

## Necrotrophic Growth of *Legionella pneumophila*

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**This study examined whether *Legionella pneumophila* is able to thrive on heat-killed microbial cells (necrotrophy) present in biofilms or heat-treated water systems. Quantification by means of plate counting, real-time PCR, and flow cytometry demonstrated necrotrophic growth of *L. pneumophila* in water after 96 h, when at least 100 dead cells are available to one *L. pneumophila* cell. Compared to the starting concentration of *L. pneumophila*, the maximum observed necrotrophic growth was 1.89 log units for real-time PCR and 1.49 log units for plate counting. The average growth was  $1.57 \pm 0.32$  log units ( $n = 5$ ) for real-time PCR and  $1.14 \pm 0.35$  log units ( $n = 5$ ) for plate counting. Viability staining and flow cytometry showed that the fraction of living cells in the *L. pneumophila* population rose from the initial 54% to 82% after 96 h. Growth was measured on heat-killed *Pseudomonas putida*, *Escherichia coli*, *Acanthamoeba castellanii*, *Saccharomyces boulardii*, and a biofilm sample. Gram-positive organisms did not result in significant growth of *L. pneumophila*, probably due to their robust cell wall structure. Although necrotrophy showed lower growth yields compared to replication within protozoan hosts, these findings indicate that it may be of major importance in the environmental persistence of *L. pneumophila*. Techniques aimed at the elimination of protozoa or biofilm from water systems will not necessarily result in a subsequent removal of *L. pneumophila* unless the formation of dead microbial cells is minimized.**

As the causative agent of Legionnaires' disease, *Legionella pneumophila* poses a substantial human health threat through colonization of water systems worldwide (13, 14, 24). Since person-to-person transmission of *L. pneumophila* has not yet been observed, measures to prevent the bacterium from spreading have concentrated on the elimination of this (opportunistic) pathogen from water sources (5). Consequently, the analysis of *L. pneumophila* in complex environmental consortia has become increasingly important (8, 19).

Despite its widespread distribution, *L. pneumophila* seems to have unusual nutritional requirements when grown in pure culture (8), and information about factors that contribute to the survival or active growth of the pathogen in its (natural) environment is very limited and divergent (5). On the one hand, it is supposed that *L. pneumophila* grows within protozoan hosts in aquatic environments and that this interaction is central to the pathogenesis and ecology of *L. pneumophila* (10, 12, 15, 16). Conversely, a number of studies suggested the protozoon-independent survival and growth of *L. pneumophila* (18, 21, 27, 32), in which case the bacterium uses biofilm matrices as a shelter and source of nutrients. Although scarce, there is evidence that a number of microorganisms in the proximity of *L. pneumophila* may favor its growth by excreting extracellular compounds as carbon and energy sources (1, 23, 29). Furthermore, sediment (mineral deposits and detritus) has also been shown to improve the survival of *L. pneumophila* by means of a synergistic effect (20), and recently, it was suggested that the required exogenous supply of amino acids may originate from other microorganisms or from decaying organic matter (5).

The prevention of *Legionella* multiplication in water systems is currently emulated by a combination of frequent heating of the water and limiting its residence time (stagnation). However, many water systems suffer from secluded spots, such as dead-end plumbing, which hamper treatment efficacy. Furthermore, *L. pneumophila* has been shown to survive extreme ranges of environmental conditions, including extensive temperatures ranging from 5 to 63°C (9). Considering these facts, it is evident that the complete eradication of *Legionella* from water systems is hard to achieve, with small numbers of residing bacteria thriving again shortly after water treatment. Recent studies using model warm water systems demonstrated significantly elevated *Legionella* concentrations ( $>10^7$  CFU/liter) following heat treatment of the water system (27, 28).

Because biofilm debris can accumulate in the periphery of distribution systems, leading to sediment accumulation and microbial proliferation (26), the hypothesis is that heat treatment induces the formation of sudden loads of dead microbial cells, which become readily available as feed to surviving *L. pneumophila*, resulting in a fast (re)colonization of the environment. This paper aimed to verify whether *L. pneumophila* is potentially necrotrophic, thereby using dead microbial cells as a source of nutrients to sustain its persistence and growth. In order to estimate the significance of this necrotrophy, a comparison was made to protozoon-mediated replication of *L. pneumophila*.

### MATERIALS AND METHODS

**Strains and cultivation.** Strains used in the course of this study were *L. pneumophila* sg1 (ATCC 33152), *Pseudomonas putida* UWC3, *Escherichia coli* ATCC 4157, *Bacillus subtilis* LMG 7135, *Lactobacillus plantarum* LMG 6907, *Acanthamoeba castellanii* ATCC 30234, and *Saccharomyces boulardii* MUCL 43341. *L. pneumophila* was grown aerobically in BYE broth or on BCYE agar plates for 72 h at 37°C (7). *P. putida*, *E. coli*, *B. subtilis*, and *S. boulardii* were grown aerobically in Luria-Bertani broth (Oxoid, Basingstoke, United Kingdom) for 24 h at 37°C (except for *P. putida*, which was grown at 28°C). *A. castellanii* was

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grown aerobically in ATCC medium 711 for 5 days at 28°C. Cell suspensions for subsequent experiments were prepared by centrifugation of a fully grown culture for 4 min at 5,000 × *g* (Minispin; Eppendorf, Hamburg, Germany) and suspension of the pellet in 0.22- $\mu$ m-filter-sterilized tap water. In order to remove all nutrients originating from the growth medium, each suspension was washed twice by centrifugation and again suspended in sterile tap water. Optical density measurements at 610 nm (ISIS9000; Dr. Lange, Berlin, Germany) were performed to obtain the desired starting concentration of each cell suspension, as determined initially by means of plate counting.

**Necrotrophic growth experiments.** In order to demonstrate necrotrophic growth of *L. pneumophila*, a number of growth experiments using mainly heat-killed (30 min at 60°C) *P. putida*, unless otherwise stated, were performed. Efficiency of the heat treatment was verified throughout the experiments by means of plating 50  $\mu$ l of the suspension on the appropriate culture medium.

**Setup 1.** Setup 1 tackled the possibility of *L. pneumophila* thriving on dead microbial cells in an aquatic environment. Sterile tubes containing 5 ml of 0.22- $\mu$ m-filter-sterilized tap water were inoculated with *L. pneumophila*, to which the following organisms were added: (i) live *P. putida*, (ii) heat-killed *P. putida* (concentrations ranging from 10<sup>3</sup> to 10<sup>9</sup> CFU/ml), (iii) heat-killed *E. coli*, (iv) heat-killed *B. subtilis*, (v) heat-killed *Lactobacillus plantarum*, (vi) heat-killed *A. castellanii*, (vii) heat-killed *S. boulardii*, and (viii) heat-treated biofilm (originating from 22-cm<sup>2</sup> polyvinyl chloride coupons installed in a cooling tower water circuit with a 211-mg chemical oxygen demand/cm<sup>2</sup>). An axenic culture of *L. pneumophila* in sterile tap water served as a negative control. Cell suspension starting concentrations, as estimated using optical density measurements, were approximately 10<sup>6</sup> CFU/ml for *L. pneumophila*, 10<sup>8</sup> CFU/ml for *A. castellanii*, and 10<sup>9</sup> CFU/ml for *P. putida*, *E. coli*, *B. subtilis*, and *S. boulardii*. All tubes were aerobically incubated at 37°C, with determination of *L. pneumophila* numbers at 0, 24, and 96 h using real-time PCR and plate counting of 10-fold dilution series on BCYE agar plates. This experiment was performed five times.

**Setup 2.** Supplemental to plate counting and real-time PCR, flow cytometry was also applied to demonstrate necrotrophic growth of *L. pneumophila*. Using a double fluorescent stain, information on the fractions of live and dead *L. pneumophila* cells, besides their total count, could be obtained. The experimental design was similar to that of setup 1, using 5 ml of filter-sterilized tap water to which the following organisms were added: (i) *L. pneumophila*, (ii) *L. pneumophila* and heat-killed *P. putida*, and (iii) *L. pneumophila* and live *P. putida*. Final concentrations were 10<sup>4</sup> CFU/ml for *L. pneumophila* and 10<sup>7</sup> CFU/ml for *P. putida*. Incubation of the mixture was done at 37°C, with flow cytometry measurements at 0, 24, and 96 h. To detect and quantify the number of live and dead *L. pneumophila* cells, 1-ml samples were stained for 10 min using the LIVE/DEAD BacLight Bacterial Viability kit (Invitrogen, Merelbeke, Belgium) according to the manufacturer's instructions. Stained samples were analyzed with a Cyan LX flow cytometer (Dakocytomation, Heverlee, Belgium) (3). The threshold trigger was set to sideward scatter, and differences in Syto 16 and propidium iodide fluorescence allowed a clear separation between live and dead *L. pneumophila* cells.

**Setup 3.** To compare necrotrophic growth of *L. pneumophila* with protozoon-mediated replication, sterile 2.2-liter polyvinyl chloride recipients were filled with 1 liter of filtered (0.22- $\mu$ m filter) tap water. Inoculation of the recipients was done with *L. pneumophila* and (i) heat-killed *P. putida*, (ii) live *A. castellanii*, or (iii) live *A. castellanii* and heat-killed *P. putida*. Again, a recipient containing only *L. pneumophila* served as a negative control. Starting concentrations for *L. pneumophila*, *P. putida*, and *A. castellanii* were approximately 10<sup>3</sup> CFU/ml, 10<sup>6</sup> CFU/ml, and 10<sup>4</sup> CFU/ml, respectively. Water samples (200 ml) for real-time PCR and denaturing gradient gel electrophoresis (DGGE) analysis were taken after 0, 1, 4, 8, and 14 days at 28°C. This experiment was performed three times.

**Setup 4.** As a final indication of necrotrophic growth, a biological oxygen demand (BOD) test was performed, which measured the oxygen consumption during microbial growth. Sterile BOD bottles (500 ml) were filled with 365 ml of sterile tap water supplemented with the following organisms: (i) 10<sup>5</sup> CFU/ml *L. pneumophila*, (ii) 10<sup>5</sup> CFU/ml *L. pneumophila* and 10<sup>8</sup> CFU/ml heat-killed *P. putida*, and (iii) 10<sup>8</sup> CFU/ml heat-killed *P. putida*. All bottles were provided with a magnetic stirrer and sealed with a cap containing an electronic pressure indicator (Oxitop; WTW, Weilheim, Germany). All cultures were washed twice using sterile water and incubated overnight to remove all internal and external medium reserves prior to transfer to the autoclaved BOD bottles. A digital oxygen pressure measurement at 37°C was performed on a daily basis during five consecutive days. The BOD value for each sample was calculated according to the instructions of the manufacturer of the Oxitop apparatus (33).

**Identification of *L. pneumophila*.** In order to verify the purity of the *L. pneumophila* culture used throughout the experiments, a latex agglutination test kit (Oxoid, Basingstoke, United Kingdom) was used according to the manufactur-

er's instructions. Furthermore, real-time PCR, flow cytometry, and DGGE analyses (4) provided additional certainty on the identity of the strain used.

**DNA extraction.** The majority of experiments provided 1-ml samples that were directly suitable for DNA extraction. In the case of setup 3, 200 ml of sample was filtered using sterile filtration units (250 ml; Nalgene, Rochester, N.Y.) equipped with a 0.22- $\mu$ m filter (47 mm; Millipore, Bedford, Mass.). Subsequent DNA extraction was performed directly on the filter paper based on the protocol described previously by Boon and coworkers (2).

**Real-time PCR.** Real-time PCR was based on the protocol described previously by Wellingshausen and coworkers (31) for the specific quantification of *L. pneumophila* by amplifying the *mip* gene. PCR was performed in 25- $\mu$ l reaction mixtures using the qPCR Core kit for SYBR Green I, according to the manufacturer's instructions (Eurogentec, Liège, Belgium), in MicroAmp optical 96-well reaction plates with optical caps (PE Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The thermal profile was as follows: 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 57°C for 1 min, and 60°C for 1 min. Amplicon dissociation curves were determined by constant fluorescent measurement during a final heating step at 60°C to 95°C at a 0.1°C/s ramping speed. The template DNA in the reaction mixtures was amplified in triplicate and monitored with an ABI Prism SDS 7000 instrument (PE Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Construction of the *mip* gene standard curve was performed by means of a dilution series of an *L. pneumophila* culture in sterile tap water. Correlation to the number of CFU and total cell count was determined by plate counting on BCYE medium and flow cytometry, respectively.

**PCR-DGGE.** PCR was performed using a *Taq* polymerase kit (Applied Bio Systems, N.J.). Primers used in this study amplified the V3 region of the 16S rRNA gene (17) and the eukaryotic 18S rRNA gene (6). PCR and subsequent DGGE analysis of the amplicons were performed, as described previously (22), by using the Dcode system (Bio-Rad). Gels were stained in 200 ml of 1× Tris-acetate-EDTA containing 16  $\mu$ l SYBR Green I nucleic acid gel stain (1:10,000 dilution; FMC BioProducts, Rockland, Maine) and immediately photographed on a UV transillumination table with a video camera module (Vilbert Lourmat, Marne-la Vallée, France). Normalization of the gels was performed using the BioNumerics software package, version 2.50 (Applied-Maths, St.-Martens-Latem, Belgium).

## RESULTS

The goal of this study was to verify whether *L. pneumophila* is capable of surviving and/or growing on dead microbial cells that are presumed to be present after heat treatment of a biofilm-containing water system.

**Setup 1.** Because of the use of one *L. pneumophila* starting suspension and equal volumes in all experiments, only the number of *L. pneumophila* cells from this starting suspension was determined at 0 h. After 96 h, real-time PCR showed significant necrotrophic growth of *L. pneumophila* when at least 10 dead *P. putida* cells were available to one *L. pneumophila* cell (10:1), whereas plate counts indicated the requirement of a 100:1 ratio (Table 1). Compared to the starting concentration of *L. pneumophila*, the observed maximum necrotrophic growth was 1.89 log units for real-time PCR and 1.49 log units for plate counting, with an average of 1.57 ± 0.32 log units and 1.14 ± 0.35 log units (*n* = 5), respectively. Table 1 contains the results at 96 h only, because no significant rise in *L. pneumophila* numbers was witnessed after 24 h in all experiments (data not shown). When the necrotrophic growth was compared to the axenic *L. pneumophila* suspension after 96 h, the actual necrotrophic growth surpasses 2 log units because of the slight decay of *L. pneumophila* in the negative control. No growth stimulation of *L. pneumophila* by live *P. putida* cells was observed and could be measured only by real-time PCR because of *P. putida* overgrowth on the BCYE plates.

Within the same experimental setup, a variety of heat-killed microorganisms as well as a biofilm sample were included in

TABLE 1. Culture-dependent and real-time PCR-monitored necrotrophic growth of *L. pneumophila*<sup>a</sup>

Inoculum	<i>L. pneumophila</i> CFU/ml (SD)		<i>L. pneumophila mip</i> gene copies/ml (SD)	
	0 h	96 h	0 h	96 h
<i>L. pneumophila</i> (approx 10 <sup>6</sup> cells)	7.8 × 10 <sup>5</sup> (2.5 × 10 <sup>5</sup> )	2.5 × 10 <sup>5</sup> (1.3 × 10 <sup>5</sup> )	2.4 × 10 <sup>6</sup> (8.6 × 10 <sup>5</sup> )	6.2 × 10 <sup>5</sup> (6.3 × 10 <sup>4</sup> )
<i>L. pneumophila</i> + 10 <sup>9</sup> live <i>P. putida</i> cells		NA		3.7 × 10 <sup>6</sup> (1.9 × 10 <sup>6</sup> )
<i>L. pneumophila</i> + 10 <sup>3</sup> dead <i>P. putida</i> cells		4.2 × 10 <sup>5</sup> (2.6 × 10 <sup>5</sup> )		3.5 × 10 <sup>6</sup> (9.6 × 10 <sup>5</sup> )
<i>L. pneumophila</i> + 10 <sup>4</sup> dead <i>P. putida</i> cells		3.8 × 10 <sup>5</sup> (9.5 × 10 <sup>4</sup> )		9.8 × 10 <sup>5</sup> (4.5 × 10 <sup>5</sup> )
<i>L. pneumophila</i> + 10 <sup>5</sup> dead <i>P. putida</i> cells		6.5 × 10 <sup>5</sup> (3.6 × 10 <sup>5</sup> )		3.5 × 10 <sup>6</sup> (1.8 × 10 <sup>6</sup> )
<i>L. pneumophila</i> + 10 <sup>6</sup> dead <i>P. putida</i> cells		4.5 × 10 <sup>5</sup> (3.1 × 10 <sup>5</sup> )		4.6 × 10 <sup>6</sup> (1.7 × 10 <sup>6</sup> )*
<i>L. pneumophila</i> + 10 <sup>7</sup> dead <i>P. putida</i> cells		6.9 × 10 <sup>5</sup> (2.6 × 10 <sup>5</sup> )		9.1 × 10 <sup>6</sup> (2.0 × 10 <sup>6</sup> )*
<i>L. pneumophila</i> + 10 <sup>8</sup> dead <i>P. putida</i> cells		5.3 × 10 <sup>6</sup> (1.1 × 10 <sup>6</sup> )*		4.8 × 10 <sup>7</sup> (9.6 × 10 <sup>6</sup> )*
<i>L. pneumophila</i> + 10 <sup>9</sup> dead <i>P. putida</i> cells		1.1 × 10 <sup>7</sup> (7.2 × 10 <sup>6</sup> )*		8.9 × 10 <sup>7</sup> (2.0 × 10 <sup>7</sup> )*
<i>L. pneumophila</i> + 10 <sup>9</sup> dead <i>E. coli</i> cells		6.3 × 10 <sup>6</sup> (2.6 × 10 <sup>6</sup> )*		6.5 × 10 <sup>7</sup> (1.3 × 10 <sup>7</sup> )*
<i>L. pneumophila</i> + 10 <sup>9</sup> dead <i>L. plantarum</i> cells		6.0 × 10 <sup>5</sup> (4.2 × 10 <sup>5</sup> )		7.5 × 10 <sup>5</sup> (5.2 × 10 <sup>5</sup> )
<i>L. pneumophila</i> + 10 <sup>9</sup> dead <i>B. subtilis</i> cells		NA		1.8 × 10 <sup>5</sup> (8.6 × 10 <sup>4</sup> )
<i>L. pneumophila</i> + 10 <sup>8</sup> dead <i>A. castellanii</i> cells		6.8 × 10 <sup>6</sup> (3.0 × 10 <sup>6</sup> )*		4.8 × 10 <sup>7</sup> (1.3 × 10 <sup>7</sup> )*
<i>L. pneumophila</i> + 10 <sup>9</sup> dead <i>S. bouardii</i> cells		5.5 × 10 <sup>6</sup> (2.8 × 10 <sup>6</sup> )*		1.1 × 10 <sup>7</sup> (8.2 × 10 <sup>6</sup> )*
<i>L. pneumophila</i> + heat-treated biofilm		NA		2.9 × 10 <sup>7</sup> (7.6 × 10 <sup>6</sup> )*

<sup>a</sup> Necrotrophic growth of *L. pneumophila* in sterile tap water on heat-killed (30 min, 60°C) microbial cells was measured. All *L. pneumophila* starting concentrations were approximately 10<sup>6</sup> CFU/ml. Quantification of *L. pneumophila* cells during 96 h was performed using plate counts on BCYE agar and real-time PCR targeting of the *mip* gene. Numbers presented are mean values of five repetitive experiments for which each separate experiment was measured in triplicate. An asterisk indicates significant necrotrophic growth of *L. pneumophila* compared to the starting concentration. NA, not available due to bacterial overgrowth.

order to determine the specificity of necrotrophic growth. As presented in Table 1, with the exception of the tested gram-positive bacteria, all organisms as well as heat-treated biofilm resulted in a significant growth of *L. pneumophila* compared to that of the axenic control strain. No large differences between the growth stimulation by *E. coli*, *P. putida*, *A. castellanii*, *S. bouardii*, and biofilm existed. Based on these results, we decided to perform further experiments using *P. putida*, an organism frequently encountered in natural biofilms.

**Setup 2.** In a separate experiment, flow cytometry was used to demonstrate necrotrophic growth and to acquire information on the ratio of live to dead *L. pneumophila* cells in each sample. A visual presentation of necrotrophic growth as recorded by flow cytometry is presented in Fig. 1. The applied heat treatment did not result in a complete lysis of the *P. putida* cells, and the dead *P. putida* population was clearly separated from both live and dead *L. pneumophila* cells, thereby facilitating a reliable quantification of the latter. After 96 h, most

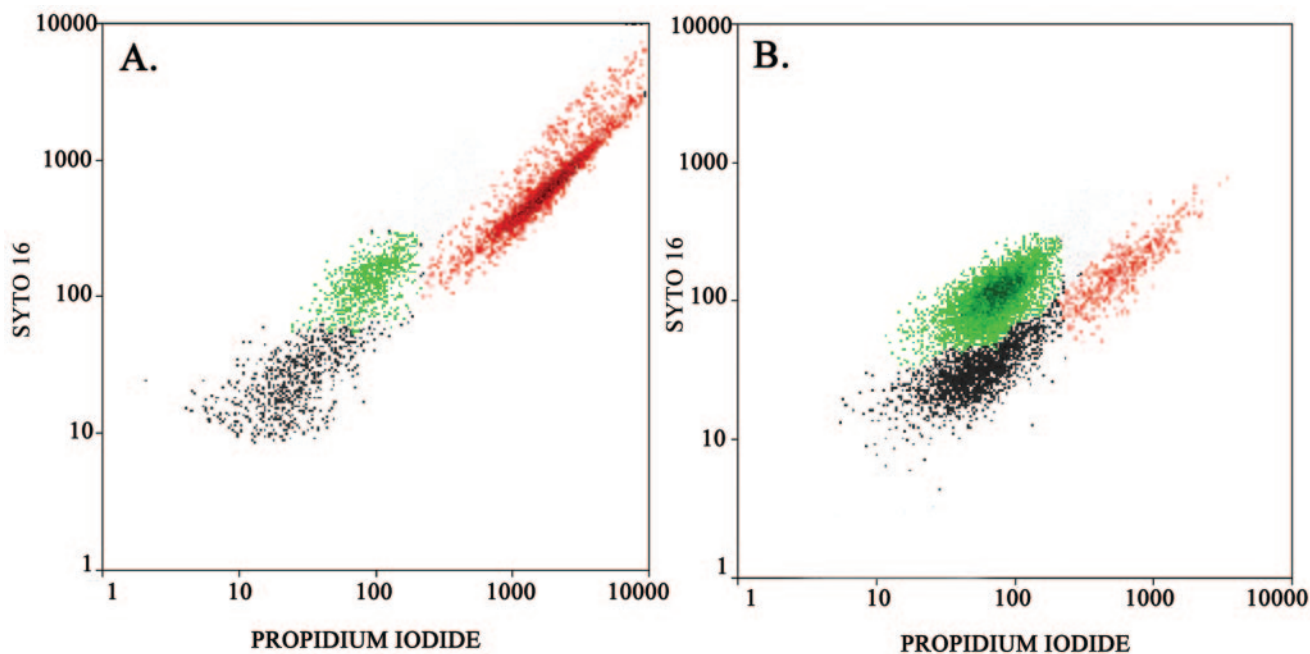


FIG. 1. Live/dead analysis of necrotrophic growth. Both plots show Syto 16 fluorescence in the ordinate and propidium iodide fluorescence in the abscissa. Red dots indicate dead *P. putida* cells, green dots indicate live *L. pneumophila* cells, and black dots indicate dead *L. pneumophila* cells (colors are for visualization purposes only and do not correspond to actual fluorescence wavelengths). (A) *L. pneumophila* plus dead *P. putida* at 0 h; (B) *L. pneumophila* plus dead *P. putida* cells at 96 h.



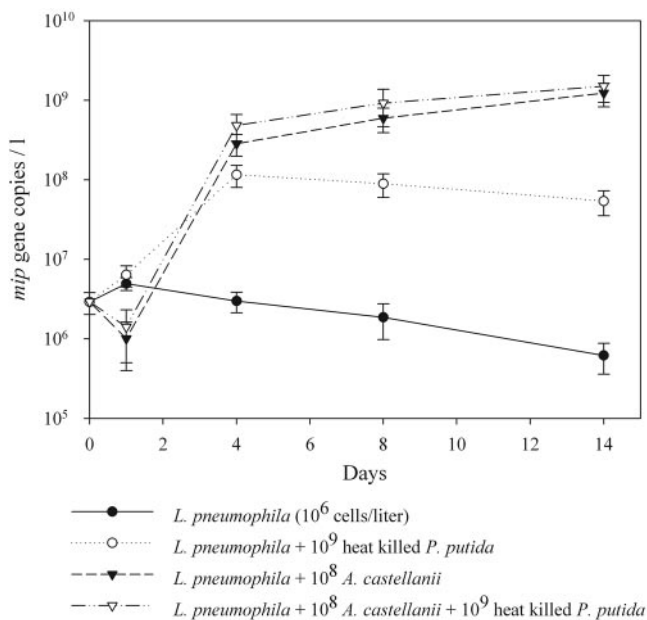


FIG. 2. Necrotrophic growth of *L. pneumophila* at 28°C in filter-sterilized tap water on heat-killed (30 min 60°C) *P. putida* cells and live *A. castellanii* cells. Quantification of *L. pneumophila* cells was performed during 2 weeks using real-time PCR targeting the *mip* gene.

dead *P. putida* cells disappeared, and the total number of *L. pneumophila* cells increased from  $2.5 \times 10^4 \pm 7.2 \times 10^3$  cells/ml to  $1.6 \times 10^6 \pm 5.5 \times 10^5$  cells/ml. In the negative control, *L. pneumophila* dropped from  $2.5 \times 10^4 \pm 6.0 \times 10^3$  cells/ml to  $9.2 \times 10^3 \pm 4.2 \times 10^3$  cells/ml. By means of viability staining, it was shown that the fraction of living cells in the *L. pneumophila* population rose from the initial 54% to 82% after 96 h. Between  $10^3$  and  $10^8$  *L. pneumophila* cells/ml, total counts by real-time PCR and flow cytometry were similar within 1 log and approximately 10 times higher than that found by plate counting. However, based on the results from live/dead staining using the flow cytometer, it could be concluded that real-time PCR slightly overestimated and plate counting slightly underestimated the number of viable *L. pneumophila* cells (data not shown). Finally, it was shown that the filter-sterilized tap water used in the experiments is a suitable matrix, with only a minor drop in viable *L. pneumophila* cells in the negative control.

**Setup 3.** One liter of filter-sterilized tap water was used to determine the importance of necrotrophic growth in larger volumes and to compare the growth with *A. castellanii*-mediated replication of *L. pneumophila*. Also, in larger volumes, necrotrophic growth of *L. pneumophila* occurs (ratio of *L. pneumophila* to *P. putida* being 1:1,000), with a maximum of 1.42 log units and an average of  $1.05 \pm 0.37$  log units ( $n = 3$ ) (Fig. 2). After 14 days, the total cell count of *L. pneumophila* was substantially lower on dead *P. putida* cells in the case of necrotrophy ( $6.2 \times 10^5 \pm 2.1 \times 10^5$  cells/liter) compared to protozoan-mediated replication ( $1.5 \times 10^8 \pm 7.3 \times 10^7$  cells/liter). However, during the first 96 h, necrotrophy kept up with protozoan-mediated growth, even demonstrating a head start during the first 48 h. The combination of dead *P. putida* cells with *A. castellanii* did not result in a significantly higher *L.*

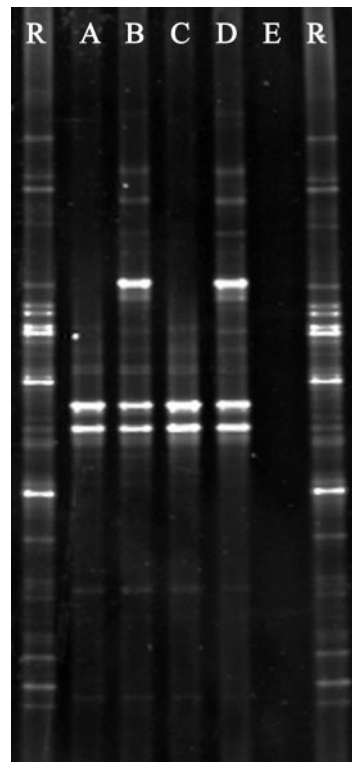


FIG. 3. Example of a prokaryotic DGGE gel to verify the bacterial composition of starting samples during a necrotrophic growth experiment in filter-sterilized tap water. R, reference pattern for normalization; A, pure culture of *L. pneumophila* strain ATCC 33152; B, *L. pneumophila* plus live *P. putida* cells; C, *L. pneumophila* in sterile tap water (negative control); D, *L. pneumophila* plus heat-killed *P. putida* cells; E, filter-sterilized tap water as a blank sample.

*pneumophila* count than that with *A. castellanii* alone. During all experiments, pro- and eukaryotic DGGE was used to verify the microbial composition of the samples as well as to confirm the axenic nature of the strains used. As an additional means of identification, the band position of *L. pneumophila* in the samples was compared to that of the type strain, as previously described by Calvo-Bado and coworkers (4) (Fig. 3). Furthermore, it was demonstrated that no protozoa besides the inoculated *A. castellanii* were present in the filtered tap water (data not shown).

**Setup 4.** Although indirect, a supplemental indication of necrotrophic growth was obtained by measuring oxygen consumption during bacterial growth of *L. pneumophila* on dead *P. putida* cells. After removing all residual nutrients originating from the culture medium, it was shown that *L. pneumophila* was unable to grow actively in sterile tap water when present as an axenic culture. Oxygen consumption remained nearly zero, with a maximum of 2 mg/liter after 5 days. In contrast, *L. pneumophila* cells supplemented with dead *P. putida* cells (ratio, 1,000:1) clearly demonstrated active growth, with levels reaching 57 mg/liter at day 5. To verify the influence of decaying organic matter, heat-killed *P. putida* in sterile tap water was monitored as well, with a maximum BOD value of 29 mg/liter, clearly lower than the value obtained with necrotrophic growth.

## DISCUSSION

Despite increasing research efforts, our understanding of the survival and/or growth of *L. pneumophila* in natural and human-made environments is still limited and divergent (5). Protozoan hosts are supposed to be crucial for replication of *L. pneumophila* in aquatic environments (10, 12, 15, 16), although alternative pathways have been suggested (5, 18, 20, 21, 27, 32). This paper demonstrates that *L. pneumophila* is able to thrive on heat-killed microbial cells present in biofilms or heat-treated water systems.

A fivefold repetition of several growth experiments using both culture-dependent and culture-independent quantification techniques clearly demonstrated significant growth of *L. pneumophila* when at least 100 dead cells are available to one *L. pneumophila* cell (Table 1). The growth yield was approximately 10%. The inclusion of live *P. putida* cells in the experiment did not result in any growth of *L. pneumophila*, indicating that the latter is not capable of actively killing other bacteria. Growth of *Legionella* on mineral deposits and detritus was previously demonstrated by Stout and coworkers (20), although it required a synergism with other bacteria. To our knowledge, the results presented in this paper are the first to demonstrate that *L. pneumophila* is able to grow on dead bacterial cells that may occur in the environment (biofilm) or originate from water disinfection measures (25, 26).

The specificity of necrotrophic growth was determined by including a taxonomic range of heat-killed microorganisms in the setup, with the eukaryotic organism *A. castellanii* inducing a slightly higher growth yield of *L. pneumophila*, most likely due to their larger cell mass. In contrast, the heat-killed gram-positive organisms *B. subtilis* and *Lactobacillus plantarum* did not result in the growth of *L. pneumophila*, probably because of the robust cell wall structure of these organisms, which prevents nutrients from becoming readily available. In the case of heat-killed *Bacillus*, growth of this organism was seen on the BCYE plates, indicating insufficient heat treatment or the survival of *Bacillus* spores. As a representative for more environmental situations, approximately 22 cm<sup>2</sup> of biofilm was collected from a cooling tower and added to *L. pneumophila* after heat treatment. Again, a significant growth of more than 1 log unit was recorded. Given the fact that a biofilm is a constantly renewing entity, containing nearly 50% dead microbial cells (25, 30), this demonstrates how *L. pneumophila* is able to survive and grow in most water systems in a protozoan-free manner.

Although plate counting and real-time PCR were mainly used to quantify *L. pneumophila* cells during the experiments, flow cytometry was also applied to validate the methodologies and verify the fractions of live and dead cells within a population. From our results, it was noted that real-time PCR produces counts that are approximately 1 log higher for *L. pneumophila* because of the detection of *mip* genes from both live and dead cells. Live/dead analysis using a flow cytometer indicated that only 18% of the *L. pneumophila* population was dead after 96 h of necrotrophic growth. This implies that real-time PCR overestimates the number of viable *L. pneumophila* cells but also that plate counting underestimates the number because of a small fraction of *L. pneumophila* cells being viable but not culturable. Despite these findings, both techniques

indicated necrotrophic growth of more than 1 log unit compared that of to their own negative control. This magnitude of growth was also confirmed using flow cytometry, which added the information that after 96 h of necrotrophic growth, 82% of the *L. pneumophila* cells were still alive. Finally, during the flow cytometric analyses, it was seen that the heat-treated *P. putida* cells did not lyse completely and that these cells may serve as nutrient packages for *L. pneumophila*, although the actual mechanism of necrotrophic growth needs to be studied in the future.

As mentioned previously, the major route for *L. pneumophila* survival and growth in the environment is considered to be replication within protozoan hosts (15). Therefore, another experiment compared necrotrophic growth to protozoon-mediated growth. It was noted that necrotrophic growth of *L. pneumophila* was also significant in larger volumes of water, as long as the ratio of at least 100 heat-killed *P. putida* cells to one *L. pneumophila* cell is maintained. Remarkably, after 24 h and 48 h, necrotrophic growth resulted in higher numbers of *L. pneumophila* than that found with protozoon-mediated growth. From 72 h on, the latter surpassed the former, resulting in numbers of *L. pneumophila* that were 1 log higher than those obtained with necrotrophic growth, which came to a halt after 96 h. The combination of *A. castellanii* and heat-killed *P. putida* in the same recipient did not significantly increase the numbers of *Legionella* cells compared to growth mediated by *A. castellanii* itself. These results indicate that the major route of *L. pneumophila* replication is by means of protozoa, although necrotrophy is far from negligible. It has to be kept in mind that the setup was batch mode; continuous feeding of the water system with heat-killed *P. putida* or protozoa may result in other growth yields of *L. pneumophila*. In this context, batch mode corresponds to sudden loads of dead cells as a result of heat treatment of (biofilm-containing) water systems (26), whereas a continuous supply of dead organics can be found within biofilm itself (25). Although it has been demonstrated that when bursting out of protozoa, *L. pneumophila* has shifted towards a virulent phenotype (11), from our results, it is not possible to conclude whether this is also the case during necrotrophic growth.

Finally, an experiment was performed to measure oxygen consumption as an indirect indication of bacterial growth. Again, compared to the negative control, *L. pneumophila* supplemented with dead *P. putida* cells demonstrated bacterial growth despite the influence of dead *P. putida* cells on the measurements. Although this experiment by itself does not provide any direct evidence for necrotrophic growth, it further supports the findings of the other experiments with specific monitoring of *L. pneumophila*.

In conclusion, this paper clearly demonstrates the possibility that *L. pneumophila* can survive and grow on dead microbial cells, as no other nutrients were sufficiently available in the experiments to achieve growth of over 1 log unit. Although this necrotrophy shows lower growth yields than replication within protozoan hosts, our findings indicate that it may have major importance in the persistence of *L. pneumophila* in the environment, such as in biofilms. Techniques aimed at the elimination of protozoa or biofilms from water systems will not necessarily result in a subsequent removal of *L. pneumophila* unless the formation of dead cells is minimized. Necrotrophy,

as demonstrated in this paper, may also explain the elevated *L. pneumophila* concentrations witnessed recently after heat treatment of a water system (27, 28). The concept of necrotrophy by *L. pneumophila* warrants further research in terms of its environmental importance and its relation to the virulence of *L. pneumophila*. It also suggests that the eradication of *L. pneumophila* from water systems may be more difficult than already expected.

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