Intracellular Proliferation of *Legionella pneumophila* in *Hartmannella vermiformis* in Aquatic Biofilms Grown on Plasticized Polyvinyl Chloride

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The need for protozoa for the proliferation of *Legionella pneumophila* in aquatic habitats is still not fully understood and is even questioned by some investigators. This study shows the in vivo growth of *L. pneumophila* in protozoa in aquatic biofilms developing at high concentrations on plasticized polyvinyl chloride in a batch system with autoclaved tap water. The inoculum, a mixed microbial community including indigenous *L. pneumophila* originating from a tap water system, was added in an unfiltered as well as filtered (cellulose nitrate, 3.0-μm pore size) state. Both the attached and suspended biomasses were examined for their total amounts of ATP, for culturable *L. pneumophila*, and for their concentrations of protozoa. *L. pneumophila* grew to high numbers (6.3 log CFU/cm^2) only in flasks with an unfiltered inoculum. Filtration obviously removed the growth-supporting factor, but it did not affect biofilm formation, as determined by measuring ATP. Cultivation, direct counting, and 18S ribosomal DNA-targeted PCR with subsequent sequencing revealed the presence of *Hartmannella vermiformis* in all flasks in which *L. pneumophila* multiplied and also when cycloheximide had been added. Fluorescent in situ hybridization clearly demonstrated the intracellular growth of *L. pneumophila* in trophozoites of *H. vermiformis*, with 25.9% ± 10.5% of the trophozoites containing *L. pneumophila* on day 10 and >90% containing *L. pneumophila* on day 14. Calculations confirmed that intracellular growth was most likely the only way for *L. pneumophila* to proliferate within the biofilm. Higher biofilm concentrations, measured as amounts of ATP, gave higher *L. pneumophila* concentrations, and therefore the growth of *L. pneumophila* within engineered water systems can be limited by controlling biofilm formation.

Legionella pneumophila is widespread in natural freshwater environments, despite its fastidious nature (16). The bacterium has also frequently been observed in engineered water systems such as warm water distributing systems, cooling towers, humidifiers, and fountains (25, 43, 50). The multiplication of the organism in water systems poses a potential human health risk when aerosolization can occur (12).

Legionellae grow in vitro only in complex media with supplements of cysteine and iron salts (11). For their multiplication in vivo, other microorganisms are required, but different species of heterotrophic bacteria (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Flavobacterium* sp.) alone do not support the growth of *L. pneumophila* (31, 45). In vitro studies using cocultures have repeatedly demonstrated the intracellular multiplication of *L. pneumophila* in amoebae (*Acanthamoeba*, *Echinamoeba*, *Hartmannella*, *Naegleria*, *Vahlkampfia*, and *Dictyostelium*) and in a ciliated protozoon (*Tetrahymena pyriformis*) (12, 15, 21, 39). Amoebae have been observed in water systems associated with Legionnaires' disease (5, 7), and *L. pneumophila* can recolonize water distributing systems within a few weeks after disinfection (26, 27). Batch experiments with tap water showed that *L. pneumophila* did not multiply in the absence of protozoa, and

growth in the presence of a protozoon (*Hartmannella*) was confirmed by the use of cocultures (14, 45).

Rogers and coworkers (35, 36) found that the growth of *L. pneumophila* on a variety of materials in contact with tap water is coupled with biofilm development. However, they concluded that (i) the numbers of *L. pneumophila* cells in the biofilms were unrelated to the total numbers of microorganisms present and (ii) the growth of *L. pneumophila* coincided with undetectable numbers of potential hosts on some materials. Furthermore, it had been concluded from several studies that the intracellular growth of *L. pneumophila* in protozoa is not always essential for the multiplication of *L. pneumophila* within a mixed bacterial consortium in water (37, 42).

This paper describes the use of a batch test system for the development of different concentrations of aquatic biofilms by using polyvinyl chloride, in both unplasticized (PVCu) and plasticized (PVCp) versions, as a support and as a source of energy and carbon. In vivo observations revealed that the intracellular multiplication of *L. pneumophila* in *Hartmannella vermiformis* was the mechanism of proliferation for *L. pneumophila* in biofilms on PVCp, which contained a high concentration of heterotrophic bacteria.

MATERIALS AND METHODS

Batch test system. Biofilms were developed on pieces of PVCu (about 9.5 cm^2 of surface area for each) and pieces of PVCp tubes (diameter, 0.80 cm; about 6.2 cm^2 of surface area for each) in a static test using heat-cleaned (550°C) Erlenmeyer flasks with a volume of 1 liter. All material pieces were pasteurized at 70°C

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for 30 min and then incubated in 600 ml of sterilized tap water (pH 7.8) at surface-to-volume ratios of 0.24 and 0.16 cm^{-1} for PVCu and PVCp, respectively. These surface-to-volume ratios were kept constant during the test; after the collection of a piece of PVCu or PVCp, a proportional volume of water was also removed from the flask. Nitrate and phosphate from autoclaved stock solutions were added to the flasks at final concentrations of 72.5 and 13.5 μM, respectively, to prevent growth limitation by these nutrients. A mixed microbial community including indigenous *L. pneumophila* serotype 1, originating from a plumbing system in The Netherlands, was used as the inoculum. Before its use as an inoculum, this mixed microbial community had been maintained in tap water containing pieces of silicone tubing at 37°C, followed by storage at −80°C. The inoculum was added in an unfiltered as well as a filtered state (3.0-μm-pore-size cellulose nitrate filter; Sartorius, Goettingen, Germany). Filtration reduced the indigenous *L. pneumophila* concentration in the inoculum from 4.1 to 2.4 log CFU/ml and the concentration of the heterotrophic bacteria from 5.1 to 4.2 log CFU/ml. Compared to that of the unfiltered inoculum, a 10-fold larger volume of the filtered inoculum was added to the test flasks to partly correct for these differences. The protozoon concentration in the unfiltered inoculum, as determined by a cultivation method, was 1.4 log protozoa/ml; no protozoa were detected in the filtered inoculum. A pure culture of the same indigenous *L. pneumophila* serotype 1 strain was also used as an inoculum after two passages on buffered charcoal yeast extract agar (11) and subsequent suspension in sterile tap water. Filter-sterilized (pore size, 0.2 μm; Schleicher & Schuell, Dassel, Germany) cycloheximide was added at a final concentration of 100 μM to two flasks with the unfiltered inoculum to prevent the growth of protozoa. All tests were conducted in duplicate, and incubation took place at 37°C without shaking for several weeks.

Biomass measurements in biofilm and planktonic phase. The attached biomass (biofilm) and the suspended biomass (planktonic phase) were analyzed for their total ATP concentrations and for the numbers of *Legionella*, heterotrophic bacteria, and protozoa at different time points during the experiments. The microorganisms in the biofilm were removed from the material pieces by six 2-min sonication steps in 10 ml of sterilized tap water (U950D; Ultrawave Limited, Cardiff, United Kingdom) at a frequency of 30 kHz and an average power input of 0.11 W/ml. The total ATP concentrations, representing the active biomasses, in the biofilm and planktonic phase were determined by ATP analysis with a commercially available ATP kit (Celsis International B.V., Landgraaf, The Netherlands). *Legionella* concentrations were determined by plating on buffered charcoal yeast extract agar and incubation at 37°C for 7 days (11). The heterotrophic bacterial concentration was determined by the heterotrophic plate count (HPC) method with R$_2$A agar (34). Protozoon concentrations were determined by cultivation, direct counting with the fluorochrome primulin, and/or fluorescent in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes. Cultivation was done according to the method described by Darbyshire and coworkers, adjusted for water samples by the addition of 50 μl of the biofilm or planktonic phase to the first wells (10). *Pseudomonas fluorescens* (about 7 log CFU/ml) was used as a food source for the protozoa. The protozoon concentration was calculated with GenStat, 6th ed. (VSN International Ltd., Oxford, United Kingdom). The cultivated protozoa were identified by use of *An Illustrated Key to Freshwater and Soil Amoebae* (32). The fluorochrome primulin (Aldrich Chemical Company, Inc., Milwaukee, Wis.) was applied to stain the protozoa, followed by direct counting as described elsewhere (6). Direct counting with FISH is described below.

FISH. At each time point, 30 ml of the planktonic phase and 50 ml of the biofilm phase were filtered over a 0.4-μm-pore-size HTTP Isopore membrane (Millipore, Bedford, Mass.) in a vacuum not exceeding 3 kPa. The samples were fixed on the filter with a 4% paraformaldehyde–phosphate-buffered saline solution at room temperature for 30 min, washed with distilled water, and dehydrated at 46°C for 5 min. The filters were placed on glass slides for hybridization by the application of 40 μl of hybridization buffer (20% [vol/vol] formamide, 0.9 M NaCl, 0.01% sodium dodecyl sulfate, 15 mM Tris-HCl [pH 7.4]), 200 ng of the fluorescein-labeled probe EUK516 (Table 1) (1), 225 ng of the Cy3-labeled probe LEGPNE1 (Table 1) (19), 38 ng of poly(U), and 76 ng of bovine serum albumin and then were covered with a coverslip. Incubation was performed at 46°C for 3 h in an isotonically humid chamber. The labeled oligonucleotides were removed by incubating the filters upside down in preheated washing buffer (40 mM NaCl, 15 mM Tris-HCl [pH 7.4], 0.01% sodium dodecyl sulfate). After incubation for 30 min at 46°C, the filters were rinsed with distilled water (room temperature) and dried at 46°C for 5 min. The filters were mounted in Vectorshield (Vector Laboratories, Inc., Burlingame, Calif.) on a glass slide. Fluorescence was detected by use of a Leica DMRXA fluorescence microscope supplied with a COHU high-performance charge-coupled device digital camera. Leica Q Fluoro, v. V1.0b, software was used to record the pictures.

TABLE 1. Primer sequences used for this study

Primer	Sequence	Reference
5′→3′ directed primers		
Euk 516	ACCAGACTTGCCCTCC	1
LEGPNE1	ATCTGACCGTCCCAGGTT	19
EUKf	ACCTGGTTGATCCTGCCAG	30
373C	GATTCCGGAGAGGGAGCCTGA	49
892C[a]	GTCAGAGGTGAAATTCTAGG	40
1262C	GTGGTGCATGGCCGTTCTTA	49
3′→5′ directed primers		
EUKr	TGATCCTTCYGCAGG TTCAC	30
373	TCAGGCTCCCTCTCCGGAATC	49
892[a]	CCTAGAATTTCACCTCTGAC	40
1262	GAACGGCCATGCACCAC	49

[a] The primer sequence was slightly modified, being adjusted to *H. vermiformis* Nijmegen (49).

PCR for detection of eukaryotes. All Erlenmeyer flasks with PVCp test pieces were checked for the presence of eukaryotes by use of a PCR assay. At each time point, 30 ml of the planktonic phase and 50 ml of the biofilm phase were filtered over a 0.2-μm-pore-size GTTP Isopore membrane (Millipore). DNA extraction of the filters was done by use of a FastDNA spin kit for soil (BIO 101, Carlsbad, Calif.) according to the instructions supplied by the manufacturer. The 18S rRNA gene was amplified by a PCR with the oligonucleotide primers EUKf and EUKr (Table 1), which were complementary to regions of conserved sequences located proximal to the 5′ and 3′ termini of known 18S rRNA genes (30). The PCR mixtures (50 μl) contained about 100 ng of template DNA, each primer at a concentration of 0.5 μM, a 0.2 mM concentration of each deoxynucleoside triphosphate, 1.5 mM MgCl$_2$, 2.5 U of *Taq* DNA polymerase (Invitrogen, Life Technologies, Breda, The Netherlands), and the PCR buffer supplied with the enzyme. PCR cycling was performed in an UNO II thermocycler Biometra (Westburg, Leusden, The Netherlands) with the following program: predenaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 60 s, and extension at 72°C for 90 s; and a final extension step at 72°C for 10 min.

Cloning of PCR-amplified products. PCR amplicons were purified by the use of a QIAquick PCR purification kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). PCR products were cloned into *Escherichia coli* JM109 by use of the Promega pGEM-T easy vector system (Promega, Madison, Wis.). PCRs were performed on cell lysates of ampicillin-resistant transformants, using the pGEM-T-specific primers T7 and Sp6 to confirm the sizes of the inserts. To establish the eukaryotic diversity between the clones obtained from the aquatic biofilm with *L. pneumophila*, we subjected the amplicons to restriction fragment length polymorphism analysis with the restriction enzymes MspI, AluI, and CfoI. The amplicons derived from plasmids containing unique inserts were purified by use of a QIAquick PCR purification kit and were subjected to DNA sequence analysis.

Sequence analysis. Sequence analysis was done by MWG-Biotech (Ebersberg, Germany). The primers used to sequence the total 18S rRNA gene are given in Table 1 (40, 49).

Phylogenetic analysis. The determined 18S rRNA gene sequences were aligned by use of the ARB software package (28). Different phylogenetic trees were constructed by different methods and by using different filters as implemented in the ARB software package.

Nucleotide sequence accession numbers. The 18S ribosomal DNA (rDNA) sequences from *H. vermiformis* KWR-1, KWR-2, and KWR-3 have been deposited in GenBank under accession numbers AY502959, AY502960, and AY502961, respectively.

RESULTS

Batch test system. A maximum ATP concentration of $9.1 \times 10^2 \pm 2.8 \times 10^1$ pg/cm^2 was reached within 3 days in the batch test system with PVCu. The HPC values showed that exponential growth occurred up to day 3. With PVCp, a maximum ATP concentration of $3.8 \times 10^4 \pm 2.7 \times 10^3$ pg/cm^2 was reached within 6 days. The total ATP concentrations declined to about
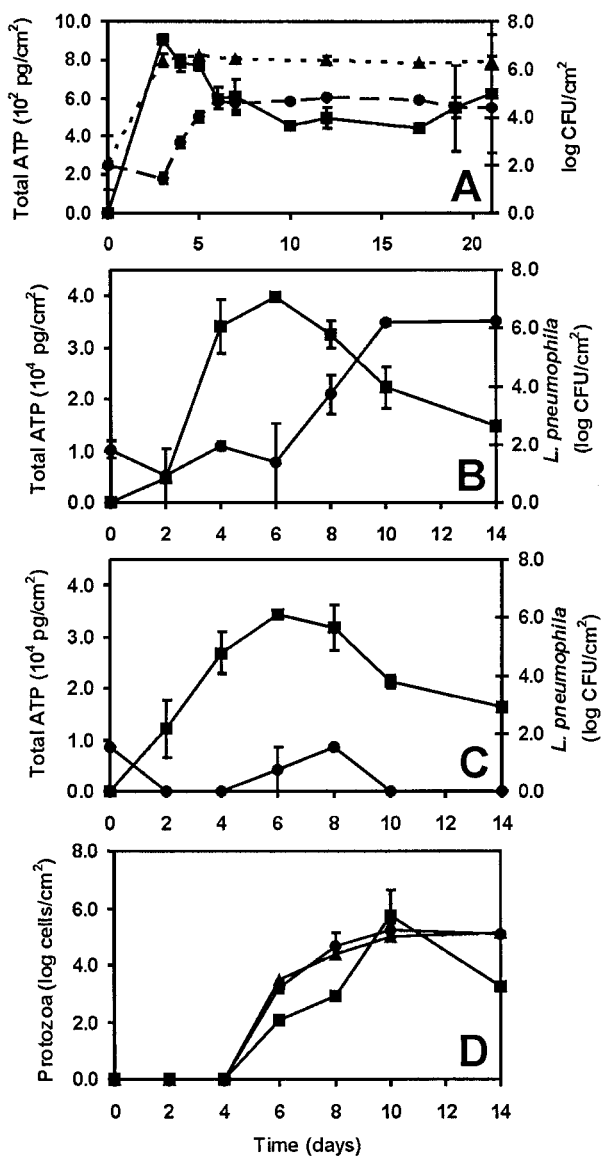
FIG. 1. (A) Concentrations of total ATP (■), *L. pneumophila* (●), and heterotrophic bacteria (▲) in flasks with the unfiltered inoculum and PVCu as the biofilm carrier. Each point represents the mean concentration from two experiments, and bars indicate standard errors. (B) Concentrations of total ATP (■) and *L. pneumophila* (●) in flasks with the unfiltered inoculum and PVCp as the biofilm carrier. Each point represents the mean concentration from two experiments, and bars indicate standard errors. (C) Concentrations of total ATP (■) and *L. pneumophila* (●) in flasks with the filtered inoculum and PVCp as the biofilm carrier. Each point represents the mean concentration from two experiments, and bars indicate standard errors. (D) Protozoon concentrations in flasks with the unfiltered inoculum. Squares (■) represent the concentrations determined by the cultivation method; circles (●) and triangles (▲) represent the concentrations determined by direct counting methods using primulin and FISH, respectively. Bars indicate standard errors. No protozoa were detected in flasks with the filtered inoculum.

$4.6 \times 10^2 \pm 0.5 \times 10^1$ pg/cm^2 on day 10 for PVCu and to about $1.6 \times 10^4 \pm 9.8 \times 10^2$ pg/cm^2 on day 14 for PVCp (Fig. 1A to C) and remained stable when we continued the experiment until day 40 (data not shown). Also, the HPC values observed

with PVCu remained at a constant level during prolonged incubations.

The *L. pneumophila* concentration increased exponentially in flasks with the unfiltered inoculum when the ATP concentrations and HPC values had reached maximal levels. Prolonged incubations showed that the *Legionella* concentration remained stable until the end of the experiment (Fig. 1A and B). The maximum *L. pneumophila* concentration for the experiments with PVCp was 6.3 ± 0.1 log CFU/cm^2 and was lower for experiments with PVCu (4.9 ± 0.1 log CFU/cm^2).

The percentage of biomass present in the biofilm relative to that in the planktonic phase depended on the material used for biofilm development. For PVCu, $57\% \pm 6\%$ of the total ATP, $77\% \pm 6\%$ of *L. pneumophila* cells, and $38\% \pm 11\%$ of the HPC value were present in the biofilm. For PVCp, these values were $37\% \pm 9\%$ for total ATP and $52\% \pm 15\%$ for *L. pneumophila*. The concentrations of total ATP and *L. pneumophila* were highest with PVCp, and this material was used in further experiments for the identification of a growth-supporting factor(s) for *L. pneumophila*.

Identification of growth-supporting factor(s) for *L. pneumophila*. Growth of *L. pneumophila* was detected in the flasks with the unfiltered inoculum (with or without cycloheximide). No proliferation of *L. pneumophila* was observed in the flasks with the filtered inoculum, although the total ATP concentrations under both conditions were similar (Fig. 1B and C). Also, no growth of *L. pneumophila* was observed when a pure culture of *L. pneumophila* serotype 1 was used as the inoculum. The addition of an untreated filter with its retentate to the flasks gave similar results to those for flasks with the unfiltered inoculum, but the addition of a pasteurized (30 min, 70°C) filter with its retentate to the flasks with the filtered inoculum did not induce the growth of *L. pneumophila* (data not shown).

Protozoa were detected by cultivation and direct counting in all of the flasks in which the growth of *L. pneumophila* was observed. No protozoa were detected ($n < 1.6$ log protozoa/cm^2 for cultivation and $n < 2.1$ log protozoa/cm^2 for direct counting) in the flasks in which no proliferation of *L. pneumophila* occurred. Based on their morphology, all of the protozoa cultivated in the 96-well plates were identified as *H. vermiformis*. The direct counting methods also revealed that only one type of protozoa with the typical features of *H. vermiformis* was present in the Erlenmeyer flasks.

From day 6 on, eukaryotic DNAs were detected by 18S rDNA-targeted PCRs in the biofilm and the planktonic phase in flasks that showed growth of *L. pneumophila* (with and without cycloheximide). Although protozoa were present in the flasks with the unfiltered inoculum before day 6, their DNAs were not detectable by PCR, indicating that the initial protozoon concentration was below the detection threshold. Cloning and subsequent sequencing confirmed that *H. vermiformis* was the only protozoon species present. No eukaryotic DNAs were detected in the flasks with the filtered inoculum (Fig. 2).

The FISH technique revealed the different stages of the intracellular proliferation of *L. pneumophila* within protozoa (Fig. 3).

Quantitative relationship between *L. pneumophila* and *H. vermiformis*. Protozoa were first detected on day 6 (Fig. 1D). The cultivation method revealed that the concentration of
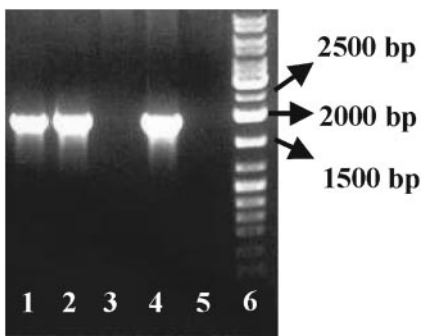
FIG. 2. Detection of eukaryotic DNA. Lane 1, DNA from biofilm from flasks with unfiltered inoculum in the presence of 100 μM cycloheximide; lanes 2 and 4, DNAs from biofilms from flasks with unfiltered inoculum; lanes 3 and 5, DNAs from biofilms from flasks with filtered inoculum; lane 6, marker (sm 0333 gene ruler DNA ladder mix; Fermentas, St. Leon-Rot, Germany).

protozoa increased to 5.8 ± 0.9 log protozoa/cm^2 by day 10 and decreased afterwards. The results of the direct counting methods using primulin and FISH were similar to the results of the cultivation method (Fig. 1D), but with these methods no decline in protozoon concentration was detected after day 10. Furthermore, it was found that $93\% \pm 4\%$ of the protozoa were present in the biofilm rather than in the planktonic phase. During the experiments, the amoebae changed from the trophozoite form on day 6 (Fig. 3A) to the cyst form on day 14 (Fig. 3E). Table 2 gives the different states of the protozoa as percentages of the total concentrations of protozoa observed by direct cell counting using primulin and FISH.

For a time interval of T (hours), the specific growth rate μ (per hour) of the protozoa was calculated as follows:

$$\mu = \frac{\ln y_T - \ln y_0}{T} \qquad (1)$$

where y_0 and y_T are the protozoon numbers at the beginning and end of the interval. During exponential growth, these numbers are related by the equation

$$y_T = y_0 \cdot e^{(\mu T)} \qquad (2)$$

The maximum growth rate of the *H. vermiformis* strain, as

observed with the cultivation method, was 0.14 h^{-1}. With both direct counting methods, a maximum growth rate of 0.15 h^{-1} was calculated.

The *L. pneumophila* concentrations observed in amoebae ranged from 1 cell per amoeba when they were just infected (Fig. 3B) to a maximum of about 100 cells per amoeba just before lysis (Fig. 3C). All but one infected amoebae were present as trophozoites (Fig. 3B and C), and only one infected cyst was detected (0.02%) (Fig. 3E). Table 2 gives the numbers of protozoa that were infected with *L. pneumophila* as percentages of all protozoa and as percentages of the trophozoites.

With the different methods for determining the protozoon concentration (cultivation and direct counting using primulin and FISH), a maximal protozoon concentration of 5.3 ± 0.4 log protozoa/cm^2 was detected on day 10 (Fig. 1D). On that day, 6.2 ± 0.1 log CFU of *L. pneumophila*/cm^2 were present (Fig. 1B). Lysis of the protozoa occurred after about 100 *L. pneumophila* cells per lysed amoeba were observed (Fig. 3C). Hence, 4.2 ± 0.1 log protozoa/cm^2 should account for the total *L. pneumophila* concentration. These lysed trophozoites were not included in the number of protozoa detected on day 10. From this estimated number of lysed protozoa, we calculated that an average of $10.5\% \pm 6.1\%$ ($n = 5$) of all protozoa had been infected between days 6 and 10. With FISH analysis, the intracellular proliferation of *L. pneumophila* within trophozoites was observed in $25.9\% \pm 10.5\%$ ($n = 2$) of the trophozoites that were present on day 10 (Table 2). This percentage was significantly different from the estimated percentage for days 6 to 10 ($P = 0.06$ by a t test). On day 14, the percentage of infected trophozoites had increased further ($>90\%$), and only 0.2% of the protozoa were present as trophozoites (Table 2).

Eukaryotic identification. The 18S rDNA-targeted PCR amplicons were cloned into *E. coli* JM109 and subsequently subjected to restriction fragment length polymorphism analysis. With this analysis, 19 different restriction patterns were obtained for 50 clones. After partly sequencing their 18S rRNA genes, we identified all 19 sequences as being from *H. vermiformis*, with $\geq 98\%$ similarities. These 19 clones could be divided into three groups (KWR-1, KWR-2, and KWR-3). The complete (1,838 bp) 18S rRNA genes for these three different operating taxonomic units were determined and showed G+C contents of 49.62, 49.51, and 49.67% for KWR-1, KWR-2, and KWR-3, respectively.

The sequences (KWR-1, KWR-2, and KWR-3) were compared by phylogenetic analysis with those of other protozoa in which the intracellular growth of *L. pneumophila* had been shown (Fig. 4). The obtained sequences fell into the group of *H. vermiformis*, consistently forming a subgroup. A total of two nucleotide variations were present in all sequence types and were different from seven other *H. vermiformis* sequences at positions 1550 and 1628 (nucleotide numbering is according to the *H. vermiformis* strain Nijmegen sequence [49]). A single base pair insertion (G) was identified between positions 1818 and 1819. In addition to the previously mentioned two mutations, only a few additional single nucleotide polymorphisms were observed among the different *H. vermiformis* strains. These observations indicate that there is a high degree of sequence similarity between *H. vermiformis* strains originating from different habitats and different parts of the world.

TABLE 2. Numbers of protozoa, as determined by direct counting using primulin and FISH, in a batch test system with PVCp as the biofilm carrier in duplicate flasks

Time point (day)	Total concn of protozoa (log cells/cm^2)[a]	% of protozoa in indicated state[a]		Trophozoites (%) containing *L. pneumophila*[a]
		Trophozoite	Cyst	
0	ND	ND	ND	ND
2	ND	ND	ND	ND
4	ND	ND	ND	ND
6	3.4 ± 0.2	100 ± 0.0	0 ± 0.0	ND
8	4.6 ± 0.2	93.9 ± 6.1	6.1 ± 6.1	ND
10	5.1 ± 0.1	5.7 ± 3.0	94.3 ± 3.0	25.9 ± 10.5
14	5.1 ± 0.1	0.2 ± 0.1	99.8 ± 0.1	92.3[b]

[a] ND, not detected: the concentration was below the detection limit ($n < 2.1$ log protozoa/cm^2).
[b] A total of 13 trophozoites were observed in three different tests, and 12 of them contained *Legionella*.
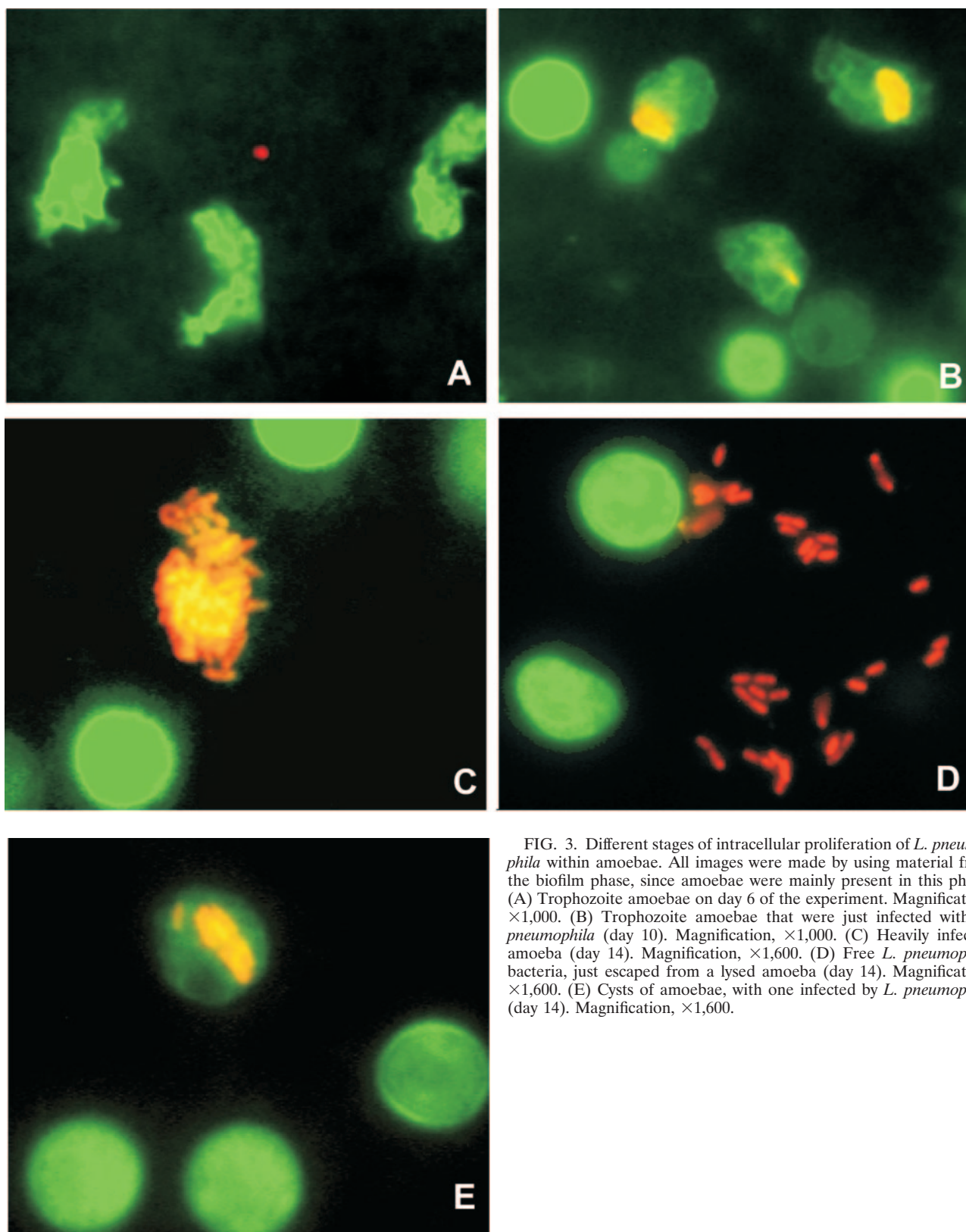
FIG. 3. Different stages of intracellular proliferation of *L. pneumophila* within amoebae. All images were made by using material from the biofilm phase, since amoebae were mainly present in this phase. (A) Trophozoite amoebae on day 6 of the experiment. Magnification, ×1,000. (B) Trophozoite amoebae that were just infected with *L. pneumophila* (day 10). Magnification, ×1,000. (C) Heavily infected amoeba (day 14). Magnification, ×1,600. (D) Free *L. pneumophila* bacteria, just escaped from a lysed amoeba (day 14). Magnification, ×1,600. (E) Cysts of amoebae, with one infected by *L. pneumophila* (day 14). Magnification, ×1,600.
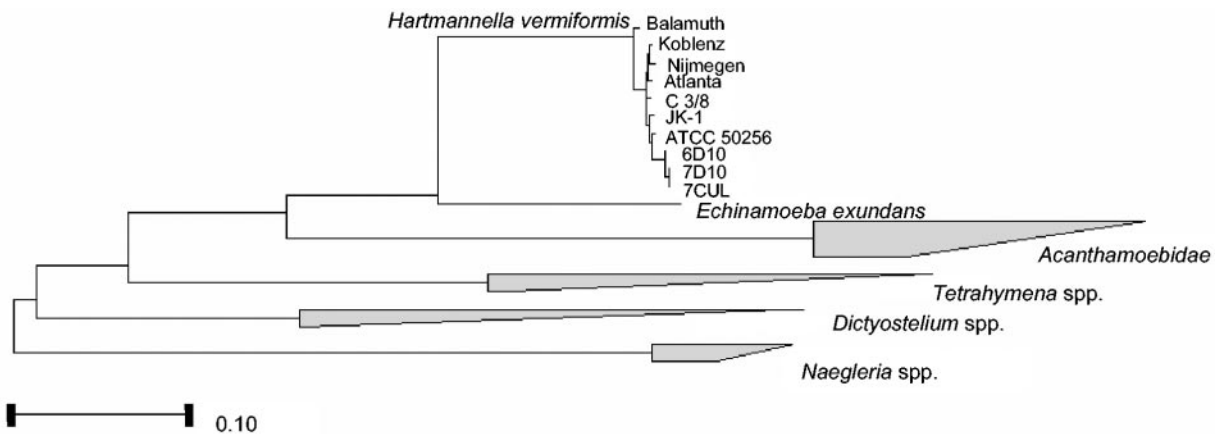
FIG. 4. Phylogenetic tree from 18S rRNA gene sequences showing the relationships of the examined sequences to those of other protozoa in which intracellular growth of *L. pneumophila* was shown (12, 15, 21, 39). Different trees were calculated, and all trees gave the same consensus, as shown in the figure. Bar, 10% sequence divergence. Strains Koblenz (X75514), Nijmegen (X75515), and Atlanta (X75513) were previously sequenced by Weekers et al. (49), strain Balamuth (ATCC 30966, M95168) was sequenced by Gunderson et al. (20), strain C3/8 (AF426157) was sequenced by Walochnik et al. (48), and strains JK-1 and ATCC 50256 were sequenced by Kuchta et al. (24). Clones: 6D10, KWR1; 7D10, KWR2; 7CUL, KWR3.

DISCUSSION

Experimental design. For the present study, we used a batch test system for the simulation of biofilm development in tap water. This batch test system has previously been compared with a dynamic model of biofilm development on different materials that are in contact with tap water (44). That previous study showed that the batch test system is well suited for the study of biofilm formation on material surfaces that are in contact with tap water. With this model, it is easy to obtain different biofilm concentrations by using different materials. ATP was used to assess the concentrations of active biomasses. The similarity between the total biomass concentrations in tests with a filtered inoculum and with an unfiltered inoculum indicates that ATP represented the bacterial biomasses. The concentrations of total ATP and *L. pneumophila* were clearly higher in the presence of PVCp than in the presence of PVCu. Obviously, the concentration of growth-supporting factors for *L. pneumophila* was higher with PVCp, thus facilitating their identification. The high biomass concentrations observed with PVCp were caused by the leaching of biodegradable plasticizers from this material (9). From the obtained percentages of attached and suspended biomasses and the applied surface-to-volume ratios (0.24 to 0.16 cm^{-1}), and assuming a biofilm thickness of 100 μm or less, it can be estimated that the biofilm concentrations were about 100 times higher than the concentrations of suspended biomass. The high proportion (>90%) of protozoa observed in the biofilm on PVCp further indicates that the biofilm was the place where the multiplication of protozoa and *L. pneumophila* occurred.

The following relationship between *L. pneumophila* and total ATP concentrations has been reported for the batch test system with incubation at 25°C: log(*Legionella* growth potential) = 0.81 log(biomass production potential) + 1.1 (44). The concentrations of *L. pneumophila* and total ATP observed in the present study after 14 days for PVCp and after 21 days for PVCu did not fit this equation. The log(*Legionella* growth potential) values in the present study were 1.4 and 1.3 times higher for PVCp and PVCu, respectively, than that calculated with the given equation. This difference was attributed to the different temperatures used for incubation of the test flasks because a temperature of 37°C is more favorable for the growth of *L. pneumophila* than is 25°C (44, 47).

Growth of *Legionella* in aquatic biofilm only in the presence of *H. vermiformis*. Inoculum filtration (3.0-μm pore size) did not influence the total biomass yield (total ATP) but removed a factor that enabled *L. pneumophila* to grow, despite the presence of this bacterium in the inoculum (Fig. 1C). Also, no growth of *L. pneumophila* was observed when it was added to the flasks as a pure culture. The inactivation of the growth-supporting factor by pasteurization indicated that a live organism served as the growth-supporting factor. These findings are in complete agreement with the observations of Wadowsky and coworkers (45), who tested the effect of inoculum filtration (pore size, 1.0 μm) on the growth of *L. pneumophila* in tap water and observed inactivation of the growth-supporting factor at 60°C.

The cultivation method for the detection of protozoa showed only *H. vermiformis*, and this organism was only present in the flasks with the unfiltered inoculum. FISH analysis revealed the in vivo intracellular multiplication of *L. pneumophila* in amoebae, which were identified as *H. vermiformis* by PCR, cloning, and sequence analysis. *H. vermiformis* has also repeatedly been identified as a growth-supporting factor for *L. pneumophila* by researchers in the United States (13, 14, 24, 31, 45, 46). Several authors have questioned the fact that protozoa are required as a host for *L. pneumophila*, and the extracellular growth of *L. pneumophila* has been reported in two studies. In one study, cycloheximide inhibited the growth of protozoa, but *L. pneumophila* proliferated (42). The other study suggested that the bacterial consortium within a biofilm supplies sufficient nutrients to enable legionellae to grow extracellularly (37). In the present study, however, growth of *L. pneumophila* was observed at a similar concentration of cycloheximide, but *H. vermiformis* was still detected by 18S rDNA-

targeted PCR (Fig. 2). Figure 3C shows a microcolony of *L. pneumophila* inside an amoeba just before lysis. The amoeba itself is hardly visible. After the lysis of amoebae, the bacteria are free in the environment, where they may occasionally cluster together (Fig. 3D).

Intracellular growth of *L. pneumophila* has been observed in vitro in *T. pyriformis*, *Acanthamoeba castellanii*, and *H. vermiformis* by the use of FISH (18, 19, 29). The protozoon cultures in these studies were inoculated with a high concentration of *L. pneumophila* (6 to 8 log cells/ml). In the present study, the development of biofilms in tap water was simulated by the use of relatively small inoculum sizes for both *L. pneumophila* and *H. vermiformis* (4.1 and 1.4 log cells/ml, respectively), thus more closely resembling the actual situation in water systems.

The present study also demonstrated the presence of *L. pneumophila* inside a cyst of *H. vermiformis* (Fig. 3E) in vivo, but the percentage of infected cysts was very low (0.02%). Kuchta et al. (23, 24) did not detect *L. pneumophila* within cysts of *H. vermiformis*, and Greub and Raoult (17) observed the bacterium within the cyst wall, but not within vacuoles or the cytoplasm, of a mature cyst of *H. vermiformis*. *L. pneumophila*-infected cysts of *Acanthamoeba palestinensis* have been observed in another study (41), and 2 to 5% of *Acanthamoeba polyphaga* cysts were found to be infected with *L. pneumophila* (38). Our observations support earlier suggestions that trophozoites and cysts may both provide a protective environment for *L. pneumophila* in engineered water systems (22, 41).

Quantitative relationship between total ATP, *L. pneumophila*, and *H. vermiformis*. Heterotrophic bacteria that contributed to the peak of the total ATP concentration (Fig. 1A) most likely served as a food source for *H. vermiformis*, since protozoa were first detected on day 6, when the maximum total ATP concentration was observed (Fig. 1D). The concentration of *L. pneumophila* increased exponentially after the first *H. vermiformis* protozoa were detected. The growth rates of *H. vermiformis* obtained in this study were 2.9-fold higher than the maximum growth rates of naked amoebae in their natural environment (3) and were similar to the highest growth rates of naked amoebae obtained under laboratory conditions (4, 8). Hence, the aquatic biofilm developing on the pieces of PVCp provided a favorable environment for the growth of *H. vermiformis*.

Our observations and calculations further showed that under the test conditions used, a significant percentage of the *H. vermiformis* population was infected with *L. pneumophila*. No infected protozoa were detected on day 8 of the experiment, when 93.9% ± 6.1% of the protozoa were present as trophozoites. On day 10, only 5.7% ± 3.0% of the protozoa were present as trophozoites, 25.9% ± 10.5% of which contained *L. pneumophila*. These percentages were even more extreme on day 14, when 0.2% ± 0.1% of the protozoa were present as trophozoites and >90% of the trophozoites were infected (Table 2). After day 10, no further growth of either *L. pneumophila* or *H. vermiformis* was observed (Fig. 1B and D). This observation supports the conclusion that the growth of *L. pneumophila* depended on intracellular proliferation. The high percentage of infected trophozoites on day 14 suggests that *Legionella* can inhibit the growth of *H. vermiformis* under batch test conditions. On the other hand, the growth of protozoa had already ceased on day 10, possibly by the depletion of pre-

ferred prey bacteria. A clarification of these processes requires further research.

Phylogenetic analysis of *H. vermiformis*. Among the protozoa in which intracellular growth of *L. pneumophila* occurs, the cluster of *H. vermiformis* showed the highest similarity with *Echinamoeba exundans*. This was also found by Amaral Zettler et al. (2). In fact, *E. exundans* was first described as a species of *Hartmannella* (33). The clones KWR-1, KWR-2, and KWR-3 clearly formed a subgroup within the *H. vermiformis* group, although there are very few sequence variations within the *H. vermiformis* cluster (49). This subgroup showed the highest similarity with *H. vermiformis* strain ATCC 50256, which was originally isolated from a hot water tank in relation with *L. pneumophila* (24). The obtained phylogenetic tree and the results of Walochnik et al. (48) do not support the previously suggested separation between European and North American strains of *H. vermiformis* (24, 49).

In conclusion, this study demonstrated that *L. pneumophila* multiplies in trophozoites of *H. vermiformis* that are present in aquatic biofilms developing on plastic materials in contact with tap water in a batch test system. Different materials (PVCu and PVCp) gave different biofilm concentrations (total ATP concentrations), and higher total ATP concentrations gave higher concentrations of *L. pneumophila*. The proliferation of *L. pneumophila* was due to its intracellular multiplication in *H. vermiformis* in the presence of a high concentration of heterotrophic bacteria in the biofilm. Prevention of the growth of *L. pneumophila* within engineered water systems thus requires the prevention of growth of protozoa in biofilms. This objective can be achieved by the removal of biodegradable compounds from the water, the selection of materials that do not support biofilm formation, and/or the maintenance of a disinfectant residual.

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