**Microbial Pathogens in Source and Treated Waters from Drinking Water Treatment
Plants in the United States and Implications for Human Health**

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ABSTRACT

An occurrence survey was conducted on selected pathogens in source and treated drinking water collected from 25 drinking water treatment plants (DWTPs) in the United States. Water samples were analyzed for the protozoa *Giardia* and *Cryptosporidium* (EPA Method 1623); the fungi *Aspergillus fumigatus*, *A. niger* and *A. terreus* (quantitative PCR [qPCR]); and the bacteria *Legionella pneumophila* (qPCR), *Mycobacterium avium*, *M. avium* subspecies *paratuberculosis*, and *M. intracellulare* (qPCR and culture). *Cryptosporidium* and *Giardia* were detected in 25% and in 46% of the source water samples, respectively (treated waters were not tested). *Aspergillus fumigatus* was the most commonly detected fungus in source waters (48%) but none of the three fungi were detected in treated water. *L. pneumophila* was detected in 25% of the source water samples but in only 4% of treated water samples. *M. avium* and *M. intracellulare* were both detected in 25% of source water, while all three mycobacteria were detected in 36% of treated water samples. Five species of mycobacteria, *M. mucogenicum*, *M. phocaicum*, *M. triplex*, *M. fortuitum*, and *M. lentiflavum* were cultured from treated water samples. Although these DWTPs represent a fraction of those in the U.S., the results suggest that many of these pathogens are widespread in source waters but that treatment is generally effective in reducing them to below detection limits. The one exception is the mycobacteria, which were commonly detected in treated water, even when not detected in source waters.

Keywords: pathogens, drinking water, treatment, source water, occurrence

1.0 INTRODUCTION

The advent of disinfection and centralized treatment has had a major impact on reducing outbreaks of disease caused by contaminated drinking water, though illnesses continue to occur, even in developed countries (Craun et al., 2010). Drinking water remains a potential source of microbial pathogens in the United States (Yoder et al., 2008) and other developed countries (Ngwenya et al., 2013) despite more than 40 years of improvements in treatment processes. Illnesses due to contaminated drinking water result in nearly one billion dollars annually in hospitalization costs alone in the U.S. (Hilborn et al., 2013).

The majority of drinking water-associated outbreaks in the U.S. in 2009-2010 were due to deficiencies in federally regulated portions of drinking water treatment plants (DWTPs) (Hilborn et al., 2013). Given this fact, few studies have evaluated drinking water treatment efficacy for microbial pathogens at DWTPs operating under real-world conditions. The Safe Drinking Water Act sets maximum contaminant levels (MCLs) or enforceable standards for some pathogens, which are published in the Code of Federal Regulations (CFR) under Title 40 CFR §141 subpart B (U.S. EPA, 2012). However many waterborne pathogens are unregulated. Monitoring directly for pathogens in treated drinking water is considered expensive and impractical, though it is recognized that monitoring for indicator bacteria (e.g. total and fecal coliforms, *E. coli*) is not useful for predicting the presence of non-fecally-related environmental pathogens. Systems using surface water or groundwater under the influence of surface water that serve > 10,000 people are required to monitor for the human pathogen *Cryptosporidium* in source water used for drinking water (40 CFR §141.700; U.S. EPA, 2002a), as are systems serving < 10,000 people when *E. coli* levels exceed prescribed thresholds. To meet federal regulations, DWTPs use a

variety of treatments to control levels of microorganisms, including filtration, chlorine (Cl₂), chloramine (NH₂Cl), chlorine dioxide (ClO₂), ultraviolet light (UV), and ozone (O₃).

A major gap exists in our understanding of the ecology of waterborne pathogens, including their occurrence and potential inactivation or removal during drinking water treatment. To begin to address this issue, this study was undertaken with the goal of obtaining occurrence information on microbial pathogens of known and emerging concern in source and treated drinking water. A secondary goal was to estimate removal, if any, of microbial pathogens from source waters by currently used drinking water treatment processes under typical plant operating conditions, and thus identify possible candidate organisms that may be amenable to enhanced reduction or removal. The occurrence of nine pathogens were estimated: two regulated protozoa, *Cryptosporidium* and *Giardia*, two bacteria listed on the U.S. Environmental Protection Agency's (USEPA) Contaminants Candidate List (U.S.EPA, 2015), *Legionella pneumophila*, and *Mycobacterium avium*, and other pathogens of concern including *Aspergillus fumigatus*, *A. niger* and *A. terreus*, *M. avium* subspecies *paratuberculosis* and *M. intracellulare*. Their occurrence was measured in paired source and treated water from 25 DWTPs located in 24 states across the U.S., representing small (501 – 3,300 people) to very large (100,000 + people) systems using surface and/or groundwater and a variety of disinfection regimes. This paper is one of a series of related papers that comprise a national study that describes the presence, persistence, and concentration of emerging contaminants in drinking water. This paper focused specifically on microbial contaminants; the other papers in the series focus on the occurrence of chemical contaminants in source and treated drinking water. A comprehensive description of the national study can be found in this issue (Glassmeyer *et al.*, 2016).

2.0 MATERIALS AND METHODS

2.1 Drinking Water Treatment Plant Selection

DWTPs were nominated for inclusion in this study by field personnel from the U.S. Geological Survey (USGS). The final selection of DWTPs maximized geographical spread and represented a range of utility sizes and drinking water treatments. Table 1 describes basic information for each DWTP regarding population served, production volume, and simplified treatment train. A paired sampling scheme was designed to evaluate the treatment process with minimal potential for regrowth of microorganisms or contamination from biofilms in the distribution system.

2.2. Sample Collection

One source (prior to entering the DWTP) and one paired treated water sample were collected from each of 25 DWTPs in sterile 1 L sample bottles for all bacteria and fungi. Most of the DWTPs were plumbed with sampling taps at different locations in the plant. These taps allowed collection either directly, or from a tap in the facility's laboratory. The DWTPs were instructed to collect the source water sample prior to any treatment or settling basin. The treated water sample was collected at a sampling point after final disinfection but prior to the clear well. Approximately 10 L of source water were collected for protozoa analysis. One L of source and 1 L of treated water were collected for *A. fumigatus*, *A. niger*, and *A. terreus* analyses. Four L of source and 4 L of treated water in total were collected for *L. pneumophila*, *M. avium*, *M. avium* subspecies *paratuberculosis*, and *M. intracellulare* analyses. Samples were shipped to laboratories in coolers containing ice and kept at 4 °C until analysis. Shipping temperatures

were monitored using an i-button (www.maximintegrated.com); shipping temperatures were on average less than 15 °C.

2.3. Quantification of *Cryptosporidium* oocysts and *Giardia* cysts

Only source water was sampled from 24 of 25 DWTPs (excluding DWTP 5) for *Cryptosporidium* and *Giardia* analyses. Samples were processed using a modified USEPA Method 1623 (US EPA, 2005). Briefly, 9-11 L were filtered in the field using the EnvirochekHV capsule (Pall-Gelman, Port Washington, NY,) using a flow meter. The last 250 ml of sample was not drained from the capsule. One sample used 2 capsules and only sampled 3.5 L because of clogging (DWTP 10). The outlet and then the inlet were capped and sent overnight on ice to the USEPA. Capsules were eluted as described in Method 1623 (US EPA, 2005). Immunomagnetic separation (IMS) was as described except heat dissociation was used (Ware et al., 2003). One IMS was performed per sample. Samples were stained using EasyStain (BTF Pty Ltd., North Ryde, NSW, Australia) as described by the manufacturer except all wash steps were omitted. Slides were then analyzed for *Cryptosporidium* oocysts and *Giardia* cysts and were enumerated as described in Method 1623 (US EPA, 2005).

2.4. Detection of *Aspergillus* species using qPCR

Methods and qPCR assays have been reported previously for detection of *Aspergillus* (Haugland et al., 2004). An aliquot of the DNA extracts, prepared according to Haugland et al. (2004), were used in this analysis. Five microliters of DNA extract, which was equivalent to

analyzing 25 ml of the original source or treated water sample, was analyzed per qPCR reaction. All primer and probe sequences used have been previously published (US EPA, 2002b).

Reactions were performed with thermal cycling conditions consisting of 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C for template denaturation and 1 min at 60°C for probe and primer annealing and primer extension. Quantification was based on standard curves generated for each microorganism. The detection limit for each assay was approximately one cell per qPCR reaction.

2.5. Detection of *L. pneumophila* using qPCR

Two assays were used to determine the presence of *L. pneumophila* in water samples. The Lp16S qPCR assay detects all *L. pneumophila* and the LpLPS assay detects serogroup 1 (SG1) strains of *L. pneumophila* only. Both assays have been previously described (Donohue et al., 2014). In general, 250 ml to 2.5 L of each source water sample and 3 L of each treated water sample were filtered and DNA extracted using a modified WaterMaster™ (Epicentre Biotechnologies, Philadelphia, PA) DNA extraction protocol (Beumer et al., 2010).

All DNA extracts were analyzed using both qPCR assays (Lp16S and LpLPS). Reactions for the Lp16S assay and the LpLPS assay were performed in duplicate. Each reaction regardless of assay was performed with 5µl of DNA extract/template, which was equivalent to analyzing 8 to 80 ml of the original source water sample or 100 ml of the original treated water sample per qPCR reaction, using a Roche LightCycler® 480 II Real-time PCR system (Roche Applied Science, Indianapolis, IN). Formulations, instrument settings, and method for estimating quantities of *L. pneumophila* were performed as described in Donohue et al. (2014). A

laboratory control, or lab blank (LB), which consisted of one filtrate of 100 ml sterile molecular biology grade water per pair of source and treated water samples from each DWTP, was prepared at the same time and processed in an identical manner as samples. A sample was considered positive if the quantification cycle (Cq) of both replicate qPCR reactions was < 40. The detection limit for the Lp16S assay was approximately one target copy per qPCR reaction and 10 copies per qPCR reaction for the LpLPS assay.

2.6. Detection of *M. avium*, *M. intracellulare*, and *M. avium* subsp. *paratuberculosis* using qPCR

Characteristics of *M. avium*, *M. avium* subsp. *paratuberculosis*, and *M. intracellulare* qPCR assays have been described previously (Beumer et al., 2010; Chern et al., 2015). All three qPCR assays were performed on 10 µl of DNA extract/template (the same extracts used for the *Legionella* qPCR assays), which was equivalent to analyzing 16 to 160 ml of the original source water sample or 200 ml of the original treated water sample per qPCR reaction, in triplicate. All reactions were carried out in an ABI Model 7900HT Sequence Detection System (Applied Biosystems, Foster City); calibration standards were run with every set of samples processed. An internal positive control (Applied Biosystems Exogenous Internal Positive Control) was added to every reaction to detect PCR inhibition. Positive qPCR assay controls included target DNA standards of known concentration. Negative qPCR assay controls included sterile H₂O added to qPCR reaction rather than DNA template. A sample was considered positive if the quantification cycle (Cq) of two of three replicate qPCR reactions was < 40, and negative controls and LBs were negative (undetermined). The detection limits of the *Mycobacterium* qPCR assays were approximately 10 target copies per qPCR reaction for *M. avium* and *M.*

intracellulare qPCR assays and 1.8 target copies per qPCR reaction for *M. avium* subspecies *paratuberculosis* assay.

Estimated quantities of mycobacteria were extrapolated from master standard curves as described in Beumer et al. (2010). A One-Way ANOVA (Kruskal-Wallis) using Sigma Plot version 12.5 (Systat Software, Inc., San Jose, CA) was performed to determine if differences in estimated quantities in source and treated water were significantly different ($p < 0.05$).

2.7. Culturing Mycobacteria

Mycobacteria were cultured from source and treated water according to section 9260M of Standard Methods for the Examination of Water and Wastewater (Eaton et al., 2005), with modifications described in Covert et al. (1999). Two 500 ml aliquots of treated water were not disinfected with cetyl pyridinium chloride (CPC) but filtered immediately as described for source water aliquots. Plates were incubated in plastic bags at 37 °C in a 10 % CO₂ atmosphere for a minimum of 8 weeks and inspected weekly. Colonies that fit the morphologies described for mycobacteria in Glover et al. (1994) were selected for identification by bidirectional sequencing of the 16S rRNA gene. Putative identification of isolates was based on a minimum of 98% similarity to known 16S rRNA sequences in the NCBI 16S rRNA database (<http://www.ncbi.nlm.nih.gov>). Twenty-three sequences were submitted to the National Center for Biotechnology Information and given GenBank Accession numbers KU686928 – KU686950.

3. RESULTS

In the current study, paired source and treated water samples were collected from 25 DWTPs across the U.S. The estimated concentration of each pathogen in source and treated water is provided, along with the limits of quantification for each method/assay, in Figure 1. No data from the molecular detection techniques were discarded due to the adverse effects of inhibition from the water matrix on a method/assay.

3.1. Source Water

All of the pathogens except *M. avium* subsp. *paratuberculosis* were detected in source water using molecular or culture methods, or microscopy for *Cryptosporidium* and *Giardia* (Table 2). *A. fumigatus* was detected in 48% (12/25) of source water samples, at a mean concentration of $1.4 \times 10^1 \pm 7.0 \times 10^0$ cells/L. *A. niger* and *A. terreus* were detected in 20% (5/25) ($\bar{x} = 1.6 \times 10^1 \pm 8.0 \times 10^0$ cells/L) and 28% (7/25) ($\bar{x} = 1.0 \times 10^3 \pm 1.4 \times 10^3$ cells/L) of source water samples, respectively. *Cryptosporidium* were detected in 25% (6/24) and *Giardia* in 46% (11/24) of the source water samples and their mean concentrations were $7.4 \times 10^{-1} \pm 2.7 \times 10^{-1}$ oocysts/L and $8.4 \times 10^{-1} \pm 6.2 \times 10^{-1}$ cysts/L, respectively.

The lab blank (LB) associated with source and treated water from DWTP 3 was positive for *L. pneumophila* using the Lp16S qPCR assay and hence, data from this plant were excluded from the analysis (Table S1). *L. pneumophila* was present in 25% (6/24) of remaining source water samples (Figure 1) and had the highest concentration of any of the nine pathogens in water entering DWTPs ($\bar{x} = 2.2 \times 10^4 \pm 4.3 \times 10^4$ target copies/L). These samples were negative for *L. pneumophila* SG1 using the LpLPS qPCR assay. The LB associated with the source water from DWTP 4 was positive for all three species of mycobacteria and therefore data from that plant

were excluded from the analysis (Table S1). No samples were deemed false negatives due to inhibition (Cq values for the exogenous internal positive control were within the acceptable range). *M. avium* and *M. intracellulare* were detected by qPCR in 25% (6/24) of remaining source water samples ($\bar{x} = 2.2 \times 10^4 \pm 3.1 \times 10^4$ target copies/L and $2.0 \times 10^3 \pm 1.8 \times 10^3$ target copies/L, respectively). Both species were detected simultaneously at four DWTPs (2, 23, 26, and 28). *M. avium*, *M. intracellulare*, and *M. avium* subsp. *paratuberculosis* were not cultured from any source water samples. In most cases, culture plates from source water samples were too overgrown by faster growing bacteria or fungi to isolate slow-growing mycobacteria. The single source water sample to yield culturable mycobacteria was from DWTP 20, where a single isolate of *M. fortuitum* was obtained (Table 3). The total plate count for this sample was >500 CFU/ml and the single isolate was plucked from the edge of a lawn of organisms growing on the plate.

The frequency of each pathogen's detection in source water ranked from highest to lowest was: *A. fumigatus* > *Giardia* > *A. terreus* > *Cryptosporidium*/ *L. pneumophila*/ *M. avium*/ *M. intracellulare* > *A. niger*. The mean concentration of each pathogen detected in source water ranked from highest to lowest: *L. pneumophila* > *M. avium* > *M. intracellulare* > *A. terreus* > *A. niger* > *A. fumigatus* > *Giardia* > *Cryptosporidium*. Therefore, while fungal and protozoan pathogens were detected more frequently in source water samples than bacterial pathogens, the bacterial pathogens were present in higher concentrations.

3.2. Treated Water

The presence of seven pathogens (detected by qPCR) and culturable mycobacteria was determined in treated water obtained from the 25 DWTPs. Treatment, independent of method, reduced the populations of all three species of *Aspergillus* to below detection limits. *L. pneumophila* was detected in three treated water samples (DWTPs 4, 10, and 25) with the Lp16S assay, but only one sample (DWTP 10, $\bar{x} = 3.0 \times 10^3 \pm 0$ target copies/L) met acceptance criteria and was considered a true positive. This sample was negative for *L. pneumophila* SG1 (LpLPS assay).

The mycobacteria provide a more complex story. *M. avium* and *M. intracellulare* were detected in 24% (6/25) of treated waters by qPCR ($\bar{x} = 2.1 \times 10^3 \pm 3.3 \times 10^3$ target copies/L and $8.0 \times 10^2 \pm 1.4 \times 10^3$ target copies/L, respectively, Table 2). Both species were detected in treated water from six DWTPs (1, 10, 20, 26, 27, and 29). Additionally, *M. avium* subsp. *paratuberculosis* was detected by both IS900 and Target 251 qPCR assays in treated water from 13% (3/24) of DWTPs with a mean concentration of $4.8 \times 10^2 \pm 4.7 \times 10^2$ target copies/L of the samples that were positive (Figure 1, Target 251 data not shown). Both *M. intracellulare* and *M. avium* subsp. *paratuberculosis* were detected at DWTP 4. *M. avium* subspecies *paratuberculosis* was not detected in any source water sample. Interestingly, there were four DWTPs where *M. avium* (DWTP 1, 20, 27, 29) or *M. intracellulare* (DWTP 4, 10, 20, 29) were detected in treated water but not source water.

As was true for source water, *M. avium*, *M. intracellulare*, and *M. avium* subsp. *paratuberculosis* were not cultured from treated water samples. However, five other species of pathogenic mycobacteria were isolated by culture and subsequently identified through 16S rRNA sequence analysis from treated water obtained from three DWTPs (Table 3). Unlike the source water samples where overgrowth commonly occurred, the treated water samples produced very

few colonies, with total plate counts ranging from 6 to 40 CFU/500 ml (Table 2). Eight isolates of *M. mucogenicum* and one isolate of the closely-related *M. phocaicum* were obtained from DWTP 25, which utilized chloramine and ozonation disinfection. One isolate of *M. fortuitum* was obtained from treated water from DWTP 12, which used chlorination as a disinfectant. The treated water sample from DWTP 11 only produced six colonies, and all were sub-cultured for identification. Five isolates were putatively identified as *M. lentiflavum* and one isolate as *M. triplex*, both slow-growing mycobacteria. DWTP 11 used chlorine and ozonation disinfection. Finally, *M. septicum* was isolated from sand filter effluent obtained from DWTP 2, the only instance sand filter effluent was sampled.

There were 13 DWTPs where no pathogens (targeted in this study) were detected by molecular methods or by culture (mycobacteria) in treated water. One targeted pathogen was detected in three DWTPs while eight DWTPs had two pathogens detected. Only one DWTP had three pathogens detected in their treated water, and no DWTPs had four or more pathogens detected in treated water (Table 1, Supplementary Material).

3.3. Effect of Drinking Water Treatment on Pathogens

While *Aspergillus* were the most frequently detected pathogens in source water samples, they were never detected in treated water samples. Additionally, *L. pneumophila* (not SG1) was detected in source water samples from six DWTPs but was only detected in treated water from one DWTP. There were six DWTPs where *M. avium* was detected in source water. After treatment, the organism was reduced at two DWTPs and not detected at four DWTPs. In addition, there were six DWTPs where *M. intracellulare* was detected in source water. The

organism was reduced by treatment at three of the DWTPs and not detected at the other three. At DWTPs where *M. avium* and *M. intracellulare* concentrations were reduced by treatment, the reduction was not statistically significant (*M. avium*, $P = 0.333$; *M. intracellulare*, $P = 0.056$). There were four DWTPs (*M. avium* and *M. intracellulare*) or three DWTPs (*M. avium* subsp. *paratuberculosis*) where mycobacteria were detected in treated water but not in source water by qPCR. Four DWTPs had no pathogens detected in either source or treated water (Table 1, Supplementary Material). Nine DWTPs demonstrated a complete reduction and eight DWTPs demonstrated a partial reduction in number of pathogens detected in treated water as compared to source water.

4. DISCUSSION

4.1. Treatment for Control of Waterborne Pathogens

The Interim Enhanced Surface Water Treatment Rule (U.S. EPA, 2002a), part of the Safe Drinking Water Act, requires DWTPs in the U.S. to treat for microbial contaminants using various treatment technologies and to maintain a disinfection residual in the finished water to prevent microbial growth during distribution (e.g. 0.2 mg/L free chlorine; 40 CFR §141, subpart H). DWTPs commonly use chlorine, chloramine, chlorine dioxide, ozone, and ultraviolet light to meet drinking water standards for microbial contaminants. While several bench-scale studies have demonstrated the effectiveness of these disinfectants at controlling microbial contaminants (Sobsey et al., 1989), data are lacking on their effectiveness at DWTPs operating under real-world conditions. To address this issue, we conducted a small survey of source and treated water from 25 small to very large drinking water treatment plants (DWTPs) located in 24 states, using

a variety of source water types and treatment technologies. Our results suggest that some pathogens are removed by treatment at these DWTPs but others are not.

4.2. Occurrence of *Cryptosporidium* and *Giardia* in Source Water

Several outbreaks of illness have been caused by *Cryptosporidium* in drinking water with the largest outbreak occurring in Milwaukee, Wisconsin in 1993 (MacKenzie et al., 1994). Treatment efficacy was not determined for protozoa in this study since only source water samples were analyzed as required under the USEPA's Long Term 2 Enhanced Surface Water Treatment Rule (LT2). Both *Giardia* and *Cryptosporidium* have unenforceable maximum contaminant level goals of zero, however, both are regulated by treatment technologies by either the Surface Water Treatment or the LT2 Rules. The low levels of *Cryptosporidium* and *Giardia* detected in source water samples in this study would presumably be removed by filtration and disinfection which has been demonstrated in several studies (Kelley et al., 1994; Jacangelo et al., 1995; Nieminski et al., 1995; Finch et al., 1997; Liyanage et al., 1997). Two large studies analyzing finished drinking water both using IMS based methodology report finding few *Cryptosporidium* oocysts. A *Cryptosporidium* monitoring program from the UK in 2008 reports finding no water samples exceeded 1 oocyst/ 10L standard in over 50,000 samples examined (DWI, 2008). Rochelle et al. (2012) report no infectious oocysts in 370 samples analyzing 349,0593 L.

4.3. Effect of Treatment on *Aspergillus*

Filamentous fungi, like species of *Aspergillus*, are common heterotrophs in nature and it is typical to find them in water. In this study, *Aspergillus* species were detected by qPCR in 56%

(14/25) of source water samples but none were detected in treated water. This is the first report describing efficacy of treatment for *Aspergillus* at DWTPs in the U.S. Though the number of DWTPs included in this study was small ($n = 25$), *Aspergillus* species were detected in source water at 14 of the DWTPs. Those 14 DWTPs represent a range of production volumes (2.07 to 133 million gallons per day) using a variety of source water types and treatment regimes, and were geographically distributed (Table 1). It is a novel finding that various treatments are effective at removing *Aspergillus* from a diversity of waters. To our knowledge, this is the first report on the occurrence of *Aspergillus* at DWTPs in the U.S., though there are reports on the effect of treatment on *Aspergillus* in other parts of the world. Warris et al. (2003) recovered 34 isolates of *A. fumigatus* by culture from untreated lake water in Oslo, Norway, and 28 isolates post treatment. The DWTP used simple treatment including aeration, filtration, and chlorination, which was insufficient at removing this fungal species. Doggett (2000) detected multiple species of filamentous fungi, including *Aspergillus*, in distribution system biofilm obtained downstream from DWTPs in the U.S. They have also been detected in home taps (Vesper et al., 2007) and may gain access through leaks.

4.4. Effect of Treatment on *Legionella pneumophila*

During 2010 – 2012, the period during which samples were collected, 58% of reported drinking-water associated outbreaks and 7% of illnesses in the U.S. were due to *Legionella* (Hilborn et al., 2013). *Legionella* caused more reported drinking water outbreaks during that year than non-*Legionella* bacteria, protozoa, and viruses combined. QPCR was used to detect the presence of *L. pneumophila* in this study. This technique can detect DNA from live cells, viable but non-culturable cells, cells within amoebae, and dead cells. While detection of dead

cells is a potential draw-back when estimating risk, qPCR detection of *L. pneumophila* provides information on the maximum levels in a sample, and is especially valuable when low levels are present, which are undetectable by culture (Donohue et al., 2014). *L. pneumophila* were detected in 25% (6/24) of source water samples and one treated water sample. The accuracy of the detection in treated water was confirmed on a separate portion of the same sample extracted for *Mycobacterium* qPCR analyses (data not shown). Though *L. pneumophila* SG1 cause most cases of Legionnaire's disease in the U.S., other serogroups of *L. pneumophila* and other species of *Legionella* are clinically significant in immunocompromised populations. In an international survey of isolates from patients with community-acquired legionellosis, only 12.8% of *L. pneumophila* isolates belonged to serogroups other than SG1 (Yu et al., 2002). Other species causing infection in the U.S. include *L. micdadei*, *L. bozemanii*, and *L. dumoffii* (Cunha et al., 2016).

DWTPs using chlorine disinfection (n = 15) were negative for *L. pneumophila* in treated water. The DWTP which tested positive for *L. pneumophila* used monochloramine disinfection at the time of sampling. Several studies have suggested that monochloramine is an effective disinfectant for controlling free-living *Legionella* (Kool et al., 1999; Flannery et al., 2006; Moore et al., 2006; Heffelfinger et al., 2003). The effect of chloramine treatment on *L. pneumophila* is unclear from this study as the number of DWTPs using chloramine disinfection participating was small (n = 10). The USEPA estimates that currently one in five individuals in the U.S. is supplied with chloramine-treated drinking water (U.S. EPA, 2009). Given the amount of illness associated with *Legionella* through drinking water, additional studies, much larger in scope, are needed to determine treatments most effective at inactivating *Legionella* under normal DWTP operating conditions.

4.5. Effect of Treatment on Mycobacteria

The illnesses associated with *M. avium* and *M. intracellulare*, and *M. avium* subsp. *paratuberculosis* are well-known (WHO, 2004). *M. avium*, *M. intracellulare*, and *M. avium* subsp. *paratuberculosis* were detected in treated water from 6, 7, or 3 DWTPs, respectively, by qPCR, in this study. There were four instances that *M. avium* and *M. intracellulare* and three instances that *M. avium* subsp. *paratuberculosis* were detected in treated water but not source water by qPCR. This could result for several reasons. A smaller volume of source water was analyzed than treated water from most DWTPs by qPCR due to clogging of membrane filters. The hydrophobic outer membrane of mycobacteria causes clumping and a heterogeneous distribution of cells in aqueous environments (Anton et al., 1996), and only a single grab sample of source and treated water was collected from each DWTP. Detection of mycobacteria in treated but not source water could be due to their growth during treatment at the DWTP. Additional studies evaluating samples at various points in the treatment train at DWTPs would be necessary to test this hypothesis.

Mycobacteria were never recovered by culture from source water samples at DWTPs where they were recovered from treated samples. In fact, only one species of mycobacteria (*M. fortuitum*) was recovered by culture from a source water sample, though treated water from this plant was positive for both *M. avium* and *M. intracellulare* by qPCR. *M. fortuitum* is a rapidly growing *Mycobacterium* species, requiring only three to four days to grow in culture. Due to extreme overgrowth, the culture plate from which it was isolated was removed from the incubator at two weeks of incubation. Isolation of *M. avium* and *M. intracellulare* requires a

minimum of eight weeks incubation, which may explain why these species were not recovered from the source water sample by culture. Overgrowth of the medium by non-target microorganisms caused considerable loss of most source water samples prior to the minimum eight week incubation period required for the slow growing *M. avium* and *M. intracellulare*.

Five potentially pathogenic species of mycobacteria were cultured from treated water obtained from three DWTPs. *M. mucogenicum* and *M. phocaicum* are emerging pathogenic mycobacteria that are highly resistant to drinking water disinfection and are the etiologic agents of catheter-associated outbreaks in hospitals, as well as central nervous system, pulmonary, and skin/soft tissue infections in immunocompetent and compromised individuals (Adekambi, 2009). *M. fortuitum* is the most frequently isolated and clinically relevant species of the rapidly growing mycobacteria, and is recognized for causing wound, skin/soft tissue infections, and pulmonary disease (Ho et al., 2012). *M. lentiflavum* and *M. triplex* infections are rare; *M. lentiflavum* is associated with cervical lymphadenitis in children and both species have caused pulmonary disease (Piersimoni et al., 2004; Marshall et al., 2011). It is important to note that culturable mycobacteria were obtained from sample volumes equivalent to those analyzed when water is collected at the end of distribution (500 ml). This study confirms that potentially pathogenic mycobacteria can be detected at the point of highest disinfection residual in DWTPs, independent of disinfectant type. DWTPs where mycobacteria were detected in treated water, either by qPCR or culture, were located in 11 geographically distributed states, used groundwater or surface water sources, and common drinking water disinfectants (chlorine, chloramine, chlorine dioxide, ozone, and ultraviolet light). Our findings are supported by laboratory studies demonstrating resistance of mycobacteria to frequently used drinking water disinfectants (Taylor et al., 2000).

This paper is one in a collection of papers describing emerging chemical and microbial contaminants in source and treated drinking waters of the U.S. This portion of the study met the primary goal of providing information on the occurrence of known and emerging microbial contaminants for assessing the potential for human exposure to these contaminants from drinking water. This study also met the second goal of understanding the effect of treatment on emerging microbial contaminants under typical plant operating conditions. The study findings suggest that drinking water treatment is effective at inactivating potentially pathogenic species of *Aspergillus*. It remains unknown if chloramination is effective at inactivating *L. pneumophila*. In addition, mycobacteria belonging the *Mycobacterium avium* complex (e.g. *M. avium*, *M. avium* subsp. *paratuberculosis*, *M. intracellulare*) and other possible pathogenic species of mycobacteria were detected in water treated with various disinfectants. For pathogens that naturally inhabit water, like mycobacteria and *Legionella*, surviving drinking water treatment has serious implications for human health if they are able to amplify downstream of the DWTP to points of use where persons at risk may be exposed, either through ingestion, inhalation, or dermal contact. These findings suggest the need for additional research to identify the scope and magnitude of DWTP treatment efficacy with respect to *Legionella* and mycobacteria.

ASSOCIATED CONTENT

Supplementary Material

Supplemental Table 1. This material is available free of charge via the Internet at

<http://pubs.acs.org>.

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Notes

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Figure and Table Legends

Figure 1. Estimated concentrations (per liter) of each target microorganism in source and treated water for each drinking water treatment plant listed in Table 1. The bold horizontal line on each graph indicates the limits of quantification for each assay, A = 1 cell, B = 10 copies, C = 1.8 copies. Note that the y-axes differ.

Table 1. Population served, production volume, and type of treatment at each drinking water treatment plant.

Table 2. Percent detection of *Cryptosporidium* spp., *Giardia*, *Legionella pneumophila*, *Aspergillus fumigatus*, *A. niger*, *A. terreus*, *Mycobacterium avium*, *M. intracellulare*, and *M. avium* subsp. *paratuberculosis* in source and treated water samples.

Table 3. Drinking water treatment plants positive by culture for non-tuberculous mycobacteria, including water sample type (source or treated water), description of treatment train, % similarity to 16S rRNA of known mycobacteria, and total plate counts.

Table 1. Population served, production volume, and type of treatment at each drinking water treatment plant.

Location	Pop Served ^a (1000s)	Production at Sampling ^a (MGD)	Residence Time of Treatment ^b (h)	Primary Disinfectant ^c	Simplified treatment train ^d
DWTP 1	>500	> 100	10	O ₃ + NH ₂ Cl	O ₃ , coag/floc, NH ₂ Cl, C, floc, C, F
DWTP 2	>500	> 100	72	Cl ₂	coag/floc, S, SF, GAC, Cl ₂
DWTP 3	50-500	10- 100	6	Cl ₂ +UV	coag/floc, C/ S, F, GAC, Cl ₂ , UV
DWTP 4	>500	10- 100	46	Cl ₂ + NH ₂ Cl	pre- Cl ₂ , coag/floc, S, secondary Cl ₂ , SF, NH ₃
DWTP 5	< 50	< 10	0.13	Cl ₂	Cl ₂
DWTP 10	50-500	> 100	7	NH ₂ Cl	coag/floc ,S, NH ₂ Cl, F
DWTP 11	<50	< 10	7	O ₃ + Cl ₂	coag/floc, S, C, O ₃ , GAC and SF, Cl ₂
DWTP 12	<50	< 10	30.72	Cl ₂	coag/floc, pre- Cl ₂ , C, GAC and SF, post- Cl ₂
DWTP 13	>500	> 100	1	Cl ₂	Cl ₂
DWTP 14	50-500	10- 100	10	ClO ₂ + Cl ₂	coag/floc, pre-ClO ₂ , GAC and SF, Cl ₂
DWTP 15	<50	< 10	1	Cl ₂	coag/floc, S, F, Cl ₂
DWTP 16	50-500	10- 100	6	NH ₂ Cl	coag/floc, S, GAC and SF, NH ₂ Cl
DWTP	<50	< 10	2	Cl ₂	C, coag/floc,

17					pre- Cl ₂ , F, Cl ₂
DWTP 18	<50	< 10	7.3	O ₃ + NH ₂ Cl	O ₃ , floc, S, Cl ₂ , GAC and SF, NH ₂ Cl
DWTP 19	50- 500	10- 100	26	NH ₂ Cl	coag/floc, PAC, S, ultrafiltration, NH ₂ Cl.
DWTP 20	>500	10- 100	30	O ₃ + Cl ₂	floc, S, O ₃ , GAC and SF, Cl ₂
DWTP 21	50- 500	10- 100	90	Cl ₂	PAC pre- Cl ₂ , coag, S, Cl ₂ , F
DWTP 22	50- 500	10- 100	10	O ₃ + Cl ₂ + UV	pre-O ₃ , coag, S, O ₃ , GAC and SF, UV, Cl ₂
DWTP 23	50- 500	10- 100	7	ClO ₂ + UV+ Cl	pre-ClO ₂ , coag/floc, S, dual media F, UV, Cl ₂
DWTP 24	50- 500	10- 100	8	NH ₂ Cl	PAC, GAC and SF, NH ₂ Cl
DWTP 25	50- 500	10- 100	13.6	O ₃ + NH ₂ Cl	Pre-O ₃ , coag, NH ₂ Cl
DWTP 26	50- 500	10- 100	24-36	Cl ₂	pre- Cl ₂ , PAC, coag, S, Cl ₂ , F, Cl ₂
DWTP 27	50- 500	< 10	4	NH ₂ Cl + UV	PAC, coag/floc, S, F, UV, NH ₂ Cl
DWTP 28	>500	> 100	1	O ₃ + NH ₂ Cl	NH ₂ Cl, O ₃ , F
DWTP 29	<50	< 10	8	Cl ₂	PAC, pre- Cl ₂ , coag/floc, S, Cl ₂ , F

^aPopulation sizes binned to give indication of drinking water treatment plant (DWTP) size variation while maintaining plant anonymity. MGD= million gallons per day.

^bDWTPs were asked to match the residence time of treatment. Some locations achieved this better than others. DWTP = drinking water treatment plant;

^cO₃= ozone; NH₂Cl= chloramine; Cl₂= chlorine; UV= ultraviolet radiation; ClO₂= chlorine dioxide.

^dMajor steps in treatment in each plant. Coag= coagulation; flocc= flocculation; C= clarification; F= filtration; S= sedimentation; SF= sand filter; PAC= powdered activated carbon; GAC= granular activated carbon.

Table 2. Percent detection of *Cryptosporidium* spp., *Giardia*, *Legionella pneumophila*, *Aspergillus fumigatus*, *A. niger*, *A. terreus*, *Mycobacterium avium*, *M. intracellulare*, and *M. avium* subsp. *paratuberculosis* in source and treated water samples.

Target	Source Water			Treated Water		
	% Detect	Mean of Positives	Median of Positives	% Detect	Mean of Positives	Median of Positives
<i>Cryptosporidium</i> spp. (oocysts/L)	25	7.4×10^{-1}	7.6×10^{-1}	Not sampled		
<i>Giardia</i> (cysts/L)	46	8.4×10^{-1}	7.3×10^{-1}	Not sampled		
<i>L. pneumophila</i> (target copy/L)	25	2.2×10^4	4.1×10^3	4	3.0×10^3	3.0×10^3
<i>A. fumigatus</i> (cells/L)	48	1.4×10^1	1.00×10^1	0	< limit of detection	< limit of detection
<i>A. terreus</i> (cells/L)	28	8.9×10^2	2.50×10^2	0	< limit of detection	< limit of detection
<i>A. niger</i> (cells/L)	20	1.6×10^1	1.00×10^1	0	< limit of detection	< limit of detection
<i>M. avium</i> (target copy/L)	24	2.2×10^4	6.99×10^3	25	2.1×10^3	6.9×10^2
<i>M. intracellulare</i> (target copy/L)	25	2.0×10^3	1.69×10^3	25	8.0×10^2	2.1×10^2
<i>M. avium</i> subsp. <i>paratuberculosis</i> (target copy/L)	0	< limit of detection	< limit of detection	8	4.8×10^2	8.6×10^0

Table 3. Drinking water treatment plants positive by culture for non-tuberculous mycobacteria, including water sample type (source or treated water), description of treatment train, % similarity to 16S rRNA of known mycobacteria, and total plate counts.

DWTP ^a	Source Water Type	Water Sample Type	Simplified Treatment Train ^c	Putative Identification (No. of Isolates Obtained)	% Similarity to 16S rRNA (GenBank ^d Accession Number)	Total CFU/Vol Filtered ^e
DWTP 2	Surface water	Sand filter effluent ^b	Coagulation/flocculation, sedimentation, sand filtration, GAC, chlorination	<i>M. septicum</i> (7)	99% (NR042916.1)	TNTC ^f /500 ml
DWTP 11	Surface water	Treated water	Coagulation/flocculation, sedimentation, clarification, ozone, GAC, sand filtration, chlorination	<i>M.</i> <i>lentiflavum</i> (5) <i>M. triplex</i> (1)	98-99% (NR041898.1) 99% (GQ153279.1)	6 CFU/500 ml
DWTP	Groundwater	Treated	Coagulation/flocculation,	<i>M. fortuitum</i>	98%	12 CFU/500 ml

12	Under the Influence of Surface Water	water	pre-chlorination, GAC, sand filtration, post- chlorination	(1)	(AF480580.1)	
DWTP 25	Surface water	Treated water	Pre-ozonation, coagulation, chloramination	<i>M.</i> <i>mucogenicum</i> (8) <i>M. phocaicum</i> (1)	98-99% (AY457075.1) 99% (HQ845985.1)	40 CFU/500ml
DWTP 20	Surface water	Source water	Coagulation/flocculation, PAC, sedimentation, ultrafiltration, chloramination	<i>M. fortuitum</i> (1)	98% (GU142933.1)	TNTC/30 ml

^aDetailed descriptions of drinking water treatment plants (DWTPs) can be found in Glassmeyer et al., this issue.

^b The only sand filter effluent sample in this study, obtained mid-way through treatment train and not disinfected.

^c For a complete description of treatment trains, see Glassmeyer et al., this issue.

^dGenBank = NIH genetic sequence database (*Nucleic Acids Research*, 2013 Jan;41(D1):D36-42).

^eCFU = colony forming units/volume water filtered. The volume of source water filtered varied due to clogging, and

was typically less than treated water.

^fTNTC = too numerous to count (>500 CFU).

Figure

Nine pathogens measured in source waters and treated drinking waters from 25 treatment plants across the US.

Source Water

Treated Drinking Water