

## Dynamics of *Legionella* spp. and Bacterial Populations during the Proliferation of *L. pneumophila* in a Cooling Tower Facility<sup>∇</sup>

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The dynamics of *Legionella* spp. and of dominant bacteria were investigated in water from a cooling tower plant over a 9-month period which included several weeks when *Legionella pneumophila* proliferated. The structural diversity of both the bacteria and the *Legionella* spp. was monitored by a fingerprint technique, single-strand conformation polymorphism, and *Legionella* spp. and *L. pneumophila* were quantified by real-time quantitative PCR. The structure of the bacterial community did not change over time, but it was perturbed periodically by chemical treatment or biofilm detachment. In contrast, the structure of the *Legionella* sp. population changed in different periods, its dynamics at times showing stability but also a rapid major shift during the proliferation of *L. pneumophila* in July. The dynamics of the *Legionella* spp. and of dominant bacteria were not correlated. In particular, no change in the bacterial community structure was observed during the proliferation of *L. pneumophila*. *Legionella* spp. present in the cooling tower system were identified by cloning and sequencing of 16S rRNA genes. A high diversity of *Legionella* spp. was observed before proliferation, including *L. lytica*, *L. fallonii*, and other *Legionella*-like amoebal pathogen types, along with as-yet-undescribed species. During the proliferation of *L. pneumophila*, *Legionella* sp. diversity decreased significantly, *L. fallonii* and *L. pneumophila* being the main species recovered.

*Legionella* species are relatively slow-growing, ubiquitous, aquatic bacteria (3). Natural freshwater environments are the major reservoirs. However, various human-made systems such as heated water in spas, showerheads, sanitary hot water networks, or cooling towers provide ideal habitats for *Legionella* species (30). In such water systems, pathogenic *Legionella* spp. responsible for acute respiratory infections can proliferate. Most cases of legionellosis can be traced to human-made aquatic environments where the water temperature is higher than the ambient temperature (17). In particular, cooling towers have been implicated in major outbreaks of legionellosis caused by *Legionella pneumophila* (7, 10, 19, 22). *Legionella* species are facultative intracellular gram-negative bacilli which multiply in protozoan hosts and can also survive within microbial biofilm communities (17). Amoebal cysts provide a protective environment for *Legionella* species, which can then withstand treatments with biocides such as chlorine (21, 34). Keeping *Legionella* under control in cooling towers is the necessary condition for reducing legionellosis outbreaks. However, due to the ecology of this bacterium, reducing the risk related to *Legionella* remains a challenge. Indeed, not only physical parameters (temperature, fouling of the network, etc.) induce a risk of proliferation, but biotic parameters such as the presence of a biofilm or amoebae also increase the risk (33). Understanding of the factors that contribute to the survival or active growth of *L. pneumophila* in the environment is still very limited. In particular, very little information is available on

microbial diversity in systems contaminated with *L. pneumophila*. Clearly, controlling the *Legionella* risk in cooling towers requires a better understanding of the dynamics of the different microbial constituents during the proliferation of pathogenic species.

In this study, the ecology of *Legionella* spp. in relation to the bacterial community was investigated in a cooling tower plant. The dynamics of the bacteria's structural diversity during the proliferation of *L. pneumophila* were analyzed. The dynamics of *Legionella* spp. were also investigated to determine the changes that arose in the *Legionella* population during the proliferation of the pathogenic species *L. pneumophila*. In fact, little is known regarding the *Legionella* species present in cooling tower systems or their dynamics within the same cooling tower (28). The growth requirements of *Legionella*, its ability to enter a viable-but-nonculturable state, the association of *Legionella* with protozoa, and the occurrence of *Legionella* in biofilms all tend to complicate its detection (7), and cultures on selective media have sometimes failed to isolate *Legionella* species from environmental samples (13). Cultivation-independent methods have thus been used to characterize the *Legionella* population in aquatic environments (12, 13, 30, 35). In the present study, PCR methods were used to quantify *Legionella* spp. and *L. pneumophila* and to analyze *Legionella* sp. diversity. The dynamics of *Legionella* spp., in relationship to those of the dominant bacteria, were followed by single-strand conformation polymorphism (SSCP) in a cooling tower plant over a 9-month period which included several weeks when *L. pneumophila* proliferated.

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### MATERIALS AND METHODS

**Cooling tower facility.** The cooling tower facility studied, located in France, had the following characteristics: a total volume of 250 m<sup>3</sup>, 16 MW of power, and

100 m<sup>3</sup> of water used per day with a flux rate of 300 to 1,800 m<sup>3</sup>/h in the cooling tower circuit. The municipal water (tap water quality) was treated in the circuit by biocide, descaling, antialgal, anticorrosion, and UV treatments. Hot water was cooled down by air after spraying at the top of the eight cooling towers. It was retrieved in a 150-m<sup>3</sup> basin. The temperature of the incoming water was around 15 to 16°C. From May to September 2005, the average temperature of the water before release at the top of the towers (sampling point) was 27°C, including an increase to 29 to 30°C in July and August.

**Chemical treatment.** Isothiazolone was used as a biocide. Volumes of 5 to 65 liters were applied weekly from 26 May 2005 to 28 September 2005 in order to reach concentrations of 100 to 250 ppm. On three dates (28 July, 11 August, and 2 September), the biocide and biodegradable detergent were added simultaneously. On these occasions, isothiazolone volumes were 65, 62, and 60 liters, respectively, while the biodegradable detergent volume added was 20 liters on each date.

**Sampling.** Water was sampled at different dates at a point located on the supply pipe common to all eight cooling towers, prior to its release at the top of the towers. One liter of water was collected, filtered through 0.45- $\mu$ m-pore-size filters (Millipore, Billerica, MA), and frozen while awaiting DNA extraction.

**ATP measurements.** For ATP measurements, the WaterGiene ATP test was used (Charm Sciences Inc., Lawrence, KS).

**DNA extraction.** DNA was extracted directly from filters with the Aquadren extraction kit (Bio-Rad, Hercules, CA). For each sample, all analyses were carried out with the same DNA extract.

**Real-time quantitative PCR.** Quantitative PCRs were performed with IQ-Check *Legionella* and IQ-Check *L. pneumophila* kits from Bio-Rad by following the stipulations of the manufacturer and using the iCycler IQ apparatus (Bio-Rad). Standard DNA curves were generated by amplification of serial 10-fold dilutions of genomic DNA of *L. pneumophila* ATCC 33152 in sterilized water and designed by an iCycler IQ apparatus. The *L. pneumophila* genome mass used was 4.3 fg of DNA. A standard DNA curve was generated for each assay. The cycle threshold ( $C_T$ ) corresponding to the number of cycles at which the reaction becomes exponential, was compared to the standard curve in order to calculate the number of genomic units (GU) in the DNA extract of the samples. The PCR equation was  $C_T = -1/[\log(1 + E)] \times (\log X_0 - \log X)$ , where  $E$  is the efficiency of the PCR,  $X$  is the concentration, and  $X_0$  is the initial concentration. The standard DNA curve was validated when the slope was between  $-3.9$  and  $-3.1$ , corresponding to 80 and 110% PCR efficiency, respectively. The intercept varied from 37 to 40. The Bio-Rad kit contained an internal control present in each amplification mixture to check the presence of inhibitory factors. In the event of PCR inhibition, the sample was further diluted and reanalyzed. Two negative controls were performed for each assay, a negative control for PCR (obtained by replacing the DNA with water) and a negative control for DNA extraction. Both controls had to be negative to validate the assay. PCR results were converted to genomic units per liter. Average values were calculated from duplicates. The detection threshold for *Legionella* spp. was 30 GU/well, which corresponded to 960 GU liter<sup>-1</sup>. In the event of PCR inhibition, the dilution of the sample brought the detection threshold to 1,920 GU liter<sup>-1</sup> (3.3 log GU liter<sup>-1</sup>). The detection threshold for *L. pneumophila* was 640 GU liter<sup>-1</sup> (2.8 log GU liter<sup>-1</sup>). The protocol of quantification by PCR of *Legionella* spp. and *L. pneumophila* followed the XP T90-471 standard elaborated by the T90E WG AFNOR (Association Française de Normalization) (6).

**PCR-SSCP.** To analyze the overall structure of the bacterial community as a whole, the V3 region of the 16S rRNA gene was amplified with primers W49 (5'-ACGGTCCAGACTCCTACGGG-3', *Escherichia coli* position F331) and 5'-fluorescein phosphoramidite-labeled W104 (5'-TTACCGCGGTGCTGGC AC-3', *E. coli* position R533) (14). PCR amplifications were performed with a Mastercycler thermal cycler (Eppendorf, Hamburg, Germany). The reaction mixtures contained 1 $\times$  polymerase buffer, 0.2 mM deoxynucleoside triphosphates (dNTPs), 130 ng of each primer, 0.5 U of *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA), 1  $\mu$ l of genomic DNA, and water added to obtain a final volume of 50  $\mu$ l. The PCR conditions were an initial denaturation step of 2 min at 94°C; 25 cycles of a three-stage program of 30 s at 94°C, 30 s at 61°C, and 30 s at 72°C; and a final elongation for 10 min at 72°C. The reactions were stopped by cooling the mixture to 4°C. The analysis of *Legionella* sp. diversity was performed after a nested PCR. First, primers specific to the 16S rRNA gene of *Legionella* spp. were used to amplify a 653-bp fragment including the V3 region, i.e., LEG-225 (5'-AAGATTAGCCTGCGTCCGAT-3') and LEG-858 (5'-GTC AACTTATCGCGTTTGCT-3') (27). The reaction mixtures contained 1 $\times$  polymerase buffer, 0.2 mM dNTPs, 200 ng of each primer, 1 U of redTaq DNA polymerase (Sigma-Aldrich, St. Louis, MO), 1  $\mu$ l of genomic DNA, and water added to obtain a final volume of 50  $\mu$ l. The PCR conditions were an initial denaturation step of 90 s at 95°C; 30 cycles of 30 s at 95°C, 60 s at 64°C, and 60 s at 72°C; and a final elongation for 5 min at 72°C. Then, 1  $\mu$ l of the PCR product

was used to amplify the V3 16S rRNA gene bacterial region with primers W49 and W104 as described above. Amplification product sizes were confirmed by electrophoresis on a 2% (wt/vol) agarose gel.

SSCP analysis permits the separation of DNA fragments of the same size but with different compositions. One microliter of further diluted PCR products was added to 18  $\mu$ l of formamide and 1  $\mu$ l of internal size standard Rox 400 HD (Applied Biosystems, Foster City, CA) diluted 10 times. The sample was then denatured for 5 min at 95°C and placed directly on ice for 10 min. SSCP was performed with the ABI 3130 genetic analyzer (Applied Biosystems) equipped with four 50-cm capillary tubes filled with 5.6% conformation analysis polymer (Applied Biosystems) in corresponding buffer and 10% glycerol. The injection of DNA in capillaries required 5 kV for 3 s. Electrophoresis was carried out at 15 kV and 32°C for 30 min per sample. Raw SSCP data were exported into the easily handled csv format with the Chroma shareware (developed by Mark J. Miller at the U.S. National Institutes of Health), and statistical analyses were performed with SAFUM (37) and the Matlab 6.5 software (MathWorks).

**Identification of *Legionella* species and eukaryotic species by cloning and sequencing.** Two libraries of *Legionella* 16S rRNA genes were built, one with DNA from the 22 June sample and the other with DNA from the sample collected on 13 July. For the eukaryotic library, the DNA extract from water collected on 13 July was used. PCR mixtures contained 1 $\times$  polymerase buffer, 0.2 mM dNTPs, 200 ng of each primer, 1 U of redTaq DNA polymerase, 1  $\mu$ l of genomic DNA, and water added to obtain a final volume of 50  $\mu$ l. A 653-bp fragment of *Legionella* 16S rRNA genes was amplified by PCR with primers LEG-225 and LEG-858 as described above, but with 35 cycles instead of 30. For amplification of a fragment of the eukaryotic 18S ribosomal DNA, primers W16 (5'-CTTAATTGACTCAACACGG-3') (20) and W176 (5'-GGGCATCACAG ACCTGTT-3') were used. The PCR conditions were an initial denaturation step of 2 min at 94°C; 35 cycles of 60 s at 94°C, 60 s at 51°C, and 60 s at 72°C; and a final elongation for 10 min at 72°C. All PCR products were purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. The purified PCR products were cloned and transformed with the pCR4-TOPO plasmid and TOP10 *E. coli* competent cells, as indicated by the manufacturer (TOPO TA cloning kit; Invitrogen, Carlsbad, CA). Recombinant cells were selected by kanamycin resistance and *ccd* gene killer inactivation before cultivation at 37°C for 24 h in LB2 $\times$  medium (tryptone at 20 g liter<sup>-1</sup>, yeast extract at 10 g liter<sup>-1</sup>, NaCl at 10 g liter<sup>-1</sup>). Sequences were obtained from clone culture (Millegen, Toulouse, France). The primer sequences were removed, and the presence of chimerical sequences was checked for with the CHECK-CHIMERA tool available at the Ribosomal Data Project (26) and the Pintail program (5). Sequences were compared with GenBank databases (www.ncbi.nlm.nih.gov/BLAST) by using the BLASTN program (4). They were imported and aligned into the January 2004 ARB database (25). The aligned sequences were added to the ARB tree by using the parsimony tool. A tree gathering sequences from environmental clones and from described *Legionella* species (613 bp) was then built by neighbor joining (29). The tree was rooted with the sequence from *Coxiella burnetii* (D89798). Bootstrap analyses (1,000 replicates) were used to assess the robustness of inferred monophyletic groups.

**Nucleotide sequence accession numbers.** The 16S rRNA gene sequences of *Legionella* spp. determined in this study were deposited in the GenBank database under accession numbers EU309480 to EU309490.

## RESULTS

Water from the cooling tower facility was collected at different dates between April 2005 and January 2006. Water was sampled since it contains the microflora which will be discharged from the towers after aerosolization. Water was sampled at a point located just before its dispersal into the air inside the eight cooling towers. This enabled us to analyze the microbiological constituents in contact with the outside and the potential microbial contamination by the plant. It is also the sampling point generally used to monitor the *Legionella* risk in cooling towers.

**Abundance of *Legionella* spp. and *L. pneumophila* and effect of chemical treatments.** Concentrations of *Legionella* spp. and *L. pneumophila* were determined by real-time quantitative PCR from April 2005 to January 2006. Results are gathered in

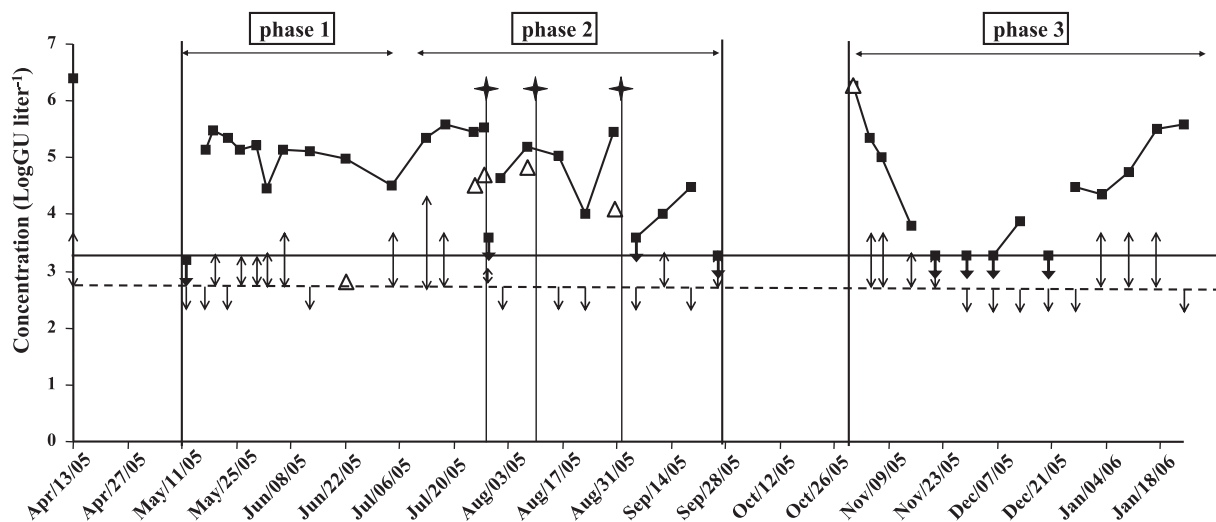


FIG. 1. Concentrations of *Legionella* spp. (■) and *L. pneumophila* (△) in a cooling tower facility as determined by quantitative PCR. When detection of *L. pneumophila* occurred but at concentrations below the quantification threshold, results are represented by double arrows. When the concentration was below the detection threshold, results are represented by single arrows. The detection thresholds of *Legionella* spp. and *L. pneumophila* are indicated, respectively, by solid and dotted lines (*Legionella* detection threshold = 3.3 log GU liter<sup>-1</sup>, *L. pneumophila* detection threshold = 2.8 log GU liter<sup>-1</sup>). Stars indicate combined biocidant and biocide treatments.

Fig. 1. The threshold of detection by quantitative PCR was 3.3 log GU liter<sup>-1</sup> for *Legionella* spp. and 2.8 log GU liter<sup>-1</sup> for *L. pneumophila*. In April 2005, the concentration of *Legionella* spp. was high (6.4 log GU liter<sup>-1</sup>) and the plant was shut down and cleaned. At the subsequent startup of the cooling tower in May, the *Legionella* sp. concentration was below 3.3 log GU liter<sup>-1</sup> and *L. pneumophila* was not detected. However, after only 5 days, the concentration of *Legionella* spp. increased to 5.1 log GU liter<sup>-1</sup> and then remained between 4.4 and 5.6 log GU liter<sup>-1</sup> for several weeks. *L. pneumophila* was detected in May and June, but its concentration remained under the quantification threshold until the end of June (phase 1). It started to rise in July, and the highest concentrations of *L. pneumophila* were obtained in July and August, at up to 4.7 log GU liter<sup>-1</sup> (phase 2). In September, concentrations of *L. pneumophila* remained low. The plant was shut down in October, and from the end of October to January (phase 3), *L. pneumophila* was quantified at a high concentration only once, when the plant was restarted.

From May, biocides were regularly injected into the system without any effect on *Legionella* sp. concentrations. After the proliferation of *L. pneumophila*, a combined biocide and biocidant treatment was carried out on 28 July, 8 August, and 2 September. This treatment reduced *Legionella* sp. and *L. pneumophila* concentrations significantly, below the detection or quantification threshold, even when the system was highly contaminated (Fig. 1). Simultaneous decreases in ATP content and biofilm size were also observed after the treatment (data not shown). The dispersal agent was effective in biofilm removal, and dispersal and biocide treatment decreased not only the total microbial population size (based on ATP levels) but also the *Legionella* sp. concentration. However, a few days after treatment, high concentrations of *Legionella* and *L. pneumophila* were observed once again, showing that although the treatment effect was real, it was only transient. *L. pneumophila* was quantified in five samples, with values ranging from 2.8

to 4.9 log GU liter<sup>-1</sup>. In these samples, the concentration of *Legionella* spp. was around 5 log GU liter<sup>-1</sup>. The corresponding percentage of *L. pneumophila* among the total *Legionella* population varied from less than 1% to up to 50%. *Legionella* spp. and *L. pneumophila* did not follow the same dynamic; the increase in the *L. pneumophila* concentration, concomitant with a constant concentration of *Legionella* spp., may indicate that the concentration of other *Legionella* spp. decreased.

**Dynamics of the microbial community. (i) Bacterial population.** The structure of the bacterial community was examined from May to September by using SSCP fingerprints. SSCP fingerprints were compared by principal-component analysis (PCA). Close SSCP fingerprints gather together on the PCA plots. The comparison is based on the totality of the SSCP signal. The first two dimensions of the PCA plots represent the two components which best highlight the differences between the SSCP fingerprints. Figure 2 presents two PCA plots, one for components 1 and 2 and one for components 1 and 3.

Six bacterial species were continuously present during the 3.5 months of the study in various amounts. In some samples, great diversity was observed, 5 to 15 peaks appearing on top of the six species. The samples displaying great diversity harbored the same bacterial species in various proportions. The increase in diversity did not seem to occur after chemical biocide and biocidant treatment. Rather, PCA analysis of the SSCP data showed that this treatment led, in some cases, to disrupted fingerprints (samples in boxes, Fig. 2b) located away from the main groupings on the PCA plot. In these samples, the six most common species were not retrieved. Figure 3 gathers three SSCP fingerprints showing the six resilient species (Fig. 3a), one high-diversity SSCP fingerprint (Fig. 3b), and one fingerprint obtained after the chemical treatment of 28 July (Fig. 3c). Overall, the system was colonized by few dominant and resilient bacterial species, an observation that held even after the community structure was strongly disrupted, and over and above this resilient flora, some more species were sometimes

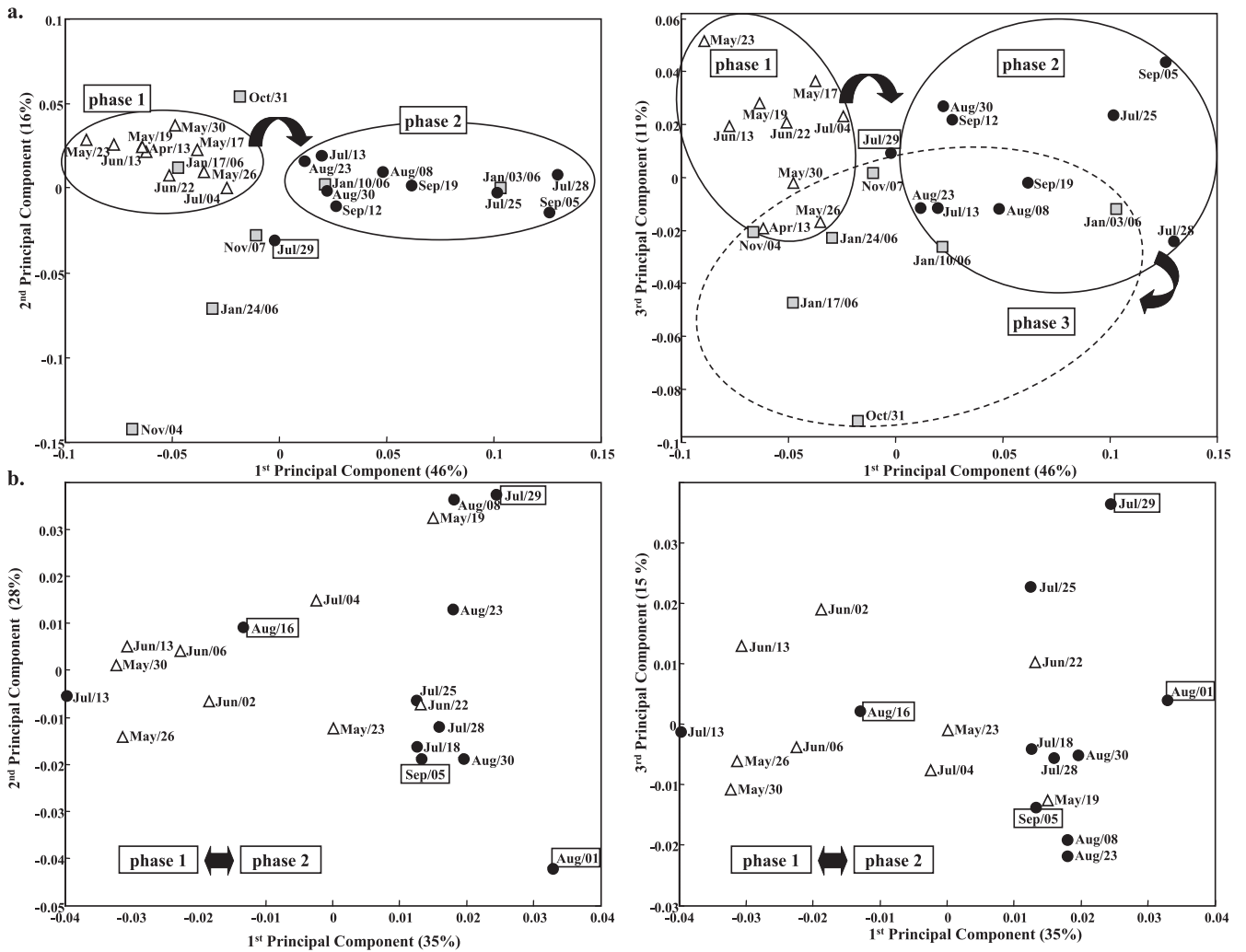


FIG. 2. PCA of SSCP fingerprints obtained for *Legionella* spp. (a) and bacteria (b) during phase 1 ( $\Delta$ ), phase 2 ( $\bullet$ ), and phase 3 ( $\blacksquare$ ). Samples in boxes were collected after chemical biocidant and biocide treatment.

present. There was no variation with the passage of time in the dynamics of the bacterial diversity. In particular, no significant change in the bacterial population was observed in July during the increase in the *L. pneumophila* concentration.

(ii) *Legionella* sp. population. The structure of the *Legionella* sp. population was determined from April 2005 to January 2006. The PCA plot of SSCP fingerprints (Fig. 2a) showed two main groupings, i.e., samples from April to July (phase 1) on the one hand and samples from mid-July to September (phase 2) on the other. The same four dominant species were observed from the beginning of April to the beginning of July (peaks A, B, C, and D in Fig. 3d). After this date, the diversity decreased and SSCP fingerprints then harbored one or two peaks (peaks A and B in Fig. 3e). Peak B, observed from May to September, was dominant during the summer, although not always recovered in October and November. SSCP fingerprints obtained during phase 3 formed another grouping on the PCA plot (Fig. 2a). Peaks A and B were present and even dominant in some of the fingerprints obtained in January. The effect of chemical treatment (biocide and biocidant) on the diversity of *Legionella* spp. could not be analyzed since the water collected just after treatment had insufficient concentrations for PCR amplification. The beginning of the proliferation of the system by *L. pneumophila* was simultaneous with a major decrease in *Legionella* sp. diversity. Indeed, changes in *Legionella* sp. community

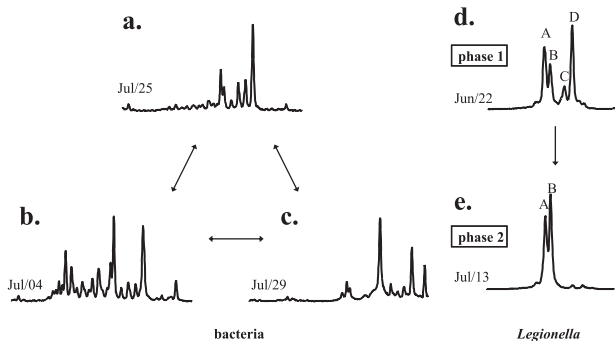


FIG. 3. SSCP fingerprints showing different bacterial community structures (resilient species [a], high-diversity SSCP fingerprint [b], and after chemical treatment on 28 July [c]) and *Legionella* sp. diversity during phases 1 (d) and 2 (e).



structure appeared mainly between the end of June and the beginning of July, which was precisely the same period when high concentrations of *L. pneumophila* were first observed.

Overall, the community structure of *Legionella* spp. varied depending on the time of year, whereas bacterial community structure was composed largely by the same group of a few dominant species and was reestablished within a few days after any disruption. *Legionella* spp. changed throughout the time period studied, with no reoccurrence of the initial population, whereas the same bacterial flora was observed during different periods of the year. Therefore, there could be no correlation between the dynamics of *Legionella* spp. and those of the dominant bacteria.

**Phylogenetic positioning of *Legionella* species.** The *Legionella* spp. present before the proliferation of *L. pneumophila* (sample collected on 22 June during phase 1) and during the proliferation (sample collected on 13 July during phase 2) were identified after cloning and sequencing of 16S rRNA genes. The 16 sequences obtained from the first sample were coded SEC, and the 34 sequences obtained during phase 2 were coded SED. All 16S rRNA gene sequences had the greatest similarity to 16S rRNA gene sequences of *Legionella* spp., confirming the specificity of the primers used. The percentages of similarity with the closest match in the GenBank database, as given by the BLASTN program, ranged from 94 to 99%; low similarity percentages were obtained for both libraries. A phylogenetic tree was built with 16S rRNA gene sequences from described *Legionella* species and from the closest environmental clones (Fig. 4). The diversity of *Legionella* spp. was high before the proliferation of *L. pneumophila*, with phylotypes differently positioned on the phylogenetic tree and distantly related. The dominant species were close to *L. fallonii* (re-named after *Legionella*-like amoebal pathogen [LLAP] 10), *L. fairfieldensis*, *L. lytica*, and other LLAPs. Several sequences (SEC10, SEC15, and SED03) were only distantly related to described species and were associated with uncultured *Legionella* spp. from drinking water, groundwater, sludge, or sediment. In the sample collected in July, *L. fallonii* and *L. pneumophila* were the main species recovered, with, respectively, 47 and 33% of the sequences. Positioning on the phylogenetic tree was not directly related to positioning on the SSCP fingerprints, since SSCP analysis was based on a smaller fragment of the 16S rRNA gene (around 200 bp). However, peak A gathered *L. lytica* and other LLAPs, as well as *L. pneumophila*, and peak B could be identified as *L. fallonii*. The other peaks corresponded mainly to environmental clones. Peak B dominated in July and August and was present from April to September.

**Identification of amoebae.** Eukaryotic diversity was analyzed in the sample from July with universal primers for the domain *Eukarya*. Of the 14 sequences analyzed, 6 were close to sequences from *Ochromonas*, a golden brown alga found mostly in freshwater, and two were associated with amoebae. For the first amoebal sequence, the closest relatives belonged to the genera *Plantamoeba* and *Vannella*. The second sequence corresponded to *Acanthamoeba*.

## DISCUSSION

This study gathers qualitative and quantitative data on the dynamics of *Legionella* species and of dominant bacteria in a

cooling tower facility over a 9-month period which included several weeks when *L. pneumophila* proliferated. Previous studies of *Legionella* in cooling towers reported quantification of *Legionella* spp. and *L. pneumophila*, but to our knowledge, none of them followed the dynamics of the *Legionella* population in relation to the bacterial microflora in the same cooling tower system.

During the spring and summer, *Legionella* sp. concentrations varied between 4.5 and 5.5 log GU liter<sup>-1</sup>. The maximal concentrations of *L. pneumophila* were obtained in July and August and were between 4.5 and 5 log GU liter<sup>-1</sup>. These concentrations are within the range of values previously reported for cooling towers (36). The high level of *L. pneumophila* obtained when the plant was restarted in October may be due to fouling of the network and considerable biofilm development during the shutdown period. This result fits in with the fact that cooling towers are implicated in outbreaks of legionellosis, particularly at startup or during construction (7, 10). It has been suggested that there may be a relationship between high *Legionella* counts in cooling towers and the occurrence of outbreaks of legionellosis (31). In this study, for similar *Legionella* sp. concentrations, some samples were highly contaminated with *L. pneumophila*, while in others the pathogenic bacterium was not detected. These results reinforce the existing assumption that the assessment of health risks from cooling towers cannot be reliably based upon single and infrequent *Legionella* tests (9, 24).

Combining biocides and biodispersants did reduce the concentration of *Legionella* spp. and of *L. pneumophila*. However, this effect was transient. Chemical treatment used to control *Legionella* in human-made water systems does not lead to total eradication of the bacterium, and recolonization occurs as soon as the treatment is interrupted (34). Legionellae are, in fact, protected inside amoebae and in biofilms and can proliferate again, recontaminating the water once the biocides lose their effect (21, 34). Amoebae present during the proliferation of *L. pneumophila* were identified after amplification of the 18S ribosomal DNA with primers universal for the domain *Eukarya*. Sequences close to those of *Plantamoeba* and *Vannella* were obtained. These flagellate amoebae are frequently found in freshwater or seawater and are affiliated with *Legionella* (32). *Acanthamoeba* was also present. This amoeba is commonly isolated from *Legionella*-contaminated plumbing systems (32) and is an important host of *L. pneumophila* in water (7). The survival of *L. pneumophila* within *Acanthamoeba* cells during biocide treatment has been reported (21), and the presence of this amoeba could explain why the effect of biocides on *Legionella* abundance was only transient.

The treatment reduced the concentration of *Legionella* spp. to such a low level that it was not possible to analyze their diversity. However, when the population grew back to its initial level, the *Legionella* spp. were the same as prior to treatment. This shows clearly that even if the treatment induced a change in the structure of the *Legionella* population, its effect was only transient. Concerning dominant bacteria, a major disruption of the community structure due to biocide and biodispersant treatment was observed (samples collected on 29 July and 1 August, i.e., 1 and 4 days after treatment). The differences observed between the other SSCP bacterial fingerprints may relate to the origin of the cells. The similar fingerprints show-



The suitability of primers LEG-225 and LEG-858 for detecting *Legionella* spp. in water has been reported previously (12, 13, 27, 35). The present study has shown that these primers can also be used to analyze *Legionella* diversity by SSCP. Concerning the phylogenetic positioning of *Legionella* spp., some relationships in the phylogenetic tree were supported moderately by bootstrap values, as previously observed by Carvalho et al. (13).

The high diversity of *Legionella* spp. in cooling towers has been clearly demonstrated (Fig. 4). This is an important result because the diversity of *Legionella* spp. in the environment, and especially in cooling tower systems, is poorly documented (28). Several sequences were close to those retrieved from treated surface water supplies and treated groundwater supplies in The Netherlands (clones Tag and S in Fig. 4) (35). Furthermore, LLAPs were particularly well represented in the cooling tower network studied. *L. fallonii*, which was constantly present in the system and dominated during the summer, was only described fairly recently (1) and has as its type strain LLAP-10<sup>T</sup>. A recent study of Dutch tap water installations also reported a large proportion of LLAPs (16). Those protozoonotic bacilli, which initially were isolated in coculture with protozoa, were named LLAPs because of their ability to infect and multiply intracellularly within amoebae in the same way that legionellae do (1, 2). Overall, a large proportion of sequences determined in the present study belong to as-yet-uncultured legionellae, a result which highlights the discrepancy between described *Legionella* species and the *Legionella* species present in the environment, including human-made systems.

*L. pneumophila* was one of the two dominant *Legionella* species during the summer (13 July). However, it was not detected by sequencing at the end of June (22 June). This rapid proliferation of *L. pneumophila*, in less than 3 weeks, may correspond to an increase in the "background" concentration of *L. pneumophila* in the environment during the summer, leading to the contamination of the system through water or air. A summer and autumn peak in incidence has been described as a characteristic epidemiological feature of Legionnaires' disease in Europe (11). Furthermore, a seasonal pattern of infection has been reported by Fliermans et al. (18), who injected guinea pigs with sample water collected monthly from a thermally altered lake, the highest frequency of infection by *L. pneumophila* occurring during the summer months. Such a seasonal variation may well be due to the facts that *Legionella* spp. multiply faster in the warmer waters of summer and that the greater use of cooling towers in summer provides opportunities for dissemination (11). During the investigation of *Legionella* colonization in 31 cooling towers in South Australia, it was found that between 60 and 75% of the cooling towers studied were colonized by *Legionella* during the summer months but only 20 to 30% were colonized during the winter (8). In the present study, the increase in *L. pneumophila* concentrations, concomitant with stability in the *Legionella* concentration, indicates that there must have been a decrease in the concentrations of other *Legionella* species during the same period. Furthermore, peak B dominated the SSCP fingerprints in phase 2, whereas it was nondominant in phase 1. This means that not only did *L. pneumophila* become one of the dominant species during phase 2, but the relative proportions of other *Legionella* species also changed significantly. In particular, peaks C and D disappeared from the SSCP finger-

prints and the apparent diversity decreased. Factors which might have led to these important changes in the structure of the *Legionella* population still remain to be identified, i.e., the antagonistic relationships between certain *Legionella* species and *L. pneumophila*, differences in temperature growth ranges, changes in amoebal hosts, etc. The results obtained thus open new perspectives for future research. It would also be interesting to analyze *Legionella* diversity during the proliferation of *L. pneumophila* in other cooling towers in order to ascertain whether the dynamics observed in the present study also occur in other systems.

The objective of this study was to determine whether the different dynamics of bacteria and of *Legionella* spp. were correlated in cooling towers. A quantitative correlation was found in some cases (a decrease in ATP levels simultaneous with a decrease in the *Legionella* sp. concentration after chemical treatment), but it was not qualitative. In particular, no change in the structure of the bacterial community was observed during the proliferation of *L. pneumophila*. Bacterial diversity was modified by the chemical treatment or the development of biofilms but did not depend on the time period. In contrast, variations in the *Legionella* population structure were observed over time, with significant changes occurring at the end of June, following a stable phase from mid-May to mid-June. In a previous study of microbial communities in a wide range of aquatic samples containing *L. pneumophila*, no relationship was found between the occurrence of *L. pneumophila* and the associated microbiota (15). It was observed, however, that the occurrence of *L. pneumophila* was possible within a certain range of species richness and diversity. The authors concluded that the relationship between the occurrence of *L. pneumophila* and the bacteriological characteristics of water is complex and that it may therefore be interesting to concentrate on specific groups of microorganisms. In this study, we used a different approach, focusing our investigation on one cooling tower facility over several months, but we also found no relationship between the dynamics of the *Legionella* population during the proliferation of *L. pneumophila* and the dynamics of the dominant bacteria in the system. It may be that the factors that regulate the occurrence of *Legionella* are different from those that govern overall bacterial populations in cooling tower systems (23).

This study has provided new input on the diversity of *Legionella* spp. in cooling towers, showing that structural changes in the *Legionella* population are linked to the time of year, displaying stable periods but also a rapid major shift during the proliferation of *L. pneumophila*. This study has also demonstrated the application of molecular techniques which enabled us to conduct a comprehensive study of the dynamics of *Legionella* in relation to the bacterial community as a whole, gathering data on abundance and population structures which cannot easily be obtained by culture-dependent approaches. Controlling the *Legionella* risk in cooling towers requires exact knowledge of which constituents of the microbial community play a role in the proliferation of the pathogenic species, as well as the importance of these microorganisms compared to abiotic parameters such as the temperature, the mineral composition of the water, or the structure of the network. In this perspective, this study has demonstrated the value of studying the dynamics of the microflora rather than using one-off analyses.

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