were 13–12,000 times lower than the Y2002E coating and because its use resulted in lower exposure of volatile compounds to personnel, additional testing proceeded with the Y2000VOC-based formulation.

Performance testing against bacteria of concern – quat-amended low-VOC coating treatment column studies

The MPN of bacteria per 100 mL of sample, plus the lower and upper CI for all samples are provided in Tables S3, S4, and S5 of Supplemental Information. In Figures 3–5 are shown the average of the three replicates with the error bars being the average of the 95% CIs. It should be noted that some of the analysis trays lacked even a single positive well, indicating a result below detection (an MPN concentration <1.0), that has been assigned a value of 0.5 MPN in the statistical analysis and in the plots, as is common practice for IDEXX analysis and other bacterial enumeration tests, representing a conservative approach to assessing the effectiveness of this technology.

For *E. faecalis* (Table S3 and Figure 3), the influent solution had an average MPN of 382.4 with the average lower and upper 95% CIs for the set of three replicates being 244.3 and 570.8. *E. faecalis* levels were found to differ significantly among the employed treatments ($4,10) = 14.42$, $p > 0.01$) with no significant differences found between influent and controls (neutralized and non-neutralized, $p = 0.90$ and $p = 0.42$, respectively), but significant reductions between influent and test (neutralized and non-neutralized, $p = 0.01$ and $p < 0.01$, respectively) as well as the test and control (neutralized and non-neutralized, $p = 0.02$ and $p < 0.01$, respectively) columns. Based on these results, it can be readily concluded that there was no discernable influence on the *E. faecalis* levels due to passage through the control media and nor is there an apparent impact of neutralization with D/E. There was no significant difference between neutralized and non-neutralized control columns

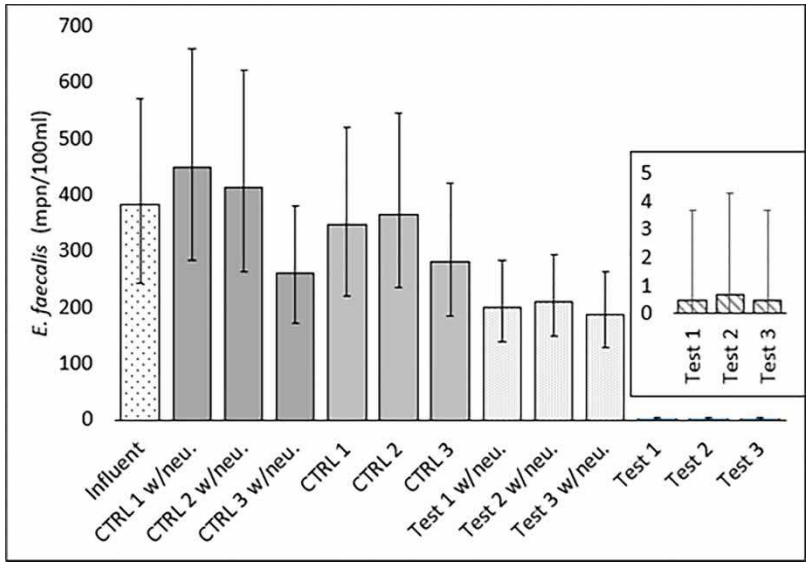


Figure 3 | Results of *Enterococcus faecalis* in column studies of low-VOC-coated media.

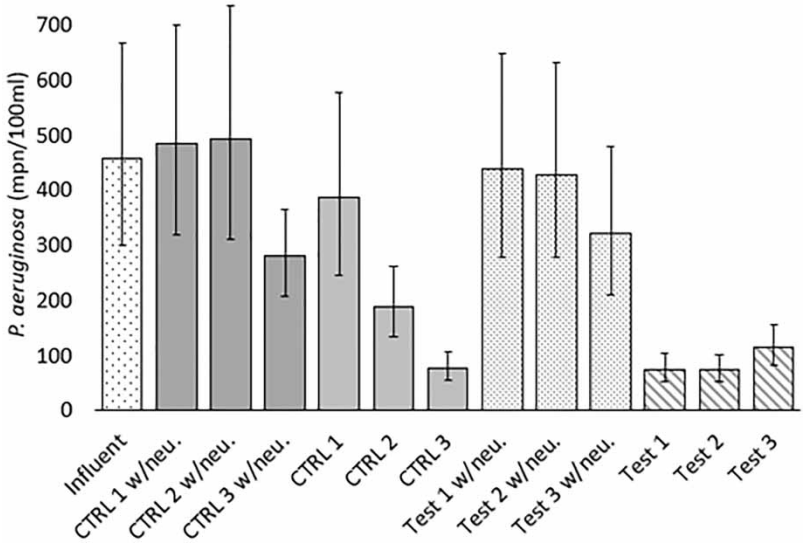


Figure 4 | Results of *Pseudomonas aeruginosa* in column studies of low-VOC-coated media.

($p = 0.49$), but the non-neutralized test columns did have significantly lower concentrations ($p = 0.01$) compared to the neutralized test column.

Comparing the average MPN concentrations, the neutralized effluent of the three test solutions was 45–51% lower than the influent solution. In the nine samples of non-neutralized effluent waters (three test columns sampled three times each), only one tray had a single positive well equating to a calculated MPN concentration of 1.0 in that sample, indicating that the percent reductions are underestimated due to the majority of final concentrations falling below the detection limit. This also shows that significant additional bacteria are being lost due to the presence of eluted component(s) of the test media or that those eluted chemicals are interfering with the IDEXX reagents. Bisphenol A has been observed to impact *E. faecalis* at concentrations above 30 μg/mL (Kyrila *et al.* 2021), but this should not be of concern given that this component release study did not observe them at levels far below that (i.e., not found above detection levels of 0.100 μg/g). However, ABDAC was found to be released which points to its likely cause as the difference in observed microbial reductions between the neutralized and non-neutralized results.

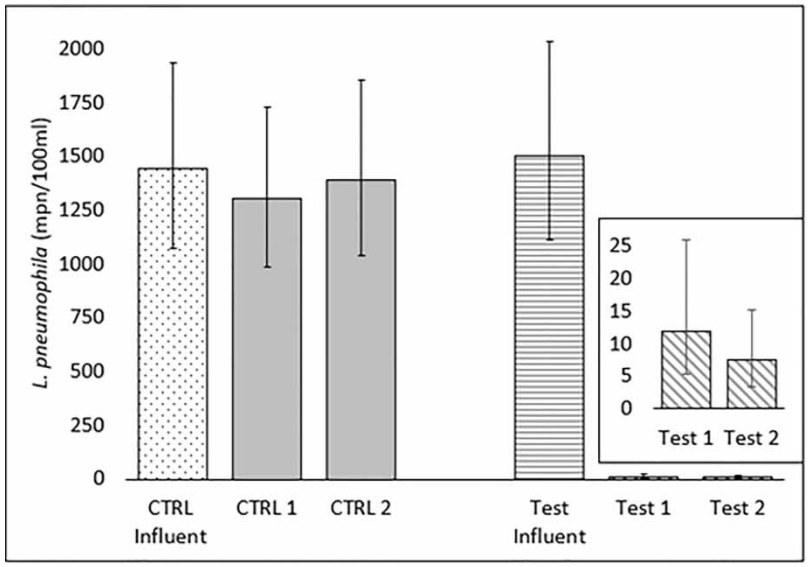


Figure 5 | Results of *Legionella pneumophila* in column studies of low-VOC-coated media.

For *P. aeruginosa* (Table S4 and Figure 4), but this should not be of concern given that this influent solution had an average MPN of 458.4 with the average lower and upper 95% CIs being 302.1 and 669.0. Given the similarities of the experimental and analytical methods, the range of uncertainty of *P. aeruginosa* in this work is similar to *E. faecalis* with the respective CIs being 34.1% below and 45.9% above the MPN, but the patterns of removal are overall more complicated. *P. aeruginosa* levels were found to differ significantly among the employed treatments ($F(4,10) = 7.772$, $p > 0.01$) with no significant differences found between influent and neutralized control ($p = 0.64$) but a significant reduction between influent and non-neutralized control ($p = 0.01$), and similarly no significant reduction between influent and neutralized test columns ($p = 0.46$) but a significant reduction between influent and non-neutralized test ($p < 0.01$). No significant differences were observed between control and test columns (neutralized and non-neutralized, $p = 0.78$ and $p < 0.13$, respectively), but significantly greater reductions were observed in neutralized compared to non-neutralized test columns ($p < 0.01$).

For the quat-amended media, there is no discernable decrease in *P. aeruginosa* for the neutralized samples with all three having their average MPN levels falling within the range of variability of the influent solution. All three of the non-neutralized samples are significantly lower than the influent with $\log_{10}$ reduction in concentration versus the average influent of 0.78, 0.79, and 0.60 for the three test columns. The interaction of neutralizer in control columns (lacking ABDAC) is difficult to understand and the overall lower performance of the porous media against *P. aeruginosa* will require additional investigation as this microorganism is of interest for some industrial water systems (Scheikl *et al.* 2014; Mittelman & Jones 2018).

The final bacteria examined was *Legionella pneumophila* (Table S5 and Figure 5). As previously stated, additional handling and cost concerns resulted in minor changes in the experimental approach. These again included different influent solutions being used for the control and test columns, sets of two rather than three control and test columns, and no neutralized column effluent samples. The average influent concentrations were 1,440.7 ($n = 3$, $\pm$ 142.3) and 1,504.2 ($n = 3$, $\pm$ 118.2) MPN/100 mL. By comparison in their examination of biocides for use in cooling towers Garcia and Pelaz (Garcia & Pelaz 2008) used $1 - 3 \times 10^7$ CFU/mL of *L. pneumophila*. By comparison, in Walser *et al.* review of *Legionella* outbreaks attributed to cooling towers (Walser *et al.* 2014), they found in the literature that measured concentrations of $10 - 10^7$ CFU/mL, but cautioned that these levels may not represent levels during outbreaks. *L. pneumophila* levels were found to differ significantly among employed treatments ($F(3,6) = 115.3$, $p < 0.01$) with no significant differences found between influent and control ($p = 0.13$), but significantly lower concentrations in test columns compared to both influent ($p < 0.01$) and control columns ($p < 0.01$).

It is worth noting that one of three Test 1 samples and 2 of 3 Test 2 samples reported no measurable levels of *Legionella*. As stated earlier, those non-detects are assigned estimated concentrations of 0.5 MPN/100 mL and using those values results in average effluent concentrations of 11.9 and 7.5 MPN/100 mL and $\log_{10}$ units of loss 2.3 and 2.7. Greater performance in absolute and log reduction may exist when challenged with a more concentrated influent solution. The performance of this technology versus *Legionella* is noteworthy because past outbreaks have resulted in commercial HVAC and cooling towers (Fitzhenry *et al.* 2017; Lapierre *et al.* 2017) systems being mandated to measure and control its levels in the state of New York (State 2016; Schoonmaker-Bopp *et al.* 2021).

CONCLUSIONS

In this project, the amendment of a commonly employed biocide, alkyl dimethyl benzyl ammonium chloride, to the epoxy marine coating on pea-gravel was shown to successfully lower bacterial levels in water streams. When combined with a low-VOC version of the coating, the stability of the material was markedly improved with significantly lower releases of bisphenol A and ABDAC compounds. In further testing, the ABDAC-amended low-VOC coating significantly reduced *E. faecalis* and *L. pneumophila* levels in water streams but demonstrated less performance against *P. aeruginosa*. These results show great potential for this technology, and with further development, it may offer an additional means for controlling microbial pollution within water systems.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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First received 26 September 2022; accepted in revised form 5 January 2023. Available online 15 March 2023