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Relationships between Free-Living Protozoa, Cultivable *Legionella* spp., and Water Quality Characteristics in Three Drinking Water Supplies in the Caribbean▽†

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The study whose results are presented here aimed at identifying free-living protozoa (FLP) and conditions favoring the growth of these organisms and cultivable *Legionella* spp. in drinking water supplies in a tropical region. Treated and distributed water (±30°C) of the water supplies of three Caribbean islands were sampled and investigated with molecular techniques, based on the 18S rRNA gene. The protozoan host *Hartmannella vermiformis* and cultivable *Legionella pneumophila* were observed in all three supplies. Operational taxonomic units (OTUs) with the highest similarity to the potential or candidate hosts *Acanthamoeba* spp., *Echinamoeba exundans, E. thermarum*, and an *Neoparamoeba* sp. were detected as well. In total, 59 OTUs of FLP were identified. The estimated protozoan richness did not differ significantly between the three supplies. In supply CA-1, the concentration of *H. vermiformis* correlated with the concentration of *Legionella* spp. and clones related to Amoebozoa predominated (82%) in the protozoan community. These observations, the low turbidity (<0.2 nephelometric turbidity units [NTU]), and the varying ATP concentrations (1 to 12 ng liter^{-1}) suggest that biofilms promoted protozoan growth in this supply. Ciliophora represented 25% of the protozoan OTUs in supply CA-2 with elevated ATP concentrations (maximum, 55 ng liter^{-1}) correlating with turbidity (maximum, 62 NTU) caused by corroding iron pipes. Cercozoan types represented 70% of the protozoan clones in supply CA-3 with ATP concentrations of <1 ng liter^{-1} and turbidity of <0.5 NTU in most samples of distributed water. The absence of *H. vermiformis* in most samples from supply CA-3 suggests that growth of this protozoan is limited at ATP concentrations of <1 ng liter^{-1}.

In tropical regions, the water temperature in drinking water distribution system is permanently about 30°C (4). In these regions, free-living protozoa (FLP), serving as hosts for pathogenic bacteria, including *Acanthamoeba* spp. (1, 38), *Hartmannella* spp. (37), and *Naegleria* spp. (38, 49), have been observed in surface water, wastewater, cooling towers, and drinking water (3, 5, 19, 45). Certain FLP with pathogenic properties, *viz. Acanthamoeba* spp. (9, 21), *Balamuthia mandrillaris* (54), and *Naegleria fowleri* (59), can proliferate in drinking water-related biofilms at elevated temperatures (36). In addition, *Legionella pneumophila*, the main etiologic agent of Legionnaires' disease (12), which proliferates in freshwater at temperatures above 25°C (57), is frequently observed in these environments (14, 31, 39).

FLP in aquatic environments feed on bacteria, fungi, other protozoa, and organic detritus in biofilms and sediments or in the planktonic phase (32). The abundance of prey organisms and detritus depends on the water composition and the hydraulic conditions in distribution systems, which therefore also affect both the FLP abundance and community composition (52, 55). Most information on community composition and abundance of FLP in freshwater environments has been obtained by using cultivation methods and microscopy. Recently, however, the presence and identities of such organisms in drinking water supplies in temperate regions have been studied by using molecular methods for detection and identification (33, 51). In two groundwater supplies in the Netherlands a total of 127 operational taxonomic units (OTUs) of FLP were identified based on their 18S rRNA gene sequences. FLP, mostly pathogens, have been characterized in only a few studies in tropical regions (3, 5, 19). In recent reviews it was concluded that more research is needed to determine which factors favor the growth of these organisms in water supplies (47, 48).

Cases of Legionnaires' disease have been reported in relation to the presence of *L. pneumophila* in drinking water supplies in the Caribbean (8, 39, 41), but information about water quality characteristics was not provided. In this study, the occurrence and identity of FLP and other small eukaryotes in treated and distributed water of drinking water supplies on three islands in the Caribbean region were investigated with molecular techniques. In these supplies, drinking water is produced from seawater by using distillation and/or reverse osmosis (RO) for desalination. The objectives of this study were (i) to determine concentrations of the protozoa *Acanthamoeba* spp. and *H. vermiformis* and cultivable *Legionella* spp. in treated and distributed water of three different water supplies, (ii) to identify the predominant FLP in these supplies, and (iii) to identify conditions favoring the growth of FLP and *Legion-*

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ella spp. by comparing the characteristics of water quality and distribution systems.

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MATERIALS AND METHODS

Drinking water supplies. The free-living protozoan communities in the drinking water supplies of three Caribbean islands (latitude and longitude, 12°6′ to 12°30′N and 68°19′ to 69°58′W) belonging to the Leeward Antilles were investigated. Water treatment plant CA-1 has a daily production of 4.4×10^4 m^3, 18% of which is produced by RO and 82% by distillation. Posttreatment with dolomite filtration to increase the hardness of the water and by the addition of corrosion inhibitors (pyrophosphate, 1.5 ppm; zinc orthophosphate, 2.5 ppm) is followed by storage in steel tanks and UV disinfection (35.99 mJ cm^{-2}) prior to distribution. Mains of copper (42%) and cement-lined cast iron (39%) lead the treated water to seven steel service reservoirs in the supply area. Supply CA-2 includes two treatment facilities with a total daily production of 5.8×10^4 m^3 drinking water. The distribution systems of both plants are interconnected. Desalination at plant CA-2a is done with RO and at plant CA-2b with RO (80%) and distillation (20%). Posttreatment of RO filtrate and distillate includes calcium hypochlorite dosage (0.3 mg $liter^{-1}$), addition of carbon dioxide, limestone and granular activated carbon (GAC) filtration, addition of fluoride (0.3 to 0.7 mg $liter^{-1}$), and disinfection with UV radiation (CA-2a, 15.4 mJ cm^{-2}; CA-2b, 7.5 to 12.2 mJ cm^{-2}). Pipes of high-density polyethylene, copper, and galvanized iron comprise about 70% of the distribution system, with seven service reservoirs. The main pipes (26%) and the transportation pipes consist of cast iron, with and without cement lining. In supply CA-3, with a daily production of 3.8×10^3 m^3, seawater is treated with RO, followed by limestone filtration and GAC filtration in parallel, addition of chlorine (0.2 mg $liter^{-1}$), and storage in two steel tanks. Treated water with a chlorine residual of <0.05 ppm is transported to five cast iron service reservoirs. The distribution system consists of pipes of cast iron (63%), polyethylene (11%), and polyvinyl chloride (9%). Maximum residence times in the distribution systems of the supplies, including the service tanks, range from 48 to about 96 h.

Sample collection. From supply CA-1, two sample series were collected, one in November 2007 and one in November 2009. Both series included four samples at different treatment stages and seven samples from the distribution system after each reservoir (6 to 12 km from the plant). From supply CA-2, samples were collected in May 2008 and in January 2009. Both series included one sample before UV from both treatment plants. In addition, 15 samples were collected from the distribution system (5 to 15 km from the plant) in 2008 and 7 samples in 2009. Treated water of supply CA-3 was sampled at the plant, and 13 samples were collected from the distribution system (5 to 10 km from the plant). All samples, contained in sterile 1-liter PE flasks, were stored on ice and processed within 24 to 72 h. The flasks for the samples of supply CA-3 contained 1 ml of sterile sodium thiosulfate (0.12 M) to neutralize the chlorine residual.

Analytical methods. Total concentrations of ATP, representing active biomass, were measured in all water samples as described earlier (27). Buffered charcoal-yeast extract (BCYE) agar, incubated at 37°C for 7 days, was used to detect cultivable cells of *Legionella* spp. in the water samples (11, 29). Subsequently, the fraction of colonies related to *L. pneumophila* was determined by an agglutination test (Legionella latex test; Oxoid, United Kingdom).

Duplicate water samples of 500 ml were filtered using an RTTP Isopore membrane (Millipore, Molsheim, France) with a 1.2-μm pore size and a 55-mm diameter. Of the samples taken from supply CA-1 in 2007, volumes of 1.75 liter were filtered. Subsequently, DNA of the organisms retained on the membrane filter was isolated as previously described (51). Concentrations of *H. vermiformis* and *Acanthamoeba* spp. in the water samples were determined using quantitative PCR (qPCR) as described earlier (23, 35). In brief, quantification of *H. vermiformis* and *Acanthamoeba* spp. was based on calibration curves which were constructed by preparing 10-fold dilutions of DNA extracted from suspensions with counted numbers of cells of *H. vermiformis* (ATCC 50237) and *Acanthamoeba castellanii* (CCAP 1501) (23). All primers were produced at Biolegio (Malden, the Netherlands). All qPCR assays were performed in duplicate, using undiluted and 10-fold-diluted DNA extracts as templates in 96-well plates in a C1000 thermal cycler (Bio-Rad, Veenendaal, the Netherlands).

The richness and composition of the eukaryotic communities in the water samples were determined by terminal restriction fragment length polymorphism (T-RFLP) analysis and cloning followed by sequence analysis of 18S rRNA gene fragments (±550 bp) as described earlier (51). Clone libraries were constructed of 2 treated water samples from plant CA-1 and 5, 10, and 6 samples of distributed water from supplies CA-1, CA-2, and CA-3, respectively. These samples were selected based on high concentrations of cultivable *Legionella* spp., low concentrations of *H. vermiformis*, and preferably a location on the periphery of the distribution systems. Approximately 45 clones per sample were analyzed, resulting in a total of 991 partial 18S rRNA gene sequences.

TABLE 1. Average values of chemical and physical water quality characteristics of treated water of supplies CA-1, CA-2, and CA-3[a]

Quality characteristic	Value for:			
	Treated water CA-1	Treated water CA-2a	Treated water CA-2b	Treated water CA-3
Temp (°C)	30	28	31.6	28.7
pH	9.3	8.2	8.3	8.5
Conductivity (μS cm^{-1})	25	132	125	420
Total hardness ($CaCO_3$ mg $liter^{-1}$)	9.9	48.2	42.2	66.0
Turbidity (NTU)[b]	0.16	0.20	0.30	0.26
Cl concn (mg $liter^{-1}$)	0.6	6.7	10.2	74
Cu concn (mg $liter^{-1}$)	0.01	5	15	0.01
Fe concn (mg $liter^{-1}$)	0.01	0.01	0.01	0.04
NPOC[c] concn (mg C $liter^{-1}$)	<0.05	<0.05	<0.05	<0.05

[a] Average values, based on routine monitoring over a period of 1 year.
[b] NTU, nephelometric turbidity units.
[c] NPOC, nonpurgeable organic carbon.

The 18S rRNA gene sequences obtained were divided into operational taxonomic units (OTUs) using a threshold of 99% sequence similarity. Subsequently, these sequences were compared to sequences in the National Center for Biotechnology (NCBI) database by BLAST search and imported and aligned into the SSU Ref SILVA98 database released in March 2009 using the ARB software package (26, 34), as previously described (51). The estimated OTU richness was determined with the ChaoI estimator from randomized data (18). The obtained eukaryotic sequences were divided into higher taxa based on the classification system of Cavalier-Smith (6) and the structure in the SILVA database (34).

Statistical analyses. The Kruskal-Wallis test was applied to determine differences in the concentrations of selected water quality parameters between the three supplies. In case of a statistical significant test result for a parameter, subsequent pairwise comparisons were used to determine which supplies differed. All tests were performed with 95% confidence and for the multiple comparisons the Bonferroni correction was applied. Linear regression analysis using log transformed concentrations was used to assess possible relationships between physical-chemical and microbiological parameters in the distributed water of the three supplies.

Nucleotide sequence accession numbers. All partial 18S rRNA gene sequences determined in this study have been deposited in GenBank under accession numbers HQ998878 to HQ999868.

RESULTS

Quality characteristics of treated and distributed drinking water. The water temperature at the treatment facilities and in the distribution systems of the three supplies ranged from about 28 to 34°C (Tables 1 and 2). The turbidity of treated water was low (≤0.3 nephelometric turbidity units [NTU]) at all facilities and remained low in the distribution system of supply CA-1. Turbidity exceeded 5 NTU at 10 locations in supply CA-2 and at two locations in supply CA-3 (Fig. 1). Similarly, the concentration of iron in treated water was low (≤0.04 mg Fe $liter^{-1}$) and remained low (<0.01 mg Fe $liter^{-1}$) in the distribution system of supply CA-1 but locally exceeded 1 mg Fe $liter^{-1}$ in supply CA-2 (nine samples) and in supply CA-3 (one sample). The turbidity of the water in distribution systems CA-2 and CA-3 correlated significantly with the iron concentration, indicating that iron is the main cause of turbidity in these supplies (Table 3). These relationships yielded

TABLE 2. Quality characteristics of water sampled from the distributions systems of supplies CA-1, CA-2, and CA-3

Parameter	Value for:								
	Supply CA-1			Supply CA-2			Supply CA-3		
	Median	Minimum	Maximum	Median	Minimum	Maximum	Median	Minimum	Maximum
Temp (°C)	32.7	31.5	34.4	31.9	27.4	32.9	29.1	27.8	31.4
Turbidity (NTU)	0.1	0.07	0.2	5.4	0.2	62.1	0.3	0.1	23.0
Fe concn (mg liter^{-1})	<0.01	<0.01	<0.01	0.7	0.03	9.5	0.03	<0.01	1.5
ATP concn (ng liter^{-1})	2.6	1.0	12.2	3.2	<1.0	55.2	<1.0	<1.0	16.1
H. vermiformis concn (cells liter^{-1})	18	3	245	4	<2	1670	<2	<2	4
Acanthamoeba spp. concn (cells liter^{-1})	<2	<2	<2	<2	<2	56	<2	<2	<2
Cultivated *Legionella* spp. concn (CFU liter^{-1})	1.3×10^4	3.0×10^2	2.5×10^5	7.1×10^3	$<2.5 \times 10^2$	1.0×10^5	7.5×10^2	$<2.5 \times 10^2$	6.5×10^4

average iron-to-turbidity ratios of 0.24 ± 0.12 mg Fe liter^{-1}/NTU^{-1} (supply CA-2) and 0.22 ± 0.13 mg Fe liter^{-1}/NTU^{-1} (supply CA-3).

In treated water of supply CA-1, a higher ATP concentration (3.7 ng ATP liter^{-1}) was observed than in treated water of supplies CA-2 and CA-3, where the concentration was below the detection limit (<1 ng ATP liter^{-1}). The elevated ATP concentration in treated water of supply CA-1 is due to an increase from <1 to 9 ng ATP liter^{-1} in the storage tanks at the plant. The ATP concentration did not increase during distribution in supply CA-1. ATP concentrations in distributed water of supply CA-2 (median, 3.2 ng ATP liter^{-1}) were significantly higher than in supply CA-3 (median <1 ng ATP liter^{-1}) and correlated with turbidity (Fig. 1 and Table 3).

***H. vermiformis*, *Acanthamoeba* spp., and cultivable *Legionella* spp. in treated and distributed water.** *H. vermiformis* (about 3 cells liter^{-1}) and cultivable cells of *Legionella* spp. (1.0×10^2 CFU liter^{-1}) were detected in the treated water of supply CA-1 after UV treatment. These microorganisms had grown in the storage tanks at the treatment plant where the concentration of *H. vermiformis* increased from <2 to 18 cells liter^{-1} and the concentration of *Legionella* spp. increased from $<1 \times 10^2$ to 1.5×10^4 CFU liter^{-1}. *H. vermiformis* was detected in all 14 samples collected from the distribution system of supply CA-1 (median value, 18 cells liter^{-1}), in 12 of 20 samples of supply CA-2 (median value, 4 cells liter^{-1}) (Fig. 2 and Table 2), and in 4 of the 13 samples from supply CA-3 at concentrations of about 3 cells liter^{-}1. The concentrations of *H. vermiformis* in the distributed water of supply CA-1 were significantly higher than in the distributed water of supplies CA-2 and CA-3 (Table 3). *Acanthamoeba* spp. were observed in 5 samples of distributed water of supply CA-2 at concentrations ranging from 2 to 56 cells liter^{-1} (median, 8.0 cells liter^{-1}) (Table 2). *H. vermiformis* was also detected in these samples.

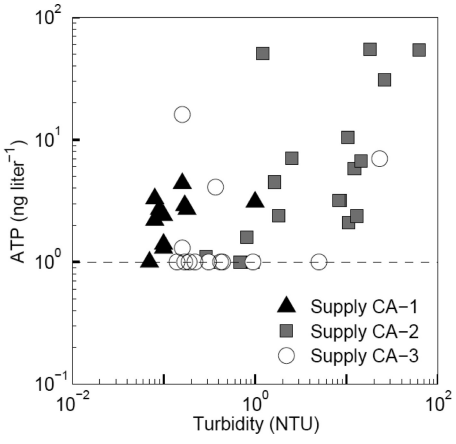

FIG. 1. Concentrations of turbidity and active biomass, measured as ATP, in the distributed water of supplies CA-1 (n = 14), CA-2 (n = 17), and CA-3 (n = 13). The detection limit for active biomass was 1 ng ATP liter^{-1}.

Legionella spp. were cultured from 41 of the 49 samples of distributed water and from 28 of these 41 samples containing *H. vermiformis* (Fig. 2). In supplies CA-1 and CA-2, *L. pneumophila* represented 80 to 100% of the cultured *Legionella* colonies and 40 to 100% in supply CA-3. The colony counts of *Legionella* spp. in the distributed water of supplies CA-1 and CA-2 were significantly higher than those in supply CA-3 (Table 3). The concentration of *Legionella* spp. correlated significantly with turbidity in the distributed water of supplies CA-2 and CA-3, with the concentrations of ATP in supply CA-2 and with concentrations of *H. vermiformis* in supplies CA-1 and CA-2. In 13 of 41 distributed water samples with cultivated *Legionella* spp., the concentrations of *H. vermiformis* and *Acanthamoeba* spp. were below the detection limit of 2 cells liter^{-1}.

Richness and identity of free-living protozoa. T-RFLP analyses using 18S rRNA gene-targeting primers revealed that the eukaryotic richness in the distributed water was higher than in the treated water at the four plants (data not shown). A total of 225 (25%) of the 908 partial 18S rRNA gene sequences in the clone libraries of the three types of distributed water clustered within FLP and represented 59 (30%) of the 195 OTUs (≥99% sequence similarity) (Table 4). Up to 11 OTUs of FLP were obtained from 0.5 liter of distributed water of supplies CA-1, CA-2, and CA-3. The highest protozoan richness was observed in supply CA-2, but the estimated total OTU richness for FLP did not differ significantly between the three supplies (Table 5).

OTUs of Amoebozoa represented a large proportion of the clones retrieved from the distributed water of supply CA-1 (Fig. 3; see also Table S1 in the supplemental material). Eukaryotic clone libraries were also constructed from the two samples of treated water before UV treatment at plant CA-1, because *Legionella* spp. were detected in the water before and after UV treatment. OTUs which clustered with Amoebozoa also predominated (>90%) in these clone libraries (see Table S2 in the supplemental material). A large proportion (39%) of

TABLE 3. Statistical analyses of differences in median concentrations (log transformed values) of selected water quality parameters of three drinking water supplies and correlations between the parameters in these supplies[a]

Parameter	Comparison between supplies		
	Supply CA-1	Supply CA-2	Supply CA-3
Turbidity (NTU)	<CA-2	>CA-1, >CA-3	<CA-2
Iron concn (mg liter^{-1})	<CA-2	>CA-1, >CA-3	<CA-2
ATP concn (ng liter^{-1})	NS[b]	>CA-3	<CA-2
H. vermiformis concn (cells liter^{-1})	>CA-2, >CA-3	<CA-1	<CA-1
Legionella spp. concn (CFU liter^{-1})	>CA-3	>CA-3	<CA-1, <CA-2
Correlation between parameters			
Turbidity vs. iron concn	NS	$R = 0.943$ ($P = 4.2 \times 10^{-10}$)	$R = 0.874$ ($P = 2.0 \times 10^{-4}$)
Turbidity vs. ATP concn	NS	$R = 0.705$ ($P = 5.2 \times 10^{-4}$)	NS
Turbidity vs. *Legionella* spp. concn	NS	$R = 0.821$ ($P = 9.1 \times 10^{-6}$)	$R = 0.834$ ($P = 7.4 \times 10^{-4}$)
ATP concn vs. *Legionella* spp. concn	NS	$R = 0.755$ ($P = 4.9 \times 10^{-6}$)	NS
ATP concn vs. *H. vermiformis* concn	NS	NS	NS
H. vermiformis concn vs. *Legionella* spp. concn	$R = 0.637$ ($P = 0.014$)	$R = 0.479$ ($P = 0.024$)	NS

[a] Only results of parameters which are significantly different ($P < 0.05$) between two or three supplies are presented.
[b] NS: not significant, $P > 0.05$.

the protozoan-related OTUs in the clone libraries of distributed water of supply CA-3 clustered within the phylum of the Cercozoa, whereas a large and even distribution of Amoebozoa (33%), Ciliophora (25%), and Cercozoa (25%) was observed in the clone libraries of distributed water of supply CA-2. A few OTUs clustering within the Choanozoa phylum were obtained from supplies CA-1 and CA-2 (Fig. 3). The OTUs related to the Stramenopiles clustered with flagellates and algae observed in freshwater or soils, although many Stramenopiles types occur in marine environments (3, 37).

Of the 59 OTUs of FLP which showed the highest similarity to *H. vermiformis* and *Hemiophrys procera*, 2 (3%) were obtained from all three supplies (see Table S1 in the supplemental material for details). Of these 59 OTUs, 9 (15%) were obtained from the clone libraries of two supplies. Approximately 30% of the OTUs of supply CA-1 were observed in more than one sample and about 20% of the OTUs in supplies CA-2 and CA-3 were observed at more than one location in the distribution system. Hence, the protozoan communities in the three supplies differed from each other and between locations within one supply. *H. vermiformis* predominated in the clone libraries of distributed water in four of five samples from supply CA-1, in two of nine samples from supply CA-2, and in one of six samples from supply CA-3. OTUs with the highest similarity to *Acanthamoeba* spp. and to candidate hosts for *L. pneumophila, viz., Echinamoeba exundans, Echinamoeba thermarum*, and *Neoparamoeba* spp., were observed in supplies CA-1 and CA-2.

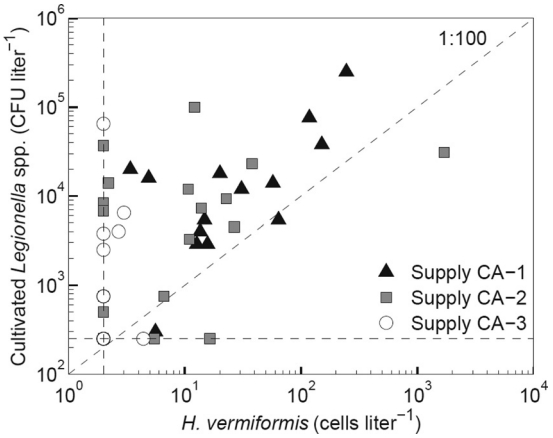

FIG. 2. Concentrations of *H. vermiformis* and cultivated *Legionella* spp. in distributed water of supplies CA-1, CA-2, and CA-3. Detection limits for *H. vermiformis* (2 cells liter^{-1}) and for cultivated *Legionella* spp. (250 CFU liter^{-1}) are shown as dotted lines.

Fungi and other small eukaryotes. A total of 83 OTUs (43%) showed the highest similarity to fungi, 46 of which clustered within the Ascomycota, the predominating fungus (≥49%) in all three supplies (Table 4; see also Table S3 in the supplemental material). One OTU with >99% similarity to the potential pathogen *Mucor racemosus* was obtained from treated water of supply CA-1, and two OTUs with >99% similarity to *M. racemosus* and the potential pathogen *Malassezia restricta* were obtained from distributed water of supply CA-2.

The metazoa were represented by 29 OTUs, 19 (66%) of which showed the highest similarity to species of nematodes (Table 4; see also Table S4 in the supplemental material). From supply CA-1 only one metazoan OTU, which clustered with Rotifera, was retrieved, and the estimated average OTU richness for metazoa in this supply (1 OTU) was significantly lower than in the supplies CA-2 (40 OTUs) and CA-3 (34 OTUs).

Ten OTUs, obtained from all three supplies, clustered within the Cryptophyta phylum and one of these OTUs, with 93% similarity to *Chroomonas* sp., was obtained from all three supplies (Table 4; see also Table S5 in the supplemental material). A total of eight OTUs, obtained from all three distributed water types, showed the highest similarity to viridiplantae. Six (3%) of the 195 obtained OTUs had similarities below 75% for described sequences in the SILVA database and remained unidentified, as described before (51).

These observations show that the eukaryotic communities and the concentrations of cultivable *Legionella* spp. differed in the investigated supplies all using seawater as a source.

TABLE 4. Classification of eukaryotic clones with >75% similarity to sequences in the SSU Ref SILVA98 database obtained from distributed water of supplies CA-1, CA-2, and CA-3[a]

Kingdom, subkingdom, or group	Distributed water CA-1			Distributed water CA-2			Distributed water CA-3			All analyzed samples		
	No. of OTUs[b]	% of OTUs	% of clones in libraries	No. of OTUs[b]	% of OTUs	% of clones in libraries	No. of OTUs[b]	% of OTUs	% of clones in libraries	No. of OTUs[b]	% of OTUs	% of clones in libraries
Free-living protozoa	23	36.5	36.0	36	33.6	25.5	13	25.0	14.6	59	30.3	24.8
Fungi	33	52.4	53.3	41	38.3	35.1	17	32.7	15.0	83	42.6	33.5
Metazoa	1	1.6	3.3	22	20.6	34.2	9	17.3	47.9	29	14.9	30.9
Cryptophyta and Viridiplantae	2	3.2	2.3	7	6.5	4.7	12	23.1	21.7	18	9.2	9.1
Sequences with <75% similarity	4	6.3	5.1	1	0.9	0.5	1	1.9	0.7	6	3.1	1.7
Total	63	100	100	107	100	100	52	100	100	195	100	100

[a] Data are totals for all analyzed samples of distributed water of the indicated supply.
[b] OTUs obtained from more than one sample are included only once. Each OTU contains 18S rRNA gene sequences with a minimum of 99% similarity.

DISCUSSION

Detection of free-living protozoa with PCR-based methods. In the present study, molecular techniques targeting the 18S rRNA gene were instrumental for the detection and identification of a large variety of small eukaryotes, including potential protozoan hosts for *Legionella* spp., in three drinking water supplies in the Caribbean at water temperatures of about 30°C. The relative abundances of different sequences in the clone libraries may not represent the community composition because different eukaryotic species can largely differ in 18S rRNA gene copy numbers, in particular in metazoa (25). For obtaining quantitative information, specific qPCR assays were used for the detection of two groups of FLP serving as environmental hosts for *L. pneumophila, viz*., *H. vermiformis* and *Acanthamoeba* spp.

H. vermiformis was observed with the specific qPCR (23) in all samples from which clones with ≥99% similarity to this organism were retrieved. However, such clones were not obtained from a few samples of supplies CA-2 and CA-3 which were positive with the qPCR for *H. vermiformis.* Obviously, the eukaryotic communities in these samples were predominantly populated by other organisms. Clones with 82 to 86% similarity to *Acanthamoeba* spp. were retrieved from several samples of supplies CA-2 and CA-3, but the specific qPCR for *Acanthamoeba* spp. (35) was negative in these samples. These 18S rRNA genes were not amplified with the *Acanthamoeba* genus-specific primers, suggesting that these sequences did not represent *Acanthamoeba* spp. These observations confirm the utility of qPCR methods for detecting specific FLP.

Conditions affecting microbial growth and protozoan richness in the three supplies. The water quality of the three supplies, using seawater as a source, is influenced by the treatment processes, e.g., type of desalination, filtration processes, softening, addition of corrosion inhibitors or chlorine, and the conditions in the distribution system, e.g., pipe materials, hydraulics, and residence time. Treated water from the examined treatment plants, all using seawater as a source, contained a very low concentration of natural organic matter (NOM, <0.1 mg C liter^{-1}) and had a low turbidity (Table 1). Still, the three supplies differed in concentrations of ATP, *H. vermiformis*, colony-forming *Legionella* spp., and compositions of communities of free-living protozoa and other eukaryotes in the distributed water (Tables 3 and 5). These parameters also varied between the different locations within one supply area, demonstrating the complexity of the interactions with environmental conditions. Comparison of the observations in the three

TABLE 5. Numbers of retrieved clones and OTUs and estimated richness of OTUs (sequence similarity of ≥99%) in distributed water of supplies CA-1, CA-2, and CA-3[d]

Type and source of organism	No. of clones	No.[a] of OTUs identified	Coverage index[b]	Total estimated OTU richness (Chao1)[c]		
				Mean	Lower limit	Upper limit
Eukaryotes in clone libraries						
Distributed water CA-1	214	63	30	129	89	236
Distributed water CA-2	427	107	25	222	165	337
Distributed water CA-3	267	52	19	118	81	209
Total	908	195	21	452	347	633
Free-living protozoa in clone libraries						
Distributed water CA-1	77	23	30	42	27	95
Distributed water CA-2	109	36	33	59	43	110
Distributed water CA-3	39	13	33	22	15	58
Total	225	59	26	111	79	190

[a] OTUs obtained from more than one sample are included only once; therefore, the sum of the OTUs in the three supplies gives excess values.
[b] Number of OTUs/number of sequences × 100%.
[c] The Chao1 index (7) was calculated with DOTUR (40). Chao1 estimation is based on the total of the clones; therefore, the sums of the estimated values per supply give other values.
[d] Data are totals for all analyzed samples of distributed water of the indicated water supply.

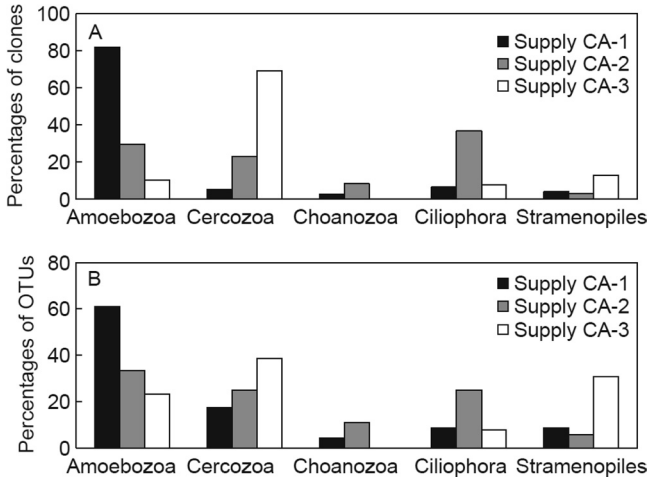

FIG. 3. (A) Taxonomic distribution of free-living protozoa, based on 18S rRNA gene clones retrieved from distributed water of supplies CA-1, CA-2, and CA-3. (B) Taxonomic distribution of OTUs with highest similarity to free-living protozoa retrieved from distributed water of supplies CA-1, CA-2, and CA-3. Data are totals for all analyzed samples of distributed water of the indicated supply.

Caribbean supplies with those in a similar study of two water supplies in the Netherlands (51) and the typical behavior of certain identified organisms enable the determination of several conditions affecting the microbial communities.

The water temperature in the Caribbean supplies was 10 to 15°C above the temperature in temperate regions. Temperature can affect the identity of the FLP in drinking water supplies, but in both regions, clones clustered within Amoebozoa, Cercozoa, Choanozoa, Ciliophora, and Stramenopiles (Fig. 3) (33, 50, 51). However, sequences related to Euglenozoa and Myzozoa were observed only in the temperate region, although certain euglenozoan types have been found in a volcanic area at a temperature above 30°C (44). The clear differences between the taxonomic distributions within the FLP communities in the three Caribbean supplies (Fig. 3) demonstrate that certain environmental conditions other than temperature affect the abundance and community composition of FLP in water supplies.

The varying ATP concentrations, combined with the low turbidity and the low iron concentration in distributed water of supply CA-1, suggest that microbial growth mainly occurs in biofilms on the walls of reservoirs and pipes of this supply (Table 2). This suggestion is supported by the relatively high concentrations of *H. vermiformis* and the large proportion and high richness of Amoebozoa in this supply, because amoebae feed much more effectively on microorganisms in a biofilm than on suspended prey (32). Furthermore, the absence of metazoa in most samples of supply CA-1 indicates that concentrations of sediments, which are needed for their growth, were low (53).

The high turbidities in supply CA-2 correlate with iron concentrations, indicating that sediments originate from corroding cast iron pipes (Tables 2 and 3). The correlations between turbidity and ATP and between turbidity and cultivable *Legionella* spp. demonstrate that these sediments support microbial growth. In comparison with supplies of CA-1 and CA-3, sequences related to Ciliophora constituted a relatively high proportion (25%) of OTUs of the FLP in supply CA-2 (Fig. 3). Ciliates feed effectively on suspended bacteria, and their relatively large cell size enables these organisms to consume a large variety of prey types, such as algae, flagellates, and other ciliates (32). In addition, a large number of metazoan OTUs, namely, 22, were observed in supply CA-2 (Table 4). Ciliates and metazoa also constituted significant proportions of the eukaryotic community in the distribution system of a groundwater supply in the Netherlands, with elevated concentrations of ATP (10 ng liter^{-1}) and NOM (8 mg C liter^{-1}) (51). Obviously, these conditions and accumulation of sediments promote the growth of metazoa, ciliates, and also cultivable *Legionella* spp.

At most locations in supply CA-3, low turbidities (<0.5 NTU) and low concentrations of iron (<0.05 mg liter^{-1}), ATP (<1 ng liter^{-1}), *H. vermiformis* (<2 cells liter^{-1}), and cultivable *Legionella* spp. (<1 × 10^3 CFU liter^{-1}) were observed (Tables 2 and 3). Elevated ATP concentrations (>4 ng liter^{-1}) at three locations, indicating local accumulation of biomass, did not all correspond with elevated turbidity. Small flagellated cercozoan types, mainly *Cercomonas* spp., predominated (69% of clones) in the free-living protozoan communities in the clone libraries of supply CA-3 (Fig. 3). These flagellates can produce pseudopodia which attach to surfaces, but they preferentially feed on suspended prey (28, 32). In an experimental distribution system, flagellates predominated in the drinking water but were absent in the related biofilm (43). Cercozoan types also predominated in the biofilm in a groundwater supply in the Netherlands with low concentrations of ATP (<1 ng liter^{-1}) and NOM (<0.5 mg C liter^{-1}), but they were a minor fraction in a groundwater supply with elevated concentrations of ATP and NOM (51).

No significant correlation was observed between the concentrations of ATP and *H. vermiformis* in the water samples collected from the three Caribbean supplies (Table 3). In supply CA-3, the *H. vermiformis* concentration was below the detection level (<2 cell liter^{-1}) in all but one of the samples with an ATP concentration below 1 ng liter^{-1}. Also, this organism was not detected in water and biofilms in the distribution system of a groundwater supply in the Netherlands with ATP concentrations of <1 ng liter^{-1} and low biofilm concentrations (51). *H. vermiformis* was observed at concentrations up to 815 cells liter^{-1} in the summer in the Netherlands in distributed water with elevated concentrations of NOM and ATP. These observations suggest that growth of *H. vermiformis* in drinking water distribution systems is limited at ATP concentrations of <1 ng liter^{-1}.

Host protozoa, pathogenic free-living protozoa, and fungi. The detection of *H. vermiformis, Acanthamoeba* spp., and *L. pneumophila* in the investigated supplies is consistent with results of other studies on drinking water systems in tropical regions (3, 5, 31, 39, 45). The current study confirmed that *H. vermiformis* is a much more common amoeba in drinking water than *Acanthamoeba* spp. (50). This difference may in part be explained by the higher yield of *H. vermiformis* than *Acanthamoeba* spp. when feeding on prey bacteria (58).

The colony counts of *Legionella* spp. in supplies CA-1 and CA-2 correlated significantly with the concentration of *H. vermiformis* (Fig. 2 and Table 3). The log value of the ratios

between the concentrations of *Legionella* spp. and *H. vermiformis* in distributed water samples containing both organisms ranged from 1.2 to 3.9. These values are below the upper tolerance limit of 4.5 as determined in biofilm batch tests using different types of freshwater inoculated with *L. pneumophila* and *H. vermiformis* and incubated at 37°C (50). Above this upper tolerance limit, protozoan hosts for growth of *L. pneumophila* other than *H. vermiformis* were observed in these tests. The observations of the present study thus confirm the prominent position of *H. vermiformis* as a host for *L. pneumophila* in freshwater environments (13, 24, 50, 56). In all three supplies, OTUs related to the described hosts *E. exundans* (13) and *Acanthamoeba* spp. (1, 38) were detected in samples with *H. vermiformis*, indicating that more than one protozoan species may have served as a host for *Legionella* spp. at these locations. Also the candidate hosts *Neoparamoeba* sp. and *E. thermarum* (50) were observed in the present study, but none of the other protozoa were identified as hosts by using *in vitro* experiments (13, 22, 42). However, certain protists belonging to the genera *Naegleria* and *Vahlkampfia*, which include hosts for *L. pneumophila* (37, 49) and/or human pathogens (59), were not amplified with the primers used. *Acanthamoeba* spp. have been identified as opportunistic human pathogens (9, 21), but it is unclear whether the sequences related to such species represent organisms with pathogenic characteristics.

Fungi, metazoa, viridiplantae, and Chryptophyta species are relatively common in drinking water distribution systems (15, 16, 20, 51). Clones with >99% similarity to the pathogenic fungi *Mucor racemosus* and *Malassezia restricta* (17, 46) were obtained from supplies CA-1 and CA-2. OTUs clustering with the genera *Basidiobolus, Candida, Pichia*, and *Penicillium*, which include pathogenic species (2, 10, 30, 60), were also obtained from the distributed water of all three supplies. However, the public health significance of the presence of fungi related to pathogenic species is not clear (16, 51).

In conclusion, highly diverse communities of free-living protozoa and other small eukaryotes were observed in the three investigated supplies. The growth of these organisms and *Legionella* spp. is enhanced by biofilms and corrosion-related sediments. An ATP concentration of <1 ng liter^{-1} in drinking water indicates growth-limiting conditions for *H. vermiformis*. Limiting the multiplication of *Legionella* spp. therefore implies reduction of the growth potential of the water and prevention of sediment accumulation.

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REFERENCES

1. **Anand, C. M., A. R. Skinner, A. Malic, and J. B. Kurtz.** 1983. Interaction of *L. pneumophilia* and a free living amoeba (*Acanthamoeba palestinensis*). J. Hyg. (Lond.) **91:**167–178.
2. **Bergman, M. M., D. Gagnon, and G. V. Doern.** 1998. *Pichia ohmeri* fungaemia. Diagn. Microbiol. Infect. Dis. **30:**229–231.
3. **Bonilla-Lemus, P., et al.** 2010. *Acanthamoeba* spp. in domestic tap water in houses of contact lens wearers in the metropolitan area of Mexico City. Exp. Parasitol. **126:**54–58.
4. **Bonnélye, V., et al.** 2007. Curacao, Netherlands Antilles: a successful example of boron removal on a seawater desalination plant. Desalination **205:** 200–205.
5. **Carlesso, A. M., A. B. Simonetti, G. L. Artuso, and M. B. Rott.** 2007. Isolation and identification of potentially pathogenic free-living amoebae in samples from environments in a public hospital in the city of Porto Alegre, Rio Grande do Sul. Rev. Soc. Bras. Med. Trop. **40:**316–320. (In Portuguese.)
6. **Cavalier-Smith, T.** 2002. The phagotrophic origin of eukaryotes and phylogenetic classification of Protozoa. Int. J. Syst. Evol. Microbiol. **52:**297–354.
7. **Chao, A.** 1987. Estimating the population size for capture-recapture data with unequal catchability. Biometrics **43:**783–791.
8. **Cowgill, K. D., et al.** 2005. Recurrence of legionnaires disease at a hotel in the United States Virgin Islands over a 20-year period. Clin. Infect. Dis. **40:**1205–1207.
9. **Culbertson, C. G.** 1961. Pathogenic *Acanthamoeba* (*Hartmanella*). Am. J. Clin. Pathol. **35:**195–202.
10. **Deng, Z., J. L. Ribas, D. W. Gibson, and D. H. Connor.** 1988. Infections caused by *Penicillium marneffei* in China and Southeast Asia: review of eighteen published cases and report of four more Chinese cases. Rev. Infect. Dis. **10:**640–652.
11. **Edelstein, P. H.** 1981. Improved semiselective medium for isolation of *Legionella pneumophila* from contaminated clinical and environmental specimens. J. Clin. Microbiol. **14:**298–303.
12. **Fields, B. S., R. F. Benson, and R. E. Besser.** 2002. *Legionella* and Legionnaires' disease: 25 years of investigation. Clin. Microbiol. Rev. **15:**506–526.
13. **Fields, B. S., et al.** 1989. Intracellular multiplication of *Legionella pneumophila* in amoebae isolated from hospital hot water tanks. Curr. Microbiol. **18:**131–137.
14. **Goh, K. T., D. L. Ng, J. Yap, S. Ma, and E. E. Ooi.** 2005. Surveillance, prevention, and control of legionellosis in a tropical city-state. Am. J. Infect. Control **33:**286–291.
15. **Hageskal, G., A. K. Knutsen, P. Gaustad, G. S. de Hoog, and I. Skaar.** 2006. Diversity and significance of mold species in Norwegian drinking water. Appl. Environ. Microbiol. **72:**7586–7593.
16. **Hageskal, G., N. Lima, and I. Skaar.** 2009. The study of fungi in drinking water. Mycol. Res. **113:**165–172.
17. **Hata, D. J., S. P. Buckwalter, B. S. Pritt, G. D. Roberts, and N. L. Wengenack.** 2008. Real-time PCR method for detection of zygomycetes. J. Clin. Microbiol. **46:**2353–2358.
18. **Hughes, J. B., J. J. Hellmann, T. H. Ricketts, and B. J. Bohannan.** 2001. Counting the uncountable: statistical approaches to estimating microbial diversity. Appl. Environ. Microbiol. **67:**4399–4406.
19. **Inglis, T. J., et al.** 2004. Preliminary report on the northern Australian melioidosis environmental surveillance project. Epidemiol. Infect. **132:**813–820.
20. **Jjemba, P. K., L. A. Weinrich, W. Cheng, E. Giraldo, and M. W. Lechevallier.** 2010. Regrowth of potential opportunistic pathogens and algae in reclaimed water distribution systems. Appl. Environ. Microbiol. **76:**4169–4178.
21. **Jones, D. B., G. S. Visvesvara, and N. M. Robinson.** 1975. *Acanthamoeba polyphaga* keratitis and *Acenthamoeba uveitis* associated with fatal meningoencephalitis. Trans. Ophthalmol. Soc. U. K. **95:**221–232.
22. **Kikuhara, H., M. Ogawa, H. Miyamoto, Y. Nikaido, and S. Yoshida.** 1994. Intracellular multiplication of *Legionella pneumophila* in *Tetrahymena thermophila*. J. UOEH **16:**263–275.
23. **Kuiper, M. W., et al.** 2006. Quantitative detection of the free-living amoeba *Hartmannella vermiformis* in surface water by using real-time PCR. Appl. Environ. Microbiol. **72:**5750–5756.
24. **Kuiper, M. W., B. A. Wullings, A. D. Akkermans, R. R. Beumer, and D. van der Kooij.** 2004. Intracellular proliferation of *Legionella pneumophila* in *Hartmannella vermiformis* in aquatic biofilms grown on plasticized polyvinyl chloride. Appl. Environ. Microbiol. **70:**6826–6833.
25. **Long, E. O., and I. B. Dawid.** 1980. Repeated genes in eukaryotes. Annu. Rev. Biochem. **49:**727–764.
26. **Ludwig, W., et al.** 2004. ARB: a software environment for sequence data. Nucleic Acids Res. **32:**1363–1371.
27. **Magic-Knezev, A., and D. van der Kooij.** 2004. Optimisation and significance of ATP analysis for measuring active biomass in granular activated carbon filters used in water treatment. Water Res. **38:**3971–3979.
28. **Myl'nikov, A. P., and S. A. Karpov.** 2004. Review of diversity and taxonomy of cercomonads. Protistology **3:**201–217.
29. **Nedelands Normalisatie Instituut.** 1991. Bacteriological examination of water: examination on the presence and the number of colony forming units

(CFU) of *Legionella* bacteria. NEN6265. Nederlands Normalisatie Instituut, Delft, the Netherlands.
30. **Odds, F. C.** 1987. *Candida* infections: an overview. Crit. Rev. Microbiol. **15:**1–5.
31. **Ortiz-Roque, C. M., and T. C. Hazen.** 1987. Abundance and distribution of Legionellaceae in Puerto Rican waters. Appl. Environ. Microbiol. **53:**2231–2236.
32. **Parry, J. D.** 2004. Protozoan grazing of freshwater biofilms. Adv. Appl. Microbiol. **54:**167–196.
33. **Poitelon, J. B., et al.** 2009. Identification and phylogeny of eukaryotic 18S rDNA phylotypes detected in chlorinated finished drinking water samples from three Parisian surface water treatment plants. Lett. Appl. Microbiol. **49:**589–595.
34. **Pruesse, E., et al.** 2007. SILVA; a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. **35:**7188–7196.
35. **Qvarnstrom, Y., G. S. Visvesvara, R. Sriram, and A. J. da Silva.** 2006. Multiplex real-time PCR assay for simultaneous detection of *Acanthamoeba* spp., *Balamuthia mandrillaris*, and *Naegleria fowleri.* J. Clin. Microbiol. **44:** 3589–3595.
36. **Rodriguez-Zaragoza, S.** 1994. Ecology of free-living amoebae. Crit. Rev. Microbiol. **20:**225–241.
37. **Rowbotham, T. J.** 1986. Current views on the relationships between amoebae, legionellae and man. Isr. J. Med. Sci. **22:**678–689.
38. **Rowbotham, T. J.** 1980. Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. J. Clin. Pathol. **33:**1179–1183.
39. **Schlech, W. F., G. W. Gorman, M. C. Payne, and C. V. Broome.** 1985. Legionnaires' disease in the Caribbean. An outbreak associated with a resort hotel. Arch. Intern. Med. **145:**2076–2079.
40. **Schloss, P. D., and J. Handelsman.** 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl. Environ. Microbiol. **71:**1501–1506.
41. **Scrimini, S., A. Junemann, and C. M. Luna.** 2007. Community acquired pneumonia in the tropics. Curr. Opin. Pulm. Med. **13:**170–176.
42. **Shadrach, W. S., et al.** 2005. *Balamuthia mandrillaris*, free-living ameba and opportunistic agent of encephalitis, is a potential host for *Legionella pneumophila* bacteria. Appl. Environ. Microbiol. **71:**2244–2249.
43. **Sibille, I., T. Sime-Ngando, L. Mathieu, and J. C. Block.** 1998. Protozoan bacterivory and *Escherichia coli* survival in drinking water distribution systems. Appl. Environ. Microbiol. **64:**197–202.
44. **Sittenfeld, A., et al.** 2002. Characterization of a photosynthetic Euglena strain isolated from an acidic hot mud pool of a volcanic area of Costa Rica. FEMS Microbiol. Ecol. **42:**151–161.
45. **Storey, M. V., C. E. Kaucner, M. L. Angles, J. R. Blackbeard, and N. J. Ashbolt.** 2008. Opportunistic pathogens in drinking and recycled water distribution systems. Water **35:**38–45.
46. **Sugita, T., M. Tajima, M. Amaya, R. Tsuboi, and A. Nishikawa.** 2004. Genotype analysis of *Malassezia restricta* as the major cutaneous flora in patients with atopic dermatitis and healthy subjects. Microbiol. Immunol. **48:**755–759.
47. **Thomas, J. M., and N. J. Ashbolt.** 2011. Do free-living amoebae in treated drinking water systems present an emerging health risk? Environ. Sci. Technol. **45:**860–869.
48. **Thomas, V., G. McDonnell, S. P. Denyer, and J. Y. Maillard.** 2009. Free-living amoebae and their intracellular pathogenic microorganisms: risks for water quality. FEMS Microbiol. Rev. **34:**231–259.
49. **Tyndall, R. L., and E. L. Domingue.** 1982. Cocultivation of *Legionella pneumophila* and free-living amoebae. Appl. Environ. Microbiol. **44:**954–959.
50. **Valster, R. M., B. A. Wullings, and D. van der Kooij.** 2010. Detection of protozoan hosts for *Legionella pneumophila* in engineered water systems by using a biofilm batch test. Appl. Environ. Microbiol. **76:**7144–7153.
51. **Valster, R. M., B. A. Wullings, G. Bakker, H. Smidt, and D. van der Kooij.** 2009. Free-living protozoa in two unchlorinated drinking water supplies identified by phylogenetic analysis of 18S rRNA gene sequences Appl. Environ. Microbiol. **75:**4736–4746.
52. **Van der Kooij, D.** 1998. Potential for biofilm development in drinking water distribution systems. J. Appl. Microbiol. **85**(Suppl.):39S–44S.
53. **Van Lieverloo, J. H. M., D. van der Kooij, and W. Hoogenboezem.** 2002. Invertebrates and protozoa (free-living) in drinking water distribution systems, p. 1718–1733. *In* G. Bitton (ed.), Encyclopedia of environmental microbiology. Wiley, New York, NY.
54. **Visvesvara, G. S., F. L. Schuster, and A. J. Martinez.** 1993. *Balamuthia mandrillaris*, N. G., N. Sp., agent of amebic meningoencephalitis in humans and other animals. J. Eukaryot. Microbiol. **40:**504–514.
55. **Volk, C. J., and M. W. LeChevallier.** 1999. Impacts of the reduction of nutrient levels on bacterial water quality in distribution systems. Appl. Environ. Microbiol. **65:**4957–4966.
56. **Wadowsky, R. M., et al.** 1988. Growth-supporting activity for *Legionella pneumophila* in tap water cultures and implication of hartmannellid amoebae as growth factors. Appl. Environ. Microbiol. **54:**2677–2682.
57. **Wadowsky, R. M., R. Wolford, A. M. McNamara, and R. B. Yee.** 1985. Effect of temperature, pH, and oxygen level on the multiplication of naturally occurring *Legionella pneumophila* in potable water. Appl. Environ. Microbiol. **49:**1197–1205.
58. **Weekers, P. H., P. L. Bodelier, J. P. Wijen, and G. D. Vogels.** 1993. Effects of grazing by the free-living soil amoebae *Acanthamoeba castellanii, Acanthamoeba polyphaga*, and *Hartmannella vermiformis* on various bacteria. Appl. Environ. Microbiol. **59:**2317–2319.
59. **Willaert, E.** 1974. Primary amoebic meningo-encephalitis. A selected bibliography and tabular survey of cases. Ann. Soc. Belg. Med. Trop. **54:**429–440.
60. **Zavasky, D. M., W. Samowitz, T. Loftus, H. Segal, and K. Carroll.** 1999. Gastrointestinal zygomycotic infection caused by *Basidiobolus ranarum*: case report and review. Clin. Infect. Dis. **28:**1244–1248.