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Water Research 38 (2004) 1457–1466

WATER RESEARCH

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Survival of *Mycobacterium avium* in a model distribution system

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Received 22 October 2002; accepted 31 July 2003

Abstract

A pilot study was designed to examine the impact of nutrient levels, pipe materials, and disinfection on the survival of *M. avium* in model drinking water distribution system biofilms. Studies showed that the survival of the organism was dependant upon a complex interaction between pipe surface, nutrient levels, and disinfectants. The findings showed that when no disinfection was applied, *M. avium* could be recovered from biofilms at nutrient levels of 50 μg/L assimilable organic carbon. *M. avium* concentrations were lower on copper pipe surfaces following disinfection with free chlorine as compared to monochloramine. However, due to the interference of corrosion products, chloramination of iron pipe surfaces controlled *M. avium* levels better than free chlorine. These data demonstrate the significance of pipe materials on the survival of *M. avium* complex in biofilms. Elimination of competitive heterotrophic bacteria on copper pipe surfaces by the application of disinfection resulted in a population of nearly 100% *M. avium*. Heat treatment of *M. avium* biofilms was affected by the pipe composition and organic content of the water. Effluent temperatures $>53^{\circ}C$ were required to control the occurrence of *M. avium* in the pipeline system. Although additional studies are required using improved detection methods, the results of this investigation suggest that reducing the biodegradable organic material in drinking water, control of corrosion, maintenance of an effective disinfectant residual, and management of hot water temperatures can help limit the occurrence of *M. avium* complex in drinking water biofilms.

Keywords: *Mycobacterium avium* complex; Biofilm; Nutrient; Disinfection; Pipe material

1. Introduction

Mycobacterium avium is an acid-fast organism that is ubiquitous in the environment and has been found in soil, house dust, water (wastewater, surface water and groundwater, and drinking water), animals, and poultry. *M. avium* is an opportunistic pathogen and the greatest increase in *M. avium* infections has been in acquired immunodeficiency syndrome (AIDS) patients, with approximately 25–50% of these patients suffering debilitating and life-threatening infections [1]. Disseminated *M. avium* infections have also been reported in patients with inherited interleukin-12 receptor deficiency [2].

Amongst immunocompetent individuals, *M. avium* is responsible for cervical lymphadenitis in children [3] and pulmonary alveolar proteinosis [4]. *M. avium* also causes pulmonary infections in persons with pre-existing pulmonary disease (e.g., pneumoconiosis, silicosis, black lung [5] and with architectural defects in lung structure [6]). Persons with pulmonary *M. avium* infections have cough, fatigue, weight loss, low-grade fever, and night sweats [5].

The bacterium can infect individuals via the gastrointestinal or pulmonary tracts, suggesting that food or water may be important routes of transmission for

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doi:10.1016/j.watres.2003.07.008

AIDS patients. It is likely that the route of infection leading to *M. avium* pulmonary disease is via aerosols. In contrast, cervical lymphadenitis and disseminated infections are likely a consequence of an oral route of infection. However, there is no direct evidence of either route of infection.

M. avium is found in natural waters [7] and drinking water distribution systems [8–11]. Data from these studies show that drinking water systems can have between 1 and 1000 CFU of *M. avium* per 100 mL water. Researchers have also noted that *M. avium* is able to grow in water samples to which no additional nutrient substrate had been added [12] and are resistant to chlorination [13]. Further, *M. avium* grows over a wide range of temperatures (e.g., 15–45°C) and salinities (e.g., 0–2% NaCl) [12]. Recent evidence suggests that the level of biodegradable organic material can influence the growth of mycobacteria in drinking water biofilms [14]. DNA fingerprinting studies have shown that single, unique strains can persist as long as 41 months in a water distribution system [10]. These characteristics suggest that drinking water may be a very hospitable environment for these organisms.

Because of their lipid cell walls, mycobacteria are hydrophobic [15] and readily colonize surfaces. The reported resistance of mycobacteria to zinc and copper [16] may aid the development of biofilms [17] on copper or galvanized (i.e., zinc coated) pipes.

With the potential for *M. avium* infections resulting from drinking water sources, water utilities will need to take proactive actions to evaluate the occurrence of these organisms and identifying means of control. Movements towards such evaluations are evident in that *M. avium* has been included the contaminant candidate list (CCL) for possible regulation by the US EPA [18]. This study was designed to provide specific information concerning the impact of nutrient levels, disinfection, pipe materials, and heat treatment on *M. avium* survival in biofilms in a pilot pipeline system.

2. Materials and methods

2.1. Description of pilot system

The pilot system has been used in previous experiments and extensively described [19,20]. The pilot system was constructed of 124 one-foot (30.48 cm) sections of pipe composed of $\frac{1}{3}$ in (2.66 cm) diameter chlorinated polyvinyl chloride (cPVC), $\frac{1}{2}$ in (1.77 cm) diameter galvanized, $\frac{1}{3}$ in (2.66 cm) diameter copper and/or $\frac{1}{2}$ in (1.77 cm) diameter black iron (Fig. 1). Pipe sections were not preconditioned and were connected using quick disconnect couplings (Lasco–Tite, Lima, OH) to allow easy removal and replacement in the pilot system. Individual pilot system materials were selected, based on specific parameters to be evaluated. The use of cPVC pipes allowed attachment of the organisms without interference from the surface itself. Galvanized surfaces were evaluated to determine impacts of zinc on mycobacterial survival. Copper pipes were used because they are commonly used in household plumbing. Black iron pipes were evaluated to simulate corroded surfaces typical in a distribution system.

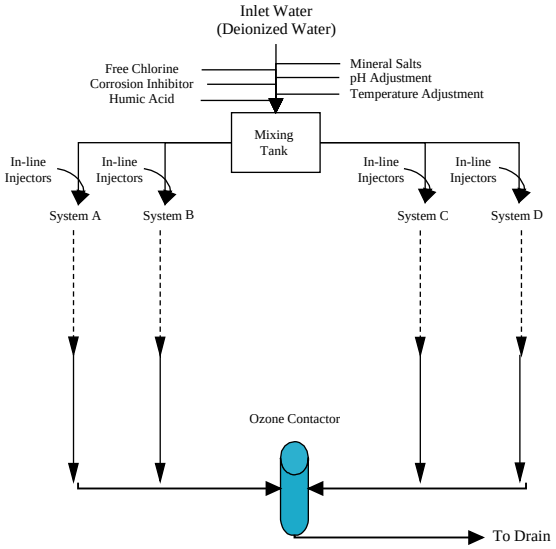

Fig. 1. Diagram of pilot distribution system.

System feed water and chemicals were mixed at a temperature of 24°C using a circulator (Model C1, HAAKE BUCHLER Instruments, Inc., Saddle Brook, NJ). For studies of heat treatment, the temperature of the circulator was progressively increased until the mix tank reached 70°C, and the pipe sections were wrapped in a foam insulation. The pH of all pilot experiments was adjusted to 7.2 using a pH metering pump (Model 7142-55, Cole-Parmer Instrument Company, Chicago, IL). Chemical additions and system flow were controlled using peristaltic pumps (Masterflex Model 7523, Cole-Parmer Instrument Company, Chicago, IL). The model was divided into four experimental distribution systems (designated as System A, System B, System C and System D). Flow rates for the distribution systems were maintained at 250 mL/min. The model was operated to flow from the bottom of each system to the top. System effluents were combined and ozonated prior to disposal into the local sewer system.

2.2. Source water and carbon source

The pilot system was fed by deionized laboratory grade water to ensure that a consistent baseline existed. Chemicals added to the feed water to simulate distributed

water included a polyphosphate corrosion inhibitor (final concentration 3.0 mg/L orthophosphate, Kjell Corporation, Benoit, WI), hardness chemicals ($MgCl_2 \cdot 6H_2O$, 10.7 mg/L; $CaSO_4$, 27 mg/L; $NaHCO_3$, 100 mg/L; Sigma Chemical Company, St. Louis, MO), and a mineral salts solution ($(NH_4)_2SO_4$, 1 mg/L; KH_2PO_4, 0.7 mg/L; K_2HPO_4, 0.3 mg/L, Sigma Chemical Company, St. Louis, MO). Corrosion rates in the system averaged 2.5 mils/year (range 0.9–3.8 mils/year). Free chlorine (5 mg/L dose) was added after 12 weeks of operation during the study to evaluate the impact of pipe materials (Table 1). Under other experimental applications, chlorine and chloramine stock solutions were added to the systems when flow began (Table 1). Free chlorine stock solutions were prepared by diluting 5% sodium hypochlorite (J.T. Baker, Phillipsburg, NJ) to 5000 mg/L. Free chlorine stocks were diluted 1:1000 for a final dose of 5 mg/L. Monochloramine was generated by combining ammonium chloride (J.T. Baker) and free chlorine in the reservoir tank at a ratio of 3:1, chlorine to ammonia for a final dose of 5 mg/L monochloramine. Disinfectant residuals were measured using the DPD test [21] and recorded as free or combined chlorine. Residence times in the reservoir tank and distribution system were 40 and 20 min, respectively.

To supplement experimental systems with carbon, humic acid stock solutions were utilized. In order to obtain target AOC levels, the stocks were prepared by dissolving humic acid (sodium salt, Aldrich Chemical Company, Milwaukee, WI) in deionized water and ozonating the stock for 40–105 min, depending on stock concentrations (model GTC-0.5 C ozone generator, Ozonia, Lodi, NJ). During the first experimental run, individual systems were supplemented with various ozonated humic acid concentrations (0.0, 3.0, 7.5 and 15 mg/L) to examine the effects of nutrient levels on the survival of *M. avium* (Table 1). The second pilot system was supplemented with 3.0 mg/L ozonated humic acid to determine the impact of pipe materials on the survival of mycobacteria. The third experimental system was supplemented with 3.0 mg/L ozonated humic acid (Systems A and B) and 10 mg/L ozonated humic acid (Systems C and D) and disinfected with free chlorine or monochloramine (5 mg/L) to evaluate survival during continuous disinfection. Each experimental system was operated for approximately 12 weeks (Table 1).

2.3. Mycobacterial strain

M. avium strain A5 was obtained from Dr. Marjorie Beggs and was isolated from an *M. avium* infected AIDS patient and was identified by DNA probe (Gen-Probe, San Diego, CA). Heterotrophic plate count bacteria were not added to the system, but occurred naturally from the water supply (geometric mean 38 CFU/mL).

2.4. Inoculation of M. avium

M. avium strain A5 was grown in Middlebrook 7H9 (M7H9, Difco Laboratories, Detroit, MI) broth for 7 days at 35°C and diluted in sterile tap water. To adapt the isolates to growth in tap water, single isolate cultures were passed weekly to sterile tap water (1:10 dilution) twice prior to inoculation into the model system. Bacterial concentrations for each isolate were estimated using optical density (600 nm) to determine necessary dilutions prior to pooling into a 20 l carboy for inoculation. Dilutions were made with system feed water to achieve *M. avium* concentrations of 6×10^4 CFU/mL, based on the average plate count data. The system was inoculated at 250 mL/min using the appropriate ozonated humic acid concentrations for each system. Following inoculation, the flow was stopped and the system was allowed to incubate in a stagnant state for 48 h.

Table 1
Summary of experimental design parameters

Experimental system	Parameter studied	Pipe composition	Ozonated humic acid concentration (mg/L)	Disinfectant type	Average reservoir disinfectant residual (mg/L)
1	Nutrient levels	cPVC	0.0, 3.0, 7.5, 15	None	0.0
2	Pipe composition	Iron, galvanized, copper, cPVC[a]	3.0	Free chlorine[b]	5.0
3	Nutrient, disinfectant	Iron and copper[c]	3.0 and 10	Free chlorine[d] Monochloramine	2.2–3.8
4	Heat	Iron and copper[c]	3.0 and 10	Free chlorine[d] Monochloramine	0.6–1.1

[a] Four separate systems.
[b] Free chlorine applied 12 weeks after system flow was induced.
[c] Four systems, each constructed of a copper pipe in the first half and iron in the second half.
[d] Each disinfectant applied to an experimental system with 3.0 and 10 mg/L ozonated humic acid.

2.5. Sampling of the model system

The first samples (water column and biofilm) were collected from the system 24 h after system flow was initiated to provide starting counts for each system. Samples were collected weekly from the system influent and effluent water column and analyzed for various water quality parameters to ensure proper operation of the system. These parameters included temperature, pH, turbidity, phosphate, nitrate, UV (254 nm), corrosion rates, assimilable organic carbon (AOC), dissolved organic carbon (DOC), biodegradable dissolved organic carbon (BDOC), and bacterial levels (HPC and *Mycobacterium* levels). Biofilm samples were collected from pipe sections periodically during the course of the experiments to monitor bacterial levels. Duplicate biofilm samples were collected from the influent and effluent of each system and microbial analyses were conducted in duplicate. Due to the more complicated setup of the third system (copper and iron pipe sections in each system, Table 1), duplicate biofilm samples were not collected.

Corrosion rates were measured with a corrator (model RCS9000, Rohrback-Cosasco Systems, Santa Fe, CA). AOC samples were analyzed by the rapid AOC method published by LeChevallier et al. [22]. BDOC levels were determined according to Joret et al. [23]. All HPC levels were determined using R2A agar (Difco Laboratories) incubated at 25°C for 7 days. Other analyses were conducted according to *Standard Methods* [21].

Biofilm samples were scraped and washed from the pipe surface using a sterile buret brush and 100 mL of sterile buffer. The samples were then homogenized for 60 s using a Polytron Model PT 1200C homogenizer (Brinkmann, Westbury, NY) and plated on R2A agar to determine HPC levels [19].

2.6. Recovery and enumeration of M. avium

Due to the high concentration of organisms seeded into the pilot system, concentration of the samples for mycobacterial analysis was not necessary. Following homogenization and/or HPC counts, water and biofilm samples were decontaminated with 0.005% cetylpyridinium chloride (CPC) for 30 min at room temperature. Dilutions were made and the samples were plated on Middlebrook 7H10 agar with oleic acid/glycerol enrichment (M7H10 + OADC, Difco) agar and incubated at 37°C for 21 days.

Following the 21-day incubation period, plates were counted and 10 isolates from each sample were streaked for purity. After 7–10 days, isolates were further characterized by acid-fast staining by the method of Zeihl-Neelson (Difco Laboratories). Isolates were identified as *Mycobacterium* spp., *M. avium,* or *M. intracellulare* using the nested PCR method for amplification of the mycobacterial 16S rRNA gene described by Wilton and Cousins [24]. Due to the large numbers of isolates to be identified using PCR, the method was adapted for analysis using 96-well plates. This adaptation allowed a large number of PCR reactions to be conducted in a short period of time, including numerous positive and negative controls. This adaptation was verified using five strains of *M. avium*, one strain each of *M. smegmatis* and *M. intracellulare*, and one strain of a non-*Mycobacterium* organism (*Klebsiella pneumoniae*). As expected, genus and species bands (genus = 1000 bp, *M. intracellulare* = 800 bp and *M. avium* = 280 bp) were identified for *M. avium* and *M. intracellulare*, and a genus band was identified for *M. smegmatis*. No bands were detected for the non-*Mycobacterium* strain. Two sets of these organisms were analyzed as controls with every 96-well plate. In all instances, only *M. avium* was recovered from the pilot distribution system.

2.7. Statistical analyses

All statistical analyses were performed using Instat-Instant Biostatistics (Version 2.0, Graphpad Software, San Diego, CA) on logarithmically transformed data. Analyses included the Wilcoxon test, paired *T*-test, unpaired *T*-test and one-way analysis of variance. All statistical calculations were based on a 95% confidence interval.

3. Results

3.1. Impact of nutrient levels on M. avium

3.1.1. Pilot system nutrient levels

To study the impact of nutrient levels on the survival of *M. avium* in biofilms, the pilot system was constructed entirely of cPVC pipe sections. Use of only cPVC sections allowed biofilm studies to be conducted on relatively inert surfaces, without interference from corrosion products. One distribution system (System A) was fed water unsupplemented with humic acid. This system provided a baseline with very low AOC levels (geometric mean 42 μg/L) by which to compare the other three systems supplemented with 3, 7.5, and 15 mg/L ozonated humic acid, (AOC levels of 85, 103, and 213 μg/L for systems B, C and D, respectively). Table 2 summarizes the average influent water quality parameters for the model system.

Nutrient levels in Systems A, B and C were relatively consistent following a 1-week system stabilization period (Fig. 2). System D, however, showed considerable variation throughout the course of the experiment. It is unlikely that this variation was due to the preparation of the ozonated humic acid stock solution because humic acid concentrations, as well as ozonation times

Table 2
Average influent water quality parameters for the model system

Parameter	System A	System B	System C	System D
Ozonated humic Acid (mg/L)	0.0	3.0	7.5	15.0
AOC (μg/L)	50±40 (42)[a]	98±65 (85)	103±30 (103)	228±87 (213)
DOC (mg/L)	0.42±0.08 (0.41)	1.11±0.41 (1.06)	1.71±0.15 (1.71)	3.46±0.46 (3.44)
BDOC (mg/L)	0.17±0.09 (0.15)	0.57±0.42 (0.47)	0.68±0.08 (0.68)	1.51±0.53 (1.41)
UV254	0.01±0.01	0.05±0.01	0.13±0.02	0.25±0.02

[a] Numbers in parentheses are geometric means. $N = 6$ for each system.

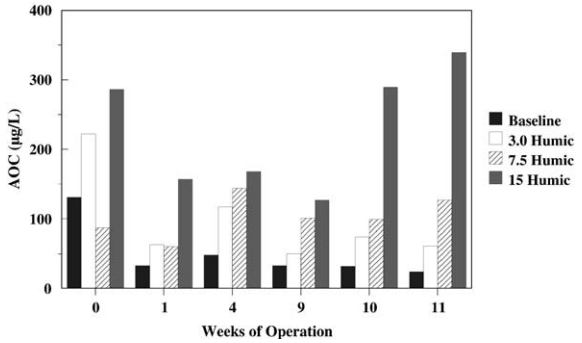

Fig. 2. AOC levels in a pilot distribution system supplied with various levels (0, 3.0, 7.5 and 15 mg/L) of ozonated humic acid.

and concentrations, were consistent throughout the experiment. DOC concentrations (average 3.46±0.46) throughout the experiment were more consistent than the AOC concentrations for System D (average 228±87 μg/L). It is possible that the nature of the carbon source or the high humic acid concentration caused some interference with the AOC analyses resulting in the large variations. For example, the samples collected from System D were visibly tinted as a result of the high humic acid concentrations. UV absorbance (254 nm) levels were 25 times higher for this system than for System A (Table 2). Color has been noted by the luminometer manufacturer (Turner Designs, Sunnyvale, CA) to interfere with ATP determinations made as part of the AOC test using ATP analyses, and could be responsible for variations in the observed results.

3.1.2. Suspended bacterial levels

In the experimental system constructed of cPVC pipe under various nutrient levels, effluent HPC bacteria and *M. avium* levels were not significantly different between experimental systems (analysis of variance (ANOVA); $p = 0.4167$ and 0.3552, respectively). However, a significant difference was noted between HPC levels at the beginning of the experiment and at the end of the experiment (ANOVA; $p = 0.0157$). The increase in bacterial counts as the experiment progressed probably occurred as a result of biofilms reaching sufficient thickness to induce sloughing of cells into the water column. *M. avium* levels did not differ significantly during the course of the experiment (ANOVA; $p = 0.1535$). Consistent *Mycobacterium* spp. levels over the course of the 12-week period in all systems were probably due to the slow growth rates of these organisms. The data show that, even under the lowest nutrient levels studied ($\geqslant 50$ μg/L AOC), suspended HPC bacteria and *Mycobacterium* spp. could be isolated from the water column. These findings indicate that when biofilms are uncontrolled by disinfection and nutrient levels are at least $\geqslant 50$ μg/L, suspended bacterial levels stabilized quickly and did not appreciably change as nutrient levels increased.

3.1.3. Biofilm levels

Pipe sections were collected from the inlet and outlet of each experimental system during every sampling campaign. Paired *t*-test analyses for all the four experimental systems revealed that bacterial biofilm counts (HPC and M7H10+OADC) from the end (outlet) of each system were not significantly different from biofilm counts from the beginning (inlet) of the system, so data are presented as daily averages for the entire system.

Biofilms of HPC bacteria and *M. avium* were rapidly formed, probably due to the large inoculum, and were relatively consistent levels for Systems A, B and C (Table 3). However, System D show an increase in *M. avium* levels from 4.7×10^3 to 9.1×10^5 CFU/cm^2 through the first 4 weeks of testing, with an unexplained decline on the last sampling date. These data show that, without disinfection, nutrient levels at least $\geqslant 50$ μg/L support substantial biofilm growth.

The study was designed to use data from nutrient experiments to determine the lowest nutrient level capable of maintaining stable *M. avium* populations for use in the evaluation of pipe materials. Bacterial levels (HPC and *M. avium*) were less consistent in System A (baseline) than System B (3.0 mg/L ozonated humic acid). Based on these findings, the ozonated

Table 3
M. avium and HPC biofilm levels in a pilot distribution system supplied with various levels of ozonated humic acid

Weeks sampled	Baseline no added humic		3.0 mg/L humic		7.5 mg/L humic		10 mg/L humic	
	HPC	*M. avium*	HPC	*M. avium*	HPC	*M. avium*	HPC	*M. avium*
1	5.27	4.24	5.04	3.86	5.62	4.41	5.08	3.68
4	5.95	4.10	6.27	3.89	6.40	4.03	6.82	4.89
9	5.99	3.17	6.08	4.44	6.64	3.85	7.16	5.36
10	5.51	4.90	6.31	4.44	6.74	4.96	6.88	5.96
11	6.51	5.07	6.33	4.27	6.50	3.91	6.98	4.04
Avg	5.85	4.30	6.01	4.18	6.38	4.23	6.58	4.79

Values are log CFU/cm^2.

humic acid concentration used in System B, 3.0 mg/L, was used for the experiments designed to examine the effects of pipe materials on *M. avium* survival in biofilms.

3.2. Impact of pipe materials on M. avium

The second pilot system study was designed to examine the effect of various pipe materials on the survival of *M. avium* in distribution system biofilms. The basic design of this system was similar to the pilot described above, except that pipeline systems were constructed of copper, cPVC, galvanized, or black iron pipe. Free chlorine (5 mg/L) was added during week 12 to determine the impact of disinfection on previously established biofilms.

3.2.1. Suspended bacterial levels

HPC bacteria and *M. avium* in pipe effluent samples averaged 2.63×10^5 and 4.79×10^4 CFU/mL, respectively, and there was no significant difference between suspended bacterial levels in the experimental pipe systems (ANOVA; $p = 0.8832$ and 0.7160, respectively). These data indicate that, under the conditions studied here (no disinfection), pipe composition had no significant impact on bacterial levels in the water column. Application of free chlorination resulted in a dramatic decrease in suspended HPC bacterial and *M. avium* levels in all systems (average 58 and 15 CFU/mL, respectively), indicating that growth on different pipe surfaces did not impact suspended bacterial levels following disinfection.

3.2.2. Biofilm levels

HPC levels were statistically different in all the four systems, with levels in the iron system being the highest (average 6.3×10^6 CFU/cm^2) and levels in the copper system being the lowest (average 1.5×10^5 CFU/cm^2) (ANOVA $p = <0.0001$).

During the first seven sampling campaigns (11 weeks of growth) from the second pilot system, *M. avium* levels

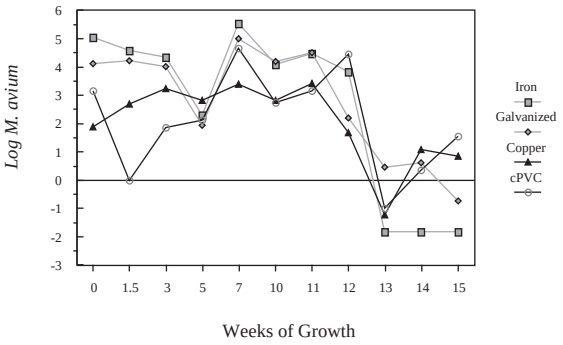

Fig. 3. *M. avium* complex biofilm levels in a pilot distribution system constructed of iron, galvanized, copper and cPVC pipe surfaces. Free chlorine (5 mg/L) was applied after week 12.

were significantly higher on iron and galvanized surfaces than on copper or cPVC surfaces (Fig. 3). After 12 weeks of testing, free chlorine (5.0 mg/L dose; 1.9–3.2 mg/L residual across the various systems) was added to the system. Following chlorination, HPC levels decreased on all pipe surfaces except iron surfaces, while a decrease in recovery of *M. avium* was noted for all pipe surfaces. Fig. 4 shows that, following system inoculation, a high percentage of isolates that initially recovered on M7H10 following decontamination were *M. avium* (iron 95%, copper 100%). As biofilms developed, the proportion of *M. avium* decreased to 20–40% of the biofilm population. However, when free chlorine was applied, the percentage of isolates identified as *M. avium* increased to 100% (Fig. 4). While HPC levels remained high on iron pipe surfaces, *Mycobacterium* spp. isolates accounted for $\leqslant 5\%$ of the organisms recovered on M7H10 agar. That behavior may have occurred as a result of changes in the level of competition between *M. avium* and HPC bacteria in each system. It is also likely that corrosion products from the iron pipe interfered with CPC decontamination, resulting in decreased sensitivity and selectivity of the mycobacterium isolation technique.

3.3. Combined impact of nutrient levels, pipe materials and disinfection on M. avium

The third pilot system was designed to examine the combined effects of nutrient levels, pipe materials, and disinfectants on the survival of *M. avium* complex organisms in distribution system biofilms. Based on differences noted in the previous study between biofilms on copper and iron pipe surfaces, this pilot system contained four individual systems, each constructed with copper pipes for the first half of each system and iron pipes on the second half. Two systems were fed water with nutrient levels supplemented by 3.0 mg ozonated humic acid/L (termed, low nutrient) and the remaining two systems were supplemented with 10 mg ozonated humic acid/L (termed, high nutrient). Two systems, one with 3.0 mg ozonated humic acid/L and one with 10 mg ozonated humic acid/L, were disinfected with free chlorine (5 mg/L dose). The remaining two systems were disinfected with monochloramine (5 mg/L dose).

3.3.1. Suspended bacterial levels

HPC levels revealed no significant differences between free chlorinated systems and the low-nutrient chloraminated system (average 1×10^3–2×10^3 CFU/mL). However, HPC levels in the high nutrient chloraminated system were significantly higher (4.6×10^4 CFU/mL) than all other test systems ($p < 0.01$). The high level of humic acid added to the high nutrient system created a substantial disinfectant demand, resulting in a chloramine residual of 1.4 mg/L in the effluent of the system (compared to 2.2 mg/L for the low-nutrient system).

Following free chlorination (effluent residual 0.3–0.6 mg/L), suspended *M. avium* levels were similar in both experimental systems (average 87 CFU/mL for the low nutrient, and 100 CFU/mL in the high nutrient system). Suspended *M. avium* levels appeared to be slightly impacted by nutrient levels in the chloraminated systems, with levels in the high nutrient system significantly higher ($p = 0.0645$) than low nutrient system levels (average 1.0×10^3 and 2.6×10^3 CFU/mL, respectively). Comparison of suspended *Mycobacterium* spp. revealed significantly lower levels in the low-nutrient, free chlorinated system than either the low- or high-nutrient, chloraminated systems (Wilcoxon test, $p = 0.0195$ and 0.0488, respectively). No differences were noted between chlorinated and chloraminated systems for suspended *Mycobacterium* spp. levels for high nutrient conditions.

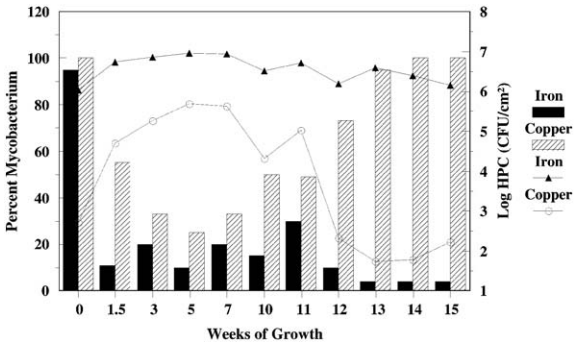

Fig. 4. Comparison of HPC biofilm levels and percentage of *M. avium* complex organisms on copper and iron pipe surfaces. Lines indicate HPC biofilm concentration in CFU/cm^2, bars indicate the percentage of mycobacteria in the biofilm population. Free chlorine (5 mg/L) was applied after week 12.

3.3.2. Biofilm levels

HPC biofilm levels on copper pipe surfaces were 3–4 logs/cm^2 lower than biofilm levels on iron pipe surfaces (Table 4). Biofilms of HPC bacteria on copper pipe exposed to free chlorine were lower than biofilms exposed to chloramines. Similarly, *M. avium* levels on copper pipe surfaces were significantly lower in free chlorinated systems compared to chloraminated systems ($p = < 0.0001$). However, chloramination of biofilms on iron pipe surfaces under low nutrient conditions resulted in significantly lower HPC bacteria and *M. avium* levels in the comparable free chlorinated system ($p = 0.0195$). Data for the high nutrient, iron pipe system are less clear, in part due to the interference of iron corrosion products on the sensitivity of the mycobacteria isolation technique.

Table 4
Impact of nutrient level, disinfectant, and pipe material on *M. avium* and HPC levels

Ozonated humic carbon level	Disinfectant type	Disinfectant residual (mg/L)	Copper pipe[a]		Iron pipe[a]	
			HPC	*M. avium*	HPC	*M. avium*
Low (3.0 mg/L)	Free chlorine	0.6	1.76	0.18	6.02	5.85
	Chloramine	2.2	2.44	2.38	5.21	4.92
High (10 mg/L)	Free chlorine	0.3	2.17	0.37	5.93	5.50[b]
	Chloramine	1.4	2.43	2.10	5.89	5.20[b]

[a] Values are log CFU/cm^2.
[b] Corrosion products interfered with these analyses.

3.4. The effect of heat treatment on suspended and biofilm *M. avium* levels

The impact of organic carbon level and pipe composition was tested on the effectiveness of heat for control of *M. avium* biofilms. The design of the study was similar to the previous system, with copper and iron pipe sections, fed with high and low nutrient levels, and treated with free chlorine or chloramines. Modifications to this system included installation of an on-demand hot water heater and heat transfer coils in the mix tank, and foam insulation of the mix tank and pipe materials. System temperatures were progressively increased from 45°C to 70°C in 1- or 2-week intervals. Despite the insulation of the system, temperatures dropped as the water flowed from the inlet to the effluent of the system (average 11.3°C, range 7–15°C). Since the inlet of the system contained the copper pipe, whereas the iron pipe was located on the back half of the pilot system, the inlet temperature was used to evaluate the impacts on copper pipe and the effluent temperature was used to evaluate the effect of heat on the iron pipe (temperatures were not measured at the mid-point).

Elevated water temperatures impacted biofilm levels on copper pipe to a greater extent than biofilms on iron pipe (Fig. 5). *M. avium* biofilms on copper surfaces did not develop to the levels observed on iron pipe, even at inlet temperatures <50°C. For iron pipe, biofilms of *M. avium* were inhibited when effluent temperatures reached 52°C (inlet temperatures were 63°C). For bacteria suspended in the water column, *M. avium* were detected at effluent temperatures up to 52°C in the low-nutrient system, but persisted at temperatures up to 53°C in the high-nutrient system. There was no significant difference between maintenance of a free chlorine residual (average residual 0.15–2.3 mg/L) or chloramines (average 0.56–0.93 mg/L).

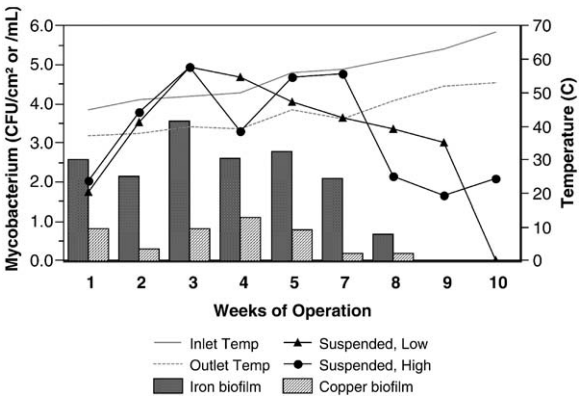

Fig. 5. Impact of heat treatment on biofilm and suspended levels of *M. avium* in water. Since there was no difference for free chlorinated or chloraminated systems, values represent average of both systems. No data were collected during week 6 of operation. Abbreviations: low, 3 mg/L ozonated humic acid carbon; high, 10 mg/L ozonated humic acid carbon.

4. Discussion

The current study demonstrates that the occurrence of *M. avium* was dependant upon a complex interaction between pipe surface, nutrient levels, and disinfectants. To implement a strategy to control the occurrence of *M. avium* in drinking water, it will be important to understand the interrelationship between these factors. Falkinham et al. [11] observed a significant correlation between increases in *M. avium* levels in eight drinking water systems and levels of AOC ($r^2 = 0.65$, $p = 0.029$) and BDOC ($r^2 = 0.64$, $p = 0.031$). Kirschner et al. [16] found the highest levels of *M. avium* in waters with high levels of organic carbon. George et al. [12] demonstrated that *M. avium* was able to grow in water samples to which no additional nutrient substrate had been added. Carson et al. [25] reported that *M. cheloneae* and *M. fortuitum* were able to multiply in commercial distilled water, achieving levels of 10^4–10^5 CFU/mL. This study showed that *M. avium* were able to colonize and grow in biofilms on pipe surfaces at AOC levels >53 μg/L and BDOC levels >0.17 mg/L. This ability of *M. avium* to grow at low nutrient levels is significant given that the geometric mean of AOC in a survey of 93 water utilities in North America was 94 μg/L, and BDOC levels had a geometric mean of 0.32 mg/L [26]. Therefore, to limit the growth of *M. avium* in drinking water supplies, AOC and BDOC levels would have to be substantially reduced. Van der Kooij et al. [27] suggested that AOC levels should be <10 μg/L to limit the growth of HPC bacteria in unchlorinated drinking water.

When biofilms were grown on non-corroded surfaces (copper or PVC pipe), free chlorine was more effective for controlling HPC and *M. avium*, but monochloramine controlled bacterial levels better on corroded iron pipe surfaces. These data are comparable to previous studies examining the survival of HPC in biofilms where corrosion products were found to interfere with free chlorine disinfection [19,20]. *M. avium* biofilm levels were higher on iron and galvanized pipe surfaces than on copper or cPVC surfaces. Significantly, when copper pipe surfaces were exposed to disinfectants, the percentage of organisms identified as *M. avium* increased to nearly 100%. This could be of significance in institutional pipeline systems when disruption of biofilms occurs. During flushing or flow reversals, disruption of biofilms containing primarily *M. avium* organisms could result in sloughing of significant numbers of these organisms leading to high exposure levels. These data indicate that disinfection of potable water alone may not be effective to control biofilms of *M. avium*.

Detection of *M. avium* in drinking water systems is problematic because suspended bacterial levels may not reflect the level of *M. avium* colonization in distribution system biofilms. Additionally, the detection methods are difficult, time-consuming, and insensitive, especially when corrosion products are present. Use of HPC bacteria as an indicator of *M. avium* colonization is not recommended because mycobacteria can vary from 0 to 100% of the total plate count population. It is important, therefore, that improved methods be developed for detection of environmental mycobacteria under real-world conditions.

Treatment of institutional plumbing with hot ($>60^{\circ}C$) water can be an effective method for minimizing the exposure to *Mycobacterium* spp. Schulze-Röbbecke and Buchholtz [28] reported a 90% reduction in *M. avium* levels by heat treatment at 60°C for 4 min under laboratory conditions, although it required 33 min to achieve the same inactivation for *M. xenopi*. Du Moulin et al. [8] recovered *M. avium* in a hospital hot water system with water temperatures between 52°C and 57°C. In this study, the effectiveness of the heat treatment was dependant upon the pipe material, the level of nutrients, and the effluent temperature. Treatment was most effective for low-nutrient, hot water (50°C), in copper pipes.

To our knowledge, this is the first study to examine the factors that influence the survival and growth of *M. avium* in drinking water systems. Although addition studies are required using improved detection methods, the results of this investigation suggest that reducing the biodegradable organic material in drinking water, control of corrosion, maintenance of an effective disinfectant residual, and management of hot water temperatures can help limit the occurrence of *M. avium* in drinking water biofilms. These tools should prove useful in reducing the risk of *M. avium* from potable water systems.

5. Conclusions

- *M. avium* were able to colonize and grow in biofilms on pipe surfaces at AOC levels $>53\,\mu g/L$ and BDOC levels $>0.17\,mg/L$. There exists sufficient biodegradable organic material in most North American distribution systems to support the growth of *M. avium* in drinking water.
- The growth of *M. avium* was influenced by the type of pipe material. Bacterial levels were higher on iron and galvanized pipe surfaces than on copper or cPVC surfaces. When copper pipe surfaces were exposed to disinfectants, the percentage of organisms identified as *M. avium* increased to nearly 100%.
- Improved methods need to be developed for detection of environmental mycobacteria, particularly for biofilms grown on iron pipe surfaces.
- Heat treatment (50°C) was most effective for low-nutrient (3 mg/L ozonated humic acid) water in copper pipes. Higher temperatures ($>53^{\circ}C$) were required for *M. avium* inactivation for cells grown in high (10 mg/L) ozonated humic acid water on iron pipes.
- The current study suggests that reducing the biodegradable organic material in drinking water, control of corrosion, maintenance of an effective disinfectant residual, and management of hot water temperatures can help limit the occurrence of *M. avium* in drinking water biofilms.

Acknowledgements

The authors would like to thank the utility subsidiaries of the American Water, Voorhees, NJ for funding and support. This project was also funded by the American Water Works Association Research Foundation. The authors gratefully acknowledge Nancy Shaw, Debbie Verges, Myra Williams and Dale Young for their technical support. The comments of Mr. Richard H. Moser were also appreciated.

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