

## Role of biofilms in the survival of *Legionella pneumophila* in a model potable-water system

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**Legionellae can infect and multiply intracellularly in both human phagocytic cells and protozoa. Growth of legionellae in the absence of protozoa has been documented only on complex laboratory media. The hypothesis upon which this study was based was that biofilm matrices, known to provide a habitat and a gradient of nutrients, might allow the survival and multiplication of legionellae outside a host cell. This study determined whether *Legionella pneumophila* can colonize and grow in biofilms with and without an association with *Hartmannella vermiformis*. The laboratory model used a rotating disc reactor at a retention time of 6·7 h to grow biofilms on stainless steel coupons. The biofilm was composed of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and a *Flavobacterium* sp. The levels of *L. pneumophila* cells present in the biofilm were monitored for 15 d, with and without the presence of *H. vermiformis*, and it was found that, although unable to replicate in the absence of *H. vermiformis*, *L. pneumophila* was able to persist.**

Keywords: *Hartmannella vermiformis*, protozoa, growth, ecology

### INTRODUCTION

Legionellae are commonly found in freshwater environments worldwide. However, these bacteria require an unusual combination of nutrients that are rarely found in aquatic environments. When such nutrients are present in the environment, they usually serve to amplify faster-growing bacteria that compete with the legionellae. Legionellae survive as intracellular parasites of free-living protozoa (Fields, 1996; Rowbotham, 1980). Rowbotham (1980) first described the ability of *Legionella pneumophila* to infect protozoa and later described these bacteria as ‘protozoonotic, i.e. naturally infecting protozoa’. Legionellae have been reported to multiply in 13 species of amoebae and two species of ciliated protozoa. Growth of legionellae in the absence of protozoa has been documented only on laboratory media (Fields, 1996). A number of studies have described the relationship between legionellae and protozoa in aquatic environments identified as potential or actual reservoirs of disease-causing strains. Protozoa naturally present in these environments can support intracellular growth of legionellae *in vitro* (Barbaree *et al.*, 1986; Newsome *et al.*, 1998). In building water systems,

microbial growth is frequently detected as biofilms on plumbing fixtures and heating, ventilating and air-conditioning equipment. Although it is generally accepted that legionellae are commonly found within these biofilms, most studies have only partially characterized the microbial flora present in the systems (Schofield & Locci, 1985; Walker *et al.*, 1994). The *Legionella* biofilm studies that have been conducted employed naturally occurring microbial communities. Several of these studies tested for the presence of protozoan organisms (Fields *et al.*, 1990; Henke & Seidel, 1986; Surman *et al.*, 1995; Thomas *et al.*, 1999) and found protozoan organisms to be present along with *Legionella*. An extensive study by Kuchta *et al.* (1998) examined interactions between *L. pneumophila* and *Hartmannella vermiformis*, and the efficacy of several disinfectants, and strongly suggested the requirement of protozoan organisms as a ‘growth factor’ for the cell replication of *Legionella*. Some investigators (Wright *et al.*, 1989) have carried out *in vitro* studies of *Legionella* biofilm formation and have observed biomass accumulation on the substrata in the absence of protozoan cells. These studies have referred to the biomass as ‘growth’; however, they failed to address whether those *Legionella* cells were actually dividing in the absence of protozoa or merely surviving, which, in our view, is a more relevant question. The purpose of this study was to determine the

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**Abbreviation:** GFP, green fluorescent protein.

ability of *L. pneumophila* to grow in a potable-water biofilm without an association with *H. vermiformis*.

## METHODS

**Design and assembly of biofilm reactors.** Rotating disc reactors (Center for Biofilm Engineering, Bozeman, MT, USA) containing 316L stainless steel coupons (1.27 cm in diameter) were used for all experiments and are shown in Fig. 1. The disc reactors were placed in a water bath to hold the temperature at 30 °C. Mixing was provided by a digitally controlled mixing plate (Mirak Thermolyne; Fisher Scientific) placed beneath the water bath. Fig. 1 shows the system set-up diagram. Initially, reactors were operated in batch mode for 72 h to establish the biofilms on the steel substrata. The medium contained 0.05 g yeast extract, proteose peptone no. 3, Casamino acids and dextrose, 0.03 g sodium pyruvate and dibasic potassium phosphate, and 0.005 g magnesium phosphate per litre of filter-sterilized reverse-osmosis water. Following the period of batch growth, the system was operated as an open system by continuously pumping a 1/10 dilution of the medium formulation given above at a flow rate of 1 ml min<sup>-1</sup> for 24 h in order to dilute the medium. The feed to the reactors was then changed to filter-sterilized dechlorinated tap water (Atlanta, GA, USA; municipal tap water dechlorinated with 0.5 ml l<sup>-1</sup> of a 15.8 g sodium thiosulfate l<sup>-1</sup> solution) at the same flow rate of 1 ml min<sup>-1</sup> (retention time of 6.7 h). This water had a pH ranging from 7.5 to 7.8. Each biofilm reactor experiment was repeated at least three times.

**Base biofilm bacterial strains.** Each reactor was inoculated with *Pseudomonas aeruginosa* (ATCC 7700), *Klebsiella pneumoniae* (DMDS Lab. No. 92-08-28a) and a *Flavobacterium* sp. (CDC-65) organism. These micro-organisms are commonly found in potable-water environments (Geldreich, 1990) and are commonly used in biofilm studies. The strains used in our studies were environmental isolates. Cultures were stored at -70 °C, transferred to R2A plates (Reasoner & Geldreich, 1979) and resuspended to a concentration equal to a 0.5 McFarland. Each reactor was inoculated with 1 ml of each cell suspension to a final concentration of approximately 5 × 10<sup>5</sup> ml<sup>-1</sup>. Base biofilms were allowed to grow for 7 d before *H. vermiformis* or *L. pneumophila* was added.

***H. vermiformis*.** *H. vermiformis* (CDC-19) stocks were grown in axenic growth medium at 35 °C without CO<sub>2</sub> (King *et al.*, 1991) and subcultured twice a week into T75 cell-culture flasks. Flasks were tapped on a solid surface to dislodge *H. vermiformis* from the growth surface, transferred to 50 ml conical tubes, centrifuged to pellet the amoebae and

resuspended in PBS. Reactors were inoculated with *H. vermiformis* for a final concentration of 10<sup>4</sup> ml<sup>-1</sup>.

***L. pneumophila*.** *L. pneumophila* (RI243) carrying the plasmid pANT4 (Lee & Falkow, 1998) encoding both kanamycin resistance and green fluorescent protein (GFP) was stored as a suspension in defibrinated rabbit blood in a liquid nitrogen (-120 °C) freezer. Fluorescence was determined by using a hand-held lamp [model UVL-21 Blak-Ray Lamp (UVP), long-wave UV 333 nm]. Four days before the isolate was needed, the mutant was cultured onto BCYE media [buffered charcoal-yeast extract agar (containing 0.1% 2-oxoglutarate)] with kanamycin and incubated at 36 °C with 2.5% CO<sub>2</sub>. After the 4 d, the isolate was resuspended in sterile water and diluted to the desired concentration. One millilitre of a suspension of *L. pneumophila* was added to each reactor for a final concentration of approximately 5 × 10<sup>5</sup> ml<sup>-1</sup>.

**Scanning electron microscopy.** Coupons were fixed by placing them into 5% glutaraldehyde (Ted Pella) in cacodylate buffer (0.067 M, pH 6.2) for fixation overnight at room temperature. Samples were then dehydrated in a graded series of ethanol (30, 50, 70, 90%) for 10 min each at room temperature and immersed in hexamethyldisilazane (Polysciences) for 4 h at room temperature. Finished specimens were mounted on aluminium stubs with silver paint, sputter-coated with 25 nm gold, and examined with a Phillips XL 30 environmental scanning electron microscope (FEI, a subsidiary of Phillips).

**Epifluorescence microscopy.** Coupons were fixed by placing them into 5% formaldehyde (J. T. Baker) in reverse osmosis water for 5 min at room temperature. Samples were then fluorescently stained with 1 µg 4',6-diamidino-2-phenylindole (Sigma) ml<sup>-1</sup> for 15 min; this was followed by rinsing in reverse osmosis filter-sterilized water. The coupon surfaces were examined with an Axioskop 2 epifluorescence microscope (Carl Zeiss) using an HBO-100 illuminator and a Zeiss Plan-NEOFLUAR ×100 1.30 oil objective with a 355/40 excitation filter, a 400/long-pass dichroic mirror and a 420/long-pass emission filter. To visualize the GFP cells, we examined the surfaces with a 480/40 excitation filter, a 505 dichroic mirror and a 510/long-pass emission filter.

**Processing for the removal of biofilms.** Coupons were removed from the reactors, dip-rinsed in phosphate-buffer water, placed into 10 ml phosphate-buffer saline, processed by three cycles of sonication for 30 s followed by vortexing for 30 s, homogenized for 1 min, and spread-plated on R2A medium for quantification of the base biofilm. For the recovery of *H. vermiformis*, 100 µl aliquots from several dilutions were plated onto non-nutritive agar that had been spread with viable *Escherichia coli*. Plates were read at 3 and 7 d for the presence/absence of *H. vermiformis* at the dilution plated. For the recovery of *L. pneumophila*, the supernate from the processing of each coupon was treated with a KCl/HCl solution, filtered through a 0.2 µm filter (part no. GTTP; Millipore), resuspended, and plated onto glycine-polymyxin B-anisomycin-vancomycin plates.

## RESULTS

Base biofilm densities of 10<sup>7</sup>–10<sup>8</sup> c.f.u. per coupon were consistently recovered from disc reactors prior to the inoculation of the system with *H. vermiformis*. Fig. 2 shows the effect of the addition of *H. vermiformis* on the base biofilm. Base biofilm counts decreased by approximately 2 logs, and biofilm-associated *H. vermiformis* increased by approximately 2 logs between day 8 and day 10; these levels were maintained for the remainder

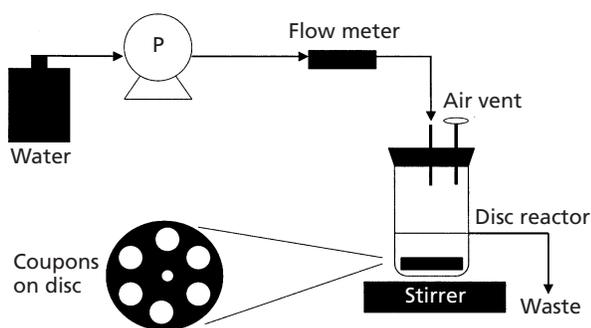
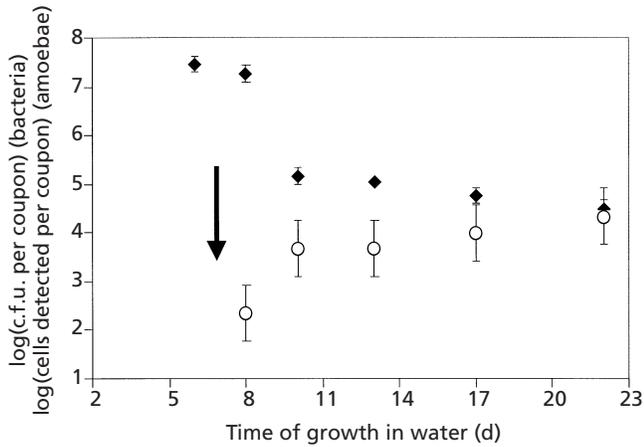
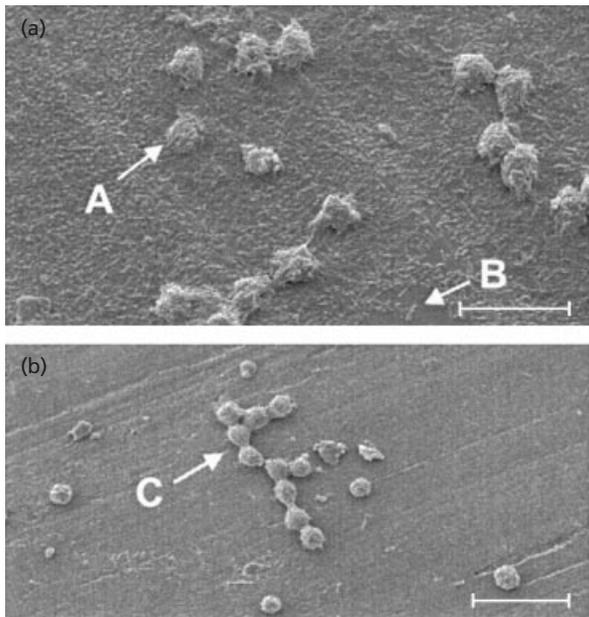


Fig. 1. Potable-water biofilm reactor set-up diagram.



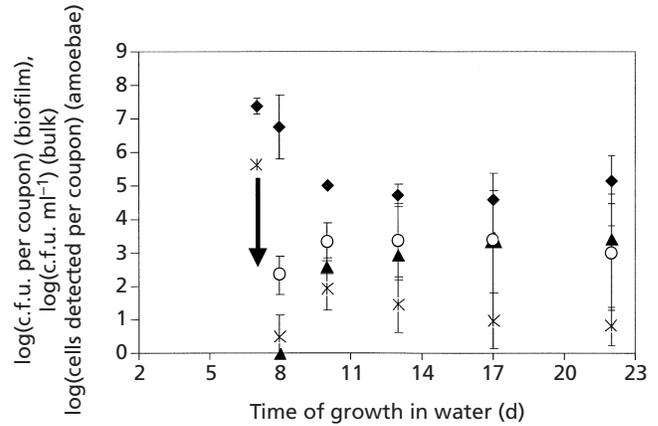
**Fig. 2.** Recovery of biofilm-associated *H. vermiformis* from a potable-water biofilm. The arrow indicates the day of inoculation with *H. vermiformis*. ◆, *P. aeruginosa*, *K. pneumoniae* and a *Flavobacterium* sp. in the biofilm. ○, *H. vermiformis* in the biofilm. Error bars indicate the SD ( $n = 3$ ).



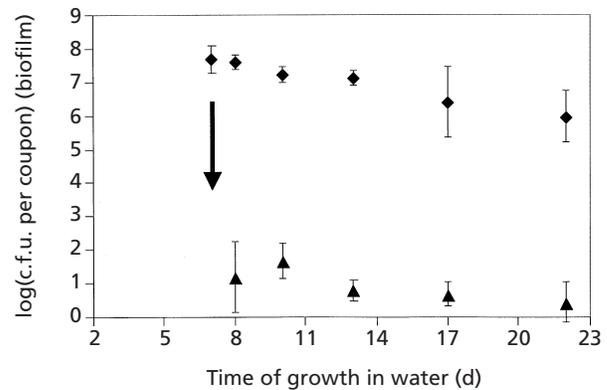
**Fig. 3.** Scanning electron micrographs of the biofilms 1 d (a) and 15 d (b) after the addition of *H. vermiformis*. A, B and C, *H. vermiformis* trophozoites covered with bacteria, bacterial cells, and *H. vermiformis* cysts, respectively. Bars, 20  $\mu\text{m}$ .

of the experiment (a total of 15 d). The reduction in the base biofilm levels was apparently due to predation by *H. vermiformis*, since the biofilm-associated *H. vermiformis* growth rates correlated with the base biofilm rate of decline, and no such rapid decline in base biofilm occurred in the absence of *H. vermiformis* (see Fig. 5).

Scanning electron micrographs of *H. vermiformis* on the base biofilm, 1 and 15 d after addition of the amoebae, respectively, are shown in Fig. 3(a, b). *H. vermiformis*



**Fig. 4.** Recovery of biofilm-associated *H. vermiformis* and *L. pneumophila* from a potable-water biofilm. The arrow indicates the day of inoculation with *H. vermiformis* and *L. pneumophila*. ◆, *P. aeruginosa*, *K. pneumoniae* and a *Flavobacterium* sp. in the biofilm. ○, *H. vermiformis* in the biofilm. ×, *L. pneumophila* in bulk liquid. Error bars indicate the SD ( $n = 3$ ).



**Fig. 5.** Recovery of biofilm-associated *L. pneumophila* from a potable-water biofilm. The arrow indicates the day of inoculation with *L. pneumophila*. ◆, *P. aeruginosa*, *K. pneumoniae* and a *Flavobacterium* sp. in the biofilm. ▲, *L. pneumophila* in the biofilm. Error bars indicate the SD ( $n = 3$ ).

trophozoites (Fig. 3a) encyst as they feed on the base biofilm and reduce it (Fig. 3b). *H. vermiformis* encysted as early as 6 d after their addition. When *L. pneumophila* and *H. vermiformis* were added to the reactor on day 7, a similar reduction in the base biofilm occurred, whereas the *H. vermiformis* and *L. pneumophila* counts increased (Fig. 4). *L. pneumophila* counts in the biofilm reached  $10^3$  c.f.u. per coupon, and remained above  $10^3$  for the duration of the experiment. Addition of *L. pneumophila* to the base biofilm without *H. vermiformis* resulted in much lower numbers of *L. pneumophila* (1–2 logs fewer) being recovered from the biofilms (Fig. 5). In addition, a comparison between Fig. 4 and Fig. 5 shows that planktonic *L. pneumophila* cells were recovered only from the disc reactors inoculated with both *H.*

**Table 1.** Validation of plasmid loss as a marker of growth in a batch study

*Legionella* recovered are expressed as mean c.f.u. ml<sup>-1</sup> for the bulk ( $n = 3$ ). The percentage GFP is presented in parentheses.

Time after inoculation (d)	With amoebae	Without amoebae
0	$1.1 \times 10^3$ (99.3)	$1.6 \times 10^3$ (99.2)
3	$8.9 \times 10^6$ (74.2)	$1.6 \times 10^3$ (99.3)
7	$3.0 \times 10^8$ (77.7)	$1.0 \times 10^2$ (93.7)

*vermiformis* and *L. pneumophila*. These results appeared to support the conclusions that *H. vermiformis* colonized the base biofilms, and that the ability of *L. pneumophila* to colonize this system was significantly improved when *H. vermiformis* was present.

To determine if *L. pneumophila* was multiplying in the absence of the amoebae, we looked at the rate of plasmid loss by *L. pneumophila* in biofilms with and without *H. vermiformis*. Loss of this plasmid can readily be determined by loss of fluorescence. Loss of the plasmid was confirmed in the nonfluorescing bacteria by loss of the ability to grow on kanamycin. To ensure that *L. pneumophila* loses fluorescence when multiplying, the bacteria were coinoculated with *H. vermiformis* into assay medium, and loss of fluorescence was determined over 7 d (Table 1, column 2). To determine the loss of fluorescence from *L. pneumophila* cells that were not in the process of multiplying, we suspended *L. pneumophila* cells in sterile water without *H. vermiformis*, and the loss of fluorescence was determined during the 7 d (Table 1, column 3). These results indicated that *L. pneumophila* numbers increased exponentially when associated with the amoebae, and that the percentage of fluorescent cells decreased steadily over the 7 d. The percentage of cells fluorescing (and total viable counts) without amoebae (column 3) was essentially unchanged when cells were suspended in sterile water. For both

datasets, the percentage of cells carrying the plasmid is shown in parentheses.

The percentage of fluorescing cells in biofilm-associated *L. pneumophila* in the biofilm reactor study is shown in Table 2. The percentage of fluorescing cells in the biofilms ranged from 82 to 89% between 1 and 15 d after inoculation with *H. vermiformis* and *L. pneumophila* (Table 2, column 3). The percentage of fluorescing planktonic *L. pneumophila* cells ranged from 71 to 100% (column 2) and, as with the biofilm cells, decreased over time. Biofilm-associated *L. pneumophila* in the absence of *H. vermiformis* showed an increase from 98% 1 d after the addition of *L. pneumophila* to 99% on day 2, and to 100% fluorescing cells for the remainder of the experiment (column 5). No planktonic *L. pneumophila* was detected [the limit of detection was  $1.3$  c.f.u. (ml bulk liquid)<sup>-1</sup>] in the reactor without *H. vermiformis*. All isolated fluorescent colonies maintained their fluorescence upon repeated transfer to fresh medium and grew in the presence of kanamycin (a measurement of plasmid-encoded kanamycin resistance). Isolates that had lost their fluorescence failed to grow in the presence of kanamycin.

Microscopic observations of the biofilm matrix showed no evidence of microcolony formation by GFP cells in either the presence or the absence of *H. vermiformis*.

Our results (Figs 4 and 5, Tables 1 and 2) showed that, in our biofilm system, *H. vermiformis* was required for *L. pneumophila* multiplication, and that the biofilm matrix in the absence of *H. vermiformis* provided an environment in which *L. pneumophila* could survive without division.

## DISCUSSION

Cell growth can be defined as the culmination of an orderly interplay between all the physiological activities of the cell. It is a complex process involving the uptake of nutrients, conversion of these nutrients into usable energy, replication of the chromosome, increases in the size and mass of the cell, and division into two daughter

**Table 2.** Assessment of *Legionella* growth, in a biofilm study, based upon GFP plasmid loss

*Legionella* recovered are expressed as mean c.f.u. per coupon for the biofilm ( $n = 3$ ). The percentage GFP is presented in parentheses. ND, Not detected.

Time after inoculation (d)	With amoebae		Without amoebae	
	Bulk	Biofilm	Bulk	Biofilm
0	–	–	ND	–
1	$4.6 \times 10^0$ (100.0)	–	ND	$4.1 \times 10^1$ (98.5)
3	$1.7 \times 10^2$ (91.8)	$4.5 \times 10^3$ (88.6)	ND	$7.8 \times 10^1$ (99.2)
6	$8.3 \times 10^1$ (81.7)	$1.2 \times 10^3$ (86.3)	ND	7.6 (100.0)
10	$1.8 \times 10^1$ (78.9)	$5.5 \times 10^3$ (87.8)	ND	6.2 (100.0)
15	$1.0 \times 10^1$ (71.4)	$3.6 \times 10^3$ (82.1)	ND	4.9 (100.0)

cells (Moat & Foster, 1995). According to this definition, active cell division and the production of daughter cells are among the prerequisites for growth. Terms such as 'viable but nonculturable' have been used by other investigators to describe cells that might be injured and, though metabolically active, incapable of cell division. Steinert *et al.* (1997) determined the recovery of *L. pneumophila* suspended in sterile water for 180 d by using routine plating media and acridine orange direct counts. They found that viable plate counts declined steadily during the first 125 d incubation, after which the cells were no longer culturable. However, the rate of decline in acridine orange direct counts was much smaller, and cells were detected even after 55 d non-culturability. When *Acanthamoeba castellanii* was added to their system containing only nonculturable cells, culturable cells of *L. pneumophila* were detected after 1 d coinoculation. Multiplication of *L. pneumophila* was evidenced by the fact that cell concentrations exceeded day 1 concentrations 3 d after inoculation with *A. castellanii*. Their results strongly support the findings of Kuchta *et al.* (1998), mentioned earlier. Because we were able to culture *L. pneumophila* cells from biofilms, it was apparent that at least a percentage of cells in our system were both viable and culturable. However, it was still unclear as to whether *L. pneumophila* cells recovered from the biofilms in the absence of amoebae were actually growing (in every sense of the definition given earlier) or simply surviving by endogenous metabolism in the early stages of the 'viable but nonculturable' state. To address this question, we used plasmid loss as an indication of cell division of *L. pneumophila*. The strain of *L. pneumophila* used carried a plasmid encoding both kanamycin resistance and GFP. A lack of fluorescence was interpreted as evidence of plasmid loss, which would serve to indicate cell division in the absence of selective pressure. After *L. pneumophila* + GFP were inoculated into a medium containing *H. vermiformis* (but without a biofilm), the *L. pneumophila* cell count increased exponentially with a steady loss of fluorescence. *L. pneumophila* suspended in water without *H. vermiformis* (and without a biofilm) showed no such growth or loss of fluorescence. Studies in the biofilm reactors confirmed the batch studies; virtually all of the *L. pneumophila* cells in biofilms without *H. vermiformis* maintained fluorescence, whereas those with *H. vermiformis* in the biofilms lost fluorescence and produced increased cell counts. These results, combined with the fact that microcolony formation was never observed, argue that although the presence of *H. vermiformis* is not required for survival, it is required for growth in our model system. Similar findings were reported at a recent international meeting (Szewzyk *et al.*, 2000). Using a continuous flow chamber described elsewhere (Szewzyk *et al.*, 1994), the investigators followed the behaviour of a *L. pneumophila* serogroup 1 strain (LP1) in the biofilm and the outflow water over 98 d. Their findings showed that *L. pneumophila* did not multiply in defined mixed biofilms with a natural water bacterium. The number of LP1 cells decreased rapidly in the outflowing water of the mixed biofilm, and no LP1 cells were detected in the

biofilm after 40 d (by fluorescent *in situ* hybridization using a *Legionella*-specific probe). In a parallel chamber *A. castellanii* (ATCC 33152) was added simultaneously to LP1. The number of LP1 cells in the outflowing water increased to several logs ml<sup>-1</sup> and remained constant for 98 d. Colonized amoebae were detected in the biofilm by fluorescent *in situ* hybridization with probes specific to eukaryotes and *Legionella*. Only half of the amoebae detected contained LP1 cells, and only 10% were heavily colonized. Some *Legionella* cells were detected outside the amoebae but were always in close proximity to the amoebae.

Our model system does not exactly replicate conditions provided in natural potable-water biofilms, and a different set of conditions could provide the necessary growth requirements for *Legionella* without associated free-living protozoa. However, the significant differences between *Hartmannella*-containing and non-*Hartmannella*-containing biofilms in terms of the *Legionella* growth rate, plasmid loss and production of daughter cells indicate that *H. vermiformis* (and probably other free-living protozoa) plays a role in the survival and growth of *Legionella* in the environment.

## Conclusions

A biofilm reactor capable of developing reproducible, steady-state bacterial biofilms in nonsupplemented potable water has been developed. We chose this model because we believe that it provides a realistic representation of the conditions and organisms that we wanted to study.

*H. vermiformis* was shown to associate with these biofilms and feed upon the bacteria within them. Under our system's conditions, viable *L. pneumophila* associated and persisted in these biofilms with and without *H. vermiformis* for a period of 15 d after inoculation.

*L. pneumophila* cells did not develop microcolonies or biofilms in the biofilm reactors, even in the presence of *H. vermiformis*. This suggests that their presence in our biofilms could be attributed to the survival of cells from the original inoculum in the biofilm matrix, plus daughter cells that resulted from cell division inside a protozoan host.

In this biofilm reactor system, higher numbers of *L. pneumophila* were recovered from the biofilm matrix in the presence of *H. vermiformis*. Also, planktonic *L. pneumophila* cells were detected only in the bulk liquid from reactors that contained *H. vermiformis*. The quantification of the GFP loss by *L. pneumophila* was used as an indication of cell replication, and demonstrated that replication occurred only in the presence of *H. vermiformis*.

On the basis of this and other similar studies, we conclude that although *L. pneumophila* was not capable of growth in the absence of the protozoan host, this organism could survive within the biofilm matrix. In

agreement with the current understanding of biofilms, we demonstrated that the presence of biofilms in potable and healthcare-facility water systems can provide a means for the survival and dissemination of *L. pneumophila*.

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