

High Diversity and Abundance of *Legionella* spp. in a Pristine River and Impact of Seasonal and Anthropogenic Effects^{∇†}

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The diversity and dynamics of *Legionella* species along a French river watershed subject to different thermal and wastewater discharges during an annual cycle were assessed by 16S rRNA gene sequencing and by a fingerprint technique, single-strand conformation polymorphism. A high diversity of *Legionella* spp. was observed at all the sampling sites, and the dominant *Legionella* clusters identified were most closely related to uncultured bacteria. The monthly monitoring revealed that *Legionella* sp. diversity changes were linked only to season at the wastewater site whereas there was some evidence for anthropogenic effects on *Legionella* sp. diversity downstream of the thermal bath. Quantification of *Legionella pneumophila* and *Legionella* spp. by culture and quantitative PCR (qPCR) was performed. Whereas only *L. pneumophila* was quantified on culture media, the qPCR assay revealed that *Legionella* spp. were ubiquitous and abundant from the pristine source of the river to the downstream sampling sites. These results suggest that *Legionella* spp. may be present at significant concentrations in many more freshwater environments than previously thought, highlighting the need for further ecological studies and culturing efforts.

Since the discovery of the human pathogen *Legionella pneumophila* in 1976, about 50 species of *Legionella* have been described. About one-half of them have been associated with human Legionnaires' disease (20), which occurs after inhalation of aerosolized water contaminated with virulent *Legionella* strains. Numerous cases of legionellosis after exposure to contaminated water from the water distribution systems of hotels, hospitals, and cooling towers have been reported (8, 9, 36). The major reservoirs of *Legionella* spp. are freshwater environments such as lakes, rivers, groundwater, and hot springs, but they can also survive in seawater and water from wastewater treatment plants (WWTPs) (12, 14, 22, 34, 38). Their presence in these different reservoirs demonstrates their ability to grow, or at least persist, under a wide range of different environmental conditions (e.g., temperature, pH). They can use a number of different strategies to survive in these different environments, including their use of free-living amoebae as hosts for intracellular replication, the protection of the cells in amoebal cysts (31), their persistence in biofilms (16), and their ability to enter a viable but nonculturable state (18, 47). These strategies have complicated the detection of *Legionella*, and there is limited knowledge of *Legionella* species diversity in the natural environment essentially due to the reliance on culture-based methods for *Legionella* sp. detection, which select for *L. pneumophila* (7, 28, 39). Further insights into *Legionella* ecology have been gained by the

development of cultivation-independent techniques using cellular approaches (immunofluorescence or fluorescence *in situ* hybridization) (6, 45) and, more recently, molecular approaches based on PCR (34, 43, 48). The construction of 16S rRNA gene clone libraries and DNA sequencing were used to study *Legionella* diversity in sand filters (10), in acidic biofilm communities in Yellowstone National Park (38), in drinking water (46), and more recently in river water in Brazil (11). However, the construction of clone libraries is labor-intensive and expensive and is not usually applied to numerous samples. A more in-depth analysis of the diversity of natural *Legionella* species requires the analysis of many samples by high-throughput screening methods. One such genetic fingerprinting technique, single-strand conformation polymorphism (SSCP), is well suited to bacterial diversity analysis of large sample sets due to its rapidity, reproducibility, and low cost and was also used recently to study the dynamics of *Legionella* spp. in water from a cooling tower plant (44).

The majority of studies have focused on the source of *Legionella* contamination in the man-made systems where they were proliferating. However, the increasing incidence of legionellosis highlights the need to better understand the origin of the *Legionella pneumophila* and non-*L. pneumophila* species in the major freshwater reservoirs and how different *Legionella* species may be impacted by environmental or anthropogenic effects.

The main objectives of this study were to better characterize *Legionella* diversity in natural water samples and to determine if there were seasonal or anthropogenic effects on *Legionella* diversity and composition. To meet these objectives, *Legionella* abundance and diversity were investigated along a river before and after thermal bath and wastewater discharges during an annual cycle. *L. pneumophila* and *Legionella* spp. were quan-

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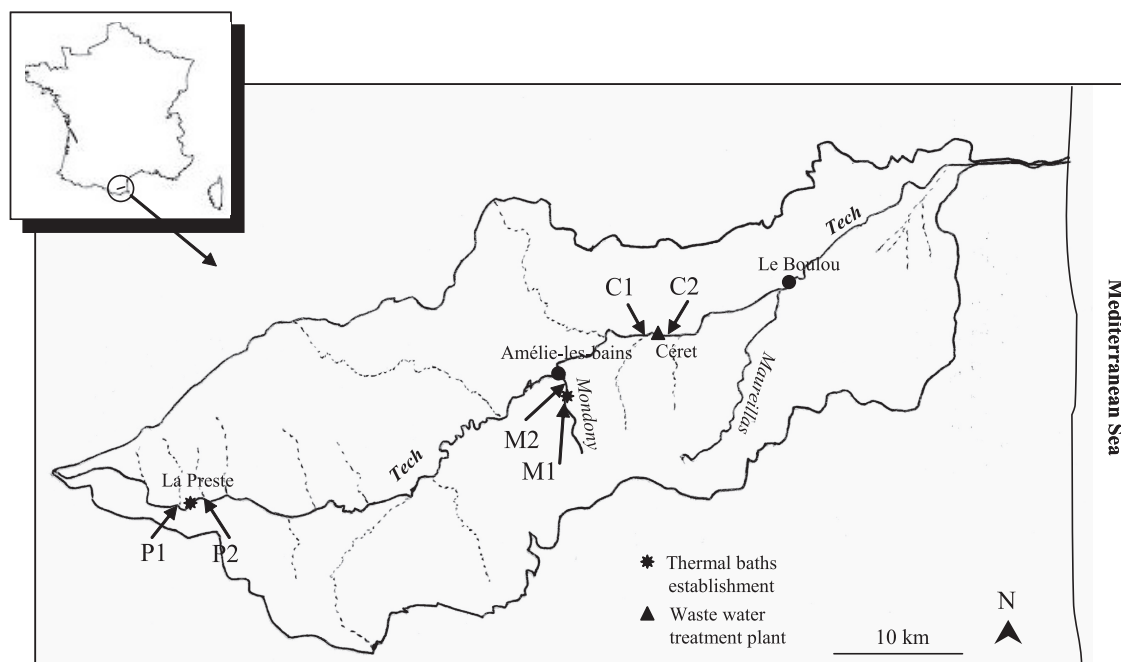


FIG. 1. Locations of sampling sites within the Tech River watershed at La Preste (P), Amélie-les-bains (M), and Céret (C), upstream (1) and downstream (2) of anthropogenic discharges.

tified by the standard culture method and by quantitative PCR (qPCR). *Legionella* diversity at the different sampling sites was characterized by cloning and sequencing of 16S rRNA gene fragments, and the dynamics of *Legionella* diversity was followed throughout the year by SSCP analysis of 16S rRNA genes.

MATERIALS AND METHODS

Strains. Bacterial strains used in this study and their sources are indicated in Table S1 in the supplemental material. *Legionella* strains were grown on buffered charcoal-yeast extract (α BCYE) agar (Oxoid, France) at 37°C for 48 to 72 h. Non-*Legionella* strains were grown on nutrient agar at 37°C for 24 h.

Study sites and sample collection. Water was sampled from the Tech River, located in the south of France, which is 84 km long from its source (Costabonne, 2,345 m high) to its estuary in the Mediterranean Sea (Fig. 1). The three sampling sites were located near to the source of the river at La Preste in a wooded mountainous area, where the water system was turbulent, from the tributary river Mondony close to Amélie-les-bains, and from Céret, 42 km downstream in an important urban area. At each sampling site, samples were taken upstream and downstream of the discharge from a thermal bath establishment and a wastewater treatment plant (Fig. 1).

For each sample, 1 liter surface water was collected in duplicate in sterile plastic bottles. Samples were treated the same day of collection or were stored at 4°C for analysis 24 h later.

Samples were collected each month, from October 2007 to September 2008, and were given designations identifying the sample site (P, La Preste; C, Céret; M, Amélie-les-bains), the sample location (1, upstream; 2, downstream), and the month. For example, P1OCT is the October upstream sample from La Preste. The water temperature was measured in each sample using a Checktemp thermometer (Hanna Instruments) (see Table S2 in the supplemental material).

Sample concentration and culture of *Legionella* species. *Legionella* species were enumerated and identified according to the Association Française de Normalisation (AFNOR) standard (AFNOR T90-431) for each sample collected (see above) (4), which is equivalent to ISO 11731. Briefly 0.1- to 1-liter water samples were concentrated by filtration through 0.45- μ m-pore-size polycarbonate filters (Sartorius). The filtered volume was dependent on the sample filterability. Membranes were transferred into 5 ml of sterile milli-Q water and

sonicated twice for 1 min (42 kHz). Volumes (100 μ l) of the concentrate were plated onto GVPC medium (Oxoid) after heat treatment (50°C for 30 min) and acid treatment (0.2 M HCl, 0.2 M KCl, pH 2, for 5 min) to eliminate non-*Legionella* organisms. Plates were incubated at 37 \pm 2°C, and colonies were counted after 3, 5, and 10 days. Colonies were examined for fluorescence under a Wood lamp. Colonies exhibiting *Legionella* morphology were transferred to α BCYE medium, α BCYE medium without cysteine, and blood agar medium (Oxoid) as controls. At least five colonies per sample were identified by *Legionella*-specific latex reagents (Oxoid). When 1 liter of water was filtered, the detection limit (DL) of the method was one colony per plate, equivalent to 50 CFU \cdot liter⁻¹, whereas the statistically significant quantification limit (QL) was based on the AFNOR recommendation of counting 5 colonies per plate, equivalent to 250 CFU \cdot liter⁻¹ (4).

DNA isolation and purification from water samples. For each sample, 0.1 to 1 liter of surface water (according to sample filterability) was filtered in duplicate through 0.45- μ m-pore-size polycarbonate filters (Sartorius). DNA was extracted directly from filters with the Aquadren extraction kit, according to the manufacturer's instructions (Bio-Rad). For each sample, qPCR and SSCP analyses were carried out with the same DNA extract.

Real-time quantitative PCR. Quantitative PCR was performed using two different iQ-Check Quanti *Legionella* kits (Bio-Rad) containing specific primers targeting the *mip* and the 5S rRNA genes for the detection of all 15 serogroups of *L. pneumophila* and *Legionella* spp., respectively. The protocol was carried out according to the French standard AFNOR XP T90-471 (5) and used the LightCycler instrument (1.0; Roche Diagnostics). Standard DNA curves were generated from genomic DNA of *L. pneumophila* ATCC 33152 provided in the kit. PCR mixtures contained 13 μ l of amplification mix, 2 μ l of the fluorescent probes per sample, and 5 μ l of sample or negative control or genomic DNA standard. The PCR protocol consisted of 2 min at 50°C, 15 min at 95°C for enzyme activation, and then 50 cycles of 15 s at 95°C, 30 s at 57°C, and 30 s at 72°C and a final elongation at 72°C for 10 min. All samples lacking a fluorescent signal were reanalyzed after being spiked with a known quantity of a genomic DNA standard provided in the kit (300 genome units [GU] of *L. pneumophila* per capillary) and compared with a positive control. Equal signals indicated an absence of inhibition of the amplification reaction.

Analyses were performed in duplicate, and results were expressed as GU per liter. When filtering one liter of sample, the DLs of qPCR methods were 128 GU \cdot liter⁻¹ and 64 GU \cdot liter⁻¹ for *L. pneumophila* and *Legionella* species, respectively. The QL was 800 GU \cdot liter⁻¹ for both methods.

PCR and capillary electrophoresis SSCP (CE-SSCP) analyses of *Legionella* spp. Two sets of primers, which targeted two different variable regions of the 16S rRNA gene of *Legionella*, were tested. The first set, JRP (phosphoramidite [TET]-5'-AGGGTTGATAGGTTAAGAGC-3') and JFP (5'-CCAACAGCTA GTTGACATCG-3') (25), amplified a 386-bp fragment of the V3-V4-V5 region, and the second set of primers, TempF (5'-CCTGGCTCAGATTGAACG-3') and TempR (TET-5'-AGGCTAATCTTAAAGCGCC-3') (41), amplified a 218-bp fragment of the V1-V2 region. The specificity of the primers was tested *in silico* (<http://rdp.cme.msu.edu/index.jsp>) and on *Legionella* and non-*Legionella* cultured strains (see Table S1 in the supplemental material). Single colonies were suspended in 100 μ l sterile physiological saline water, and cells were lysed at 95°C for 15 min.

PCR mixtures (25 μ l) contained approximately 3 to 10 ng DNA, 1 μ M primers, 0.8 mM deoxynucleoside triphosphates (dNTPs), and 1 to 1.5 U of *Pfu* DNA polymerase (Promega). The PCR protocol included an initial denaturation of 5 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 55°C (TempF/TempR) or 57°C (JFP/JRP), and 1 min at 72°C, with a final extension of 10 min at 72°C. Amplification products were verified by agarose gel electrophoresis, and their concentrations were estimated by comparison with molecular size markers. Dilutions of the PCR products were made in molecular grade water, and 1 μ l of each dilution was mixed with 18.8 μ l of Hi-Di formamide (Applied Biosystems) and 0.2 μ l of the internal size standard GeneScan-400HD (ROX) (Applied Biosystems). Samples were denatured at 94°C for 5 min and cooled rapidly in a water-ice bath for 10 min. Fragments were separated by CE-SSCP as described previously (17) using an ABI310 genetic analyzer (Applied Biosystems). To check the reproducibility of the SSCP peak profiles, duplicate PCRs from duplicate extractions were carried out for 6 samples (P1FEB, P2FEB, C1FEB, C2FEB, M1FEB, and M2FEB) and then analyzed by CE-SSCP. Identical profiles were generated for each duplicate sample (data not shown), and therefore single reactions were carried out for the remaining samples.

The raw data generated by the Genescan analysis software of the ABI310 genetic analyzer were exported using Chromagna (19) into csv files. These files were then imported into the SAFUM program (49), which normalizes the total area of the SSCP profiles and the mobilities between different runs using the internal standard. The similarities of the SSCP profiles were compared by first calculating a Bray-Curtis resemblance matrix and then by constructing dendrograms with the unweighted-pair group method using average linkages (UPGMA) or by plotting nonmetric multidimensional scaling (nMDS) graphs using Primer Enterprises (Primer-E) software (13).

Identification of *Legionella* species by cloning and sequencing. The primers TempF and TempR were used to amplify genomic DNA from samples M2JAN, P2DEC, and C2JUN based on the method described previously (1). For each DNA sample, 10 replicate PCRs were made to reduce PCR bias. Each 25 μ l of PCR mixture contained approximately 2 ng DNA together with 0.3 μ M primers, 0.5 mM dNTPs, 1 U of Super-*Taq* polymerase, and 1 \times Super-*Taq* buffer (HT Biotechnology, Cambridge, United Kingdom). The reactions were cycled using the following parameters: an initial denaturation of 5 min at 95°C followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, with a final extension of 10 min at 72°C. The PCR products were pooled and gel purified (gel extraction kit; Qiagen) before cloning the same day using the TOPO TA cloning kit (pCR2.1) according to the manufacturer's instructions.

Plasmid DNA (96 clones for each of the 3 libraries) was sequenced with the BigDye Terminator kit and the 3730xl automatic sequencer (Macrogen, Seoul, South Korea) using the M13 primer. Sequences were manually corrected using the sequence editing software Bioedit (23) and aligned using the MEGA software (40) and the ARB program (30). Clone sequences were submitted to Blastn (2) to identify the closest relatives. Clusters or operational taxonomic units (OTUs) were defined from the aligned sequences at 99% similarity by using the software Clusterer with the UPGMA setting (26).

Statistical tests. The ANOSIM (analysis of similarities) routine of Primer-E was used to test the null hypothesis that there were no differences in *Legionella* diversity between the sampling sites or that there were no differences in diversity between the different seasons.

ANOSIM is a nonparametric test of significant difference between two or more groups that uses the underlying Bray-Curtis resemblance matrix to rank similarities among *a priori*-defined groups. ANOSIM calculates a global *R* statistic from the fraction of the difference between average rank similarity within groups and average rank similarity between groups as a function of the number of samples. The resulting *R* value usually ranges between 0 and 1, with high values indicating a large degree of discrimination among groups whereas values near 0 indicate that there is little difference among the groups (13). The significance level or *P* value is computed by permutation of group membership, with 1,000 replicates. The observed value of *R* is then referred to its permutation

distribution. If the observed value is unlikely to come from the null distribution, the null hypothesis can be rejected.

To test for significant differences in cell concentrations, the data were analyzed by the nonparametric Wilcoxon test. All statistical tests were done using XL stat software (Addinsoft, France).

Nucleotide sequence accession numbers. The sequence data from this study have been submitted to the GenBank database (<http://www.ncbi.nlm.nih.gov/GenBank/index.html>) under the accession numbers GU180682 to 180770 (Céret), GU180771 to 180840 (Amélie-les-bains), and GU180841 to 180919 (La Preste).

RESULTS

Detection and quantification of *Legionella* spp. in river water samples. The quantification of *Legionella* spp. present in the water samples collected along the Tech River (Fig. 1) was conducted by the standard culture method and by quantitative PCR. After being cultured on the GVPC medium, only 15 samples out of 72 (20.8%) were positive for *Legionella* spp. Among them, 9 (12.5%) were collected at P2 and 6 (8.3%) at M2, whereas there were no *Legionella* spp. detected at Céret. All selected colonies were identified as *L. pneumophila*. Concentrations at P2 ranged from 143 to 500 CFU \cdot liter⁻¹ all year except in December, April, and May. At M2, the concentrations ranged from 100 to 583 CFU \cdot liter⁻¹ in the winter months, June, and July. For all the positive samples, plate count results were between the detection limit (DL; 50 to 250 CFU \cdot liter⁻¹) and the quantification limit (QL; 250 to 1,250 CFU \cdot liter⁻¹) of the method, except for the P2SEP sample, where the plate counts were higher than the QL (250 CFU \cdot liter⁻¹). Concentrations at P2 ranged from 143 to 500 CFU \cdot liter⁻¹ all year except in December, April, and May. At M2, the concentrations ranged from 100 to 583 CFU \cdot liter⁻¹ in the winter months, June, and July.

For the qPCR analyses, PCR inhibitors were detected in all the water samples from nondiluted DNA extracts. This problem was resolved by repeating the analyses with 10-fold dilutions of the DNA extracts. However, this decreased the sensitivity of the qPCR assays and increased the quantification limits of the qPCR method from 8×10^3 to 8×10^4 GU \cdot liter⁻¹ (Fig. 2). No *L. pneumophila* organisms were detected by qPCR in any of the samples, but *Legionella* spp. were detected above the QL of the method for all the different sampling sites analyzed throughout the year (Fig. 2). The highest concentrations of *Legionella* spp. were detected at the Céret sampling site and ranged from 1.24×10^5 GU \cdot liter⁻¹ to 9.36×10^5 GU \cdot liter⁻¹. Slightly lower concentrations were found at the Amélie-les-bains site (5.01×10^4 GU \cdot liter⁻¹ to 5.82×10^5 GU \cdot liter⁻¹) and at the La Preste site (7.39×10^3 GU \cdot liter⁻¹ to 2.62×10^5 GU \cdot liter⁻¹). Pairwise Wilcoxon tests revealed that for the Céret and Amélie-les-bains sampling sites the *Legionella* sp. concentrations of the upstream and downstream sample sites were not significantly different ($P > 0.05$), whereas there was a significant difference for La Preste ($P < 0.05$). Over the year, mean temperature variations between water taken upstream and downstream of anthropogenic discharges varied according to the sampling site location. The average temperature variations were 2.7°C, 6°C, and 0°C for the La Preste, Amélie-les-bains, and Céret sampling sites, respectively (see Table S2 in the supplemental material).

Specificity testing of *Legionella*-specific PCR primers. Two sets of primers for the amplification of fragments located in

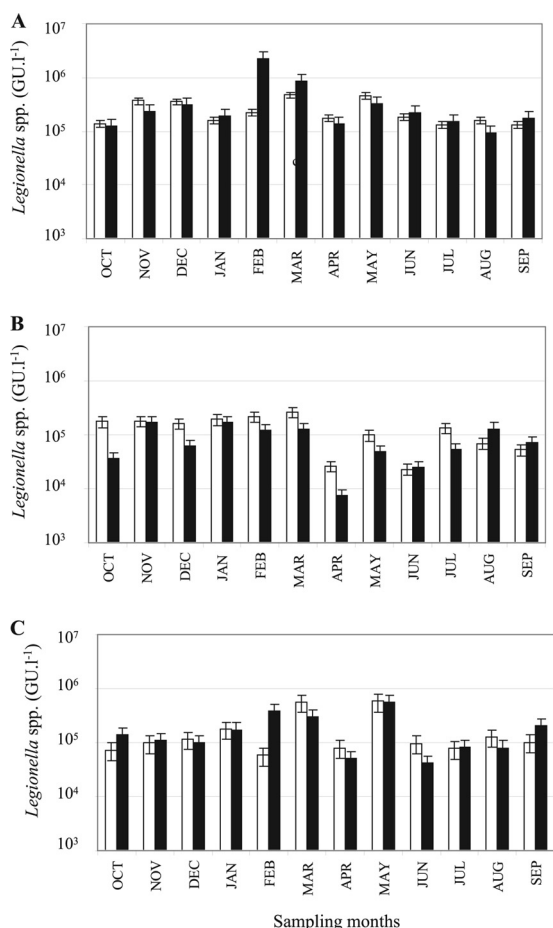


FIG. 2. Temporal change of *Legionella* sp. concentrations at the three sampling sites, Céret (A), La Preste (B), and Amélie-les-bains (C) upstream (white) and downstream (black) of discharge, as measured by qPCR.

two different hypervariable regions of the 16S RNA of *Legionella* were tested. The first set (25) allowed the amplification of fragments located in the hypervariable regions V3, V4, and V5, whereas the second set (41) amplified fragments located in the hypervariable regions V1 and V2. The primer sets were first tested with a range of *Legionella* strains and non-*Legionella* strains (see Table S1 in the supplemental material) to verify their specificity. All the *Legionella* strains tested were amplified by both sets of primers (TempR/TempT and JRP/JFP), whereas the non-*Legionella* species were not (data not shown). The amplification products from the two sets of primers producing two different fragment sizes were then analyzed by CE-SSCP to determine which primer set allowed better discrimination of the 42 different *Legionella* strains tested (27 *Legionella* spp. and 15 *Legionella pneumophila* strains comprising serogroups 1 to 15). The primer set TempR/TempF, which gave the shortest fragment, allowed better discrimination of the *Legionella* species than the other primer set, JRP/JFP, for which the SSCP profiles were not clearly distinguishable due to significant overlapping (see Fig. S2 in the supplemental material). These differences can be explained by the low resolution of large fragments with the applied electrophoretic conditions

(3). Consequently, all further analyses of *Legionella* diversity in the different environmental samples were done using the TempR/TempF primer set.

Diversity of *Legionella* 16S rRNA gene fragments. To characterize the *Legionella* diversity and to verify the specificity of the PCR primers, SSCP PCR products obtained from 3 different sampling sites after water discharge (M2JAN, P2DEC, and C2JUN) were cloned and sequenced.

A total of 238 sequences were obtained: 89 sequences from Céret, 79 from La Preste, and 70 from Amélie-les-bains. All sequences had the greatest similarity to *Legionella* spp., confirming the specificity of the primers used in this study. Similarities between the 16S rRNA gene sequences and the *Legionella* sequences deposited in the GenBank database ranged from 87% to 100% for the three sampling sites (Table 1). However, even the sequences with lower similarities had *Legionella* species as their nearest relatives. Sequences were then clustered into operational taxonomic units (OTUs) at 99% similarity, giving 29 clusters of 163 sequences (68% of the total sequences) and 75 singletons. The 99% threshold was chosen since this permitted different cultured *Legionella* spp. to be placed into different OTUs. The closest relatives of the representative sequences from each cluster together with the relative abundances of sequences in a given cluster at each of the sampling sites are shown in Table 1.

For all sampling sites, the major clusters were cluster 3 and cluster 8, accounting for 17.6% and 9.7%, respectively, of the total sequences, and for Céret, cluster 3 accounted for nearly one quarter of the sequences. The closest relative of the most dominant cluster, cluster 3, was an uncultured *Gammaproteobacteria* sequence isolated from the Columbia River (15), and the nearest *Legionella* species was only 94% similar. For cluster 8, the closest identified sequence was “*Candidatus Legionella jeonii*,” at 96% similarity. This species was previously described as an obligate endosymbiotic bacterium in *Amoeba proteus* (35) and was placed apart from *Legionella*-like amoebal pathogens (LLAP) in phylogenetic analyses. The representative sequence from cluster 2 was 100% identical to *L. pneumophila*, and this cluster was the third most abundant when all sequences were considered. Interestingly, *L. pneumophila* sequences were recovered from all sites, even at the most pristine site at La Preste. Clusters 13, 16, and 9 accounted for 3.4%, 3.8%, and 2.9%, respectively, of the total sequences, and the closest relatives identified by Blastn were uncultured clones isolated from surface water in South Africa and from a corroded sewer system (32). The other clusters represented between 0.8% and 2.1% of the total sequences. Among them, clusters 14 and 21 had high similarities (99% to 100%) with the already-described species *Legionella cincinnatiensis* and *Legionella feeleii*. Cluster 15 also included sequences with high similarity (99%) to an uncultured clone isolated from groundwater (M. W. Fields et al., unpublished data). It is interesting to note that, out of 29 clusters, 17 were most closely related to uncultured bacteria, and these included the major OTUs, highlighting the large diversity of clones representing not yet described *Legionella* species. The high diversity of *Legionella* OTUs was also observed in rarefaction curves (see Fig. S3 in the supplemental material), which had not reached a plateau.

TABLE 1. Relative abundances of clone sequences in each cluster (defined at 99% similarity) for the individual libraries and out of all sequences, presented with the closest relatives in GenBank

Cluster no.	% clones/cluster/library for site ^a :			% clones out of all sequences	Closest relative in GenBank	% identity	Accession no.
	C	M	P				
3	23.6	15.7	12.7	17.6	Uncultured gammaproteobacterium clone CRO-2	98	AF141558
8	7.9	1	11.4	9.7	" <i>Candidatus</i> <i>L. jeonii</i> "	96	AY598719
2	4.5	5.7	2.5	4.2	<i>L. pneumophila</i>	100	M59157
16	2.2	1.4	7.6	3.8	Uncultured bacterium clone EV818EB5CPSAJJ36	96	DQ337039
13	3.4	5.7	1.3	3.4	Uncultured bacterium clone EV818SWSAP64	96	DQ337087
9	6.7	1.4		2.9	Uncultured bacterium clone IC-85	97	AB255083
19	4.5	1.4		2.1	LLAP14	97	U66104
23	1.1	2.9	2.5	2.1	Uncultured gammaproteobacterium clone WCB127	96	AY217479
6	3.4	1.4		1.7	Uncultured soil bacterium clone 1_H3	96	EU589279
7		1.4	3.8	1.7	Uncultured bacterium clone EV818SWSAP64	97	DQ337087
11	1.1		2.5	1.3	LLAP8	94	X97361
14		2.9	1.3	1.3	<i>L. cinцинатиensis</i> (ATCC 43753)	100	X73407
15	1.1		2.5	1.3	Uncultured bacterium clone 300G-C11	99	AY662002
17	1.1	1.4	1.3	1.3	LLAP11	96	X97362
18			3.8	1.3	Uncultured bacterium clone EV818SWSAP64	97	DQ337087
21		1.4	2.5	1.3	<i>L. feeleeii</i> (ATCC 35072)	99	X73395
28	3.4			1.3	Uncultured gammaproteobacterium clone ADK-HDe02-55	92	EF520570
29	1.1	2.9		1.3	Uncultured <i>Legionella</i> sp. clone CL1.B267	95	FM175311
1		2.9		0.8	<i>Legionella oakridgensis</i> (ATCC 33761)	96	X73397
4	2.2			0.8	Uncultured bacterium clone IC-85	97	AB255083
5			2.5	0.8	Uncultured gammaproteobacterium clone ADK-BTh02-70	92	EF520563
10		1.4	1.3	0.8	<i>Legionella beliardensis</i>	96	AF122884
12	1.1	1.4		0.8	Uncultured bacterium clone P7X3b1C06	87	EU491023
20	1.1		1.3	0.8	Uncultured bacterium clone TSAF31	97	AB186839
22	1.1		1.3	0.8	<i>Legionella wadsworthii</i> (ATCC 33877)	97	X73401
24		1.4	1.3	0.8	<i>Legionella beliardensis</i>	94	AF122884
25	1.1	1.4		0.8	<i>Legionella jordanis</i> (ATCC 33623)	95	X73396
26	2.2			0.8	Uncultured gammaproteobacterium clone ADK-BTh02-70	94	EF520563
27	2.2			0.8	Uncultured gammaproteobacterium clone CRO-2	98	AF141558

^a C, Céret; M, Amélie-les-bains; P, La Preste.

Seasonal and anthropogenic effects on *Legionella* diversity.

Considering all the water samples, a high number of SSCP peaks were observed, varying from 6 to 15, for all sampling sites throughout the year. The SSCP peak profiles obtained for June and December for each sampling site before and after anthropogenic discharge are presented in Fig. 3 and show clear differences in the number of peaks and in their relative abundances for the different sampling sites and for the different seasons for a given sampling site. The similarities of the SSCP profiles, taking into account the areas under the profiles, were compared to determine if the distribution of *Legionella* species was site specific. Considering all profiles from the whole year, the *Legionella* populations were not separated according to sample site, as indicated by the overlap in the similarities of many of the SSCP profiles (see Fig. S1 in the supplemental material). An ANOSIM test also gave an *R* value close to 0 (*R* = 0.103, *P* < 0.001), indicating that the *Legionella* diversities at the sample sites were not significantly different. However, the wide separation of many of the Amélie-les-bains samples and some Céret samples in the nMDS plot (see Fig. S1 in the supplemental material) suggests that, for some time points, the *Legionella* diversities at these two sampling sites were different. Interestingly, the similarities of the monthly profiles for La Preste were more closely clustered than those for Céret and Amélie-les-bains (see Fig. S1 in the supplemental

material), which could suggest that there was less variability in *Legionella* diversity at this pristine site.

nMDS plots for the monthly samples for the individual sample sites (Fig. 4A to C) and the dendrograms (Fig. 5) showed that for Céret the samples appeared to be clustered roughly according to season whereas such a clustering was less clear for La Preste and Amélie-les-bains. ANOSIM tests confirmed the good separation of the seasonal groups for Céret (*R* = 0.512, *P* < 0.001) but less separation for La Preste (*R* = 0.277, *P* < 0.001) and Amélie-les-bains (*R* = 0.152, *P* < 0.001). In addition, at Céret, pairwise ANOSIM tests indicated that, whereas the spring and the summer profiles were not well separated from each other (*R* = 0.1, *P* < 0.17), they were very different from the winter and fall profiles (*R*_{spring/winter} = 0.957, *P* < 0.002; *R*_{summer/winter} = 0.954, *P* < 0.002; *R*_{fall/winter} = 0.607, *P* < 0.002).

To determine the impacts of anthropogenic discharges on *Legionella* population structure, the CE-SSCP profiles obtained from samples collected upstream and downstream of water discharges were compared in dendrograms and by calculating the similarity of the profiles for each time point (Fig. 5). At the Céret sampling site, 10 of 12 CE-SSCP profiles obtained during the year were very similar (more than 82% similarity). Only two, obtained in October and December, showed quite different peak patterns (40 and 56% similarity), suggesting that different *Legionella* species

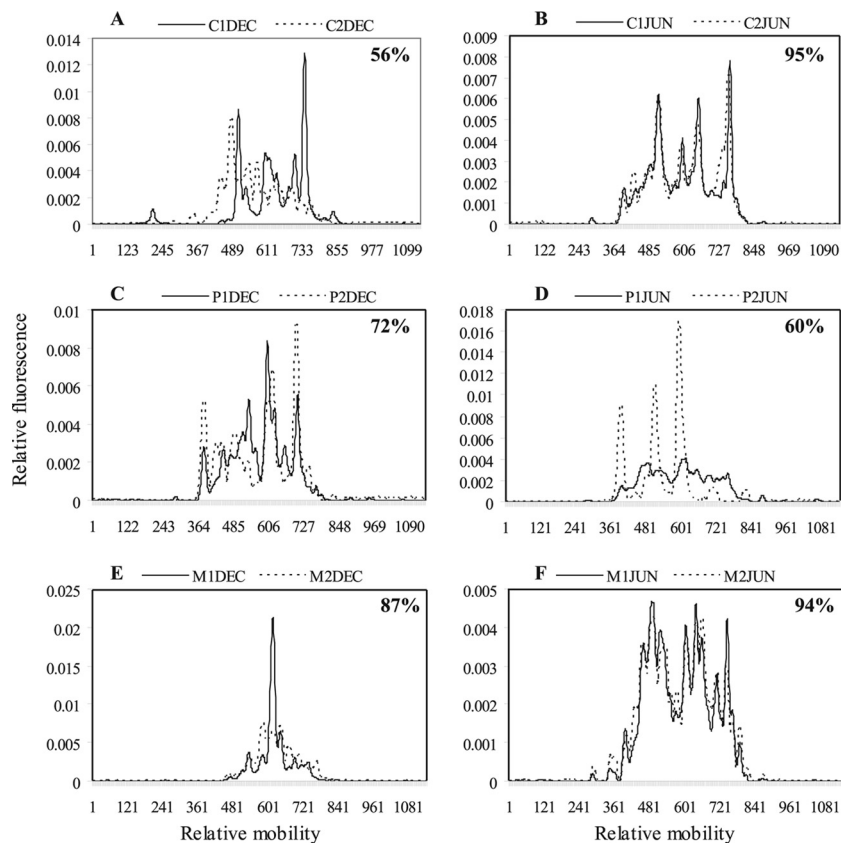


FIG. 3. Examples of temporal and spatial variation of *Legionella* CE-SSCP profiles for the three sampling sites, Céret (A and B), La Preste (C and D), and Amélie-les-bains (E and F), upstream (solid lines) and downstream (dashed lines) of the water discharges. Percent similarities between the upstream and downstream profiles are indicated.

were present at different relative abundances. At La Preste in November and June, the CE-SSCP profiles after the discharge of the thermal baths were very different from the ones obtained upstream. Indeed three major peaks dominated the SSCP fingerprint, whereas the upstream pattern possessed the usual 12 to 14 peaks (June) (Fig. 3D). Percentages of similarity obtained at these 2 dates were the lowest of the year (55 and 60% similarity), followed by December (72% similarity), which showed similarity differences more in the relative abundance of peaks than in the absence or presence of peaks. When considering all profiles of the year, we could observe that the discharge of the thermal baths of La Preste appeared to have an occasional impact on *Legionella* population, especially in the fall (November and December) and June. At the Amélie-les-bains sampling site, only 4 profiles (March to June) were very similar (>94%) before and after the thermal bath discharge, whereas the other profiles obtained upstream and downstream of the thermal baths for the rest of the year were less similar (<80 to 87%). Indeed, in fall and winter, the upstream and downstream profiles were very different (<80% similarity). Therefore, whereas there appeared to be an occasional effect of the water discharges from Céret and La Preste on *Legionella* diversity, this effect was more marked for the thermal baths on the Mondony River (Amélie-les-bains).

DISCUSSION

Although a few limited studies have revealed the presence of *Legionella* spp. in different aquatic environments (11, 38, 46), little is known about how these natural populations are affected by environmental and anthropogenic factors. Therefore, research into *Legionella* sp. ecology is essential to better understand their sources in the natural environment, the mechanism of their entry into man-made water systems, and the factors enabling their survival and growth in aquatic habitats. To address this need, this work investigated the dynamics of natural *Legionella* sp. populations along a river before and after water discharges from thermal bath establishments and a wastewater treatment plant during an annual cycle. To our knowledge this is the first time that such a comprehensive study of natural *Legionella* diversity and abundance has been performed during a whole year using both culture and culture-independent methods.

Whereas no *Legionella* spp. other than *Legionella pneumophila* were quantified on culture media, a real-time qPCR assay enabled us to detect and to quantify *Legionella* spp. in all samples throughout the year. Indeed, it is widely recognized that some *Legionella* species, such as *L. lytica*, and other *Legionella*-like amoebal pathogens (LLAPs) remain unculturable on the medium normally used to grow *Legionella* and on other media tested (29, 39). Although *L. pneumophila* could be

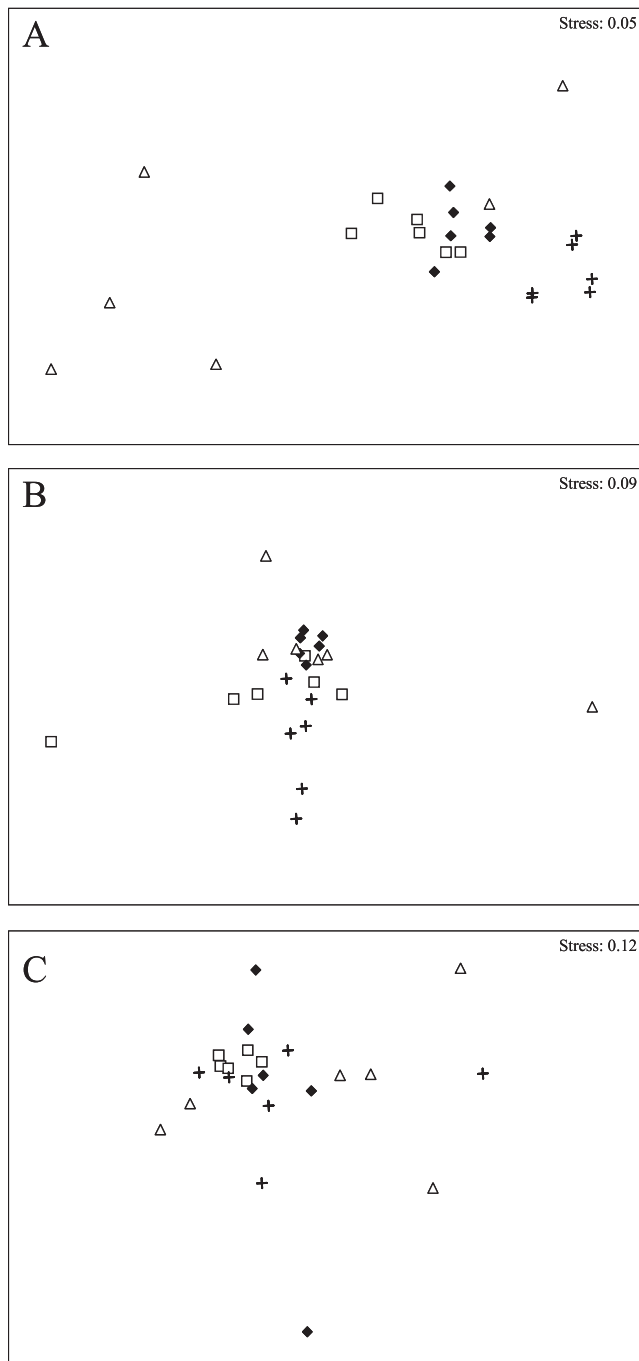


FIG. 4. nMDS plots showing the definition of seasonal groups from the CE-SSCP profiles for Céret (A) but not for La Preste (B) or Amélie-les-bains (C). Δ , fall; +, winter; \square , spring; \blacklozenge , summer.

quantified by the culture method, this species could not be quantified reliably by qPCR because the concentrations fell below the QL. The QL for the qPCR method was higher than the culture one, and qPCR was therefore less sensitive because the DNA samples had to be diluted 10-fold to overcome PCR inhibition. However, for *Legionella* spp., qPCR abundances were as high as 1×10^5 GU \cdot liter $^{-1}$ for the majority of the sampling dates for all sampling sites along the Tech River,

even close to the source of the river, where there is no human activity. This could suggest that the *Legionella* spp. originate from the source of the river or that they could come from groundwater and/or atmospheric inputs, as has been proposed previously (14, 37). Very few studies have quantified *Legionella* abundance in their natural reservoirs, and the majority of these used culturing methods. Studies using qPCR quantification of *Legionella* have been mostly restricted to man-made systems such as drinking water distribution systems (hot sanitary) and cooling towers implicated in legionellosis outbreaks. Surprisingly, the *Legionella* abundances found close to the source of the Tech River, which could be considered a pristine environment, were comparable to those found in cooling towers (44).

Another important result from this study was the high diversity of *Legionella* for all sampling sites, shown both by sequencing of the 16S rRNA gene V1-V2 fragment and by SSCP analysis, which showed a high number of different OTUs and peaks, respectively.

This is in contrast to a phylogenetic study of *Legionella* species performed from a hypervariable short fragment of 16S rRNA gene (V3-V4-V5 region) in a river in the Brazilian rain forest that found a low diversity of *Legionella* species (11). Only *L. pneumophila* was detected in the pristine sampling site, and a dozen species of *Legionella* were found downstream, where the anthropogenic effect was greater (11). In addition to finding high *Legionella* diversity, this study found that the majority of the OTUs were most closely related to clones of uncultured bacteria than to already-described *Legionella* strains.

A high number of sequences affiliated with not yet described *Legionella* species were also found in cooling towers (44), suggesting that cultured representatives are lacking for *Legionella* not only in natural reservoirs but also in man-made systems. Interestingly, although the *Legionella* community was initially diverse in these cooling towers, the *Legionella* sp. diversity was dramatically reduced during the proliferation of *L. pneumophila*. The quantification data and the clone sequences in our study all show that *L. pneumophila* was not the most abundant member of the *Legionella* community in the Tech River. This may suggest that this species is able to proliferate and dominate the *Legionella* community only under specific conditions (e.g., higher temperatures characteristic of man-made systems), as already reported by Wéry et al. (44).

To better understand the dynamics of the *Legionella* populations, SSCP was used to analyze monthly samples at the different sampling sites before and after the anthropogenic discharges. No changes in *Legionella* populations were observed in relation to their position upstream or downstream in the river. Indeed, the high diversity was preserved throughout the year along the Tech River, and no SSCP profile was specific to a given station. Consequently, we could conclude that a large number of *Legionella* species were able to survive along the river in spite of the environmental pressure. Nevertheless, SSCP analyses of the dynamics of *Legionella* populations during a year revealed that some changes in the *Legionella* diversity could be related to the season. This was observed especially in Céret, where fingerprints of *Legionella* detected in fall and winter were different from those obtained in spring and summer. We did not find the same seasonal effect at La Preste and Amélie-les-bains, where different groups could be identi-

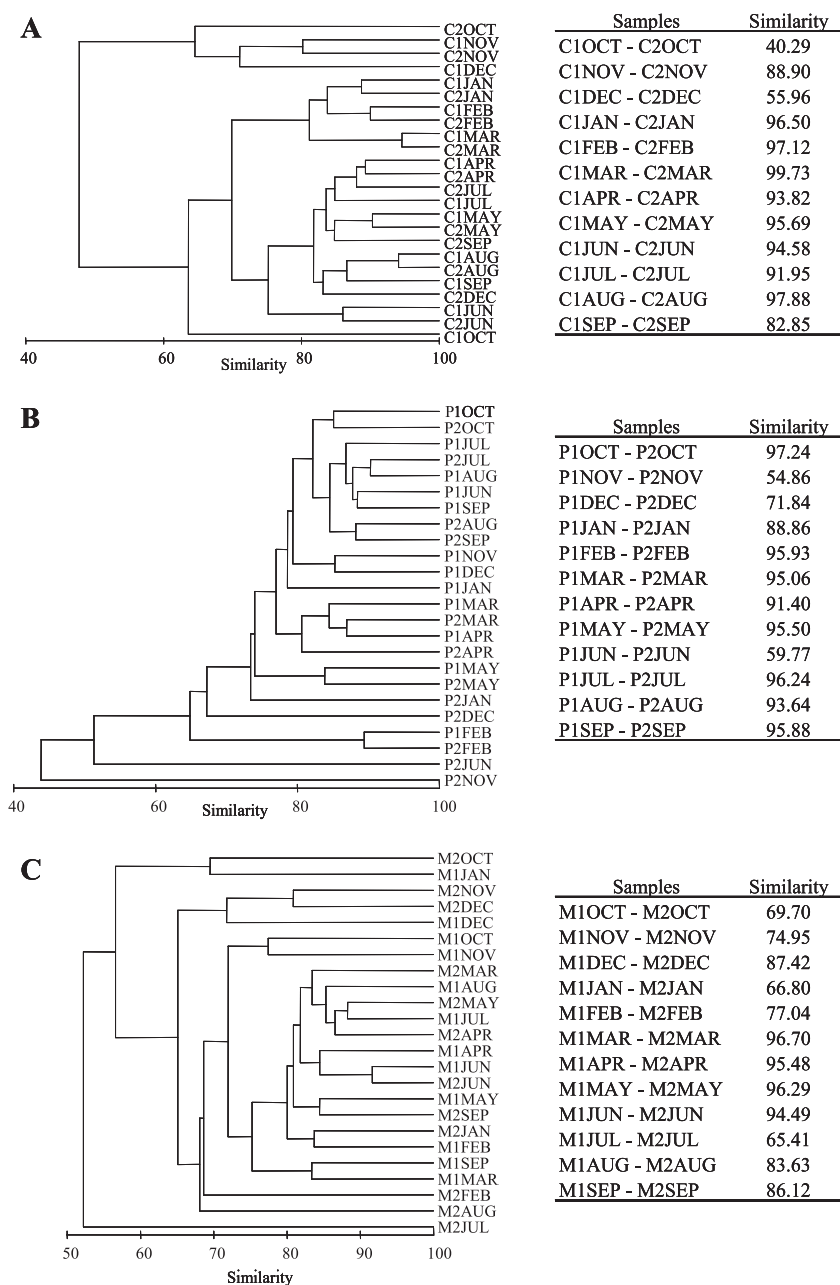


FIG. 5. Dendrograms and similarity comparisons of CE-SSCP profiles before (1) and after (2) wastewater (Céret [C]) or thermal bath (La Preste [P] and Amélie-les-bains [M]) discharge for all monthly samples from the 3 sampling sites. (A) Céret; (B) La Preste; (C) Amélie-les-bains.

fied but were unrelated to the seasons. We suggest that seasonal variations were less marked at La Preste than at Céret and may be explained by different environmental factors. Biotic and abiotic factors (e.g., grazing, UV radiation, temperature, sedimentation, etc.) (21) may have had more impact at Céret, where the river bed was wider and shallower than at La Preste, where the river was narrow and the flow turbulent.

Comparison of the SSCP profiles upstream and downstream of the water discharges throughout the year suggested that the WWTP discharges had little impact on *Legionella* diversity. However, further studies should be carried out to determine the impact of other WWTPs on *Legionella* diversity and abun-

dance to confirm that the effect of the wastewater discharge is limited. In contrast, at the thermal bath establishment sampling sites, the differences in *Legionella* sp. diversity between the upstream and downstream sites were more marked for a greater number of sampling dates (3 sampling dates for La Preste and 5 to 8 for Amélie-les-bains). The change in *Legionella* sp. diversity could be due to a direct addition of different *Legionella* spp. into the river population from the natural hot spring water or the water distribution system in the thermal bath establishment (24, 33). The thermal discharge water could also modify the *Legionella* sp. populations in the river by altering the physicochemical properties of the river water. The

average temperature variations were 2.7°C and 6°C for La Preste and Amélie-les-bains, respectively, whereas no temperature difference was observed at Céret (see Table S2 in the supplemental material). The more marked changes in *Legionella* sp. diversity observed downstream of the thermal bath stations could be explained by the higher temperatures since temperature changes can influence the distribution of *Legionella* species (38). In addition to modifying *Legionella* sp. diversity via temperature effects, the discharge water could modify diversity via other abiotic and biotic factors such as pH or the presence of amoebae and biofilms, which could improve the survival of *Legionella* in the river (16, 27, 42).

However, we cannot rule out the possibility that the discharge waters of the two sites could be differently contaminated (in terms of *Legionella* diversity and abundance), explaining the different impacts on the *Legionella* population. In terms of *Legionella* sp. abundance, the qPCR data indicated little difference between the upstream and downstream sites except for La Preste, where 7 out of 12 monthly samples gave lower concentrations of *Legionella* spp. downstream of the site. Possible explanations of this observation could be a greater dilution effect from the discharge water (and assuming a low concentration of *Legionella* spp. in this water) at this site or perhaps an inhibitory effect of the discharge water on the natural *Legionella* sp. populations.

In order to confirm anthropogenic effects on *Legionella* populations, further studies involving higher numbers of replicate samples upstream and downstream of the site of interest and the sampling of the discharge water itself will be required.

In conclusion, our results showed a high abundance and diversity of *Legionella* species at all the sampling sites, even near the source of the river. This is an important result because it suggests that *Legionella* reservoirs may be much more widespread than previously thought. The dominance of sequences most closely related to uncultured *Legionella* demonstrates the urgent need to isolate these different *Legionella* species to better understand their persistence in aquatic environments and to determine their pathogenicity. A necessary step will be to study the interaction of *Legionella* with the protozoa, which are often essential for their proliferation and survival in the environment.

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