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Occurrence and Genetic Diversity of Uncultured *Legionella* spp. in Drinking Water Treated at Temperatures below 15°C

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Representatives of the genus *Legionella* were detected by use of a real-time PCR method in all water samples collected directly after treatment from 16 surface water (SW) supplies prior to postdisinfection and from 81 groundwater (GW) supplies. *Legionella* concentrations ranged from 1.1×10^3 to 7.8×10^5 cells liter^{-1} and were significantly higher in SW treated with multiple barriers at 4°C than in GW treated at 9 to 12°C with aeration and filtration but without chemical disinfection. No Legionellae (<50 CFU liter^{-1}) were detected in treated water by the culture method. *Legionella* was also observed in untreated SW and in untreated aerobic and anaerobic GW. Filtration processes in SW and GW treatment had little effect or increased the *Legionella* concentration, but ozonation in SW treatment caused about 1-log-unit reduction. A phylogenetic analysis of 16S rRNA gene sequences of 202 clones, obtained from a selection of samples, showed a high similarity (>91%) with *Legionella* sequences in the GenBank database. A total of 40 (33%) of the 16S rRNA gene sequences obtained from treated water were identified as described *Legionella* species and types, including *L. bozemanii*, *L. worsleiensis*, *Legionella*-like amoebal pathogen types, *L. quateirensis*, *L. waltersii*, and *L. pneumophila*. 16S rRNA gene sequences with a similarity of below 97% from described species were positioned all over the phylogenetic tree of *Legionella*. Hence, a large diversity of yet-uncultured Legionellae are common members of the microbial communities in SW and GW treated at water temperatures of below 15°C.

Reported annual incidences of Legionnaires' disease, a life-threatening pneumonia, typically range from about 5 per million (e.g., in the United States) to nearly 20 per million (in several European countries), but many cases are either not diagnosed or not reported (34, 61). Sporadic community-acquired cases with unidentified origin of the etiologic agent prevail, but large outbreaks continue to occur as well (19). The outbreak of legionellosis among visitors and participants at a flower show in The Netherlands in 1999 caused 31 deaths and more than 200 cases of disease. This demonstrated once again the potential public health risk of the presence of *Legionella pneumophila* in water, especially when usage leads to aerosolization. A whirlpool on display at the entrance of the flower show was identified as the source of the outbreak (15). *L. pneumophila* is responsible for more than 90% of the reported cases of legionellosis (17, 61). Currently, about 50 *Legionella* species have been defined, nearly half of which have been associated with cases of disease (e.g., *L. pneumophila*, *L. micdadei*, *L. bozemanii*, *L. longbeachae*, and *L. dumoffii*) (17, 35, 40).

L. pneumophila is ubiquitous in natural freshwater environments, including hot springs, and is also a common inhabitant of engineered water systems, such as treated sewage, cooling towers, and hot water systems (17, 18, 42, 44, 50, 58). The organism has been isolated from these environments at temperatures below 10°C (surface water) to 60°C (engineered water systems), but growth of *L. pneumophila* is restricted to temperatures between 20 and 43°C (49, 59). *L. pneumophila* has been detected only sporadically by use of the cultivation method in untreated groundwater (GW) and in treated water at temperatures below 20°C (4, 14, 20, 24, 56). However, cultivation-independent techniques, including immunological methods, fluorescent in situ hybridization (FISH), and PCR-based methods, clearly revealed the common presence of *Legionella* species in aquatic environments, even at temperatures below 20°C (11, 12, 14, 44, 50). In addition, FISH methods showed that undefined *Legionella* species may represent up to 7% of biofilms grown in treated water (52). 16S rRNA gene sequences related to *L. parisiensis*, *L. maceachernii* and *Legionella*-like amoebal pathogen (LLAP) species were observed in slow sand filters operating at temperatures below 20°C and were used for fungal plant pathogen suppression (13).

GW, both aerobic and anaerobic, and surface water (SW) are used as sources for the production of drinking water in The Netherlands at a ratio of approximately two to one. GW is treated and distributed without chemical disinfection. SW is treated with multiple barriers against chemical and microbiological contaminants and distributed without or with a very low disinfectant residual. Contamination of drinking water in the distribution system is prevented by a series of protective measures, including maintenance of high pressure and cross-connection control. Regrowth is limited by a far-reaching removal of growth-promoting compounds from the water to achieve biological stability (57). A number of reports showed that *L. pneumophila* multiplying in hot-water systems in buildings originated from the water supply (27, 27, 37). The aim of our study was to elucidate the role of drinking water in the distribution of *Legionella.* For this purpose, an investigation on the presence and identity of *Legionella* in treated water was conducted using a quantitative real-time PCR method as well as a standardized culture method to ensure that all types of *Legionella* were included in the analysis. Furthermore, the genetic diversity of

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Legionella was studied to determine (i) the presence of pathogenic *Legionella* species and (ii) the diversity of the indigenous *Legionella* types.

MATERIALS AND METHODS

Water sampling sites. Samples of treated water were collected from a total of 82 GW treatment plants and 16 SW plants, covering 67% of the total drinking water production in The Netherlands. The GW plants included 72 plants using anaerobic GW, which in most cases is treated with aeration to introduce oxygen and to remove methane. Subsequently, water is treated by one or two stages of rapid sand filtration to remove ammonia, iron, and manganese. Aerobic GW is either distributed without treatment or aerated to add oxygen and to remove CO_2, followed by limestone filtration. No chemical disinfectant is used in GW treatment and distribution. The concentration of dissolved natural organic matter, measured as nonparticulate organic carbon, in treated GW ranges from below 0.5 mg liter^{-1} in aerobic GW to about 7 mg liter^{-1} in an anaerobic GW supply. The temperature of treated GW ranges from 9 to 12°C throughout the year, attaining a maximum of 15°C in a few plants, whereas the temperature of treated SW ranges from about 3°C in winter to 22°C in summer. SW treatment includes either storage in open reservoirs or soil passage, followed by a combination of the following processes: coagulation and sedimentation, chlorination, ozonation, dual medium filtration, granular activated carbon (GAC) filtration, and slow sand filtration. In one treatment plant, reverse osmosis is used as the final treatment process. Postdisinfection with a low residual concentration (<0.1 mg liter^{-1}) of chlorine dioxide or chlorine is applied when GAC filtration is the final treatment step. Each water sample was taken directly after the final treatment step but before postdisinfection when applicable. In addition, SW samples were collected from different rivers and from open basins for storage or collection of pretreated SW. Untreated GW was sampled from anaerobic and aerobic sources.

Water filtration and DNA extraction. At each sampling location a volume of 2 liters was collected in a glass container, which had been heat treated (4 h at 150°C) to ensure the absence of DNA contamination. The samples were stored at 4°C and processed within 24 h. DNA-free water was analyzed in each experiment to check for possible DNA contamination during filtration, DNA extraction, and PCR amplification. Samples were filtered through a 25-mm polycarbonate filter (0.22-μm pore size, type GTTP; Millipore, Amsterdam, The Netherlands) in volumes of approximately 1 liter for treated water and GW and 10 ml for samples of SW and open storage reservoirs. Subsequently, DNA was isolated and purified using a MagNA Pure LC DNA isolation kit III following the instructions of the manufacturer (Roche Diagnostics, Almere, The Netherlands). In brief, filters were transferred to a six-well multidish (Nuclon; Nalge Nunc International, Neerijse, Belgium). To each well 120 μl lysis buffer and 30 μl proteinase K were added, and the dishes were incubated at 65°C for 10 min. Following incubation, the suspension of lysed cells was transferred from each well to a new microtube. The filters in the wells of the multidish were washed with 300 μl binding/lysis buffer, and this buffer was transferred to the same microtubes. Washing of the filter with the binding/lysis buffer was performed to maximize DNA recovery. Magnetic glass beads (150 μl) were added to bind DNA. After incubation for 10 min at room temperature, the magnetic glass particles with bound DNA were concentrated using a magnet (Dynal Biotech S.A., Compiègne, France). Subsequently, beads were washed twice with washing buffer. Finally, the DNA was eluted from the beads in 100 μl elution buffer and analyzed by PCR.

Detection of *Legionella* in SW and reservoir water. A semiquantitative (dilution) PCR was applied for assessing the concentrations of DNAs of *Legionella* and *L. pneumophila* in SW samples and samples from open basins. In this test, undiluted and decimal dilutions (10^0, 10^{-1}, 10^{-2}, and 10^{-3}) of the isolated DNA were analyzed in duplicate in the PCR. A second dilution series (10^{-4} and 10^{-5}) was analyzed when all dilutions were positive in the first series. *Legionella* was detected using LEG-225 (5′ AAGATTAGCCTGCGTCCGAT) and LEG-858 (5′ GTCAACTTATCGCGTTTGCT) *Legionella*-specific primers (38) targeting the 16S rRNA gene. The amplification resulted in a DNA fragment of approximately 654 bp, enabling genetic analysis to determine the diversity of the detected *Legionella*. The primers L*mip*L920 (5′ GCTACAGACAAGGATAAG TTG) and *Lmip*R1548 (5′ GTTTTGTATGACTTTAATTCA), targeting the *mip* gene, were used for the specific detection of *L. pneumophila* (32). Identical amplification mixtures were used for both specific reactions. A 25-μl reaction mixture contained 10 μl of each template DNA solution, 3 U of Platinum *Taq* DNA polymerase (Invitrogen, Breda, The Netherlands) with the supplied PCR buffer, 0.2 μM of primers LEG-225 and LEG-858 or L*mip*L920 and *Lmip*R1548, 0.2 mM of each deoxynucleoside triphosphate, 3 mM of $MgCl_2$, and 0.4 mg ml^{-1} bovine serum albumin (PCR grade; Roche Diagnostics, Almere, The Netherlands). Amplification was performed in an ABI 9700 thermocycler (Applied Biosystems, Foster City, Calif.) with a PCR thermal profile consisting of an initial incubation for 2 min at 94°C; 40 cycles of 20 s at 94°C, 30 s at 60°C, and 40 s at 72°C; and finally a postamplification step of 2 min at 72°C. Subsequently, PCR products were analyzed by agarose gel electrophoresis. The initial DNA concentration was calculated from the highest DNA dilution that yielded a PCR product.

The semiselective buffered charcoal yeast extract (BCYE) agar medium supplemented with antibiotics was used to detect culturable *Legionella* (16, 41). Untreated SW and water from open storage reservoirs contain nonspecific bacteria which hamper the growth of *Legionella* on this medium. Therefore, no concentration step was included in the procedure, and sample volumes of 100 μl were spread directly over 10 plates of BCYE agar medium, resulting in a detection limit of 1,000 CFU/liter. The plates were examined for the presence of typical *Legionella* colonies after 5 and 7 days of incubation at 37°C.

Detection of *Legionella* in GW and in treated water. Untreated GW and all samples of treated GW and SW were analyzed for the presence of *Legionella* species (DNA) by using real-time PCR with SYBR green. The above-mentioned dilution PCR method was used to detect the presence of *L. pneumophila*, and the culture method was also used with these samples. In the *Legionella* species real-time PCR assay, the primers LEG-225 and LEG-858 were also used. Amplification, detection, and data analysis were performed with the iCycler IQ real-time detection system (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). The amplification mixture of 50 μl contained 25 μl of 2× Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Breda, The Netherlands), 10 μl of DNA template, 0.2 μM of primers LEG-225 and LEG-858, and 0.4 mg ml^{-1} bovine serum albumin (PCR grade; Roche Diagnostics, Almere, The Netherlands). The number of PCR cycles after which the amplified DNA is detected in the real-time PCR system (threshold cycle) is used to quantify the concentration of *Legionella*. This threshold cycle depends on the initial concentration of the target DNA. The undiluted sample and the 10^{-1} dilution of each DNA sample were analyzed in duplicate, resulting in four different PCRs. The PCR thermal profile was identical to the profile used for the dilution PCR method.

The average number of threshold cycles for each water sample was transformed into numbers of cells by using a calibration curve obtained with known concentrations of *L. pneumophila* cells. For this purpose a freshly grown colony was collected from the BCYE medium and suspended in distilled water free of DNase and RNase (Invitrogen, Breda, The Netherlands). The cells were enumerated by the acridine orange direct count method using epifluorescence microscopy (22). Volumes of 1.5 ml of the suspension were stored at −80°C. For each sample series, 1 ml of the reference cell suspension containing $3.5 \times 10^4 \pm 9.1 \times 10^3$ *Legionella* cells was filtered, and DNA was isolated by the procedure described above. Subsequently, DNA was eluted in 50 μl DNA-free water, and 10 μl was added to each PCR. A dilution series containing approximately 10^4, 10^3, 10^1, 10^2, and 1 cells was prepared to generate a calibration curve, and each DNA dilution was analyzed in duplicate. The calibration curve was calculated automatically with the software supplied by the iCycler IQ real-time detection system (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands), according to the average threshold cycle and the highest possible r^2 value. The concentration of the initial *Legionella* DNA in each sample was calculated using the threshold cycle values of the duplicates of the undiluted and diluted DNA solutions and the calibration curve.

The culture method with BCYE medium was also used to detect *Legionella* bacteria in treated water (41). For this purpose, 500 ml of the sample was filtered through a 47-mm polycarbonate filter with pore size of 0.2 μm (Sartorius Technologies BV, Nieuwegein, The Netherlands). The filters were sonicated upside down in 5 ml of sample water for 2 min using an ultrasonic water bath (42 KHz, 135 W) (Branson 5510; FMH Medical, Veenendaal, The Netherlands). Volumes of 100 μl of the obtained suspension were spread over BCYE medium with and without antibiotics and cysteine, as described above (detection limit, 50 CFU liter^{-1}).

Cloning and sequencing of PCR products. The genetic diversity of the *Legionella* community in SW, storage basins, and treated water was determined by cloning and sequence analysis of the PCR products of the genus-specific primer pair LEG-225 and LEG-858. The PCR products obtained from the undiluted target DNA sample were cloned into pGEM-T Easy Vector System II (Promega, Leiden, The Netherlands) according to the instructions of the manufacturer. Briefly, the PCR products were ligated overnight into the pGEM-T plasmid at 4°C and were subsequently transferred to competent *Escherichia* coli JM109 cells by heat shock treatment. The transformed cells were incubated overnight at 37°C on standard LB medium with ampicillin, IPTG (isopropyl-β-D-thiogalactopyranoside), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). White

TABLE 1. *Legionella* concentrations in untreated and treated water as determined with the quantitative real-time PCR method

Water type (no. of samples)	*Legionella* concn (cells liter^{-1})		
	Minimum	Median	Maximum
Surface water (10)[a]	2.0×10^4	1.0×10^6	2.5×10^6
Storage basin (4)[a]	2.5×10^3	1.9×10^4	1.4×10^5
Anaerobic groundwater (3 out of 7)	$<2.0 \times 10^2$	5.5×10^2	2.4×10^3
Aerobic groundwater (8 out of 9)	$<2.0 \times 10^2$	8.3×10^3	2.5×10^4
Treated surface water (16)	2.1×10^4	4.1×10^4	7.8×10^5
Treated anaerobic groundwater (70)	1.1×10^3	1.4×10^4	1.7×10^5
Treated aerobic groundwater (11)	2.9×10^3	2.3×10^4	9.8×10^4

[a] Water samples analyzed with the dilution PCR method (concentrations in 16S rRNA gene copies liter^{-1}).

colonies were screened for the correct insert size by PCR using the standard primers T7 and SP6 located outside the cloning site of the pGEM-T plasmid, followed by agarose gel electrophoreses. The DNA insert was sequenced in both the forward and reverse directions using primers T7 and SP6. If possible, the sequences of 5 clones of each selected sample of treated water and 10 clones of each selected SW sample were analyzed.

Nucleotide sequence data analysis. The two sequences of a cloned 16S rRNA gene sequence were assembled and edited by using the software package DNAStar Seqman II (Lasergene Inc., Madison, Wis.). The edited sequences were aligned and manually checked using ClustalW (http://www.ebi.ac.uk/clustalw/). A BlastN search (4a) was performed to analyze the similarity of the clone sequences to 16S rRNA gene sequences available in the GenBank database. The primer sequences at both sides of the sequence were removed, and the obtained sequences of approximately 614 bp were imported and aligned into the 16S rRNA ARB database of January 2004 (31). The aligned sequences were added to the main tree by using the parsimony tool with local optimization included in the ARB program. Sequences of species of *Piscirickettsia*, *Wolbachia*, and *Coxiella* which were most closely related to the *Legionella* cluster were used as an outgroup in this tree. 16S rRNA gene sequences with 99% and more similarity were grouped.

Nucleotide sequence accession numbers. All partial 16S rRNA gene sequences from the 16S rRNA gene sequences determined in this study have been deposited in GenBank under accession no. AY923985 to AY924186.

RESULTS

***Legionella* in SW and in open storage basins.** *Legionella* species were detected by the dilution PCR method in all 10 SW samples and in all 4 samples from open basins (Table 1). These samples were all collected in the winter period (January and February) at a water temperature of 3 to 4°C. The concentration of *Legionella* in the river water samples was in most cases greater than 10^6 16S rRNA gene copies liter^{-1}. In the open storage basins, containing pretreated SW, clearly lower concentrations were observed, ranging from 2.5×10^3 to 1.4×10^4 16S rRNA gene copies liter^{-1}. *L. pneumophila* was detected at concentrations of between 1.0×10^3 and 2.0×10^4 *mip* gene copies liter^{-1} in four SW samples, comprising 0.1 to 1% of the total *Legionella* concentration. In the other samples the *L. pneumophila* concentration was below the detection limit of 1×10^3 16S rRNA gene copies liter^{-1}. Culturable *Legionella* bacteria were not detected in any of these samples (detection limit, 1,000 CFU liter^{-1}).

***Legionella* in GW and in treated water.** A total of 16 samples of untreated GW, 82 samples of treated GW, and 16 samples of treated SW taken directly after treatment were quantitatively analyzed for the presence of *Legionella* by using a real-time PCR method. The amplification efficiency as determined with dilutions of the suspension of *L. pneumophila* was above 85% in all sample series, and the correlation coefficient (r^2 value) of the standard curve was high (0.99). Each DNA sample was analyzed undiluted and as a 10^{-1} dilution, both in duplicate. The relative standard deviations (RSDs) of the concentrations obtained for the PCR duplicates and for the two DNA dilutions were calculated to assess the quality of the quantitative measurements. The average RSDs of the obtained concentrations were 13% (for the undiluted duplicates; $n = 97$), 26% (for the 10^{-1} duplicates; $n = 79$) and 6% (for the two DNA concentrations; $n = 79$). These values demonstrate that the quantification of the DNA concentration is highly reproducible.

Legionella was detected in three out of seven samples of anaerobic GW at a maximum concentration of 2.4×10^3 cells liter^{-1} (Table 1). In one sample the *Legionella* concentration was below the detection limit of 2.0×10^2 cells liter^{-1}, and in three water samples the PCR was inhibited, as was demonstrated by spiking with the *L. pneumophila* suspension (results not shown). The *Legionella* concentrations in eight of nine samples of aerobic GW were above the detection limit and ranged from 2.7×10^3 to 2.5×10^4 cells liter^{-1} (Table 1). *Legionella* was detected in 33 of 34 samples of water collected from different treatment stages in 25 treatment plants (8 SW and 17 GW). *Legionella* was below the detection limit in water sampled after reverse osmosis. Increased *Legionella* concentrations (>1 log unit) were observed directly after aeration and after sand filtration of anaerobic GW. Effects of granular activated carbon filtration, applied in SW treatment, ranged from a decrease to no effect or an increase (1 log unit) of the *Legionella* concentration. Ozonation caused a clear (>1-log-unit) reduction (results not shown).

All samples of treated water taken from 97 different treatment plants contained *Legionella* species at concentrations ranging from 1.1×10^3 to 7.8×10^5 cells liter^{-1} (Fig. 1). The highest *Legionella* concentration was observed in treated SW, with GAC filtration as the final treatment step (before post-disinfection). *L. pneumophila* was not detected in any of these water samples by using the dilution PCR method (detection limit, 100 *mip* gene copies liter^{-1}). Furthermore, no culturable *Legionella* organisms were detected (detection limit, 50 CFU liter^{-1}) in treated water. The concentrations in treated SW

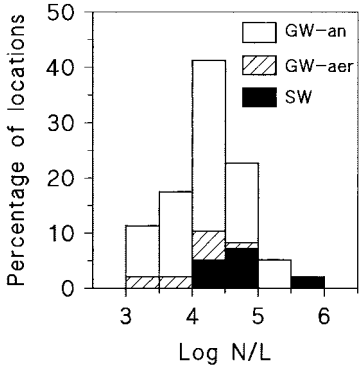


FIG. 1. Frequency distributions of *Legionella* concentrations (N, cells/16S rRNA gene copies per liter) in treated water from 97 treatment plants analyzed by a real-time PCR method using the 16S rRNA gene-targeting primers LEG-225 and LEG-858. GW-an, groundwater supply with anoxic source water; GW-aer, groundwater supply with aerobic source water; SW, surface water supply.

TABLE 2. *Legionella* species or types related to 16S rRNA gene sequences (similarities of >97%) obtained from untreated surface water, pretreated surface water from open storage basins, treated surface water, and treated groundwater, in decreasing frequency of occurrence

Species or type	Human pathogen[a]	No. of sequences obtained from:					Total no. of sequences (%)
		SW	Open storage basin	Treated SW	Treated anaerobic GW	Treated aerobic GW	
L. worsleiensis	−	2	2	1	5	1	11 (5.5)
L. bozemanii	+	1	1	3[b]	5 (3[b])		10 (5.0)
L. lytica (LLAP 9)	+	1	1	3[b]			5 (2.5)
L. pneumophila	+	3	1		1		5 (2.5)
L. waltersii	−		1	3[b]		1	5 (2.5)
L. quateirensis	−		1		1	3[b]	5 (2.5)
L. donaldsonii	−	1	1	1			3 (1.5)
LLAP 2[c]	−	1			1	1	3 (1.5)
LLAP 7[c]	−	2	1				3 (1.5)
L. adelaidensis	−				1	1	2 (1.0)
L. dumoffii	+		1		1		2 (1.0)
L. londiniensis	−	1		1			2 (1.0)
LLAP 4[d]	+	1		1			2 (1.0)
L. rowbothamii (LLAP 6)	−	2					2 (1.0)
L. anisa	+			1			1 (0.5)
L. fairfieldensis	−				1		1 (0.5)
L. drozanski (LLAP 1)	+	1					1 (0.5)
LLAP 3[c]	+		1				1 (0.5)
LLAP 8	−		1				1 (0.5)
L. fallonii (LLAP 10)	+			1			1 (0.5)
LLAP 11[d]	−	1					1 (0.5)
L. micdadei	+					1	1 (0.5)
L. steigerwaltii	−				1		1 (0.5)
L. wadsworthii	−		1				1 (0.5)
Total identified (%)		17 (34.7)	13 (44.8)	15 (30)	17 (34.7)	8 (32)	70 (34.7)
Total isolated		49	29	50	49	25	202 (100)

[a] Species associated with cases of disease. −, no reports; +, references 17, 33, and 35.
[b] Number of 16S rRNA gene sequences from a single sample.
[c] Related to *L. lytica* (LLAP 9).
[d] Related to *L. drancourthii* (LLAP 12).

differed significantly ($P < 0.05$) from those in treated aerobic and anaerobic GW, which did not significantly differ from each other.

Sequence diversity of detected *Legionella* 16S rRNA genes. Cloning and sequencing of the PCR products of the *Legionella* genus-specific PCR primers LEG-225 and LEG-858 (654 bp) resulted in 49 16S rRNA gene sequences from seven SW samples and 29 sequences from three storage basins. Furthermore, a total of 123 16S rRNA gene sequences were obtained from treated water samples collected at eight SW treatment plants (50 sequences), 10 anaerobic GW treatment plants (49 sequences), and 6 aerobic GW treatment plants (25 sequences). All 202 16S rRNA gene sequences exhibited the greatest similarity to 16S rRNA-encoding genes from defined *Legionella* spp., confirming the specificity of the PCR primers used. Similarities between the 16S rRNA gene sequences and *Legionella* sequences deposited in the GenBank database ranged from 91% to a maximum of 99%. Even 16S rRNA gene sequences with a similarity value of 91% had *Legionella* species as the nearest relative. Sequences with a similarity equal to or above 97% are considered to represent the same species (55). A total of 30 (38%) 16S rRNA gene sequences obtained from SW and open collection basins and 40 (32%) of the 16S rRNA gene sequences from treated water samples showed similarity values of ≥97% with 18 *Legionella* species and a number of LLAP types (Table 2). Relatedness to *L. pneumophila* was observed in four 16S rRNA gene sequences originating from three samples of untreated SW (97 to 99% similarity) and in one from a sample of treated GW (98% similarity). Most (18 out of 20) 16S rRNA gene sequences related to LLAP types had SW-related origins, viz., untreated SW (9 sequences), open storage basins (4 sequences), or treated SW (5 sequences). Other *Legionella* species with high similarities to 16S rRNA gene sequences from SW(-related) samples were *L. worsleiensis* (5 sequences), *L. bozemanii* (5 sequences), and *L. donaldsonii* (3 sequences). *Legionella* species most frequently related to 16S rRNA gene sequences from treated GW were *L. worsleiensis* (6 sequences), *L. bozemanii* (5 sequences), *L. quateirensis* (4 sequences), and *L. adelaidensis* (2 sequences). The 16S rRNA gene sequences related to *L. bozemanni* predominated in two samples, viz., the GAC filtrate of an SW supply and the rapid sand filtrate of an anaerobic GW supply. Furthermore, 16S rRNA gene sequences related to *L. lytica* and *L waltersii* predominated in GAC filtrates of SW supplies, and *L. quateirensis* predominated in the limestone filtrate of an aerobic GW supply.

A total of 65% of the 202 16S rRNA gene sequences had similarities of below 97% with defined *Legionella* species and may belong to not-yet-described *Legionella* types. A phylogenetic tree was calculated using the ARB program (31) (Fig. 2). Parts of the tree, e.g., LLAP-related sequences, are presented separately (Fig. 2B and C). The various 16S rRNA gene se-

quences created a highly diverse tree without major clusters. A total of 44 groups, containing 16S rRNA gene sequences with similarities of ≥99%, are present in the tree. Sixteen of these groups contain a total of 38 16S rRNA gene sequences originating from identical water samples, and 28 groups contain sequences from different water samples. Hence, certain groups predominated in specific samples, e.g., groups 3 and 4 in treated aerobic GW (limestone filtrate); groups 8 and 19 in treated anaerobic GW supplies (filtrates of rapid sand filters); and groups 13, 27, 31, 40, and 41 in treated SW (GAC filtrates). Groups 22 to 25, with a total of 14 16S rRNA gene sequences, mostly originating from SW samples and samples of treated SW, formed a distinct branch in the tree (Fig. 2C). This branch, with a minimal internal sequence similarity of 96% and a minimal 91 to 93% similarity with *Legionella* sequences deposited in the GenBank database, possibly represents a surface water phylotype. Another distinct branch is formed by groups 26, 27, 28, and 29. The 16S rRNA gene sequences in these groups all originated from different types of SW or treated SW, with no 16S rRNA gene sequences from untreated SW and treated SW at specific plants. In most water samples, a variety of different 16S rRNA gene sequences were detected. The observations show that certain defined *Legionella* species as well as distinct, yet-unknown *Legionella* species are common members of the microbial communities in SW and GW, both untreated and treated at temperatures below 15°C.

DISCUSSION

Detection of *Legionella* in aquatic environments. A steadily increasing number of *Legionella* species, presently a total of about 50, have been defined since the discovery of *L. pneumophila* in 1976. About half of these species are associated with disease, but *L. pneumophila* still is by far the most prominent reported pathogen (17, 61). A variety of methods, including guinea pig inoculation, fluorescent-antibody (FA) techniques, culture techniques, FISH, and PCR-based assays, have been applied to detect *L. pneumophila* in environmental samples. Culture methods, which enable the quantitative detection of culturable bacteria and isolation of strains, are commonly used but have a number of limitations, viz., a long incubation period, growth of competing background bacteria, and recovery reduction by antibiotics and sample treatment (10, 16, 28, 48, 60). Reduced recoveries or inability to grow on solid media strongly limit the use of these methods for detection of non-*L. pneumophila* species (28, 39). FA-based methods are available for rapid and specific detection of *L. pneumophila*, resulting in concentrations exceeding the culturable counts, but do not differentiate between dead and viable cells (3, 5, 9). A number of defined culturable, non-*L. pneumophila* species can also be detected with FA-based methods (42). Uncultured yet undefined *Legionella* types, however, remain undetected with this method. Several so-called LLAPs can be isolated in amoebal coculture, but this method is not attractive for quantitative detection (26, 51). FISH methods have a potential for the detection of individual *Legionella* species and differentiate between active and inactive (dead) cells, but their application is laborious and the detection level is relatively high (12, 62). PCR-based methods, which enable quantitative detection even at low concentrations, therefore were used to determine the presence of *L. pneumophila*, non-*L. pneumophila* species, and yet-undefined *Legionella* types in treated water, despite the inability to differentiate between dead and viable cells.

Quantitative real-time PCR analysis. The primers LEG-225 and LEG-858 were used to detect *Legionella* by a semiquantitative dilution PCR method and by a quantitative real-time PCR method. The suitability of the primer set in detecting *Legionella* in environmental samples has unequivocally been demonstrated (13, 38, 45). The results of the present investigation shows that real-time PCR is a suitable method for selective and quantitative detection of representatives of the genus *Legionella*, both defined species and yet-undefined types. In three of seven anaerobic GW samples, however, major PCR inhibition was observed, which might have resulted in false-negative PCR results. The high concentrations of iron (up to 9 mg liter^{-1}) and manganese (0.5 mg liter^{-1}) in these water samples could be the cause of the observed inhibition. Amplification and detection of the 16S rRNA genes of *Legionella* were highly effective and reliable for the other samples, as was demonstrated by the small RSDs of the concentrations obtained from the collected DNA solutions and the decimal dilutions. A suspension of *L. pneumophila* was used for calibration and calculation of the concentration of *Legionella* cells from the threshold cycle values. This approach incorporates the efficiency of the DNA extraction in the method. As indicated above, the composition of the water may have an impact on the efficiency of the DNA extraction; tests with samples spiked with *L. pneumophila* showed that the efficiency of isolation of DNA from drinking water did not differ much from that of the control (data not shown), but random variations were observed. In this respect, the method resembles the culture method (10, 48), and improvement requires further research.

***Legionella* in raw and in treated water.** The present study confirmed earlier reports that *L. pneumophila* is a common member of the microbial community of SW, even at low temperatures. The ubiquitous presence of *L. pneumophila* in rivers and lakes in the United States at a temperature range of 10 to 29°C, with concentrations ranging from 10^4 to 10^7 cells liter^{-1}, has been demonstrated for the first time with an FA-based method (18). Concentrations of up to 10^8 cells liter^{-1} have been observed with this method in subtropical environments at temperatures between 23 and 30°C (42). *L. pneumophila* does not multiply at temperatures below 20°C but can survive for very long periods in water at low temperature (46). Consequently, *L. pneumophila* organisms detected in SW at low temperatures may be survivors from the summer period when water temperatures above 20°C are reached in The Netherlands, and/or they may originate from discharges in surface water of treated and untreated sewage with relatively high *Legionella* concentrations (36, 43, 50). In the present study, *L. pneumophila* was not detected in samples of treated SW and was detected in only one sample of treated GW (Table 2). Multiple-barrier SW treatment in The Netherlands causes a 3- to 6-log-unit reduction of vegetative cells (*E. coli*) and spores (sulfite-reducing clostridia) (21). Hence, in the absence of growth, *L. pneumophila* is not likely to be detectable in treated SW. *L. pneumophila* has been detected by use of the culture method in water from GW wells in the United States (11, 47) but was only rarely observed in GW samples (1%) in Germany

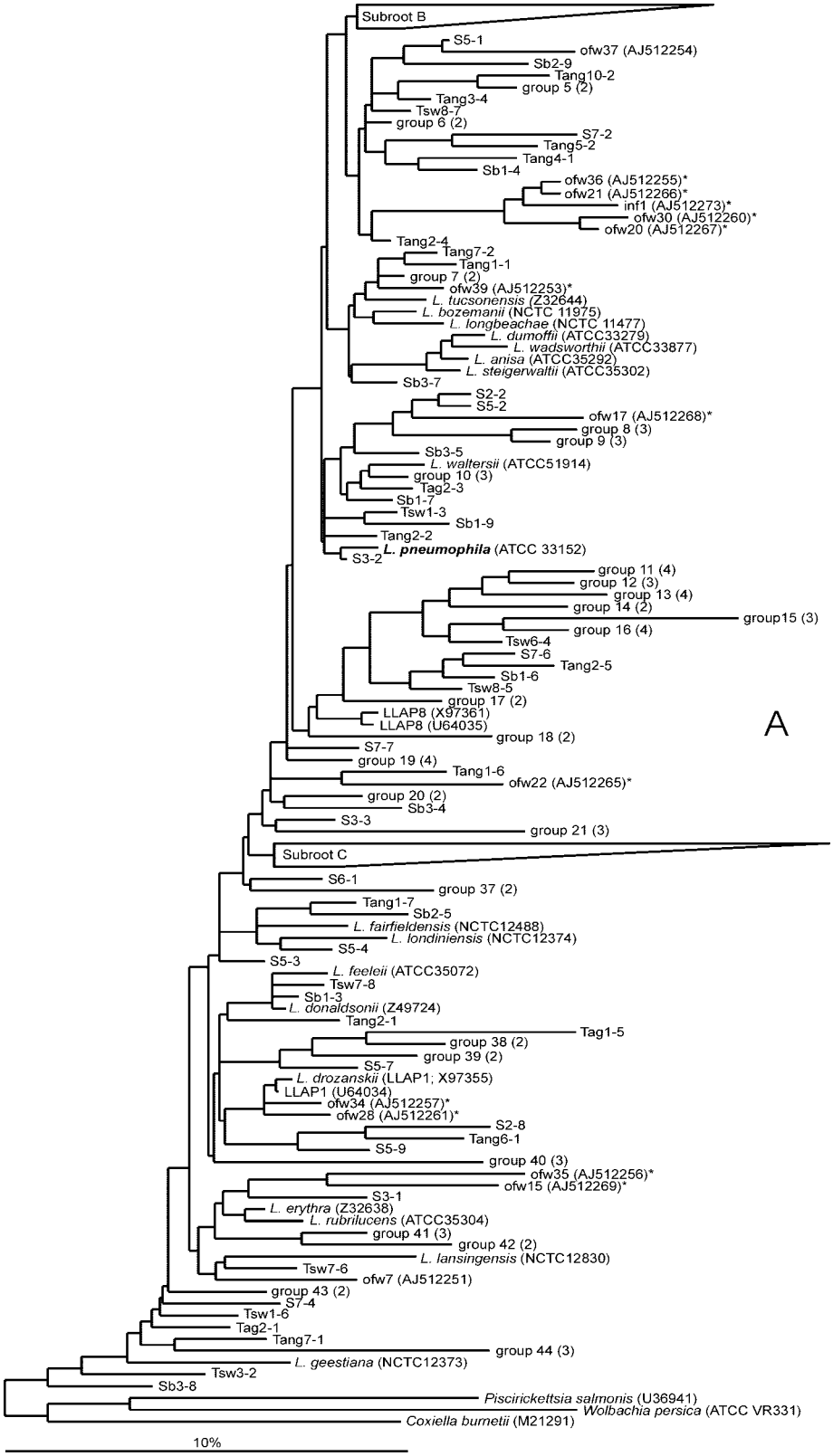
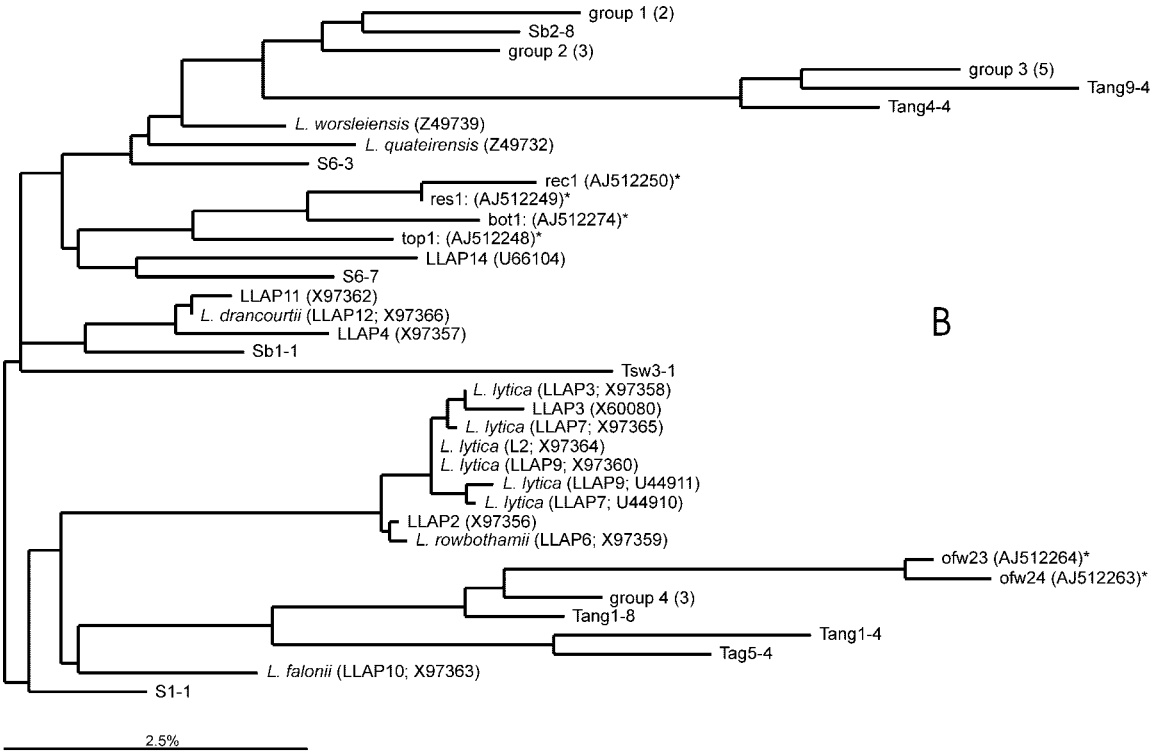


FIG. 2. Phylogenetic trees showing the positions of the 202 16S rRNA gene sequences of the partial 16S RNA gene amplified using *Legionella*-specific primers LEG-225 and LEG-858. The subtree in panel B includes mostly LLAP-related 16S rRNA gene sequences, and that in panel C contains sequences mostly originating from SW and samples from treated SW. The 16S rRNA gene sequences shown in the trees are derived from seven SW samples (S1 to S7), three storage basins (Sb1 to Sb3), eight SW treatment plants (Tsw1 to Tsw8), ten anaerobic GW treatment plants (Tang1 to Tang10), and eight aerobic GW treatment plants (Tag1 to Tag8). The trees were constructed by using the parsimony tool with local optimization. The bar represents percentage sequence difference. Asterisks indicate 16S rRNA gene sequences from slow sand filters (13). Origin of 16S rRNA gene sequences included in groups (≥99% similarity): 1, S2- and S7-1; 2, Tang10-3, Tag3-3, and Sb3-10; 3, Tang8-5, Tag4-1, Tag4-2, and Tag4-5; 4, Tag3-1, Tag3-2, and Tag3-5; 5, Tsw5-3 and Tang6-2; 6, Tang10-4 and Tang7-3; 7, Tsw8-1 and Tsw8-2; 8, Tang8-1, Tang8-3, and Tang8-4; 9, Tang8-2, Tsw1-8, and Tang4-5; 10,



Tsw4-2, Tsw4-4, and Tsw4-5; 11, Tag2-4, Tag2-5, Tag6-1, and Tang6-3; 12, Tsw7-4, Tag3-4, and Sb1-2; 13, Tsw5-2, Tsw5-4, Tsw5-5, and Tsw4-3; 14, Sb3-3 and Sb3-6; 15, Tang9-3, Tag5-3, and Tang2-3; 16, Tang3-1, Tang5-1, Tang5-3, and Tang5-5; 17, Tang10-5 and Sb3-1; 18, S2-1 and S2-6; 19, Tang9-2, Tang3-2, Tang3-3, and Tang3-5; 20, Sb3-2 and Sb3-9; 21, Tang5-4, Tang1-2, and Tang1-5; 22, S4-1, Tang4-3, and S4-8; 23, S7-3 and Tsw1-5; 24, Tsw3-4 and Tag1-4; 25, Sb2-2 and Sb2-6; 26, S5-5, S6-2, S4-9, and S2-9; 27, Tsw6-1, Tsw6-6, and Tsw6-9; 28, Tsw6-7, S1-2, S1-3, and Tsw6-8; 29, Tsw6-3 and Tsw6-5; 30, S5-6 and S5-8; 31, Tsw1-1, Tsw1-4, and Tsw1-7; 32, S2-4 and S2-5; 33, Tsw8-3 and Tsw8-4; 34, S2-3 and S7-9; 35, S4-5, S4-3, and S4-7; 36, Tsw5-1, Tang9-1, and Tsw3-3; 37, Sb1-8 and Sb1-10; 38, Tag5-1 and Tag5-2; 39, S6-4 and S6-5; 40, Tsw7-1, Tsw7-2, and Tsw7-3; 41, Tsw7-5, Tsw7-7, and Tsw7-9; 42, Tsw2-1 and Tsw2-2; 43, Tsw2-3 and Tag1-1; and 44, Sb2-1, Sb2-4, and Sb2-7.

(53). The results of this study using PCR-based methods show that *L. pneumophila* is not a common member of the microbial community in raw or treated GW in The Netherlands at temperatures of <12°C. In contrast, non-*L. pneumophila* species and types were observed in all samples (Table 1). Aerobic GW originates from sand soils and is highly oligotrophic, with low concentrations of dissolved organic carbon (DOC) (<0.5 mg liter^{-1}) and inorganic nutrients and very low heterotrophic plate count values, and it is free from fecal contamination. The presence of *Legionella* spp. in untreated aerobic GW, with a median concentration of 8.3×10^3 cells liter^{-1} and with even higher concentrations after limestone filtration, demonstrates the adaptation of these organisms to oligotrophic conditions. Examples of such organisms include 16S rRNA gene sequences related to *L. quateirensis* (Table 2) and groups 3, 4, 11, and 38 (Fig. 2A and B). *Legionella* was also detected at relatively low concentrations (median value, 5.5×10^2 cells liter^{-1}) in raw anaerobic GW at three different sites. Untreated anaerobic GW contains methane, ammonia, iron, and manganese and has a DOC concentration of up to 7 mg liter^{-1}. All cultured *Legionella* species need molecular oxygen for multiplication, and therefore the most likely explanation for its detection is the introduction of (trace amounts of) oxygen at the well head, the transportation pipe, or the sampling location (tap), enabling some local growth.

Representatives of uncultured *Legionella* spp. were detected in treated SW and in treated GW at all locations. Given the above-mentioned effects of multiple-barrier SW treatment, it must be concluded that these organisms most likely multiply during water treatment. This conclusion is supported by the differences between the 16S rRNA gene sequences isolated from SW and treated SW at the involved treatment plants. The presence of *Legionella* in untreated GW in the absence of fecal contamination and its presence in treated GW also demonstrate its ability to multiply in the involved aquatic environments. Filtration processes, including dual-medium filtration, rapid sand filtration, GAC filtration, or slow sand filtration, which are operated without chemical disinfectant, are the environments for growth. Such growth occurs at a wide range of concentrations of dissolved organic carbon (nonparticulate organic carbon concentrations of <0.5 to 7 mg liter^{-1}). No significant correlation was observed between the DOC concentration and the *Legionella* concentration in treated GW (results not shown). Growth of the *Legionella* species detected in treated water occurs at relatively low temperatures (<5°C for SW and <12°C for GW). These observations correspond with results of other studies where PCR was used to quantify *Legionella*. In these studies, it was demonstrated that *Legionella* species contributed to 7% of the total biomass in biofilms grown on pieces of polyvinyl chloride and polyethylene exposed to treated surface water and multiplied at temperatures of <20°C in slow sand filters used for horticulture as well as in experimental sand columns (13, 29, 52). The ability of *Legionella* spp. to multiply in biofilms in low-pH environments has recently been demonstrated, and those authors also concluded that water temperature affected species composition (54).

Sequence diversity of the detected *Legionella*. The genus *Legionella* presently includes about 50 defined species. This number increases continuously with newly described species, including LLAPs (1, 2, 6, 17, 23, 26, 30). LLAPs, which initially were isolated in coculture with protozoa (51), have been obtained from various environmental sources. Several of these organisms create a monophyletic subgroup within the phylogenetic tree of the genus *Legionella* (7). The LLAP species are presented in Table 2 and in Fig. 2 in connection with 16S rRNA gene sequences obtained in this study. In the present study all 202 16S rRNA gene sequences of a 654-bp fragment showed the greatest similarity to 16S rRNA-encoding genes of *Legionella* sequences deposited in the GenBank database. None of the 16S rRNA gene sequences were more than 99% similar with cultured *Legionella* species, and 65% of the 16S rRNA gene sequences had similarity values of below 97% (Table 2). The predominance of 16S rRNA gene sequences related to defined species, e.g., *L. bozemanii*, *L. lytica*, *L. quateirensis*, and groups of unidentified types in specific situations, may provide information about the conditions favoring the growth of the organisms harboring these 16S rRNA gene sequences. *L. quateirensis* was also observed in GW from wells in the United States and Canada (11), but most *Legionella* species observed in these wells differed from those described in the present study. Furthermore, *Legionella* species and sequences identified in slow sand filters (13) differed from those obtained in this study (Fig. 2). The high genetic diversity of *Legionella* phylotypes multiplying in aquatic environments at low temperature is further demonstrated by (i) positioning of the obtained 16S rRNA gene sequences all over the phylogenetic tree and (ii) different sequence types being obtained from individual samples. The groups of 16S rRNA gene sequences identified in the present study may represent yet-undefined *Legionella* spp. Hence, continuing studies using PCR-based methods most likely will cause a large increase of the number of species within the genus *Legionella*.

Public health significance. *L. pneumophila* is not commonly detected in treated water and represented only a small fraction (<1%) of the total number of Legionellae detected in this study. Still, the organism has been observed in many hot water installations, and the large majority of reported cases of legionellosis are caused by *L. pneumophila* (17, 61). A number of non-*L. pneumophila* species, including LLAP types, have been observed in association with disease or antigen titer increase (1, 17, 33, 35). Sequences with a high similarity to the disease-associated *L. bozemanii* predominated in several samples of treated water (Table 2). However, it is not clear if these or other organisms represent potential pathogens, because representatives of *L. pneumophila* serogroups and genotypes also show distinct differences in infectivity (8, 17, 25, 61). Furthermore, none of these organisms were detected with the BCYE medium. Some of the organisms may have been nonviable, but other explanations include (i) absence of certain specific growth requirements, (ii) inhibition of growth by certain medium components, or (iii) inability to multiply at 37°C. In a recent study, more isolates were obtained from GW samples incubated at 30°C than from those incubated at 35°C (11). Bacteria unable to multiply at body temperature are not likely to be human pathogens. The *Legionella* types observed in this study may include psychrophilic species that are unable to multiply at elevated temperatures. Elucidation of the properties of these organisms is needed to assess their potential public health significance and explain the conditions favoring their growth in the aquatic environments including water treatment.

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