

Detection of Protozoan Hosts for *Legionella pneumophila* in Engineered Water Systems by Using a Biofilm Batch Test^{∇†}

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Legionella pneumophila proliferates in aquatic habitats within free-living protozoa, 17 species of which have been identified as hosts by using *in vitro* experiments. The present study aimed at identifying protozoan hosts for *L. pneumophila* by using a biofilm batch test (BBT). Samples (600 ml) collected from 21 engineered freshwater systems, with added polyethylene cylinders to promote biofilm formation, were inoculated with *L. pneumophila* and subsequently incubated at 37°C for 20 days. Growth of *L. pneumophila* was observed in 16 of 18 water types when the host protozoan *Hartmannella vermiformis* was added. Twelve of the tested water types supported growth of *L. pneumophila* or indigenous *Legionella anisa* without added *H. vermiformis*. In 12 of 19 BBT flasks *H. vermiformis* was indicated as a host, based on the ratio between maximum concentrations of *L. pneumophila* and *H. vermiformis*, determined with quantitative PCR (Q-PCR), and the composition of clone libraries of partial 18S rRNA gene fragments. Analyses of 609 eukaryotic clones from the BBTs revealed that 68 operational taxonomic units (OTUs) showed the highest similarity to free-living protozoa. Forty percent of the sequences clustering with protozoa showed ≥99.5% similarity to *H. vermiformis*. None of the other protozoa serving as hosts in *in vitro* studies were detected in the BBTs. In several tests with growth of *L. pneumophila*, the protozoa *Diphyllia rotans*, *Echinamoeba thermanum*, and *Neoparamoeba* sp. were identified as candidate hosts. *In vitro* studies are needed to confirm their role as hosts for *L. pneumophila*. Unidentified protozoa were implicated as hosts for uncultured *Legionella* spp. grown in BBT flasks at 15°C.

Legionella pneumophila, the causative agent of Legionnaires' disease, is a common inhabitant of natural freshwater environments and human-made water systems, including cooling towers, whirlpools, air-conditioning systems, and installations for warm tap water (14). In the aquatic environment *L. pneumophila* proliferates within certain free-living protozoa, which serve as its hosts (15, 30, 59). Environmental factors favoring the growth and survival of *L. pneumophila* in freshwater systems include a water temperature between 20°C and 45°C (41, 60) and the presence of biofilms and sediments on which the protozoan hosts can graze (30, 41, 56).

Rowbotham (44) was the first to report the growth of *L. pneumophila* within free-living amoebae, which belonged to the genera *Acanthamoeba* and *Naegleria*. *In vitro* studies with cocultures have revealed that 14 species of amoebae, viz., *Acanthamoeba* spp. (1, 35, 44, 53), *Balamuthia mandrillaris* (47), *Echinamoeba exundans* (15), *Hartmannella* spp. (43), *Naegleria* spp. (38, 44, 53), and *Vahlkampfia jugosa* (43); the slime mold *Dictyostelium discoideum* (20, 48); and two species of the ciliate genus *Tetrahymena* (15, 26) can serve as hosts for *L. pneumophila*. Recently, it has been reported that *L. pneumophila* can also replicate within the intestinal tract of the microbiovorous nematode *Caenorhabditis elegans* (3).

A number of the free-living protozoa mentioned above and

others, e.g., *Vannella* spp. and *Saccamoeba* spp., have been observed in aquatic environments from which *L. pneumophila* was cultivated or in which it was detected with PCR (4, 42, 51, 52). However, it remains unknown which of these protozoa actually serve as hosts for *L. pneumophila* in the aquatic environment, including human-made water systems. Moreover, it cannot be excluded that free-living protozoa other than those tested *in vitro* can serve as hosts for *L. pneumophila* as well. Information is also lacking about protozoan hosts for *Legionella anisa* (13, 49), which is frequently present in water installations in temperate regions (11, 62). Furthermore, it is unknown which free-living protozoa serve as hosts for uncultured *Legionella* bacteria that can grow at temperatures of about 15°C (61; B. A. Wullings, G. Bakker, and D. van der Kooij, submitted for publication).

L. pneumophila can proliferate in samples of surface water, effluent of wastewater treatment plants, potable water, and water from cooling towers incubated at 25°C, 35°C, or 37°C (28, 45, 56). Consequently, incubation of freshwater samples can be used to amplify protozoan hosts for *L. pneumophila* and other *Legionella* spp. In this study, different human-made water types were investigated using a biofilm batch test (BBT) system to (i) amplify and subsequently identify predominating, known, and yet-undescribed hosts for *L. pneumophila* and (ii) identify potential protozoan hosts for *Legionella* bacteria that can grow at 15°C.

MATERIALS AND METHODS

Experimental setup of the BBT system. A biofilm batch test (BBT) was used to amplify and subsequently identify protozoan hosts for *L. pneumophila*. The BBT consists of well-cleaned, heat-sterilized (4 h at 150°C) Pyrex glass Schott flasks (1 liter) with 600 ml of test water and polyethylene cylinders that were

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TABLE 1. Characteristics of the water types tested and results for the controls in the BBT incubated at 37°C

Origin of sample	Temp (°C) ^a	Concn of ATP (ng liter ⁻¹) ± SD ^{a,d}	Concn of <i>H. vermiformis</i> (cells liter ⁻¹) ± SD ^{a,d}	Growth of <i>L. pneumophila</i> in control flask of BBT ^b	<i>L. pneumophila</i> / <i>H. vermiformis</i> ratio (log) in control flask of BBT ^c
Drinking water supplies					
Groundwater supply A					
Treated water	11	<1	<0.5	ND ^f	NA ^g
Biomass from filter bed (limestone)	11	4.6 ± 0.04 ^e	<2 ^e	+	2.1; 2.5
Installation A1	39	1.9 ± 0.3	51 ± 14	+	3.6
Installation A2	37	3.3 ± 0.2	8.2 ± 2.2	+	2.9
Groundwater supply B					
Treated water	11	6.7 ± 0.3	<0.5	ND	NA
Biomass from filter bed (sand)	11	52.6 ± 0.7 ^e	11.4 ± 7.6 ^e	+	2.4; 3.5
Installation B1	35	4.1 ± 0.2	6.8 ± 2.5	+	2.4
Installation B2	37	7.8 ± 0.9	1,530 ± 290	+	3.3
Flushed tap water	12	16.0 ± 3.3	805 ± 74	+	3.0
Groundwater supply C; tap water	18	3.3 ± 0.4	<4	+	3.1
Surface water supply D					
Biomass from filter bed (granular activated carbon)	5	22.1 ± 1.3 ^e	5.0 ± 1.8 ^e	+	2.6
Biomass from filter bed (sand)	5	55.5 ± 4.3 ^e	12.3 ± 1.7 ^e	+	2.0
Surface water					
Storage reservoir for surface water supply D	5	14.2 ± 0.5	<6.7	+	3.5
Water from Rhine River (autumn)	10	83.2 ± 5.4	<10	ND	NA
Water from Rhine River (winter)	4	61.1 ± 2.4	<10	+	3.0
Treated sewage	15	705.8 ± 4.9	28.0 ± 10.8	+	1.9
Water from cooling tower					
Cooling tower 1 (pH 7.3)	27	101.8 ± 1.5	<6.6	+	2.6
Cooling tower 2 (pH 8.5)	9	35.7 ± 0.3	<2	+	3.2
Cooling tower 3 (pH 7.8)	24	19.0 ± 0.1	<2	-	-
Cooling tower 4 (pH 6.6)	19	14.7 ± 0.6	<2	-	-
Cooling tower 5 (pH 8.5)	47	55.8 ± 4.9	19 ± 1.6	+	2.9

^a At time of sampling.

^b +, significant growth ($P < 0.05$); -, no growth.

^c Log-transformed ratio between the maximum concentrations of *L. pneumophila* and *H. vermiformis*.

^d Standard deviations of the analysis are also shown.

^e Concentration per gram (wet weight) of filter bed material.

^f ND, not determined.

^g NA, not applicable.

incubated to develop a biofilm of indigenous and inoculated microorganisms. Initial tests were incubated with 12 cylinders of cross-linked polyethylene (PE-Xa) (diameter, 16 mm; each with surface area of about 9 cm²). In subsequent tests each BBT flask contained six gamma-irradiated (Isotron, Ede, Netherlands) polyethylene (PE-80) cylinders (diameter, 20 mm; each with a surface area of about 10 cm²). Nitrate and phosphate were added to each BBT flask at final concentrations of 72.5 μM and 13.5 μM, respectively, to prevent growth limitation by these nutrients.

L. pneumophila was inoculated at a concentration between 4.2×10^3 and 5.0×10^4 *mip* gene copies liter⁻¹ (6) to ensure the presence of this organism. Each test was carried out in duplicate flasks. The BBT flasks were incubated at 37°C (±1°C) in the dark for at least 20 days without shaking. The concentration and diversity of *Legionella* and eukaryotic communities in the planktonic phase and biofilm were monitored during the incubation period. Control BBTs in which flasks were inoculated with *L. pneumophila* and the protozoan host *Hartmannella vermiformis* were performed to verify if growth of *L. pneumophila* occurs under the test conditions in the investigated water type. *H. vermiformis* was inoculated at a concentration between 3.3×10^5 and 4.8×10^5 cells liter⁻¹. Control tests were done in single flasks.

A number of BBT flasks were additionally incubated at 15°C with tap water and with filtered tap water (3.0-μm-pore-size and 47-mm-diameter TSTP Isopore membrane [Millipore, Molsheim, France]) from groundwater supply C to test if growth of indigenous *Legionella* spp. occurred in the presence and the absence of free-living protozoa (Table 1). Furthermore, BBT flasks inoculated

with biomass from the filter beds of groundwater supplies A and B were incubated at 15°C to determine whether growth of indigenous *Legionella* bacteria occurred in the presence and the absence of inoculated *H. vermiformis*.

Preparation of inoculum for *L. pneumophila* and *H. vermiformis*. A suspension of *L. pneumophila* serogroup 1, grown for 7 days on buffered charcoal-yeast extract (BCYE) agar plates (12) at 37°C, was diluted in autoclaved tap water and used for inoculation (600 μl per flask). *H. vermiformis* (ATCC 50237) was axenically cultivated in modified PYNFH medium (ATCC 1034) for 2 weeks at 30°C (16) and was used as inoculum (600 μl per flask). Total direct cell counts of the inoculum suspensions of *L. pneumophila* and *H. vermiformis* and suspensions of protozoa for calibration curves were determined using acridine orange and epifluorescence microscopy (21).

Sample collection and preparation. Samples included biomass from four filter beds and treated water from three different drinking water supplies (A, B, and D), six tap water types from three supply areas (A, B, and C), two surface water types, the effluent of a wastewater treatment plant, and five cooling tower samples (Table 1; see Fig. S1 in the supplemental material for details of the treatment systems for the four drinking water supplies). The samples were collected in containers of sterile glass or polyethylene, stored at 4°C, and processed within 24 h.

In supply A, aerobic groundwater is aerated to remove CO₂, followed by limestone filtration to increase the pH and the hardness of the water (see Fig. S1 in the supplemental material for details). In supply B, anaerobic groundwater is treated by intensive aeration, rapid sand filtration, caustic dosage followed by

pellet softening, aeration, and a second stage of rapid sand filtration (54). In supply C, anaerobic groundwater is aerated, followed by rapid sand filtration. In supply D, seepage water is treated with iron(III) chloride, storage in a lake, rapid sand filtration, ozonation, pellet softening, granular activated carbon filtration, and slow sand filtration. All four drinking water types are treated and distributed without chemical disinfection (57).

The biomass from the filter beds of supply A (limestone filter), supply B (slow sand filter), and supply D (granular activated carbon filter and slow sand filter) was collected by adding 45 g (wet weight) of filter bed material to 900 ml of the associated treated water or autoclaved tap water, followed by low-energy sonication as previously described (33). Subsequently, the biomass suspension was diluted to obtain an initial ATP concentration in the BBT flask of 10 times the ATP concentration in the related treated water.

Biomass in samples of warm tap water (about 40 liters) was concentrated to about 5 liters with ultrafiltration using the hemoflow method (58). The effluent from a sewage plant was diluted 5-fold with autoclaved tap water, and the sample of flush water was diluted 3-fold before incubation in the BBT flask. All other samples were directly incubated in the BBT flask.

Microbiological analyses. The attached microorganisms were removed from the polyethylene cylinders and suspended in autoclaved tap water by using low-energy sonication as described elsewhere (30). Total ATP concentrations in the planktonic phase and in the biomass suspension, representing the active biomass, were determined as previously described (33).

Direct plating on BCYE medium and incubation at 37°C for detection of culturable *Legionella* bacteria (12, 37) were used in the initial BBT flasks inoculated with biomass from the filter beds of supplies A and B.

For DNA isolation, volumes of 50 to 200 ml of the planktonic samples and 50 to 100 ml of biofilm suspensions were filtered through a 0.22- μ m-pore-size and 55-mm-diameter polycarbonate Track-Etch membrane (Sortorius, Goettingen, Germany) to isolate the microorganisms. Subsequently, DNA was isolated according to previously described procedures (54). *L. pneumophila* bacteria were quantified at days 0, 3, 5, 10, and 20 of the incubation by quantitative PCR (Q-PCR) by applying the primers LpneuF and LpneuR and the specific TaqMan probe LpneuP as described earlier (Wullings et al., submitted). *Legionella* spp. were quantified with Q-PCR on the same sample days as was *L. pneumophila* with the primers LEG-225 and LEG-858 as described earlier (36). *L. anisa* was detected with the primers LaF (5'-CAATGTCTACTGTAATGGCAGC-3') and LaR (5'-AACCGCTTGGAGTACCGT-3') and the specific TaqMan probe LaP (5'-AGACGGAATGTCTGGTGCCCAATTGA-3') targeting the *mip* gene. The thermal cycling conditions were similar to the conditions of Q-PCR for *L. pneumophila*. All primers and probes were produced at Biolegio (Malden, Netherlands), and all Q-PCR assays were performed in 96-well plates in an I-cycler real-time PCR detection system (Bio-Rad, Veenendaal, Netherlands). Quantification was based on plasmid-based calibration curves. The concentrations of *Legionella* spp. are expressed in genome units (GU) liter⁻¹ or GU cm⁻². The concentrations of *L. pneumophila* and *L. anisa* are measured in *mip* gene copies liter⁻¹ or *mip* gene copies cm⁻².

Q-PCR assays for detection of *Acanthamoeba* spp. were performed with the primers AcantF900 and AcantR1100 (40) at days 0 and 10 after incubation and for *H. vermiformis* were performed with the primers Hv1227F and Hv1728R at all sample days (29). The quantification of *Acanthamoeba* spp. and *H. vermiformis* was based on calibration curves which were constructed by preparing 10-fold dilutions of DNA extracted from suspensions with known numbers of cells of *Acanthamoeba castellanii* (CCAP 1501) and *H. vermiformis* (ATCC 50237).

The diversity and composition of the eukaryotic communities in the planktonic and biofilm samples were determined by terminal restriction fragment length polymorphism (T-RFLP) analysis and sequence analysis of 18S rRNA gene fragments (\pm 550 bp) as described earlier (54). T-RFLP analysis was done for all BBT flasks at day 0 and after 20 days of incubation in planktonic samples of 50 to 200 ml and/or in biofilm suspensions of 50 to 100 ml. For each water type, this analysis was performed on the same day to minimize the experimental variation. From the BBT flasks incubated at 37°C and 15°C, a total of 820 eukaryotic clones, varying from 5 to 59 clones per library depending on the complexity of the band pattern of T-RFLP fingerprints, were analyzed. All 18S rRNA gene sequences obtained were grouped in operational taxonomic units (OTUs) with 99% similarity (46). The composition of the *Legionella* community in the biofilm of the BBT flasks incubated at 15°C was determined by sequence analysis of genus-specific 16S rRNA gene fragments (\pm 650 bp) of clones retrieved as described earlier (61). All 16S rRNA gene sequences obtained were grouped in OTUs with 97% similarity. From each BBT, about 45 16S rRNA gene sequences were analyzed.

The sequences obtained were compared to sequences in the National Center for Biotechnology (NCBI) database by BLAST search and imported and aligned

into the SSU Ref SILVA94 database released in April 2008 by using the ARB software package as previously described (32, 39). The eukaryotic sequences obtained were organized into taxa based on the classification system of Cavalier-Smith (5) and the structure in the SILVA database (39).

Statistical analysis. The analysis of variance (ANOVA) *F* test was used for the comparison of the concentrations of *L. pneumophila* and *Legionella* spp. measured with Q-PCR with the concentrations of cultivated *Legionella* bacteria in the BBT flasks with biomasses of supplies A and B. The statistical significance of the growth of *L. pneumophila*, *L. anisa*, and *H. vermiformis* populations in the BBT flasks between day 0 and day 10 (or day 20 depending on the time point of maximum concentration) was determined with the *t* test by using log-transformed concentrations. The Shapiro-Wilk normality test was applied to the log₁₀-transformed ratios of the maximum concentrations of *L. pneumophila* and *H. vermiformis* (*L. pneumophila*/*H. vermiformis* ratio) observed in the BBT flasks. From the normally distributed values for the *L. pneumophila*/*H. vermiformis* ratio of the control group results, the upper limit (tolerance limit) of the one-sided 95% confidence interval of the 99th percentiles was calculated.

Nucleotide sequence accession numbers. The 18S rRNA gene sequences determined in this study have been deposited in GenBank under accession numbers GU970094 to GU970913. The sequences of the *Legionella* 16S rRNA gene have been deposited in GenBank under accession numbers GU970914 to GU971083.

RESULTS

Development of the biofilm batch test (BBT) system. Despite the presence of *H. vermiformis*, no growth of *L. pneumophila* was observed in initial BBT flasks incubated at 37°C with biomass from the filter beds of supply A (initial concentration of 7.9 ng ATP liter⁻¹) and supply B (131.7 ng ATP liter⁻¹), without inoculation with *L. pneumophila* (Fig. 1). Furthermore, inoculated *L. pneumophila* did not multiply in the BBT flasks with two types of treated water without exogenous addition of *H. vermiformis*. In contrast, significant growth ($P < 0.05$) of *L. pneumophila* was observed in both BBT flasks with water from cooling tower 1 in which *H. vermiformis* was not detected at day 0 (< 20 cells liter⁻¹) (Fig. 2). Within a few days of incubation, a significant growth ($P < 0.05$) of *H. vermiformis* also was observed in this water, as was growth of *L. pneumophila*, which reached its maximum level of growth after about 10 days. Based on these observations, *L. pneumophila* was added to all BBT flasks to ensure the presence of this organism in the test, and control flasks with *L. pneumophila* and *H. vermiformis* were included in each test to verify if the water under investigation supports its growth in the presence of a host protozoan.

Significant growth ($P < 0.05$) of *L. pneumophila* was observed in 16 of 18 control BBT flasks (Table 1). In these flasks, at least a 2-log-unit increase of the *L. pneumophila* concentration was observed in the planktonic phase and in the biofilm within 10 days of incubation. Maximum concentrations of colony-forming cells of *Legionella* and maximum concentrations of *Legionella* spp. and *L. pneumophila* as measured by Q-PCR were not significantly different ($P < 0.05$) in the BBT flasks with biomass from the filter beds of supplies A and B incubated at 37°C. Therefore, Q-PCR was used in subsequent tests to quantify growth of *L. pneumophila* and *Legionella* spp. The log-transformed *L. pneumophila*/*H. vermiformis* ratios for the controls ranged from 1.9 to 3.6 (average, 2.8 ± 0.5) and were normally distributed according to the Shapiro-Wilk test ($P = 0.577$). From these data an upper tolerance limit of 4.5 was derived, above which it is most likely that protozoan hosts for growth of *L. pneumophila* other than *H. vermiformis* are present in the involved BBT flask.

L. pneumophila did not proliferate in the control BBT flasks

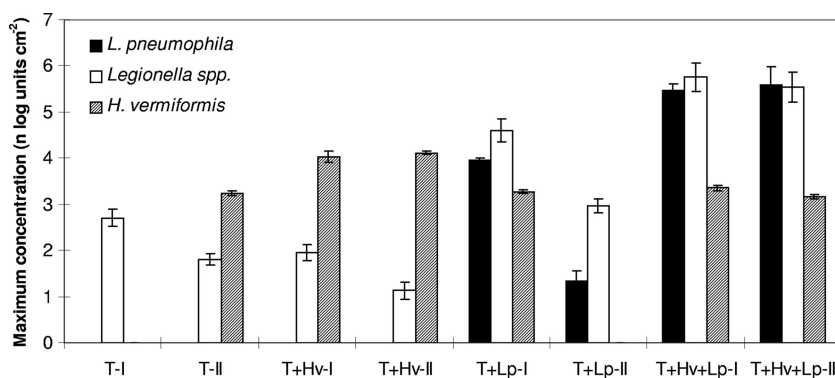


FIG. 1. Maximum concentrations of *L. pneumophila* (*mip* gene copies cm⁻²), *Legionella* spp. (GU cm⁻²), and *H. vermiformis* (cells cm⁻²) in the biofilm on PE-Xa in the BBT flasks with biomass from a limestone filter bed in treated water of supply A incubated at 37°C for 20 days. Initial concentrations of *L. pneumophila* (about 2 log units of *mip* gene copies cm⁻²) and *H. vermiformis* (about 3 log units of cells cm⁻²) are converted from units liter⁻¹ to units cm⁻². Abbreviations: T-I and T-II, blank test flasks (no inoculation); T+Hv-I and T+Hv-II, duplicate test flasks inoculated with *H. vermiformis*; T+Lp-I and T+Lp-II, duplicate test flasks inoculated with *L. pneumophila*; T+Hv+Lp-I and T+Hv+Lp-II, duplicate test flasks inoculated with *L. pneumophila* and *H. vermiformis* (controls). Error bars indicate standard deviations of the analysis.

with water from cooling towers 3 and 4 (Table 1). Probably, a biocide residual inhibited biofilm development and/or inactivated *L. pneumophila* because the ATP concentration decreased from 40 ng ATP liter⁻¹ to 12 ng ATP liter⁻¹ during the incubation in the flask with water from cooling tower 3. In the other controls, the maximum ATP concentrations in the water varied between 9.3×10^2 and 3.7×10^3 ng ATP liter⁻¹. In the tests, the maximum ATP concentration in water varied between 5.4×10^1 and 5.1×10^2 ng ATP liter⁻¹ and the maximum concentration of the biofilm on the surface of the PE-80 cylinders ranged from 1.3 to 9.3 ng ATP cm⁻².

Growth of *Legionella* spp. in the BBT flasks at 37°C. The concentration of *L. pneumophila* increased significantly ($P < 0.05$) by 1 to 3 log units in one or both BBT flasks with 11 of 21 water types inoculated with this organism (Table 2; see also Tables S2.1 to S2.3 in the supplemental material). The maximum concentration of *L. pneumophila* ranged from 5.3×10^3 to 5.7×10^7 *mip* gene copies liter⁻¹ and from 4.9×10^2 to 5×10^5 *mip* gene copies cm⁻² in the biofilm depending on the

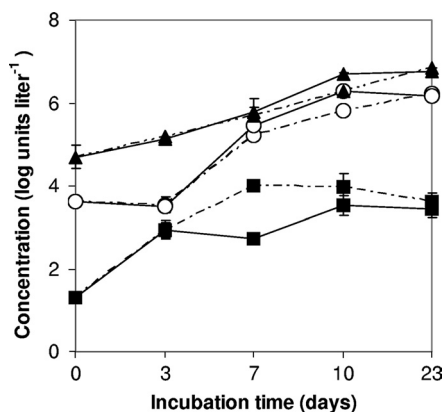


FIG. 2. Growth of inoculated *L. pneumophila* in two biofilm batch test flasks with water from cooling tower 1 during incubation at 37°C for 23 days. Symbols: ○, *L. pneumophila* (*mip* gene copies liter⁻¹); ▲, *Legionella* spp. (genome units [GU] liter⁻¹); ■, *H. vermiformis* (cells liter⁻¹). The detection limit for *H. vermiformis* is 20 cells liter⁻¹. Error bars indicate standard deviations of the analysis.

water type. In these BBT flasks the maximum concentration of *Legionella* spp. was similar to the maximum concentration of *L. pneumophila*, indicating that the *Legionella* community was dominated by *L. pneumophila*. Growth of *Legionella* spp. was observed, but *L. pneumophila* did not multiply in two of four BBT flasks with water originating from two tap water installations in the distribution system of supply B. In these water types significant growth ($P < 0.05$) of indigenous *L. anisa* to maximum concentrations ranging from 2.1×10^6 to 2.6×10^7 *mip* gene copies liter⁻¹ was observed (Table 2). No growth of *Legionella* spp. was observed in 9 of 21 water types.

Growth of *H. vermiformis* and *Acanthamoeba* spp. in the BBT flasks at 37°C. *H. vermiformis* was detected at day 0 in 10 of 21 water types at concentrations ranging from 6.8 to 1,530 cells liter⁻¹ and from 6.2 to 12.3 cells g (wet weight)⁻¹ of filter bed material (Table 1). Significant growth of indigenous *H. vermiformis* in the BBT flasks was observed in 11 of 21 water types tested, with significant growth of *L. pneumophila* or *L. anisa* in 9 of these 11 water types (Table 2). The concentration of *H. vermiformis* increased by 0.6 to 4 log units in the planktonic phase and in the biofilm depending on the water type and the initial concentration. In four water types, *H. vermiformis* was not observed at day 0 but appeared within a few days of incubation. *Acanthamoeba* spp. were detected in water collected from tap water installation A2 and in water from cooling tower 4 at concentrations of 0.3 cells liter⁻¹ and 1.7 cells liter⁻¹, respectively. However, in none of the BBT flasks was growth of *Acanthamoeba* spp. observed.

The *L. pneumophila*/*H. vermiformis* ratios in 12 of 19 BBT flasks with growth of *L. pneumophila* did not exceed the derived tolerance limit, and in 11 of these 12 BBT flasks significant growth of *H. vermiformis* was observed (Table 2). In BBT flask II with cooling water 5, no significant growth of *H. vermiformis* was observed, but this protozoan was detected during the incubation and the *L. pneumophila*/*H. vermiformis* ratio did not exceed the derived tolerance limit. These observations indicate that *H. vermiformis* served as a host for *L. pneumophila* in these 12 BBT flasks. In seven BBT flasks with growth of *L. pneumophila*, the *L. pneumophila*/*H. vermiformis* ratio did exceed the derived tolerance limit and in six of these seven

TABLE 2. Predominant 18S rRNA gene sequences clustering with free-living protozoa obtained from the biofilm in BBT flasks with growth of *L. pneumophila* and/or *L. anisa* after ≥ 20 days of incubation at 37°C

Water type	BBT flask	Growth in flask ^a		<i>L. pneumophila</i> / <i>H. vermiformis</i> ratio ^b (log ₁₀)	% of clones clustering with:		Predominant OTU clustering with protozoa		
		<i>L. pneumophila</i>	<i>H. vermiformis</i>		Protozoa	<i>H. vermiformis</i>	Organism with highest similarity (accession no.)	Similarity (%)	% of clones
Biomass from limestone filter bed (supply A)	I	+	+	1.1	100	100	<i>Hartmannella vermiformis</i> (AY680840)	99.5	100
Installation A2 (supply A)	II	+	+	3.0	100	93	<i>H. vermiformis</i> (AY680840)	100	93
Biomass from sand filter bed (supply B)	I	+	-	>6.8 ^f	91	<2	<i>Rhinosporidium</i> sp. (AY477020)	89.3	73
	II	+	+	2.0	97	93	<i>H. vermiformis</i> (AY680840)	99.8	97
	III	+	-	>6.6 ^f	16	<3	Uncultured cercozoan clone (AY620304)	>97.7	80
Installation B1 (supply B)	II	- ^c	+	1.9 ^e	100	27	<i>Sphaeroeca volvox</i> (Z34900)	83.6	73
Installation B2 (supply B)	I	+ ^c	+	2.0 (2.3 ^e)	100	100	<i>H. vermiformis</i> (AY680840)	100	100
	II	- ^c	+	2.9 ^e	100	100	<i>H. vermiformis</i> (AY680840)	100	100
Biomass from granular activated carbon filter bed (supply D)	I	+	+	3.4	100	<13	<i>Nuclearia simplex</i> (AF484687)	100	100
Biomass from sand filter bed (supply D)	II	+	+	1.6	92	92	<i>H. vermiformis</i> (AY680840)	100	100
Water from Rhine River (autumn)	I	+	-	>5.6 ^f	15	<5	<i>Neoparamoeba</i> sp. (AF371972)	95.6	67
	II	+	-	>5.7 ^f	87	<4	Uncultured eukaryote from treated water of groundwater supply B (EU860860)	93.9	100
Water from Rhine River (winter)	I	+	+	5.5 ^f	<3	<3	No protozoan observed		
	II	+	+	2.1	61	52	<i>H. vermiformis</i> (AY680840)	99.8	86
Treated sewage	II	+	-	>4.6 ^f	52	<4	<i>Diphyllia rotans</i> (AF420478)	99.3	100
Cooling tower 1	I	+	+	2.7	42	21	<i>H. vermiformis</i> (AF426157)	100	50
	II	+	+	2.1	50	17	<i>H. vermiformis</i> (AF426157)	99.8	33
Cooling tower 5	I	+	-	>4.5 ^f	64	<4	<i>Echinamoeba thermanum</i> (AJ489262-AJ489268) ^g	96.2-98.7	79
	II	+	- ^d	3.6	89	7	<i>E. thermanum</i> (AJ489262-AJ489267-AJ489268) ^g	96.2-98.9	42

^a +, significant growth ($P < 0.05$); -, no growth.

^b Log-transformed ratio between the maximum concentration of *L. pneumophila* and the maximum concentration of *H. vermiformis*.

^c Significant growth ($P < 0.05$) of *L. anisa* in the BBT flask.

^d *H. vermiformis* was detected, but no significant growth was observed.

^e Log-transformed ratio between the maximum concentration of *L. anisa* and the maximum concentration of *H. vermiformis*.

^f Log-transformed *L. pneumophila*/*H. vermiformis* ratio exceeds the tolerance limit of *L. pneumophila*/*H. vermiformis* ratio for the control group (4.5).

^g More than one OTU with highest similarity to *E. thermanum*.

BBT flasks no significant growth of *H. vermiformis* or *Acanthamoeba* spp. was observed (detection limit, <20 cells liter⁻¹) (Table 2). The absence of *H. vermiformis* and/or the high *L. pneumophila*/*H. vermiformis* ratio indicates that free-living protozoa other than *H. vermiformis* served as hosts for *L. pneumophila* in these seven BBT flasks.

Identity and diversity of eukaryotes and free-living protozoa predominating in the BBT flasks at 37°C. T-RFLP fingerprints revealed complex eukaryotic communities in the water types directly after sampling. During incubation, the eukaryotic diversity in the planktonic phase decreased and different eukaryotic communities developed in duplicate BBT flasks (Fig. 3).

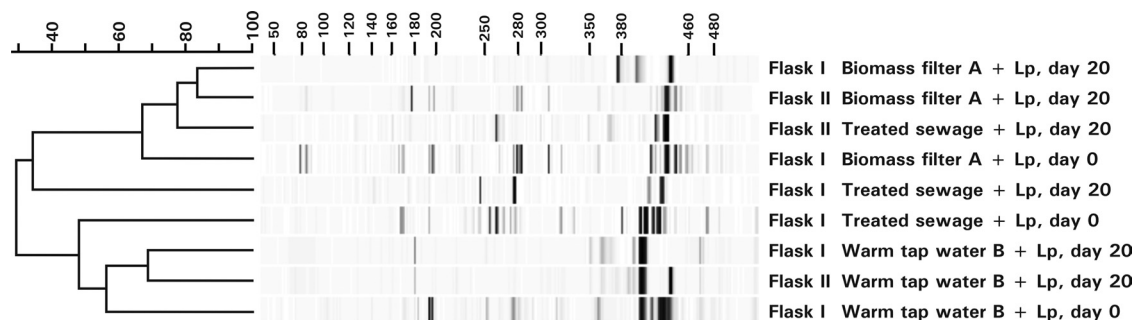


FIG. 3. UPGMA (unweighted-pair group method using average linkages) dendrogram of T-RFLP fingerprints of the BBT flasks with (i) biomass from a limestone filter bed of supply A (biomass filter A), (ii) treated sewage, and (iii) water from warm tap water installation B1 in distribution area of supply B (warm tap water B) at day 0 and after 20 days of incubation at 37°C with *L. pneumophila* (+ Lp). Sample volumes, 50 to 200 ml of the planktonic sample and 50 to 100 ml of the biofilm suspensions. The numbers above the dendrogram represent percent similarity, and those above the fingerprints represent fragment length (number of nucleotides).

TABLE 3. Classification of eukaryotic clones retrieved from the biofilm in the BBT flask after ≥ 20 days of incubation at 37°C with samples from drinking water supplies, surface water, and cooling towers inoculated with *L. pneumophila*

Kingdom or subkingdom	Drinking water supplies		Surface water		Cooling towers		All analyzed samples	
	No. ^a of OTUs (%)	% of clone libraries	No. of OTUs (%)	% of clone libraries	No. of OTUs (%)	% of clone libraries	No. of OTUs (%)	% of clone libraries
Free-living protozoa	35 (40.4)	66.4	7 (25.0)	34.8	30 (56.6)	57.4	68 (41.2)	57.7
Amoebozoa	8 (22.2)	58.2	4 (57.1)	57.4	21 (70)	66.7	29 (42.6)	59.7
Cercozoa	8 (22.2)	8.4	3 (42.9)	42.6	5 (16.7)	15.2	17 (25)	14.2
Choanozoa	11 (30.6)	25.9	0 (0.0)	0	3 (10)	16.7	14 (20.6)	20.7
Euglenozoa	2 (5.6)	5.0	0 (0.0)	0	0 (0.0)	0	2 (2.9)	3.4
Myzozoa	5 (13.9)	2.1	0 (0.0)	0	0 (0.0)	0	5 (7.4)	1.4
Stramenopiles	1 (2.8)	0.4	0 (0.0)	0	1 (3.3)	1.5	2 (2.9)	0.6
Fungi	25 (28.1)	15.8	18 (64.3)	60.7	17 (32.1)	31.3	60 (36.4)	28.7
Metazoa	14 (15.7)	10.8	0 (0)	0	2 (3.8)	4.3	14 (8.5)	7.2
Protophyta and plants	3 (3.4)	1.1	3 (10.7)	4.4	2 (3.8)	3.5	8 (4.8)	2.3
Organisms with <75% similarity	12 (13.5)	5.8	0 (0)	0	2 (3.8)	3.5	15 (9.1)	4.1
Total	89 (100)	100	28 (100)	100	53 (100)	100.0	165 (100)	100

^a OTUs obtained from more than one sample type are included only once.

Free-living protozoa constituted the largest proportion of the OTUs (>40%) and clones (>56%) in the BBT flasks with water samples from drinking water supplies and cooling towers, but OTUs (64%) and sequences (61%) with the highest similarity to fungi predominated in the BBT flasks with surface water (Table 3). A total of 68 OTUs representing 58% of the clones showed the highest similarity to free-living protozoa (Table 3; see also Tables S2.1 to S2.3 in the supplemental material for details). Almost 40% of these clones represent one OTU, which showed $\geq 99.5\%$ similarity to *H. vermiformis*, and were obtained from all three water types. Only three other OTUs were obtained from two water types, and all 64 of the other OTUs were observed only once in a single BBT flask.

Sequences clustering with free-living protozoa predominated in 16 of 19 clone libraries of the BBT flasks with growth of *L. pneumophila* or *L. anisa* (Table 2; see also Tables S2.1 to S2.3 in the supplemental material for details). *H. vermiformis* constituted more than 50% of the clones clustering with free-living protozoa in 8 of the 19 clone libraries of BBT flasks with growth of *L. pneumophila* and/or *L. anisa*. The *L. pneumophila*/*H. vermiformis* ratios in these BBT flasks varied between 1.1 and 3.0 and did not exceed the derived tolerance limit (Table 2). The *L. pneumophila*/*H. vermiformis* ratio also did not exceed the tolerance limit in four other BBT flasks, *viz.*, with water from tap water installation B1, with biomass from the granular activated carbon filter bed of supply D, and with water from cooling towers 1 and 5. Obviously, *H. vermiformis* served as host for *L. pneumophila* without predominating in these clone libraries.

The predominating OTUs in the clone libraries of the seven BBT flasks with *L. pneumophila*/*H. vermiformis* ratios exceeding the tolerance limit showed the highest similarity to *Diphyllia rotans* (99.3%), an uncultured cercozoan clone (97.7%), *Echinamoeba thermanum* (>96%), *Neoparamoeba* sp. (95.6%), an uncultured eukaryote from treated water of supply B (93.9%), and *Rhinosporidium* sp. (89.3%) (Table 2). These free-living protozoa may have served as hosts for *L. pneumophila* in these BBT flasks. No sequences related to free-living protozoa were obtained from the BBT flask with water from

the Rhine River (winter) in which growth of *L. pneumophila* was observed.

Sequences related to *Sphaeroeca volvox* (>83.6% similarity) predominated in the clone libraries of two BBT flasks with water from tap water installation B1, in one of which growth of *L. anisa* and *H. vermiformis* was observed (Table 2; also see Table S2.1 in the supplemental material for details). This observation suggests that the free-living protozoan related to *S. volvox* did not serve as a host for *L. anisa* or for *L. pneumophila*. Most clone libraries of the BBT flasks without growth of *L. pneumophila* and *Legionella* spp. were dominated by OTUs clustering with fungi and metazoa. Predominating OTUs of the protozoan community in these BBT flasks had the highest similarities to *Ichthyophonus irregularis* (89.2%), *Platyamoeba stenopodia* (93.9%), an uncultured cercozoan (>98.9%), and an uncultured eukaryote retrieved from treated water from supply A in an earlier study (54) (see Tables S2.1 to S2.3 in the supplemental material for details). Obviously, these free-living protozoa did not serve as hosts for *L. pneumophila* under the test conditions.

Transfer of one PE cylinder with biofilm and 60 ml of the planktonic phase from BBT flasks with the different water types which had been incubated for at least 50 days to freshly prepared BBT flasks with autoclaved tap water and five PE cylinders, followed by incubation at 37°C, did not induce growth of *L. pneumophila* in most flasks. Growth of *L. pneumophila* in freshly prepared BBT flasks was observed only in the presence of *H. vermiformis*, *e.g.*, in the flasks inoculated from the BBT flask with treated sewage and with biomass from the granular activated carbon filter bed of supply D. The *L. pneumophila*/*H. vermiformis* ratios in these BBT flasks indicated that *H. vermiformis* served as a host for *L. pneumophila*.

Growth of *Legionella* spp. and eukaryotes in the BBT system at 15°C. Growth of indigenous *Legionella* spp. was observed in one of the BBT flasks with tap water of supply C and in the BBT flask inoculated with *H. vermiformis* but not in the membrane-filtered (3.0- μ m) sample. At least a 2-log-unit increase of the *Legionella* community was observed in all flasks inoculated with biomass from filter beds of supplies A and B, but no

colonies were observed on the BCYE medium. The inoculated *L. pneumophila* did not multiply in the BBT flasks during incubation at 15°C. The *Legionella* community (maximum concentration, 3.1×10^5 GU cm⁻²) grown in the BBT flasks with biomass from the limestone filter bed of supply A with and without addition of *H. vermiformis* was dominated (>92% of the clone library) by OTUs clustering with sequences obtained from treated water and raw water from supply A in an earlier study (Wullings et al., submitted). These sequences differ from the species described (data not shown). The *Legionella* community (maximum concentration, 3.0×10^4 GU cm⁻²) in the BBT flasks with biomass from a sand filter bed of supply B was dominated by one OTU (84% of the clone libraries), which differs from reported sequences.

Acanthamoeba spp. were not observed in the BBT flasks incubated at 15°C, and *H. vermiformis* was observed only in the BBT flasks inoculated with this protozoan. Changes in the T-RFLP fingerprints of the eukaryotic communities in the biofilm during incubation at 15°C indicated that growth of indigenous eukaryotes occurred (results not shown). Sequences clustering with free-living protozoa were obtained from all four eukaryotic clone libraries of the BBT flasks with biomass from filter beds of supplies A and B incubated at 15°C. A total of 51 OTUs showed the highest similarity to protozoan phyla, viz., Amoebozoa (7 OTUs), Cercozoa (16 OTUs), Choanozoa (17 OTUs), Ciliophora (1 OTU), Euglenozoa (7 OTUs), Myzozoa (2 OTUs), and Stramenopiles (1 OTU). More than 80% of the clones clustering with free-living protozoa that were retrieved from the BBT flasks inoculated with biomass from the limestone filter bed of supply A showed the highest similarity with the Cercozoa phylum (see Tables S3.1 and S3.2 in the supplemental material for details). Sequences clustering with Choanozoa and Euglenozoa predominated in the clone libraries of the BBT flasks with biomass from a sand filter bed of supply B. Most (57%) of the OTUs that clustered with free-living protozoa showed the highest similarity to sequences which differ from already-described species retrieved in an earlier study from treated water or distribution system biofilms of supplies A and B (54). These observations indicate that yet-undescribed protozoa serve as hosts for yet-uncultured *Legionella* bacteria.

DISCUSSION

Performance of the biofilm batch test (BBT) system. Only a few *in vivo* studies have shown that certain free-living protozoa serve as hosts for *L. pneumophila* (15, 30, 59), but a large number of free-living protozoa have been identified as hosts for *L. pneumophila* by using *in vitro* studies (17, 26, 35, 38, 43, 44, 47, 53). Random *in vitro* testing of free-living protozoa to determine whether they serve as hosts for *L. pneumophila* is time-consuming, and only a minor fraction of the free-living protozoa is available in culture collections. Furthermore, it is unknown if free-living protozoa which serve as hosts when tested *in vitro* with a pure culture of *L. pneumophila* serve as hosts in aquatic environments. Therefore, a batch test with biofilm growth in combination with molecular techniques for detection and identification was used to identify potential protozoan hosts for *L. pneumophila* in different freshwater types (Table 1).

The present study shows that *Legionella* spp. and free-living

protozoa multiplied in the BBT flasks at 37°C and also at 15°C. Therefore, this approach can be used to amplify and subsequently identify free-living protozoa that can serve as hosts for *L. pneumophila* and other *Legionella* spp. in freshwater types of different origins. The maximum concentrations of active biomass on the surface of the PE-80 cylinders varied between 1.3 and 9.3 ng ATP cm⁻² and are in the same range as biofilm concentrations measured in tap water installations (55). Therefore, the BBT design represents the environment in water installations. *L. pneumophila* serogroup 1 was added to ensure the presence of this organism in the BBT flasks (Fig. 1). Growth of indigenous *L. anisa* was observed in two water types despite the inoculation with a relatively high number of *L. pneumophila* bacteria. *L. anisa* is a common inhabitant of engineered water systems (11, 62) and also needs free-living protozoa for growth in aquatic environments (13, 49).

Growth of *H. vermiformis* in the BBT flasks was followed by growth of *L. pneumophila* (Fig. 2). The *L. pneumophila*/*H. vermiformis* ratio in the test in combination with the tolerance limit for this ratio derived from the controls can be used as an indicator for the role of *H. vermiformis* as protozoan host in the BBT flasks. The obtained *L. pneumophila*/*H. vermiformis* ratios, which ranged from 1.9 to 3.6 log units, are consistent with the average ratios of 2.5 to 2.6 log units obtained in a biofilm batch model system with autoclaved tap water inoculated with pure cultures of *L. pneumophila* and *H. vermiformis* (28) (Table 1). The *Legionella*-to-host ratio may be different for other protozoan hosts, e.g., *Acanthamoeba* spp., depending on their cell size.

In more than 50% of the tested water types, growth of *L. pneumophila* or *L. anisa* was observed only in one of two BBT flasks incubated at 37°C. Different protozoan communities can develop in duplicate samples (Fig. 3 and Table 2), indicating that slight differences in environmental conditions result in predominance of other free-living protozoa. This observation is consistent with the observation of highly diverse free-living protozoan communities at different locations within drinking water distribution systems (54).

The procedure for the identification of the free-living protozoa in clone libraries, as applied in this study, has some limitations. First of all, 18S rRNA gene sequences of a few genera within the phyla Amoebozoa, Euglenozoa, and Percolozoa were not amplified with the primers used. These organisms include *Naegleria* spp. (38, 44) and *Vahlkampfia jugosa* (43), which have been identified as hosts for *L. pneumophila* by using *in vitro* tests. Furthermore, the composition of the clone libraries does not exactly reflect the composition of the involved eukaryotic communities, because 18S rRNA genes are present in different copy numbers in each eukaryotic species (31). Moreover, although one OTU related to free-living protozoa predominated in the clone libraries of most BBT flasks with growth of *L. pneumophila* (Table 2), it cannot be excluded that more than one protozoan type served as hosts for *L. pneumophila* in one BBT flask. Despite these limitations, a number of candidate hosts for *L. pneumophila* were detected.

Identity of protozoan hosts for *L. pneumophila* and *L. anisa*. The observation of *H. vermiformis* in 14 of the 21 water types tested (before or during incubation) is consistent with the ubiquitous presence of this organism in drinking water supplies (34, 42, 51, 52, 54), cooling towers (28), and surface water (29,

34, 52) (Tables 1 and 2). In 12 of the 19 flasks with growth of *L. pneumophila* or *L. anisa*, growth of *H. vermiformis* also was observed. The *L. pneumophila*/*H. vermiformis* ratios in 11 of these 12 BBT flasks did not exceed the derived tolerance limit. OTUs with the highest similarity to *H. vermiformis* predominated in 8 of the associated clone libraries, thus confirming that *H. vermiformis* served as host for *L. pneumophila* or *L. anisa* in these BBT flasks.

Significant growth of *H. vermiformis* was observed in two water types without growth of *L. pneumophila* and other *Legionella* spp., and also no growth of *L. pneumophila* was observed in the control for one of these water types. All sequences with the highest similarity to *H. vermiformis* showed a minimum of 99.5% similarity to each other and to the *H. vermiformis* strain (ATCC 50237) used in the controls. The absence of growth of *Legionella* spp. in the presence of *H. vermiformis* remains unexplained.

Acanthamoeba spp. were observed at low initial concentrations in 2 of the 21 water types, indicating that this organism is much less common in freshwater in temperate regions than is *H. vermiformis*. Still, *Acanthamoeba* spp. are frequently used to study proliferation and growth of *L. pneumophila* within free-living protozoa and numbers between $>1.0 \times 10^2$ and 4.5×10^4 CFU of *L. pneumophila* per amoeba or vesicle have been reported (18, 22, 24, 43, 44). Growth of *Acanthamoeba* spp. was not observed in the BBT flasks, although most species of this genus can multiply at 37°C (9, 19). Of the free-living protozoa, the slime mold, and the metazoan which have been identified as hosts for *L. pneumophila* by using *in vitro* studies, only *H. vermiformis* was identified as a host for *L. pneumophila* or *L. anisa* in the present study. The prominent position of *H. vermiformis* as a host for *L. pneumophila* in aquatic environments is consistent with results of other *in vivo* experiments (15, 30, 59). However, the potential of the other listed organisms to serve as hosts for *L. pneumophila* in the aquatic environment needs confirmation using *in vivo* tests.

L. pneumophila did not proliferate in the absence of *H. vermiformis* in freshly prepared BBT flasks inoculated with the microbiota grown in BBT flasks with selected water types, and no attempts were made to culture free-living protozoa from these BBT flasks. Consequently, not yet-recognized protozoan hosts for *L. pneumophila* were not isolated in this study. The composition of a number of eukaryotic clone libraries and the values of the *L. pneumophila*/*H. vermiformis* ratio suggest that testing of certain protozoa in coculture with *L. pneumophila* may lead to the identification of novel protozoan hosts for *L. pneumophila* (Table 2). The involved organisms include (i) *Diphylleia rotans* (99.3% similarity), an algivorous heterotrophic flagellate that feeds on cyanobacteria (27); (ii) *Echinamoeba thermarum* (>96%), an extremely thermophilic protozoan (2) related to *E. exundans*, which is described as a host for *L. pneumophila* (15); and (iii) *Neoparamoeba* sp. (95.6% similarity) of the class Flabellinea with the genera *Platyamoeba* and *Vannella*, which are affiliated with *Legionella* spp. (50). These candidate hosts were observed in BBT flasks with surface water, water from a cooling tower, and biomass from a sand filter bed of a groundwater supply, but not from tap water (Table 2).

Free-living protozoa growing at 37°C were dominated by OTUs clustering within Amoebozoa, while the clone libraries

of the BBT flasks at 15°C were dominated by OTUs with the highest similarity to cercozoan and choanozoan types. Also in drinking water supplies (<20°C), only a minor fraction of OTUs of free-living protozoa clustered with Amoebozoa (54). OTUs related to free-living protozoa with pathogenic properties, viz., *Acanthamoeba* spp. (7, 25) and *Balamuthia mandrillaris* (23, 47), were observed in several water types (see Tables S2.1 to S2.3 in the supplemental material for details). However, it is unclear whether these sequences represent pathogenic organisms. In most BBT flasks without growth of *Legionella* spp., OTUs that showed the highest similarity to fungi and metazoa predominated. The free-living protozoa related to *Sphaeroeca volvox*, *Ichthyophonus irregularis*, and *Platyamoeba stenopodia* and an uncultured cercozoan did not serve as hosts for *L. pneumophila* under the test conditions (see Tables S2.1 to S2.3 in the supplemental material for details).

Identity and diversity of *Legionella* spp. and free-living protozoa in the BBT system at 15°C. *Legionella* bacteria, including several yet-uncultured species, are ubiquitously present in freshwater (8, 10, 61; Wullings et al., submitted). Growth of *Legionella* spp. at 15°C was observed in the BBT system but not with membrane-filtered water (3.0 µm), confirming that these bacteria require free-living protozoa for growth. The yet-undescribed *Legionella* (sequence) types predominating in the BBT flasks with biomass from a limestone filter bed of supply A clustered with several sequence types previously obtained from raw and treated water of the associated supply (Wullings et al., submitted). The sequences of the protozoa predominating in the BBT flasks with *Legionella* spp. growing at 15°C clustered with sequences related to cercozoan types retrieved from the treated water of supplies A and B in an earlier study (54). However, inoculated *L. pneumophila* did not grow at 15°C, nor did it grow in one of the BBT flasks incubated at 37°C with biomass from a limestone filter bed of supply A, despite the predominance of cercozoan types (see Tables S2.1 and S3.1 in the supplemental material for details). Growth of unidentified *Legionella* spp. at 15°C in the presence of free-living protozoa related to choanozoan and euglenozoan types in the BBT flasks with biomass from a sand filter bed of supply B suggests that also these protozoa can serve as hosts. One choanozoan type (*Rhinosporidium* sp.) also predominated in the clone library of a BBT flask with growth of *L. pneumophila* at 37°C (*L. pneumophila*/*H. vermiformis* ratio, >6.8), suggesting that this organism also served as a host for *L. pneumophila* (Table 2).

Most (57%) OTUs of free-living protozoa in the BBT flasks inoculated with biomass from filter beds and incubated at 15°C showed the highest similarity to sequences obtained from treated water or distribution system biofilms of supplies A and B in an earlier study (54). Obviously, either the many free-living protozoa present in the treated water and biofilms in the distribution systems have their origin in the filter beds and/or the environmental conditions in the filter bed resemble those in the biofilm in the distribution systems.

In conclusion, our observations confirm the prominent position of *H. vermiformis* as a host for *L. pneumophila*, whereas none of the other protozoa serving as hosts in *in vitro* studies were observed in the BBT system. A few protozoa, e.g., *Diphylleia rotans*, *Echinamoeba thermarum*, *Neoparamoeba* sp., and *Rhinosporidium* sp., were identified as candidate hosts for *L.*

pneumophila, but *in vitro* studies with these organisms are needed for confirmation.

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