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Disinfection of bacterial biofilms in pilot-scale cooling tower systems

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Abstract

The impact of continuous chlorination and periodic glutaraldehyde treatment on planktonic and biofilm microbial communities was evaluated in pilot-scale cooling towers operated continuously for 3 months. The system was operated at a flow rate of 10,080 l day^{-1}. Experiments were performed with a well-defined microbial consortium containing three heterotrophic bacteria: *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Flavobacterium* sp. The persistence of each species was monitored in the recirculating cooling water loop and in biofilms on steel and PVC coupons in the cooling tower basin. The observed bacterial colonization in cooling towers did not follow trends in growth rates observed under batch conditions and, instead, reflected differences in the ability of each organism to remain attached and form biofilms under the high-through flow conditions in cooling towers. *Flavobacterium* was the dominant organism in the community, while *P. aeruginosa* and *K. pneumoniae* did not attach well to either PVC or steel coupons in cooling towers and were not able to persist in biofilms. As a result, the much greater ability of *Flavobacterium* to adhere to surfaces protected it from disinfection, whereas *P. aeruginosa* and *K. pneumoniae* were subject to rapid disinfection in the planktonic state.

Keywords

pilot-scale cooling towers; chlorination; glutaraldehyde; disinfection; bacterial biofilms; microbial community

Introduction

Cooling towers are used in most large commercial and residential buildings, industrial power generation units, and chemical, petrochemical and petroleum industries to reject waste heat to the environment. These water systems provide highly favorable environments for microbial growth (Liu et al. 2009; Pagnier et al. 2009), and have been attributed to be a source for dissemination of human pathogens, notably *Legionella* spp., but also a variety of

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other pathogenic bacteria, protozoa and viruses (Yamamoto et al. 1992; Breiman 1996). Cooling towers generally have large-volume water reservoirs open to the atmosphere, within which the temperature is typically maintained between 25°C and 35°C. In addition to receiving inputs of carbon and nutrients from atmospheric sources and the building water loop, many traditional water treatment chemicals used in cooling towers, such as antiscalants and corrosion inhibitors, also provide nutrients that support microbial growth (Kusnetsov et al. 1993; ASHRAE 2000). Nutrients and organic matter that enter cooling towers are concentrated by evaporation and recycling within these systems. As a result of these favorable environmental conditions, bacteria grow rapidly in cooling towers. Further, the plentiful availability of surfaces in the evaporative fill material, water reservoir, and cooling water loop supports extensive growth of surface-attached microbial communities (biofilms) (Kusnetsov et al. 1993).

Currently, continuous application of oxidizing biocides is recommended to maintain microbial populations at low numbers, and an occasional or periodic dose of an additional non-oxidizing biocide is used as a supplemental control measure whenever elevated bacterial numbers are detected (ASHRAE 2000; Pagnier et al. 2009). Despite the widespread use of this type of control scheme, the long-term efficacy of this approach has not been evaluated in detail for cooling tower environments, based on the current authors' search of the peer-reviewed literature. Although the effectiveness of various biocide application strategies in cooling towers has been evaluated, most studies focused only on the total planktonic bacterial numbers recirculating in the water system (Kim et al. 2002). Few studies have specifically focused on characterizing biofilm communities within the water reservoir, piping, and evaporative fill material, which represent a majority of the bacterial biomass in cooling towers. It should be noted that biofilm development in cooling towers is a particular health concern, as biofilms formed by nonpathogenic bacteria can harbor a variety of pathogens and protect them from biocides (Hall-Stoodley et al. 2004). Little information is available on the impact of cooling tower operation and disinfection conditions on biofilm community structure.

Previous studies have shown that biocides that kill planktonic microorganisms may not be fully effective in removing biofilms owing to protection provided by the extracellular polymeric substances (EPS) that form the biofilm matrix (Costerton et al. 1995; Stewart 2002; Hall-Stoodley et al. 2004; Fux et al. 2005). Beyond chemical interactions with EPS, slow diffusion through the biofilm matrix also reduces delivery of disinfectants to bacteria deep in the biofilm (Stewart 2003; Stewart and Franklin 2008). While extensive biofilm growth has been observed in cooling towers, the efficacy of different biocide application regimens on biofilm communities in cooling towers is not known. In addition, persistent survival of even a small number of organisms in biofilms can serve as a source of rapid recolonization of the system following any interruption of biocide treatment (Hosni et al. 2009), but the extent and rate of bacterial re-growth from these communities following treatment also is not known.

Cooling towers support complex microbial ecosystems encompassing a wide variety of ecological niches that behave quite differently than small, homogeneous laboratory culture devices. Batch systems and chemostats, which are often used to examine the effectiveness of

disinfectants in the laboratory, clearly are not representative of the diverse hydrodynamic and habitat conditions found in cooling tower systems (Wright et al. 1991; Green 1993; Murga et al. 2001; Donlan et al. 2005). However, it is extremely difficult to conduct well controlled biofilm disinfection studies in full-size industrial cooling towers because of the large volume and open nature of these systems (Kurtz et al. 1982). In general, field studies are not reproducible and are highly dependent on local conditions, making it extremely difficult to generalize results. This has made it difficult to evaluate recommended industrial treatments for control of microbial communities in cooling towers. To address these limitations, experiments were performed in a set of pilot-scale cooling tower systems that were constructed following industrial standards using exactly the same materials as found in conventional full-size cooling tower systems (Liu et al. 2009). The pilot-scale systems described here replicate all essential features found in industrial cooling towers, and provide a realistic distribution of local environmental conditions for study of microbial growth and removal in cooling towers while also providing the necessary degree of control for experimental investigations of microbial growth processes. The objective of the present study was to evaluate the impact of both oxidative (chlorine) and non-oxidative (glutaraldehyde) disinfection treatments on microbial biofilm population and community structure in cooling tower environments. This work provides useful new insight into strategies for successfully managing cooling towers to protect public health.

Materials and methods

Pilot-scale cooling towers

A detailed description of cooling tower system configuration and operation is provided in Liu et al. (2009), and is briefly summarized here. As shown in Figure 1, each cooling tower consists of a chamber (56 l) containing a water basin (10.8 l) and three blocks of commercial cooling tower fill material ($0.2 \times 0.3 \times 0.5$ m^3) composed of polyvinyl chloride (PVC) horizontally packed in an open, highly porous configuration (Brentwood Industries, PA). Water was pumped from the basin through a heat exchanger, and then to the top of the tower, where it was distributed *via* a perforated plate over the top deck of the PVC fill. The system was operated at a flow rate of 10080 l day^{-1}. Air was drawn through the tower horizontally at the rate of 372 ± 17 m min^{-1} to cool the falling water. As in standard industrial practice, each test cell was drained at a constant rate in order to maintain a steady concentration of dissolved solids in each cooling tower. Laboratory tap water was used as make-up water to exactly match water losses from evaporation plus the drainage (blow-down). The make-up water was dechlorinated by means of UV irradiation provided by two submerged BL-15 lamps (UVP LLC, CA). Throughout the experiment, the recirculating water was heated to a constant temperature of 32 ± 0.5°C, and the temperature in the cool water basin (after passing through the evaporation section of the cooling tower) was 28 ± 1.6°C.

Prior to the study, each cooling tower test cell was disinfected by treatment with glutaraldehyde at a concentration of 10 mg l^{-1}. Numerous coupons for characterization of biofilm growth were then inserted vertically in the water basin and subsequently removed for biofilm analysis. Two coupon materials were used, corresponding to the major materials

of the cooling tower: stainless steel, identical to the material used for the cooling tower housing and water basin (cold rolled steel, Inland Steel Co. IL) (0.5 × 0.5 × 0.1 cm) and PVC, identical to the material used in the evaporative fill (Brentwood Industries, PA) (0.5 × 0.7 × 0.2 cm). Prior to use, all coupons were disinfected following the steps reported in Liu et al. (2009). The hydrophobicity of the coupons was determined by sessile drop contact angle measurements using a drop shape analysis instrument (Newport Optical Inc., RI).

Inoculation of cooling towers

Microbial growth was initiated in the cooling towers using a defined consortium of three biofilm-forming bacteria, *Pseudomonas aeruginosa* (ATCC 7700), *Klebsiella pneumoniae* (DMDS Lab. No. 92-08-28a) and *Flavobacterium* sp. (CDC-65). The stored strains were streaked onto R2A agar plates and incubated at 28°C for 16 h (*K. pneumoniae* and *P. aeruginosa*) or 36 h (*Flavobacterium*). A single colony was then transferred into 5 ml of R2A broth and grown in a shaker incubator at 200 rpm and 28°C until the bacterial cells reached the stationary phase (16 h for *K. pneumoniae*; 20 h for *P. aeruginosa*; 30 h for *Flavobacterium*). R2A growth medium was prepared by dissolving 0.5 g l^{-1} yeast extract, proteose peptone no. 3, casamino acids, and dextrose, 0.3 g l^{-1} sodium pyruvate and dibasic potassium phosphate, and 0.05 g l^{-1} magnesium sulfate in reverse osmosis (RO) purified water.

Cooling towers were seeded with bacteria by adding sufficient volumes of the stationary-phase cell suspensions necessary to achieve initial *K. pneumoniae, P. aeruginosa* and *Flavobacterium* concentrations of 10^8 CFU ml^{-1} for each of the species. R2A broth was added to reach 10% of the stock medium concentration described previously.

Cooling tower operation and disinfection

As shown in Table 1, five cooling tower test cells (TC1-5) were operated in parallel for 3 months. At the beginning of the experiment (day 0), the bacterial strains described above were inoculated into all five test cells. In order to observe whether colonization of a pre-established biofilm would yield different results than those from initial colonization of the system, the three bacterial cultures were reinoculated into each cooling tower test cells on day 16. One of the test cells (TC1) was selected to be continuously treated with bleach (sodium hypochlorite) to maintain a residual free chlorine concentration of 2 ± 0.28 ppm. The other four test cells (TC2-5) were not treated during the first 48 days of the experiment in order to observe colonization of the cooling towers by the introduced bacteria. After 48 days, two of the inoculated test cells, TC4 and TC5, were treated with continuous chlorination (free chlorine concentration of 2 ± 0.28 ppm), and the remaining two test cells (TC2 and TC3) were used as positive controls for biofilm growth (never disinfected).

Glutaraldehyde (Bio-Source Inc., GA) treatment was initiated at day 58 in chlorine-treated cells TC1, TC4 and TC5. Subsequently, glutaraldehyde was added weekly to each of these test cells (days 65, 72, 79, 86). Each treatment consisted of adding 200 ml of concentrated glutaraldehyde solution (550 mg l^{-1}) directly into the cooling water basin to achieve an average glutaraldehyde concentration of 10 mg l^{-1} throughout the treated cooling tower.

Chemical and physical testing in cooling tower system

The water conductivity and temperature were measured daily using a conductivity meter coupled with a thermister probe (ES-12, HORIBA, Ltd, Japan). pH was measured twice a week with a pH triode (Orion, Thermo Fisher Scientific, Inc., MA) connected to a digital pH scale (Orion 720A, Thermo Fisher Scientific, Inc., MA). A colorimeter (DR/890, Hach Company, CO) was used to analyze total chlorine, free chlorine, phosphonate, and silica concentrations using Hach reagents. Total and free chlorine measurements were performed daily, while phosphonate and silica concentrations were measured once a week. Alkalinity was tested once a week using a HACH digital titrator (model 16900, HACH, CO). Airflow measurements were performed twice a week using a hand-held anemometer (Davis, model LCA30-VT). For each cooling tower, airflow readings were collected at the top, middle and bottom of the air inlet. The average of the three readings was then recorded as the overall cooling tower airflow rate. Samples for all analyses were collected from the water basin of each cooling tower.

Bacterial enumeration and biofilm analysis

The abundance of planktonic and surface-attached bacterial cells in the cooling towers was monitored periodically. Biofilm growth was monitored on sampling coupons placed in the cooling water basin. To remove surface-attached bacteria and EPS, the sampling coupon was placed in a 2 ml centrifuge tube containing 1 ml of PBS, ultrasonicated for 10 min (FS 20H, Fisher Scientific Inc., IL), and then vortexed (Genie 2, Fisher Scientific Inc., IL) at the maximum speed for 30 s. This ultrasonication-vortexing process has previously been proven to efficiently dissociate bacteria from coupons (Liu et al. 2007; Liu and Li 2008).

Viable bacterial cell counts were obtained using the drop plate method (Liu et al. 2007). A series of 10-fold dilutions was performed and 10 μl of each dilution were plated on R2A agar plates in triplicate. Heterotrophic bacteria plate counts (HPC) were obtained after incubating the plates at 28°C for 1 week. The three types of inoculated bacteria were readily distinguished by the appearance of their colonies: *K. pneumoniae* colonies are white, *Flavobacterium* are yellow, and *P. aeruginosa* are light green. Moreover, *K. pneumoniae* and *P. aeruginosa* are faster-growing, so their colonies were counted 16 h after plating while slower-growing *Flavobacterium* colonies were counted 36 h after plating. Non-inoculated bacteria (environmental immigrants) were detected in the cooling tower system after operation for 20 days. Total colonies and the number of colonies for each organism were counted after 1 week. The lower limit of this detection method is ~10^2 colony-forming units (CFU) ml^{-1}.

In addition, immigrant species were identified by the 16S rRNA gene sequencing analysis following the procedure described in a previous publication (Liu et al. 2009). The identification of inoculated strains in the consortium was confirmed using the same protocol. Briefly, single colonies were selected from R2A agar plates based on their distinctive morphology, transferred to different R2A plates, and incubated under 28°C. Cells were then grown in 5 ml of growth medium at 28°C until they reached stationary phase. Genomic DNA was extracted using the phenol/chloroform method (Zoetendal et al. 2006). Partial 16S rRNA gene fragments were amplified from the genomic DNA by PCR using primer pair 63f

(5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 1387r (5′-GGG CGG WGT GTA CAA GGC-3′) (Marchesi et al. 1998). PCR was run with an initial hot start at 95°C for 3 min, followed by 36 cycles of 30 s at 95°C, 45 s at 56°C and 90 s at 72°C, and then a final extension at 72°C for 10 min. The PCR products were purified before sequencing using QIAquick Spin Columns (QIAGEN Sciences, MD) and sequenced at the University of Chicago Cancer Research Center DNA Sequencing Facility (Chicago, IL). Each sequence was matched against the NCBI nr nucleotide database using the nucleotide BLAST program.

The structure of surface-attached biofilms in the cooling tower water basins was evaluated by analyzing coupon samples using an upright Leica confocal laser scanning microscope (CLSM) model DM RXE-7 (Leica Microsystems, Germany). Concanavalin A conjugated with Texas Red was used to stain biofilm EPS, and SYTO 9 was used to stain bacteria (Invitrogen Corporation, CA). Stained coupons were placed in PBS buffer, and visualized under a 40× water immersion objective. At least five images were examined for each sample and all CLSM images were collected within 3 h after the sample coupons were removed from the cooling towers.

Batch experiments

Additional batch experiments were performed to evaluate the impact of bacterial growth rate on their adhesion to PVC and steel coupons. The mixed bacterial culture was inoculated in test tubes with pre-inserted sterilized sampling coupons. The same water used for the cooling tower make-up supply was used as the solution medium for the batch studies. The initial cell and nutrient concentrations in the test tubes were identical to those inoculated in the cooling tower systems.

Results

Chemical and physical properties in cooling towers (TC1-5)

The chemical and physical conditions in the recirculating water in the cooling towers are reported in Table 2. The operational conditions remained essentially constant throughout the experiment.

Planktonic and biofilm bacteria in cooling towers without treatment (TC2 and TC3)

Figure 2 shows the persistence of heterotrophic bacteria in each cooling tower basin throughout the experiment. Three heterotrophic bacteria, *P. aeruginosa*, *K. pneumoniae*, and *Flavobacterium* sp. were inoculated simultaneously in each cooling tower and the persistence of heterotrophic bacteria was monitored in the bulk and biofilm phases. As shown in Figure 2a, the concentrations of all three bacteria decreased two-to-four orders-of-magnitude 3 h after the initial injection of the bacteria. The loss of bacteria is primarily attributed to wash-out associated with the high rate of water outflow (blow-down), and can also result from bacterial cell lysis due to the sudden dilution (Liu et al. 2009). Planktonic *K. pneumoniae* and *P. aeruginosa* reached concentrations of 10^4 CFU ml^{-1} and 10^3 CFU ml^{-1}, respectively, 1 day after initial injection. The concentrations of both species decreased < 10^2 CFU ml^{-1} (the sensitivity limit of the analytical method) after 5 days. The *Flavobacterium* concentration decreased by three orders-of-magnitude in 5 days, and then remained at a

constant concentration of 10^5 CFU ml^{-1} for the remainder of the experiment (Figures 2a and 3a).

The observed differences in the planktonic concentrations of *Flavobacterium* relative to *K. pneumoniae* and *P. aeruginosa* are explained by differences in their ability to form biofilms in the cooling towers. Figure 2b and 2c depict the colonization of the PVC coupons and the steel coupons by the three test strains. As shown in Figure 2b and 2c, *P. aeruginosa* and *K. pneumoniae* both showed a low degree of adherence to coupons surfaces, and were washed out of the cooling tower systems after 4 days of continuous operation. Conversely, *Flavobacterium* rapidly colonized the sampling coupons and became the dominant microorganism in the cooling tower biofilms. This observation can be explained by the fact that *Flavobacterium* had a much less negative zeta potential than the other two bacteria (*Flavobacterium*: −8.75 mV; *K. pneumoniae*: −18.83 mV; *P. aeruginosa:* −20.77 mV), indicating a less negatively charged cell surface. According to the Derjaguin, Landau, Verwey and Overbeek (DLVO) theory, more negatively charged bacterial surfaces pose higher repulsion to the negatively charged steel coupon surfaces (Liu et al. 2009).

An additional set of batch experiments was carried out to evaluate the adhesion of each strain to steel and PVC surfaces under chemical conditions similar to those found in cooling towers. These experiments were performed in a shaker incubator at 100 rpm. As shown in Figures 2 and 3, substantial differences were observed in the microbial communities that developed in the closed batch systems compared to those in the cooling towers. Under the closed batch condition, while *Flavobacterium* still had a high degree of adherence to sampling coupons, *P. aeruginosa* grew much better than *Flavobacterium* in mixed-species biofilms and ultimately became the dominant organism. However, the lower degree of adherence caused *P. aeruginosa* and *K. pneumoniae* to be rapidly washed out of the open cooling tower systems, and *Flavobacterium* became the dominant microorganism in the cooling towers. In addition, the batch and cooling tower systems differed in air flow conditions, which may contribute to the difference in biofilm formation by *P. aeruginosa* and *K. pneumonia.* These results indicate that adhesion, retention, and growth on solid surfaces all play important roles in the bacterial community that develops in cooling tower systems.

In both cooling tower and batch experiments, higher bacterial concentrations were observed on the PVC coupons than on steel, indicating PVC presents a more favorable surface for bacterial colonization than steel. Previous testing of the bacteria showed that all three bacteria are hydrophilic (Liu et al. 2009), and PVC coupon surfaces are more hydrophobic than steel surfaces under the condition tested (water contact angle on steel = 84.2° ± 0.4°; PVC = 93.0° ± 0.5°). These results are in agreement with the extended DLVO theory, which suggests that bacterial adhesion is favored as the material surface hydrophobicity increases.

The three bacterial cultures were re-introduced at day 16 to determine whether colonization of pre-established biofilms would yield different results than initial colonization of the system. Re-inoculation times are indicated by arrows in Figure 2. *P. aeruginosa* disappeared from the cooling tower system within 1 day after re-injection, while re-injected *K. pneumoniae* only persisted in the system for 2 days. Similar results have been reported

previously (Liu et al. 2008), and can be explained by the high biofilm surface hydrophilicity and polymeric interactions between the biofilm and the newly injected bacteria, which discourage bacterial adhesion to biofilm surfaces. The average *Flavobacterium* concentration became relatively constant after continuous cooling tower operation for 20 days, with some fluctuations. The constant concentration of both surface-attached and planktonic bacteria indicates the ecological stability of the cooling tower system for the steady physical and chemical conditions imposed here.

CLSM images provide direct observations of the extent of colonization of different surfaces in the cooling tower water basin (Figure 4). On the steel coupons, the attached cells were widely dispersed over the coupon surfaces with an average thickness of 7.3 ± 1.2 μm on day 1. Large cell clusters developed later in the experiment, reaching thicknesses up to 80.5 ± 3.7 μm on day 22. The structure of the biofilms remained stable for the remainder of the experiment, indicating that the spatial organization of biofilms did not change after the period of initial colonization and biofilm formation. Colonization of PVC coupons was similar to that observed on the steel coupons. The biofilm became thicker on PVC than on steel ($p < 0.005$), reaching 100 μm after day 22. These results are consistent with the plate count results.

Impact of chlorination: continuous chlorination from system startup (TC1)

Although surface attached biomass was less abundant with chlorination treatment (TC1) than without (TC2 and TC3), bacterial biofilms developed on both PVC and stainless steel coupon surfaces even under the continuous chlorine treatment. The concentration of suspended *Flavobacterium* decreased from 10^8 CFU ml^{-1} to ~10^3 CFU ml^{-1} one day after inoculation under chlorine treatment (Figure 2g), compared to 10^5–10^6 CFU ml^{-1} without treatment (Figure 2a, d). Bacterial counts in the chlorine-treated test cell then decreased further to ~10^2 CFU ml^{-1} after 7 days, and then stabilized at that level. Similarly, total biofilm concentrations stabilized at 10^3–10^4 CFU cm^{-2} under chlorine treatment (Figure 2h, i), compared to 10^5–10^7 CFU cm^{-2} without treatment (Figure 2b, c, e, f). Therefore, while chlorine treatment successfully reduced planktonic and biofilm concentrations by several orders of magnitude, it was not sufficient to keep the cooling towers free of bacteria.

Bacterial re-growth was observed at day 20 and day 35 following two temporary shut-downs of the chlorination system for 24 h and 28 h on day 19 and day 34, respectively. These short outbreaks of bacterial growth demonstrate that cessation of chlorine disinfection for even a few hours has an important effect on water quality, even when continuous chlorination otherwise provides good containment of bacterial populations. The fact that the re-growth was extremely fast indicates that chlorine treatments commonly used to control bacteria in cooling tower systems do not truly eradicate bacteria from biofilms, or fully suppress their activity, even under 2 mg l^{-1} of free chlorine residual.

Impact of chlorination on mature biofilms (TC4 and TC5)

As shown in Figure 2d–f, the concentrations of planktonic and biofilm bacteria became steady after ~10 days of continuous operation, indicating that the microbial ecosystem in the cooling tower was mature and stable after this time. This stability of the community

structure persisted even after re-inoculation with the three test strains at day 16. After 48 days, the impact of chlorination on biofilms was tested in the two cooling tower test cells by continuous injection of sodium hypochlorite into the test cell basins to achieve a constant free chlorine concentration of 2 ppm. The concentrations of planktonic and surface-attached bacteria decreased by one-to-two log units within 3 h after chlorination. These concentrations decreased further by approximately an additional log unit over the following 2 days. However, biofilm re-growth was observed beginning 3 days after the chlorine treatment was initiated. Within a few days, the planktonic concentration reached pre-treatment values, ~10^5 CFU ml^{-1} (Figure 2d), while biofilm bacterial numbers reached 10^4 CFU cm^{-2} and 10^3 CFU cm^{-2} on the PVC and steel coupons, respectively, approximately one order-of-magnitude less than before chlorination (Figure 2e, f). The bacterial regrowth in the face of continuous chlorination can be explained by the rapid adaptation of established bacterial communities to chemical stresses (Simoes et al. 2008), as well as by protection of the biofilm community from disinfection.

Impact of glutaraldehyde (TC1, TC4, and TC5)

Following typical recommendations for long-term disinfection of cooling towers (ASHRAE 2000), glutaraldehyde was added weekly to cooling towers TC1, TC4, and TC5 in conjunction with the continuous chlorine treatment beginning on day 58. As shown in Figure 2d–f, each pulse of glutaraldehyde greatly reduced planktonic and biofilm bacterial counts immediately after injection. However, the residence time of glutaraldehyde is relatively short in this system, and regrowth occurred 4–5 days after each treatment, again most probably from sheltered biofilm communities. Repeat introduction of glutaraldehyde every week resulted in similar trends, indicating that the chlorine + glutaraldehyde treatment is also not effective in eliminating the biofilm community. Thus, the combination of continuous chlorination and episodic glutaraldehyde treatment must be continued indefinitely to control bacterial populations in the cooling towers.

Microbial community in the cooling towers (TC1–5)

Microbial community composition in cooling tower test cells was monitored to confirm that the test organisms were retained in the system and to identify immigrant populations. Sequencing results showed that *Methylobacterium*, *Sphingomonas*, and *Rhodococcus* spp. successfully colonized the cooling towers. These bacteria are all common microbes in soils and aquatic systems, and are known to be able to survive under low nutrient conditions (Liu et al. 2009). The long-term persistence of the introduced *Flavobacterium* strain also was confirmed using 16S rRNA gene sequencing. Although the community composition of the biofilms was observed to change over time, *Flavobacterium* was the dominant community member at all times and in all test cells.

Discussion and conclusions

Following recommended industrial practice, a non-oxidizing biocide was used in conjunction with chlorine to evaluate the efficacy of disinfection treatments in pilot scale cooling towers. Glutaraldehyde was used for this purpose, as it is commonly employed in industrial and in hospital environments since it has a broad range of activity and is non-

corrosive to metals, rubber and optical lenses (Pereira et al. 2001). The biocidal effect of glutaraldehyde is attributed to its two aldehyde groups, which can interact with microbial cell constituents, primarily by reacting with ammonia and primary amines and more slowly with secondary amines (Simoes et al. 2003). The results reported here show that the combination of continuous chlorination and weekly application of the non-oxidizing biocide glutaraldehyde effectively controlled bacterial populations in the cooling towers, but did not eradicate bacteria from the system.

The three-species consortium tested in this study are abundant in biofilms found in industrial equipment, building water systems, and cooling towers, and this consortium has previously been used as a model biofilm for laboratory studies of *Legionella* growth and biofilm colonization of cooling towers (Murga et al. 2001; Liu et al. 2009). *P. aeruginosa* and *K. pneumoniae* are also opportunistic pathogens that cause diverse infections, and are of particular concern in hospitals and building water systems and in other facilities that house vulnerable populations. The current study demonstrates that bacterial communities in cooling tower systems are greatly affected by differences in the ability of introduced organisms to adhere to surfaces because of the high degree of wash-out (blow-down) associated with operation of these systems. Cooling towers having extensive stagnant regions are more likely to become colonized by organisms that adhere poorly to surfaces. The cooling tower microbial communities were found to be very robust and stable, most likely due to the extremely steady operational conditions that were imposed. Re-inoculation of the three bacterial strains had only a very transitory effect on the community composition.

With continuous chlorination, both *P. aeruginosa* and *K. pneumoniae* were not detectable in the cooling tower test cells from the beginning of the study. Considering the low degree of attachment of these two types of bacteria and their resulting lack of persistence in biofilms, the non-detection of *P. aeruginosa* and *K. pneumoniae* can be explained by the fact that chemical disinfection is much more effective against planktonic microorganisms than surface-attached microorganisms (Costerton et al. 1995; Costerton et al. 1999). On the other hand, inoculated *Flavobacterium* remained the dominant organism throughout all experiments despite colonization of the biofilms by environmental bacteria. To better understand these results, batch experiments were performed to test the effects of chlorine on planktonic bacteria under identical chemical conditions as used in the cooling tower systems. Fluorescence signals from *P. aeruginosa* and *K. pneumoniae* were not detected by CLSM on either PVC or steel coupons from the cooling tower basins, based on at least 10 microscope fields examined, which can be attributed to the low bacterial cell density on these coupons. With an initial concentration of 10^8 CFU ml^{-1} and 2 ppm free chlorine, all three bacteria had similar rates of inactivation, with 95% of the bacteria inactivated in 3 h (data not shown). Therefore, the high degree of difference in the persistence of the three strains in the cooling towers under disinfection can be attributed to differences in their ability to form biofilms in this system. Specifically, the much greater ability of *Flavobacterium* to adhere to surfaces protected it from disinfection, whereas *P. aeruginosa* and *K. pneumoniae* were subject to rapid disinfection in the planktonic state. In full-scale systems, variability in operating conditions and/or periodic shut-downs are likely to increase

the diversity of the microbial community, as will regular introduction of a variety of organisms from the atmosphere.

It was found that biofilms protect bacteria in cooling towers from disinfection, preventing eradication of microbial populations and supporting rapid re-growth following cessation of treatment. Continuous chlorination at 2 ppm free chlorine was not sufficient to control bacterial populations in cooling towers because of the resistance of the biofilm community. Biofilm populations persisted at 10^3–10^4 CFU cm^{-2} under 2 ppm of free chlorine. Further, extremely rapid regrowth from these populations was observed after temporary cessation of chlorination. Similar regrowth was observed following episodic addition of glutaraldehyde. While weekly additions of glutaraldehyde, in conjunction with continuous chlorination, successfully reduced bacterial populations to below detectable concentrations, re-growth was observed to occur within ~ 5 days after each injection of glutaraldehyde owing to the relatively short hydraulic residence time within the cooling towers.

These results emphasize the need for continuous disinfection and monitoring programs in order to guarantee the microbiological safety of cooling towers. The recommended protocol of continuous chlorination plus weekly application of an additional, non-oxidizing biocide should be effective at controlling microbial populations in clean cooling towers. However, this treatment regimen does not eradicate biofilm communities, so that microbiological outbreaks can occur rapidly (within days) in the event that treatment is interrupted. Further, the results reported here were obtained under nearly ideal conditions for disinfection, with clean laboratory conditions and only a minor amount of deposition from the atmosphere (most likely from local sources of dust). In full-scale building cooling towers, atmospheric inputs of organic matter should be expected to hinder disinfection by reducing free chlorine while simultaneously providing additional substrates and nutrients for microbial growth, making it imperative to ensure that these systems are maintained in a clean condition.

The combination of the potential for rapid outbreaks from biofilm regrowth, ongoing inputs of new bacteria from the atmosphere, and reduced disinfection efficiency by organic matter suggests that cooling towers should be equipped with continuous on-line monitoring equipment to guarantee that adequate disinfection is maintained at all times. Further, both continuous chlorination and periodic application of an additional, non-oxidizing biocide must be maintained indefinitely in these systems in order to control bacterial populations at low numbers. In summary, cooling towers provide good conditions for microbial growth, and support development of extensive biofilms by organisms that can remain attached to surfaces under high-throughflow conditions. Further, it is extremely difficult to eradicate these biofilms by any reasonable level of chemical disinfection. Therefore, cooling towers should be viewed as having active microbial populations at all times, requiring building water systems managers to rigorously maintain anti-microbial treatment protocols in order to adequately control these populations.

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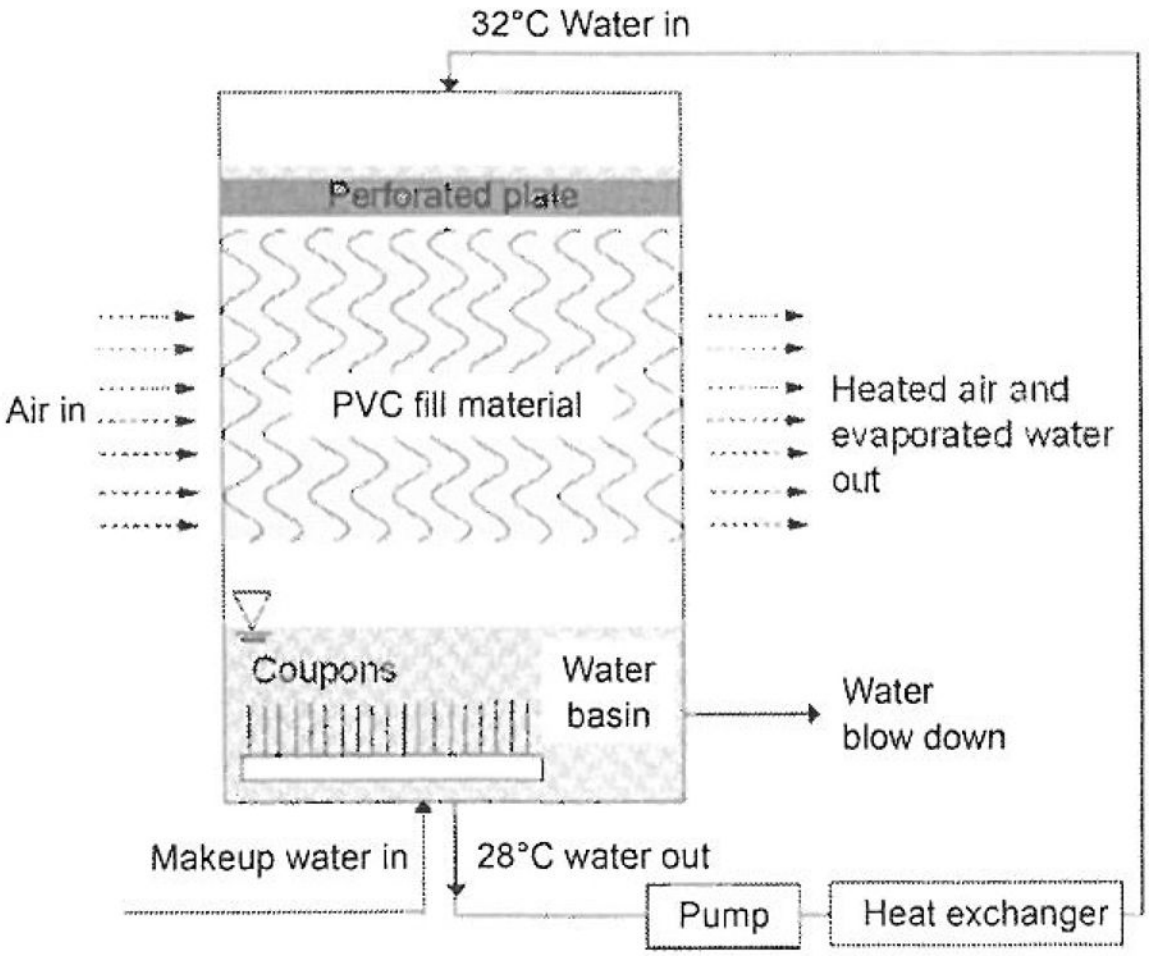

Figure 1.
Schematic of the laboratory cooling tower system. Only one test cell is illustrated here.

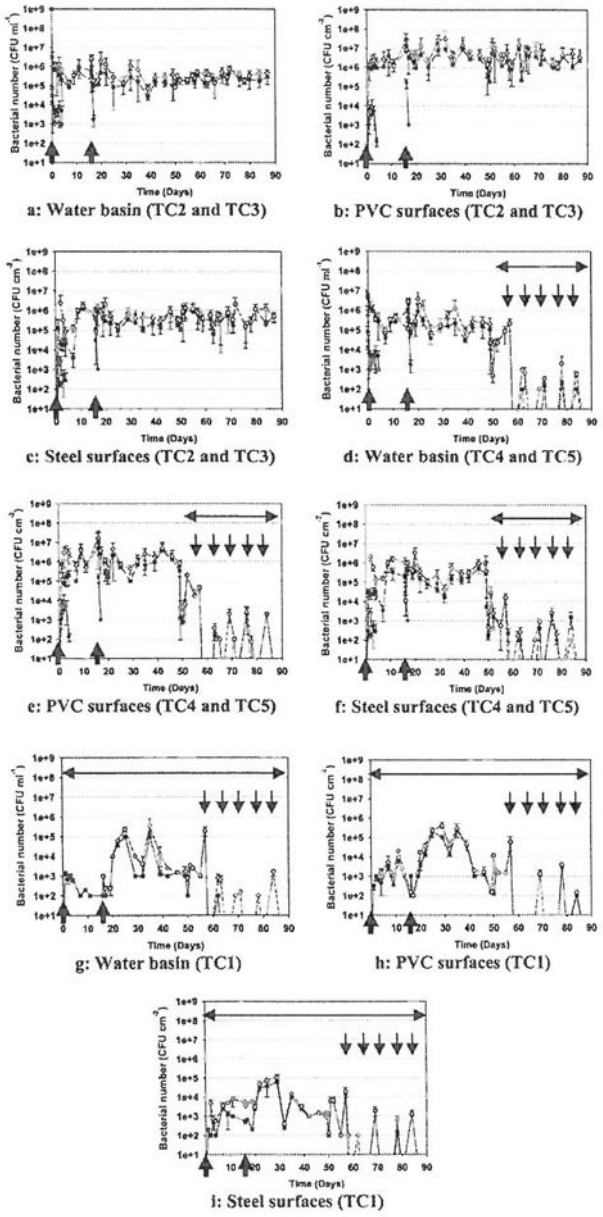

Figure 2.

Concentration of three introduced bacterial strains and total bacteria in the cooling tower water basin (a, d, g) and on PVC (b, e, h) and steel (c, f, i) coupon surfaces. Test cells TC2 and TC3 (a–c) were never disinfected; TC4 and TC5 (d–f) were disinfected with chlorine starting on day 48 and with weekly additions of glutaraldehyde starting on day 58; and TC1 (g–i) was disinfected continuously with chlorine starting on day 0 and with weekly additions of glutaraldehyde starting on day 58. Error bars represent SDs of triplicate measurements. (Arrows ↔ indicate periods of continuous chlorination. Each ↓ indicates application of a single dose of glutaraldehyde. Each ↓ indicates an inoculation of bacteria. Bacterial strains: —●— *K. pneumoniae*, —▽— *P. aeruginosa* —■— *Flavobacterium* sp., —◇— total bacteria).

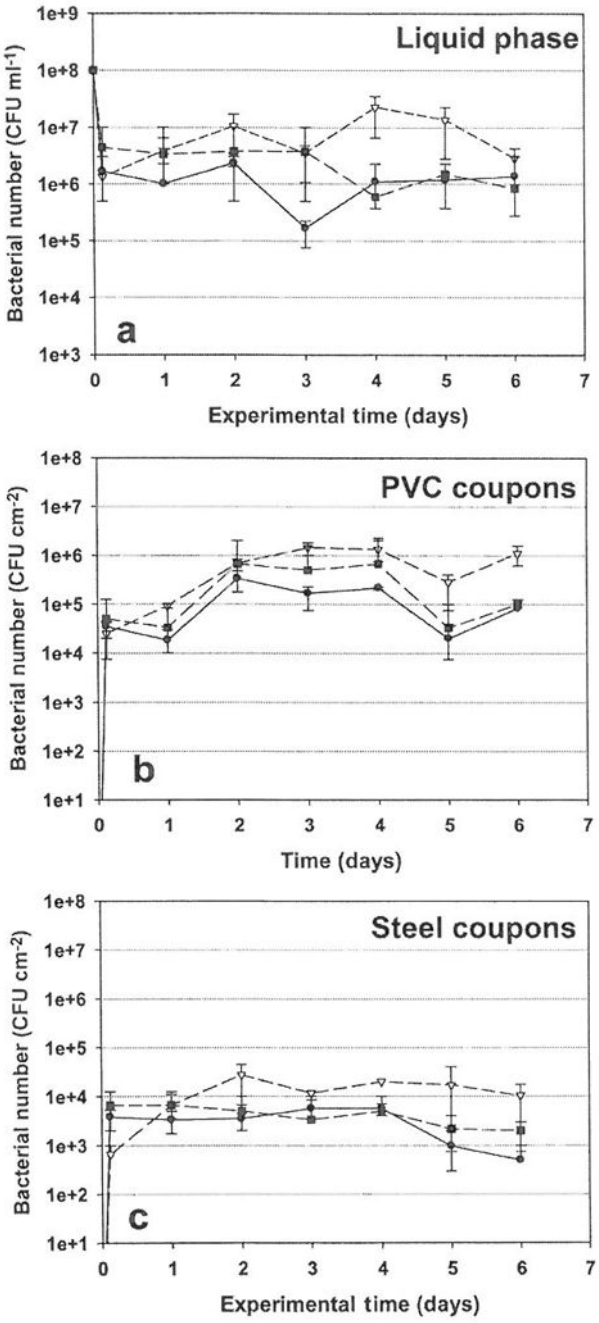

Figure 3.

Concentration of the three introduced bacterial strains in liquid phase and on coupon surfaces observed in batch studies. Error bars represent the SDs of triplicate experiments. (—●— *K. pneumoniae,* —▽— *P. aeruginosa,* —■— *Flavobacterium* sp.).

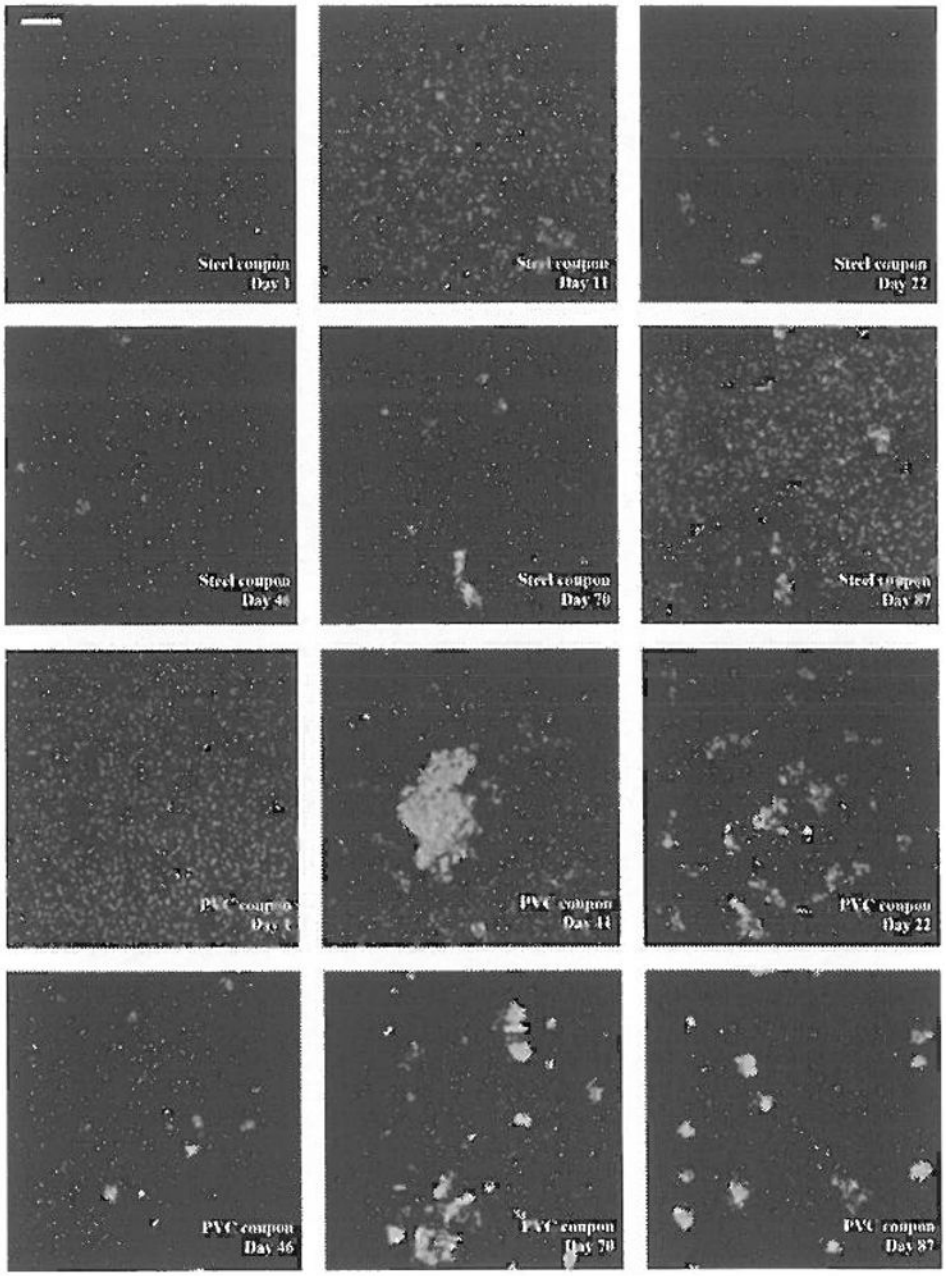

Figure 4.

Representative confocal images of biofilms on steel and PVC coupons in cooling tower test cells TC2 and TC3 (no treatment). Bar = 50 μm.

Table 1

Experiments performed in five cooling tower test cells.

		Time (days)												
TC	**Description**	**0**	**7**	**14**	**21**	**28**	**35**	**42**	**49**	**56**	**63**	**70**	**77**	**84**	**91**
1	Disinfected from day 0	Chlorine treatment													
2	Never disinfected	No treatment													
3	Never disinfected														
4	Disinfected from day 48	No treatment									Chlorine treatment				
5	Disinfected from day 48														

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Table 2

Physical and chemical conditions in cooling tower test cells.

Chemical and physical parameters	TC1	TC2 and TC3	TC4 and TC5
Alkalinity (ppm)	379 ± 19	465 ± 28	415 ± 33
Conductivity (mS)	0.91 ± 0.14	0.94 ± 0.04	1.12 ± 0.21
Phosphonate (ppm)	6.90 ± 1.93	8.20 ± 0.73	7.00 ± 2.44
pH	9.2 ± 0.05	9.0 ± 0.03	8.9 ± 0.36
Airflow (m min^{-1})	116.2 ± 0.8	118.5 ± 1.9	110.4 ± 2.8
Si (mg l^{-1})	4.90 ± 1.13	3.65 ± 0.32	5.20 ± 0.30