AEM
Journals.ASM.org

Nontuberculous Mycobacteria, Fungi, and Opportunistic Pathogens in Unchlorinated Drinking Water in the Netherlands

Paul W. J. J. van der Wielen,[a] Dick van der Kooij[a]

KWR Watercycle Research Institute, Nieuwegein, the Netherlands[a]

The multiplication of opportunistic pathogens in drinking water supplies might pose a threat to public health. In this study, distributed unchlorinated drinking water from eight treatment plants in the Netherlands was sampled and analyzed for fungi, nontuberculous mycobacteria (NTM), and several opportunistic pathogens by using selective quantitative PCR methods. Fungi and NTM were detected in all drinking water samples, whereas *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, and *Aspergillus fumigatus* were sporadically observed. *Mycobacterium avium* complex and *Acanthamoeba* spp. were not detected. Season had no influence on the occurrence of these organisms, except for NTM and *S. maltophilia*, which were present in higher numbers in the summer. Opportunistic pathogens were more often observed in premise plumbing water samples than in samples from the distribution system. The lowest number of these organisms was observed in the finished water at the plant. Thus, fungi, NTM, and some of the studied opportunistic pathogens can multiply in the distribution and premise plumbing systems. Assimilable organic carbon (AOC) and/or total organic carbon (TOC) had no clear effects on fungal and NTM numbers or on *P. aeruginosa*- and *S. maltophilia*-positive samples. However, *L. pneumophila* was detected more often in water with AOC concentrations above 10 μg C liter^{-1} than in water with AOC levels below 5 μg C liter^{-1}. Finally, samples that contained *L. pneumophila*, *P. aeruginosa*, or *S. maltophilia* were more frequently positive for a second opportunistic pathogen, which shows that certain drinking water types and/or sampling locations promote the growth of multiple opportunistic pathogens.

Most microorganisms are nonpathogenic to humans, but certain microbial species are highly virulent, causing disease in immunocompetent humans. Other microorganisms are moderately to weakly virulent and can cause disease in immunocompromised humans. These organisms are known as opportunistic pathogens, and some of them can grow in drinking water or drinking water-related biofilms (1). *Legionella pneumophila* is the best known example of an opportunistic pathogen that can multiply in drinking water-related biofilms and cause disease in (immunocompromised) humans (2–5). In addition, certain species of nontuberculous mycobacteria (NTM) (e.g., *Mycobacterium avium*, *Mycobacterium kansasii*), *Acanthamoeba*, and *Burkholderia pseudomallei* isolated from drinking water can also be genotypically identical to strains isolated from patients (6–10). Others have shown that certain strains of *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, and *Burkholderia cepacia* complex isolated from sinks, taps, shower heads, bathtubs, and bathing toys have been identical to strains isolated from patients (11–16). These observations imply that drinking water may be a vehicle for the spread of several opportunistic pathogens.

Two trends might impact the occurrence of these opportunistic pathogens in drinking water. First, global warming, due to climate change, results in higher surface and drinking water temperatures (17). It is expected that growth of most opportunistic pathogens in drinking water will be enhanced when water temperatures rise above 20 to 25°C (18). Second, the number of immunocompromised humans, who are vulnerable for infection with opportunistic pathogens, will increase further in the First World because of the aging of the population and the longer life spans of patients who suffer from serious diseases (e.g., HIV, cancer, cystic fibrosis) (19). Both trends rationalize the need for further research on the occurrence of opportunistic pathogens in drinking water.

In the Netherlands, drinking water is distributed without a disinfectant residual but with very low concentrations of biodegradable organic carbon. Whether these low concentrations of biodegradable organic carbon prevent regrowth of opportunistic pathogens in the distribution and premise plumbing system is not known. Based on a literature study that investigated the potential impact on public health of several opportunistic pathogens capable of multiplying in drinking water-related biofilms, priority for further research in the Netherlands was given to *L. pneumophila*, NTM, pathogenic fungi, *P. aeruginosa*, and *S. maltophilia* (18). Molecular methods have been developed to detect these opportunistic pathogens in drinking water, and in a preliminary study that investigated the distributed unchlorinated drinking water from two treatment plants in the Netherlands, some opportunistic pathogens were found (18). Consequently, a more comprehensive study on the occurrence of these organisms in the unchlorinated drinking water in the Netherlands was conducted. The objectives of this study were to determine (i) the effects of the source (surface water versus groundwater), the season (winter versus summer), and the premise plumbing system on the occurrence of opportunistic pathogens (*L. pneumophila*, NTM, *M. avium* complex, fungi, *Aspergillus fumigatus*, *P. aeruginosa*, *S. maltophilia*, and *Acanthamoeba* spp.) in drinking water and (ii) the relationship

Received 6 September 2012 Accepted 13 November 2012
Published ahead of print 16 November 2012
Address correspondence to Paul W. J. J. van der Wielen, paul.van.der.wielen@kwrwater.nl.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.02748-12.

doi:10.1128/AEM.02748-12

TABLE 1 Water source used for drinking water production, TOC and AOC concentrations in treated water, and heterotrophic plate counts on plate count agar at 22°C in distributed drinking water

Plant	Source	Yearly mean (± SD)		
		TOC (mg liter^{-1})	AOC (μg C liter^{-1})	HPC (CFU ml^{-1}) (yearly geometric mean ×/ geometric SD)
SW1	Surface water	2.5 ± 0.7	18.4 ± 6.9	13.7 ×/0.7
SW2	Surface water	1.6 ± 0.1	14.7 ± 4.0	8.3 ×/1.0
SW3	Surface water	1.7 ± 0.2	28.2 ± 8.0	8.5 ×/1.0
SW4	Surface water	2.3 ± 0.3	4.5 ± 0.9	3.3 ×/0.8
SW5	Surface water	3.1 ± 0.5	20.4 ± 7.7	7.5 ×/0.8
GW1	Groundwater	0.5 ± 0.1	3.1 ± 1.9	1.1 ×/1.0
GW2	Groundwater	2.1 ± 0.1	ND[a]	3.1 ×/0.6
GW3	Groundwater	7.9 ± 0.6	14.8 ± 5.9	13.3 ×/0.5

[a] ND, not determined.

between opportunistic pathogens in drinking water and other parameters (temperature, water composition, ATP, total cell numbers, and numbers of *Aeromonas* CFU).

MATERIALS AND METHODS

Sampling. The distributed unchlorinated drinking water from five treatment plants that used surface water (plants SW1, SW2, SW3, SW4, and SW5) and three treatment plants that used groundwater (plants GW1, GW2, and GW3) were analyzed. The drinking water produced at these plants differed in total organic carbon (TOC) and easily assimilable organic carbon (AOC) concentrations, and the heterotrophic plate counts (HPC) in the distributed drinking water differed as well (Table 1). These eight distribution systems were sampled in the winter (January through March) and the summer (August through September) of 2010. Drinking water samples (2 liters each) were taken from the kitchen cold-water tap of different houses (plant SW1, 13 houses; SW2, 13 houses; SW3, 11 houses; SW4, 11 houses; SW5, 13 houses; GW1, 12 houses; GW2, 10 houses; GW3, 10 houses) connected to the distribution system of each treatment plant, and the water temperature was measured immediately after each sample was taken. One sample was also taken of the treated water at each treatment plant in the summer, except for plant SW3. Samples at the tap were taken according to the Dutch drinking water decree so that they would represent drinking water from the distribution system. In short, each tap was flushed until the water temperature remained stable for 30 s, and the drinking water was subsequently sampled. Exceptions to this procedure were made for samples collected from the distribution systems of plants SW2 and SW3 in the summer, which were taken directly from the tap without prior flushing. These samples represented drinking water from the premise plumbing systems. The water samples were transported and stored at 4°C and processed within 24 h after collection.

DNA isolation. A volume of 1,000 ml was filtered through a 25-mm polycarbonate filter (0.22-μm pore size, type GTTP; Millipore, the Netherlands). The filter and a DNA fragment of an internal control were added to phosphate-MT buffer in a lysing matrix E tube of the FastDNA spin kit for soil (Qbiogene, Inc., Carlsbad, CA) and stored at −20°C. The internal control was used to determine the recovery efficiency of DNA isolation and PCR analysis (20). DNA was isolated according to the supplier's protocol. The filter, DNA fragment, and buffer were processed for 30 s at speed 5.5 in a FastPrep instrument. Subsequently, the lysing matrix E tubes were centrifuged for 30 s at 14,000 × *g*. The supernatant was transferred to a clean tube, and 250 μl protein precipitation solution (PPS) reagent was added and subsequently mixed by hand for 10 min. The tubes were centrifuged for 5 min at 14,000 × *g*. Subsequently, the supernatant was transferred to a clean 15-ml tube, and 1 ml binding matrix suspension was added. Tubes were subsequently inverted by hand for 2 min and placed in a rack for 3 min. Five hundred microliters of supernatant was carefully removed and discarded. Approximately 600 μl of the mixture was added to a spin filter and centrifuged for 1 min at 14,000 × *g*. Subsequently, the catch tube was emptied, and the remaining supernatant was added to the spin filter and centrifuged again for 1 min at 14,000 × *g*. Next, 500 μl salt-ethanol wash solution (SEWS-M) was added to the spin filter and subsequently centrifuged for 1 min at 14,000 × *g*. Subsequently, the spin filter was replaced in the catch tube and centrifuged for 2 min at 14,000 × *g*. Afterward, the spin filters were placed in fresh kit-supplied catch tubes, and the filters were air dried for 5 min. After drying, 200 μl DNA elution solution (DES) was added, and the matrix on membrane was gently stirred with a pipette tip. Finally, the spin filters and the catch tubes were centrifuged for 1 min at 14,000 × *g*. The eluted DNA was subsequently kept at −80°C.

Quantitative PCR analyses. The numbers of gene copies of the opportunistic pathogens in the drinking water samples were determined with previously developed quantitative PCR (qPCR) analyses for drinking water samples (18). These qPCR methods target the macrophage infectivity potentiator (*mip*) gene of *L. pneumophila*, the 16S rRNA gene of *Mycobacterium* spp., the 16S rRNA gene of *M. avium* complex, the 18S rRNA gene of fungi, the 28S rRNA gene of *Aspergillus fumigatus*, the *regA* gene of *P. aeruginosa*, the *chiA* gene of *S. maltophilia*, and the 18S rRNA gene of *Acanthamoeba* spp. (18). Reaction mixtures for PCR analyses contained 25 μl of 2× IQTM SYBR green supermix (Bio-Rad Laboratories BV, Veenendaal, the Netherlands), 0.2 μM each primer and, if applicable, probe, 0.4 mg ml^{-1} bovine serum albumin, and 10 μl DNA template in a total volume of 50 μl. Amplification, detection, and data analysis were performed in an iCycler iQ real-time detection system (Bio-Rad Laboratories BV). Primer-probe sequences and the amplification programs are shown in Tables S1 and S2 in the supplemental material. The PCR cycle after which the fluorescence signal of the amplified DNA is detected (threshold cycle [C_T]) was used to quantify the gene copy concentrations. Quantifications were based on comparison of the sample C_T value with the C_T values of a calibration curve based on known copy numbers of the respective gene from the different microorganisms.

Other microbiological parameters. The ATP concentration in each drinking water sample was determined by measuring the amount of light produced in the luciferin-luciferase assay as described by van der Wielen and van der Kooij (21). The numbers of total and membrane-intact cells were determined using a flow cytometer. In short, 1 ml of a water sample was incubated with 10 μl SYBR green (100× dilution of a 10,000× concentrate) (Invitrogen, Carlsbad, CA) and 10 μl propidium iodide (50 μg/ml) (BD) and stained for 15 min at room temperature. Subsequently, membrane-intact and membrane-disrupted cells were counted on a FACSCalibur flow cytometer (BD, Breda, the Netherlands). The numbers of *Legionella* CFU were determined using buffered charcoal yeast extract agar according to NEN standard 6265 (22). The numbers of *P. aeruginosa* CFU were determined using *Pseudomonas* agar base supplemented with CN agar according to ISO standard 16266 (23). *Aeromonas* was determined because *Aeromonas* has to be monitored as an indicator for regrowth in drinking water according to the Dutch drinking water decree. The numbers of CFU were determined using ampicillin dextrin agar (24).

Statistical analyses. The log-transferred values of the 18S rRNA gene copy numbers of fungi and the 16S rRNA gene copy numbers of mycobacteria were normally distributed according to the Kolmogorov-Smirnov test. Consequently, significant differences in these gene copy numbers between winter and summer samples were determined using the *t* test, whereas significant differences between treatment plants were determined using analysis of variance (ANOVA) with the Bonferroni *post hoc* test. Whether the percentage of samples positive for *L. pneumophila*, *P. aeruginosa*, *S. maltophilia*, or *A. fumigatus* was statistically different between winter and summer samples was determined using the nonparametric Mann-Whitney test. Pearson correlation analyses were performed on parameters that were normally distributed according to the Kolmogorov-

Smirnov test. Differences and correlations were considered statistically significant when the P value was <0.01.

RESULTS

Occurrence. The percentage of drinking water samples that tested positive for opportunistic pathogens and the range of the observed gene copy numbers for each opportunistic pathogen are shown in Table 2. The 18S rRNA gene of fungi and the 16S rRNA gene of NTM were observed in all drinking water samples, and the numbers varied between 1.9×10^4 to 8.7×10^6 and 2.0×10^4 to 1.3×10^7 gene copies liter^{-1}, respectively. In contrast, the 16S rRNA gene of *M. avium* complex and the 18S rRNA gene of *Acanthamoeba* spp. were not observed in any of the drinking water samples, meaning that gene copy numbers were below 200 per liter. The 28S rRNA gene of *A. fumigatus* was detected only in drinking water from the distribution system of plant SW5, with gene copy numbers ranging from <200 to 7.9×10^2 per liter.

The *mip* gene of *L. pneumophila* was detected in the distribution system of five of the eight treatment plants (SW1, SW2, SW3, SW5, and GW3), although most distribution locations at four of these five plants were negative (Table 2). Drinking water samples taken from the distribution system of plant SW2 in the summer were all positive for the *mip* gene of *L. pneumophila*. However, *L. pneumophila* could not be cultivated from any of the drinking water samples (<100 CFU liter^{-1}).

The *regA* gene of *P. aeruginosa* was observed in the distribution system of plants SW2, SW3, SW4, GW2, and GW3, with gene copy numbers ranging from <200 to 4.4×10^3 liter^{-1} (Table 2). Although the *regA* gene of *P. aeruginosa* was observed in five drinking water samples in the summer, *P. aeruginosa* could not be cultivated from these samples (<10 CFU liter^{-1}). In contrast, *P. aeruginosa* was cultivated from two distributed drinking water samples (10 CFU liter^{-1}) from plant GW1, where the *regA* gene of *P. aeruginosa* was below the detection limit (<200 gene copy numbers liter^{-1}). The *chiA* gene of *S. maltophilia* was observed in several samples of the distribution system from plants SW2, SW3, SW4, SW5, and GW3, and gene copy numbers ranged from <412 to 1.1×10^4 liter^{-1} (Table 2).

Effect of season. The drinking water temperatures were significantly higher in the summer in the distribution systems of all treatment plants ($P < 0.01$). These temperatures were, on average, 8.7 to 14.8°C higher in the summer than in the winter (see Fig. S1 in the supplemental material). The 18S rRNA gene copy numbers of fungi in the distributed drinking water of most treatment plants were higher in the summer than in the winter (Fig. 1A). However, only at treatment plants SW3 and SW5 were the gene copy numbers of fungi in the distribution systems significantly higher in summer than in winter ($P < 0.01$). The 16S rRNA gene copy numbers of NTM were significantly higher in summer than in winter in the distribution systems of plants SW1, SW2, SW3, GW1, GW2, and GW3 ($P < 0.01$) (Fig. 1B). Thus, the gene copy numbers of mycobacteria were affected by season and/or temperature in most of these distribution systems.

The percentages of drinking water samples that were positive for the *mip* gene of *L. pneumophila* were higher in the summer than in the winter for plants SW1, SW2, and SW3 (Fig. 2). In contrast, lower numbers of drinking water samples in the distribution systems of plants SW5 and GW3 were positive for the *mip* gene of *L. pneumophila* in the summer than in the winter. However, only at plant SW2 were these differences significant ($P < 0.01$). The

TABLE 2 The occurrence of *L. pneumophila*, *Mycobacterium* spp., *M. avium* complex, *P. aeruginosa*, *S. maltophilia*, fungi, *A. fumigatus*, and *Acanthamoeba* spp. in drinking water sampled in the winter and summer of 2010 from the distribution systems of eight different treatment plants in the Netherlands

Plant	No. of samples	*L. pneumophila*		*Mycobacterium* spp.		*M. avium* complex		*P. aeruginosa*		*S. maltophilia*		Fungi		*A. fumigatus*		*Acanthamoeba* spp.	
		Positive (%)	Range (gene copies liter^{-1})	Positive (%)	Range (gene copies liter^{-1})	Positive (%)	Range (gene copies liter^{-1})	Positive (%)	Range (gene copies liter^{-1})	Positive (%)	Range (gene copies liter^{-1})	Positive (%)	Range (gene copies liter^{-1})	Positive (%)	Range (gene copies liter^{-1})	Positive (%)	Range (gene copies liter^{-1})
SW1	27	7.4	<200–4.4×10^3	100	5.3×10^4–4.5×10^6	0.0	<200	0.0	<200	<412	<412	100	2.2×10^4–2.0×10^6	0.0	<200	0.0	<200
SW2	27	48.1	<200–5.2×10^4	100	4.9×10^4–1.0×10^7	0.0	<200	14.8	<200–4.4×10^3	22.2	<412–1.1×10^4	100	4.1×10^4–8.7×10^6	0.0	<200	0.0	<200
SW3	22	13.6	<200–1.3×10^4	100	4.7×10^4–3.6×10^6	0.0	<200	17.4	<200–1.4×10^3	36.4	<412–5.3×10^3	100	4.3×10^4–1.9×10^6	0.0	<200	0.0	<200
SW4	23	0.0	<200	100	1.4×10^5–1.1×10^7	0.0	<200	4.3	<200–8.5×10^2	8.7	<412–3.8×10^3	100	2.6×10^4–2.1×10^6	0.0	<200	0.0	<200
SW5	27	11.1	<200–9.2×10^3	100	7.7×10^4–1.5×10^6	0.0	<200	0.0	<200	3.7	<412–2.9×10^3	100	5.8×10^4–2.5×10^6	14.8	<200–7.9×10^2	0.0	<200
GW1	25	0.0	<200	100	2.0×10^4–9.7×10^5	0.0	<200	0.0	<200	0.0	<412	100	7.9×10^4–2.9×10^6	0.0	<200	0.0	<200
GW2	22	0.0	<200	100	1.0×10^5–1.3×10^7	0.0	<200	4.5	<200–1.2×10^3	0.0	<412	100	1.9×10^4–2.6×10^6	0.0	<200	0.0	<200
GW3	20	5.0	<200–1.1×10^4	100	1.5×10^5–3.9×10^6	0.0	<200	30	<200–3.3×10^3	5.0	<412–1.0×10^4	100	1.2×10^5–3.6×10^6	0.0	<200	0.0	<200

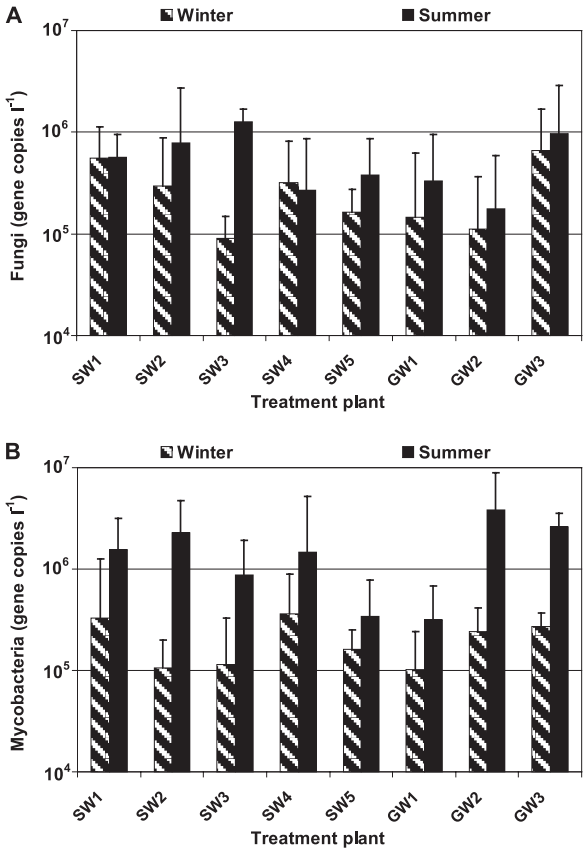

FIG 1 Geometric means (± standard deviation) of the 18S rRNA gene copies of fungi (A) and the 16S rRNA gene copies of mycobacteria (B) in drinking water from the distribution systems of eight different treatment plants sampled in the winter and the summer of 2010. The geometric means (± standard deviation) are based on 13 (SW1, SW2, SW5), 12 (GW1), 11 (SW3, SW4), or 10 (GW2, GW3) samples.

numbers of samples positive for the *regA* gene of *P. aeruginosa* were higher in the summer than in the winter in the distribution systems of plants SW3 and GW2, whereas the number of *P. aeruginosa*-positive samples was higher in the winter in the distribution systems of plants SW2, SW4, and GW3 (Fig. 2). Only in the distribution system of plant GW3 were these differences significant ($P < 0.01$). In general, no clear seasonal effects on the occurrence of *L. pneumophila* and *P. aeruginosa* were observed, similar to the results for fungi.

The percentages of samples that were positive for the *chiA* gene of *S. maltophilia* in the distribution systems of plants SW2 and SW3 were significantly higher in the summer than in the winter ($P < 0.01$; Fig. 2). The *chiA* gene of *S. maltophilia* was observed in the distribution systems of plants SW4, SW5, and GW3 as well, but again, only in the summer samples. The 28S rRNA gene of *A. fumigatus* was also observed in drinking water samples obtained in the summer, but only in the distribution system of plant SW5.

Differences among treatment plants. In the winter, the 18S rRNA gene copy numbers of fungi in drinking water sampled from the distribution systems of plants SW1 and GW3 were the highest (Fig. 1A). These values were significantly higher than fungal gene copy numbers in the distribution systems of plants SW3 and GW2 ($P < 0.01$). In the summer, fungal 18S rRNA gene copy numbers were high in the distribution systems of plants SW1, SW2, SW3, and GW3. Gene copy numbers in the distributed drinking water from plant SW3 were significantly higher than in the water from plants SW4 and GW2 ($P < 0.01$). The 16S rRNA gene copy numbers of mycobacteria did not differ significantly between drinking water samples from the different treatment plants in the winter period (Fig. 1B). In the summer, the 16S rRNA gene copy numbers of NTM in the distributed drinking water samples from plants GW1 and SW5 were significantly lower than in distributed water samples from plants SW1, SW2, GW2, and GW3 ($P < 0.01$).

L. pneumophila, *P. aeruginosa*, *S. maltophilia*, and *A. fumigatus* were rarely or not detected in drinking water samples from the

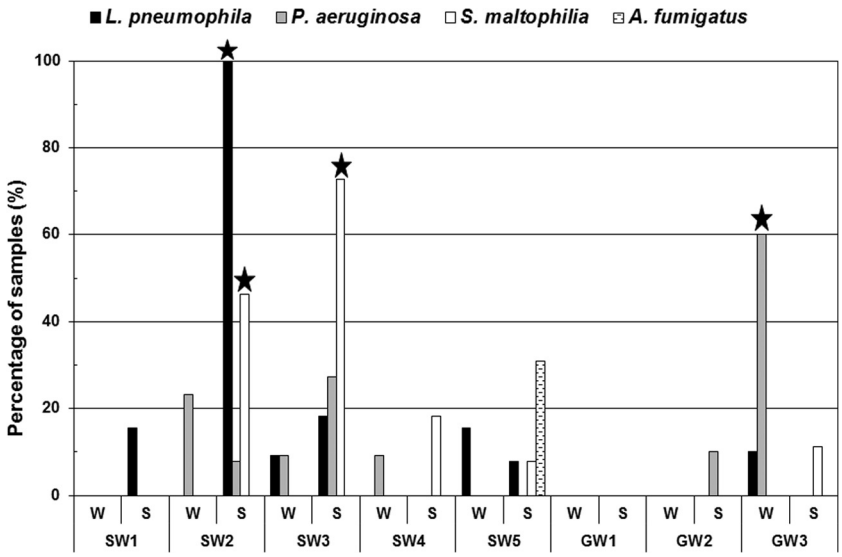

FIG 2 Percentages of drinking water samples positive for *L. pneumophila*, *P. aeruginosa*, *S. maltophilia*, and *A. fumigatus*. Drinking water was sampled from the distribution systems of eight different treatment plants in the winter (W) and the summer (S) of 2010. The stars indicate percentages of positive samples in the winter and the summer that were significantly different ($P < 0.01$).

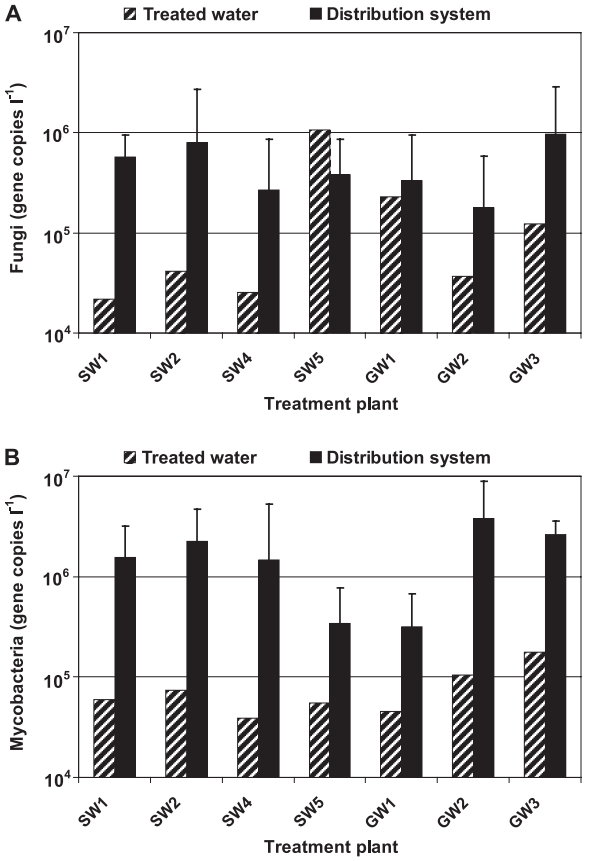

FIG 3 Geometric means (± standard deviation) of the 18S rRNA gene copies of fungi (A) and the 16S rRNA gene copies of mycobacteria (B) in finished drinking water of seven different treatment plants and in their corresponding distribution systems. The geometric means (± standard deviation) are based on 13 (SW1, SW2, SW5), 12 (GW1), 11 (SW4), or 10 (GW2, GW3) samples taken from the distribution systems.

distribution systems of plants SW1, GW1, and GW2, whereas distributed drinking water from the other plants was more often positive for *L. pneumophila* (SW1, SW2, SW3, SW5, and GW3), *P. aeruginosa* (SW2, SW3, SW4, and GW3), *S. maltophilia* (SW2, SW3, GW2, and GW3), and/or *A. fumigatus* (SW5) (Fig. 2). These results make it difficult to determine whether the numbers of these opportunistic pathogens differ significantly between the distribution systems because the organisms were not or only sporadically detected in drinking water sampled from them.

Effect of distribution. The treated water at the plants was also sampled in the summer and compared to distributed water. In six of the seven treatment plants, the 18S rRNA gene copy numbers of fungi were higher in the distribution system than in the treated water (Fig. 3A). These gene copy numbers were, on average, 1.5 to 26 times higher in the distribution system than in the treated water. The 16S rRNA gene copy numbers of mycobacteria were also higher in the distribution system than in the treated water, with gene copy numbers in the distribution system being, on average, 6 to 38 times higher than in the treated water (Fig. 3B). These results demonstrate that fungal and mycobacterial numbers increase during distribution, which suggests that these organisms multiply in the distribution system.

L. pneumophila, *P. aeruginosa*, *S. maltophilia*, and *A. fumigatus* were not detected in the treated water sampled prior to distribution. In contrast, several samples in the distribution systems of a number of these plants were positive for these organisms. Although treated water could only be tested at one location at each plant, the combined results imply that *L. pneumophila*, *P. aeruginosa*, *S. maltophilia*, and *A. fumigatus* proliferate in the drinking water distribution systems of some treatment plants.

Relationship with other microbiological parameters. The ATP concentrations, cell counts, and numbers of *Aeromonas* CFU are shown in Table S3 in the supplemental material. Most relations between ATP, total cell number, and total membrane-intact cell number or number of *Aeromonas* CFU and fungal 18S rRNA or mycobacterial 16S rRNA gene copy numbers were statistically significant, but the R^2 values were low in general (<0.3) (Table 3). Hence, relationships are weak, and more than 70% of the variations in gene copy numbers of fungi or mycobacteria are not reflected in the variations of the other microbiological parameters.

To investigate whether there was an increased risk that samples contained multiple opportunistic pathogens (*L. pneumophila*, *P. aeruginosa*, *S. maltophilia*, or *A. fumigatus*), the numbers and percentages of drinking water samples in the distribution systems that tested positive for a certain opportunistic pathogen were used to calculate the numbers of samples that would be expected to be positive for two opportunistic pathogens. These calculated samples were compared to the numbers of samples in which two opportunistic pathogens were detected (Table 4). The results demonstrate that the numbers of drinking water samples positive for both *P. aeruginosa* and *L. pneumophila* or *P. aeruginosa* and *S. maltophilia* were approximately two times higher than expected. Moreover, the numbers of drinking water samples positive for *L. pneumophila* and S. *maltophilia* were four times higher than expected (Table 4).

DISCUSSION

Occurrence of opportunistic pathogens. Despite the differences in water quality at the eight treatment plants, all analyzed drinking water samples contained the 18S rRNA gene of fungi and the 16S rRNA gene of mycobacteria. Moreover, gene copy numbers increased in the distribution systems, indicating that these organisms were capable of multiplying in all analyzed drinking water distribution systems. Thus, fungi and mycobacteria seem to be adapted to the oligotrophic drinking water ecosystems in the Netherlands. Previous studies in other countries have also identified fungi in unchlorinated and chlorinated drinking water (25–

TABLE 3 Data from correlation analysis between log-transformed gene copy numbers of fungi or mycobacteria and ATP or log-transformed total cells, total membrane-intact cells, and *Aeromonas* CFU that were measured in drinking water samples from the distribution systems of eight different treatment plants in the winter and summer of 2010

Parameter 1	Parameter 2	*P* value	R^2
ATP	Gene copies of fungi	<0.01	0.09
Total cells	Gene copies of fungi	<0.01	0.05
Total membrane-intact cells	Gene copies of fungi	>0.01	—[a]
Aeromonas CFU	Gene copies of fungi	<0.01	0.29
ATP	Gene copies of mycobacteria	>0.01	—
Total cells	Gene copies of mycobacteria	>0.01	—
Total membrane-intact cells	Gene copies of mycobacteria	<0.01	0.07
Aeromonas CFU	Gene copies of mycobacteria	<0.01	0.17

[a] —, no R^2 calculated.

TABLE 4 Numbers of expected and observed samples that were positive for a second opportunistic pathogen

Samples positive for:	*L. pneumophila*		*P. aeruginosa*		*S. maltophilia*		*A. fumigatus*	
	Expected	Observed	Expected	Observed	Expected	Observed	Expected	Observed
L. pneumophila			2.0	4	2.2	9	0.5	0
P. aeruginosa	2.0	4			1.6	3	0.4	0
S. maltophilia	2.2	9	1.6	3			0.4	0
A. fumigatus	0.5	0	0.4	0	0.4	0		

34). Some of these studies reported lower numbers of samples positive for fungi or lower fungal numbers in drinking water derived from groundwater than in drinking water derived from surface water, which is in contrast to our findings. This apparent difference can be explained by the different methods used to quantify fungi. In the previously published studies, cultivation methods were used to quantify fungi, and not all fungal species from drinking water can be cultivated yet (18). Another possible explanation is that fungal 18S rRNA gene copy numbers might give an overestimation of fungal cells in drinking water samples, since fungal cells can contain up to 100 copies of the 18S rRNA gene (35).

The presence of fungi in drinking water does not imply the occurrence of opportunistic pathogenic fungi, since most fungal species are nonpathogenic to humans. Still, a previous study identified 18S rRNA genes of opportunistic pathogenic fungi in unchlorinated drinking water samples in the Netherlands (e.g., *Aspergillus restrictus*, *Cladosporium cladosporioides*) (18). Moreover, we observed the opportunistic fungal pathogen *A. fumigatus* in some distribution samples from treatment plant SW5. Other studies, using cultivation-based methods, have also identified *A. fumigatus* in drinking water samples (25, 26, 28, 30, 36). An epidemiological link between *A. fumigatus* strains from patients and drinking water has been suggested (25, 36, 37), but additional studies are required to determine whether *A. fumigatus* strains from drinking water samples are identical to clinical strains.

NTM were observed in drinking water by others using cultivation methods, but the numbers of positive samples ranged from 15% to 83% (8, 38–43), lower than the 100% positive drinking water samples observed with qPCR in our study. A recent study demonstrated that cultivation methods give an underestimation of NTM in drinking water compared to estimation with qPCR (40). Thus, the use of qPCR methods in our study explains the differences with the other studies where cultivation methods were used to quantify NTM. qPCR detects cultivable, viable but noncultivable, and dead NTM, which could be the reason for the higher numbers detected with qPCR than with cultivation-based methods. Only a limited number of mycobacterial species are opportunistic pathogens (e.g., *Mycobacterium abscessus*, *M. avium*, *Mycobacterium intracellulare*, *Mycobacterium chelonae*, *Mycobacterium cosmeticum*, *Mycobacterium fortuitum*, *Mycobacterium gordonae*, *Mycobacterium kansasii*, *Mycobacterium mucogenicum*, *Mycobacterium phocaicum*, *Mycobacterium malmoense*, *Mycobacterium margeritense*, *Mycobacterium massiliense*, *Mycobacterium simiae*, and *Mycobacterium xenopi*) (44, 45). Consequently, it remains uncertain whether opportunistic pathogenic mycobacteria were present in the drinking water samples analyzed in our study. In addition, we did not observe the opportunistic pathogen *M. avium* complex in any of the unchlorinated drinking water samples, which is in contrast to studies on drinking water in the United States (6, 39, 42, 46, 47). Bacteria belonging to the *M. avium* complex are resistant to chlorine or chloramine (48, 49), and the use of these disinfectants in drinking water in the United States might select for these bacteria. In the Netherlands, drinking water is distributed without a disinfectant residual; therefore, selection for disinfectant-resistant bacteria does not occur. However, other factors (e.g., temperature, AOC concentration) might play roles as well in the establishment of *M. avium* complex in drinking-water-related ecosystems (39).

Around 300 to 400 cases of Legionnaires' disease are reported annually in the Netherlands, and warm tap-water installations have been identified as a source for dissemination of *L. pneumophila* (2, 3). In this study, the *mip* gene of *L. pneumophila* was sporadically detected in drinking water from the distribution systems. However, *L. pneumophila* could not be cultivated from drinking water samples that were positive for the *mip* gene of *L. pneumophila*. Similar results were reported for unchlorinated drinking water sampled from the distribution systems of two groundwater treatment plants in the Netherlands (50). However, in contrast to our findings, the *mip* gene copy numbers observed in that study were below the detection level of the culture method, which could explain the observed differences between qPCR and cultivation results. In our study, we observed gene copy numbers of *L. pneumophila* that were above the detection limit for the culture method. It might be that the *mip* gene detected in our study belonged to *L. pneumophila* cells that were inactive or dead, since qPCR cannot discriminate between viable and dead cells. Consequently, it remains uncertain whether the presence of the *mip* gene of *L. pneumophila* in drinking water alone can be linked to cases of Legionnaires' disease.

The *regA* gene of *P. aeruginosa* and the *chiA* gene of *S. maltophilia* were also sporadically detected in the drinking water samples analyzed in this study. The amount of samples positive for *P. aeruginosa* was comparable to those found in German studies, where cultivation methods were used to detect *P. aeruginosa* in drinking water (51, 52). In addition, the number of drinking water samples positive for *P. aeruginosa* and the number of cultivable *P. aeruginosa* seem to be higher in countries with warmer climates (e.g., Togo and Israel) (53–55). To our knowledge, a comprehensive study on the occurrence of *S. maltophilia* in drinking water has not been performed, but the organism has been isolated from drinking water in the past (4, 16, 56, 57). We demonstrated that up to 36.4% of the drinking water samples in a drinking water distribution system can be positive for the *chiA* gene of *S. maltophilia*. It is not clear from our study whether the *P. aeruginosa* and *S. maltophilia* strains observed in drinking water pose a risk for public health, although it has been noticed that certain *P. aeruginosa* and *S. maltophilia* strains from drinking water or drinking water environments (e.g., sinks, taps, showers) have been identical to strains isolated from patients (13, 15, 56).

In a recent study, the occurrence of *Mycobacterium* spp., *M. avium*, *L. pneumophila*, *P. aeruginosa*, and *Acanthamoeba* spp. in chloraminated drinking water sampled from eight locations in the distribution systems of two treatment plants in the United States was determined by qPCR methods as well (58). The percentages of samples positive for *Mycobacterium* spp., *L. pneumophila*, and *P. aeruginosa* were similar to those observed in our study. The gene copy numbers observed for these organisms in a chloraminated drinking water supply from one treatment plant in Virginia were also similar to the gene copy numbers observed in our study, but gene copy numbers of these three organisms in the water samples of a treatment plant in Florida were 100 times higher. This difference might have been a result of the drinking water temperatures observed in Florida, which were higher (26.8 ± 0.9°C) than those in Virginia (19.7 ± 2.4°C) and in the Netherlands (18.7 ± 0.9°C). The percentages of samples positive for *M. avium* were higher in the chloraminated drinking water samples from the two supplies in the United States, and gene copy numbers were at least 10 to 100 times higher in those samples than in the nonchlorinated drinking water samples from the Netherlands. These differences suggest that the use of chloramine as a disinfectant residual might select for *M. avium* in the distributed drinking water.

A limitation of using qPCR methods to quantify microorganisms in environmental samples is that qPCR cannot discriminate between viable and nonviable cells. Especially when microorganisms are inactivated by certain treatments in the distribution or premise plumbing system, e.g., disinfection, qPCR might overestimate the number of viable cells. However, in the Netherlands, drinking water is distributed without a disinfectant residual, and point-of-use treatment was not applied in the premise plumbing of the houses that were sampled. Consequently, it is unlikely that a dominant part of the microorganisms is dead. Two observations are consistent with this statement. First, flow cytometry showed that, in general, 70% to 99% of the bacterial cells in the drinking water samples had intact membranes (data not shown), which some consider to be a viability discriminator (59). Second, the numbers of fungi and NTM increased during distribution in the distribution systems of all treatment plants, and water sampled at the treatment plants was always negative for opportunistic pathogens, indicating that these organisms multiplied in the distribution systems. Moreover, cultivation methods, which have been the "golden standard" in the past, also suffer from a similar limitation. Obviously, cultivable microorganisms are viable, but most microbial species in the environment cannot yet be cultivated in the laboratory due to unknown cultivation requirements or because the selective conditions of the cultivation method are too stringent for growth (60). In addition, it has been suggested that some bacterial species that can be selectively cultivated might enter a viable but noncultivable state (61). It is likely, therefore, that cultivation methods underestimate the number of viable opportunistic pathogens in the environment. As such, an ideal method for specifically quantifying certain microorganisms and reliably discriminating between viable and nonviable cells is not yet available.

Factors affecting occurrence of opportunistic pathogens. A seasonal effect on the occurrence of opportunistic pathogens in drinking water was most clearly seen in the distribution systems of plants SW2 and/or SW3, where gene copy numbers of fungi and mycobacteria and the occurrence of *L. pneumophila* and *S. maltophilia* were significantly higher in the summer than in the winter. In contrast, no clear effect of season on opportunistic pathogens, except NTM and *S. maltophilia*, was observed for the distribution systems of the other treatment plants. The water temperatures in all the distribution systems were significantly higher in the summer than in the winter, but temperatures in the distribution systems of plants SW2 and SW3 were not significantly higher than those in the distribution systems of the other plants. Consequently, the higher water temperatures alone cannot explain the higher fungal numbers or higher numbers of *L. pneumophila*- and *S. maltophilia*-positive samples in the distribution systems of plants SW2 and SW3.

Drinking water sampled in the summer from the distribution systems of plants SW2 and SW3 were taken directly from the tap without flushing. In all other cases, samples were taken at the tap after flushing until the water temperature remained stable for 30 s. This means that the drinking water sampled in the summer from plants SW2 and SW3 was stagnant water from the premise plumbing system, whereas in the other cases, water came from the distribution system and had flown through only the premise plumbing system. Hence, the higher numbers of fungi and the higher percentages of *L. pneumophila*- and *S. maltophilia*-positive samples in drinking water sampled from the distribution systems of plants SW2 and SW3 in the summer could be attributed to the growth of these organisms in the premise plumbing installations. Others have also demonstrated that growth of mycobacteria, fungi, and *L. pneumophila* occurs in the in-house plumbing system (2, 3, 8, 29, 46, 62). It is likely that the higher surface/volume ratio, different pipe materials, and/or higher temperatures in the premise plumbing may have contributed to the growth of these organisms.

Mycobacteria were detected in significantly lower numbers in the winter than in the summer for most distribution systems. In addition, *S. maltophilia* was not detected in drinking water sampled in the winter, but the organism was detected in distributed drinking water samples from five different treatment plants (SW2, SW3, SW4, SW5, and GW3) in the summer. These results indicate a seasonal effect on the occurrence of mycobacteria and *S. maltophilia* in drinking water. Higher temperatures in the summer are probably advantageous for the growth of these organisms in the distribution systems and in premise installations. However, *S. maltophilia* was not observed in drinking water from the distribution system of SW1, GW1, or GW2, indicating that factors other than temperature play a role in multiplication of *S. maltophilia* in the distribution system. *A. fumigatus* was observed only in the summer as well, but since it was detected in one distribution system, it is not possible to conclude whether seasonal effects influence the occurrence of this organism in drinking water distribution systems.

Treated water from the selected plants originated from different sources (groundwater and surface water) and differed in TOC concentrations, AOC concentrations, and heterotrophic plate counts (Table 1). Despite these differences in water quality, the 16S rRNA gene copy numbers of mycobacteria in the distributed water in the winter were not significantly different between the plants. Mycobacterial gene copy numbers were significantly higher in the distribution systems of plants SW1, SW2, GW2, and GW3 than in the distribution systems of plants SW5 and GW1 in the summer. However, the AOC and TOC concentrations in drinking water differed considerably between plants SW5 and

GW1, indicating that AOC and TOC levels do not influence the mycobacterial numbers in drinking water. Moreover, these results demonstrate that species belonging to the genus *Mycobacterium* can grow in drinking water (biofilms) at different but low levels of (easily biodegradable) organic carbon in the water. In contrast, by using cultivation methods, it was observed in Finland and the United States that mycobacterial numbers are positively correlated with AOC concentrations (39, 43). This apparent discrepancy might be explained by differences in AOC levels, which were considerably higher and had larger ranges in the U.S. and Finnish studies (17 to 234 μg C liter^{-1} and 38 to 350 μg C liter^{-1}, respectively) than in our study (3.1 to 28.2 μg C liter^{-1}). In addition, drinking water in the United States and Finland is chlorinated or chloraminated, whereas in the Netherlands, drinking water is distributed unchlorinated, which might influence the mycobacterial population as well (48, 49). Furthermore, we used qPCR methods to quantify mycobacteria, whereas cultivation methods were used in the other two studies.

Fungi were present in significantly higher numbers in distributed drinking water from plant GW3 than in distributed drinking water from plant GW2. Treated groundwater from plant GW3 had much higher TOC and AOC concentrations than treated groundwater from plants GW1 and GW2 (Table 1). The relatively high TOC and AOC concentrations in the drinking water of plant GW3 are the likely cause for the relatively high number of fungi in the water. Drinking water produced from surface water at five different treatment plants had similar fungal numbers in the distribution system, although AOC concentrations varied as well. However, the TOC concentrations in the treated water were similar between these five treatment plants. These results suggest that fungal growth might be more related to TOC concentrations in drinking water than AOC concentrations, which might be in agreement with the hypothesis that fungi are more specialized in degrading complex organic carbon substrates than easily degraded organic compounds that will be measured with the AOC test (63).

AOC concentrations above 10 μg C liter^{-1} were observed in treated water from treatment plants SW1, SW2, SW3, SW5, and GW3 (Table 1). Drinking water samples from the distribution systems of those plants were sporadically positive for *L. pneumophila*. In contrast, treated water from plants SW4 and GW1 had considerably lower AOC levels (4.5 and 3.1 μg C liter^{-1}, respectively), and *L. pneumophila* was not observed in the distribution systems of those two plants. These results suggest that the AOC levels in drinking water relate to the presence of *L. pneumophila*. Our results are consistent with results from a previous study where *L. pneumophila* was detected in a distribution system that was fed with drinking water with a high concentration of natural organic matter (NOM) but not in a distribution system that was fed with drinking water having a low NOM concentration (50). *P. aeruginosa* and *S. maltophilia* were also sporadically observed in drinking water, but these organisms were present in drinking water with high or low concentrations of AOC and TOC. Thus, it cannot be concluded that the water composition influenced the occurrence of *P. aeruginosa* and *S. maltophilia* in unchlorinated drinking water in the Netherlands. Still, the results indicate that *P. aeruginosa* and *S. maltophilia* are capable of growing at low AOC concentrations in drinking water, as has been observed previously (64).

The 18S rRNA gene copy numbers of fungi and the 16S rRNA gene copy numbers of mycobacteria showed only weak correlations with ATP or total cell numbers. These results are consistent with those from other studies (38, 43, 62). Allegedly, conditions that influence the growth of the dominant microbial community differ from conditions that influence the growth of fungi and mycobacteria in drinking water. In addition, the results from our study show that different opportunistic pathogens can be present in the same water sample. It was also observed that samples that contained *L. pneumophila*, *P. aeruginosa*, or *S. maltophilia* have a higher risk of containing another of these three opportunistic pathogens than samples that did not contain any of these three organisms. Hence, certain drinking water types and/or sampling locations in the distribution or premise plumbing system might promote growth of different opportunistic pathogens. The growth potential of the water, pipe material, temperature, and hydraulics influence biofilm growth on each location in the drinking water distribution system and premise plumbing system and might influence the growth of some opportunistic pathogens in drinking water (5, 54, 65). Therefore, a more comprehensive study on these local factors in the distribution system can identify mechanisms that are responsible for the growth of multiple opportunistic pathogens in drinking water distribution systems.

Overall, the results from our study demonstrate that fungi and NTM are commonly present in unchlorinated drinking water in the Netherlands, whereas *L. pneumophila*, *P. aeruginosa*, *S. maltophilia*, and *A. fumigatus* are sporadically present. Gene copy numbers of these organisms increase in the distribution system and in the premise plumbing system, which stresses the need for further research to identify distribution and premise plumbing factors that are responsible for these increasing numbers. Furthermore, the type of source used for drinking water production, the season, and water composition had no large influence on the occurrence of these opportunistic pathogens in unchlorinated drinking water. However, locations that are positive for one opportunistic pathogen are more likely to harbor other opportunistic pathogenic species as well, which implies that certain "hot spots" in the distribution or premise plumbing system might pose a higher risk to public health than other locations.

ACKNOWLEDGMENT

This work was financed by the joint research program (BTO) of the Dutch drinking water companies.

REFERENCES

1. **Bartram J, Cotruvo C, Exner M, Fricker C, Glasmacher A.** 2003. Heterotrophic plate counts and drinking-water safety. IWA Publishing/World Health Organization, London, United Kingdom.
2. **Garcia-Nuñez M, Sopena N, Ragull S, Pedro-Botet ML, Morera J, Sabria M.** 2008. Persistence of *Legionella* in hospital water supplies and nosocomial Legionnaires' disease. FEMS Immunol. Med. Microbiol. **52**: 202–206.
3. **Luck PC, Schneider T, Wagner J, Walther I, Reif U, Weber S, Weist K.** 2008. Community-acquired Legionnaires' disease caused by *Legionella pneumophila* serogroup 10 linked to the private home. J. Med. Microbiol. **57**:240–243.
4. **Rusin PA, Rose JB, Haas CN, Gerba CP.** 1997. Risk assessment of opportunistic bacterial pathogens in drinking water. Rev. Environ. Contam. Toxicol. **152**:57–83.
5. **van der Kooij D, Veenendaal HR, Scheffer WJ.** 2005. Biofilm formation and multiplication of *Legionella* in a model warm water system with pipes of copper, stainless steel and cross-linked polyethylene. Water Res. **39**: 2789–2798.
6. **Aronson T, Holtzman A, Glover N, Boian M, Froman S, Berlin OG, Hill H, Stelma G, Jr.** 1999. Comparison of large restriction fragments of *Mycobacterium avium* isolates recovered from AIDS and non-AIDS pa-

tients with those of isolates from potable water. J. Clin. Microbiol. **37**: 1008–1012.
7. **Currie BJ, Mayo M, Anstey NM, Donohoe P, Haase A, Kemp DJ.** 2001. A cluster of melioidosis cases from an endemic region is clonal and is linked to the water supply using molecular typing of *Burkholderia pseudomallei* isolates. Am. J. Trop. Med. Hyg. **65**:177–179.
8. **Engel HW, Berwald LG, Havelaar AH.** 1980. The occurrence of *Mycobacterium kansasii* in tapwater. Tubercle **61**:21–26.
9. **Kilvington S, Gray T, Dart J, Morlet N, Beeching JR, Frazer DG, Matheson M.** 2004. *Acanthamoeba* keratitis: the role of domestic tap water contamination in the United Kingdom. Invest. Ophthalmol. Vis. Sci. **45**: 165–169.
10. **von Reyn CF, Maslow JN, Barber TW, Falkinham JO, III, Arbeit RD.** 1994. Persistent colonisation of potable water as a source of *Mycobacterium avium* infection in AIDS. Lancet **343**:1137–1141.
11. **Buttery JP, Alabaster SJ, Heine RG, Scott SM, Crutchfield RA, Bigham A, Tabrizi SN, Garland SM.** 1998. Multiresistant *Pseudomonas aeruginosa* outbreak in a pediatric oncology ward related to bath toys. Pediatr. Infect. Dis. J. **17**:509–513.
12. **Denton M, Todd NJ, Kerr KG, Hawkey PM, Littlewood JM.** 1998. Molecular epidemiology of *Stenotrophomonas maltophilia* isolated from clinical specimens from patients with cystic fibrosis and associated environmental samples. J. Clin. Microbiol. **36**:1953–1958.
13. **Doring G, Jansen S, Noll H, Grupp H, Frank F, Botzenhart K, Magdorf K, Wahn U.** 1996. Distribution and transmission of *Pseudomonas aeruginosa* and *Burkholderia cepacia* in a hospital ward. Pediatr. Pulmonol. **21**: 90–100.
14. **Nelson JW, Doherty CJ, Brown PH, Greening AP, Kaufmann ME, Govan JR.** 1991. *Pseudomonas cepacia* in patients with cystic fibrosis. Lancet **338**:1525.
15. **Vochem M, Vogt M, Doring G.** 2001. Sepsis in a newborn due to *Pseudomonas aeruginosa* from a contaminated tub bath. N. Engl. J. Med. **345**: 378–379.
16. **Weber DJ, Rutala WA, Blanchet CN, Jordan M, Gergen MF.** 1999. Faucet aerators: a source of patient colonization with *Stenotrophomonas maltophilia.* Am. J. Infect. Control **27**:59–63.
17. **Solomon S, Qin D, Manning M, Marquis M, Averyt K, Tignor MMB, Miller HL.** 2007. Climate change 2007: the physical science basis. Cambridge University Press, Cambridge, England.
18. **van der Wielen PWJJ, Italiaander R, Wullings B, Heijnen L, van der Kooij D.** Opportunistic pathogens in drinking water in the Netherlands. *In* van der Kooij D, van der Wielen PJJ (ed), Microbial growth in drinking water distribution systems: problems, causes, prevention and research needs, in press. IWA Publishing, London, United Kingdom.
19. **Glasmacher A, Engelhart S, Exner M.** 2003. Infections from HPC organisms in drinking-water amongst the immunocompromised, p 137–145. *In* Bartram J, Cotruvo J, Exner M, Fricker C, Glasmacher A (ed), Heterotrophic plate counts and drinking-water safety. IWA Publishing/World Health Organization, London, United Kingdom.
20. **van der Wielen PW, Medema G.** 2010. Unsuitability of quantitative *Bacteroidales* 16S rRNA gene assays for discerning fecal contamination of drinking water. Appl. Environ. Microbiol. **76**:4876–4881.
21. **van der Wielen PWJJ, van der Kooij D.** 2010. Effect of water composition, distance and season on the adenosine triphosphate concentration in unchlorinated drinking water in the Netherlands. Water Res. **44**:4860–4867.
22. **Nederlands Normalisatie Instituut (Dutch Normalisation Institute).** 2007. NEN 6265. Bacteriological examination of water: examination on the presence and the number of colony forming units (CFU) of *Legionella* bacteria. NEN, Delft, the Netherlands.
23. **International Organisation for Standardisation.** 2008. ISO 16266. Water quality—detection and enumeration of *Pseudomonas aeruginosa*—method by membrane filtration. ISO, Geneva, Switzerland.
24. **Havelaar AH, During M, Versteegh JF.** 1987. Ampicillin-dextrin agar medium for the enumeration of *Aeromonas* species in water by membrane filtration. J. Appl. Bacteriol. **62**:279–287.
25. **Anaissie EJ, Stratton SL, Dignani MC, Lee CK, Summerbell RC, Rex JH, Monson TP, Walsh TJ.** 2003. Pathogenic molds (including *Aspergillus* species) in hospital water distribution systems: a 3-year prospective study and clinical implications for patients with hematologic malignancies. Blood **101**:2542–2546.
26. **Arvanitidou M, Kanellou K, Constantinides TC, Katsouyannopoulos V.** 1999. The occurrence of fungi in hospital and community potable waters. Lett. Appl. Microbiol. **29**:81–84.
27. **Franková E, Horecká M.** 1995. Filamentous soil fungi and unidentified bacteria in drinking water from wells and water mains near Bratislava. Microbiol. Res. **150**:311–313.
28. **Grabinska-Loniewska A, Konillowicz-Kowalska T, Wardzynska G, Boryn K.** 2007. Occurrence of fungi in water distribution system. Pol. J. Environ. Stud. **16**:539–547.
29. **Hageskal G, Gaustad P, Heier BT, Skaar I.** 2007. Occurrence of moulds in drinking water. J. Appl. Microbiol. **102**:774–780.
30. **Hageskal G, Knutsen AK, Gaustad P, de Hoog GS, Skaar I.** 2006. Diversity and significance of mold species in Norwegian drinking water. Appl. Environ. Microbiol. **72**:7586–7593.
31. **Kanzler D, Buzina W, Paulitsch A, Haas D, Platzer S, Marth E, Mascher F.** 2008. Occurrence and hygienic relevance of fungi in drinking water. Mycoses **51**:165–169.
32. **Pedro-Botet ML, Sanchez I, Sabria M, Sopena N, Mateu L, Garcia-Núñez M, Rey-Joly C.** 2007. Impact of copper and silver ionization on fungal colonization of the water supply in health care centers: implications for immunocompromised patients. Clin. Infect. Dis. **45**:84–86.
33. **Warris A, Voss A, Abrahamsen TG, Verweij PE.** 2002. Contamination of hospital water with *Aspergillus fumigatus* and other molds. Clin. Infect. Dis. **34**:1159–1160.
34. **Zacheus OM, Martikainen PJ.** 1995. Occurrence of heterotrophic bacteria and fungi in cold and hot water distribution system using water of different quality. Can. J. Microbiol. **41**:1088–1094.
35. **Maicas S, Adam AC, Polaina J.** 2000. The ribosomal DNA of the zygomycete *Mucor miehei.* Curr. Genet. **37**:412–419.
36. **Warris A, Voss A, Verweij PE.** 2001. Hospital sources of *Aspergillus species*: new routes of transmission? Rev. Iberoam. Micol. **18**:156–162.
37. **Anaissie EJ, Stratton SL, Dignani MC, Summerbell RC, Rex JH, Monson TP, Spencer T, Kasai M, Francesconi A, Walsh TJ.** 2002. Pathogenic *Aspergillus* species recovered from a hospital water system: a 3-year prospective study. Clin. Infect. Dis. **34**:780–789.
38. **Covert TC, Rodgers MR, Reyes AL, Stelma GN, Jr.** 1999. Occurrence of nontuberculous mycobacteria in environmental samples. Appl. Environ. Microbiol. **65**:2492–2496.
39. **Falkinham JO, III, Norton CD, LeChevallier MW.** 2001. Factors influencing numbers of *Mycobacterium avium*, *Mycobacterium intracellulare*, and other mycobacteria in drinking water distribution systems. Appl. Environ. Microbiol. **67**:1225–1231.
40. **Hussein Z, Landt O, Wirths B, Wellinghausen N.** 2009. Detection of nontuberculous mycobacteria in hospital water by culture and molecular methods. Int. J. Med. Microbiol. **299**:281–290.
41. **Le Dantec C, Duguet JP, Montiel A, Dumoutier N, Dubrou S, Vincent V.** 2002. Occurrence of mycobacteria in water treatment lines and in water distribution systems. Appl. Environ. Microbiol. **68**:5318–5325.
42. **Pryor M, Springthorpe S, Riffard S, Brooks T, Huo Y, Davis G, Sattar SA.** 2004. Investigation of opportunistic pathogens in municipal drinking water under different supply and treatment regimes. Water Sci. Technol. **50**:83–90.
43. **Torvinen E, Suomalainen S, Lehtola MJ, Miettinen IT, Zacheus O, Paulin L, Katila ML, Martikainen PJ.** 2004. Mycobacteria in water and loose deposits of drinking water distribution systems in Finland. Appl. Environ. Microbiol. **70**:1973–1981.
44. **Collins CH, Grange JM, Yates MD.** 1984. Mycobacteria in water. J. Appl. Bacteriol. **57**:193–211.
45. **van Ingen J, Blaak H, de Beer J, de Roda Husman AM, van Soolingen D.** 2010. Rapidly growing nontuberculous mycobacteria cultured from home tap and shower water. Appl. Environ. Microbiol. **76**:6017–6019.
46. **Feazel LM, Baumgartner LK, Peterson KL, Frank DN, Harris JK, Pace NR.** 2009. Opportunistic pathogens enriched in showerhead biofilms. Proc. Natl. Acad. Sci. U. S. A. **106**:16393–16399.
47. **von Reyn CF, Waddell RD, Eaton T, Arbeit RD, Maslow JN, Barber TW, Brindle RJ, Gilks CF, Lumio J, Lahdevirta J, Ranki A, Dawson D, Falkinham JO, III.** 1993. Isolation of *Mycobacterium avium* complex from water in the United States, Finland, Zaire, and Kenya. J. Clin. Microbiol. **31**:3227–3230.
48. **Berry D, Horn M, Xi C, Raskin L.** 2010. *Mycobacterium avium* infections of *Acanthamoeba* strains: host strain variability, grazing-acquired infections, and altered dynamics of inactivation with monochloramine. Appl. Environ. Microbiol. **76**:6685–6688.
49. **Taylor RH, Falkinham JO, III, Norton CD, LeChevallier MW.** 2000.

Chlorine, chloramine, chlorine dioxide, and ozone susceptibility of *Mycobacterium avium*. Appl. Environ. Microbiol. **66**:1702–1705.
50. **Wullings BA, Bakker G, van der Kooij D.** 2011. Concentration and diversity of uncultured *Legionella* spp. in two nonchlorinated drinking water supplies with different concentrations of natural organic matter. Appl. Environ. Microbiol. **77**:634–641.
51. **Schubert R, Blum U.** 1974. Zur Frage der Erweiterung der hygienischen Wasserkontrolle auf den Nachweis von *Pseudomonas aeruginosa*. Gas Wasserfach **115**:224–226.
52. **Wingender J, Hambsch B, Schneider S.** 2009. Mikrobiologisch-hygienische Aspekte des Vorkommens von *Pseudomonas aeruginosa* im Trinkwasser. DVGW energie/wasser-praxis **60**:1–7.
53. **Reitler R, Seligmann R.** 1957. *Pseudomonas aeruginosa* in drinking water. J. Appl. Bacteriol. **20**:145–150.
54. **Schubert R, Scheiber P.** 1975. Das Vorkommen von *Pseudomonas aeruginosa* im Grundwasser, Oberflächenwasser und in Wasserversorgungsleitungen in tropischen Gebieten. Gas Wasserfach **116**:413–415.
55. **Shehabi AA, Masoud H, Maslamani FA.** 2005. Common antimicrobial resistance patterns, biotypes and serotypes found among *Pseudomonas aeruginosa* isolates from patient's stools and drinking water sources in Jordan. J. Chemother. **17**:179–183.
56. **Verweij PE, Meis JF, Christmann V, Van der Bor M, Melchers WJ, Hilderink BG, Voss A.** 1998. Nosocomial outbreak of colonization and infection with *Stenotrophomonas maltophilia* in preterm infants associated with contaminated tap water. Epidemiol. Infect. **120**:251–256.
57. **Wilkinson FH, Kerr KG.** 1998. Bottled water as a source of multiresistant *Stenotrophomonas* and *Pseudomonas* species for neutropenic patients. Eur. J. Cancer Care (Engl.) **7**:12–14.
58. **Wang H, Edwards M, Falkinham JO, III, Pruden A.** 2012. Molecular survey of the occurrence of *Legionella* spp., *Mycobacterium* spp., *Pseudomonas aeruginosa*, and amoeba hosts in two chloraminated drinking water distribution systems. Appl. Environ. Microbiol. **78**:6285–6294.
59. **Boulos L, Prevost M, Barbeau B, Coallier J, Desjardins R.** 1999. Live/Dead BacLight: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. J. Microbiol. Methods **37**:77–86.
60. **Rappe M, Giovannoni S.** 2003. The uncultured microbial majority. Annu. Rev. Microbiol. **57**:369–394.
61. **Oliver JD.** 2010. Recent findings on the viable but nonculturable state in pathogenic bacteria. FEMS Microbiol. Rev. **34**:415–425.
62. **Gottlich E, van der Lubbe W, Lange B, Fiedler S, Melchert I, Reifenrath M, Flemming HC, de Hoog S.** 2002. Fungal flora in groundwater-derived public drinking water. Int. J. Hyg. Environ. Health **205**:269–279.
63. **van der Kooij D.** 1992. Assimilable organic carbon as an indicator of bacterial regrowth. J. Am. Water Works Assoc. **84**:57–65.
64. **van der Kooij D, Oranje JP, Hijnen WA.** 1982. Growth of *Pseudomonas aeruginosa* in tap water in relation to utilization of substrates at concentrations of a few micrograms per liter. Appl. Environ. Microbiol. **44**:1086–1095.
65. **Schulze-Robbecke R, Janning B, Fischeder R.** 1992. Occurrence of mycobacteria in biofilm samples. Tuber. Lung Dis. **73**:141–144.