Free-living amoebae, *Legionella* and *Mycobacterium* in tap water supplied by a municipal drinking water utility in the USA

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ABSTRACT

Legionella and *Mycobacterium* can proliferate within free-living amoebae (FLA) where they are protected from disinfectants at concentrations that can kill bacteria but not protozoa. Despite effective treatment of drinking water, microbes can enter water utility distribution systems (DS) and hence the plumbing within building premises. Additionally, biofilm formation may account for the persistence of microbes in the DS. In the present study a domestic water tap in north-central United States (USA) was sampled in March and September 2007 and analysed for FLA, *Legionella* and *Mycobacterium*. Identification of organisms was determined by growth on specific culture media, light and electron microscopy, and amplification of DNA probes specific for each organism. In both the spring and fall samples, amoebae, *Legionella* and *Mycobacterium* were detected. However, *Acanthamoeba* was prominent in the spring sample whereas *Vahlkampfia* and *Naegleria* were the amoebae detected in the autumn. Bacterial proliferation in laboratory cultures was noticeably enhanced in the presence of amoebae and biofilms rapidly formed in mixed amoebae and bacteria cultures. It is hypothesized that temperature affected the dynamics of FLA species population structure within the DS and that pathogenic bacteria that proliferate within FLA, which are themselves opportunistic pathogens, pose dual public health risks.

Key words | *Acanthamoeba*, cysts, *Naegleria*, opportunistic infections, trophozoites, *Vahlkampfia*

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ABBREVIATIONS

DS	distribution system
FLA	free-living amoebae
ITS	internal transcribed spacer
MIP	macrophage infectivity potentiator
MAC	*Mycobacterium avium intracellulare* complex
NNA	non-nutrient agar
OI	opportunistic infection
SEM	scanning electron microscopy
TEM	transmission electron microscopy
WBDO	waterborne disease outbreaks

INTRODUCTION

The free-living amoebae (FLA) *Acanthamoeba*, *Naegleria*, *Balamuthia* and *Vahlkampfia* have been associated with infections of the central nervous system and eye in humans (Marciano-Cabral & Cabral 2003, 2008; Visvesvara *et al.* 2007; Khan 2008). These amoebae can be acquired from lakes and ponds, recreational waters and domestic water supplies. Furthermore, bacterial pathogens such as *Legionella pneumophila* and members of the *Mycobacterium avium intracellulare* complex (MAC) are taken up by protozoa such as amoebae and ciliates without undergoing

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digestion. While *Mycobacterium* can proliferate either free in the environment or within eukaryotic cells, *L. pneumophila* in the natural environment may require eukaryotic cytoplasmic environments for replication. These bacteria multiply within select protozoa thus increasing the probability of causing infections in humans (Abu Kwaik *et al.* 1998; Marciano-Cabral 2004). The intracellular lifestyle of these bacteria confers protection from disinfectants at concentrations that normally kill bacteria but not protozoa (Kilvington & Price 1990; Miltner & Bermudez 2000; Thomas *et al.* 2004; Molmeret *et al.* 2005). Also, there is evidence that bacteria released from amoebae exhibit altered properties and enhanced virulence (Barker *et al.* 1993; Cirillo *et al.* 1994). The cysts of amoebae have thick, impermeable walls and are much more resistant to desiccation and chemical penetration than are trophozoites. Bacterial pathogens can remain within amoebae during encystation providing the bacteria with additional protection from biocides and fostering their persistence within drinking water distribution systems (DS) (Kilvington & Price 1990; Thomas *et al.* 2004). Thus, despite effective treatment of drinking water, after the water enters the DS the quality of the water can change with respect to its microbial ecology.

FLA and bacterial organisms have been found in biofilms and water columns of drinking water distribution systems and tap water in several countries (Rohr *et al.* 1998; Brown & Barker 1999; Barbeau & Buhler 2001; Thomas *et al.* 2004; Langmark *et al.* 2005; Loret *et al.* 2005). The presence of a number of pathogenic microorganisms including *Legionella* and *Mycobacterium* within water DS has been reported (Covert *et al.* 1999; Falkinham *et al.* 2001; Le Dantec *et al.* 2002; September *et al.* 2004, 2007; Saby *et al.* 2005). It has been reported, also, that these bacteria are more resistant to standard disinfection methods and are not as easily inactivated by drinking water and wastewater treatment processes (Loret *et al.* 2005).

Aerosols generated from water containing *Legionella* and *Mycobacterium* may lead to the development of legionellosis or mycobacterial infections in susceptible humans. *L. pneumophila* can cause severe disease and death even in immunocompetent individuals (Barbaree *et al.* 1986). Immunocompromised individuals are susceptible to pneumonitis caused by members of the *Mycobacterium avium intracellulare* complex (MAC). Thus, these bacteria are considered agents of opportunistic infections (OIs). Indeed, there are increasing numbers of humans who are at risk for developing life-threatening OIs, such as AIDS patients, individuals undergoing therapy for solid organ transplant or those undergoing cancer chemotherapy (Marciano-Cabral & Cabral 2003; Visvesvara *et al.* 2007; Khan 2008). Thus, the presence of opportunistic FLA and pathogenic bacteria in drinking water DS and plumbing systems within individual premises is an important public health issue. However, there is a paucity of information on the microbial ecology in DS and tap water in different premises and geographic locations in the United States. In the present study, we analysed samples of tap water in a municipal drinking water system in the USA for the presence of FLA, *L. pneumophila,* and MAC sampled at two different seasons of the year.

METHODS

Water samples

A domestic water tap in a community drinking water system in north-central USA was sampled in March and September 2007. The water utility supplying the water uses surface water and treats it with free chlorine for primary and secondary disinfection. The typical chlorine concentration of water leaving the treatment plant was 1.1–1.2 mg l^{-1}. The temperature of the DS water ranged from 4 to 28°C over the period of the study. The water utility has always been in compliance with the water quality standards set forth in the Safe Drinking Water Act (www.epa.gov/safewater/sdwa/index.html). The water tap source was a utility sink in a building that receives intermittent use, which was approximately 3 miles from the treatment plant. The water age in the DS at the building site was estimated at 5 h. The water main serving the building was composed of cast iron and its installation was estimated to have been in 1936. A copper service line provided water from the main line into the building and the internal plumbing was also copper.

Prior to sampling, the tap was opened for 1–2 min. Then, 1,000 L of water was filtered through an Envirochek^{TM}HV sampling capsule (Pall Corp., East Hills, New

York) at a flow rate of 1 L/min (approximately 18 h total). Sterile sodium thiosulfate (0.01%) was added to the water to eliminate chlorine residuals. The thiosulfate solution was aseptically introduced to the tap water via a proportioning pump during the entire filtration time so that dechlorinated water always entered the filter. The capsules were maintained at ambient temperature during sampling and at 37°C in the laboratory. Prior to analysis, the contents of the capsules were shaken vigorously by hand to suspend organisms present in the filter. Aliquots were obtained for culturing and assessment for bacteria and amoebae. Page amoeba saline (Page 1976) was added as needed to maintain the water volume in capsules kept under laboratory conditions.

Electron microscopy

Aliquots of water samples were concentrated by centrifugation and the resultant pellets were processed for transmission electron microscopy (TEM). Cell pellets were fixed in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.2), post-fixed in osmium tetroxide and processed for TEM (Pettit *et al.* 1996). Samples were examined in a JEM 1230 transmission electron microscope (JEOL Ltd, Tokyo, Japan) operating at an accelerating voltage of 80 kV. Additionally, aliquots (100 μl) of capsule water were placed directly on glass coverslips for 24 h to 48 h and incubated at 37°C for identification of organisms by scanning electron microscopy (SEM). The glass coverslips containing organisms were fixed with glutaraldehyde, post-fixed with osmium tetroxide and processed for SEM (Pettit *et al.* 1996).

Culture of amoebae

To isolate amoebae, aliquots (0.2 ml) of water were removed from the capsules and placed on 1.5% non-nutrient agar (NNA) plates containing a layer of heat-killed *Escherichia coli* as a food source for the amoebae. The plates were incubated at 37°C and at 42°C. Following incubation for 2–3 days, the plates were examined using an inverted microscope to allow for detection of amoebae. Plates containing amoebae were scraped and the amoebae harvested by centrifugation and identified by PCR assays.

PCR for identification of amoebae

Samples cultured on NNA plates were harvested and the pellet suspended in 100 μl of deionized, sterile water. The resuspended material was tested for *Naegleria fowleri* using a nested PCR assay that allows for discrimination of *N. fowleri* from other species of amoebae in environmental samples including the closely related *Naegleria lovaniensis* (Reveiller *et al.* 2002). PCR was performed by amplifying a portion of a gene (mp2cl5) that is unique to *N. fowleri* using the forward primer, Mp2Cl5.for (5′-TCTAGAGATCCA-ACCAATGG-3′) and the reverse primer, Mp2Cl5.rev (5′-ATTCTATTCACTCCACAATCC-3′) to yield a 166-bp fragment of the Mp2Cl5 gene. To increase the sensitivity of the assay, nested primers Mp2Cl5.for-in (5′GTACATTGTT-TTTATTAATTTCC-3′) and Mp2Cl5.rev-in (5′GTCTTTG-TGAAAACATCACC-3′) were used to amplify a 110-bp fragment of Mp2Cl5 in a second round of PCR. Plasmid DNA (10 ng) purified from *E. coli* clone Mp2CL5 served as a positive control, while PCR-grade water lacking DNA template served as a negative control.

For identification of *Acanthamoeba* spp., DNA was extracted from amoebae using a Qiagen DNeasy kit (Valencia, California). Genomic DNA was subjected to a PCR assay that amplifies a fragment of small subunit rDNA using the forward primer, JDP1(5′-GGCCCAGATCGTT-TACCGTGAA-3′) and the reverse primer, JDP2 (5′-TCTCA-CAAGCTGCTAGGGAGTCA-3′) to yield a fragment of approximately 500 bp in size (Schroeder *et al.* 2001). DNA (10 ng) purified from laboratory-grown *Acanthamoeba astronyxis* served as a positive control, while PCR-grade water lacking DNA template served as a negative control.

A third PCR assay was performed to detect other FLA. DNA was extracted using the Qiagen DNeasy kit and subjected to an internal transcribed spacer (ITS) PCR assay that allows for identification of various amoebae including *Naegleria* spp., *Hartmannella* spp., *Vahlkampfia* spp. and *Willaertia* spp. (Pelandakis & Pernin 2002). This PCR assay allows for amplification of the complete ITS region using the forward primer, ITS1 (5′- GAACCTGCGTAGGGAT-CATTT-3′) and the reverse primer, ITS2 (5′-TTTCTTTT-CCTCCCCTTATTA -3′) located in the small-subunit and the large-subunit rRNA genes. DNA (10 ng) extracted from laboratory-grown *N. fowleri and Hartmannella* spp. served

as positive controls, while PCR-grade water lacking DNA template served as a negative control. All amplified PCR products were run (100 V) on a 1.5% Gene Pure (8.2 cm) agarose gel (ISC Bioexpress, Kaysville, Utah) and the gel was stained with ethidium bromide. All PCR products were sent to the MCV-VCU Nucleic Acids Research Facility and sequenced. Sequences were analysed further using the National Center for Biotechnology Information's (NCBI, www.ncbi.nlm.nih.gov/blast/Blast.cgi) Basic Local Alignment Search Tool (BLAST).

Culture and isolation of bacteria

The capsules were shaken vigorously then the water was aseptically removed and used for the isolation of *Legionella* spp., *Mycobacterium* spp. and other bacteria. *Legionella* was isolated using buffered charcoal yeast extract differential agar plates containing the dyes bromothymol blue and bromocresol purple, polymyxin B, anisomycin, along with vancomycin (BCYE with DPAV, Remel, Lenexa, Kansas). *Legionella* was also isolated using buffered charcoal yeast extract selective agar plates containing the dyes bromothymol blue and bromocresol purple, glycine, vancomycin and polymycin B (BCYE with DGVP, Remel, Lenexa, Kansas). Plates were incubated at 40°C for 4 days. Isolated colonies were re-plated onto BCYE plates until pure colonies were obtained.

Mycobacterium avium complex organisms were isolated using Lowenstein–Jensen slants inoculated with capsule water to promote growth of *Mycobacterium* spp. The slants were incubated at 37°C with 10% CO_2 for 12 days. Colonies were transferred to Middlebrook 7H9 (Remel) broth with glycerol and incubated for an additional 5 days.

PCR for detection of bacteria

Colonies isolated on BCYE plates, as well as cultures of bacteria grown on Lowenstein–Jensen agar or in Middlebrook 7H9 broth, were harvested by centrifugation, and DNA was extracted using a Qiagen DNeasy kit (Valencia, California). PCR assays were performed using different primer sets. A PCR Primer Amplification Kit (Maxim Biotech, San Francisco) using the forward primer, For (5′-GCTGCAACCGATGCCACATC-3′) and the reverse primer, Rev (5′-ACTACCGTTTCGCATGACGACTTA-3′) to yield a fragment of approximately 232 bp in size was used according to the manufacturer's instructions to specifically amplify the gene for macrophage infectivity potentiator (MIP) protein found in *Legionella pneumophila*. DNA from *L. pneumophila* was provided to use as a positive control, while PCR-grade water lacking DNA template served as a negative control.

For detection of *Mycobacterium avium*, cultures of bacteria grown on Lowenstein–Jensen agar and transferred to Middlebrook 7H9 broth were harvested by centrifugation and DNA was extracted. PCR was performed using a PCR Primer Amplification Kit (Maxim Biotech, San Francisco) according to the manufacturer's instructions that specifically amplifies the 16S rRNA gene present in *M. avium* using the forward primer, For (5′-GGAAAGGCCTCTTCGGAGG-3′) and the reverse primer, Rev (5′-AGTTCTGCGTACAGAAGACCAC-3′) to yield a fragment of approximately 134 bp in size. DNA from *M. avium* was provided to use as a positive control, while PCR-grade water lacking DNA template served as a negative control.

To identify other bacteria cultured on BCYE plates, PCR was performed using universal primers for small subunit rRNA gene using the forward primer, UniversFwd (5′-AGAGTTTGATCCTGGCTCAG-3′) and the reverse primer, UniversRev (5′-GGACTACCAGGGTATCTAAT-3′) to yield a fragment of approximately 750 bp in size (Tokajian *et al.* 2005). PCR-grade water lacking DNA template served as a negative control. Amplified PCR products from all samples were run (100 V) on 1.5% Gene Pure(8.2 cm) agarose gels (ISC Bioexpress, Kaysville, Utah) and the gels were stained with ethidium bromide. For verification of PCR results, all PCR products were subjected to a Qiagen QiaQuick PCR purification kit and sent to the Medical College of Virginia/Virginia Commonwealth University sequencing facility.

RESULTS AND DISCUSSION

Drinking water distribution system

Waterborne diseases constitute a major human health problem worldwide. Although waterborne disease outbreaks

(WBDO) are less common in the USA than in developing countries, outbreaks in the USA have been associated with drinking water. The incidence of WBDO is likely greater than that which has been reported in the surveillance system database (Blackburn *et al.* 2004; Liang *et al.* 2006; Reynolds *et al.* 2008). Although studies of water DS have been performed in several countries, many of these have been based on evaluation of water quality in simulated model systems to assess for the effects of disinfectants on pathogens in drinking water (Norton *et al.* 2004; Williams *et al.* 2004; Chauret *et al.* 2005; Donlan *et al.* 2005; Loret *et al.* 2005; Van der Kooj *et al.* 2005). Microorganisms can enter the DS via cross-connections between drinking water and sewer lines, backflows, breakthroughs in drinking water, wastewater treatment plant operations, and leaking pipes, valves, joints and seals as well as contamination of the tap by the end users. Also, the presence of microbes in tap water is thought to occur within the plumbing of building premises by regrowth of organisms that survive disinfection. It has been shown that in some countries, treated drinking water held in storage tanks which supply the home (Seal *et al.* 1992), and recirculating water used for aerosols, serve as major sources for these microbial pathogens. In the present study, water samples collected directly from a tap with water supplied by a municipal drinking water system in the USA, were found to contain FLA and pathogenic bacteria.

FLA

The occurrence of FLA in domestic water supplies, dental unit waterlines and water systems of hospitals has been reported previously (Rohr *et al.* 1998; Barbeau & Buhler 2001; Marciano-Cabral *et al.* 2003; Blair *et al.* 2008; Shoff *et al.* 2008). In the present study, amoebae and bacteria were observed in water taken directly from the capsule within 24 h of collection prior to culturing. TEM of pelleted material following centrifugation of capsule water documented the detection of these microbes (Figure 1a). Trophozoites and cysts with morphological characteristics of *Acanthamoeba* were observed by SEM (Figure 1b and c). Cysts containing bacteria were also observed (Figure 1c). Additionally, amoebic trophozoites were detected on NNA plates from water samples taken in both

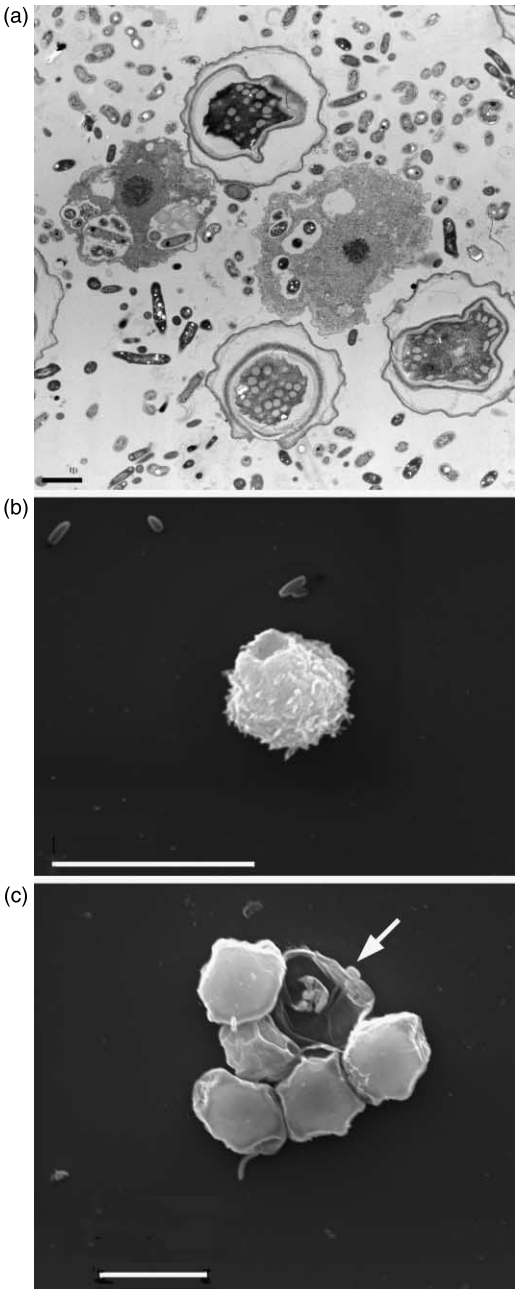

Figure 1 | Direct examination of the concentrated tap water sample by electron microscopy. (a) An aliquot of water from the Envirochek capsule was centrifuged and processed for transmission electron microscopy; trophozoites containing bacteria and amoebic cysts were observed. (b) and (c) Aliquots of water placed directly onto glass coverslips and processed for scanning electron microscopy. (b) Amoebic trophozoite and extracellular bacteria. (c) Amoebic cysts one of which contains bacteria (arrow); bar designations: (a) 2 μm; (b) and (c) 10 μm.

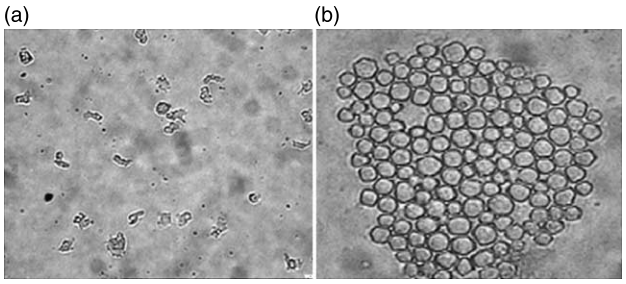

Figure 2 | Light microscopic examination of water contents following growth on non-nutrient agar (NNA) containing heat-killed *E. coli* as a food source. (a) Numerous trophozoites were observed after 48 h growth. (b) Cysts were observed after 7 days. Amoebic trophozoites were harvested and used for PCR analysis.

March and September after 3 days of incubation at 37°C (Figure 2a). Cysts were also present on NNA plates after one week of culture at 37°C (Figure 2b).

In order to identify the amoebae and bacteria present in the water samples, PCR assays were performed. The water sample taken in March 2007 was shown by PCR to be positive for *Acanthamoeba* spp. but negative for *Naegleria fowleri* (Figure 3) using different assays specific for each amoeba. A 500-bp PCR product for *Acanthamoeba* was observed on the gel. The water sample taken in September was positive by ITS PCR for *Vahlkampfia* and *Naegleria* spp. but not for *Acanthamoeba* (Figure 4). Although the ITS PCR assay does not allow for detection of *Acanthamoeba*, the PCR assay for *Acanthamoeba* using specific primers did not yield a detectable amplification product in the autumn sample (data not shown).

One of several possible explanations for not detecting *N. fowleri* in the spring sampling is probably the lower

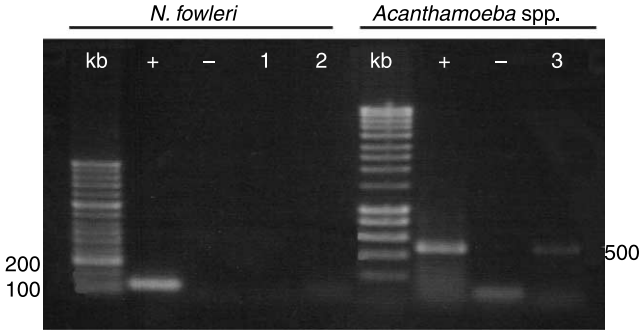

Figure 3 | PCR results of amoebae harvested from NNA cultures of water obtained in March 2007. The sample was negative for *N. fowleri* using a nested PCR assay. The sample was positive for *Acanthamoeba* using an *Acanthamoeba*-specific PCR assay. Lane designations: bp, base pair markers; +, positive control; –, negative control; 1–3, NNA harvest samples subjected to the respective PCR reactions. Note the band in lane 3 of approximately 500 bp.

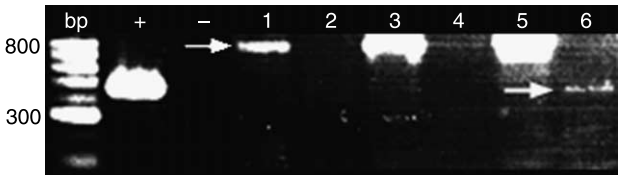

Figure 4 | Internal transcribed spacer PCR for free-living amoebae. Aliquots of water obtained in September 2007 were cultured on NNA-*E. coli* plates, and subjected to PCR using primers for the entire internal transcribed spacer region of ribosomal RNA genes. Lane designations: bp, base pair markers; +, positive control for *N. fowleri*; –, negative control; 1–6, water samples from six replicate culture plates. Samples 1, 3 and 5 were positive for *Vahlkampfia* spp. (arrow) as confirmed by sequence analysis and lane 6 was positive for *Naegleria* spp. (arrow).

temperatures in the DS over the winter compared with the summer. As these amoebae are known to thrive at higher temperatures their numbers might be higher in the DS following a warm summer season during which *N. fowleri* were able to proliferate. In contrast, their numbers might be low in the DS in the spring following the colder winter temperatures and hence were not found in that particular spring sampling at levels detectable by the protocols used in the present study. In contrast, *Acanthamoeba*, which has a lower growth temperature optimum than *Naegleria fowleri* (Griffin 1972), was detected in the spring, but not autumn sampling. The higher temperatures in the DS over the warm summer months might have limited the proliferation of *Acanthamoeba* and hence their numbers might have been sufficiently low to avoid detection in the autumn sampling. *Hartmannella vermiformis* was not detected in our study, although it has been reported to be the most common amoeba found in some hot water DS (Wadowsky *et al.* 1991; Sanden *et al.* 1992; Donlon *et al.* 2005). However, similar to the present study, those earlier reports indicated the presence of *Vahlkampfia* and *Acanthamoeba* in the samples that were analysed.

Bacteria

Growth of bacterial colonies of four different colours (green, grey, brown and tan) was observed when water samples collected in March and September were cultured on BCYE plates (data not shown). The colonies were isolated and *Legionella pneumophila* was identified by PCR on the grey-coloured colonies using specific primers (Figure 5). A PCR assay using universal primers was then used to allow for discrimination of the other bacteria grown

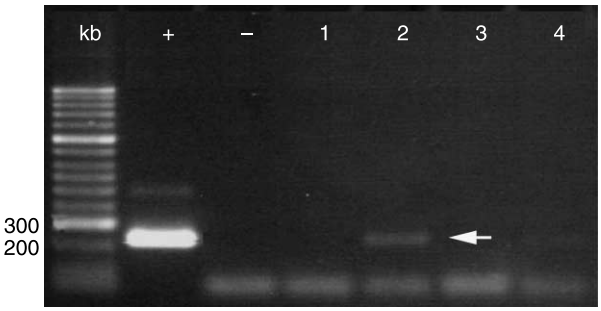

Figure 5 | PCR results of colonies grown on BCYE plates for *L. pneumophila*. Aliquots of water from the tap water samples were streaked on BCYE plates. Colonies exhibiting four different colours were observed: green, brown, grey and tan. Colonies were selected, re-grown, and subjected to PCR to identify bacteria. The grey colony was positive for *L. pneumophila* using primers for the macrophage infectivity potentiator protein (MIP) gene. Lane designations: bp, base pair markers; +, positive control for *L. pneumophila*; −, negative control; 1, green colony; 2, grey colony; 3, brown colony; 4, tan colony. Note the band in lane 2 of approximately 232 bp.

on the BCYE plates based on their genus designation. Table 1 lists other bacteria that were detected using universal primers on bacteria grown on BCYE agar plates. Bacteria belonging to the genera *Sphingomonas*, *Brevundimonas*, *Burkholderia*, *Rhizobium* and an alpha-proteobacterium were identified by sequencing the PCR products. Bacteria cultured on Lowenstein–Jensen agar were found to be acid-fast (data not shown). The bacteria were transferred to Middlebrook medium and were identified as *Mycobacterium avium* by a specific PCR assay (Figure 6). The September water sample, again, was PCR-positive for *L. pneumophila* and *M. avium* (Figure 7).

Legionella pneumophila and *M. avium* are commonly detected in drinking water systems (Lin *et al.* 1998; Covert *et al.* 1999; Falkinham *et al.* 2001; Le Dantec *et al.* 2002; Vaerewijck *et al.* 2005). These were also found in the water samples we obtained both in March and September in the present study suggesting they persist in the municipal DS and/or premises plumbing over the entire year. *L. pneumophila* can survive a broad range of temperatures including the temperatures of this DS, which can be as low as 4°C and remains < 20°C for approximately half the year. It was recently reported that, under laboratory conditions, *L. pneumophila* did not replicate within *Acanthamoeba castellanii* at temperatures below 20°C (Ohno *et al.* 2008). However, the recovery of this bacterium in both the spring and autumn DS samplings is consistent with findings of *L. pneumophila* and FLA in low-temperature waters in the environment (Söderberg *et al.* 2008). This suggests that, during periods when the DS temperature is low, *L. pneumophila* might replicate in other protozoan host species and/or are within biofilm matrices as viable but non-culturable organisms that were resuscitated (Garcia *et al.* 2007) under the experimental conditions used in this study.

Mycobacteria reportedly tolerate wide ranges of pH and temperature, and are more resistant to chlorine than other bacteria found in DS (September *et al.* 2007). In an attempt to determine the source of *Mycobacterium avium complex* (MAC) infections in AIDS patients, von Reyn *et al.* (1993)

Table 1 | Bacteria identified in tap water in this study

Bacterium	Growth medium*	Colony colour†	Primers‡	Sequence identity§ (%)
Legionella pneumophila	BCYE	Grey	MIP‖	100
Mycobacterium mageritense	BCYE	Grey	Universal¶	96
Sphingomonas sp.	BCYE	Green	Universal	96
Brevundimonas sp.	BCYE	Brown	Universal	95
Burkholderia sp.	BCYE	Brown	Universal	98
Rhizobium sp.	BCYE	Tan	Universal	95
Alpha-proteobacteria	BCYE	Tan	Universal	100
Mycobacterium avium	L-J**	Yellow	16S rRNA gene††	100

*Water samples were plated on BCYE agar to determine the presence of *L. pneumophila*.
†Four different colonies observed on BCYE agar, were isolated and identified by PCR, using a Universal Primer Set (Tokajian *et al.* 2005); all PCR products were sequenced.
‡Primers used in PCR assays to identify bacteria.
§Sequence identity determined by BLAST nucleotide search.
‖MIP macrophage infectivity potentiator protein, primer set specific for identification of MIP of *L. pneumophila*.
¶Universal Primer Set (Tokajian *et al.* 2005).
**Water samples cultured on Lowenstein–Jensen (L–J) slants and transferred to Middlebrook 7H9 broth for DNA isolation prior to PCR.
††16S rRNA gene present in *M. avium.*

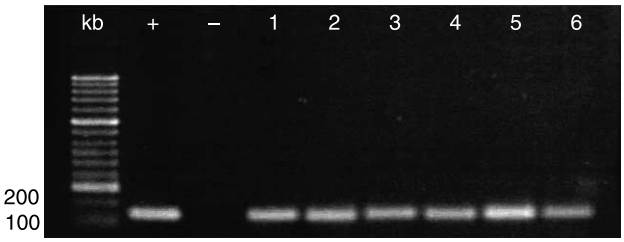

Figure 6 | PCR results of colonies grown on Lowenstein–Jensen slants for identification of *Mycobacterium avium complex*. Water samples from the distribution system were streaked onto slants, transferred to 7H9 medium and maintained at 37°C or 42°C. All samples were positive for *M. avium*. Lane designations: bp, base pair markers; +, positive control for *M. avium*; −, negative control; 1–6, water samples. Note the bands in lanes 1–6 of approximately 134 bp.

examined 91 water samples in the USA, Finland and sub-Saharan Africa. Interestingly, the prevalence of MAC-positive water supply systems was shown to be higher in the USA and Finland than in Africa (32% vs. 5%). Twenty percent of hospital water supplies in the USA tested positive for MAC suggesting a need for more information on the apparent high prevalence and implications for public health of this opportunistic pathogen in drinking water in developed countries.

In addition to *M. avium*, *Mycobacterium mageritense* was also identified in the present study by PCR and nucleotide sequence analysis. This opportunistic bacterium has been isolated from a municipal water supply and has been shown to cause furunculosis in patrons of nail salons who receive footbaths prior to pedicures. *M. mageritense* was isolated from the same whirlpool footbaths used by these patrons (Gira *et al.* 2004). Other culturable bacteria found in the DS in this study include *Sphingomonas* sp., *Brevundimonas* sp., *Burkholderia* sp., *Rhizobium* sp. and an unidentified α-proteobacterium. The incidence of sphingomonads in DS is apparently common (White *et al.* 1996; Williams *et al.* 2004; Tokajian *et al.* 2005; Simões *et al.* 2007).

Although microbes cannot totally and indefinitely be eliminated from DS, drinking water utilities should be cautious when considering changes in treatment regimens. For example, when a municipal water utility in the USA switched from chlorine to monochloramine as a disinfectant, this was accompanied by marked changes in microbial community composition of water in the DS characterized by a decrease in many types of bacteria and the appearance of others (Pryor *et al.* 2004). In particular, *Sphingomonas* became more dominant in the bacterial community following the switch to chloramines. However, in the present study, the water utility used chlorine as the disinfectant suggesting that sphingomonads are common in spite of the water treatment used. The occurrence of both *Sphingomonas* and *Burkholderia* in the DS has been reported in other drinking water systems (Zanetti *et al.* 2000). The presence of sphingomonads in DS could present a health problem since it has been associated with bacteraemia in neutropenic patients (Perola *et al.* 2002). *Sphingomonas* has also been associated with septic shock bacteraemia in an immunocompromised patient undergoing hematopoietic stem cell transplantation (Al-Anazi *et al.* 2008). *Brevundimonas* spp., also identified in the present study, has been reported in association with *Sphingomonas* in water from dental unit reservoirs and other water systems (Mergaert *et al.* 2001; Szymanska 2007).

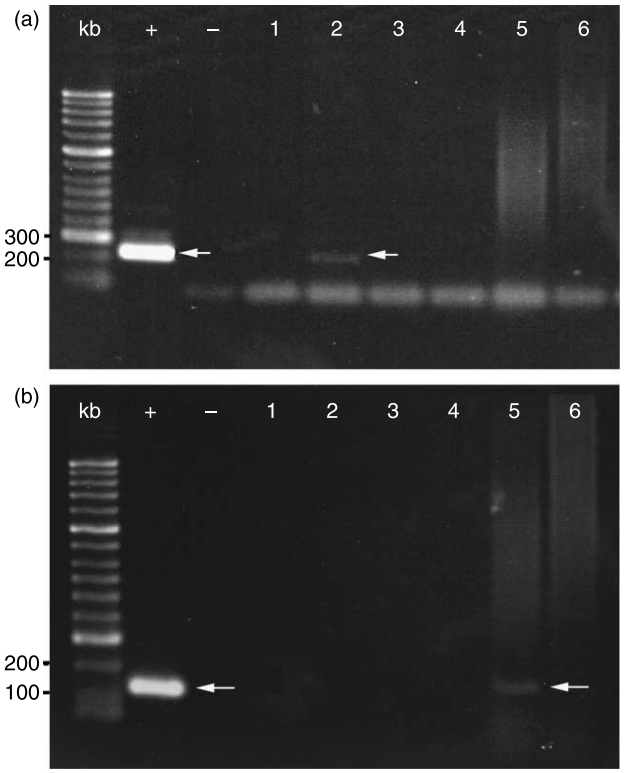

Figure 7 | PCR results of aliquots of water obtained in September 2007. Water samples were streaked onto BCYE agar plates or Lowenstein–Jensen slants to identify *L. pneumophila* or *M. avium*, respectively, and colonies were assessed by PCR. (a) PCR results from BCYE plates to assess for *L. pneumophila*. (b) PCR results from Lowenstein–Jensen slants to assess for *M. avium*. Both *L. pneumophila* and *M. avium* were identified by PCR. Lane designations: bp, base pair markers; +, positive control; −, negative control; 1–4, supernatant samples; 5–6, pellet samples.

Bacteraemia (Gilad *et al.* 2000; Chi *et al.* 2004), meningitis (Mondello *et al.* 2006), cutaneous infections (Panasiti *et al.* 2008) and infective endocarditis (Yang *et al.* 2006), caused by the opportunistic bacterium, *Brevundimonas* sp., have been reported.

The presence of *Rhizobium* spp., a symbiotic nitrogen-fixing bacterium found in nodules of the roots of certain leguminous plants, suggests soil contamination within the DS studied. Species of *Rhizobium* produce exopolysaccharides that influence biofilm formation (Fujishige *et al.* 2006; Rudrappa *et al.* 2008). Microbial biofilms found in a DS were studied using metagenome and phylogenetic analysis (Schmeisser *et al.* 2003). The metagenome survey of drinking water networks determined that a significant fraction of genes identified was similar to those of *Rhizobium* spp.

Biofilms

Scanning and transmission electron microscopy of water samples demonstrated the presence of biofilm formation by amoebae and bacteria. Aggregates of bacteria attached to the amoeba surface were observed by SEM (Figure 8a) in water taken directly from the capsule and placed on glass coverslips for 24 h. Additionally, aggregates of bacteria were observed attached to amoebae and amoebic cysts by TEM (Figure 8b).

In the present study, biofilms consisting of amoebae with clusters of attached bacteria were observed by electron microscopy in water samples taken directly from the Envirochek capsules. Amoebic trophozoites in these aggregates also harboured intracellular bacteria (Figure 8b). This interaction and the extracellular matrix of biofilms have been reported to protect bacteria from the biocides present in the DS (Barbeau & Buhler 2001; Storey *et al.* 2004; Thomas *et al.* 2004; Loret *et al.* 2005). In an assessment of the microbial ecology of DS, Simões *et al.* (2007) reported the mutualistic/synergistic interaction in biofilm formation between *Sphingomonas* and *Burkholderia* and suggested a cooperative interaction of the two species in a low nutrient environment that could include intergeneric metabolic cooperation. Bacteria and their protozoan hosts can resist disinfection through formation of biofilms (Langmark *et al.* 2005; Berry *et al.* 2006). It is known that multiple

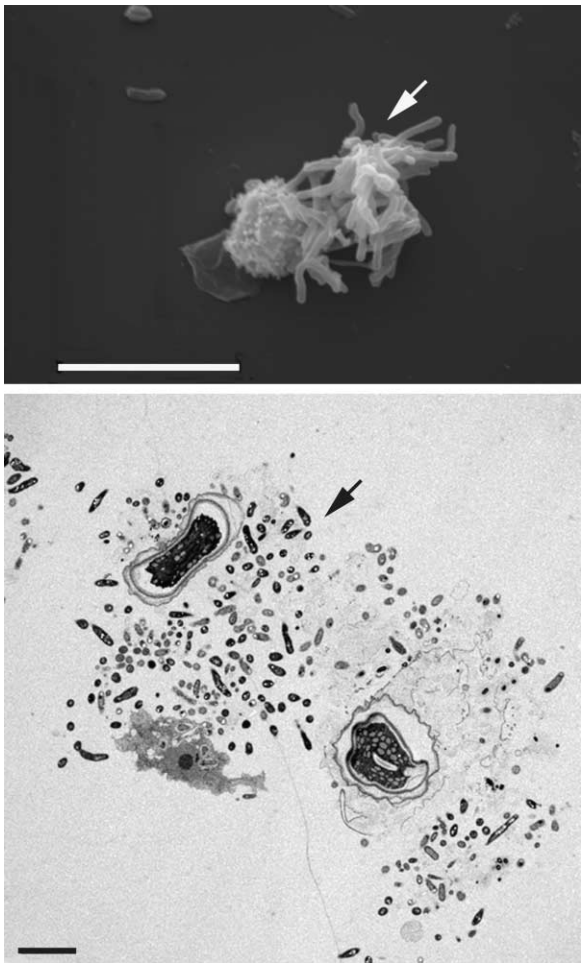

Figure 8 | Biofilm formation observed in water taken directly from the Envirochek capsule within 24 h of collection. Clusters of bacteria (arrows) were attached to the surface of the amoebae. (a) Scanning electron micrograph; (b) transmission electron micrograph. Note the presence of intracellular bacteria in the trophozoite stage; bar designations: (a) 10 μm; (b) 2 μm.

microorganisms coexist in biofilms within DS which increases their resistance to disinfectants such as chlorine and chlorine dioxide. Thus, in addition to their intracellular existence in amoebae, the persistence of *Legionella*, *Mycobacteria* and other bacteria in water systems appears to be enhanced by biofilm formation.

CONCLUSIONS

This study provides a better understanding of the microbial ecology of DS by detecting and identifying bacterial pathogens and opportunistic FLA in this specific DS, providing new information that might be used for

improving water quality and safety. The observations reported here indicate that the population dynamics of FLA species in tap water is apparently influenced by the temperature in the DS. Hence the high temperature species *Naegleria fowleri* was not detected in the spring whereas *Acanthamoeba*, which has a lower temperature growth optimum (Griffin 1972; Ohno *et al.* 2008) was not detected in the autumn. Among several worthwhile questions to address in future studies are whether there are both spatial and temporal differences in the microbial flora within this relatively extensive water utility DS and whether there are detectable quantitative changes in the various pathogens within the water samples in real time (upon collection) over the year. As the properties of water vary from source to source, analyses of other water utility DS should be similarly evaluated and compared with the findings in this report.

The public health significance of FLA in DS and/or plumbing systems within buildings is twofold. First, FLA serve as reservoirs for pathogenic bacteria including *L. pneumophila* and *M. avium* (Lin *et al.* 1998; Brown & Barker 1999; Marciano-Cabral 2004; Berry *et al.* 2006; Visvesvara *et al.* 2007; Marciano-Cabral & Cabral 2008). Second, some FLA are themselves opportunistic pathogens in humans. People with defective immune systems are especially vulnerable; the demographics of these populations are changing (Kaneshiro & Dei-Cas 2009) and there is currently no good therapy for clearing opportunistic FLA infections.

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