Investigation of opportunistic pathogens in municipal drinking water under different supply and treatment regimes

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Abstract Changing regulations to lower disinfectant byproducts in drinking water is forcing utilities to switch disinfection from chlorine to monochloramine. It is generally unknown whether this will impact positively or negatively on the microbiological quality of drinking water. A utility in Florida, using water with relatively high organic carbon levels from deep wells in several wellfields, made the decision to change its disinfection regime from chlorine to chloramine in order to meet the new regulations. To assess the impacts of such a change on the microbiology of its water supplies, it undertook a number of studies before and after the change. In particular, the presence of the opportunistic pathogens *Legionella* and *Mycobacterium*, and also the composition of drinking-water biofilms, were examined. A preliminary synthesis and summary of these results are presented here. *Legionella* species were widely distributed in source waters and in the distribution system when chlorine was the disinfectant. In some samples they seemed to be among the dominant biofilm bacteria. Following the change to monochloramine, legionellae were not detected in the distribution system during several months of survey; however, they remained detectable at point of use, although with less species diversity. A variety of mycobacteria (21 types) were widely distributed in the distribution system when chlorine was the disinfectant, but these seemed to increase in dominance after chloramination was instituted. At point of use, only four species of mycobacteria were detected. Other changes occurring with chloramination included (a) an altered biofilm composition, (b) increased numbers of total coliforms and heterotrophs and (c) nitrification of water storage tanks. The results suggested that consideration should be given to the microbiological effects of changing disinfection regimes in drinking-water and distribution system biofilms.

Keywords Biofilms; drinking-water disinfection; *Legionella*; *Mycobacterium*; opportunistic pathogens

Introduction

Regulations in the US (Stage 1 Disinfection-Byproducts Rule – promulgated 1 January, 2002) lowered the Maximum Contaminant Levels (MCL) for total trihalomethanes from 100 μg/L to 80 μg/L. Also included in the rule was a MCL for haloacetic acids of 60 μg/L. Similar or even lower limits are likely to be implemented in Canada. These new regulations are forcing many drinking-water utilities to make the switch in disinfectant from chlorine to chloramine, because it has been proven that such a change in chemical treatment will lower the levels of these regulated Disinfection-Byproducts (DBP). Utilities in Florida are in danger of exceeding the DBP limits because of the high levels of naturally occurring organic carbon in the ground- and surface waters. However, does switching to chloramines to lower DBP levels have effects on microbiological water quality?

Drinking-water distribution pipe biofilms contain numerous species resistant to the applied disinfectant residual, including opportunistic pathogens. Legionellae and mycobacteria are prominent among those that flourish in the niches provided in biofilm growth and, perhaps, benefit from a reduced competition for nutrients when a disinfectant residual is present. Pathogenic strains of certain *Legionella* and *Mycobacterium* species

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can present special risks for the immunocompromised when biofilm fragments are shed into drinking water. Showering and other aerosol generating activities may be a particular risk. Both legionellae and mycobacteria are ubiquitous in surface waters, but less is known about their presence and distribution in groundwater and systems supplied by groundwater, especially when no direct surface links are known that influence deep wells. However, these bacteria tend to have complex interactions with grazing protozoa, whereby carriage of the bacteria in the protozoa may aid in bacterial survival and distribution.

What is the fate of potential pathogens such as *Legionella* and *Mycobacterium* when a switch from chlorine to chloramines is made? Do other parameters that serve as indicators of water quality change? One utility in Florida has attempted to answer these questions by additional in-house monitoring and by participating in several collaborative projects to better understand the effects of changing disinfection regimes and/or water sources on system microbiology. These studies, using both culture and molecular techniques, were focused on (a) the presence, types and numbers of legionellae in the groundwater production wells serving the utility, (b) the presence and types of legionellae and mycobacteria in the distribution system before and after the change to chloramination, (c) changes in microbial communities in drinking-water distribution system biofilms and (d) after the switch to chloramination, the presence and types of legionellae and mycobacteria at point-of-use outlets in the distribution system. Some early data, prior to the switch to monochloramine, have been presented previously (Pryor *et al*., 2001), but a preliminary synthesis and summary of all the microbiological studies relevant to the switch in disinfectant is presented here.

Materials and methods

Location and system changes

The drinking-water system examined in this study was at Pinellas County Utilities (PCU) (Florida), which had historically used groundwater with chlorine as a disinfectant. Multiple wells, from three groundwater sources with similar water quality, fed a municipal distribution system. An average TOC level in PCU's groundwater was 4.0 mg/L and wells were more than 700 feet (213 m) deep. Treatment and supply changes were as follows: (a) a hydrogen sulphide plant was brought on line, (b) then, in May 2002, chloramine was substituted for the previously used chlorine, and (c) finally the source was switched from groundwater to surface and/or mixed groundwater/surface supplies. Data reported here were obtained from samples collected before, during and after the transition to chloramination, over a time frame of approximately 2–3 years. No data are included following the switch to surface water, as these studies are still ongoing. Microbiological investigations included the presence, extent and speciation of legionellae in the production wells, legionellae and mycobacteria in distribution system biofilms and composition of microbial biofilm communities and assay of conventional water-quality indicators at point of use.

Samples

Water (1 L samples) from production wells (PW) and distribution system (DS) sites, or biofilms obtained at PW (PVC pipe sections, water meters, or end-cap scrapings) or in DS (scrapings from backflow valves, master meters, home water meters and sample stations; PVC and iron coupons from annular reactors; experimental bead devices) were shipped on ice by courier and processed within 24 h of collection. Independent of biofilm type, samples were shipped in water from the sample site. Standard water parameters, including metals and microbial water quality indicators, were measured. Temperature in PW was approx. 23–25°C, and in the DS varied about 10°C during the year (21–31°C).

Legionella and *Mycobacteria*

Water was filtered through 0.45 μm-porosity polycarbonate filters. Biofilm samples were scraped and resuspended in source water before vortexing and filtering through 0.45 μm-porosity polycarbonate filters. For culture, pre-treatment(s) to suppress other indigenous bacteria was necessary for both genera.

Legionella spp. Legionellae were cultured on three types of semi-solid media (BCYEα, GVP, CCVC) (Riffard *et al*., 2003), all based on buffered charcoal yeast extract (BCYEα) either untreated or after heat or acid treatment. Duplicate plates from all media were incubated at both 30°C and 35°C for <14 d. Presumptive colonies were confirmed by failure to grow on either BCYEα without L-cysteine or blood agar. DNA was isolated from presumptive colonies, and partial nucleotide sequencing of the 16S rRNA gene was used for identification of the *Legionella* species. Sequences obtained were compared with available sequences in the DDBJ, EMBL and GenBank databases with the Gapped BLAST program (*http://www.ncbi.nlm.nih.gov*). Legionellae were also detected using semi-nested PCR with LEG 225 and LEG 858 oligonucleotides enclosing 654 bp of the 16S rRNA gene in the first step and LEG 448 and LEG 858 in the second step reaction. Absence of PCR products triggered a test for PCR inhibitors. An immunomagnetic separation step was also developed in an attempt to enhance recovery of legionellae. *Legionella* samples at point of use were analysed by the CDC (Fields *et al*., 2003).

Mycobacterium spp. Mycobacteria were cultured for up to several months on 7H10 agar at ambient (approx. 22°C), 30°C, 37°C and 42°C after sample filtration (0.45 μm) and pre-treatment with 0.8% cetyl pyridinium chloride (CPC). Colonial characteristics were recorded, and suspect colonies were sub-cultured and PCR conducted on these colonies for a 439bp fragment of the hsp65 gene. PCR products were subjected to restriction enzyme analysis using *Bst E II* and *Hae III*; the images were analysed by AlphaEase software and compared to a mycobacteria library.

Direct characterisation of microbial communities

Phospholipid fatty acid analysis (PLFA) and denaturing gradient gel electrophoresis (DGGE) were used to characterise microbial biofilm communities in the distribution system. PLFA gave the relative viable biomass of prokaryotic and eukaryotic community members, as well as general information on community structure and physiological status. DGGE determined specific members of the microbial community. The 1540 bp fragment of the 16S rRNA gene common to bacteria was amplified and the products electrophoresed on a denaturing gel. Predominant DGGE bands were sequenced and phylogenetic affiliations determined from comparison of DNA sequences with those of known bacteria in RDP or GenBank.

Protozoa analysis

Protozoa analysis was performed on some PW samples by direct microscopic examination and cultivation on modified PYNFH medium especially formulated for the cultivation of small, free-living amoebae, including the amoebae commonly associated with *Legionella*, i.e. *Acanthamoeba*, *Hartmanella*, *Naegleria* and *Tetrahymena*. Detection of amoebae in point-of-use samples was performed by the Centres for Disease Control and Prevention (Fields *et al*., 2003).

Results and discussion

Legionella in production wells

These samples were analysed in two phases. In the first phase, single samples were analysed from six wells. The results by culture showed legionellae in 4/6 water samples and 3/6 biofilm samples, but only one well was negative in both water and biofilm. Using PCR, 2/6 water samples and 3/6 biofilm samples were positive for legionellae; three wells appeared negative. The second phase was targeted at following the occurrence of legionellae in a few wells, using monthly samples, to examine variations over time. In addition to culture and PCR, some samples were analysed by also including IMS-culture and IMS-PCR. Overall, the majority of production wells (10/12) were sources of legionellae. Individual PW yielded qualitatively and quantitatively different results over time. Culture consistently performed better than PCR, with more legionellae isolated at 30°C than the traditionally used 35°C. Acid treatment was the most successful sample pre-treatment. However, in spite of the sample pre-treatments, many of the culture plates were too overgrown to be able to visually identify and isolate suspected *Legionella* colonies. In PCR quite a few samples were shown to contain PCR inhibitors; this was a particular problem with biofilm samples. Therefore, the legionellae isolated must be considered an absolute minimum of those present. The addition of IMS recovered an occasional culture isolate in samples negative by other methods, but the results obtained with it were not consistent.

Multiple autofluorescent and non-autofluorescent *Legionella* species, including some recognised pathogens, were cultured from both water and biofilm samples from all three water sources. Table 1 shows only the legionellae isolated by culture, where partial nucleotide sequencing of the 16S rRNA gene from the isolated colony was performed and that could be unambiguously identified when compared with on-line databases. In some samples, multiple species were found concurrently, and several species were isolated in multiple samples. Two species were isolated from biofilms that were not found in the water samples. Several of the species found have been implicated in human disease, but *L. pneumophila* was not found in the production well samples. For the identified species, numbers ranged from 25 to 46,000/L in water and from 1 to 500 CFU/cm^2 in biofilms.

Mycobacteria appeared to be absent from PW, although only a limited number of samples were tested and a more detailed study would be needed to confirm or refute their absence. Analysis of protozoa in a limited number of PW samples showed a variety to be present, including some that were known to support the growth of legionellae – *Hartmanella*, *Naegleria* and other amoebic cysts. Data from the PW samples formed part of a larger study investigating the occurrence of legionellae in groundwaters across North America. Only two utilities submitted samples that were apparently free of legionellae which were found regardless of well depth or water temperature. PW at PCU are more than 700 ft (213 m) deep, with no known specific surface influences.

Table 1 *Legionella* species identified in production wells

Species identified	Implicated in human disease	Species identified	Implicated in human disease
*L. anisa**	Y	*L. gresilensis*	N
L. dumoffii	Y	*L. parisiensis*	Y
L. erythra	N/Y	*L. quateirensis*	N
L. fallonii	N	*L. santicrucis*	N
L. feelei	Y	*L. waltersii*	N
L. geestiana	N	LLAP-2	?

* found only in biofilm samples; some legionellae could not be identified

***Legionella* and *Mycobacterium* in the distribution system**

In DS samples legionellae were also common when chlorine was used, being found in permanent sample stations, backflow valves, master meters, flushing samples and home water meters. Twelve different species of *Legionella* were identified in biofilms and water samples, but *L. pneumophila* was never detected in these distribution samples. Dead-end streets and neighbourhoods that received water flow from more than one direction seemed to be especially prone to colonisation with *Legionella*, with the rate of colonisation estimated at 20%.

Five sample sites distributed throughout the distribution system were tested monthly for *Legionella*. During the first year of testing (May 1999 through 2000), one site was *Legionella*-positive every month; two other sites were positive for one or two sampling events. Engineering interventions that improved water flow through the area of greatest concern appeared to reduce the rate of colonisation. A chlorine analyser and feed system also kept chlorine residuals of 0.8–1.2 mg/L. The following year (2001) the *Legionella* positive rate dropped to 3.3%. However, during this same year (2001), 20 additional sample sites were selected as potential problem spots. The *Legionella*-positive rate based on this single sample event was again estimated at 20%, although only non-autofluorescent species were found. The most frequently positive site, which also had the lowest pH and chlorine residual (average 0.6mg/L), as well as the highest turbidity, iron and TOC, was at the end of the DS where residence time was long and water flow was low. Other positive sites were in "backwash" areas that may have increased the age of the water. One negative site had a chlorine residual of >3.0 mg/L.

PCR also detected legionellae but was less successful than culture, perhaps due to PCR inhibitors in some samples. Surprisingly, in 10 DS biofilm samples, legionellae appeared to be the predominant organisms detected by DGGE. DGGE analysis also suggested legionellae were present throughout the DS even though they are not necessarily culturable. Additional species detected in DS samples included: *L. longbeachae*, which is known to cause human disease, and *L. fairfieldensis*, which is not. The latter appeared to be among the predominant species identified by DGGE analysis, but was not cultured. In cooling towers, *L. pneumophila* serotype 1 was the predominant *Legionella* species, but *L. bozemanii* was also isolated. Annular reactor coupons supported *L. beliardensis, L. bozemanii, L. donaldsonii, L. dumofii*, and *L. gormanii*. The experimental beads captured *L. waltserii*, which was also found in production wells. Following the switch to monochloramine, legionellae were apparently absent from DS biofilm communities both by culture and DGGE analysis. However, lack of detection may be simply due to a reduction in numbers rather than elimination.

Of 32 DS sample sites, 12 tested culture positive for mycobacteria (12/32 water and 12/25 biofilm), including some known pathogens. Mycobacteria were found throughout the DS in main lines, backflow valves, home water meters, and hose bibs. Due to their resistance to chlorine, mycobacteria were isolated from sites where the chlorine residual was >3.0 mg/L. Following the switch to chloramine, mycobacteria appeared more common, as shown by predominant bands from DGGE gels. This may perhaps have been due to reduced competition, as many other species were reduced or absent.

Table 2 shows the species of mycobacteria isolated from DS samples. Counting different species types separately, 21 types of *Mycobacterium* were cultured and identified. Some of these species are considered to be pathogens, including *M. avium* (found only once) and *M. intracellulare* (the most frequently isolated). Because of their slow growth, pre-treatment methods for samples to detect mycobacteria are critical to being able to detect the target bacteria. However, the pre-treatment method used may have (a) prevented the detection of some particular mycobacteria species and (b) also minimised the number of samples found positive and the number of colonies seen. It was impossible to tell what

Table 2 Mycobacteria found in the distribution system and point-of-use showerheads

Species	DS	Shower	Number of hits
M. avium complex	X	X	1
M. brumae	X		1
M. chlorophenolicus	X		2
M. flavescens type 1	X		2
M. flavescens type 3	X		4
M. fortuitum	X		1
M. genavense	X		1
M. gordonae type 1	X	X	34
M. gordonae type 2	X	X	28
M. gordonae type 3	X	X	8
M. gordonae type 4	X	X	2
M. gordonae type 7	X	X	2
M. gordonae type 9	X	X	2
M. haemophilum	X		1
M. intracellulare type 1	X	X	29
M. kansasii	X		1?
M. malmoense	X		1
M. nonchromogenicum	X	X	2
M. senagalense	X		1?
M. simiae	X		1?
M. smegmatis	X		1
M. szulgai	X		3
M. vaacae	X		1
M. JS621	X		2

would have been detected with other pre-treatments. The pre-treatment method used was developed in comparison with other published methods and was found to be optimum for the recovery of mycobacteria in highly contaminated samples (unpublished).

Microbial community analyses in DS

Microbial community analyses showed clear differences between the relatively simple PW microbial communities and the more complex ones established in biofilms exposed to chlorine and monochloramine, although both were dominated by Gram-negative bacteria. Protozoa, including some known to host legionellae, were also found in PW samples. The switch from chlorine to monochloramine was accompanied by marked changes in community composition, with the apparent disappearance of many predominant types, and the appearance of others. Other changes of note included (a) *Thiothrix,* shown to be present in PW by DGGE, which became dominant and caused increased turbidity following installation of the hydrogen sulphide plant, (b) *Sphingomonas* and *Pseudomonas*, which became more dominant in the microbial communities following the switch to chloramines and (c) a deterioration in water quality, as measured by total coliforms, was apparent in DS samples following the switch to chloramines. In 2002, two samples were positive for total coliforms before the switch to chloramination, and 21 were positive after the switch.

Legionella and *Mycobacterium* at the point of use before and after switching to chloramines

Studies performed by the Centre for Research in Environmental Microbiology (CREM), the Centres for Disease Control and Prevention (CDC), and PCU were combined to compare the colonisation rate of *Legionella* and *Mycobacterium* before and after switching to chloramines. Biofilm and water samples were collected from hot water heaters and showerheads. The *Legionella* colonisation rate in showerheads was measured at 20% when chlorine was still used as the disinfectant. After switching to chloramines, the colonisation rate of *Legionella* species dropped to about 6.2%. However, the numbers of *L. pneumophila*

remained the same; their population seemed to be unaffected by a change in disinfectant. Although *L. pneumophila* was never found directly in a distribution system sample, only in cooling towers, it was found in 29% of the *Legionella*-positive hot water heater and showerhead samples when chlorine was used as a disinfectant, and in 78% (7/9) of the *Legionella*-positive samples after switching to chloramines. Cooling towers, hot water heaters and showerheads are all likely to exert similar temperature selection pressures that may allow the *L. pneumophila* strains to become predominant. This is supported by the occurrence of *L. pneumophila* in almost pure culture in one groundwater source where the water temperature was 40°C (Riffard *et al*., 2003).

Eight different species of *Legionella* were cultured in the chlorine phase of this study, and only three species of *Legionella* were identified in the chloramines phase of the study. Amoebae were also found in about 15% of point-of-use sites, and their numbers remained unchanged before and after switching to chloramines (Fields *et al*., 2003).

The rate of colonisation by *Mycobacterium* was 19.1% when chlorine was used as a disinfectant. In contrast to *Legionella*, populations of *Mycobacterium* in biofilms and water samples from showerheads appeared to increase when the disinfectant changed from chlorine to chloramines. Overall numbers of positive sites changed from 19.1% (chlorine) to 42.2% (chloramines). The increase in *Mycobacterium*-positive biofilm samples (from 17.7% to 27.1%) was less than in bulk water (from 20.8% to 57.3%). In the previous DS studies discussed above, many species of *Mycobacterium* were identified in the distribution system. Much less diversity was seen in showerhead samples, where *M. gordonae* and *M. intracellulare* were the two predominant species.

Legionella and *Mycobacterium* by DGGE and band sequencing

Other evidence that the numbers of *Legionella* may have decreased after switching to chloramines was seen in the DGGE analyses done by Microbial Insights. Two of three sites selected for the Annular Reactor study showed prominent bands (>1% of total population), identified as *Legionella*, when chlorine was the disinfectant. After switching to chloramines, these bands were no longer present in the DGGE gels. In contrast, no bands identified as *Mycobacterium* were present when chlorine was used as a disinfectant. Almost immediately after the switch to chloramines, very prominent bands identified as *Mycobacterium* spp. appeared on DGGE gels.

Coliform bacteria and heterotrophic bacteria in the distribution system

Following the switch to chloramination, the number of positive coliform samples and the levels of heterotrophic bacteria (HPCs) also rose quite dramatically. Although no direct correlation has been seen between the presence of pathogens and increased levels of HPCs or positive coliform samples, an increase in these parameters did demonstrate a favourable environment for bacteria to grow. In spite of a number of system improvements, the total number of positive compliance samples more than doubled for the conversion year of 2002 compared to previous years. As stated above, only two samples were out of compliance prior to the switch to chloramines in May, whereas 21 were out of compliance between May and December 2002. At many of the monthly sample locations that are monitored for water quality purposes, the heterotrophic plate counts increased 50-fold (at some sites 250-fold) after conversion to chloramines.

Nitrification in the distribution system

Since the conversion from chlorine to chloramines, water storage tanks at four of six locations have nitrified. Loss of total chlorine residual and monochloramines was shown to occur relatively quickly over a several-day period.

Conclusions

The results of this study provided some insight into the presence of opportunistic pathogens, particularly legionellae and mycobacteria, in the source and distribution of a municipal water supply before and after switching to chloramination. Although switching from chlorine to chloramines may have some benefits, such as lower regulated disinfection-byproducts and a decreased variety of *Legionella* species, there was no indication of a decrease in *L. pneumophila* at the point of use. Other measures of water quality may also not be as beneficially impacted, especially when the organic carbon content of the source water was relatively high. This again raised the difficult issue of balancing DBP and microbial risks, and potentially suggested that a decision to switch to chloramination needed to be justified by local data. This would require an understanding of both DBP formation and concentration of the most harmful/mutagenic chemical species, as well as the microbial communities within the source water and distribution system. The results of this study also confirmed the lack of value of conventional water-quality indicators for opportunistic pathogens such as legionellae and mycobacteria.

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